Synthesis of Novel Prodrugs for Targeted Photodynamic Therapy

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Mots clefs:
l'acide-δ-aminolevulinic (ALA), thérapie photodynamique (PDT), cibler, Protoporphyrine (PpIX), prodrogue, glucide, cellules cancéreuses, acides aminés, vitamines, nucleoside, porphobiliogène.

Key words:
Aminolevulinic acid (ALA), Photodynamic therapy (PDT), targeted, Protoporphyrin (PpIX), prodrug, carbohydrate, glycoside, cancer cells, amino acids, vitamin, nucleoside, porphobilinogen.

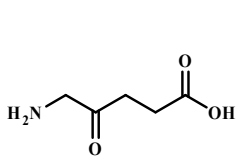
Abbreviations

APCI : Atmospheric Pressure Chemical Ionization ;aq. : aqueous ; atm.: atmosphrere, BnBr: Benzyl bromide, cat. : catalytic ; TLC : Thin layer chromatography; GC: gas chromatography; DIAD : diisoprpyl azodicarboxylate ; DPTS : 4-(diméthylamino)pyridinium toluene-para-sulfonate ; Bpt :Boiling point; F: freezing point; EDCI : 1-ethyl-3-(3-diméthylaminopropyl)carbodiimide ; EI : electronic impact ; eq. : equivalent(s) ; ESI : Electron Spray Ionization ;
FID : Flame Ionization Detector ; h : hour (s) ; HR-MS : *High*
Resolution Mass Spectroscopy ; IR : infra-red ; ppm : parts per million ;
NMR : Nuclear Magnetic Resonanmce ; *R*f : retention factor; rt: room temperature;

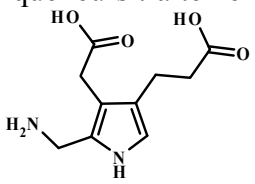
Résumé

L'existence d'effets secondaires dans les traitements de la médecine moderne constitue actuellement l'une des principales préoccupations du grand public. La mise au point de thérapies ciblant spécifiquement les cellules malades représente l'un des challenges de la lutte contre le cancer. Mis à part quelques exceptions, la plupart des thérapies anticancéreuses ne permettent pas d'éradiquer véritablement la maladie, on parle plutôt de période de rémission qui ne font qu'allonger la durée de vie des patients.

La thérapie photodynamique (PDT) ciblant l'acide-δ-aminolevulinic (ALA) représente une méthode pour le traitement du cancer. ALA est une molécule naturellement présente dans nos cellules, elle est à l'origine de la biosynthèse de l'hème, un constituant des globules rouges. Dans le cas des cellules cancéreuses, ce mécanisme est perturbé et conduit à l'accumulation d'un composé intermédiaire Protoporphyrine (PpIX) qui a la particularité d'être photosensible et fluorescent. Par conséquent l'utilisation d'ALA comme 'prodrogue' conduit à une surproduction de ce composé photosensible au sein des cellules malades et permet leurs détection ainsi que leurs traitement.

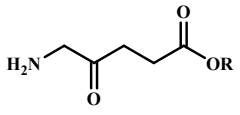


5-aminolevulinic acid (ALA)



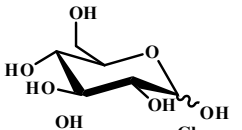
porphobilinogen (PBG)

De nouvelles 'prodrogues' plus sélectives que ALA sont cependant nécessaires. Nous avons donc synthétisé des dérivés où une molécule d'ALA est connectée à différentes biomolécules, comme des glucides, des acides aminés, des glycérols ou encore des vitamines.

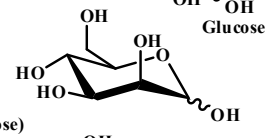


5-aminolevulinic acid derivatives

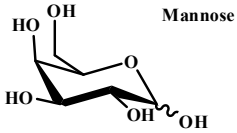
R= sugars (glucose, mannose and galactose)
or
R= vitamins
or
R= natural alcohol
or
R= nucleosides



Glucose



Mannose



Galactose

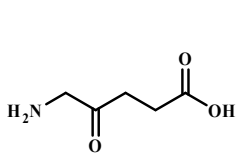
Tous les produits synthétisés ont été testés sur différents types de cellules cancéreuses, les dérivés ALA-glucide se sont révélés particulièrement efficaces et non cytotoxiques. En effet certains d'entre eux induisent une production plus importante de composés photosensibles que ALA et de plus ils s'avèrent très sélectifs envers certains types de cellules cancéreuses.

Ce travail de thèse a finalement permis la synthèse d'une nouvelle variété de prodrogues plus efficaces que celle disponible sur le marché (ALA), contribuant à la détection des cellules cancéreuses et dans certains cas à leur élimination.

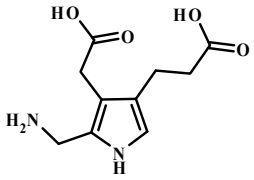
Summary

One of the concerns of the general public about modern day medicine is ‘side effects’. Therapies targeting only the diseased cells are the solution for this. The targeted delivery of drugs is one of the main challenges that all cancer therapies are dealing with. With very few exceptions, almost all the therapies for cancer today are only extending the life of patients by few months instead of really targeting cancer cells and cure patients.

Delta aminolevulinic acid (or 5-aminolevulinic acid or shortly ALA) based photodynamic therapy (PDT) is one among the therapies for cancer. ALA is a natural product which is synthesized in the body of all human beings which transforms in series of steps to heme. One of the intermediates in heme pathway is Protoporphyrin IX (PpIX) which is a photosensitizer. ALA, when administered externally induces preferentially higher PpIX production in cancer cells. The fluorescent nature of PpIX is used for detection of cancer cells besides therapy.

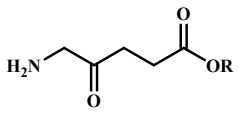


5-aminolevulinic acid (ALA)



porphobilinogen (PBG)

Novel prodrugs for inducing higher PpIX generation than ALA and having more selectivity towards cancer cells are required. We synthesized novel derivatives of ALA where ALA is conjugated with monosaccharides (sugars), natural alcohols, vitamins and aminoacids. Also derivatives of porphobilinogen (PBG) which is an advanced intermediate in heme pathway were synthesized. We synthesized different ALA-sugar derivatives where not only different sugars were utilized for conjugation, but also, ALA was substituted in different hydroxyl groups of sugars.



5-aminolevulinic acid derivatives

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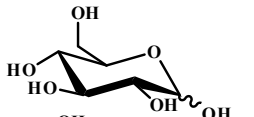
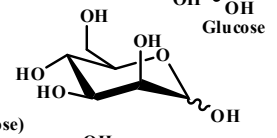
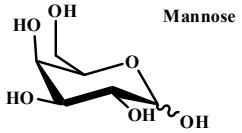


Glucose



Mannose



Galactose

All the novel prodrugs were evaluated for PpIX induction using various cancer cell lines. We found that all these novel prodrugs are not cytotoxic; some of them induce higher generation of PpIX than ALA. But the selectivity towards cancer cells is not better than ALA.

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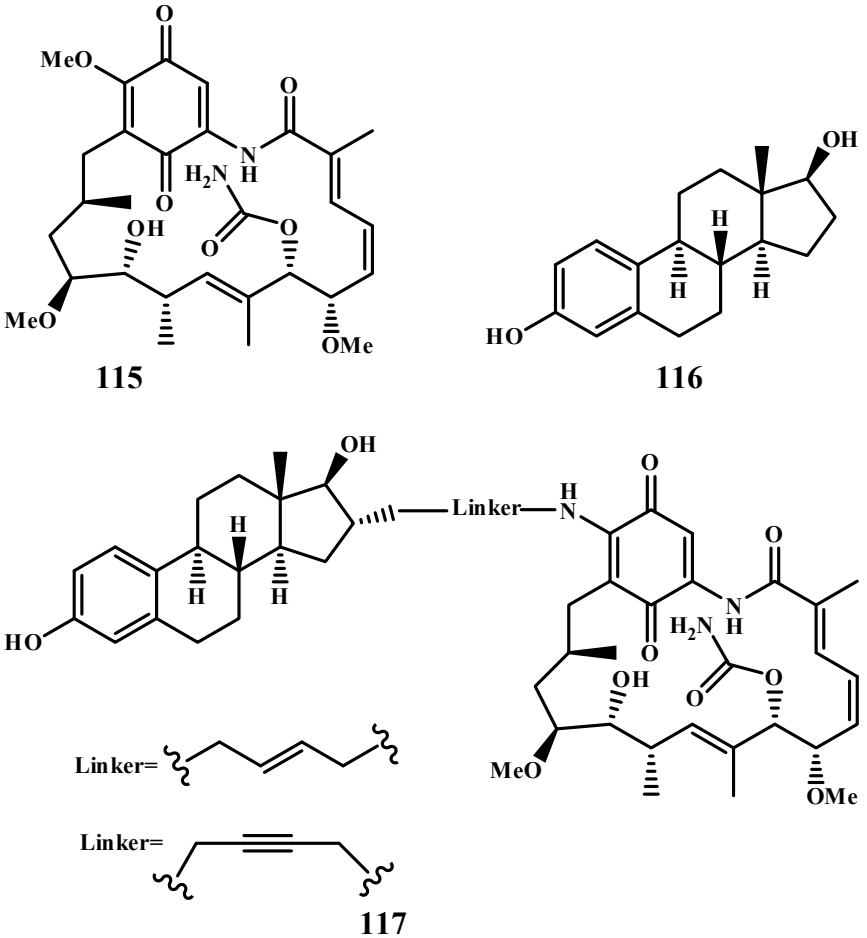
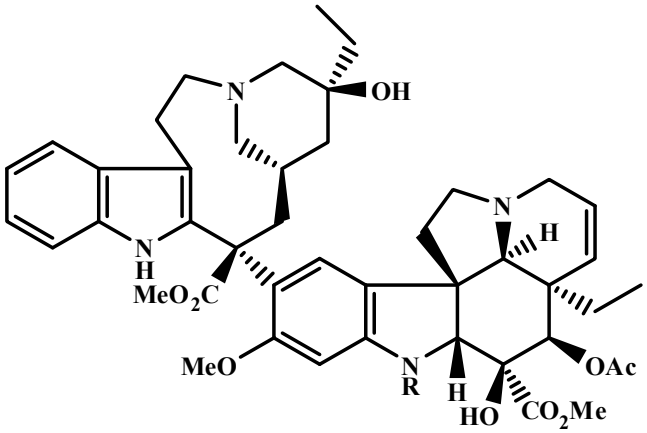
1. Introduction

One of the most fruitful concepts applied with increasing success in recent years in bioorganic chemistry may be the synthesis and study of natural product hybrids. A natural product hybrid is a synthetic compound having two or more natural products derived fragments joined at least by one carbon-carbon bond. Using the enormous number of natural products known and available, an unlimited number of natural-product hybrids as combination of different natural products can be synthesized. This new synthetic approach has proven to be very promising in the development of leads for medicinal applications, as the biological activity of several new hybrids exceeds that of the parent compounds. An example is geldanamycin- estradiol hybrid **117**. Geldanamycin **115**, a natural antibiotic binds and causes the degradation of several important signalling proteins. Estradiol **116** is a natural hormone and novel estradiol-geldanamycin hybrids have been synthesized and evaluated in cancer cells for their ability to induce the selective degradation of tumor-relevant proteins in breast (estrogen receptor (ER)). The hybrid compounds are not only active but also more selective than the parent causing degradation of ER (estrogen receptor), but not other targets [1].



Scheme 1: Example of a natural product hybrid

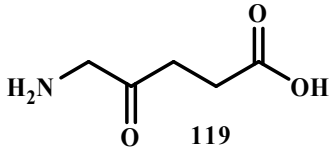
The advantage of this natural product hybrid concept over a combinatorial chemistry approach is the high diversity and the inherent biological activity of the hybrids [2, 3]. Actually, from a general view, the approach is not quite new, since even Nature employs such a strategy; Nature makes natural products of diversity and complexity and these are generally derived through specific biosynthetic pathways like shikimate, polyketide or mevalonate, leading to a particular class of compounds. Many biologically active natural products are also derived through mixed biosynthesis [4, 5]. An example of naturally occurring natural product hybrid can be the indole alkaloid vincristine **118b** which is a famous drug for lymphatic leukemia. This is a dimeric indole alkaloid consisting of vindoline—an alkaloid of the Aspidosperma subgroup—and catharanthine—a member of the Iboga subgroup of indole alkaloids. It is of special interest that both monomeric alkaloids do not express any pronounced or useful biological activity. Artificial natural product hybrids have not yet been used as drugs, as this idea is quite new, but several novel compounds of this type developed in the last years show promising biological activity.



118a; R=Me Vinblastine
118b; R=CHO Vincristine

Scheme 2: A natural product hybrid synthesized by Nature

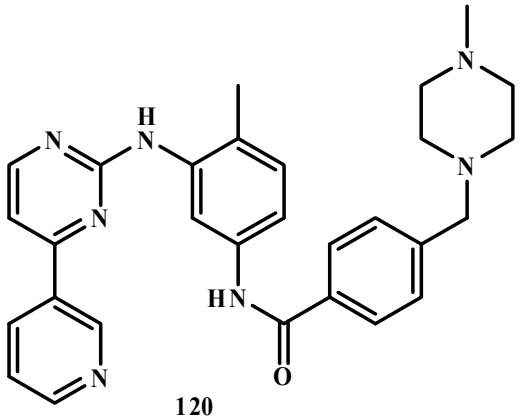
The starting point of our work is the use of natural product hybrids in photodynamic therapy (here after referred as PDT). All the compounds synthesized in this thesis can be classified as natural-product hybrids. Each compound is derived from two natural products, namely a sugar or vitamin or nuceloside and an aminolevulinic acid **119** (here after referred as ALA) moiety. How this natural product hybrid is synthesized and how its biological activity exceeds the efficiency of the natural product (aminolevulinic acid) alone in the field of photodynamic therapy (PDT) of cancer is the main focus of the work reported here.



Scheme 3: 5-aminolevulinic acid (ALA)

Cancer is one of the dreaded diseases which affect the human population. Almost 7 million people died of cancer in the year 2005 alone as per the World Cancer Report, done by the World Health Organization [6]. The report states also that 11 million new cases were diagnosed during the same period. Cancer claims twice as many lives worldwide as AIDS. In fact, more than 12% of all deaths (60 million cases of deaths due to all causes) every year is caused by cancer (7 million cases of cancer) [7]. This figure is more than AIDS, tuberculosis, and malaria put together which are 6.8 million cases [8]. In industrialized countries, cancer is the leading cause of premature mortality, and the same trend is emerging in developing countries. Cancer rates could further increase by 50% to 15 million new cases in the year 2020, according to the World Cancer Report, the most comprehensive global examination of the disease to date [6].

Chemotherapy, surgery, radiotherapy, hormone therapy and many other therapies are used today to treat cancer patients. Most of these treatments help to relieve the symptoms to prolong the life expectancy sometimes only by months in the better cases by years, but most of the actual treatment do not lead to a real cure (for exceptions see the use of Gleevec® **120**) [9, 10]. Many of the cancer therapies used lead to severe side-effects, which would be unacceptable in any other modern treatment. Photodynamic therapy (PDT) is one of the especially mild treatments of cancer, which thereby has the potential to lead to a real cure.



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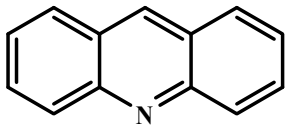
Scheme 4: Structure of Gleevec®

1.1. History of Photodynamic therapy

Light has been used as therapy for more than three thousand years. Ancient Egyptian, Indian and Chinese civilization used light to treat various diseases including psoriasis, rickets, vitiligo and skin cancer [11]. In one of India's sacred books *Atharva-veda* (1400 BC) the use of seeds of the plant *Psoralea corylifolia* for the treatment of vitiligo is described. Psoralens are the photoactive components of these seeds, just as in the extracts of the plant *Ammi majus*, which grows on the banks of the Nile. The Egyptians used *Ammi majus* to treat vitiligo.

In 1898, O. Raab showed the cytotoxic effects of the combination of acridine **121** and light on infusoria (*Paramecium caudatum*) [12].



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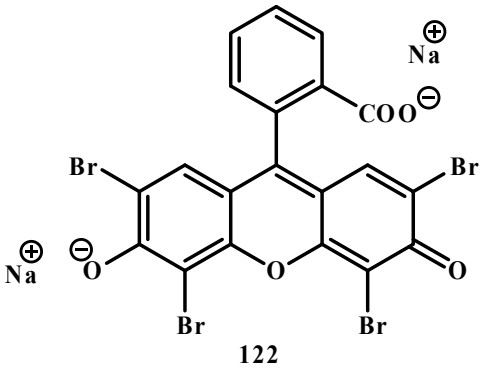
Scheme 5: Acridine

'Phototherapy' or the use of light to treat disease was developed systematically during 1900s by a scientist from Denmark named N. Finsen. He was awarded a Nobel Prize in 1903 for his work in phototherapy [13, 14]. He invented the red light treatment for small pox [15] and also used ultraviolet light to treat tuberculosis. The term 'photodynamic action' was introduced by A. Jesionek and H.v.Tappeiner (Professor of O.Raab) and who had treated skin tumors with topically applied eosin **122** and white light in 1903 [16].



122

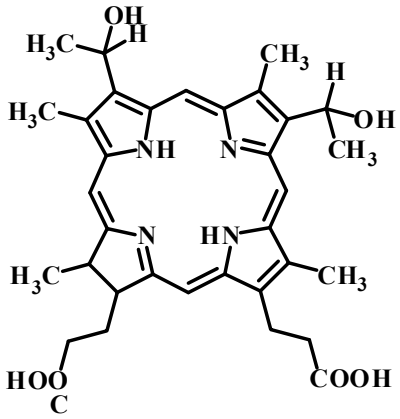
Scheme 6: Eosin

F. Meyer-Betz investigated as early as 1913 porphyrins, the class of compounds most often used today, for their application in photodynamic therapy (PDT). He studied the accumulation of hematoporphyrin (HP) and its derivatives in rat tumors and PDT effects following systemic administration [17]. The fluorescence from these compounds was further investigated for diagnostic and tumor margin delineation in the late 1940s and 1950s by F.H.J. Figge and colleagues [18]. Modern photodynamic therapy (PDT) was initiated by R.L. Lipson and E.J. Blades. They established that an impurity in HP was the tumor-localizing agent, and not the parent compound. This led to the "synthesis" of hematoporphyrin derivative (HPD; **123**), a mixture of porphyrins produced by the acid treatment of HP [19]. The exact chemical composition and structure of this mixture remains unclear, although there is general consensus that the active portions consist of porphyrin oligomers with ether and/or ester linkages along with monomeric porphyrins. HPD was further developed for laboratory and clinical investigations through the efforts of T. J. Dougherty and colleagues in 1970s and 1980s [20]. The history of photodynamic therapy can be depicted as shown in the table1.

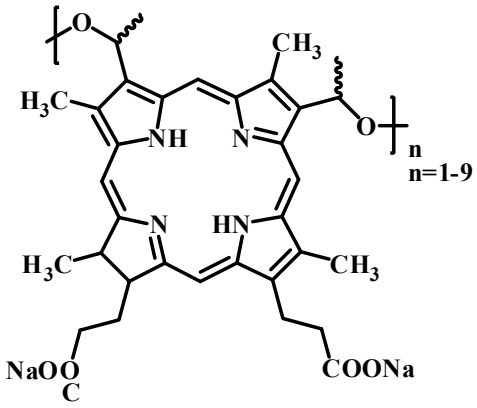
Table 1: History of Photodynamic therapy – Time line

HISTORY OF PHOTODYNAMIC THERAPY: PERIOD 1900 – present		
Year of discovery	**Scientist (University or company)**	**Discovery, commercial application or important event**
1900	O. Raab (Zeitschrift fuer Biologie, Munich, Germany)	The cytotoxic effects of the combination of acridine and light on infusoria (*Paramecium caudatum*).
1901	N. Finsen (University of Copenhagen, Denmark)	Light treatment of smallpox and cutaneous tuberculosis
1903	H. von Tappeiner and A. Jesionek (Zeitschrift für Biologie, Munich, Germany)	Topical eosin application and white light treatment of malignant melanomas
	N. Finsen (University of Copenhagen, Denmark)	Nobel Prize for his work on phototherapy.
1907	H. vonTappeiner (Director, Pharmacological institute of the Ludwig-Maximilian University, Munich) and A.Jodlbauer	Introduction of the term 'photodynamic'
1911	W. Hausmann (Physiol. Inst., Hochschule für Bodenkultur, Vienna, Austria)	Phototoxic effects of haematoporphyrin on the skin of mice
1913	F. Meyer–Betz (Chief Physician in Königesberg, Germany)	First human tests. PDT using porphyrins. Self-experiment using his own hands
1955	S. Schwartz (University of Minnesota, Minneapolis, USA)	Development of haematoporphyrin derivative (HPD) by acetylation and reduction of haematoporphyrin.
1960	R. L. Lipson and E. J. Baldes (Mayo clinic / University of Vermont College of Medicine, U.S.A)	Accumulation of HPD in tumors and its use in the photodetection of tumors
1972	I. Diamond (University of California, U.S.A)	Demonstration of the phototoxicity of haematoporphyrin against gliomas in vivo and in vitro
1975	T.J. Dougherty (Photodynamic Therapy Center, Roswell Park Cancer Institute, New York, U.S.A)	Successful treatment of skin cancer in patients
	J. F. Kelly (St. Mary's Hospital Medical School, London, United Kingdom)	Treatment of bladder cancer in humans ; using HPD tumor regression is observed
1978	T.J. Dougherty (Photodynamic Therapy Center, Roswell Park Cancer Institute, New York, U.S.A)	First controlled clinical study in humans of PDT for the treatement of skin tumor
1999	QLT Phototherapeutics, Vancouver, Canada and American Cyanamid Co. Pearl River, New York,	Approval of the first PDT drug in Canada

Tumors in virtually every anatomic site have been treated with PDT, and most are responsive to this therapy to some extent. Although, to date, several thousand patients have been treated with PDT for a variety of neoplasms, randomized clinical trials of this mode of cancer treatement were initiated only in 1987, using a purified form of HPD, called porfirmer sodium (Brand name-Photofrin®) [21]. These first randomized trials, were sponsored by Quadra Logic Technologies, Inc. (now QLT Phototherapeutics, Vancouver, Canada) and American Cyanamid Co. (Pearl River, New York), and compared the efficacy of PDT with that of other forms of therapy for bladder, oesophagal, and lung cancers. Within the past 5 years significant progress has been made worldwide in obtaining regulatory approval for a variety of indications. Currently, PDT with Photofrin® **124** is approved in 10 countries.



123
Hematoporphyrin



124
Photofrin

Scheme 7: First generation photosensitizers

The understanding of the biology of PDT has advanced, and efficient, convenient, and inexpensive systems of light delivery are now available. Moreover, encouraging results from randomized phase III trials are becoming published, and improved photosensitizing drugs are under development. Besides, Porfirmer sodium (Photofrin®), temoporfin (also called meso-tetra (hydroxyphenyl) porphyrin (m -THPP) (brand name - Foscan®) has now been approved for systemic administration, and aminolevulinic acid and methyl aminolevulinate have been approved for topical use. PDT has several potential advantages over surgery and radiotherapy: it is less invasive, it can be targeted accurately, repeated doses can be given without the total-dose limitations associated with radiotherapy, and the healing process results in little or no scarring. PDT can usually be done in an outpatient or day-care setting and has few side effects, improving patient convenience.

1.2. Mechanism of Photodynamic therapy

PDT is the result of combination of three non-toxic elements namely light, oxygen and photosensitizer. In the following paragraphs, the essentials of each of these components will be described.

1.2.1. Light

Light is a form of electromagnetic radiation that covers a wide range of wavelengths, λ, between radio wavelengths in the meter (m) range to gamma rays with wavelengths around 10^{-11} m. Visible light, most relevant to PDT, covers the limited range of 4 to 7 × 10^{-7} m (400–700 nm). The energy content (*E)* of light is related to the wavelength of absorption by $E = h\gamma = hc/\lambda$, where *h* is Planck's constant (6.63 x 10^{-34}Js), γ is frequency, *c* is the speed of light in vacuum (3.0 x 10^{6} m/s), and λ is a single wavelength.

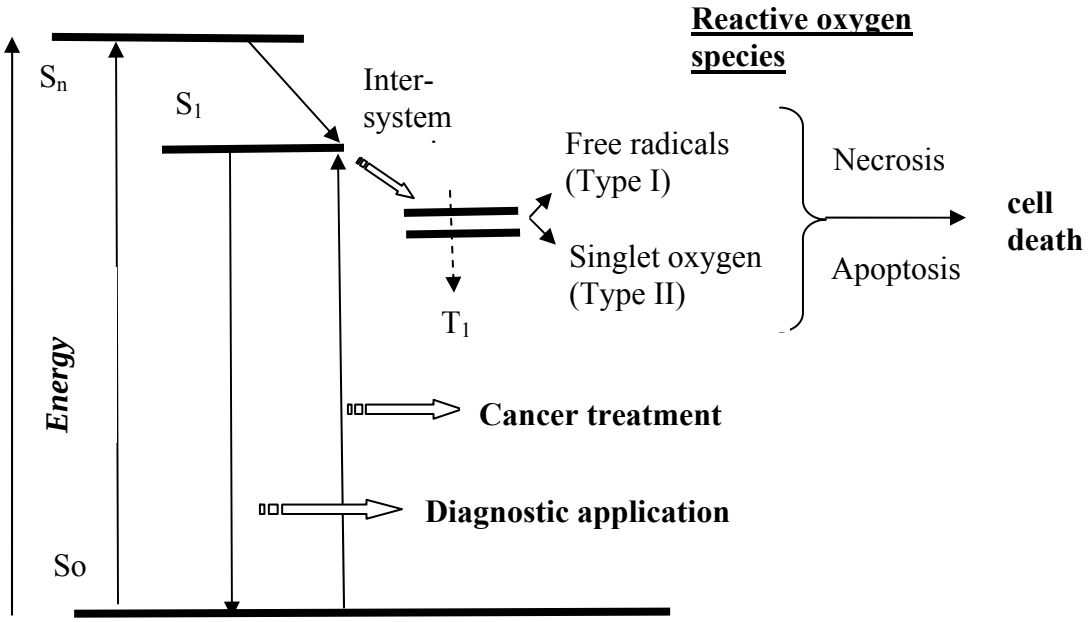
Conventional gas discharge lamps were initially used for the photosensitization process. The introduction of lasers equipped with optical fibers revolutionized photosensitization and expanded its applicability in medicine, enabling the endoscopic delivery of light to almost every site of the human body. Photodynamic treatment in dermatology is simplified by the accessibility of the skin to light application and allows using any light source with the appropriate spectrum e.g.: metal halogen lamps, which emit 600 to 800 nm radiation at high power density, short-arc xenon lamps, tunable over a bandwidth between 400 and 1200 nm. The broad light beam produced by incoherent lamps is useful for the treatment of large lesions. In contrast to traditional incandescent lamps, lasers permit to select the exact wavelengths and to apply the light beam with high precision. Pulsed lasers, such as the gold vapor laser (GVL) and the copper vapor laser or pumped dye lasers (GVDL), produce brief light pulses of millisecond to nanosecond duration [22]. The comparison of the efficacy of PDT using continuous-wave light sources and pulsed lasers has shown no difference. Tunable solid-state lasers, such as the neodymium doped YAG laser, are particularly useful for PDT. The above-listed laser systems are expensive, relatively immobile, and require frequent repair. The development of semiconductor diode lasers is a novel approach to circumvent these disadvantages. Portable diode lasers, such as the gallium-aluminium-arsenide laser, produce light in the range from 770 to 850 nm, which corresponds to the absorption peaks of many new photosensitizers [23].

1.2.2. Oxygen and photochemistry

Numerous investigations supported the idea that the efficacy of photosensitization is directly related to the yield of singlet oxygen ($^1O_2$) created in the tumor environment and the yield of singlet oxygen ($^1O_2$) depends on the concentration of ground state oxygen (which is the triplet state) in the tissue [24]. Hypoxic cells are very resistant to photosensitization and the photodynamic reaction mechanism itself may consume oxygen at a rate sufficient to inhibit further photosensitization effects. It has been suggested, that hyperbaric oxygen might enhance the photosensitization effect.

When light is absorbed, the energy of the absorbed photons causes the absorbing molecule to be electronically excited. This excitation energy may be converted into heat (kinetic energy) by the collision of the excited molecule with surrounding molecules by radiationless decay. Alternatively, it may be re-emitted as fluorescence. The electronic energy levels between which transitions occur by absorption of ultraviolet-visible light (λ = 200–700 nm) may be represented by the simplified energy level diagram presented in Fig 1.

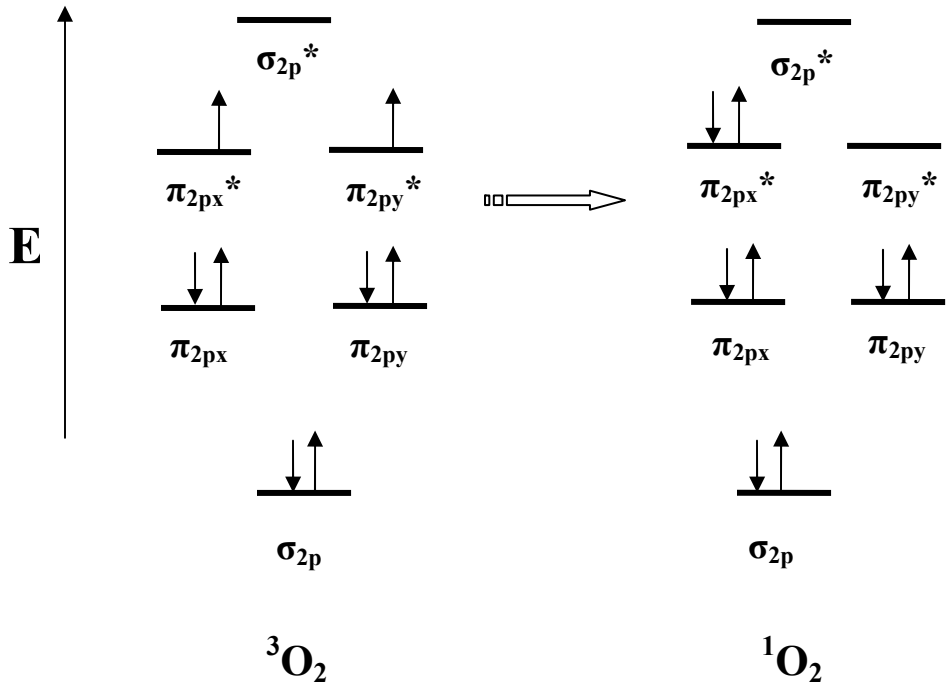
Figure 1: Jablonski energy level diagram for photodynamic therapy



The electronic states of the photosensitizer are the singlet states S_0 to S_n and the triplet states T_1 and T_n. The ground state of the photosensitizer S_0 can only be a singlet state, all electron spins are paired. In the excited states the electron spin of the two unpaired electrons can either be antiparallel S_n, singlet states, or parallel T_n, triplet states. With conventional light sources, a photosensitizer molecule will typically absorb a single photon exciting the molecule to the first excited singlet state S_1. From this excited state the molecule may directly initiate photochemistry (depending on the chemical structure) or undergo intersystem crossing to an electronically different excited state, the first triplet state T_1. From S_1, the excited molecule may also relax back to S_0 by radiationless decay and generate heat or may re-emit radiation as fluorescence, which may be used for diagnostic purposes. In general, T_1 is longer lived and chemically more reactive so that the biologically relevant photochemistry is often mediated by this state. T_1 can initiate photochemical reactions directly, giving rise to reactive free radicals, or transfer its energy to the ground state oxygen molecules ($^3O_2$) to give rise to excited singlet state oxygen molecules $^1O_2$. This excitation responsible for the production of $^1O_2$ requires excitation energy of at least 20 kcal/mole, which places limits on the wavelength of

absorption of the photosensitizer. If the energy of T_1 is appropriate, photo-oxidative reactions may occur mediated by the formation of $^1O_2$ (see Fig.1 and 3).

Figure 2: Molecular orbital diagram of oxygen showing the electron distribution of singlet and triplet oxygen



The excited triplet of the photosensitzer can undergo two kinds of reactions (Fig.2). The photoinduced oxidation processes have been classified by Macdonald *et al* as [25] : Type I reaction is a *radical* or *redox* reaction. The excited triplet can react directly with substrates, such as molecules forming the cell membrane or other molecules (A) of the cell content, and abstract an electron to form radical cations of the molecules contained in the cells. These radical cations interact with oxygen to produce oxygenated products (eqn1).

Equation 1

$$^3S + A \longrightarrow S^{\bullet -} + A^{\bullet +}$$

$$A^{\bullet +} + {}^3O_2 \longrightarrow A_{ox}$$

Alternatively, the radical anion of excited photosensitizer can transfer their electron to oxygen to produce superoxide radical anions which in turn can react with the substrate such as molecules contained in the cell membrane to give oxygenated products. There exists another possibility of forming superoxide radical anions from oxygen. Triplet oxygen can react directly with excited triplet state of a photosensitizer to give superoxide radical anions and a radical cation of the photosensitizer. Those two reactive species can

react with substrate molecules e.g. contained in the cell membrane to produce oxygenated products and at the end of this process the photosensitizer is back in the ground state (eqn 2).

Equation 2

$$S^{\bullet -} + {}^3O_2 \longrightarrow S_0 + O_2^{\bullet -}$$

$$O_2^{\bullet -} + A \longrightarrow A_{ox}$$

Or

$${}^3S + {}^3O_2 \longrightarrow S^+ + O_2^{\bullet -}$$

$$S^+ + A \longrightarrow S_0 + A^{\bullet +}$$

The second kind of reaction called type II reaction is an *energy transfer process*. Here, the excited triplet can transfer its energy directly to oxygen, to form singlet oxygen — a highly reactive oxygen species (ROS) as shown in eqn 3.

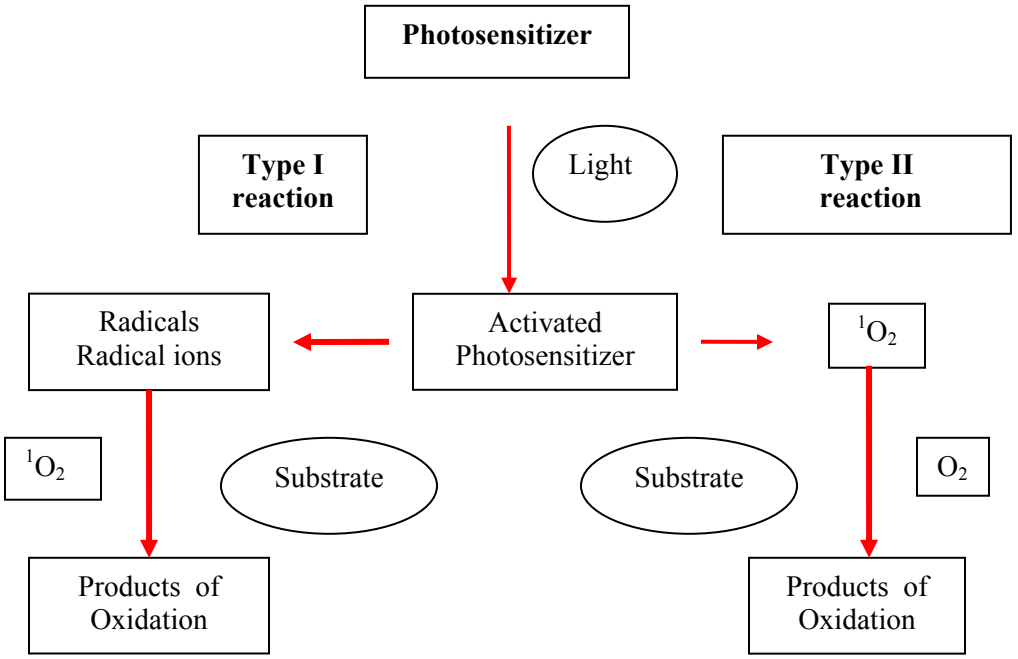
Equation 3

$${}^3S + {}^3O_2 \longrightarrow S_0 + {}^1O_2$$

$$A + {}^1O_2 \longrightarrow A_{ox}$$

Independent of the sort of photosensitizer used for PDT, the efficiency of the treatment is oxygen dependent. Photosensitization typically does not occur in anoxic areas of tissue. *In vivo* studies showed that induction of tissue hypoxia, by blood vessel clamping, abolished the PDT effects of porphyrins [26]. Both type I and type II reactions occur simultaneously, and the ratio between these processes depends on the type of sensitizer used, the concentrations of substrate and oxygen, as well as the binding affinity of the sensitizer for the substrate. Because of the high reactivity and short half-life of the ROS, only cells that are proximal to the area of the ROS production (areas of photosensitizer localization) are directly affected by PDT [27]. The half-life of singlet oxygen in biological systems is <0.04 μs, and the radius of the action of singlet oxygen is <0.02 μm. The extent of photodamage and cytotoxicity is multifactorial and depends on: the type of sensitizer, the extracellular and/or intracellular localization, the total dose administered, the total time of light exposure, the light flux, the availability of oxygen and the time between the administration of the drug and light exposure. All of these factors are interdependent [28] and need to be carefully optimized.

Figure 3: Reactions of Photosensitizer on Exposure to Light [28].



1.2.3. Mechanism of tumor destruction by PDT

There are three main mechanisms by which PDT mediates tumor destruction [29]. In the first case, the ROS that is generated by PDT can kill tumor cells directly. PDT also damages the tumor-associated vasculature, leading to tumor infarction. Finally, PDT can activate an immune response against tumor cells. These three mechanisms can also influence each other. The relative importance of each one of them for the overall tumor response has yet to be defined. It is clear, however, that the combination of all these three processes is required for long-term tumor control.

1.2.3.1. Direct killing of tumor cells

In vivo exposure of tumors to PDT has been shown to reduce the number of clonogenic tumor cells, through direct photodamage [30]. However, complete tumor eradication is not always fully realized by this mechanism alone. One reason is the non-homogenous distribution of the photosensitizer within the tumor. Furthermore, in 1995, Mladen Korbelik *et al* showed that both intravenously administered photosensitizer accumulation and the level of tumor cell killing decrease with the distance of tumor cells from the vascular supply [31]. Another parameter that can limit direct tumor-cell destruction is the availability of oxygen within the tissue that is targeted by PDT. Oxygen shortage can arise as a result of the photochemical consumption of oxygen during the photodynamic process, as well as from the immediate effects of PDT on the tissue microvasculature. Rapid and substantial reduction in the tissue oxygen tension during and after illumination of photosensitized tissue have been reported [32, 33]. Depending on the localization of

the photosensitizer at the time of illumination, oxygen tension can increase transiently [34]. Although the developments of microvascular damage and hypoxia after PDT have been shown to contribute to the long-term tumor response, the reductions in oxygen that occur during PDT can limit the response. There are two ways to overcome this problem. One is to lower the light flux to reduce the oxygen consumption rate, and the other is to fractionate the PDT light delivery to allow re-oxygenization of the tissue [35, 36]. The extent of modulation by light intensity is dependent on the localization of the photosensitizer [37].

1.2.3.2. Vascular damage

The viability of tumor cells also depends on the amount of nutrients supplied by the blood vessels. In turn, formation and maintenance of blood vessels depend on growth factors produced by tumor or host cells [38, 39]. Targeting the tumor vasculature is therefore one promising approach to cancer treatment. In the past 15 years, there have been a number of reports of PDT causing microvascular collapse [40-42], leading to severe tissue hypoxia and anoxia. As early as 1989, Barbara Henderson *et al* showed in a fibrosarcoma mouse model that Photofrin-based PDT (Photofrin is a photosensitizer produced by Axcan Pharma, Montreal, Canada) induced vascular shutdown, limiting the oxygen supply to the tumor [43]. Pre-clinical *in vivo* studies, performed last year with the photosensitizer MV6401 — a pyropheophorbide derivative (Miravant Medical Technologies, Santa Barbara, California) —, showed a biphasic vascular response following PDT. The first, immediate response was vasoconstriction. After three hours, a second, long-term response, characterized by thrombus formation, occurred [40]. This response could be inhibited with heparin. These vascular effects were associated with a delay in tumor growth. Previous studies with other photosensitizers, such as a benzoporphyrin derivative (BPD) [41] , HPD [42] and Photofrin [41] also reported vascular constriction, thrombus formation and inhibition of tumor growth. On the other hand, expression of vascular endothelial growth factor (VEGF) and cycloxygenase (COX)-2 — both potent angiogenic factors — were upregulated during PDT [44]. These effects were presumably due to the ROS formation and hypoxia that was induced by PDT. Further studies are required to determine the long-term effects of PDT on tumor vasculature.

1.2.3.3. Immune response

Studies in the late 1980s and early 1990s also reported infiltration of lymphocytes, leukocytes and macrophages into PDT-treated tissue, indicating activation of the immune response [45, 46]. Differences in the nature and intensity of the inflammatory reaction between normal and cancerous tissues could contribute to the selectivity of PDT-induced tissue damage. The inflammatory process is mediated by factors such as vasoactive substances, components of the complement and clotting cascades, acute phase proteins, proteinases, peroxidases, ROS, leukocyte chemoattractants, cytokines, growth factors and other immunoregulators. The inflammatory cytokines interleukin (IL)-6 and IL-1, but not tumor necrosis factor- α (TNF-α), have been shown to be upregulated in response to PDT [47]. In 1996, Wil de Vree and colleagues also reported that PDT activated neutrophil

accumulation, which slowed tumor growth [48]. Depletion of neutrophils in tumor-bearing mice decreased the PDT-mediated effect on tumor growth.

In 1996, Mladen Korbelik *et al* compared the long-term effects of PDT on tumor growth in normal Balb/C and immunodeficient mice. Whereas short-term tumor responses to Photofrin PDT were similar, long-term effects were quite different, as tumor recurrence occurred frequently. These results indicate that, whereas the direct effects of PDT can destroy the bulk of the tumor, the immune response is required to eliminate the surviving cells [49]. In 1999, Korbelik *et al* reported that PDT generated tumor-sensitized immune cells that could be recovered from lymphoid sites distant to the treated tumor at different time intervals after PDT [50]. An interesting observation was also made by Barbara Henderson *et al*, who reported that a tumor-cell lysate that was isolated following PDT with Photofrin could be used to vaccinate mice against the development of further tumors, indicating the induction of tumor-specific immunity [51]. This vaccination approach has been shown to be more effective at activating an immune response than lysates made from tumors that were exposed to ultraviolet or ionizing irradiation. These PDT vaccines seem to induce a cytotoxic T-cell response that involves induction of IL-12 expression. Studies with PDT and tumor-cell lysates indicate that PDT could have potential as a systemic immune therapy [51]. Further experiments are required to determine whether similar results can be obtained in patients who receive PDT.

1.3. Photosensitizers

1.3.1. First Generation Photosensitizers

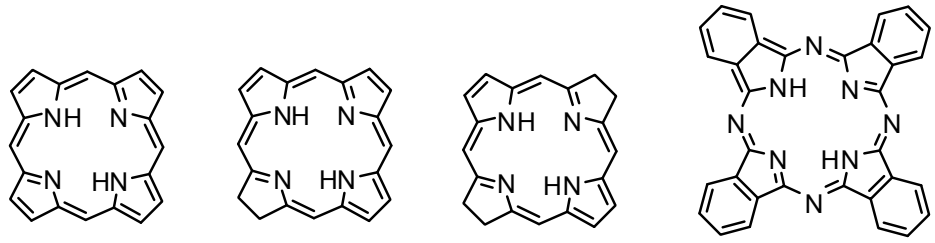
Hematoporhyrin and its derivatives comprise the first generation of photosensitizers. The first generation photosensitizers starts in the 1960s, when R. Lipson *et al* initiated the modern era of PDT at the Mayo Clinic [19]. These studies involved a compound that was developed by S. Schwartz called 'haematoporphyrin derivative' (HPD) [52]. To prepare this derivative, crude haematoporphyrin was treated with acetic and sulphuric acids, filtered and then neutralized with sodium acetate. The precipitate was then purified to produce HPD. R.L. Lipson and E.J. Baldes then showed that HPD localized to tumors, where it emitted fluorescence. Because this derivative could also be administered at much smaller doses than crude haematoporphyrin, it held promise as a diagnostic tool. The mechanisms by which photosensitizers such as HPD selectively accumulate in tumors are complex and not fully understood. It is presumably because of the high vascular permeability of the agents, as well as their affinity for proliferating endothelium and the lack of lymphatic drainage in tumors [21]. HPD has been partially purified to remove the less-active porphyrin monomers, to form Photofrin® (also called porphymer sodium) [20], a very widely used photosensitizer in clinical PDT. Photofrin® is the first photosensitizer for which marketing clearance was obtained. It was first approved for prophylaxis of bladder cancer in Canada on April 16, 1993. Since then, it has been approved in several countries for the treatment of not only bladder but also oesophageal, gastric, cervical, and lung cancers [20, 53] . The suitability of Photofrin® for the treatment of several other cancerous diseases is being studied in phase I, II, or III clinical trials. For example, Photofrin® has been studied to treat head and neck cancers and, in a phase I clinical trial,

to treat chest-wall recurrences of breast cancer. In addition, Photofrin® PDT was performed after surgery in the treatment of intraperitoneal cancer in a phase I clinical trial and colorectal cancer in a phase III clinical trial. Photofrin fits some of the criteria for ideal photosensitizers but suffers from several drawbacks. First, it is a complex mixture of porphyrins with various monomeric and oligomeric forms, so poorly characterized chemically. Secondly, its long wavelength falls at 630 nm, which lies well below the wavelength necessary for maximum tissue penetration. Finally, it induces prolonged cutaneous photosensitivity, and patients are advised to keep away from direct sunlight (at least for a month or so) after treatment. These drawbacks stimulated the research for the synthesis and testing of new and efficient photosensitizers which forms the class of second generation photosensitizers.

1.3.2. Second Generation Photosensitizers

During the last 10 years, various new porphyrin based long wavelength absorbing photosensitizers related to chlorins, bacteriochlorins and phthalocyanines (Table 2) have been reported. The basic structure of these classes are shown in scheme 8. These, second generation photosensitizers are pure synthetic compounds, which can absorb light of longer wavelengths and exhibit a strong absorption band in the red wavelength region. Like HpD, they are composed of an aromatic macrocycle such as porphyrins and benzoporphyrins, chlorins, bacteriochlorins or phtalocyanins. The properties that were aimed for in the development of these sensitizers were improved selectivity, longer wavelengths of light absorption, and increased extinction coefficient (molar absorptivity) at these wavelengths. Many second-generation photosensitizers have been studied in phase I, II, or III clinical trials for various indications. Among the new photosensitizers, marketing clearance has been obtained for the BPD-MA (Benzoporphyrin derivative-monoacid ring A), the *m*-THPC (*meta*-tetrahydroxyphenyl chlorine) chlorin and the Tin etiopurpurin ($SnET_2$) metallochlorin. PDT treatments with BPD-MA and *m*-THPC have also been approved in Europe, Canada and in the USA [21, 53]. Besides the porphyrin based photosensitizers, a precursor for endogenous photosensitizer (Protoporphyrin – shortly referred as PpIX) called δ- aminolevulinic acid (ALA) was also developed as a second generation photosensitizer. These second generation photosensitizers can be classified in three categories: anionic lipophilic photosensitizers, cationic photonsensitizers and 5-aminolevulinic acid based PDT.

Scheme 8 Chemical structures of Porphyrin, Chlorin, Bacteriochlorin and Phthalocyanine



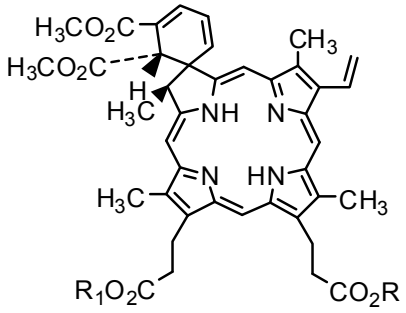
125 Porphyrin 22 π electrons

126 Chlorin 20 π electrons

127 Bacteriochlorin 18 π electrons

128 Phthalaocyanine

Scheme 9 Photosensitizers under clinical and preclinical trials



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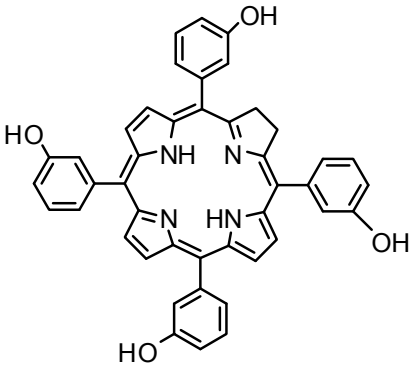
BPD-MA (Verteporfin™)



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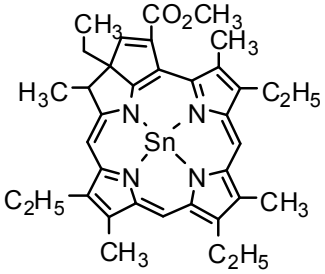
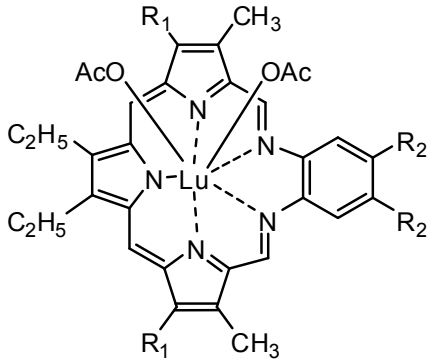
***m*-THPC (Foscan®)**



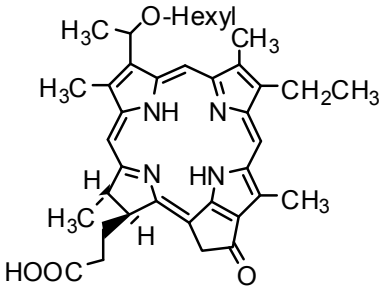
131

Tin Etiopurpurin (Purlytin™)



132

Lutetium Texaphyrin (Lu-tex) ®



133

HPPH (Photoclor) ®

Table 2: Porphyrin, chlorin, Phthalocyanine based Photosensitizers

Modified from [54] Allison R.R. *et al, Photosensitizers in clinical PDT*, Photodiagnosis and Photodynamic Therapy, 2004, Vol 1, issue 1, p27-42 and [28] Dolmans D.E.J.G.J *et al, Photodynamic therapy for cancer*, Nature Reviews Cancer. Vol 3, issue 5, p 380-387.

Drug (Brand name)	Active ingredient	Manufacturer	Diseases treated	Activation wavelength
Photofrin®	HpD	Axcan Pharma	Cervical, endobronchial, Oesophagal , bladder and gastric cancer and brain tumors	630 nm
Visudyne®	Vertiporfin (Benzoporphyrin derivative monoacid A)	Novartis Pharmaceuticals	Age-related macular degeneration, NMSC	690 nm
Foscan®	Temoporfin (m-THPC)	Biolitec Pharma Ltd	NMSC of head and neck	652 nm
Antrin®, Lutex®	Lutexaphyrin	Pharmacylics	Cervical, prostate and brain tumors	732 nm
Purlytin®	SnET2	Miravant Medical Technologies	Cutaneous metastatic breast cancer, basal-cell carcinoma, kaposi's sarcoma, prostate cancer	664 nm
LS11	Talaporfin (Taporfin sodium)	Light Science	Solid tumors from diverse origins	664 nm
Photochlor	HPPH	RPCI	Basal-cell carcinoma	665 nm
Photosens®	mixture of sulphonated Al-phthalocyanines	General Physics Institute	Cutaneous / subcutaneous lesions from diverse solid tumor origins	670 nm
Pc 4®	Silicon phthalocyanine	---	Solid tumors from diverse origins	670 nm

NMSC-Non-melonama skin cancer
HPPH- Hexyl Ether Derivative of Pyropheophorbide
RPCI-Roswell Park Cancer Institute

1.3.2.1. Anionic lipophilic photosensitizers

These compounds all of which are tetrapyrrole compounds are lipophilic due to tetrapyrrole core and also are anionic. These newer compounds show somewhat improved selectivity for tumor over normal tissue compared to photofrin and consequently have reduced cutaneous phototoxicity. For some, the pharmacokinetics is reasonably rapid with plasma half-lives that are often biphasic, with values ranging from a few hours to up to a few days. They also have superior photochemical properties in terms of the absorption at longer wavelengths and corresponding extinction coefficients. For example, the chlorins have red-shifted absorption spectra (λ_{max} 650–670 nm, compared with 630 nm for photofrin [55] and extinction coefficients in the 3 to 5 x 10^4 $M^{-1}cm^{-1}$ range compared with the estimated values of 1.5 x 10^3 $M^{-1}cm^{-1}$ for photofrin. Some of the most important anionic lipophilic photosensitizers are listed in Scheme 9.

1.3.2.1.1. Benzoporphyrin Derivative Monoacid (BPD-MA, Verteporfin, Visudyne®)

Of the newer photosensitizers, the most advanced as far as clinical application is concerned is benzoporphyrin derivative monoacid A **129** (BPD-MA) [56-59]. The BPD chromophores have absorption maxima around 690 nm. This chlorin-type compound which is a mixture of two diasteroisomers (ring A Diels–Alder adduct as monocarboxylic acid) with essentially equivalent photosensitizing properties is formulated in liposomes. The drug is currently in phase I/II trials for treatment of skin cancer and in a phase I/II clinical trial for treatment of basal cell carcinoma and metastatic skin lesions. Patients with basal cell carcinoma were found to respond better than those with metastatic skin lesions. However, due to its effectiveness in the obliteration of neovessels, this compound is being aggressively developed by QLT Phototherapeutics and Ciba-Vision as a first-line treatment for age related macular degeneration (AMD) of the eye [60] . This direction was stimulated by a series of preclinical studies, [61] where intraocular tumors implanted in rabbit eyes were used as a model for neovasculature. These studies showed that the very efficient destruction of these tumors could be achieved.
The filings have been made in Europe, Canada, and the United States for the approval of BPD-MA in the treatment of AMD, approval was granted in Switzerland and a panel of the FDA recommended approval in the USA.
***Advantages*:** (i) Long-wavelength absorption; (ii) No skin phototoxicity.
***Disadvantages*:** (i) Difficult to separate the A and B ring Diels–Alder products from the mixture, and only the ring A isomer is effective; (b) Clears rapidly

1.3.2.1.2. Tetra (*m*-hydroxyphenyl)chlorin (*m*-THPC, Foscan®)

meta-tetrahydroxyphenyl chlorin **130** (*m*-THPC), tradenamed Foscan®, is the most active photosensitizer in a series of tetrahydroxyphenyl analogues. This photosensitizer has a

singlet-oxygen quantum yield comparable to other chlorines [57, 62, 63] and appears to be the most active, requiring in some cases only a dose as low as 0.1 mg kg^{-1} and a light dose of 10 J $cm^{-2.}$ In a preliminary trial, patients have been treated for early stage squamous cell carcinoma of the aerodigestive tract, Barrett's esophageous with superficial adenocarcinoma, and bronchial and oesophagal tumors [64]. In patients with early stage tumors, no recurrence was seen during follow-up of 3–35 months. This drug is also in phase II/III clinical trials for head and neck cancers in Europe and the United States.

***Advantages*:** (i) Easy to synthesize; (ii) Long–wavelength absorption at 650 nm; (iii) Effective photosensitizer.

***Disadvantages*:** (i) Skin phototoxicity which lasts for several weeks; (b) For optimal activity, light treatment is required 7–9 days after injection of the drug.

1.3.2.1.3. Tin Etiopurpurin (SnEt2, Purlytin®)

Tin etiopurpurin **131** (SnEt2), tradenamed Purlytin®, is a chlorin photosensitizer being investigated for use in PDT [57]. Purlytin® has a tin atom in the center of the macrocycle and has an absorption maximum near 650 nm. It has been reported that Purlytin1 localizes into the skin and can produce a photoreaction for 7 to 14 days after administration [65, 66]. This compound is currently in phase II clinical trials for age related macular degeneration and prostate cancer. In preliminary trials for basal cell carcinoma and metastatic breast cancer, patients were treated with 1.2 mg kg^{-1} of drug, then 24 h post-injection with 200 J cm^{-2} of light at 660 nm. Photosensitivity reactions were seen in patients 1–2 months post-treatment.

***Advantages*:** (i) Slightly deeper light penetration into tissue compared to Photofrin; (ii) Availability of diode lasers at 660–665 nm.

***Disadvantages*:** (i) Insoluble in water, hence formulated in lipids, which causes some dark toxicity and (ii) Delayed phototoxicity—a major drawback.

1.3.2.1.4. Lutetium Texaphyrin (Lutex®)

Lutetium texaphyrin **132**, tradenamed Lutex®, is a non-porphyrin photosensitizer currently in Phase-II clinical trials for the treatment of cancer [67]. Lutex® has been used to treat malignant melanoma [68] because it is highly selective for tumor tissue and it can be activated with 732 nm light that penetrates more deeply into pigmented lesions than the light needed to excite Photofrin®, Verteporfin® or Foscan®. Lutex® is also being used to prevent re-stenosis of vessels after cardiac angioplasty [69] by photo-inactivating foam cells that accumulate within arteriolar plaques. For this indication Lutex® has been given the tradename Antrin®. A phase II/III trial using Lutex® for treating certain cancers is under way.

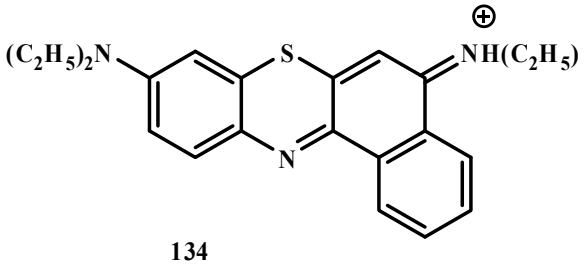
***Advantages*:** (i) Strong absorption at 732 nm; (ii) Limited skin phototoxicity; (c) Effective photosensitizer.

***Disadvantages*:** (i) Severe pain during light treatment; (ii) Multi-step synthesis.

1.3.2.1.5. Hexyl Ether Derivative of Pyropheophorbide-a (HPPH, Photoclor®)

Among the chlorophyll derivatives prepared so far, the alkyl ether analogs of methyl pyropheophorbide [64] have attracted the most attention. In this series of compounds it was observed that the biological activity increased by increasing the length of the alkyl ether carbon chain, being maximum in compounds with *n*-hexyl **133** and *n*-heptyl chains. It possesses a strong absorption at 660 nm with a high singlet oxygen quantum yield. It is highly selective and has reduced phototoxicity compared to Photofrin. HPPH has been approved by the United States Food and Drug Administration for phase I/II clinical trials at Roswell Park Cancer Institute, Buffalo. Five patients with oesophagal cancer have already been treated with excellent results and no skin phototoxicity was observed.
***Advantages*:** (i) Easy to synthesize; (ii) Long-wavelength absorption near 665 nm; (iii) Excellent efficacy at very low doses; (iv) No skin phototoxicity.
***Disadvantages*:** (i) Poor solubility in physiological medium (ii) Pain during treatment

1.3.2.2. Cationic photonsensitizers



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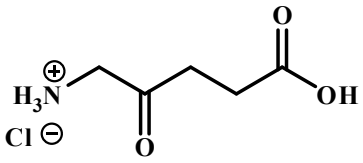
Scheme 10: A benzophenothiaze based sensitizer

A different group of photosensitizers that merits a brief mention are the cationic photosensitizers. In contrast to the porphyrins, which derive their PDT effect in large part via destruction of the tumor vasculature, cationic photosensitizers are suggested to be cellularly localized molecules and to act at the tumor cell level. It is believed that the basis for their preferential accumulation in tumor tissue is that the electrical potential across the mitochondrial membrane in tumor cells is much steeper than in normal cells [70]. This steep gradient leads to a high accumulation in tumor cells of compounds with a delocalized positive charge. The best developed of the series are the benzophenothiazinium dyes [71, 72]. In systematic investigations of these dyes, Cincotta and colleagues showed high cure rates in two animal models of sarcoma, using the cationic photosensitizer 5-ethylamino-9-diethylaminobenzo (a) phenothiazinium chloride **134** (EtNBS) activated with 652 nm irradiation [71]. Cellular uptake of these compounds appears to occur rapidly, within seconds. In an attempt to combine vascular and cellular effects, a benzophenothiazinium sensitizer and BPD-MA were used in PDT of EMT-6 tumors in Balb/c mice. The treatment produced a synergistic effect, compared with the single treatments [73]. Using the iodinated form of the cationic photosensitizer, it was shown that the antitumor effect was mediated by both T cells and NK cells, indicating that PDT can elicit antitumor protective immunity [74]. These preclinical studies may be useful in various clinical settings and are currently under development.

1.4. 5-Aminolevulinic acid based PDT (ALA-PDT)

A new approach to PDT based on the use of endogenous photosensitizer protoporphyrin IX (fig. 4), a precursor in the biosynthesis of heme was introduced in 1990. It made use of 5-Aminolevulinic acid **119** (5-ALA, also called δ-ALA or simply ALA), the first intermediate for the biosynthesis of heme.

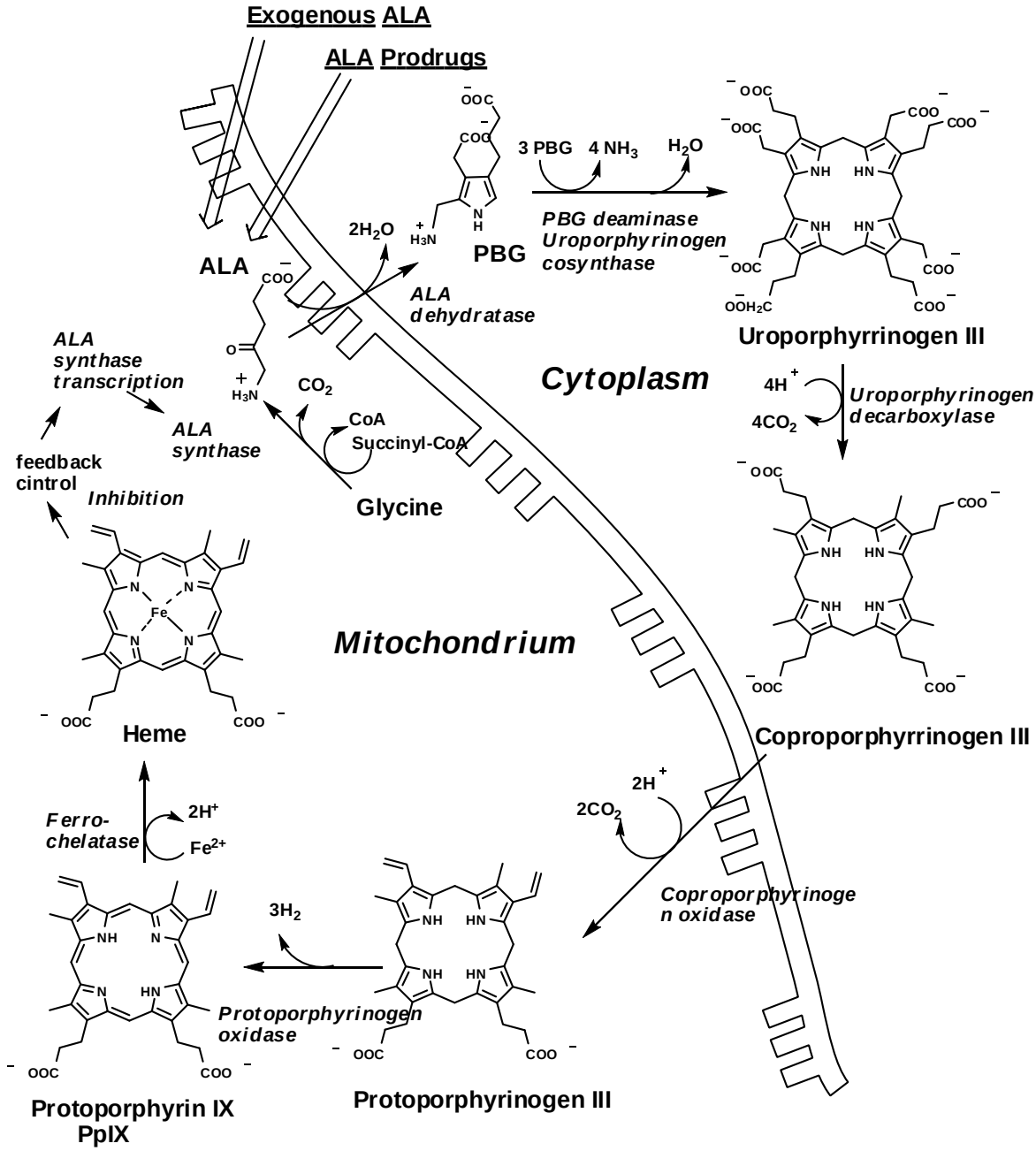


119

1.4.1. Heme Biosynthesis

Almost all cells in the body have a requirement for heme. Heme forms the reactive centre of a number of enzymes and proteins including proteins which are widely present such as cytochrome P450 and the respiratory chain cytochromes. These cells must maintain a capacity for heme biosynthesis and this process occurs by the now-classical porphyrin biosynthetic pathway which is shown in Figure 4. Although the starting components are the common metabolites succinate and glycine, the first dedicated intermediate in the porphyrin biosynthetic pathway is 5-aminolevulinic acid (ALA), an unusual amino acid **119**. The details of this pathway have been well studied over many years, partly because of its relevance in the porphyria. The pathway is very tightly regulated by a number of control points, with feedback inhibition by heme being an important component of this regulation. The process is not only under strict enzymatic control but it is also regulated through the fact that different subcellular compartments are responsible for different parts of the biosynthesis. The synthesis of ALA occurs in the mitochondria by the condensation of glycine and succinyl CoA, catalyzed by ALA-synthase. Once synthesized ALA is exported into the cytosol, where it undergoes a series of synthetic steps forming porphobilinogen (PBG), then porphyrinogens (the colourless precursors of porphyrins), protoporphyrin IX and finally heme. Eight molecules of ALA form four molecules of PBG using the enzyme porphobilinogen synthase .These four molecules of PBG in turn form one molecule of Uro III which is shown in Fig 4. These precursors of porphyrin intermediates are colourless because they have a reduced chromophor system compared to the porphyrins and lack thereby the delocalized electron system seen in the porphyrin macrocyclic ring. In the final stages of heme biosynthesis the intermediate coproporphyrinogen is transported from the cytosol into the mitocondria and converted to the first true porphyrin, protoporphyrin IX (PpIX). The last step requires the insertion of iron into PpIX to produce haem. PpIX is a very powerful photosensitizer. It does not normally cause significant photodynamic damage (except in certain examples of the porphyrias), since it does not accumulate but is simply used as an intermediate in low concentrations in the pathway to heme.

Figure 4: Heme Biosynthesis: Endogenous ALA is synthesized by ALA Synthase. Exogenous ALA comes from outside the cell and/or from ALA-prodrugs and allows bypassing the negative feedback of Heme on ALA synthase. Gly: glycine, Succ-CoA: succinyl-coenzyme A, PBG: porphobilinogen and URO III: uroporphyrinogen III.



1.4.2. Mechanism of ALA-PDT

The slowest and thereby rate limiting step in heme biosynthesis is the formation of 5-aminolevulinic acid (ALA) from succinyl-coenzyme A and glycine catalyzed by the enzyme ALA synthase. Under physiologic conditions, cellular heme synthesis is regulated in a negative feedback control of the enzyme ALA synthase by free heme.

The ALA-PDT depends on the generation of the only photosensitizing intermediate of heme biosynthesis, which is protoporphyrin (PpIX). Under normal conditions PpIX is an intermediate and does not accumulate in the pathway. The only way to generate larger quantities of PpIX is by administering ALA exogenously (Fig 4). So, when exogenous ALA is given to cells with an active heme biosynthetic pathway, the pathway becomes temporarily overloaded, the control mechanism is bypassed, and downstream metabolites are synthesized in excess. Under these conditions, ferrochelatase, which catalyzes iron insertion into PpIX, becomes the rate-limiting enzyme. Following the addition of exogenous ALA, the low physiologic rate of iron insertion by ferrochelatase is unable to compensate for the excess PpIX that is formed. PpIX, therefore, accumulates in cells and renders them photosensitive. The build-up of porphyrins on addition of ALA to cells has been known for many years, but it is only in the last decade that this has been developed as an effective form of PDT [75]. Following 5-ALA-induced synthesis in mitochondria, PpIX selectively accumulates in mitochondria. Some investigators have indicated that the primary cause of cell death after PDT is mitochondrial phototoxicity (47–49). Some investigators have shown that ALA induced PpIX formation induced an apoptotic responses [76]. It has been established that ALA uses the intestinal and renal apical peptide transporters for entering into epithelial cells [77] and in certain type of cells ALA is transported by system BETA transporters [78]. This explains the excellent intestinal absorption of ALA when it is adminstered by oral route. The production rate of PpIX increased with cell size and with increasing ALA concentration [79].

At present, our knowledge of the mechanisms involved in ALA-based PDT is limited. One of the important open questions is the following: the reason for preferential ALA uptake and conversion by tumors and dysplastic tissue is not clear. Various theories have been put forward to account for the selectivity of PpIX formation in tumors and other lesions:

- It has been suggested that tumors contain lower levels of ferrochelatase (the enzyme responsible for the insertion of iron into PpIX) than the surrounding normal tissue, resulting in less effcient conversion of PpIX into heme within tumor cells and hence the build-up of PpIX.
- Tumors may have less readily accessible iron than surrounding normal tissue which would result in poorer conversion of PpIX into heme.
- It is well established that tumors have a lower pH than normal cells and this may result in more PpIX retention in the tumor cells due to the various protonated species which may be formed from PpIX.

- Selective accumulation of PpIX may be related to selective uptake of ALA since the skin or epithelium overlying the tumor or lesion has greater permeability than non-tumoral tissue.
- There is some indication that porphobilinogen deaminase the enzyme that converts four porphobilinogen molecules into the protoporphyrin precursor, uroporphyrinogen III, may have a regulatory role in the 5-ALA induced increase of PpIX.
- Porphyrin biosynthesis capacity is 20 fold enhanced in cancer cells at least from the stages of porphobilinogen (PBG) formation up to that of coporphyrinogen, when compared with its original normal tissue [80].

The reason why ALA administration results in the build-up of PpIX rather than other porphyrin intermediates is also not well understood. Simple arguments such as the supposed rate-limiting effects of ferrochelatase have been advanced, but it is now clear that this represents an oversimplification of the true picture. For example, it is known that other intermediates in the pathway do indeed accumulate [81] but that these intermediates may be more readily excreted from cells than PpIX. Another possibility is that the immediate precursor of PpIX, known as protoporphyrinogen IX, may also accumulate and be converted non-enzymatically into PpIX. Finally, the subcellular compartmentation of the heme biosynthetic pathway may be important, since excess PpIX may escape from the mitochondria and therefore might not be available for the iron insertion step to make heme. The effects of exogenous ALA cannot be imitated by administration of pre synthesized PpIX.[82] Along with deficiencies in the metabolic pathway of heme, other factors including increased ALA uptake, limited availability of ferrous iron, cell cycle activation, proliferative activity, mitochondrial density, increased temperature and lower pH values have been suggested to be responsible for the alteration in the biosynthesis of heme in neoplastic cells.

1.4.3. Advantages of ALA

- ✓ 5-ALA in contrast to porphyrins is a small, soluble molecule able to penetrate the abnormal stratum corneum overlying skin tumors. So it can be applied topically unlike other photosensitizers.
- ✓ In contrast to most tetrapyrrole photosensitizers, ALA-PpIX localizes in cells (where it is incorporated into the biosynthesis) rather than in the tumor vasculature.
- ✓ ALA and ALA-PpIX are rapidly cleared from the system, which results in an acceptably short period (1-2 days compared to 1-1.5 months with HpD and Photofrin®) of cutaneous photosensitivity or skin phototoxicity. This is viewed as an advantage over some of the other photosensitizers where light protection may be required for several weeks. This allows multiple treatment regims and thereby increases the efficacy of PpIX.
- ✓ ALA can be easily synthesized unlike other porphyrin based photosensitizers.
- ✓ ALA-PDT is noninvasive, can be used to treat multiple lesions by short treatment sessions, produces excellent cosmetic results by not causing damage to

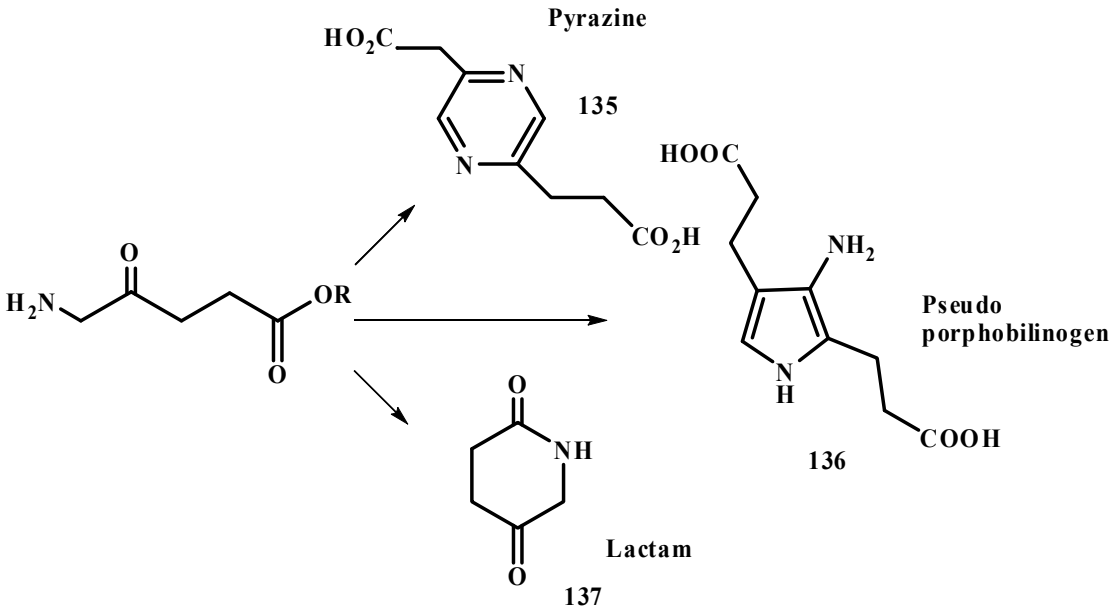
surrounding tissue, has no side effects beyond slight pain during irradiation, and is well accepted by patients [83].

- ✓ ALA can be easily formulated. So ALA can be topically applied which gave promising results in the treatment of superficial cutaneous malignancies.

1.4.4. Disadvantages of ALA

- o A significant shortcoming of ALA is its limited ability to cross certain biological barriers (e.g. cellular membranes) due to its low lipid solubility [84].
- o PpIX is active at 630 nm, which should give adequate depth penetration; when topically administered, ALA does not penetrate to great depth; it has limited depth penetration (up to about 1 mm).
- o No matter how good the delivery of ALA may be, the formation of PpIX requires the activity of several enzymes (Figure 4)
- o Pain associated with PDT; when administered systemically: transient liver abnormalities have been observed and patients have reported mild nausea after ALA administration [85].
- o Though multiple treatment regimens are possible, the efficacy of PpIX generation is decreased in the subsequent regimen if taken within 24 hours [86].
- o ALA is stable at acidic pH as low as 2-2.5. In aqueous solutions buffered to a physiological pH (pH=7), ALA dimerises to give pyrazine derivatives **135** while at higher pH pseudo-porphobilinogen **136** may be formed [87-91] and conversion to ester derivatives appears to worsen these problems. Esters are known to increase the potential for formation of lactam type derivatives **137** (See Scheme 11).

Scheme 11: ALA degradation products



1.4.5. ALA derivatives

In order to overcome the shortcomings of ALA and improve its bioavailability, ALA can be derivatized. ALA has two principal functional groups, a carboxylic acid and an amino moiety which are both easily accessible for derivatization. The simplest way to alter the lipophilicity of ALA is via esterification of acid moiety. The use of ALA derivatives as good candidates for the treatment of skin cancer has been extensively reviewed [92, 93]

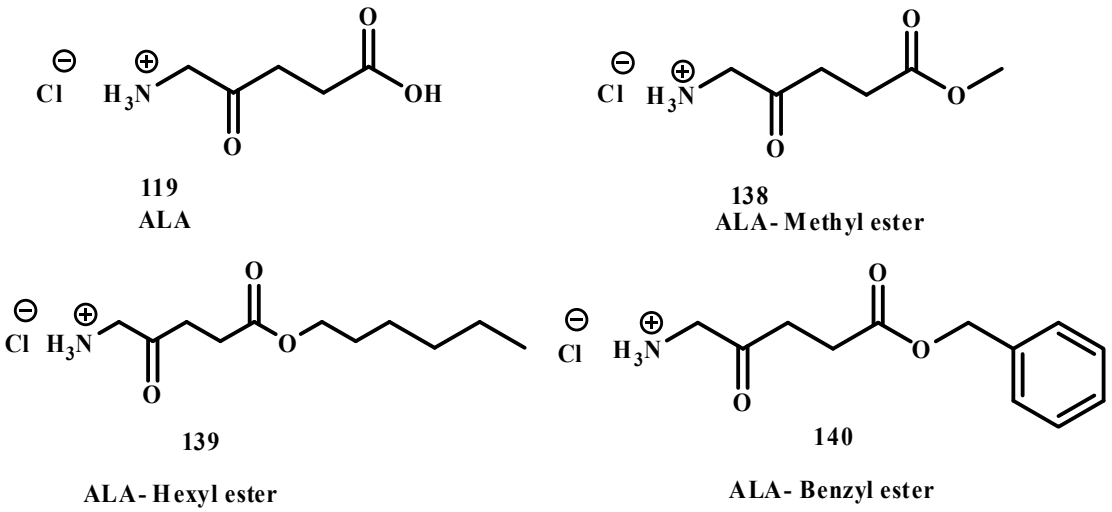
1.4.5.1. ALA esters

ALA esters were originally studied as potential PDT drugs on the basis that they may have improved pharmacological properties compared with ALA itself. For example, since they are more hydrophobic / lipophilic than ALA, it was believed that they might be taken up into cells by different mechanisms, which might improve the efficiency of the therapy. The rationale was, that once inside cells ALA esters may be hydrolysed to ALA, which could then enter the heme biosynthetic pathway. ALA esters were first developed by Peng *et al* [94, 95] and Kloek *et al* [84, 96]. Esters with various chain lengths were prepared by these investigators (Table 3). They concluded that esters with chain length of C5- C7 (pentyl, hexl and heptyl) generate very high PpIX formation; esters longer than C7 length produce less efficient PpIX formation. Also, 2-(hydroxy methyl) tetrahydrofuranyl, 2-(hydroxy methyl) tetrahydropyranyl esters and benzyl esters of ALA were found to generate higher amount of PpIX. The esters have indeed proved to be effective in both phototherapy and photodetection [97, 98]. However, the mechanisms involved in the production of PpIX from ALA esters may not be as simple as first thought. Certainly the uptake is different from that of ALA itself [78]. Increased cellular uptake of lipophilic ALA esters resulting in enhanced PpIX concentrations has been demonstrated in a number of *in vitro* and *in vivo* systems [94, 96, 99] . Though these esters are incorporated into the cells at higher rate than ALA, studies conducted show that they also efflux at an increased rate mediated by passive diffusion [100].

The octanol-water partition coefficients of ALA esters (log P) have been determined to obtain a parameter related to lipophilicity. The influence of this parameter lipophilicity along with concentration, time and pH dependence on PpIX formation was studied. But no direct relationship between lipophilicity and total PpIX build-up has been found, indicating that two different processes, uptake and ester cleavage are necessary for efficient PpIX formation [101]. These long chain ALA esters require 30-150 fold lower concentration than ALA for comparable amounts of PpIX production. However, the use of these esters resulted in a non-specific distribution of ALA in all cell types, with a certain preference for tumor cells [84, 99, 101]. Berger *et al* synthesized and tested various ethylene glycol esters of ALA **141** (Scheme 13) with varying chain length. They found that these esters are designed to target cellular esterases. They induce high levels of PpIX, but they are also less toxic than 5-ALA hexyl esters over a wide range of concentrations [102]. Brunner *et al* synthesized and tested a series of ALA esters carrying electron withdrawing groups to enhance PpIX generation. These electron withdrawing groups enhance the ester hydrolysis and should so help to generate higher intracellular PpIX. They found that ALA nonafluorohexylester hydrochloride, ALA thiohexylester

hydrochloride and ALA dibenzyldiester dihydrochloride **142** (Scheme 13) produced higher levels of PpIX than that produced by ALA, ALA hexyl ester and ALA benzyl esters [103]. The data indicates that the altered properties further improve the diagnostic and therapeutic potential significantly beyond what is achieved with ALA-based PDT [104, 105].

Scheme 12: ALA esters under clinical trials and / or approved drugs



Scheme 13: ALA esters efficient in PpIX induction

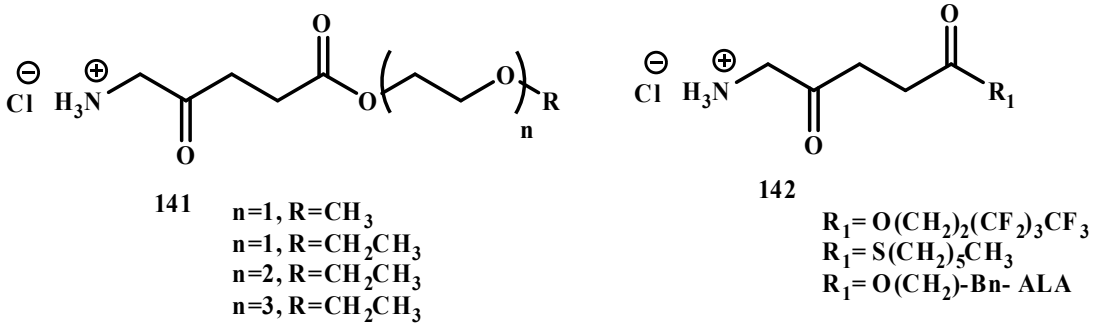
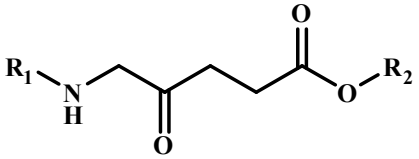
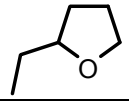
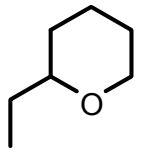
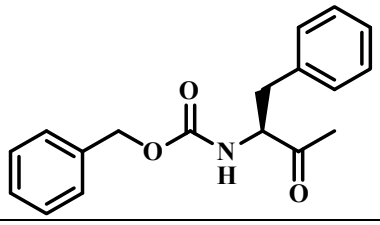
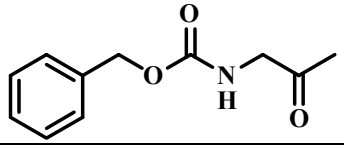
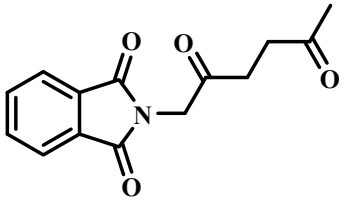
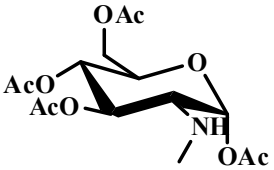
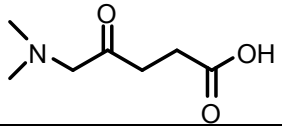
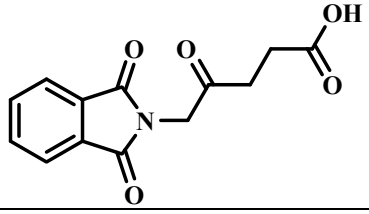


Table 3: Other ALA derivatives (selected)

Adapted from Lopez, R.F.V., *et al*., *Photodynamic therapy of skin cancer: controlled drug delivery of 5-ALA and its esters.* Advanced Drug Delivery Reviews, 2004. **56**(1): p. 77-94.



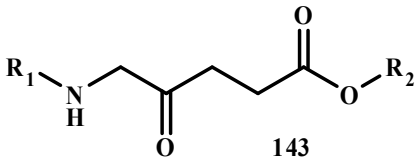
Molecule	R_1	R_2
ALA-Ethyl	-H	$-C_2H_5$
ALA-Propyl	-H	$-C_3H_7$
ALA-Butyl	-H	$-C_4H_9$
ALA-Pentyl	-H	$-C_5H_{11}$
ALA-Octyl	-H	$-C_8H_{17}$
R,S- ALA-2-(hydroxymethyl) tetra- hydrofuranyl	-H	
R,S- ALA-2-(hydroxymethyl) tetra- hydropyranyl	-H	
N-acetyl-ALA	CH_3CO	-H
N-acetyl- ALA-ethyl	CH_3CO	$-C_2H_5$
N-acetyl-ALA-butyl	CH_3CO	$-C_4H_9$
N-butanoyl-ALA	$CH_3(CH_2)_2CO$-	-H
N-pentyl-ALA	$CH_3(CH_2)_3CO$-	-H
N-hexanoyl-ALA	$CH_3(CH_2)_4CO$-	-H
N-heptanoyl-ALA	$CH_3(CH_2)_5CO$-	-H
Carbobenzoylloxy-glycinyl-ALA-hexyl		$-(CH_2)_5CH_3$
Carbobenzoyloxy-D-phenylalanyl-ALA-ethyl ester		$-CH_2CH_3$
Carbobenzoyloxy-glycinyl-ALA-ethylester		$-CH_2CH_3$
N-phthalylimido-ALA-glucosamine-tetraacetate		
N,N-dimethyl-ALA		
N-phthalylimido-ALA		

1.4.5.2. ALA – pseudo peptide derivatives

ALA with N-terminal blocked by acetyl, butanoyl, pentyl, hexanoyl groups were synthesized. All these N-terminal blocked ALA failed to induce high quantities of PpIX in vitro [84, 106] though a carbobenzyloxy phenylalanyl ALA ethyl ester has been shown to induce considerable amounts of PpIX in human and rat skin explants [106].

ALA-amino acid pseudo peptide derivatives were also synthesized and tested on various cancer cell lines by different research groups [102, 106-108] . These can be potential substrates for cytoplasmic amino peptidases (acidic or neutral or basic) and/ or peptide and amino acid transporters. It was observed by Berger *et al* that the compounds blocked at the N-terminal of amino acid were no good precursors measured by the amount of PpIX formed. Also the pseudo peptides containing acidic or basic amino acid moiety were not active. Only ALA pseudo peptides containing neutral amino acids like Alanine, Phenylalanine with the acid moiety of ALA either free or protected as esters generate comparable amount of PpIX than that ALA. Later it was found that N-blocked or N-free dipeptides-ALA-ethyl esters **143** (Scheme 14), but not tripeptides–ALA-ethyl esters were substrates for cellular peptidases and were metabolized to ALA. The precursors were hydrolyzed intracellularly involving serine-proteases and metalloproteases. Cell selectivity for human endothelial or carcinoma cells was observed for some of this dipeptides-ALA.
In general ALA-pseudo peptide derivatives do not induce high PpIX quantities unlike that of the simpler ALA ester derivatives. This difference might be due to low cellular peptidase activity.

Scheme 14: ALA -peptides efficient in PpIX induction



143

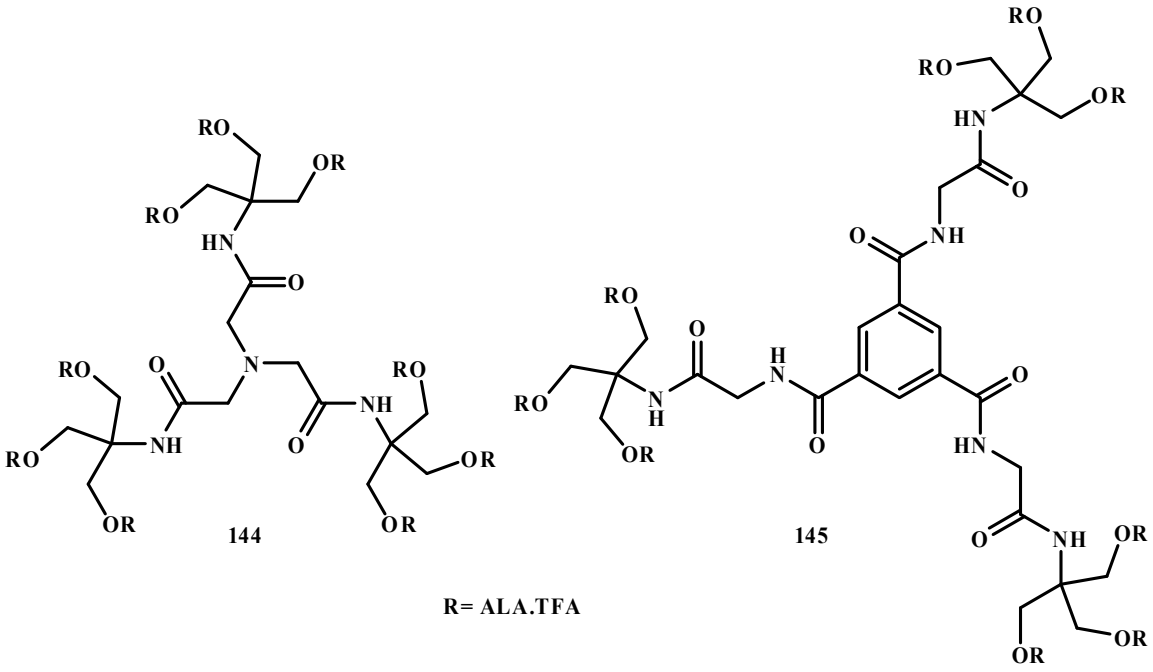
R_1= Gly-Gly-, R_2=Et
R_1= Gly-Pro-, R_2=Et
R_1= Gly-Gly-, R_2=$O(CH_2CH_2O)_2CH_2CH_3$
R_1= Gly-Gly-, R_2=$O(CH_2CH_2O)_3CH_2CH_3$
R_1= Gly-Gly-Gly-, R_2=Me
R_1= Gly-Gly-, R_2=Me

1.4.5.3. ALA dendrimers

Recent advances in polymer chemistry now allow the synthesis of structurally defined, hyperbranched polymers (or dendrimers) which can be conjugated with drug molecules [109]. The drug can either be bound to a preformed dendrimer, in which case the exact loading of the drug cannot easily be controlled, or the drug can be incorporated into the structure of the dendrimer during synthesis. With this approach, Battah et al synthesized

dendrimers bearing 6 or 9 ALA residues by attaching a tris (Boc-protected ALA)-containing wedge to a di- or tripodent aromatic, or tripodent aliphatic core **144** and **145** (Scheme 15). Two second generation 18-ALA-containing dendrimers were also synthesized using a 3, 3'-iminodipropionic acid spacer unit between the wedge and the aromatic core. These compounds differed only in the distance between the core and the linker unit. The results show that the ALA-containing dendrimers are clearly able to pass through the cell membrane, release ALA intracellularly and generate comparable quantities of PpIX as that of ALA; however, there is no direct correlation between the number of ALA molecules attached to the dendrimer and PpIX accumulation [110]. Rhe same group recently evaluated in vivo and in vitro the efficacy of the dendron, aminomethane tris-methyl ALA (containing 3 ALA residues) in PpIX generation. Although this dendron is capable of being taken up by cells and liberate the active ALA thereby induce PpIX, in vivo it was not possible to improve the efficacy of ALA-induced PpIX formation [111].

Scheme 15: ALA dendrimers efficient in PpIX induction



1.4.6. ALA formulations

The major obstacle of ALA is its limited penetration depth and consequently low induction of PpIX. The simplest approach is to introduce so-called penetration enhancers, such as DMSO, oleic acid, azacycloalkane derivatives, and ethanol into the formulation. Malik *et al* found that ALA with 2% DMSO enhances PpIX production significantly [112].

Also, glycerol monooleate, a lipid permeation enhancer, increased in vitro permeation and retention of ALA [113]. Another approach is to introduce additives that act on heme biosynthesis, e.g. by removing ferrous iron. This can be achieved by adding iron chelating substances such as ethylene diamine tetracetic acid (EDTA), desferrioxamine (DFO), and 3-hydroxypyridin-4-ones (HPOs). In vitro, the iron specific DFO is superior to EDTA [114] at equal concentration with respect to PpIX formation.

1.4.7. Clinical and Pre-clinical Aspects of ALA-PDT for Diagnosis and Therapy

Besides therapeutic indications which are expanding, the use of ALA-induced PpIX (ALA-PpIX) has gained much interest in diagnostics. (table 4). PpIX fluorescence is being used for the detection of early malignancies, carcinomas in situ, and cancer precursor lesions. By minimally invasive diagnostic procedures, accessible organs, such as the bladder [115], oral cavity [116], lungs [117], upper and lower gastrointestinal tract [118], and cervix [119], to name a few, are being viewed with optical devices, and ALA-PpIX fluorescence is used to detect suspicious areas for guided biopsy and therapy. In urology, ALA-PpIX is used for the early diagnosis of urothelial dysplasia and carcinoma. In this case, the ALA is administered locally by instillation and remains for about 2 h in the bladder. Subsequently, the bladder wall is examined by using a modified cystoscope that allows regular illumination and blue-light excitation for observation of porphyrin fluorescence. With white light, the lesion can be barely distinguished, while blue light-induced ALA-PpIX fluorescence clearly demonstrates the outline of the neoplasia. In larger patient groups, this use of ALA significantly enhanced the cystoscopic detection of malignant and dysplastic lesions, and in the application of imaging following surgical resection it has been shown to be a useful “second-look” tool to find remaining dysplastic tissues, thereby significantly reducing the local recurrence rate following surgery. ALA-PpIX fluorescence is also useful for determining tumor margins. In a similar manner, during brain surgery ALA-PpIX fluorescence-guided resection of malignant gliomas reportedly results in prolonged patient survival [120].

Patients with Barrett’s esophagus, precancerous dysplasia and early oesophagal carcinomas are being efficiently treated with oral ALA-based PDT [121]. An ingenious and highly successful blue light system employing ALA for actinic keratosis with outpatient treatment as devised by DUSA Pharmaceuticals Inc has FDA approval. Given the excellent cosmetic outcome, one might predict this system will become widespread in cosmetic and plastic surgery circles. ALA also has had success for head and neck tumors, though invasive lesions do not achieve complete response. Given its limited penetration, ALA would more likely to show higher efficiency for dysplastic and in situ lesions, which are rather common in the oralcavity. PhotoCure ASA, a Norwegian company has employed methylated ALA (Metvix®) for a wide variety of lesions. The drug is topically applied and then about 3 h later red light illumination is employed. The drug/light therapy is approved in many European countries for treatment of actinic keratosis. The same company produces Hexvix® for photodiagnosis and likely photodynamic therapy. Currently this drug is infused in the bladder and 30-60 min later blue light is employed to

induce fluorescence abnormal tissues. Benzvix® is the drug Photocure ASA believes will allow for diagnosis and treatment of early oesophagal and GI tract lesions. ALA has been successful for oesophagal treatment and with an oral form of drug available this is convenient treatment.

Table 4: ALA esters as Prodrugs- Disease treated and/or under clinical trials

Drug (Brand name)	Active ingredient	Manufacturer	Diseases treated	Activation wavelength
Levulan®	ALA	DUSA Pharmaceuticals	Actinic keratosis, Basal cell carcinoma, Bowen's disease, psoriasis, hair removal, acne, bladder cancer detection	632 nm
Metvix®	ALA-Methyl ester	Photocure ASA Galderma	Actinic keratosis, Basal cell carcinoma	632 nm
Hexvix®	ALA Hexyl ester	Photocure ASA	Bladder cancer detection	632 nm
Benzvix®	ALA Benzyl ester	Photocure ASA	Bladder cancer detection	632 nm

1.5. Third Generation Photosensitizers or Targeted Photodynamic therapy

Most therapeutic drugs distribute through the whole body, which results in general toxicity and poor acceptance of the treatments by patients. The targeted delivery of chemotherapeutics to defined cancer cells is one of the main challenges and a very active field of research in the development of treatment strategies to minimize side-effects of drugs. Disease-associated cells express molecules, including proteases, receptors, or adhesion molecules, that are different or differently expressed than their normal counterparts. Therefore one goal in the field of targeted therapies is to develop chemically derivatized drugs or drug vectors able to target defined cells via specific recognition mechanisms [122-124]. This approach should also be able to overcome biological barriers.

Four different strategies of improving selectivity (targeting) in PDT have been practiced so far [125]. First strategy for selective photosensitizer (PS) delivery utilizes targeting moieties, such as monoclonal antibodies (MAbs), directed against antigens or ligands that

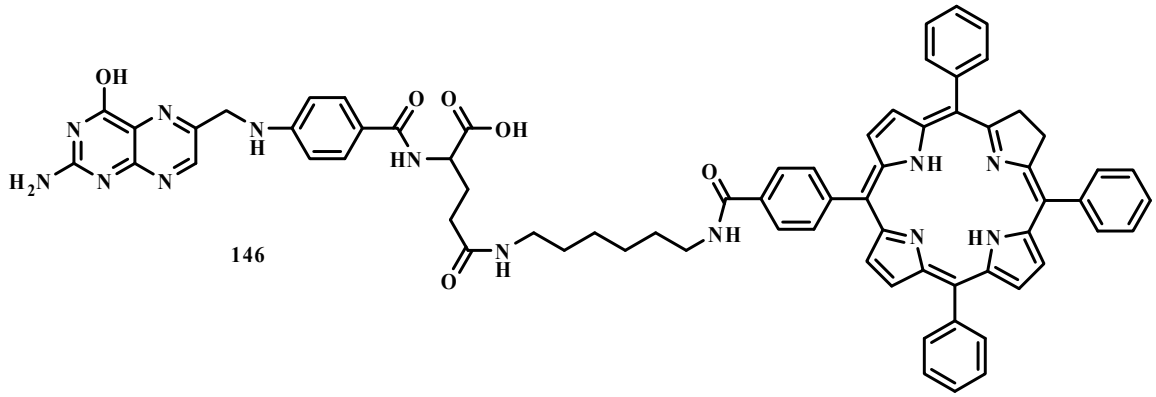
are specifically overexpressed on cancer cells. An important determinant of successful PDT is the localization of the PS at the time of irradiation. More precise drug targeting is therefore desirable in order to ensure success but also to reduce toxicity to uninvolved tissues and organs in complex sites, such as in the abdominal cavity. It is important to note that contrary to conventional targeted chemotherapy where the drug has to be released from the carrier moiety to elicit a response; this is not a prerequisite when carrier molecules are used for delivery of PS in PDT if PS is not ALA. Antibodies raised against epitopes of cancer cells have also been coupled to photosensitizers (photoimmunotherapy = PIT). Hp, Ce6 or phthalocyanins have been bound to monoclonal antibodies recognizing cancer cells and PIT was found to be active against cancer cells [126].

A second strategy which is commonly used, to improve the delivery of PS to target tissue involves their encapsulation in colloidal carriers, such as liposomes, oil-dispersions, polymeric particles, and polymers to facilitate drug delivery [127, 128].

Third strategy is to conjugate photosensitizer (PS) with specific receptors which are over expressed in cancer cells. This helps in receptor mediated endocytosis. The expression of low density lipoproteins (LDL) receptors is increased in many cancer cells. Hydrophobic photosensitizers were incorporated into LDL and targeted to their receptors [129]. Chlorin e6 (Ce6) was also conjugated to LDL. The cellular uptake of the conjugate and efficiency of PDT were increased in retinoblastoma and fibroblasts compared to free Ce6 assuming that the conjugate will be carried by LDL and internalized by an LDL-receptor dependant mechanism.

Nuclear targeting was also investigated by coupling a porphyrin to the nuclear localization sequence (NLS) [130]. Several types of cancer cells exhibit increased expression of transferin receptors and therefore, hematoporphyrin (Hp) was conjugated to transferin. The uptake of the Hp-transferin in human colon carcinoma HT29 cells was shown to be receptors mediated. Another receptor overexpressed on cancer cells is epidermal growth factor (EGF) receptors. EGF has been coupled to various photosensitizers to target specially cancer cells. Metal-disulfonated phtalocyanins bound to EGF were 10 times more active than free phtalocyanins in human breast carcinoma MCF7 cells [131]. Estradiol, a steroid hormone, has also been evaluated to target estrogen receptors (ER). The hormone has been conjugated to Tetraphenylporphyrin (TPP) and it has been shown that the molecule has still a strong affinity for ER [132].

The main problem of these protein-based conjugations may reside in their potential immunogenicity. Therefore, non-proteic ligands have also been evaluated such as folic acid which is vitamin B9. Tetraphenylporphyrin (TPP) has been conjugated to folic acid **146** (Scheme 16) to target tumor cells overexpressing its receptors [133]. Cellular uptake and photodynamic activity of the conjugate on human nasopharyngeal cell line were greatly increased compared to free TPP and competitive assays using free folic acid demonstrated folate-receptor dependent uptake of the conjugate.



Scheme 16: Tetraphenylporphyrin (TPP) has been conjugated to folic acid

Fourth strategy to selectively enhance PS levels in a disease site is to facilitate PS uptake more in the target tissue than the surrounding normal areas. For example in the case of ALA- based PDT, there are various formulations of ALA designed to increase its uptake as discussed in chapter 1.4.6.

Alternative ways of targeting PS to a specific cell population need to take advantage of certain properties of these cells, which either distinguish them from other cell or tissue types, or differentiate, malignant from normal cells. An approach that was used by Zhang *et al*., [134] is based on the altered sugar metabolism of cancer cells. Rapidly growing tumors are able to maintain high glucose catabolic rate by upregulation of the enzyme hexokinase. This enzyme phosphorylates glucose to glucose-6-phosphate, which is then retained in the cell. Photosensitizers have been also functionalized with a large quantity of different sugars. Saccharides-porphyrin or –chlorin conjugates are probably the most active field of research in the targeting of photosensitizers [135]. The efficiency of the saccharide composition has been evaluated using xylosyl, arabinosyl, glucosyl, galactosyl or 2-aminoglucosamide groups [134]. When linked to a photosensitizer, uptake and accumulation in the endoplasmic reticulum and photoxicity were increased in cancer cells. The length of the oligosaccharide is also an important factor for the membrane penetration [136].

1.6. ALA - Other applications

ALA has a variety of agricultural applications not only as an herbicide, insecticide and growth promoting factor, but also based on its ability to confer salt and cold temperature tolerance in plants [137].

1.6.1. Agricultural applications

1.6.1.1. Biodegradable herbicide

The practical application of ALA as a biodegradable herbicide was demonstrated by Rebeiz *et al*. [138]. This was quite significant because ALA is a selective, not harmful

and biodegradable material. The mechanism of ALAs function as "photodynamic herbicide," as proposed by Rebeiz *et al*. is as follows: Plants which are treated with a high concentration of ALA accumulate an excess amount of protoporphyrin IX (PpIX) at the stage preceding chlorophyll biosynthesis. When such plants are exposed to light, the excess PpIX produces active oxygen, which oxidizes unsaturated fatty acids on the cell surface thereby damaging the plant. ALA can be used as a safe substitute for highly toxic herbicides such as paraquat. However, the herbicide effects of ALA were not as great as those of chemical herbicides. Therefore, ALA was applied as a herbicide accelerator for diphenyl-ether-type herbicides such as NIP (2,4-dichlorophenyl-4-nitrophenyl ether), which act by a mechanism similar to the photodynamic herbicide effect of ALA [137, 139]

1.6.1.2. Growth promoting factor for plants

ALA application in a low concentration increased chlorophyll content and accelerated the growth of plant tissue and rice seedling [140]. Foliage, soil and root treatment with ALA was considered to be effective for increasing the yield of crops [137, 139]. The effects were particularly strong for root crops, such as potatoes, and garlic [141]. It was observed that ALA application enhanced photosynthetic activity and CO_2 uptake in radishes. The ability of ALA to enhance photosynthesis (photosystems I and II) has also been observed in *Spirulina platensis* [142].

1.6.1.3. Increased salt and cold temperature tolerance

All over the world, large areas of farmland are being lost due to salt accumulation in the soil. ALA was found to increase salt tolerance in cotton [143]. Cotton seedlings treated with ALA (30–100 mg/l) were able to grow in soil containing 1.5% (w/w) NaCl, and Na^+ uptake by the plants was suppressed by ALA application. In addition, when applied at 0.1–1.0 mg/l, ALA increased the survival of rice plants at 5 °C by 40–50% compared to the control (without ALA application). In the cells, polysaccharide-like compounds were accumulated and seemed to protect the plants by enhancing photosynthesis.

Further agricultural applications of ALA, such as color-intensifying effects for apples, quality improvement of vegetables (nitrate content reduction) and green-color maintenance for grass, are summarized in Table 5.

1.6.2. Medical (other than PDT) applications

Diagnosis of heavy-metal poisoning & other medical applications

It has long been known that ALA accumulates in the urine of patients with heavy-metal poisoning, and the ALA concentration in the urine is frequently measured for detection and screening [144, 145]. ALA dehydratase is inhibited by heavy metals, therefore, accumulation of ALA is observed. Accurate ALA detection is important for the early diagnosis of heavy-metal poisoning. The quantity of ALA in the urine gives an indication

for diagnosing porphyria [146, 147]. The application of ALA for treating rheumatoid arthritis has been reported [148]. A method for hair removal from a skin area (photodynamic depilation) consisting of selective photoinactivation and using derivatives of aminolevulinic acid (ALA) with lipophilic chains has been reported [149], although the mechanism is not known, heme compounds are assumed to play a role. Study of ALA mediated PDT for killing bacteria which are capable of synthesizing porphyrins (*Haemophilus parainfluenzae*) has been evaluated [150]. Other medical applications, such as treatment of mycosis and cosmetic and dermatological applications (prevents skin penetration of UV), are summarized in Table 5.

1.6.3. Biotechnology application

ALA applications in the biotechnology fields such as heme containing enzyme production [151], vitamin B12 production [152] and bacterial culture [153] are also summarized in Table 5.

Table 5 Applications of ALA in agriculture, medicine (other than cancer) and biotechnology

Field	Application	Reference
Agriculture	Biodegradable herbicide	[138]
	Biodegradable insecticide	[154]
	Herbicide accelerator	[137, 139]
	Growth promoting factor and yield enhancement	[141]
	Salt tolerance	[143]
	Cold temperature tolerance	[141]
	Quality improvement of vegetables, Color intensifying effects	[141]
	Growth and green –color maintenance of grass	[141]
Medicine	Diagnosis of heavy – metal poisoning	[144, 145]
	Diagnosis of porphyria	[146, 147]
	Treatment of rheumatoid arthritis	[148]
	Photodynamic depilation	[149]
	Photodynamic destruction of bacteria	[150]
	Treatment for mycosis	[155]
	Cosmetic and dermatological applications	[156]
Biotechnology	Heme-containing enzyme production	[151]
	Vitamin B12 production	[152]
	Genetically treated bacteria culture	[153]

2. Biological background behind prodrugs' design -A review of literature knowledge

2.1. Sugars in targeted PDT

Carbohydrates are an essential part of cell surface molecules and are crucial in cell surface recognition and information transfer. Oligosaccharides are attached to proteins or lipids to form glycoproteins or glycolipids that comprise part of the lipid bilayer as shown in the schematic view below (Figure 1) adapted from Seeberger, P.H. and D.B. Werz, *Automated synthesis of oligosaccharides as a basis for drug discovery.* Nature Reviews Drug Discovery, 2005. **4**(9);vp. 751-763 [157]. However, the lack of carbohydrate-based drugs is not surprising considering that our understanding of fundamental glycobiology is a recent development. With the advent of automated solid-phase synthesis of oligosaccharides as a basis for drug discovery, the days of carbohydrate-based pharmaceuticals are not very far [158, 159].

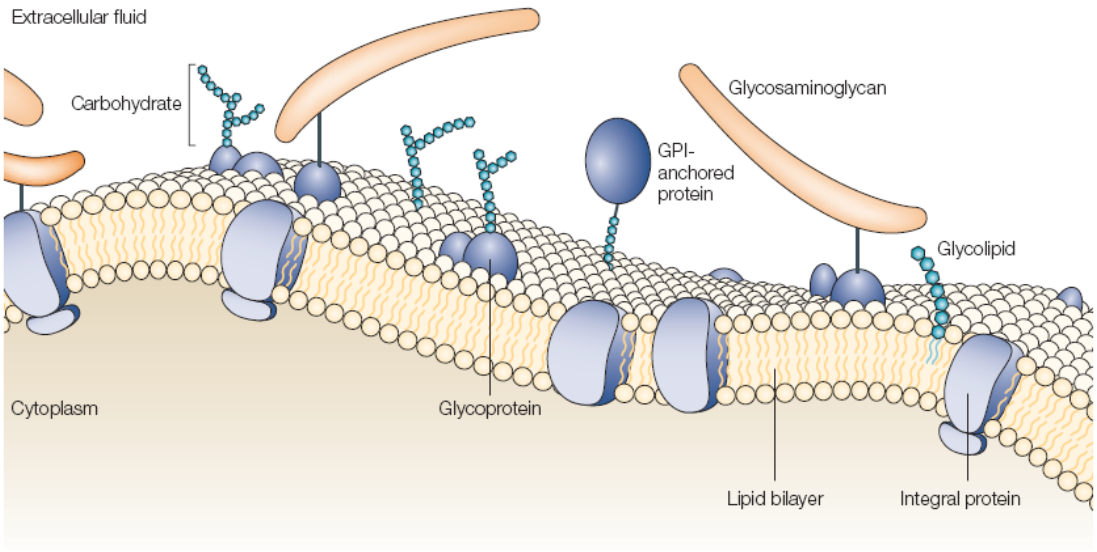
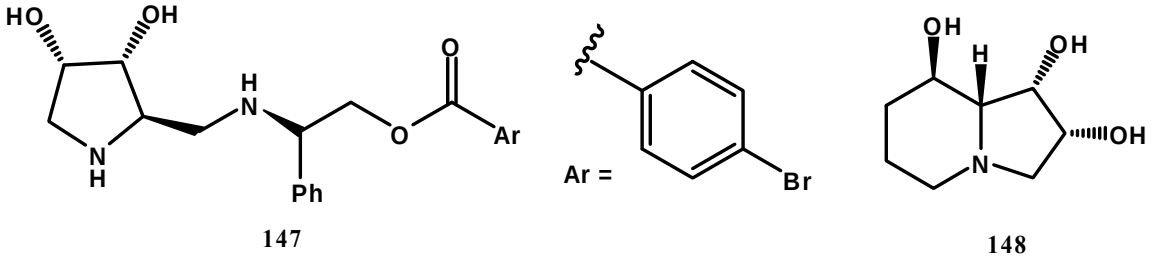


Figure 5: Schematic view of a cell membrane with glycoproteins, glycolipids and glycosylphosphatidylinositol (GPI)-anchored proteins.
Adapted from Seeberger, P.H. and D.B. Werz, *Automated synthesis of oligosaccharides as a basis for drug discovery.* Nature Reviews Drug Discovery, 2005. **4**(9): p. 751-763.

2.1.1. Altered glycosylation in cancer cells- Potential target

Glycans, which decorate all eukaryotic cell surfaces, undergo changes in structure with the onset of diseases such as cancer and inflammation. The term glycan refers to polysaccharides or oligosaccharides. Glycans may also be used to refer to the carbohydrate portion of a glycoconjugate, such as a glycoprotein, glycolipid etc. Glycans usually consist of monosaccharides linked to each other via O-linkages or N-linkage. As the bioconjugates synthesized in our research are of the type O-glycosidic monosaccharides we will concentrate our discussion on this type of glycans.

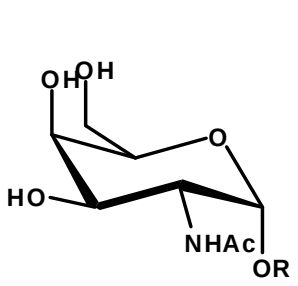
Aberrant glycosylation is a hallmark of cancer cells. Altered glycosylation patterns in cancer cells result in modified expression of glycans. These altered glycans are very typical and provides an indication of cancer. Glycosidases and glycosyltransferases in these glycosylation pathways represent potential innovative targets for drug development in cancer therapies [160]. Functionalized pyrrolidine derivatives like 4-bromobenzoyl derivative of pyrrolidine **147** have been found to inhibit α-mannosidases more effectively than the conventional ones like swainsonine **148** (Figue 2). Pyrrolidine derivative **147** was found to show a promising inhibition of glioblastoma and melanoma cells whereas it was less effective on healthy human fibroblasts [161].



147 **148**

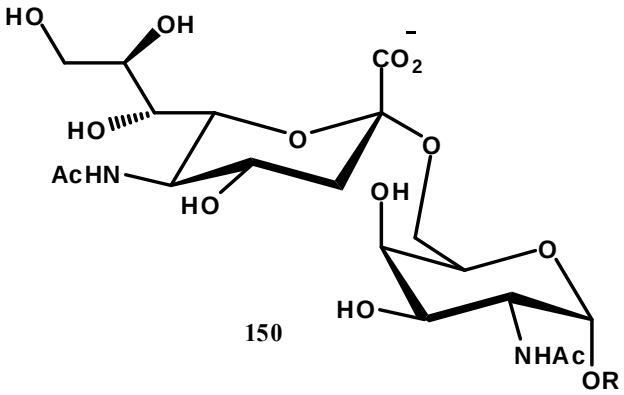
Scheme 17: α-mannosidase inhibitors

Glycoproteins act as critical cell surface communication markers [162, 163] and therefore it does not come as a surprise that change in glycosylation pattern occurs in cancer cells. The induced change in glycan pattern can be detected in certain motifs such as the Thomsen-Friedenreich (Tf) antigen, which are associated with various cancer cell lines [164]. Glycosyltransferases (for example sialyltransferases) involved in linking terminating residues on glycans tend to be overexpressed in tumour tissue. The increase in activity of these glycosyltransferases in turn leads to the overexpression or altered pattern of certain terminal glycans. Examples of terminal glycan epitopes commonly found on cancer associated cells include Tn **149**, sialyl Tn (sTn) **150**, sialyl Lewis a (sLe a)**151** (Tn is a precursor of tumour associated antigen called T antigen or Thomsen-Friedenreich antigen) etc [165] (Figure 3).



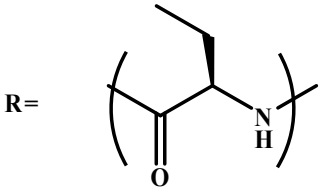
149

Tn

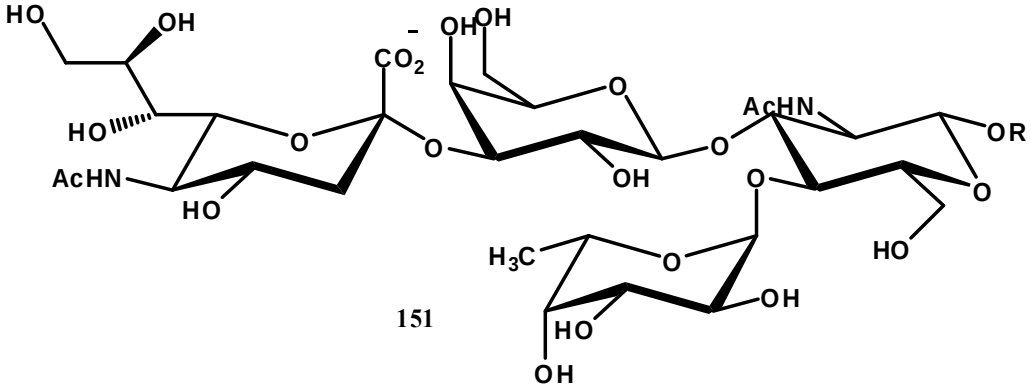


150

Sialyl Tn (sTn)



Serine-linked peptide



151

Sialyl Lewis a (sLe[a])

Scheme 18: Cancer- associated glycans

With the ability to engineer the cell surface oligosaccharides by replacing the N-acetyl group by an N-levulinamide group a ketone group was introduced on the cell surface. This unnatural sialic acid with ketone group allowed to fix biotin via hydrazone formation with the keto function. Selective drug delivery (indirectly) utilizing this biotin has been achieved through biotin-avidin complex formation where the drug is conjugated to avidin. This work was reported by Bertozzi *et al* [166].

2.1.2. Enhanced glycolysis in cancer cells- a target for transporting glycoconjugates?

Many tumor cells have a very distinctive metabolism, characterized by a high rate of aerobic glycolysis. Significantly, the glycolytic capacity of a given tumor is characteristic of its state of differentiation [167]. One of the biochemical "hallmarks" of malignancy is enhanced tumor glycolysis, which is primarily due to the overexpression of glucose transporters (GLUTs) and the increased activity of mitochondria-bound hexokinase in tumors [168]. This hexokinase enzyme is responsible for catalyzing glucose to glucose 6-phosphate which in a further series of reaction breaks down to pyruvic acid and high energy molecules.

The ability to sustain an enhanced glycolytic rate represents one of the most consistent and profound biochemical phenotypes of many cancer cells. This high rate of glycolysis is important for rapidly growing tumors, as they may obtain as much as 50% of their energy from this process [169]. Enhanced rates of glucose intake and turnover and alterations of cellular levels and kinetic properties of key glycolytic enzymes have been suggested to account, at least in part, for the enhanced glycolysis of many tumors. For example, in comparison to normal cells, the activity of hexokinase is markedly elevated in rapidly growing tumors that exhibit the high glycolytic phenotype [167, 170].

2.1.2.1. Glucose transporters

Glucose is a basic source of energy for mammalian cells whose metabolism provides ATP under both anaerobic and aerobic conditions. The maintenance of a relatively constant blood glucose concentration to sustain cerebral metabolism and the delivery of glucose to peripheral tissues for storage and utilization are key metabolic processes and, in many situations, transport of D-glucose across cell membranes plays a key role in their regulation and control. Cell membranes are effectively impermeable to D-glucose, so movement of glucose across cell membranes must be mediated by protein transporters. The transport of glucose across the plasma membrane is carried out by members of two distinct transporters.

There are two types of sugar transporters. One is energy-independent facilitated diffusion transporter. E.g. the family of hexose transporters, mainly GLUT (glucose transporters) those are present on the surface of cells. They have Km values for glucose uptake in the 2-20mM range. A second type of transporter is an energy-dependent variety such as sodium-glucose transporters (SGLT). These are typically found in epithelial cells of intestine and in kidney tubules where they absorb monosaccharide derived from the diet or retrieve glucose from the kidney filtrates, respectively. They are more efficient than GLUT transporters and have Km values of less than 1mM.

2.1.2.1.1. Sodium-dependent glucose co-transporters

The sodium-dependent glucose transporters, also known as co-transporters or symporters, are integral membrane proteins that mediate the transport of glucose and galactose across the plasma membrane by an active transport mechanism [171]. This transport process is a co-transport of glucose molecules and sodium ions. The SGLT1 protein is a high-affinity, low-capacity sodium-glucose symporter with sodium to glucose coupling ratio of 2:1. The SGLT2 protein is in contrast to SGLT1 protein a low-affinity, high-capacity sodium-glucose symporter with a sodium-to-glucose coupling ratio of 1:1. Deficiencies of these transporters can result in glucose/galactose malabsorption [172].

2.1.2.1.2. The GLUT family

Glucose transport by glucose transporters facilitators (GLUT) follows its gradient. The human genome contains 14 members of the GLUT family [171]. The different characteristics of the members allow a complex and specific regulation of glucose uptake according to the cellular requirements and the physiologic conditions of substrate supply. For the basic supply of glucose, GLUT1 is expressed ubiquitously in all cells but at very high expression levels in erythrocytes and endothelial cells of the brain. GLUT2 is a low-affinity glucose transporter with predominant expression in pancreatic cells, liver, kidney, and small intestine. In all these tissues, the uptake of glucose is not dependent on the number and activity of the glucose transporters but on the blood glucose concentration. Thus, transport activity of GLUT cannot be saturated by physiologic blood glucose concentrations [171]. GLUT3 is a high-affinity glucose transporter with predominant expression in tissues with a high glucose requirement (e.g., brain). GLUT4 is a high-affinity glucose transporter expressed in insulin-sensitive tissues (heart, skeletal muscle, adipose tissue). GLUT7 is a high-affinity transporter for glucose and fructose and it can be detected in small intestine, colon, testis, and prostate. GLUT8 is a high-affinity glucose transporter which also transports D-fructose and D-galactose. GLUT8 is predominantly expressed in testis. GLUT9 exhibits highest expression levels in kidney and liver; lower levels were detected in small intestine, placenta, lung, and leukocytes. GLUT10 is predominantly expressed in the liver and pancreas. GLUT12 is predominantly expressed in heart and prostate and it can also transport D-galactose and to a lesser extent by D-fructose. In addition, GLUT12 seems to sustain the increased glucose consumption in prostate carcinoma and breast cancer. Defects in primary glucose transport appear to be extremely rare. One has to admit that not all possible deficiencies have been identified so far [171].

2.1.2.2. Mannose Transport

In humans part of mannose transport is using the glucose transporters. High-affinity glucose transporters GLUT1, GLUT2, GLUT3 and GLUT4 proteins can also mediate the mannose transport with relatively good affinities [173]. Because mannose is not commonly found as terminal residues in mammalian glycoproteins but is frequently

found on the glycoprotein that decorate the surface of many microorganisms, mannose receptors are generally a recognition element for the immune response. There are two C-type lectins of the mannose receptor family that recognize mannose derivatives. They are called the mannose receptor (MR) and the M-type Endo180 protein [174]. The MR (also called CD206) plays a role in the innate and adaptive immune system and is found on macrophages. Endo180 is a recycling endocytic receptor involved in remodelling of the extracellular matrix and is generally found in stromal cells, endothelial cells and also in macrophages. There is another C-type lectin, the mannan- (or mannose-) -binding lectin (MBL), which can also bind mannose saccharides [175]. However, this protein is secreted by the liver into the bloodstream and its role is to bind sugar arrays found on many microbial surfaces and activate the complement via associated serine proteases. [176].

2.1.2.3. Galactose Transporters

In humans part of galactose transport appears to use the glucose transporters in an arrangement similar to the one already described for the mannonse transport. The sodium-dependent glucose transporters SGLT1 and SGLT2 are active transporters that can also mediate the galactose transport [171]. GLUT8 and GLUT12 proteins which are both high affinity glucose transporters, can also mediate the galactose transport [171]. Galectin proteins can also recognize extracellular β-galactosides with their carbohydrate recognition domain (CRD) [177]. This family of proteins is composed of 14 different galectins both intracellular and extracellular proteins. Galectins can have various functions; they can modulate cell-cell and cell-matrix interactions and cell adhesion. They can act on cell division, apoptosis, pre-mRNA splicing, metastasis, cell maturation and differentiation [177]. Their expression varies among different adult tissues, changes during development and is dramatically altered upon neoplastic transformation [178].

Some extracellular C-type (Calcium ion-dependant) lectins different of galectins can internalize β-galactosides via a receptor-mediated endocytosis mechanism. The hepatic asialoglycoprotein receptor (ASGP-R) was the first described and is one of the best characterized proteins responsible for receptor-mediated endocytosis [179]. The ASGP-R mediates the endocytosis and degradation of a wide variety of desialylated glycoproteins and neoglycoproteins that contain terminal galactose (Gal) or N-acetylgalactosamine (GalNAc) residues on their N-linked carbohydrate chains. The ASGP-R has been used for targeted gene delivery [180]. Other Gal/GalNAc binding proteins sharing extensive amino acid sequence homology to the hepatic ASGP-R are found in various tissues or cell types, such as macrophages, intestinal epithelial cells, testis and thyroid glands [179]. They could also be used as target for controlled delivery as the testis galactose binding protein in the delivery of antioxidant compounds [181].

The cumulated literature evidence shows that cancer cells possess a modified metabolism of sugar molecules. The observed differences in sugar metabolism between cancer and normal cells are significantly large that the hope to use these differences in the detection and recognition of cancer cells has a good foundation.

2.1.3. Glycoconjugates

Glycoconjugation can be a potentially effective strategy for targeting and transporting photosensitizers towards tumor cells. Glycosylation provides the possibility for specific interaction of the resulting conjugate with lectin type receptors which are overexpressed in certain malignant cells [182, 183]. Lectins are typically sugar-binding proteins which show a remarkable specificity in their binding of multivalent complex carbohydrate structures. Hydrogen bonding between lectin and carbohydrate, protein-bound calcium ions which can coordinate with vicinal carbohydrate groups and van der Waals' interactions between typically aromatic side chains of protein and hydrophobic 'patches' on carbohydrates (Figure 4) are responsible for the specific binding of lectins. [184] .

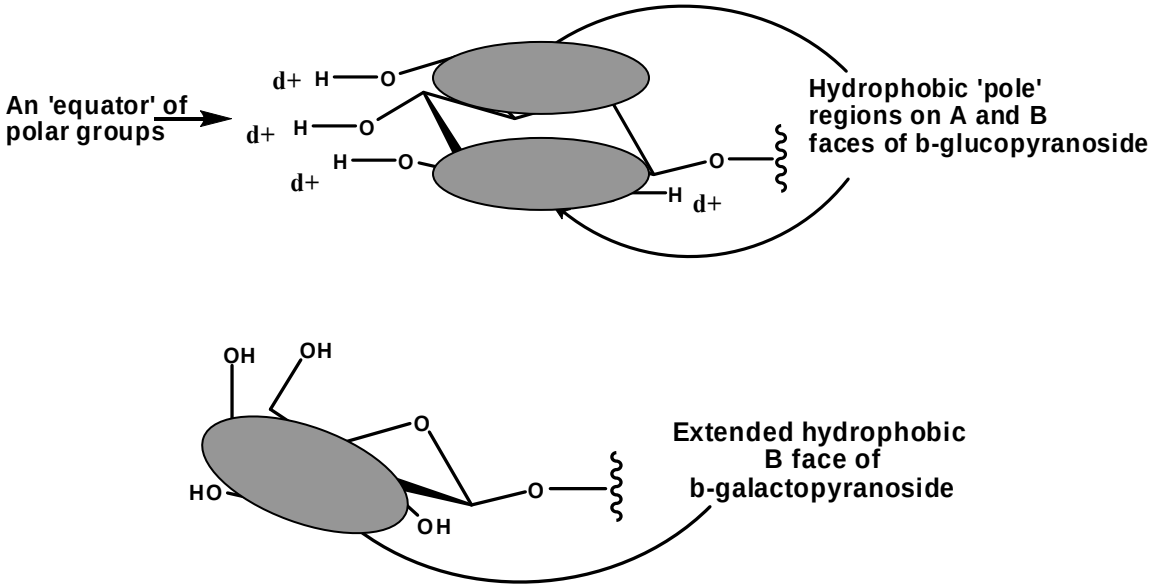
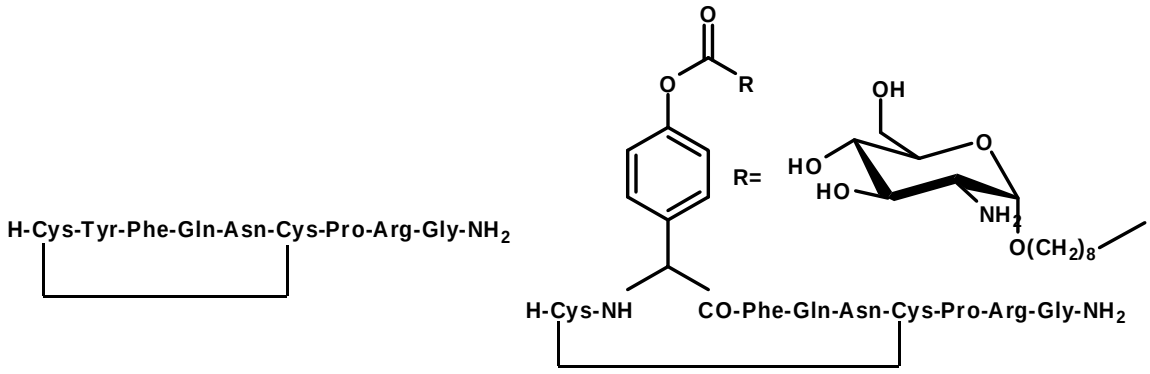


Figure 6: Hydrophobic and 'polar' patches on carbohydrates

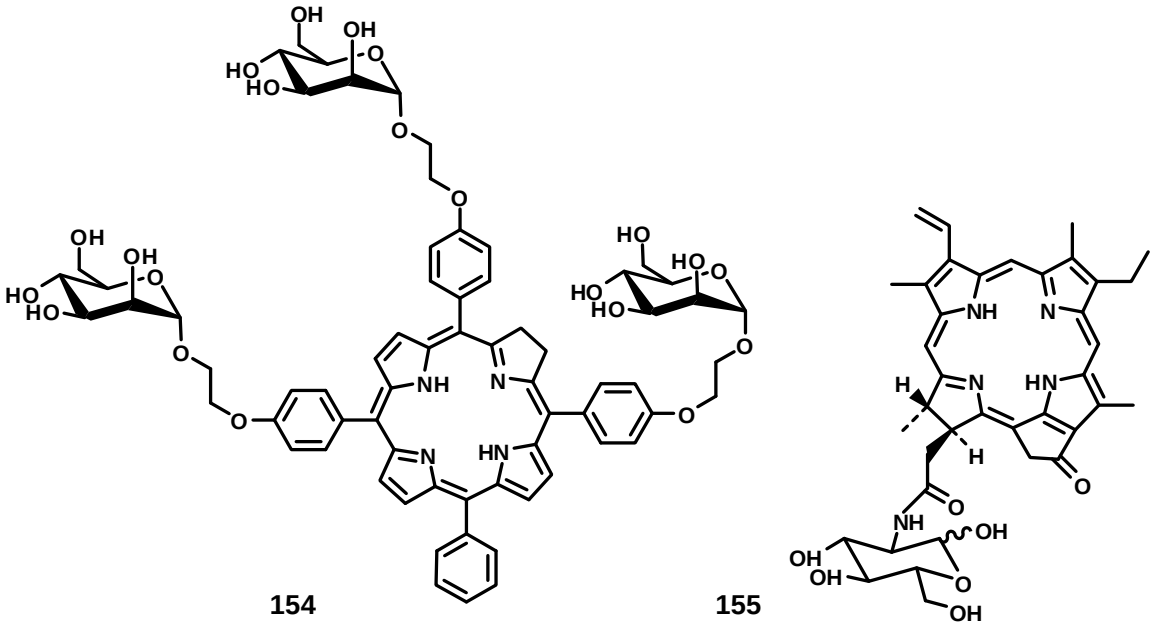
Glycoconjugate compounds exhibit a number of useful properties due to their amphiphilicity and multiple functionalities. In particular, their high solubility in water and other polar solvents makes them suitable for intravenous administration and widens the possibilities of their use in medicine. There are many examples of proteins and peptides whose biological activity is enhanced by conjugation to carbohydrates. For example, the activity of the anti-diuretic nonapeptide arginine-Vasopressin **152** is almost doubled through glycosylation or galactosylation **153** [185] (Scheme 19). Carbohydrate moieties can interact with specific receptors on the cell surface membrane [186] which may help internalize such conjugates by the cell due to the receptor-mediated endocytosis and enhance the selectivity of their accumulation in tumor tissues. Griegel and co-workers [187] established that human retinoblastoma cells express sugar receptors that exhibit a preferential affinity for galactose and mannose residues



152 **153**

Scheme 19: Arginine-Vasopressin **152** and glucosylated Arginine-Vasopressin **153**

In PDT, glycoconjugated porphyrins where *m*-tetraphenyl porphyrins (TPPs) conjugated with galactose or mannose via ethylene glycol linker had been synthesized (Eg. TPP(*p*-Meg-O-R-ManOH)$_3$ **154** in Scheme 20) and their preferential cellular uptake had been demonstrated [188]. Nowadays, the interest in the use of glycoporphyrins as efficient sensitizers for PDT is growing [189] and synthesis of glycoporphyrins has been reviewed [190] . The unsubstituted porphyrins are not soluble in water and only sparingly soluble in organic solvents. In contrast to the normal prophyrins the glycoporphyrins can be easily disolved in water. The water solubility allows intravenous administration of these amphiphilic glycoporphyrins and thereby considerably widens their use in medicine. Photosensitizers targeted at glucose transporters (GLUTs) like pyropheophorbide 2-deoxyglucosamide **155** (Pyro-2DG) (Scheme 20) had been synthesized and their efficiency of the intracellular trappings by tumor cells has been evaluated [134].



154 **155**

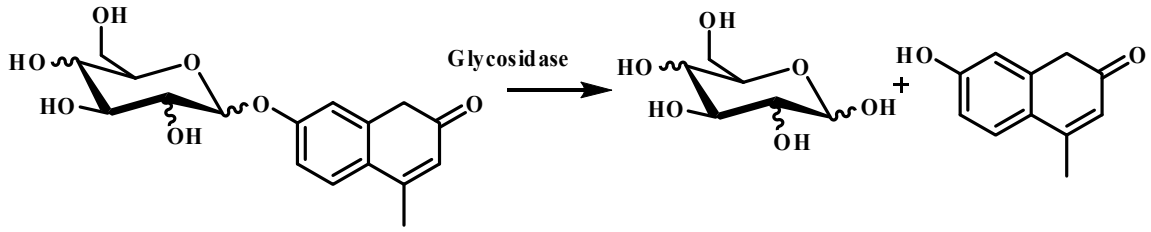
Scheme 20: Glycoconjugated *m*-tetraphenyl porphyrins (TPPs) **154** and Pyropheophorbide 2-deoxyglucosamide (Pyro-2DG**) 155.**

The reasons cited above justify the recent interest in the synthesis and use of glycoporphyrins as efficient sensitizers for the photodynamic cancer therapy [134, 188, 190]. Using glycoconjugates of ALA instead of porphyrins for targeting tumour cells has not been attempted so far. This is all the more surprising because besides specifically targeting cancer cells through the sugar part of the bioconjugate, the various sugar transporters often overexpressed in cancer cells can also be used for the effective internalization of glycoconjugate of ALA.

2.1.4. Glycosidase activities

For a successful application of glycoconjugates in PDT the following biological processes must occur in sequence and with high efficiency: specific recognition of the glycoconjugate (= prodrug) by the cancer cells, transport of the glycoconjugate through the cell membrane, cleavage of the glycoconjugate and transformation of the biological precursor to the active chromophore. In view of this complex sequence each step has to be understood and if possible to be optimized. Therefore the understanding of glycosidase activities is essential for the design glycoconjugates of ALA which can target specifically tumour cells.

Studies with the goal to characterize the glycosidase activities were carried out by the group of Dr.L. Juillerat-Jeanneret and her PhD student F.Schmitt, University Institute of Pathology, CHUV (Centre Hospitalier Universitaire Vaudois), Lausanne, Switzerland. [191] .

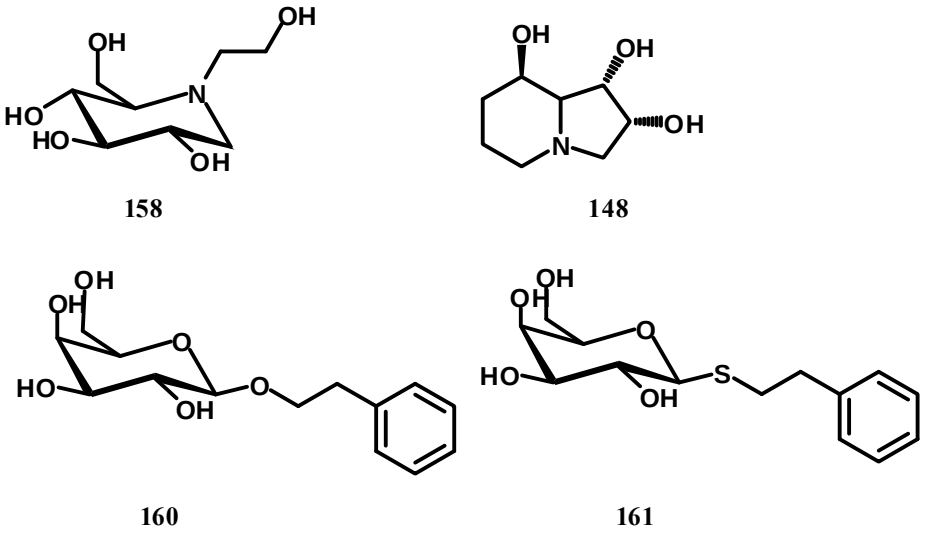


156 **157**

Scheme 21: Glycosidase activities on cancer cells using 4-methylumbelliferyl glycoside **144**

Glycosidase activities of various cancer cell lines were studied using 4-methylumbelliferyl glycoside derivatives **156**. Glycosidase activities in the cell supernatants were negligible. α-glucosidase, α-mannosidase and β-galactosidase activities in cellular extracts were studied. Kinetic studies were performed for all cell lines and in all cell lines, fluorescence due to 4-methylumbelliferone **157** was strong for α-glucosidase activity, medium for α-mannosidase and weak for β-galactosidase. Commercially available inhibitors of these activities were also tested: Miglitol **158** as an inhibitor of α-glucosidase, swainsonin **148** of α-mannosidase, phenylethyl-β-galactoside **159** and phenylethyl-β-thiogalactoside **160** of β-galactosidase. Miglitol and swainsonin were strong inhibitors of α-glucosidase and α-mannosidase respectively, whereas phenylethyl-β-galactoside and phenylethyl-β-thiogalactoside did not inhibit β-galactosidase. α-glucosidase, α-mannosidase and β-galactosidase activities of HT29 cells.

The tests showed a higher fluorescence for α-glucosidase activity than for α-mannosidase and β-galactosidase.



Scheme 22: Structures of glycosidase inhibitors : Miglitol **158**, Swainsonin **148**, phenylethyl β-galactoside **160** and phenylethyl β-thiogalactoside **161**.

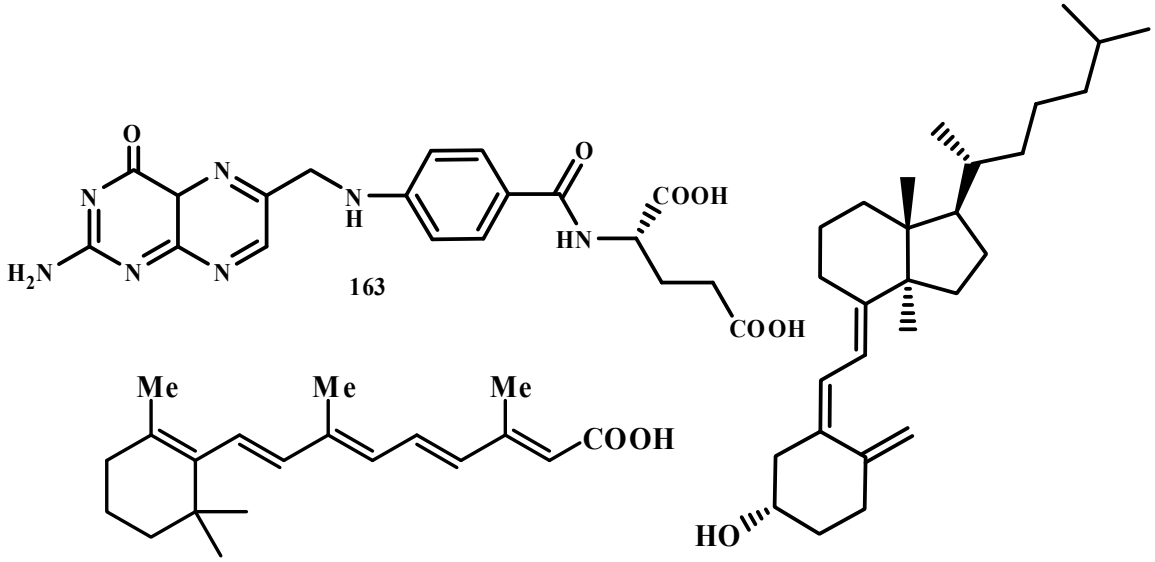
These results indicate that α-glucosidase, α-mannosidase and β-galactosidase are potential targets for the treatment of cancer cell lines as they have increased activities compared to normal cells. ALA glycoconjugate derivatives connecting ALA as an ester to the C1 or C6 hydroxyl group of the carbohydrates have the potential to be a totally novel approach to photodynamic therapy. These novel prodrugs should be designed taking into account α-glucosidase, α-mannosidase and β-galactosidase as receptors or targets which will then release ALA as biological precursor to generate PpIX.

2.2. Vitamins in targeted PDT

Dietary habit is instrumental in about 40-60% of human colon cancer. Fruit and vegetable consumption is associated with decreased risk of several types of cancer, including colonic malignancy. Fruits and vegetables contain many non-nutritive as well as nutritive compounds such as carotenoids, dithiolthiones, flavonoids, glucosinolates, indoles, isothiocyanates, monoterpenes, polyphenols and vitamins (including vitamin C, vitamin E, and folate). These non-nutritive compounds are known as 'chemopreventive agents'. Many of them are anti-oxidants and might suppress carcinogenesis [192].

Retinoic acid **162** (Figure 9) metabolized from Vitamin A has been shown to inhibit the growth of a variety of tumor cells [193]. Retinoic acid **162** is being used clinically to treat promyelocytic leukemia, head and neck tumors as well as cervical dysplasia [193]. Also, 1, 25-dihydroxy vitamin D_3, the metabolized form of vitamin D_3 **71** (also called cholcalciferol, figure 9) has been recently shown to inhibit tumor cell replication and stimulate differentiation of selected tumor types. 1, 25-Dihydroxy vitamin D_3 exerts its

effects via the vitamin D receptor (VDR). The antiproliferative action makes 1,25-dihydroxyvitamin D_3 and its analogs a possible therapeutic tool to treat hyperproliferative disorders [194]. Also, Vitamin D enhances ALA-induced protoporphyrin IX production and photodynamic cell death in the cultures of keratinocytes [195] .

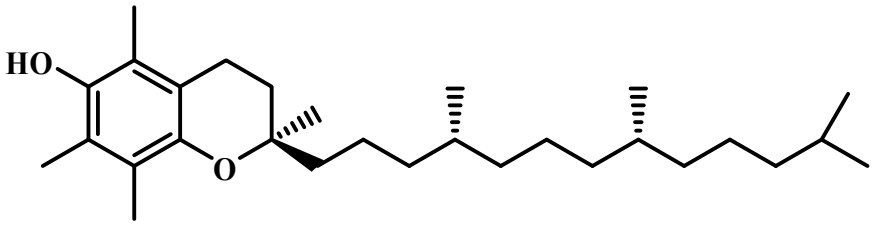


163 162 71

Scheme 23: Structures of retinoic acid **162,** vitamin D_3 (cholcalciferol) **71** and Folic acid **163**

Vitamin B_9 called pteroylglutamic acid **163** or more commonly folic acid are daily required for many biosynthetic processes. Traditionally, the folate leucovorin (folinic acid), for instance, is used to reduce antifolate toxicity in leukemia or to enhance the effect of the fluoropyrimidine 5-fluorouracil in some solid tumors. More recently, it has also been noted that folic acid has the ability to increase antitumor activity of several structurally unrelated regimens, such as alimta/pemetrexed and cis-platin. It has been demonstrated that the folates could modulate the expression and the activity of multidrug resistance (MDR) transporters (MRP1/ABCC1) and the breast cancer resistance protein BCRP/ABCG2. [196]. These crucial and specific cell membrane-associated transporters mediate drug resistance by increasing the rate of excretion of a wide spectrum of toxic agents. Thus, folate supplementation may have differential effects on chemotherapy: (1) reduction of toxicity, (2) increase of antitumor activity, and (3) modulation of MRP1 and BCRP associated cellular drug resistance. It is well known that folate receptors present in the cells has the ability to transport folic acid and folate derivatives.

Folate receptors (FRs) exhibit limited expression on healthy cells, but are often present in large numbers on cancer cells. For example, FRs are overexpressed on cancers of the ovary, uterus, cervix, mammary gland, colon, lung, prostate, nose, throat, and brain. Targeted photodynamic therapy (PDT) using a photosensitizer covalently bound with folic acid via a small linker had been described. Selective recognition of the conjugate by the human nasopharyngeal cells (KB) which overexpress FR, was revealed [133].



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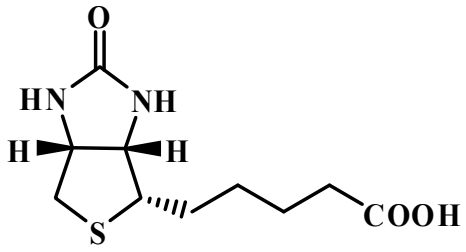


67

Scheme 24: Structures of vitamin E (tocopherol) 62 and Biotin 67

Conjugation of vitamins with photosensitizer or precursors of photosensitzers has not yet been tried to the best of our knowledge (searched in Scifinder programme in chemical abstract database by (1) structure search and (2) key words search containing "vitamins" and "photosensitizer", then looking the hits for synthetic molecule combining vitamin and photosensitizer). Hydroxyl functions of vitamins like vitamin E (tocopherol) **62** and vitamin D_3 (cholecalciferol) **71** can be conjugated with ALA as ester and acid moiety of vitamins like folic acid **163** and biotin **67** can be coupled with ALA as a pseudo peptide. Considering the vitamin based receptors as target and considering ALA part of the bioconjugate as being effective photodynamic detection and therapy agent, the conjugation of ALA with various vitamins can provide not only an innovative approach but also a synergistic approach towards target oriented ALA-photodynamic therapy.

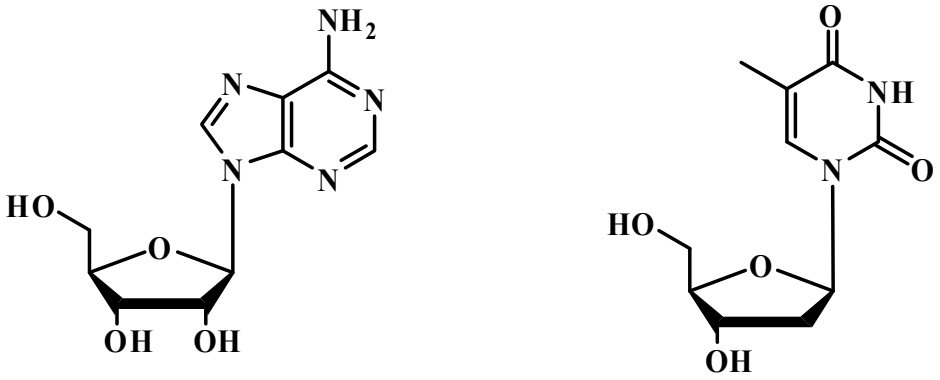
2.3. Nucleosides in targeted PDT

Though the application of nucleoside analogs has been in use for chemotherapy, the conjugation of nucleoside with any photosensitizer has not yet been reported so far (searched in Scifinder programme in chemical abstract database by (1) structure search and (2) key words search containing "nucleosies" and "photosensitizer", then looking the hits for synthetic molecule combining nucleoside and photosensitizer). Adenosine **164** one of the four nucleosides is found to be released from a variety of cells throughout the body, as the result of increased metabolic rates, in concentrations that can have a profound impact on the vasculature, immunoescaping, and growth of tumor masses. Adenosine acts as a regulatory molecule by binding to specific G-protein-coupled A1, A2A, A2B, and A3 cell surface receptors. It is recognized that the concentrations of this nucleoside are increased in cancer tissues [197]. It has been recently demonstrated that adenosine induces a differential effect on tumor and normal cells [198]. Adenosine **164** acts as a potent regulator of normal and tumor cell growth. While inhibiting in vitro tumor cell growth, adenosine stimulates bone marrow cell proliferation. Studies have

shown that A3 adenosine receptor agonist, exhibits systemic anticancer, antimetastatic, and myeloprotective effects in colon carcinoma [199].

As measured by receptor binding assays, the density of A3 receptor was higher in colon carcinomas as compared with normal mucosa originating from the same individuals [200]. A3 receptor plays a role in colon tumorgenesis and, more importantly, can potentially be used as a diagnostic marker or a therapeutic target for colon cancer. Nucleosides play an essential role in intermediary metabolic and biosynthesis, as well as signal transduction through interaction with purinergic receptors [201]. Inhibitors of the active site of thymidine kinase 1 (TK1) enzyme have a high potential as therapy for cancer [202].

These studies indicate that adenosine receptors like A3 receptors and thymidine kinase (TK1) can be a potential substrate for targeting tumour cells. The occurence of thymidine receptors and thymidine transporters suggests that ALA can be conjugated with thymidine **165** besides adenosine **164** by coupling with the hydroxyl group of ribose sugar in nuceloside could pave a new way for targeted ALA-PDT.



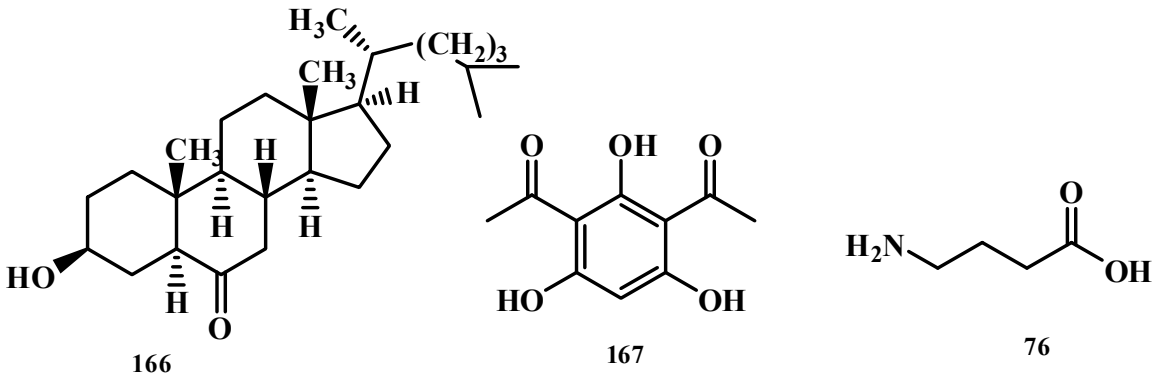
164 **165**

Scheme 25: Structures of adenosine **164** and thymidine **165.**

2.4. Novel ALA esters and peptides of natural products

An important limiting factor for topical delivery of 5-ALA to the targeted cancer tissue is the skin. One strategy to enhance the transport through the skin of charged drugs like 5-ALA, can be the alteration of the dipole potential over the skin membranes due to incorporation of molecules with inherent dipole moments into the membrane double layer. In studies of model membranes addition of 6-ketocholestanol increased the dipole potential of the membrane bilayers 6-Ketocholestanol increased the crossing rate of anions and reduced the rate of cations in these model phospholipids membranes [203]. The ability of 6-ketocholestanol **166** to increase the skin permeation of the prodrug aminolevulinic acid (5-ALA) was investigated already [204]. Another possibility could be to couple 6-ketocholestanol **166** with ALA so that the bioconjugate ester penetrates more easily than ALA. The bioconjugate could then be cleaved by esterases to release the prodrug ALA. It is well documented that glycerol or long chain glycerides are widely

used in drug formulations to enhance its permeability [205]. From this emerge the idea of coupling all the three hydroxyl of glycerol with ALA. The ALA glycerides should be able to cross the plasma membrane much more easily. In this approach not only crossing the lipid bilayer should be enhanced but a single ALA glyceride is able to generate three ALA molecules.

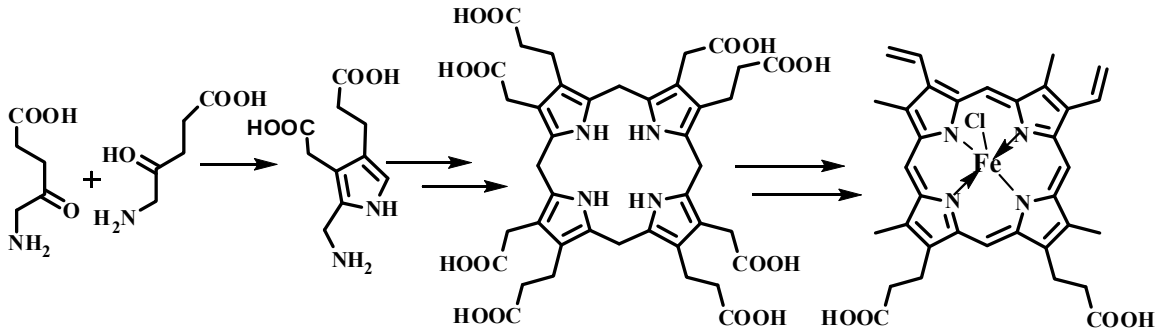


Scheme 26: Structures of 6-Ketocholestanol **166**, 2,4-Diacetyl Phloroglucinol **167** and GABA **76**

It has been reported that 2,4-diacetyl phloroglucinol (2,4-DAPG) **167** is an antibiotic with broad-spectrum antibacterial and antifungal activities. 2,4-DAPG **167** is a major determinant in the biological control of several plant diseases [206]. 2,4-DAPG is a polyphenol (three hydroxyl groups) which can act as antioxidant essential for chemoprevention of cancer besides being an antibiotic. Esterifying all the phenolic groups of 2,4-DAPG with the acid group of three ALA molecules which might have synergistic approach for the destruction of tumor cells. The unsubstituted phloroglucinol has also been studied for its cytoprotective effects against oxidative stress induced cell damage in Chinese hamster lung fibroblast (V79-4) cells [207].

Gamma-aminobutyric acid (GABA) **76** is the inhibitory neurotransmitter in the brain, also playing a role in diseases like epilepsy. Due to its structural similarity with GABA, ALA is incorporated into the cells by a carrier protein which is a GABA transporter [208, 209]. GABA receptors have been detected on epithelial colon cancer cells. The presence of GABA receptors on colon cancer cell lines (KM12SM, HT29) has been demonstrated [210]. GABA can also reduce migratory activity in SW 480 colon carcinoma cells [211] . ALA can be coupled with GABA through an amide bond. The ALA-GABA pseudo peptide should be easily transported by GABA transporters and should also be recognized by GABA receptors. Besides ALA- GABA, ALA pseudo peptides with tyrosine, ALA coupled with ALA itself to give oligo ALA peptides (dimmer, hexamer, octamer etc) would be attractive goals to be studied for their potential in PDT.

2.5. PBG as precursor for PpIX generation in PDT



Scheme 27: Heme biosynthesis: ALA–starting material; PBG–advanced intermediate

In heme biosynthesis, four molecules of porphobilinogen (PBG) **168** are formed from eight molecules of ALA **119** catalysed by the enzyme called ALA dehydratase or porphobilinogen synthase [212, 213]. Porphyrin biosynthesis capacity is twenty fold enhanced at least from the porphobilinogen (PBG) formation up to that of coporphyrinogen, when compared with the original biosynthetic capacity of normal tissue [80]. ALA-dehydratase regulates PpIX synthesis and thereby impact the effectiveness of ALA-mediated photosensitization [214]. This suggests that if ALA is replaced by a more advanced intermediate of heme biosynthesis the rate limits imposed by ALA-dehydratase could be surpassed. Such an approach should generate considerably higher amounts of PpIX than that generated from exogenous ALA. Enhancing the PpIX production might be achieved by giving exogenously PBG instead of ALA. The major obstacle to test this approach is the difficulty of synthesizing PBG in useful quantities and also the instability of PBG [215].

To the best of our knowledge, there has not been a single publication with PBG as precursor of photosensitizer PpIX (searched in Scifinder programme in chemical abstract database by (1) structure search and (2) key words search containing "porphobilinogen" and "photosensitizer", then looking the hits for synthetic molecule composed of PBG structure). This makes a strong case to develop stable PBG lipophilic derivatives which should be easy to be internalized into cancer cells. Once inside the cancer cell, the bioconjugate might release PBG which in turn should induce an efficient PpIX production. The reported synthesis of PBG yields the natural compound without any protecting group [215-218]. In order to couple PBG with an amino acid the acid of PBG have to be protected e.g. as methyl esters so that the amino group of the side chain can be coupled with a judiciously protected amino acid. In this approach one obtains a PBG bioconjugate in the form of a stable and easily releasable derivative. The only uncertainty in this strategy might be the transport of the bioconjugate through the membrane.

3. Goal

Nowadays in cancer, a therapy with total destruction of the cancer tissue together with some of the healthy tissue surrounding the cancer is still an acceptable treatment despite the heavy side effects inflicted on the patients. In view of the progress made in molecular biology, a more selective approach to cancer treatment must be possible.

PDT has the potential to become a more selective treatment of cancer, because visible light is much less aggressive than X-ray radiation used for radiotherapy. Guiding visible light precisely onto cancer tissue is possible using modern laser technologies. Systemic injection of the clinically used photosensitizer leads to relatively higher retention of the compounds in tumors than in the surrounding normal tissue, resulting in increased accumulation of the photosensitizer in tumors. However, this accumulation in tumor is not discriminating enough and results in false positive detection and damage to the surrounding tissue in PDT protocols. Thus improvement in the targeting of photosensitizers is needed. So the major challenge for progress is to develop photosensitizer or precursors of photosensitizer which possess sufficient specificity for cancer cells.

In this context it was our task to design, synthesize and test novel bioconjugates of ALA and their application in targeted PDT. To be effective, the novel ALA- bioconjugates should be designed keeping in mind the following criteria:

1. Transport: The bioconjugates should allow modulating the physico chemical and physiological properties of these novel ALA- bioconjugates which could be transported intracellularly.

2. Target: These ALA-bioconjugates should be able to make use of the specific cellular pathways or metabolisms related to cancer to target specifically cancer cells. The specificity of these bioconjugates for cancer cells, their metabolism in cancer cells etc in comparison to normal cells is discussed in chapter “Biological background for Prodrugs’ design- A review of literature knowledge”.

3. Release active ingredient: The enzymes found in the cells should be able to release the active ingredient ALA from the ALA-bioconjugates.

A major advantage of the approach chosen is the opportunity to use the specific recognition sites of the partner conjugated with ALA as a means to increase the specificity for cancer cells.

In view of this reflection, the goal of this thesis was to synthesize different classes of ALA/PBG-bioconjugates, then evaluate them for ALA-PDT. To be more efficient than the standard ALA, the bioconjugates of ALA should satisfy the following requirements:

a) Conjugates should be stable and easy to synthesize.

b) Increase the efficiency of Protoporphyrin (PpIX) generated from ALA-bioconjugates compared to that of ALA as precursor for the application in PDT (measure PpIX fluorescence).

c) Selectively target cancer cells - compare PpIX fluorescence due to cancer cells and normal cells (fibroblast cells).

d) Should not be cytotoxic (measured by MTT or alamar blue assay) in the absence of exposure to light.

We planned, synthesized and tested (all the conjugates are not yet tested) the following bioconjugates:

1. Sugar-ALA derivatives

2. Vitamin-ALA derivatives

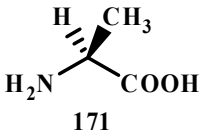
3. Nucleoside - ALA derivatives

4. ALA ester/pseudopeptide derivatives

5. PBG peptide derivatives

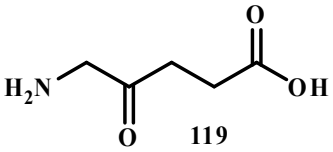
4. Chemistry of ALA

δ-aminolevulinic acid (ALA) is a natural amino acid, but not a common amino acid. The most common amino acids are α-amino acids which forms the building block of all proteins. As shown in the structure of α-amino acid here, the R represents a side chain specific to each amino acid. The central carbon atom called Cα is a chiral atom (except glycine). δ-aminolevulinic acid (ALA) is an amino acid where the amino group is at the delta or 4 carbons away from the acid moiety. ALA can be classified in the class of δ-amino acids as well as in the class of α-amino ketones.



171

Alanine (α-amino acid)



119

δ-aminolevulinic acid (ALA)

Scheme 28: Structure of a α-amino acid and δ-Aminolevulinic acid (ALA)

4.1. Similarities and dissimilarities between ALA and α-amino acids

Similarities

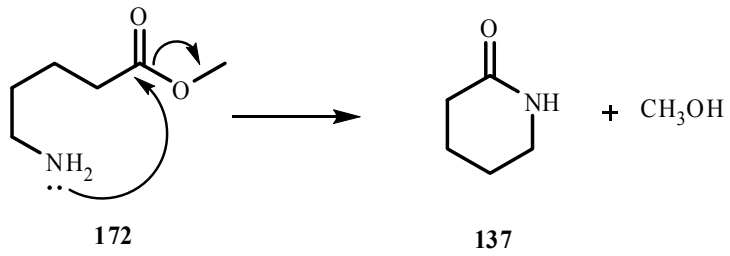
1. Like α-amino acids, ALA is white crystalline solid and water soluble
2. ALA has the properties of ionic salts.
3. ALA is a natural amino acid and is a product of biosynthesis in living organisms.
4. The aqueous solution of ALA can conduct electric current like α-amino acids.
5. Similar to α-amino acids, the COOH groups and NH_2 groups in ALA are ionizable. As in the case of α-amino acid, when ALA is at physiological pH (pH=7.4), the carboxyl groups are unprotonated and amino groups of ALA are protonated and exists in the electrically neutral form called zwitter ion.
6. Like α-amino acids, the amino group of ALA can react with an acid moiety to form peptide bond and the carboxyl groups of ALA can react with an amino group of any molecule to form amide or peptide bond. Also the carboxyl group of ALA similar to α-amino acids can form esters.

Dissimilarities

1. There is no chiral carbon in ALA where as α-amino acids due to the presence of chiral carbon rotate linearly polarized light (optical rotation).
2. ALA lacks any side chain compared to α-amino acids. Depending on the polarity of the side chain, α-amino acids vary in their hydrophilic or hydrophobic character. This character determines the tertiary structure of protein.

3. ALA is not used in the protein biosynthesis and it is synthesized from glycine, a α-amino acid.

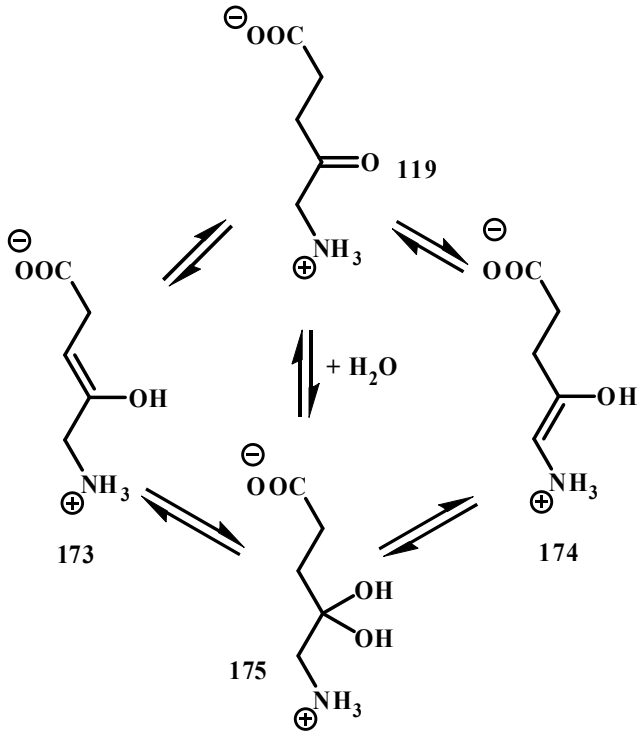
4. The amino group in ALA is 4 carbons far away from the acid group which makes the ALA (with free amine) prone to intramolecular condensation [108] to form stable six membered lactam **137** as shown in scheme 29.



172 **137**

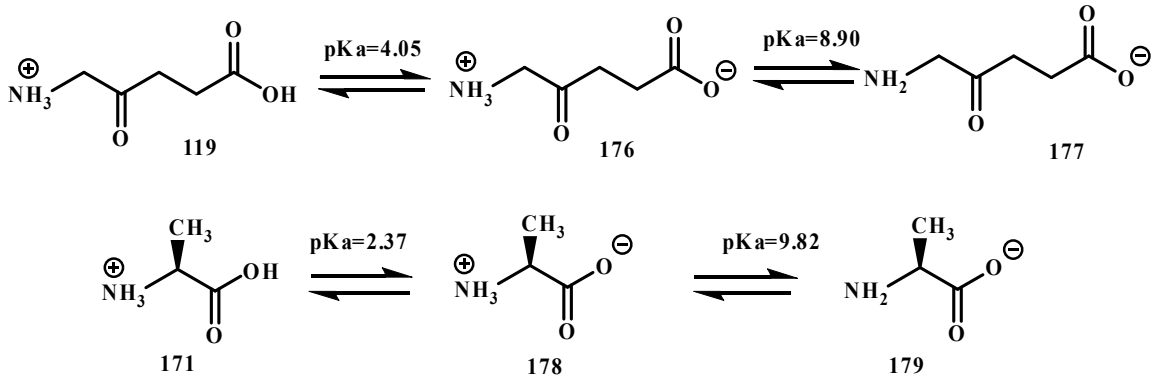
Scheme 29: Intramolecular condensation of ALA ester to form lactam

5. ALA belongs to the class of α-amino ketones. α-amino ketones readily dimerize to dihydropyrazines and are never isolated although the ammonium salts such as the hydrochlorides of these compounds are stable [219]. The dihydropyrazines thus formed from α-amino ketones are easily oxidized even by air to form pyrazine. ALA being an α-amino ketone, gets dimerized (inter molecular condensation/cyclization) to form dihydropyrazines as per scheme 34. The NMR and kinetic studies [88] suggest that under physiologic conditions, ALA rapidly equilibrates between the ketone, the hydrate at C4 and two enol forms (C3-C4 and C4-C5) as shown in scheme 30.



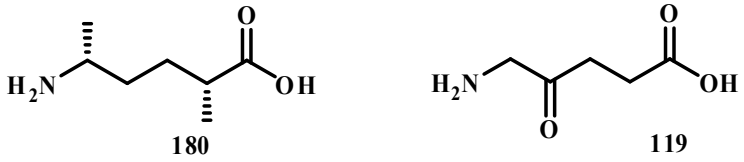
Scheme 30: Equilibrium of ALA between hydrated form and enol forms

6. The acidity constant (pKa) values of ALA and α-alanine are different. The first pKa value of ALA is 4.05 and the second pKa value is 8.90. In the case of alanine, the first pKa value is 2.35 and the second pKa values is 9.87. The first pKa value of α-amino acids ranges from 1.80 (histidine) to 2.46 (Tryptophan).



Scheme 31: pKa comparison between ALA and alanine.

4.2. Similarities and dissimilarities between ALA and other δ- amino acids



δ-amino acid (D-*Ala*-L-*Ala* isostere) δ-aminolevulinic acid (ALA)

Scheme 32: Structure of a simple δ-amino acid and δ-aminolevulinic acid (ALA)

Similarities

1. Similar to δ-amino acids, ALA when protonated exists in the electrically neutral form called zwitter ion.

2. ALA is white crystalline solid and water soluble.

3. Like δ-amino acids, the amino group can react with an acid moiety to form peptide bond and the carboxyl groups of ALA can react with an amino group of any molecule to form amide or peptide bond. Also the carboxyl group of ALA similar to α-amino acids can form esters.

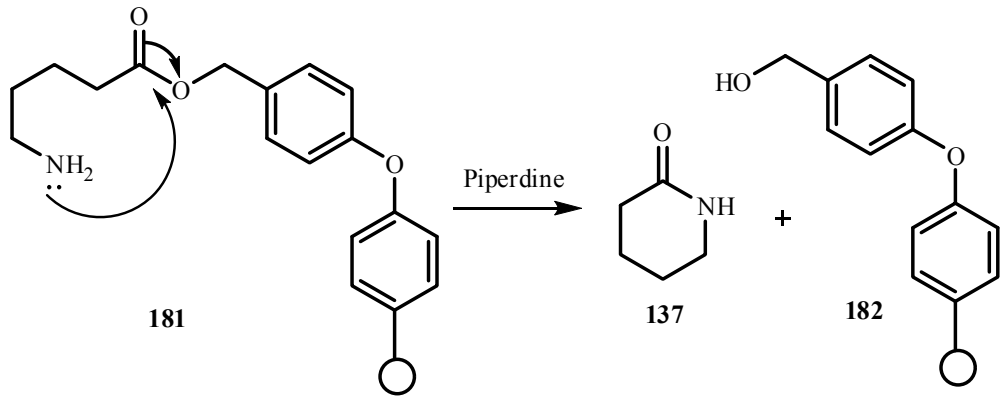
4. At pH=7, both exists as zwitterions.

Dissimilarities

1. ALA is a natural product and the starting intermediate of heme biosynthesis. It is biosynthesized in living organisms where as δ-amino acids are synthetic.

2. δ- Peptides with well defined and stable secondary and tertiary structures are known. These synthetic δ- amino acids which are isosteric replacement of α-dipeptide unit, attracted considerable interest nowadays in the design of peptidomimetics [220, 221]. Furthermore, δ-amino acids have gained importance as building blocks for backbone generation of peptide nucleic acid (PNA) structures as well as for foldamers, since the six-atom length of these amino acid homologues corresponds to the optimal distance to mimic the ribose unit found in RNA and DNA [222-225].

   Similar long chain peptides having repetitive ALA monomer unit has not yet been synthesized using ALA which might be due to two reasons: A) Many of the δ-Peptide libraries have been synthesized using the methodology of Merrifield's solid phase synthesis which was well established for peptides based on α-amino acids . But the same methodology of solid phase synthesis was not successful for the construction of peptide libraries based on ALA which is also a δ-amino acid [226]. The Fmoc strategy required for the standard solid phase synthesis was impossible for ALA due to the stability problems associated with ALA in the presence of nucleophilic and / or basic moieties [102, 226]. Fmoc-ALA on Fmoc deprotection/coupling step of solid phase synthesis, got cylized (intra-molecular cyclization) to form stable six-membered 2,5-dioxo piperidine. Similar observation was made for Fmoc δ- amino valeric acid also, which got cyclized to form valerolactam while deprotecting Fmoc from wang linker [226] as shown in scheme33. B) Another reason of difficulty with ALA is dimerisation of two molecules of ALA leading to intermolecular cyclization in the presence of base to give various products like dihydro pyrazine, pyrazine, pseudo porphobilinogen etc as shown in scheme 34. This difference of reactivity in ALA compared to other δ-amino acids is due to its unique keto group.



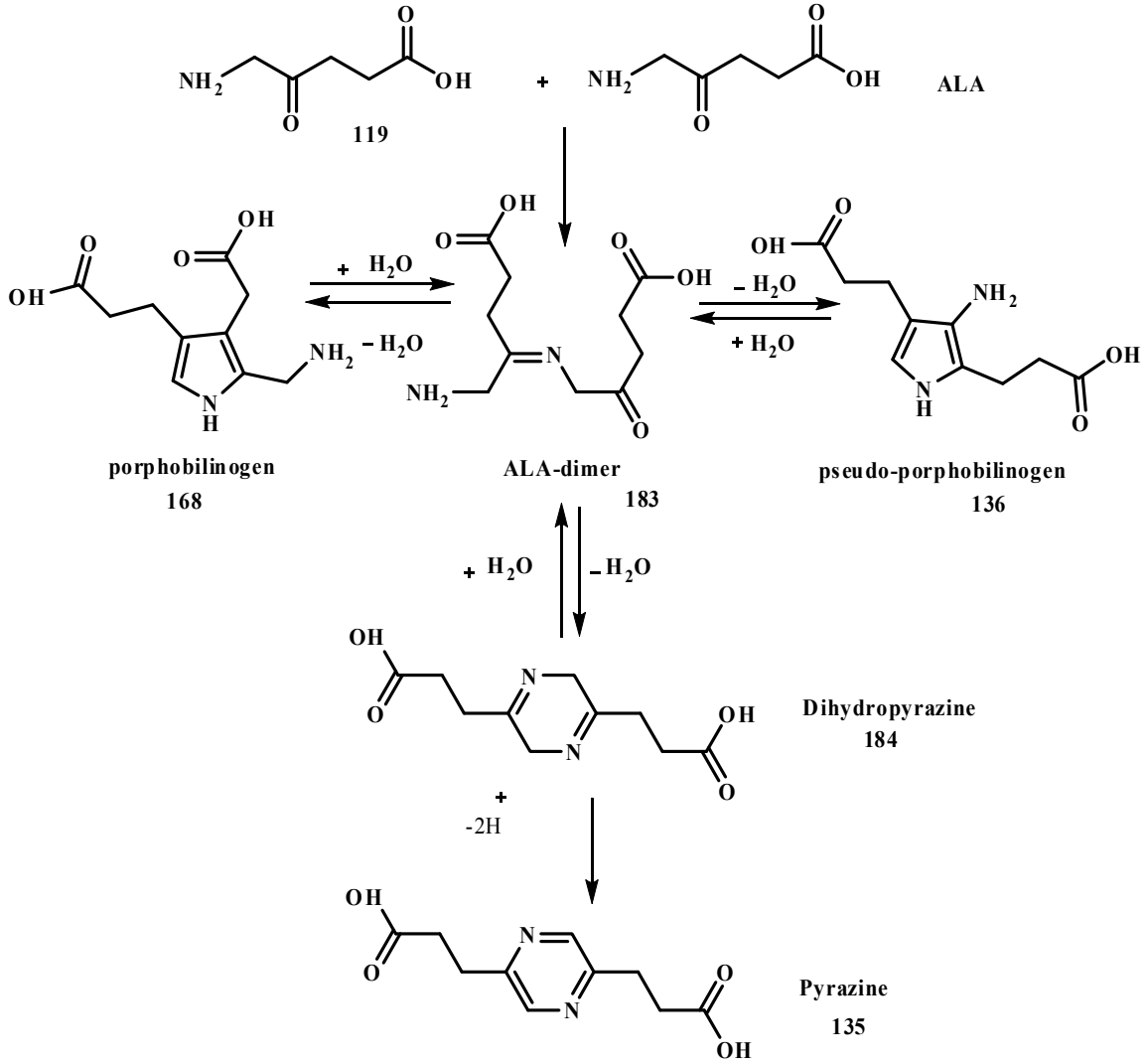
Scheme 33: Valerolactam formation from aminovaleric acid joined to Wang linker

3. The synthesis of amino group protected ALA- acid chloride was not achieved whereas similar acid chlorides have been reported for various synthetic δ- amino acids except for δ- amino valeric acid. The synthesis of acid chlorides from Fmoc

δ- amino valeric acid and benzoyl δ- amino valeric acid were either impossible or very difficult [227-229].

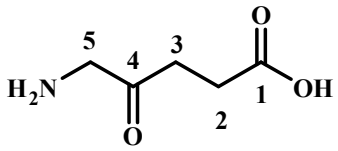
4.3. Stability of ALA and ALA derivatives

Several studies indicated that ALA reacts in aqueous solutions to yield several condensation products [87-91, 230-233]. A 1% solution of ALA at pH 7.53 can be predicted to have a shelf-life ($t_{90\%}$, the time it takes for the substance to degrade 10%) of 1.9 h at 20^0C. The shelf-life ($t_{90\%}$) of 10% ALA at pH 7.53 can be predicted to be as short as 10 min at 20^0C [91]. Even in freezers at -20^0C breakdown of ALA occurs [89]. Conversion to ester derivatives does not eliminate the problems of instability, introducing the potential formation of lactam type derivatives as shown in scheme 2 [108]. In aqueous solutions buffered to a physiological pH (pH=7), ALA dimerises to give pyrazine derivatives while at higher pH pseudo-porphobilinogen may be formed. Analytical methods like reverse-phase chromatography, capillary electrophoresis etc were developed to quantify ALA and other degradation products [90, 234].



Scheme 34: Condensation products of ALA on dimerisation

4.4. ALA functionalization



Scheme 35: Numbering of carbons in δ-aminolevulinic acid (ALA)

1. C1 (Acid moiety): The C1 carbon is sp^2 hybridized. The acid moiety can be derivatized as ester by reacting with an alcohol. Also it can be coupled with an amino group to form peptide. Though, the acid moiety can be converted to thiol acid or dithio acid or thiol ester or phosphonate or unsaturated alkyl ester, there are very few literature reported on these derivatives [103, 235, 236] . Majority of the reported literature on ALA deals with esterification of the acid moiety of ALA. This is because of the presence of cellular esterases enzymes which can release ALA from ALA derivative prodrug.

2. C2, C3 and C5 (methylene moiety): Though transformations on these sp^3 hybridized carbons are possible, they are not performed as the compounds have to be stable and need a mechanism to release ALA molecule inside the cells for photodynamic activity.

3. C4 (keto moiety): Ketone group can be either transformed into alcohol or thio ketone. But as the keto group in ALA is essential for the formation of PBG, the next intermediate in heme biosynthesis, there is not a single report in the literature (searched Scifinder Scholar with structure mode) where these derivatives are used for ALA-PDT.

4. Amino moiety: The peptidic coupling of the amino group is the useful transformation for ALA-PDT as the peptidases enzymes in cells can cleave this and release ALA. When the amino group is not protected, it is always synthesized in salt form. All the literature reported so far in ALA-PDT keeps the free amine either as hydrochloride salt or trifluoroacetate salt except one where a p-toluene sulfonate salt was made [237]. The salt formation of the free amine is done to prevent any intramolecular cyclization due to free amino group as shown in scheme 34.

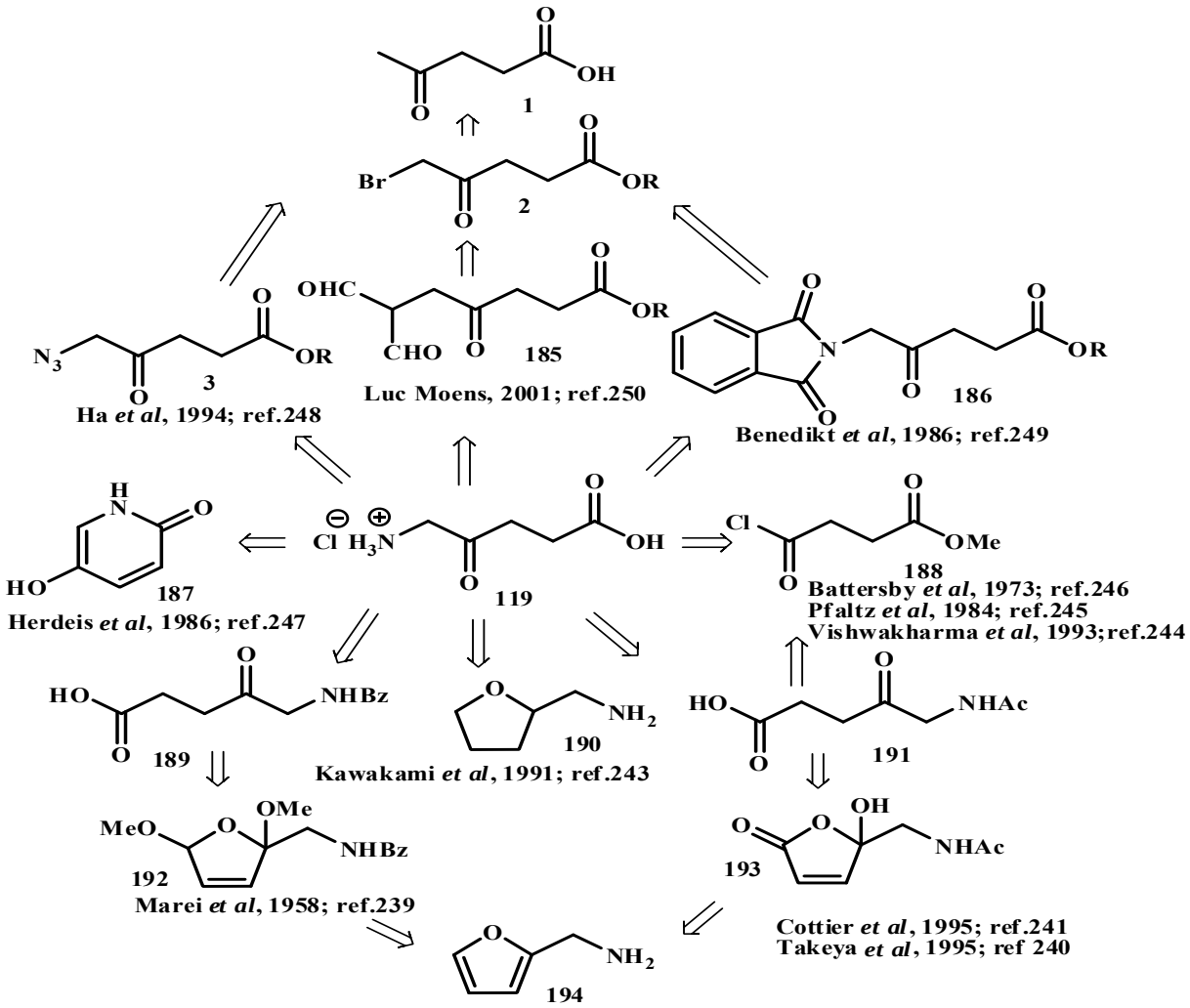
We followed the carbon mumbering of ALA as mentioned here in scheme 35, for all our ALA derivatives.

4.5. Synthesis of ALA-based starting material

In the synthesis of ALA derivatives as prodrugs for ALA-PDT, most of the reported literature utilized aminolevulinic acid hydrochloride (ALA-HCl) as starting material with the exception of two reports [103, 110] where they utilized Boc-aminolevulinic acid (Boc-ALA).

4.5.1. ALA-HCl as starting material

In the literature, synthesis of ALA-HCl has been carried out by various methods as shown in scheme 36.



Scheme 36: Synthesis of ALA by various routes- An overview

ALA can be synthesized (scheme 36) from the following starting materials: (A) furfuryl amine **194** (B) tetrahydrofurfuryl amine **190** (C) succinic acid (D) 2-hydroxy pyridine (E) levulinic acid **1**. One of the first and relatively simple routes was conversion of N-benzoylfurfurylamine to ALA [238]. This route starts with the formation of dihydrodimethoxyfuran **192** by an electrochemical variant of the Clauson-Kass oxidation. Catalytic hydrogenation of this hemiacetal led to a tetrahydrofuran that after oxidation and hydrolysis resulted in the formation of ALA. A similar pathway is the oxidation of 5-acetamido-methylfurfural [239-241]. This generates a butenolide **193** which after selective reduction of the hydroxyl group followed by ring opening gives acetyl protected

ALA. Also ring opening of the phthalimide of tetrahydrofurfurylamine **190** by oxidation led to ALA [242]. Another precursor for ALA is succinoyl chloride monomethyl ester **188** which can be transformed to ALA via acyl cyanide [243-245] . A strategy involving hydrolytic ring opening of lactam which was obtained by hydrogenation of 5-hydroxy-3-pyridone **187** has been reported [246]. This 5-hydroxy-3-pyridone can be synthesized from 2-hydroxy pyridine. Starting from levulinic acid **1**, activation of C5-position has been made by direct bromination. From 5-bromolevulinate **2**, there are three ways to synthesize ALA: via azide derivative **3** [247], via phthalimide derivative **186** [248] and via diformylamide derivative **185** [249].

The synthesis of ALA-HCl was performed in our group [250] in 3 steps (overall yield=25%) as per the method of Benedikt *et al* [248]. The first step was bromination of levulinic acid in methanol solvent to give major product 5-bromo methyl levulinate which was then converted to 5-(phthalimido) methyl leulinate. This on acidic hydrolysis afforded ALA-HCl.

4.5.2. Disadvantages of ALA-HCl

With our group's experience in the field of ALA prodrug synthesis, we found that ALA-HCl as starting material has following disadvantages for synthesizing ALA derivatives:

1. ALA-HCl is a highly polar compound, which makes the common purification techniques like column chromatography, crystallization etc very difficult once it is derivatized as ester.
2. ALA-HCl being highly hygroscopic has difficulty in handling. It has to be stored in freezer.
3. It is less stable even at neutral pH conditions and tends to form side products like pyrazine, pseudo porphobilinogen etc [87, 89-91, 108].
4. Besides the above mentioned problems, ALA-HCl has reactivity problem e.g.: For the efficient synthesis of esters, one requires activated form of acids which can be stable and be storable as well.

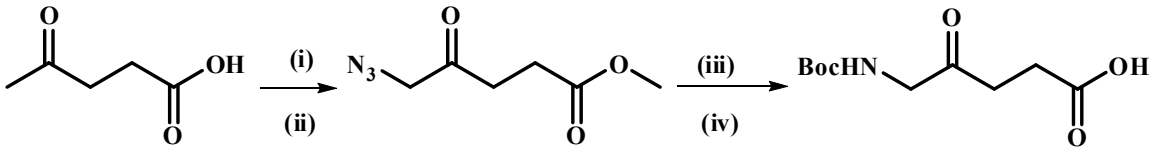
ALA acid chlorides though active, are not stable and could not be either isolated or stored as it could form lactam-type impurities[108]. Our trials to synthesize activated ester (pentafluoro phenyl ester) of ALA-HCl were futile.

5. Results and Discussion

5.1. Boc-ALA and activated esters as starting material

As ALA-HCl has above mentioned disadvantages, we wanted to synthesize stable, easy to handle and also reactive species of ALA-HCl which could be used for synthesizing ALA based derivatives. A solution for this is to synthesize amino protected ALA. As mentioned in chapter 4.1.2, Fmoc protected ALA is not an attractive solution because ALA gets cyclized when Fmoc group is deprotected. A solution for this can be a protecting group which is stable, easy to synthesize and stable during transformations of the acid moiety of ALA. Also the protecting group should be easy to cleave without affecting esters /amides functionalities since ALA is derivatized usually as esters or amides. 'Boc' protecting group is the best choice in this case. The 'Boc' group protection of ALA renders it stable, easy to handle and helps in purification techniques. The 'Boc' group is also easy to cleave under acidic conditions which transform the free as well very reactive amine of ALA to stable ammonium salt. Besides this, Boc-ALA can be converted to stable 'Boc' protected activated ester of ALA which can be efficient in coupling with wide variety of alcohols. So we decided to synthesize Boc-ALA

We synthesized Boc-ALA in four steps (scheme 37) starting from levulinic acid (overall yield = 33% to 30%) which is efficient and also different from the reported ones. The reported literatures synthesized Boc-ALA from ALA-HCl in 60% yield, but the best yield available in literature to synthesize ALA-HCl from levulinic acid is 40%. Therefore, with the reported literatures, an overall yield of 24% can be achieved for synthesizing Boc-ALA from levulinic acid where as our scheme achieved 30 to 33% overall yield.

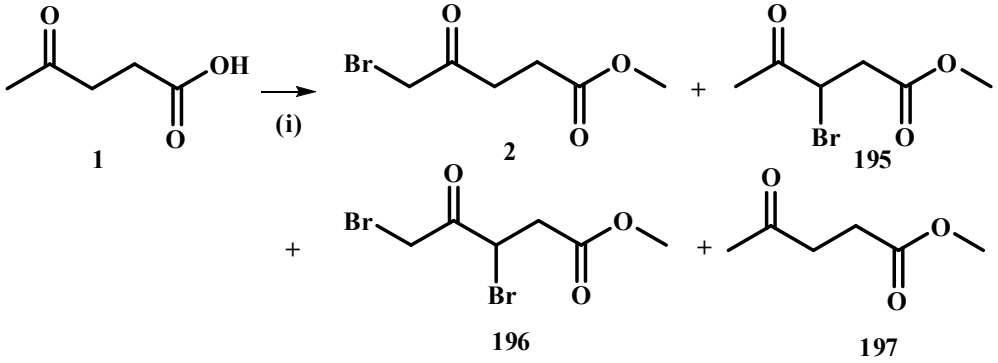


1 3 5

Reagents and conditions: (i) Br_2, MeOH, reflux, 1.5 h, 57%; (ii) NaN_3, rt, 1 h, 95%; (iii) H_2/Pd, Boc_2O, 12 h, rt, 74%; (iv) *Pig liver esterase* (PLE), phosphate buffer, pH=8, rt, 7 h, 85%;

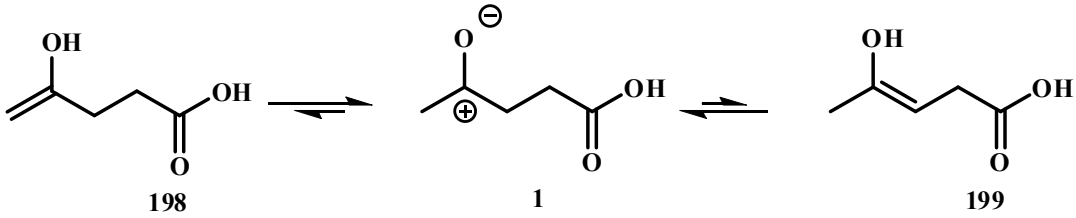
Scheme 37: Synthesis of Boc-ALA and Azidolevulinic acid

5.1.1. 5-Bromo methyl levulinate

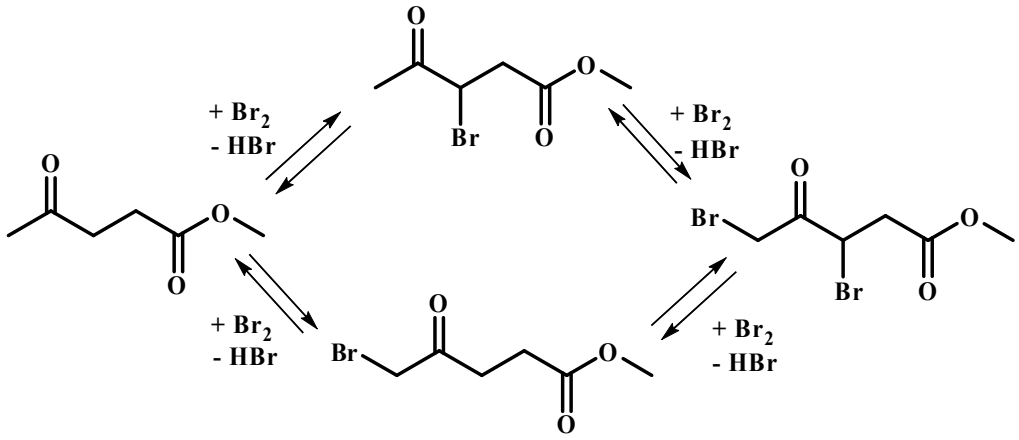


Reagents and conditions: (i) Br_2, MeOH, reflux, 1.5 h, 57%.
Scheme 38: Bromination of levulinic acid

Levulinic acid **1** was brominated with MeOH as solvent (scheme 38). In a typical reaction, levulinc acid was dissolved in MeOH, cooled and then bromine was added drop wise. After, the reaction mass was refluxed for 1.5h. [247, 249, 251-253]. The bromination is induced by traces of acid (HBr) that catalyze enolization of keto group (scheme 39), the 5-bromo ester **2** is probably formed first (kinetic product), whereafter it slowly isomerizes to the 3-bromo isomer **195** under the influence of the dissolved HBr. Other by-products from this reaction are 3,5-dibromo ester **196** and methyl levulinate **197**. All the products are obtained as methyl esters as a result of HBr catalyzed esterification. There are reports which found that the selectivity of bromination is controlled by an equilibrium (scheme 40) that is supported by the presence of HBr in solution [254-256].



Scheme 39: Enolization of levulinic acid



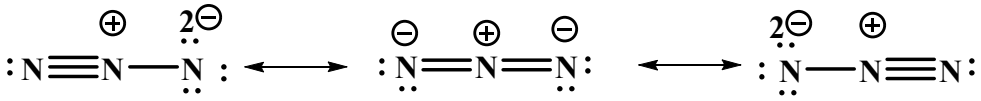
Scheme 40: Equilibrium of brominated products of levulinic acid

Besides, the problem of erratic fluctuations of boiling points which we experienced (experienced by other researchers also [249] which they attribute due to traces of HBr), we also found that some of the fractions collected at the end contained methyl levulinate along with our desired 5-bromo ester. This is bizarre as the difference between the boiling point of methyl levulinate and 5-bromo ester was approximately 30^0C at 0.05mbar and it normally gets collected during the initial fractions. This can be explained from the study carried out by Rappe [254] on rearrangement of 3-bromo levulinic acid to 5-bromo levulinic acid. The first step of this rearrangement is replacement of a halogen atom by a hydrogen atom, a reduction performed by HBr. Thus, one can say that the presence of methyl levulinate along with the 5-bromo ester factions was due to the reduction occurring at high temperature by traces of the dissolved HBr.

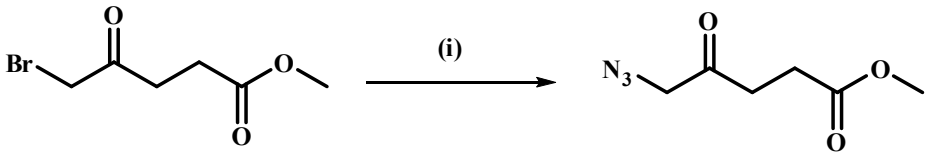
The kinetically favorable product 5-bromo methyl levulinate **2** was isolated by a fractional distillation using a widmer column as the difference between the boiling points of 5-bromo methyl levulinate and 3-bromo methyl levulinate was approximately 10^0C at 0.05mbar. We also tried to avoid this very critical fractional distillation by attempting crystallization at low temperatures (-10 to -30^0C), which were not successful. The ratio of methyl levulinate: 5-bromo methyl levulinate: 3-bromo methyl levulinate: 3, 5-dibromo methyl levulinate after the reaction was 5: 26: 65: 4. We followed the procedure of Manny *et al* [253] who studied this bromination using various solvents and reaction conditions. The authors concluded that ratio of 5-bromo methyl levulinate: 3-bromo methyl levulinate was highest with MeOH as solvent, using 1 molar equivalent Br_2 and refluxing the reaction for 1.5 h. Though there are other reported methods [256-259] of synthesizing 5-bromo methyl levulinate some of which are efficient, they were not favored because they either use starting material which is not commercially available [257] or use diazomethane [256, 258, 259] whose handling requires special apparatus. The bromination using NBS instead of Br_2 is not efficient [260]. Recently, there was a report where unsymmetrical ketones were non selectively di brominated and then selectively debrominated to give terminally brominated compound [261]. This method might be able to give selectively 5-bromo methyl levulinate.

5.1.2. 5-Azido methyl levulinate

There are many ways of introducing amino group to the halogen group like via nitro, via azide etc. The requirement of a nitrogen nucleophile is such that the alkylation of amino group stops with the first alkylation as amines are prone to polyalkylation. The azide ion N_3^- ion is a good nucleophile in this respect and also stabilized by resonance forms. This azide anion is a weak base (pKa (HN_3) = 4.68) and is a good nucleophile and could react with halogen easily by a SN2 attack.



Scheme 41: Resonance forms of azide ion

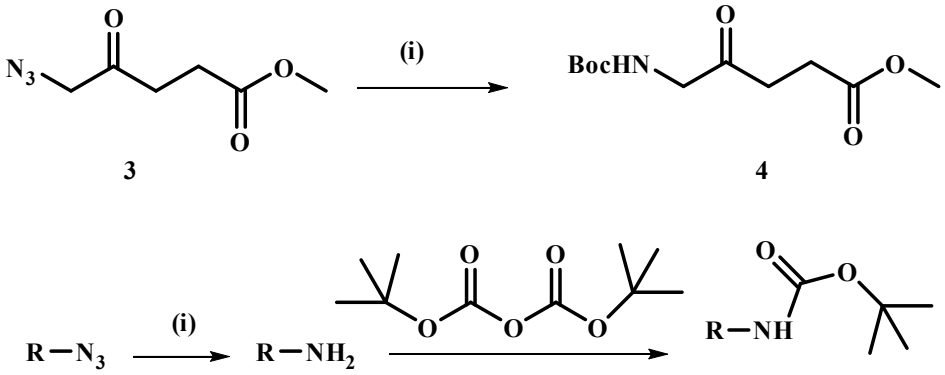


2 **3**

Reagents and conditions: (i) NaN_3, rt, 1 h, 95%.
Scheme 42: Synthesis of 5-azido methyl levulinate

Then, 5-bromo methyl levulinate was converted to 5-azido methyl levulinate **3** by a SN2 attack of azido on the carbon bearing bromine with an excellent yield of 95% (scheme 42). The excess NaN_3 used in this reaction need to be destructed whose protocol is available in the experimental section.

5.1.3. 5-(Boc) amino methyl levulinate



Reagents and conditions: (i) H_2/Pd, Boc_2O, 12 h, rt, 74%.
Scheme 43: Hydrogenation and Boc protection of 5-azido methyl levulinate

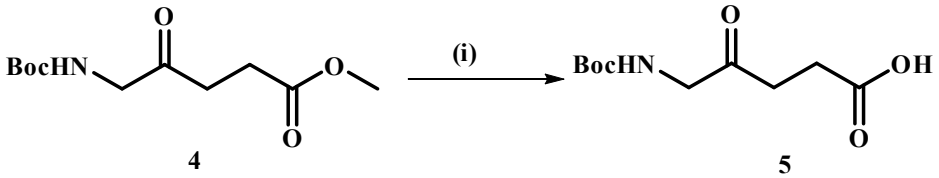
200 **201**

Boc-ON t-butyl aminocarbonate
(2-(tert-Butoxycarbonyloxyimino)-2-phenylacetonitrile)
Scheme 44: Other reagents for Boc protection

There are many reports of converting azido to Boc amino compound in one pot reactions [262-265]. The method of staudinger reaction [264] using triphenly phosphine had been tried before in our group [250] which gave very poor yield (47%). We followed the method of Saito *et al* [265] where azide is reduced by hydrogenolysis using H_2 and Pd over carbon in the presence of di-t-butyl dicarbonate (Boc_2O). This method (scheme 43) was very clean and efficient to afford 5-(Boc) amino methyl levulinate **4** in 70% yield. We also tried other reagents(scheme 44) such as Boc-ON **200**, t-butyl aminocarbonate

201 [266] instead of Boc_2O and also Boc_2O in the presence of DMAP in order to achieve higher yield, but were not successful.

5.1.4. 5-(Boc) aminolevulinic acid (Boc-ALA)



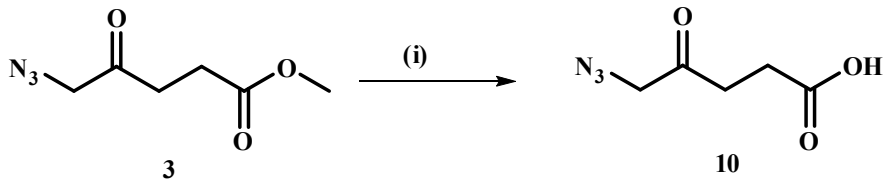
4 5

Reagents and conditions: (i) *Pig liver esterase* (PLE), phosphate buffer, pH=8, rt, 7 h, 85%.

Scheme 45: Hydrolysis of Boc-ALA-OMe

The hydrolysis of 5-(Boc) amino methyl levulinate (scheme 45) was performed in the presence of enzyme *Pig liver esterase* (PLE). 5-(Boc) amino methyl levulinate was treated with PLE in phosphate buffer (containing 0.6 g NaH_2PO_4 + 13.5 g Na_2HPO_4 in 1 litre deionised water). The liberation of acid was neutralized using 0.1N NaOH solution by means of a pH-stat (pH=8). The reaction was connected with pH-stat was stirred at ambient temperature till the disappearance of starting material by TLC. The crude on purification by column chromatography led to 5-(Boc) aminolevulinic acid (Boc-ALA) **5** in 63% yield.

5.1.5. Azidolevulinic acid as starting material



3 10

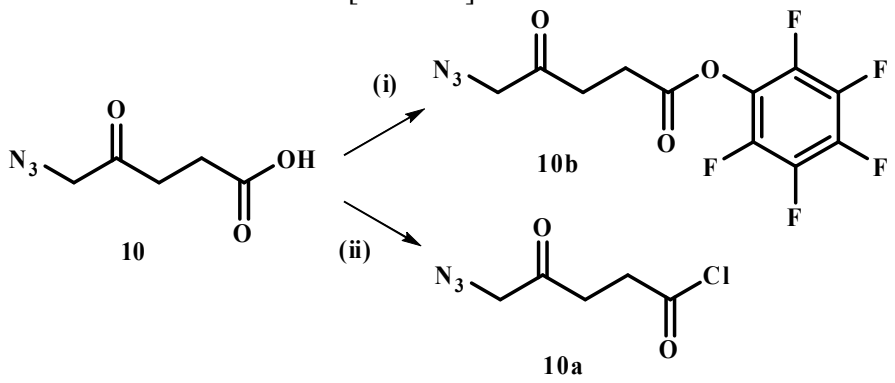
Reagents and conditions: (i) *Pig liver esterase* (PLE), phosphate buffer, pH=8, rt, 7 h, 87%.

Scheme 46: Azidolevulinic acid from azido methyl levulinate

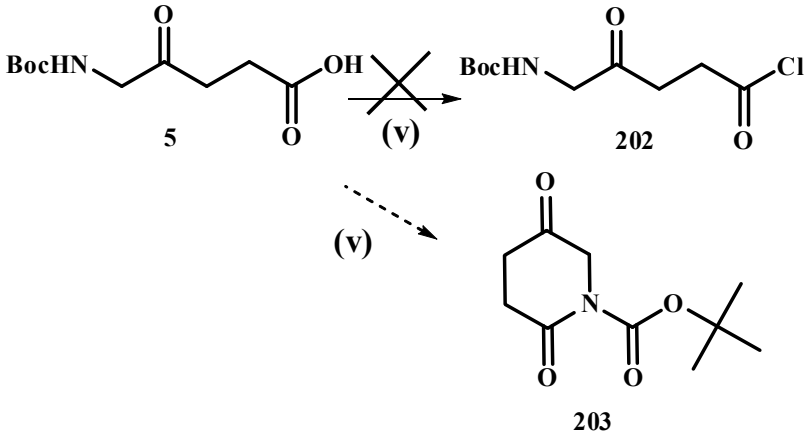
One of the intermediates in synthesizing Boc ALA is 5-azido methyl levulinate which can be utilized for making efficient peptide bond with any activated ester when subjected to catalytic hydrogenation. The hydrolysis product of this ester (scheme 46), azidolevulinic acid **6** can also be used in esterification reactions because azido group is a masked functionality of amine so that the amine group could be obtained by catalytic hydrogenation. The hydrolysis of 5-azido methyl levulinate was performed in the presence of enzyme *Pig liver esterase* (PLE). 5-azido methyl levulinate was treated with PLE in phosphate buffer. The liberation of acid was neutralized using 0.1N NaOH solution by means of a pH-stat (pH=8). The crude upon purification by column chromatography, led to 5 in 83 % yield.

5.1.6. Activated esters

We synthesized activated ester of azidolevulinic acid, pentafluorophenyl ester of azidolevulinic acid **10b** (scheme 47). The azidolevulinic acid was easily converted to the more reactive acid chloride **10a** [267]. This can be used for coupling reactions though this acid chloride could not be isolated because of its thermal instability. Our attempts to synthesize Boc-ALA-acid chloride **202** (scheme 48) were not successful even though acid chlorides of many Boc protected α-amino acids are very common and reported [268-270]. Boc-ALA-acid chloride is not stable and it could get cyclized to form 1-Boc-2,5-dioxopiperidine **203** (lactam) as similar cyclization were reported to be occurred when Fmoc-aminovaleric acid and Benzoyl aminovaleric acid were subjected for their transformation to acid chlorides [227-229].



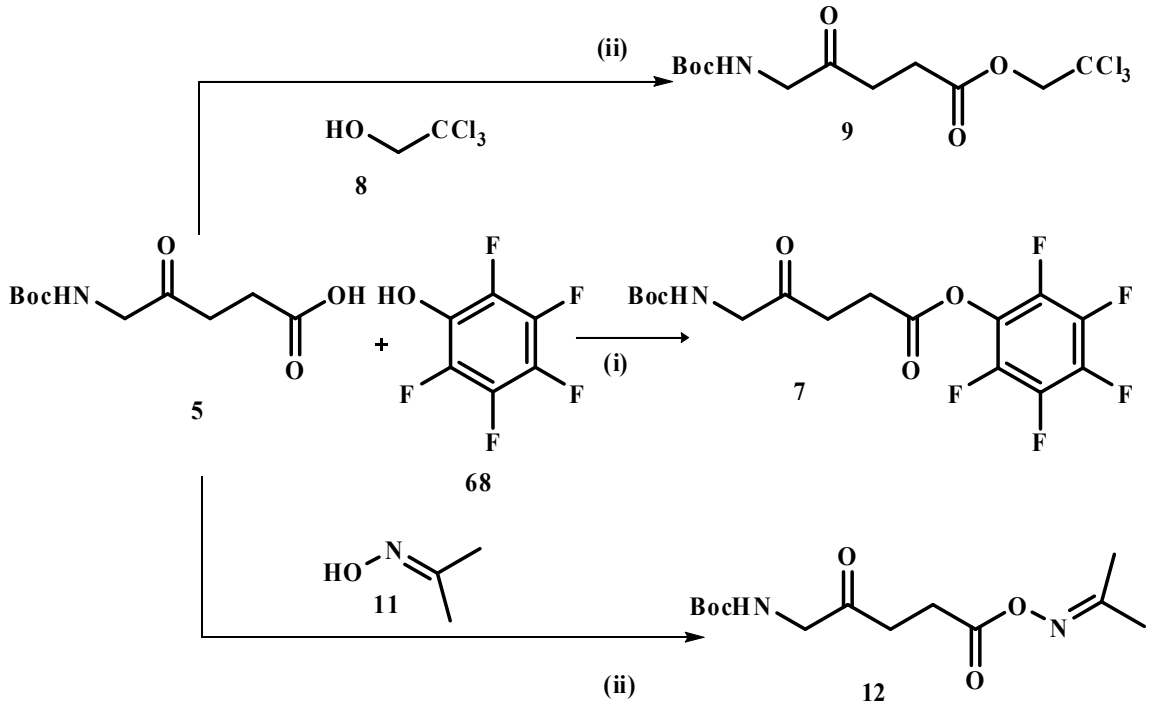
Reagents and conditions: (i) DCC, rt, 2 h, C_6F_5OH; (ii) $SOCl_2$, reflux, 1 h.
Scheme 47: Activated ester and acid chloride of azido levulinic acid



Reagents and conditions: (i) $SOCl_2$, reflux, 1 h.
Scheme 48: Failed reaction of Boc-ALA acid chloride

We synthesized three activated esters from Boc-ALA **5** (Scheme 49): Boc-ALA-trichloroethyl ester **9** (Boc-ALA-TCE ester), Boc-ALA-pentafluorophenyl ester **7** (Boc-ALA-PFP ester) and Boc-ALA-oxime ester **12**. Boc-ALA-PFP ester was synthesized by DCC mediated coupling of Boc-ALA **5** with pentafluorophenol **68**. Boc-ALA-TCE ester

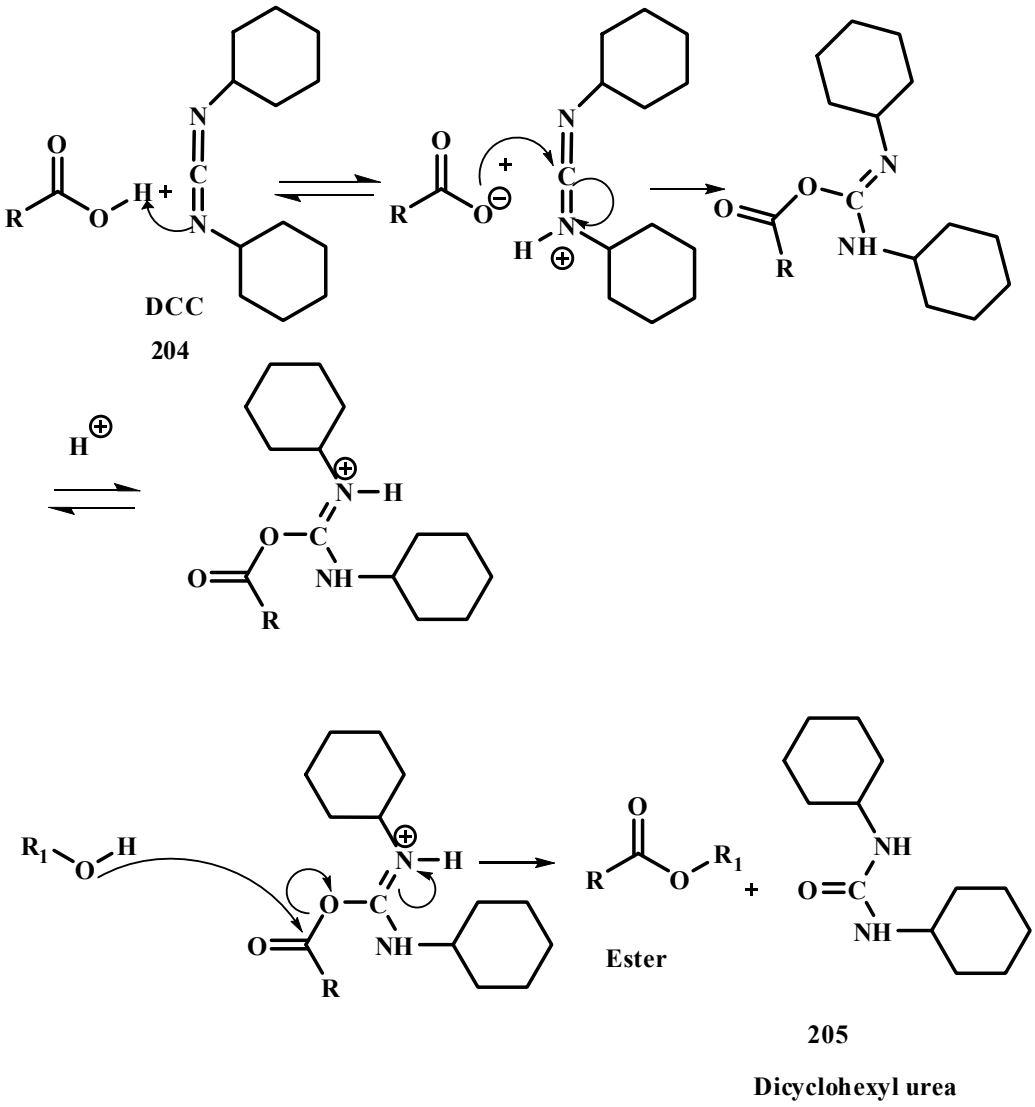
and Boc-ALA-oxime ester were synthesized by EDCI /DMAP mediated coupling of Boc-ALA with trichloro ethanol **8** and acetone oxime **11** respectively. All these activated esters which were synthesized for the first time were utilized for various esterification reactions below to obtain various ALA-glycoconjugates, Vitamin-ALA conjugates, nucleoside-ALA conjugates, ALA esters, ALA-pseudo peptides etc.



Reagents and conditions: (i) DCC, rt, 2 h; (ii) EDCI, DMAP, rt, 6 h.
Scheme 49: Activated esters of Boc-ALA synthesis

5.1.6.1. Mechanism of DCC coupling:

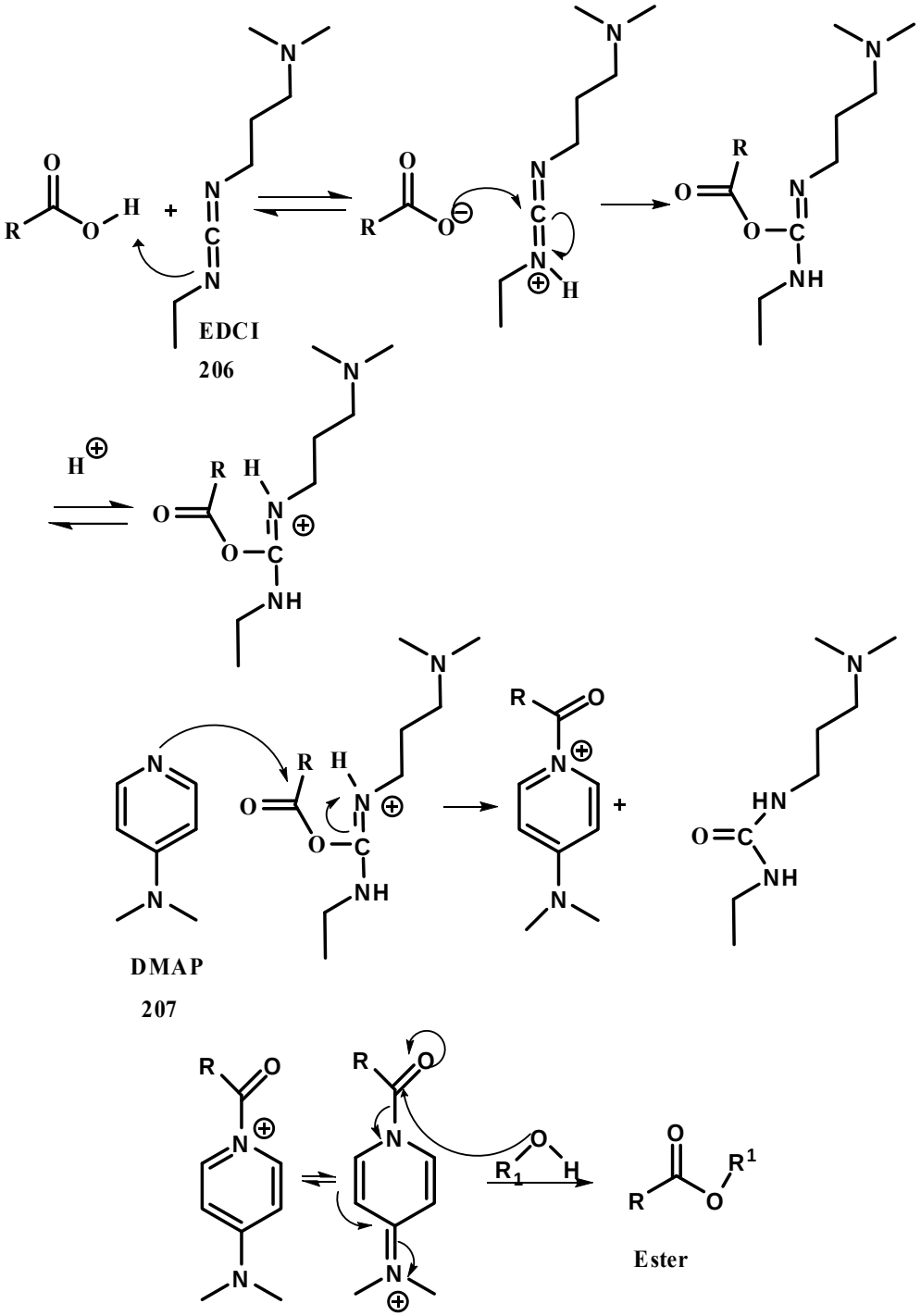
By DCC **204** (dicyclohexyl carbodimide) mediated coupling, the acid is converted to a better leaving group. The mechanism (scheme 50) is similar to nucleophilic catalysis mechanism. DCC is converted to DHU **205** (dicyclohexyl urea). Evidence for this mechanism is the O-acyl ureas synthesized gave esters when they reacted with alcohols catalyzed by acids.



Scheme 50: DCC coupling mechanism

5.1.6.2. Mechanism of EDCI/ DMAP coupling:

This mechanism using EDCI **206** (scheme 51) is similar to DCC except that instead of the direct nucleophilic attack of alcohol on acid-urea complex, here the attack comes from DMAP **207**. This reactive amide of DMAP is later attacked by alcohol to form ester.



Scheme 51**:** EDCI / DMAP coupling mechanism.

5.2. Biological Procedures and tests

The biological tests for the evaluation of PpIX induction by all the precursors we synthesized were performed by the group of Dr.L.Juillerat-Jeanneret and her PhD student Mr.F.Schmitt, University Institute of Pathology, CHUV (Centre Hospitalier Universitaire Vaudois), Lausanne, Switzerland.

5.2.1. Cell lines and Culture Conditions

Human colon adenocarcinoma HT29 and SW480, breast carcinoma T47D and MCF7, lung carcinoma A549, glioblastoma LN18, human microvascular endothelial HCEC cell lines and human primary fibroblasts were used for testing all ALA-conjugates.Cells were grown in DMEM medium (Gibco BRL) with phenol red containing 10 % FCS (Gibco BRL) and penicillin/streptomycin, at 37 °C and 6 % CO_2. They were routinely maintained every week in 25 cm² flasks using trypsinization for plate detachment. All the handling with cell plates was performed in sterile hood.

5.2.2. PpIX Fluorescence Measurements

Cells were cultured in 48 (8 × 6) well plate (Corning, NY, USA) 48 h prior to experiments in complete culture medium. The cell plates were taken out from the incubator and the growth medium above the cells from each well of the plate were removed by sterile pipette. Confluent cell layers in 48-well plates were then exposed to the indicated concentrations (3 wells per concentration) of the different PpIX precursors in 250 μL/well DMEM medium without phenol red and free of FCS. Increase of fluorescence was measured in a fluorescent multi-well reader CytoFluor Series 4000 (PerSeptive Biosystems, MA, USA) at 37 °C using excitation filter at λ = 409 nm and emission filter at λ = 640 nm.

5.2.3. Determination of Cell Viability

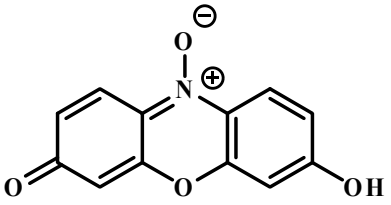
Cell viability was determined after 24 h exposure to ALA derivatives using the Alamar Blue (AB) assay or the MTT assay.

5.2.3.1. Alamar Blue assay

Alamar Blue (Serotec) assay is the most simple and rapid test, whereby 10% of the commercially available solution is added to the cell medium and measured either by colourimetry or fluorimetry. Alamar Blue is nontoxic to cells. The oxidized form **208** which is nonfluorescent, is reduced to a pink fluorescent dye **209** in the medium by cell activity (likely to be by oxygen consumption through metabolism).There is a direct correlation between the reduction of Alamar Blue **208** (or resazurin) in the growth media and the quantity/proliferation of living organisms. Unlike MTT assay, no cell lysis is necessary for alamar blue assay.

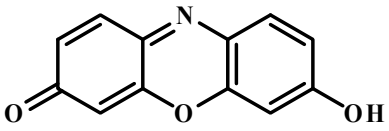
The basic assay steps in a fluorescent multi-well reader CytoFluor Series 4000 (PerSeptive Biosystems, MA, USA) are as follows: (A) 25 μL/well of resazurin solution were added on cells in culture medium in 48-well plates. (B) Incubation for 2 h at 37°C.

(C) Measurement of the fluorescence using excitation filter at λ = 530 nm and emission filter at λ = 580 nm.



Resazurin
(oxidised form)

208



Resorufin
(reduced form)

209

Scheme 52: Structures of resazurin and resorufin (alamar blue assay)

5.2.3.2. MTT assay

MTT assay evaluates the mitochondrial succinate dehydrogenase activity by the reduction of 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazolium bromide **210** (MTT) to formazan **211**, an insoluble compound which absorbs at 540 nm (scheme 53). MTT was added to each well at 250 µg/mL and incubation was continued at 37 °C for 2 h. The supernatant was removed, the cell layer was dissolved in *iso*-propanol/HCl 0.04 N and absorbance at 540 nm was quantified in a multiwell plate reader iEMS Reader MF (Labsystems, Bioconcepts, Switzerland).

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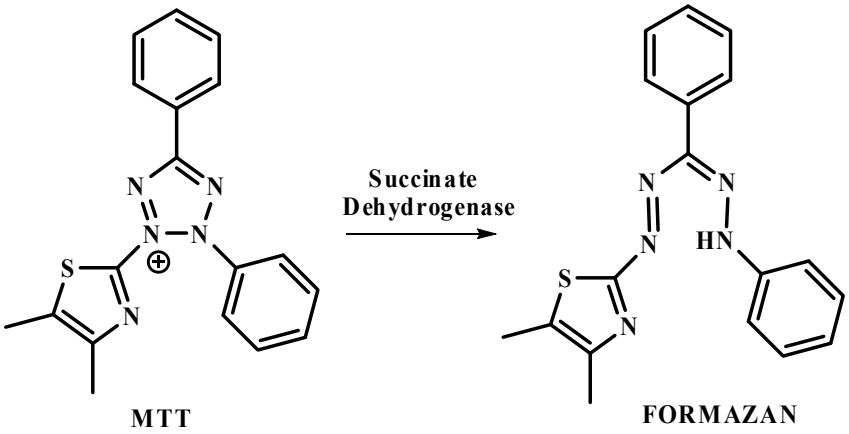


MTT

210

FORMAZAN

211

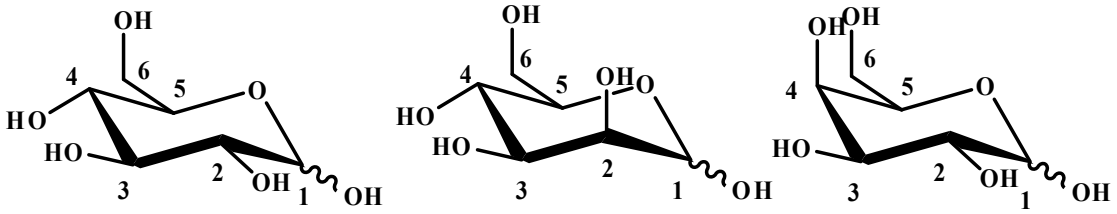
Scheme 53: Structures of MTT and Formazan (reduced form)

5.2.4. Microscopy

SW480 cells were grown on histological slides in complete medium until 25% of confluence and then, 1-α-Glc-ALA (2mM) was added for further 24h. Slides were washed with PBS, mounted and analyzed on a fluorescent microscope Axioplan2 (Carl Zeiss AG, Feldbach, Switzerland) using 535 ± 25 nm excitation light (BP 510-560, FT 580, LP 590).

5.3. Synthesis of ALA-Glycoside Derivatives (Sugar-ALA derivatives)

For the substitution of ALA in sugars, we chose three simple sugars namely glucose; mannose and galactose because various transporters of these sugars are available in cells (refer chapter 2 "Biological background behind Prodrugs' design-A review of literature knowledge"). These transporters can help for the effective internalization of ALA - glycoconjugates. Three types of ALA glycoside derivatives were synthesized. First, the acid moiety of ALA was esterified with C6 hydroxyl group of the three sugars mentioned above. Second, ALA was esterified with C1 hydroxyl group of sugars to obtain α-glucoside, α-mannoside and β-galactoside derivatives. Finally, ALA was esterified with C1 hydroxyl group of sugars via an ethylene glycol linker to obtain α-glucoside, α-mannoside and β-galactoside derivatives.



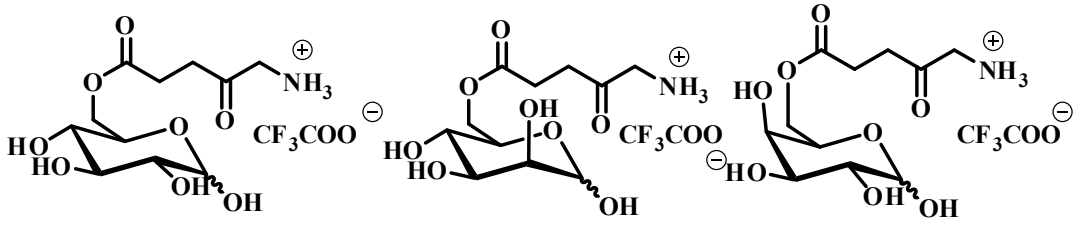
13 24 27

Glucose (α or β) Mannose (α or β) Galactose (α or β)

Scheme 54: Monosaccharides chosen for ALA substitution

5.3.1. Synthesis of 6′-O-(ALA) sugars (First generation ALA-glycoconjugates)

We decided to synthesize our first generation ALA- glycoconjugates where ALA is linked as an ester with C6 hydroxyl group of the three monosaccharides namely glucose, mannose and galactose (scheme 55).



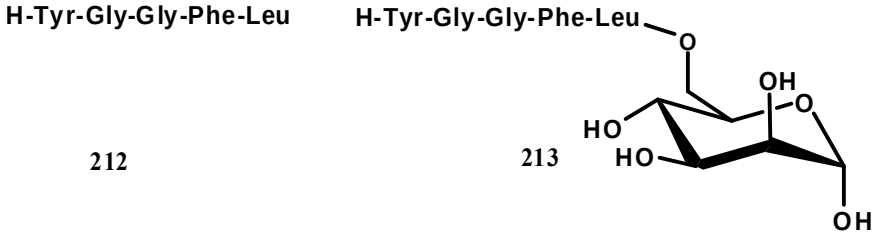
15 26 29

6′-*O*-(ALA) glucose 6′-*O*-(ALA) mannose 6′-*O*-(ALA) galactose

Scheme 55: 6′-O-(ALA) sugars (first generation ALA-glycoconjugates)

Regioselective acylation of sugars is a fundamental and difficult task in organic chemistry [271]. Even preferential acylation of primary (C6) over secondary hydroxyl groups can only rarely be efficiently carried out with free sugars [271, 272]. Although many reports are available in literature for the synthesis of ester-carbohydrate linkages

over C6 hydroxyl group by either chemical or enzymatic ways [273-317], there are very few reports of synthesis of amino acid esters of carbohydrates [318-323]. This is more surprising given the fact that several biofunctional molecules, such as enkephalin-carbohydrate conjugates **213** (scheme 56) that influence opioid activity [275, 308-310, 324], exhibit antiviral activity against HIV-1 [325] and also modulate fibroblast and melanoma growth [326], are α-amino acid esters of carbohydrates. Enkephalins **212** are pentapeptides containing either Tyr-Gly-Gly-Phe-Leu or Tyr-Gly-Gly-Phe-Met sequence. They are found in the thalamus of the brain and in some parts of the spinal cord that transmit pain impulses. They act as analgesics and sedatives in the body and appear to affect mood and motivation. Carbohydrate–peptide conjugates connected by potentially metabolisable, sacrificial linkages, such as esters, have high potential utility as prodrugs in which the glycan moiety affords both protection and specific transport properties [324].



Scheme 56: Structure of enkephalin **212** and enkephalin-carbohydrate conjugates **213**

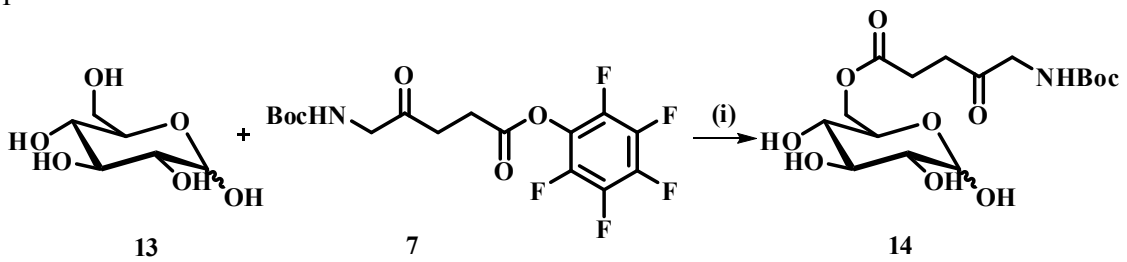
There are four main ways of carrying out the synthesis of ester-carbohydrate linkages over C6 hydroxyl group: **A)** condensing an unprotected sugar with an acid by DCC coupling [312] or using tributylstannyloxide [284-286] or via cobalt chelates [282, 283] or via mitsunobu reaction [279-281] **B)** condensing an unprotected sugar with an activated ester using imidazole [274, 275, 307-311] or using base catalysis [304-306, 327] **C)** Following stepwise protection methodology with protecting groups [313, 314] such as benzyl [302, 303], boronate [297-300], isopropylidine [273, 294-296], benzylidine [293] etc. keeping the C6 hydroxyl group free for coupling with an acid moiety. **D)** Enzymatic regioselective acylation on C6 hydroxyl group by an activated ester [276-278, 287-292, 301, 316] and more specifically by an activated ester of an amino acid [318, 320, 321].

5.3.1.1. Synthesis of 6´-O-(ALA) glucose

We planned to synthesize the substitution of Boc-ALA on the primary hydroxyl (C6) of glucose and then deprotect the 'Boc' group to obtain the required 6´-*O*-(ALA) glucose as the 'Boc' group facilitates not only the ease of purifying the product by column chromatography but also in handling the product.

5.3.1.1.1. Preliminary attempts for 6′-O-(ALA) glucose (Imidazole catalyzed)

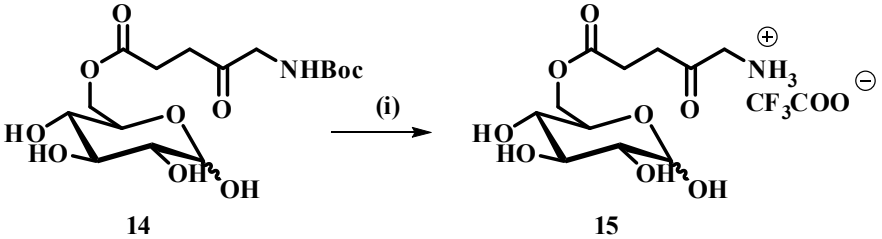
In the first attempt to synthesize 6′-*O*- (Boc-ALA) glucose **14**, Boc-ALA-PFP ester **7** was coupled with D-glucose **13** promoted by imidazole, an approach termed "accelerated activated ester" method [307-310]. In this method, 3 equivalents of sugar and 5 equivalents of imidazole were taken for each equivalent of activated ester. By this method, the attempts to isolate the pure product 6′-*O*- (Boc-ALA) glucose from the excess imidazole which had the same retention factor in TLC, have been failed. This was despite the various trials like crystallization, column chromatography, aqueous work-up, ion-exchange complexation etc which were either unsuccessful or hydrolyzes the product.



13 7 14

Reagents and conditions: (i) catalytic imidazole, rt, 12 h, 70%.
Scheme 57: Imidazole catalyzed synthesis of 6′-O-(ALA) glucose

Later, as a modified attempt of the first, Boc-ALA-PFP ester was coupled successfully with D-glucose in the presence of imidazole which was added in a catalytic quantity instead of excess (scheme 57). This coupling also works well with catalytic DMAP. Though this attempt of using either catalytic imidazole or DMAP takes longer reaction time (~12h), it was easy to isolate the product 6′-*O*- (Boc-ALA) glucose **14** unlike the former attempt. But this method gave the product 6′-*O*- (Boc-ALA) glucose approximately of 75% purity, rest of which contained other regioisomers Though this method did not give the pure regioselective product, it provided a quick manner to conduct preliminary biological tests in various cancer cell lines which was necessary to demonstrate if our fundamental concept of ALA –sugar conjugation ('proof of principle') can generate PpIX, so that we could proceed in this direction. 6′-*O*- (Boc-ALA) glucose **14** synthesized by the above method was deprotected for the removal of Boc group by TFA (scheme 58), to give 6′-*O*- (ALA) glucose **15**. This product, 6′-*O*- (ALA) glucose was tested in various cancer cell lines for the induction of PpIX generation and also cell survival (MTT evaluation) after 24 h exposure. 6′-*O*- (ALA) glucose **15** was able to generate higher levels of PpIX than that generated from ALA.



14 15

Reagents and conditions: (i) TFA, rt, 1h, 98%.
Scheme 58: 'Boc' deprotection

5.3.1.1.2. Preliminary evaluation of PpIX induction by 6′-*O*- (ALA) glucose

PpIX production was evaluated for 6′-*O*- (ALA) glucose **15** by measuring increase in fluorescence intensity in living cells at different times by incubating cells in medium containing ALA conjugate at concentrations from 0.1mM to 3 mM. Cells used for experiments were Human colon carcinoma HT-29 and SW 480, human breast T47D and MCF7 carcinoma, A549 lung carcinoma, LN18 glioblastoma cell lines and HCEC human immortalized brain endothelial cells

6′-*O*- (ALA) glucose induced strong PpIX production in all cell lines. The PpIX produced after 24h (figure7) show that all the concentrations of 6′-*O*- (ALA) glucose except 0.1mM induced higher level of PpIX than that induced by 3mM ALA. Also, the increase in PpIX level for 3 h (figure8) shows that 6′-*O*- (ALA) glucose with concentrations 3mM, 2mM and 1mM produce higher level of PpIX than that induced by standard ALA at any point of time.

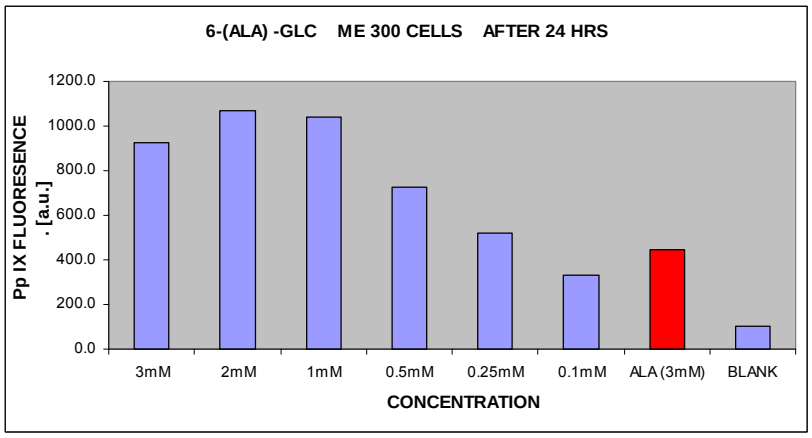


Figure 7: Comparison of PpIX induction between 6′-O-(ALA)-glucose and ALA after 24h

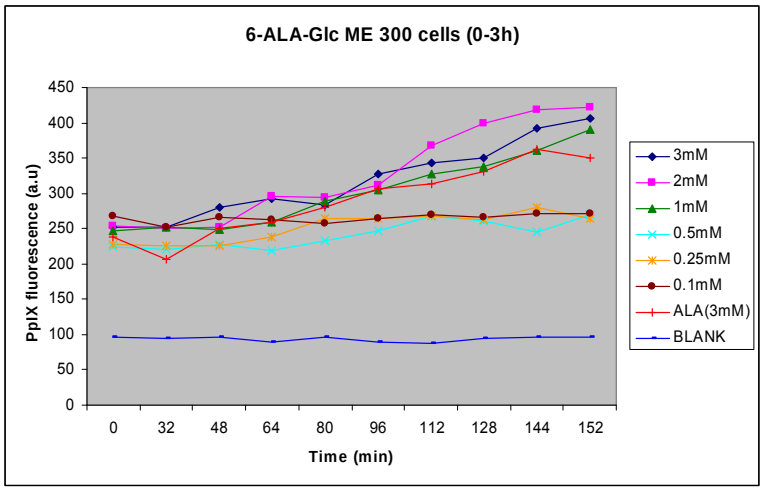
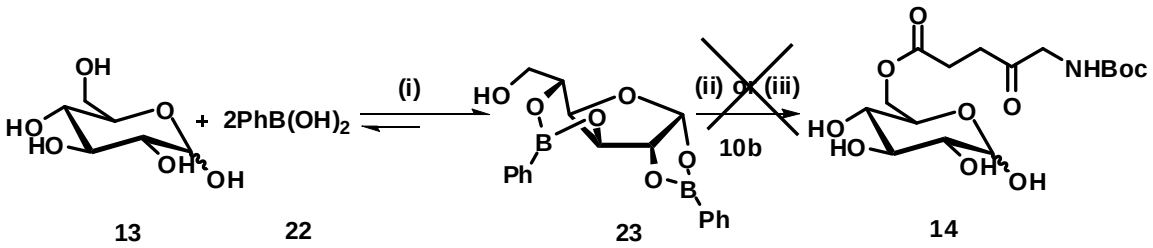


Figure 8: Increase in PpIX level between 6′-*O*-(ALA)-glucose and ALA during 3h

5.3.1.1.3. Attempts for pure regio isomer 6′-*O*-(ALA) glucose (Protection method 1)

Once this concept of ALA-sugar conjugation was demonstrated for PpIX generation, attempts to synthesize pure regioisomer 6′-*O*- (Boc-ALA) glucose **14** without any other regio isomer were tried using protection-deprotection methdology. As a first trial of this approach, easily soluble α-D-glucofuranose 1,2:3,5-bis phenyl boronate **23** was synthesized (scheme 59) [297-300]. This boronate protected glucose was attempted for condensing with Boc-ALA-PFP ester using either enzyme *Porcine pancreatic lipase* [301, 317] or DMAP which were not successful (scheme 59).

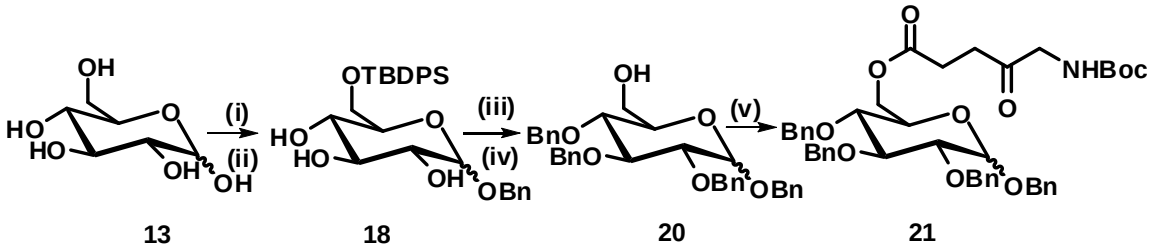


Reagents and conditions: (i) toluene, reflux, 2 h; 45% (ii) enzyme *Porcine pancreatic lipase* (PPL), rt, 7 h (iii) catalytic DMAP, rt, 7 h.

Scheme 59: 'Boronate' protection of glucose

5.3.1.1.4. Attempts for pure regio isomer 6′-O-(ALA) glucose (Protection method 2)

As a second approach of this protection-deprotection methodology, glucose **13** was protected in a stepwise manner (scheme 60) via Benzyl glucose **17**, Benzyl 6′-O-TBDMS -D-glucose **18**, Benzyl 6′-O-TBDMS -2′,3′,4′-tri-O-benzyl-D-glucose **19**, Benzyl 2′,3′,4′-tri-O-benzyl-D-glucose **20** etc [303]. Although by this benzyl protection-deprotection method, it was possible to condense Boc-ALA with benzyl 2, 3, 4-tri benzyl glucose **20** to obtain pure benzyl 6′-O-(Boc-ALA)- 2′,3′,4′-tri-O-benzyl-D-glucose **21** (scheme 32), we felt that this approach is time-consuming and less efficient if we try to follow this procedure for other sugars as well. So this approach was laid back in our goal of synthesizing pure regioisomer 6′-O- (Boc-ALA) glucose **14**.



Reagents and conditions: (i) BnOH, dry HCl, 100^0C, 3 h, 58%; (ii) TBDPSCl, imidazole, -30^0C to rt, 3.5 h, 90%; (iii) NaH, BnBr, TBAI, rt, 7 h, 67%; (iv) TBAF, 0^0C to rt, 9 h, 74% (v) **5**, EDCI, DMAP, rt, 4 h, 74%.

Scheme 60: 'Benzyl' protection of glucose

5.3.1.1.5. Enzyme catalyzed method for pure region isomer 6´-O-(ALA) glucose

Enzymes possess a unique place as selective catalysts in organic chemistry. They can function not only in aqueous solutions but also in dry organic solvents. For example, lipases, the most widely used group of enzymes have been used in anhydrous organic solvents for a wide range of stereoselective and regioselective transformations.

Carbohydrates molecules with multiple hydroxyl groups represent a challenge for regioselecetive modifications. Sugars are soluble in only a few, very hydrophilic organic solvents such as pyridine, DMF etc. Although there are reports of pancreatic porcine lipase regioselectively acylating the primary hydroxyl group of monosaccharides, unfortunately, there are only very few commercially available enzymes which are catalytically active in hydrophilic organic solvents such as pyridine, DMF etc [328].

Regioselective enzyme catalyzed acylation (or trasnesterification) of sugars is well documented in the literature [276-278, 287-292, 301, 329]. Besides the activity of the enzyme, the regioselectivity of enzyme catalyzed acylation depends on many factors: 1) substrate solubility 2) length of acyl group 3) nature of activated ester and others such as temperature of reaction etc.

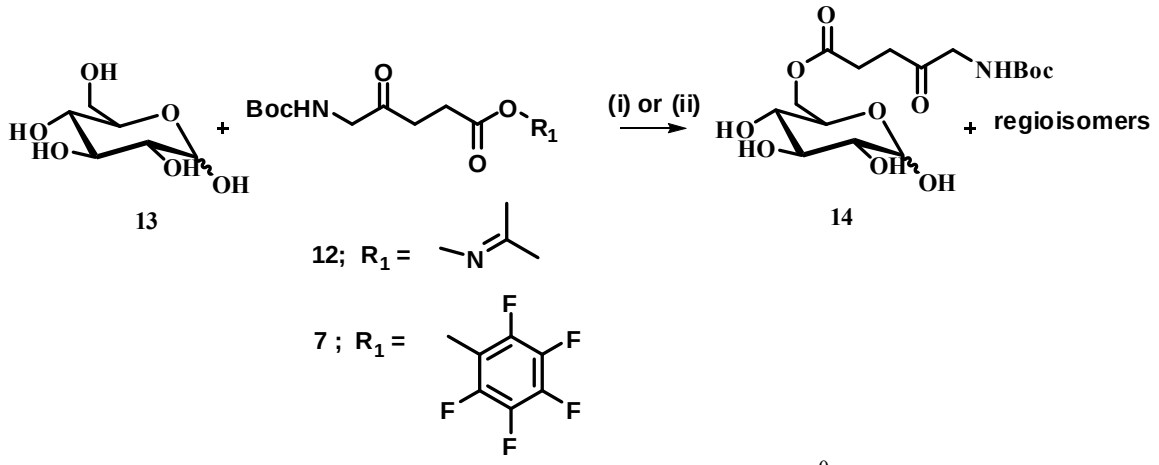
1) Substrate solubility : Although enzyme as a catalyst controls the regioselectivity of a reaction, with poorly soluble substrates such as glucose, the relative solubility of the product compared to substrate also contributes to the regioselectivity [278]. Glucose has poor solubility compared to alkyl glucosides, which explains the fact that higher regioselectivity by enzyme is always obtained for glucosides compared to free glucose when they are used as substrates. In glucosides, the product 6-O-acyl glucoside and the substrate has similar solubility and do not affect regioselectivity which is not the case for glucose [278]. This might be one of the reason that many reports are available for acylating glucosides but not for glucose [316, 321, 330-333]. Glucose remains suspended solid and the initial 6-O-acylation yields a more soluble compound, which then undergoes further acylation to the 3,6-diacyl derivative [278].

2) Length of acyl group: This factor influences the above mentioned factor ie solubility of the product. Acylation with shorter chains (e.g C2 to C6) gives a mixture of regioisomers because the initially formed 6-O-acyl derivative is soluble and undergoes further acylation. *Pig pancreatic lipase* (PPL) catalyzed acylation of glucose in pyridine with activated acetyl esters gave 5.6:1 ratio of 6-substituted : other mixture of regioisomers [288] whereas acylation with lauryl esters (C12) gave a 20:1 ratio of 6-substituted : other mixture of regioisomers [288].

3) Nature of activated ester: Due to the reversible nature of the reaction (transesterification) when catalyzed by an enzyme, the activated ester should render transesterification essentially irreversible. An example for such ester is enol esters [334]. The enol freed on transesterification rapidly tautomerizes to the corresponding volatile aldehyde or ketone preventing back reactions. Also oxime esters are well known as irreversible acyl transfer for enzyme catalyzed transesterifications [335-337]. Activated

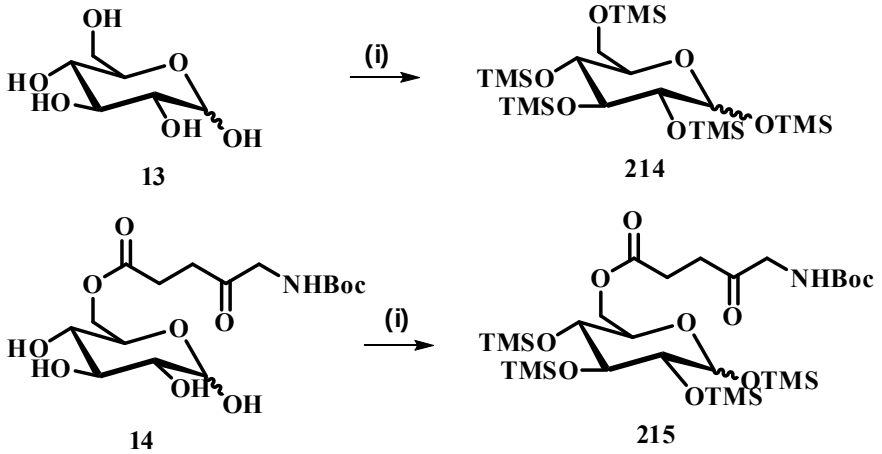
esters of pentafluorophenol are stable and provide a tool to monitor the reaction by ^{19}F NMR spectroscopy.

This approach of regioselective enzyme catalyzed acylation was tried to obtain pure 6′-*O*- (Boc-ALA) glucose **14** without any other regio isomers. In the case of glucose, the method of Therisod *et al* [288] using the enzyme *Porcine pancreatic lipase* (PPL) (scheme 34) and the method of Gotor *et al* [287, 291] using the enzyme *Candida antarctica* lipase (Novozyme 435) alongwith Boc-ALA-oxime ester (scheme 61) were successful but still did not give high regioselectivity. We had problems to quantify the other regiosiomers which will be described below. Although the ^{1}H-NMR of products of these above methods did not show any significant impurities, the ^{13}C-NMR (espically DEPT) showed minor extra peaks due to regioisomers.



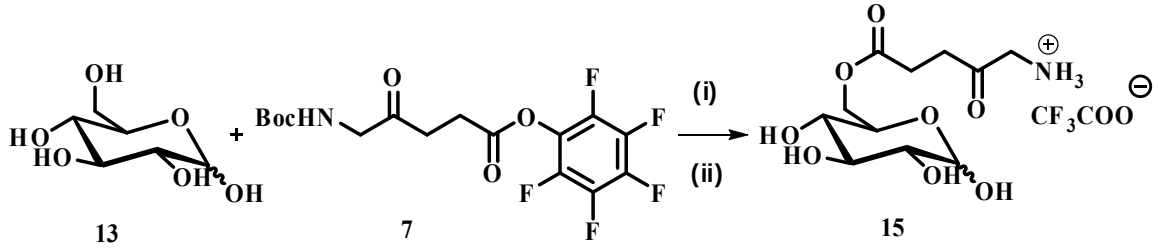
Reagents and conditions: (i) **10a**, *Porcine pancreatic lipase*, 50^0C, 48 h (ii) **10b**, *Candida antarctica* lipase (Novozyme 435), 50^0C, 48 h.
Scheme 61: Enzymatic acylation of glucose with poor regioselectivity

We tried to estimate the composition of regioisomers of the reaction by GLC (Gas-liquid chromatography). There are reports of derivatizing carbohydrates into volatile trimethyl silyl (TMS) derivatives which can be easily analyzed by GLC [316, 338, 339]. We derivatized our compound 6′-*O*- (Boc-ALA) glucose by heating with hexamethyl disilazane and trimethysilyl chloride (scheme 62). When this derivatized sample was injected, the chromatogram showed major peak corresponding to the derivative of glucose. Even after repeated trials like derivatizing at room temperature etc, changing columns, changing temperature gradient etc showed the same peak as that of glucose in GLC whereas the same sample by NMR proved it to be 6′-*O*- (Boc-ALA) glucose. The reason could be either our columns were not able to give separation between glucose and 6′-*O*- (Boc-ALA) glucose or the product 6′-*O*- (Boc-ALA) glucose is getting cleaved while making the derivative. So we abandoned this GLC method.



Reagents and conditions: (i) HMDS, TMS-Cl, reflux, 5min
Scheme 62: Silyl derivatization of glucose and 6′-*O*-(Boc-ALA) glucose

The method of Riva *et al* [320] using the enzyme *subtilisin proteinase* (scheme 63) gave exclusive regioselectivity for 6′-*O*- (Boc-ALA) glucose with a reasonable yield (50%). *Bacillus subtilis* proteinase is stable and active in organic solvents such as pyridine, DMF etc. The product of this method showed only peaks corresponding to 6-regioisomer (not even traces of any other regioisomer) in ^{1}H-NMR, ^{13}C-NMR and DEPT.



Reagents and conditions: (i) *subtilisin proteinase*, 50^{0}C, 24 h, 48%; (ii) TFA, rt, 0.5 h, 92%.
Scheme 63: Successful enzymatic regioselective acylation of glucose

The position of acylation at C6-hydroxyl group was confirmed by 13C NMR, taking the cue from Yoshimoto at al who had described the additivity of shift parameters in 13C NMR for O-acyl glucoses (see table 6) [340]. Yoshimo *et al* studied 13C NMR of all positional isomers of various acyl glucoses dissolving in various NMR solvents. He found that the acylation shift parameters are independent of the solvent and the kind of acyl group. So these shift values proposed by him could be regarded as universal set of additive values for any acyl glucoses. We found that the chemical shift of 6′-*O*-(Boc-ALA) glucose also matched this universal set of additive values with a change of ±0.2 ppm. We also confirmed the position of acylation using COSY techniques.

Compound	Carbon number					
	C-1	C-2	C-3	C-4	C-5	C-6
1-*O*-Acyl α	-0.4	-2.0	0	-1.1	+3.3	-0.7
1-*O*-Acyl β	-2.9	-2.6	-0.1	-0.9	+1.0	-0.8
2-*O*-Acyl α	-2.9	+1.6	-3.0	+0.3	0	-0.1
2-*O*-Acyl β	-2.1	+0.3	-2.1	+0.4	+0.4	-0.1
3-*O*-Acyl α	0	-1.7	+2.3	-2.3	+0.1	-0.5
3-*O*-Acyl β	+0.1	-1.8	+1.0	-2.0	+0.1	-0.4
4-*O*-Acyl α	-0.1	+0.3	-2.5	+1.0	-2.0	-0.4
4-*O*-Acyl β	+0.2	+0.3	-2.5	+1.0	-2.0	-0.4
6-*O*-Acyl α	+0.1	0	0	-0.1	-2.7	+2.0
6-*O*-Acyl β	+0.1	+0.1	-0.2	-0.2	-3.1	+2.2

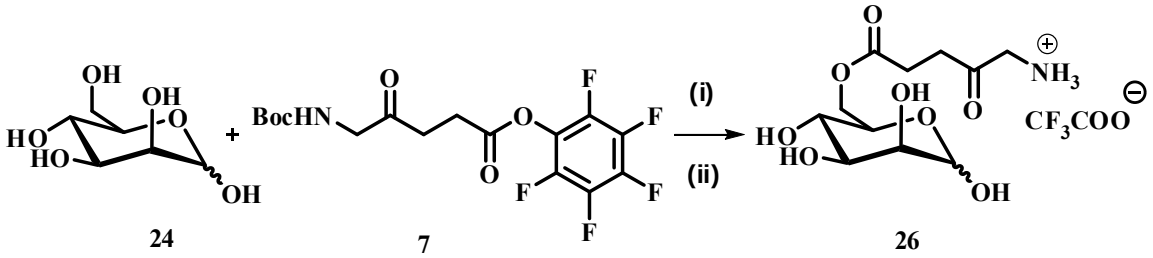
Table 6: Acylation shift parameters for D-glucopyranoses

In the enzymatic regioselective acylation, for the activated ester we used pentafluoro phenyl ester (Boc-ALA-PFP ester) instead of trichloroethyl ester which was mentioned in the literature [320]. Our method employed 0.5 g (approximately 2.5 eq. per eq. of glucose) of *subtilisin proteinase* (activity = 5units per mg of protein) and 3 eq.of Boc-ALA-PFP ester per eq. of glucose (Scheme 63). The suspension (as *subtilisin proteinase* was not soluble in pyridine) was stirred at 50^0C. After 20h, no free glucose was left. Then the enzyme was removed by filtration over celite, the product was purified by silica gel flash chromatography followed by crystallisation.
6´-*O*- (Boc-ALA) glucose **14**, thus prepared by enzymatic regioselective transesterification was deprotected by TFA to give pure regioisomer 6´-O- (ALA) glucose **15** (Scheme 58). This pure 6´-O- (ALA) glucose was further tested in various cancer cell lines for the induction of PpIX.

5.3.1.2. Synthesis of 6´-O-(ALA) mannose

In the case of mannose also, regioselective enzyme catalyzed acylation (or trasnesterification) was employed for the selective substitution of ALA on C6 hydroxyl group. A good regioselectivity on C6 hydroxyl group and a reasonable yield (68%) were obtained by the method of Therisod *et al* using the enzyme *porcine pancreatic lipase* (PPL) [288]. For the activated ester, we used pentafluoro phenyl ester (Boc-ALA-PFP ester) instead of trichloroethyl ester as mentioned in the literature. Our method (scheme 64) employed 0.325 g (approximately 8eq. per eq. of mannose) of porcine pancreatic lipase (activity = 24 units per mg of protein) and 2 eq.of Boc-ALA-PFP ester per eq. of mannose (scheme 64). The suspension was stirred at 50^0C. After 48h, no free mannose was left. Then the enzyme was removed by filteration over celite and the product was isolated and purified by silica gel flash chromatography followed by crystallization.

The position of acylation at C6-hydroxyl group was confirmed by ^{13}C-NMR and ^{1}H-NMR values which was matching the values of similar 6-O-acyl mannoses mentioned in literature [288, 308].



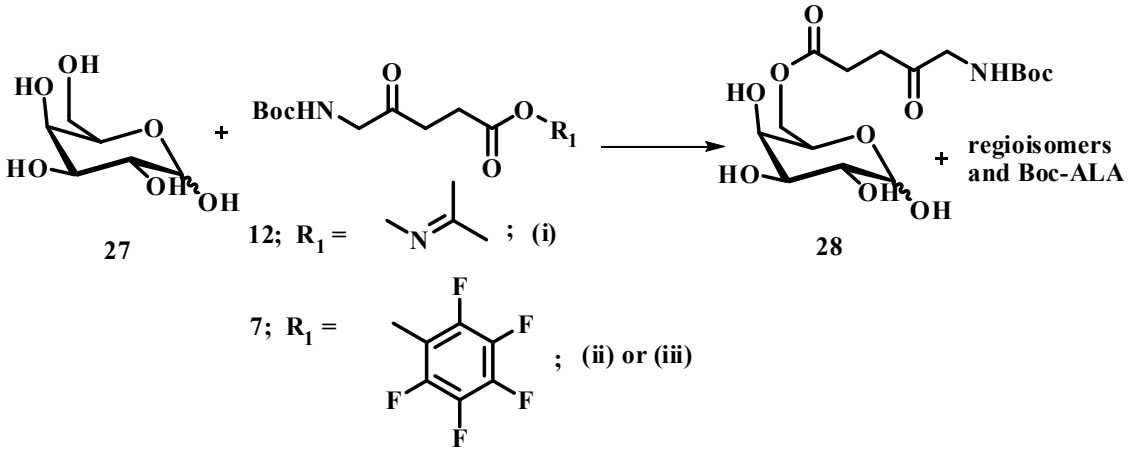
Reagents and conditions: (i) *Porcine pancreatic lipase*, 50^0C, 24 h, 69%; (ii) TFA, rt, 0.5 h, 91%.

Scheme 64: Synthesis of 6′-*O*-(ALA) mannose

6′-*O*- (Boc-ALA) mannose **25**, thus prepared was deprotected by TFA to give pure regioisomer 6′-*O*- (ALA) mannose **26** (scheme 64) which was tested in various cancer cell lines for the induction of PpIX.

5.3.1.3. Synthesis of 6′-O-(ALA) galactose

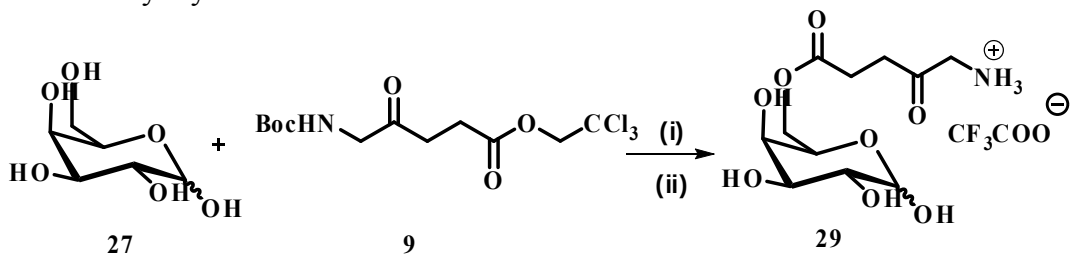
The protocol applied in the case of mannose which is using Boc-ALA-PFP ester as activated ester and the enzyme *porcine pancreatic lipase* (PPL) did not provide good regioselectivity for galactose (scheme 65) [288]. When we applied the protocol which was used for synthesizing 6′-*O*- (Boc-ALA) glucose (using Boc-ALA-PFP ester and the enzyme *subtilisin proteinase*) [320], we were not able to achieve good regioselectivity (scheme 38). The products of all these failed methods besides failing in high regioselectivity also contained sufficient Boc-ALA, which had been hydrolyzed from Boc-ALA-PFP ester. We tried the regioselectivity using the enzyme *Candida antarctica* lipase (Novozyme 435) along with Boc-ALA- PFPester (scheme 65) [287, 291]. Although we were able to achieve exclusive regioselectivity on C6 using this enzyme with moderate yield (49%), we had to use large amount of this enzyme; 1.5 g (activity=10 units/mg protein) for 1 mmole of galactose. Since this enzyme is expensive (~150 CHF for 1g) and also since we needed more amount of 6′-*O*- (Boc-ALA) galactose **28,** we had to search for other procedures for regioselectivity.



Reagents and conditions: (i) *Candida antarctica* lipase (Novozyme 435), 50^0C, 48h. (ii) *Porcine pancreatic lipase*, 50^0C, 48h; (iii) *Subtilisin proteinase*, 50^0C, 48h.

Scheme 65: Enzymatic acylation of galactose with poor regioselectivity

Finally, exclusive regioselectivity for obtaining 6′-*O*- (Boc-ALA) galactose **28** was achieved by regioselective enzyme catalyzed acylation (or trasnesterification) using the enzyme *porcine pancreatic lipase* (PPL) but performing the reaction with Boc-ALA-TCE ester **9** instead of Boc-ALA-PFP ester **7** [288] (scheme 66). Although the literature mentioned trichloro ethyl ester as activated ester, we were using initially Boc-ALA-PFP ester, because this was used for various ALA ester formations including for synthesizing 6′-*O*- (Boc-ALA) mannose **25.** Our method employed 0.95 g (approximately 19eq. per eq. of galactose) of *porcine pancreatic lipase* (activity = 20 units per mg of protein) and 6.4 eq.of Boc-ALA-TCE ester per eq. of galactose. The suspension was stirred at 50^0C. After 48h, no free galactose was left. Then the enzyme was removed by filtration over celite and the product was isolated and purified by silica gel flash chromatography followed by crystallisation.



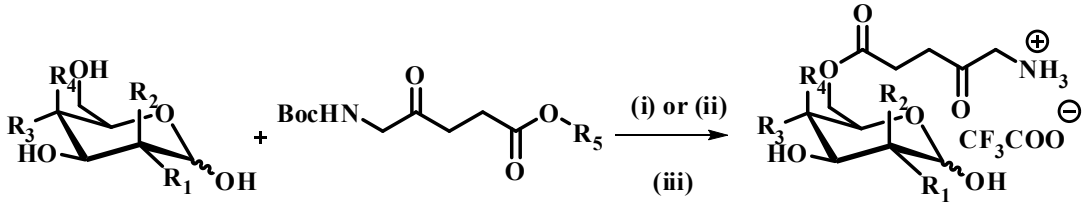
27 9 29

Reagents and conditions: (i) *Porcine pancreatic lipase*, 50^0C, 60h, 44%; (ii) TFA, rt, 0.5 h, 93%.

Scheme 66: Enzymatic regioselective acylation of galactose

6′-*O*- (Boc-ALA) galactose **28** (Yield=44%), thus prepared was deprotected by TFA (scheme 66) to give pure regioisomer 6′-*O*- (ALA) galactose **29** which was tested in various cancer cell lines for the induction of PpIX.

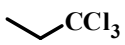
In summary, as shown in scheme 67 we acylated regioselectively on C6 hydroxyl group of glucose, mannose and galactose. We used a proper combination of an enzyme and an activated ester to achieve this regioselectivity.

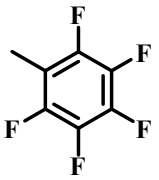


13; R_1=OH, R_2=H, R_3=OH, R_4=H

24; R_1=H, R_2=OH, R_3=OH, R_4=H

27; R_1=OH, R_2=H, R_3=H, R_4=OH

9; R_5 = 

7; R_5 = 

15; R_1=OH, R_2=H, R_3=OH, R_4=H

26; R_1=H, R_2=OH, R_3=OH, R_4=H

29; R_1=OH, R_2=H, R_3=H, R_4=OH

Reagents and conditions: (i) *Subtilisin proteinase*, 50^0C, 24 h; (ii) *Porcine pancreatic lipase* (PPL), 50^0C, 60h; (iii) TFA, rt, 0.5 h.
Scheme 67: Summary of enzymatic regioselective (C6) acylation of glucose, mannose and galactose by ALA.

We observed that obtaining regioselectivity in the case of galactose was more difficult than in the case of mannose and glucose. We found that the ease of regioselectivity and efficiencies of the formation was in the following order: Mannose > glucose > galactose which was same as reported by Boyer *et al* for synthesizing 6-*O*-phenylalanine-carbohydrate conjugates using enzyme protease subtilisin where he observed the preference based only on the stereochemistry of the parent carbohydrate [318].

5.3.1.4. Evaluation of PpIX production by cells by first generation ALA-sugar conjugates - 6′-O- (ALA) glucose, 6′-O-(ALA) mannose and 6′-O-(ALA) galactose

5.3.1.4.1. Efficiency- PpIX generation

PpIX production was evaluated for all the regiopure 6′-*O*- (ALA) glucose **15**, 6′-*O*-(ALA) mannose **26** and 6′-*O*- (ALA) galactose **29** by measuring increase in fluorescence intensity in living cells at different times by incubating cells in medium containing ALA conjugate at concentrations from 0.1mM to 2 mM. Cells used for experiments were Human colon carcinoma HT29, breast T47D carcinoma, A549 lung carcinoma and HCEC human immortalized brain endothelial cells.
6′-*O*- (ALA) glucose, 6′-*O*- (ALA) mannose and 6′-*O*- (ALA) galactose induced strong PpIX production in all cell lines (the graphs of increase in PpIX level by certain selected cell lines are shown here). The increase in PpIX level from 0h to 3h (figure 9 to 11) shows that in all cell lines except T47 D, 6′-*O*- (ALA) glucose produces higher level of PpIX than that induced by standard ALA at any point of time. In T47D cells, 6′-*O*-(ALA) galactose produced highest level of PpIX than others.

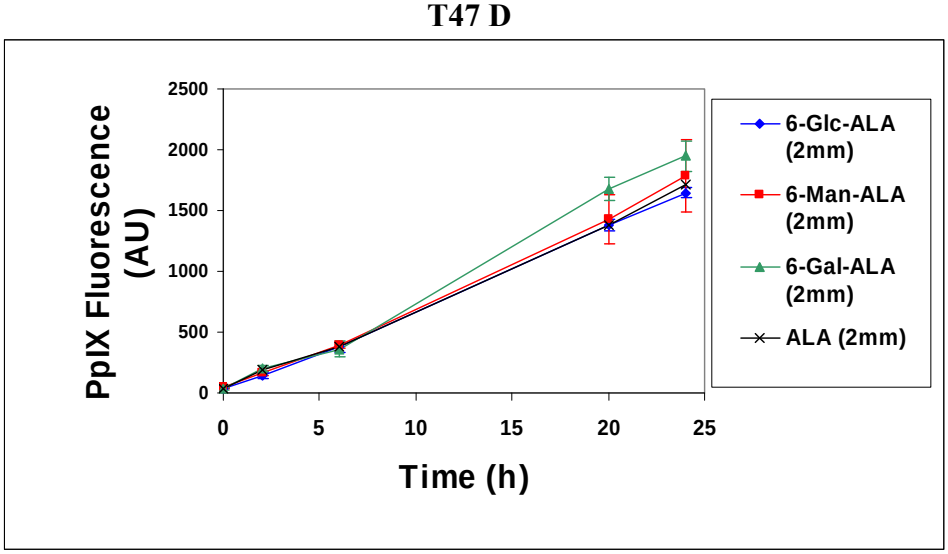


Figure 9: Increase in PpIX level due to 6′-*O*-(ALA) sugars and ALA in T47 D cells

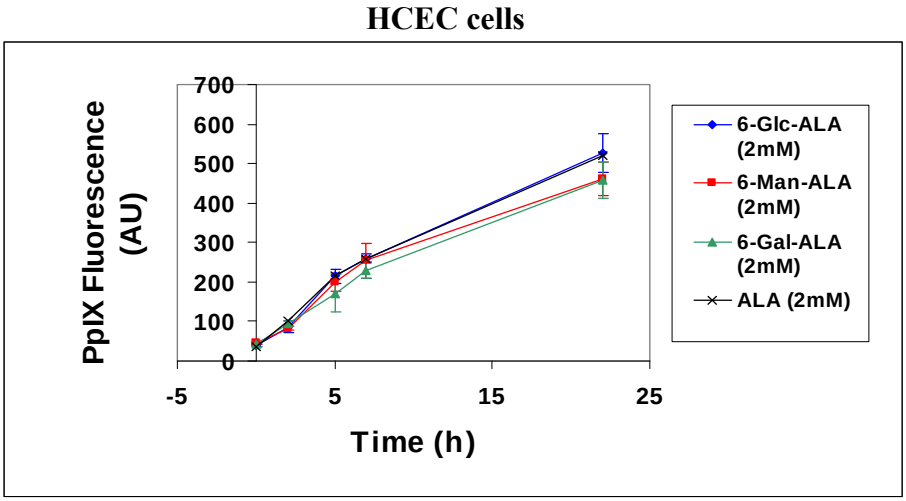


Figure 10: Increase in PpIX level due to 6′-*O*-(ALA) sugars and ALA in HCEC cells

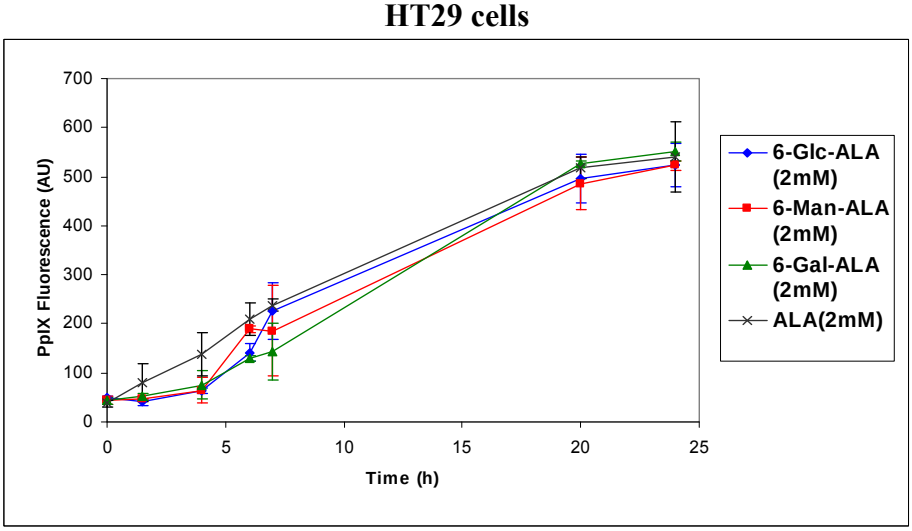


Figure 11: Increase in PpIX level due to 6′-*O*-(ALA) sugars and ALA in HT29 cells

5.3.1.4.2. Cytotoxicity

The alamar blue assay showed that all the 6′-*O*-(ALA) sugar conjugates are not cytotoxic in the absence of exposure to light. The data of this assay is explained in chapter 4.3.4 where the alamar assay of all the ALA glycoconjugates was described.

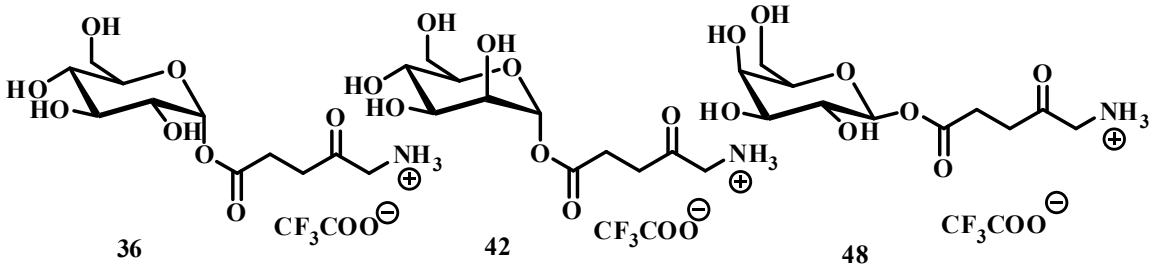
5.3.1.4.3. Selectivity

The PpIX generation due to 6′-*O*-(ALA) sugar conjugates were compared with ALA in fibroblasts (VS 59). The PpIX generated by 6′-*O*-(ALA) sugar conjugates was in the same level as that of ALA. This means the selectivity of 6′-(ALA) sugar conjugates towards cancer cells is not better than ALA. The PpIX level is shown in chapter 4.3.4 where the PpIX generation due to all the ALA glycoconjugates in fibroblasts is shown.

5.3.2. Synthesis of 1´-*O*-(ALA) sugars (Second generation ALA-glycoconjugates)

As explained in the "metabolism of cancer cells" chapter 2 (Biological background behind Prodrugs design), the study on glycosidase activities performed by the group of Dr.L.Juillerat-Jeanneret and her PhD student Mr.F.Schmitt, Institute of Pathology at CHUV, Lausanne indicate that α-glucosidase, α-mannosidase and β-galactosidase are the potential targets in cancer cell lines as they have strong activities in cancer cell lines compared to normal cell lines.

Based on this, we decided to synthesize specific anomeric ALA glyconjugates; 1´-*O*-(ALA) α-glucose, 1´-*O*-(ALA) α-mannose and 1´-*O*-(ALA) β-galactose where ALA is linkes as an ester with C1 hydroxyl group of monosaccharides namely glucose, mannose and galactose (conjugated in a specific anomeric position) (scheme 68).

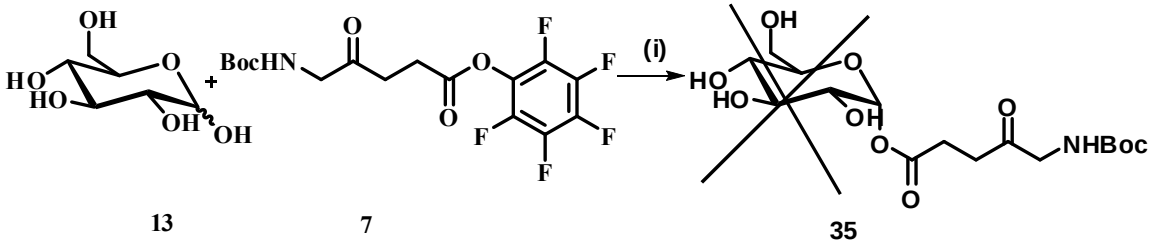


1´-*O*-(ALA) α-glucose 1´-*O*-(ALA) α-mannose 1´-*O*-(ALA) β-galactose.
Scheme 68: 1´-*O*-(ALA) sugars (Second generation ALA-glycoconjugates)

5.3.2.1. Synthesis of 1´-O-(ALA) α-glucose

The regioselective acylation of C1 hydroxyl group of sugars can be carried out in a stepwise manner: first, synthesizing properly protected sugar keeping C1 hydroxyl group free; second, coupling the protected sugar with an acid and then deprotecting the groups without hindering the ester functionality synthesized. Another way is coupling an unprotected sugar with an activated ester [341, 342], but the available reported literature on this transformation are few.

5.3.2.1.1. Attempted trials using unprotected glucose

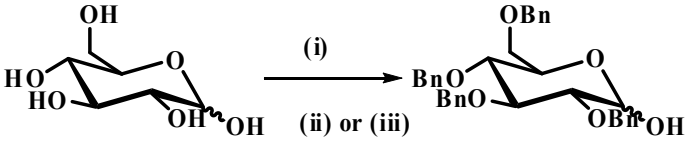


Reagents and conditions: (i) catalytic NaH, rt, 12h.
Scheme 69: Failed reaction using unprotected glucose for synthesizing 1´-*O*-(ALA) glucose

The regioselective methods using unprotected glucose rely on the acidity of C1 hydroxyl group in the carbohydrates [343]. The reported literature on these transformation used activated reactive amides or reactive azole derivatives for coupling with β-glucose. These reactive amides were reported to be synthesized by treating acyl chlorides with 8-hydroxyquinoline or imidazole/ triazole /tetrazole. Then reactive amides (1eq) were treated with β-glucose (3eq) in the presence of catalytic NaH (0.16 eq). We attempted this protocol using activated ester Boc-ALA-PFP ester instead of reactive amides as mentioned in the literature, because we were using Boc-ALA-PFP ester for many coupling reactions. Also, those reactive amides were synthesized from acyl chloride which is not possible in our case, as the synthesis of Boc-ALA acid chloride was not feasible. First, we attempted (scheme 69) using 0.16 eq. NaH, Boc-ALA-PFP ester and α-glucose and found that the conversion was poor. Second, we attempted using 0.32 eq NaH and the rest being the same. Although the conversion was better, to our surprise we found that the major product isolated comprised of 6′-*O*-(Boc-ALA) α-glucose. Repeated attempts by changing the NaH equivalents did not yield desired result and so we abandoned this route.
Then, we decided to follow protection-deprotection methodology for synthesizing 1′-*O*-(ALA) α-glucose.

5.3.2.1.2. Protection-deprotection methodology

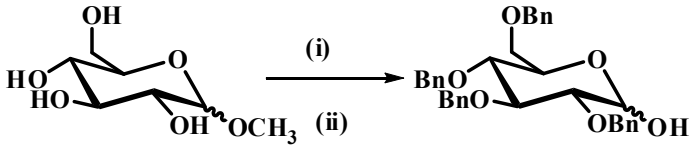
5.3.2.1.2.1. Synthesis of 2, 3, 4, 6-tetra-O-benzyl glucose (TBG)



13 31

Reagents and conditions: (i) NaH, BnBr, TBAI, rt, 8 h, 74% (ii) CH_3COOH, 6N HCl, 60^0C, 4 h, 58%; (iii) Pd over alumina, $HCOONH_4$, 10h, rt, 61%.
Scheme 70: Synthesis of 2, 3, 4, 6-tetra-O-benzyl glucose (TBG)

This methodology starts with synthesizing 1,2,3,4,6 - Penta-O- benzyl glucose (major β anomer) **30** from glucose **13** (scheme 70). Then, selective deprotection [344-346] at C1 provided 2, 3, 4, 6-tetra-O-benzyl glucose (TBG) **31** α- anomer being major (scheme 43). The selective deprotection of benzyl group at C1 was carried out using either acidic hydrolysis (acetic acid/6N HCl) [346] or using catalytic hydrogenolysis (Pd over alumina) with ammonium formate as hydrogen source [345]. Attention should be paid while performing both these methods as longer duration of reaction maintenance will lead to deprotection of benzyl group substituted on other hydroxyl groups besides C1. Also, Pd over alumina was air sensitive and catches fire easily when it is dry. On larger scale, we preferred the selective deprotection using acetic acid/6N HCl as this protocol was not only safe to handle but also yielded consistent and better yield (60%).



33 31

Reagents and conditions: (i) NaH, BnBr, TBAI, rt, 8 h, 91% (ii) CH_3COOH, CF_3SO_3H, 80^0C, 7 h, 58%.

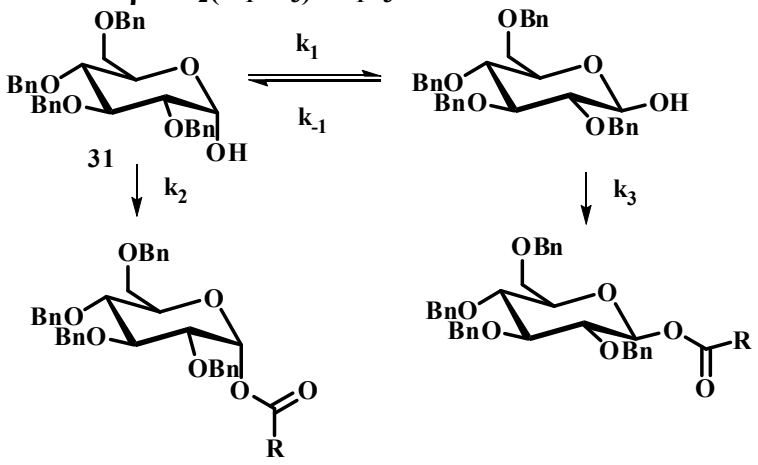
Scheme 71: Synthesis of TBG from α-methyl glucoside

The synthesis of TBG was also performed via protection of all hydroxyl groups of methyl glycoside by benzyl groups and then hydrolyzing methyl group at C1 using trifluoromethanesulfonic acid [347] (scheme71).

5.3.2.1.2.2. Coupling of TBG with Boc-ALA

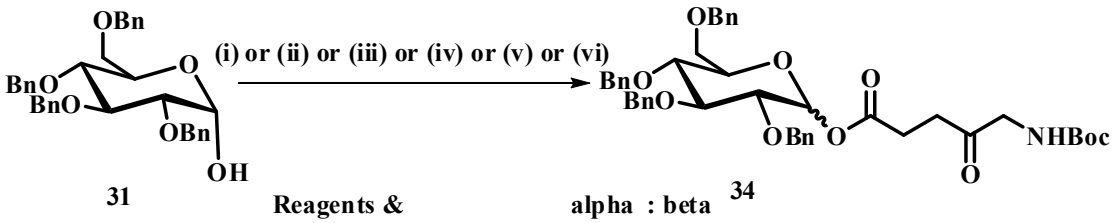
There exists in literature, various coupling procedures for obtaining 1-O-acyl glucose predominantly either in α form [348-353] or in β form [351, 354-363]. It is well known that the sterically hindered acid achlorides form mostly β ester on coupling with TBG [356]. The α:β ratio of the product depends on the first-order rate constants according to equaton 1. Since equatorial alcohols are acylated faster than axial [364], k_3 would be expected to be larger than k_2. Thus if k_1 and k_{-1} were small (slow mutarotation) and k_2 & k_3 were large (fast acylation) the reaction would lead predominantly to α ester ($\alpha:\beta \sim k_2.k_3 / k_1.k_3$), while the reverse (fast mutarotation and fast acylation) would result in predominantly β ester ($\alpha:\beta \sim k_2k_{-1} / k_3k_1$) (scheme 72).

Equation 4 $$\alpha/\beta = k_2(k_{-1}+k_3) / k_1k_3$$



Scheme 72: Equlibrium between TBG anomers and acylation of the respective anomers

We followed the coupling methods which can give predominantly α anomer product as we required product with α configuration.



Reagents & Conditions	alpha : beta
(i) 5, EDCI, DMAP, rt, 5 h	0.28 : 1
(ii) 10b, BuLi, -20^{0}C to rt, 1 h	2 : 1
(iii) 10b, NaH, -20^{0}C to rt, 1 h	2.7 : 1
(iv) 7, BuLi, -20^{0}C to rt, 1 h	5.6 : 1
(v) 10b, pyridine, rt, 5 h	1 : 2
(vi) 5, DEAD, PPh_3	1 : 1

Scheme 73: Trials of various couplings of TBG and Boc-ALA for synthesizing mjor α-anomer product

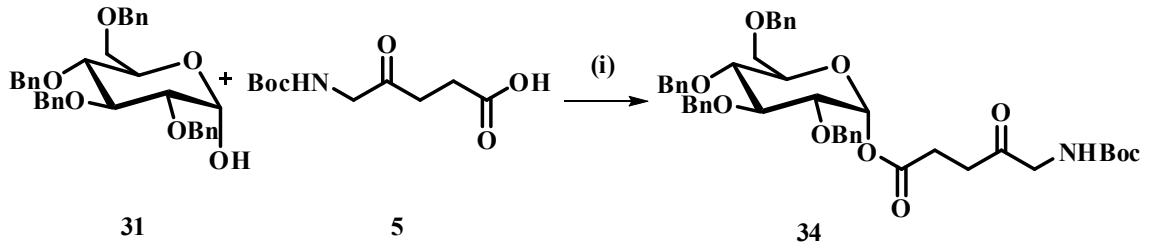
First, TBG was subjected to carbodiimide coupling using EDCI and DMAP [103], the predominant product was β anomer of 1′-*O*-(Boc-ALA)- 2′, 3′, 4′, 6′-tetra-O-benzyl glucose **34** instead of the required α anomer (scheme 73). Though β anomer product is not what we desired, we had taken this product for further steps which will be explained later.

Metallation of TBG by n-butyl lithium followed by acylation with acyl halides is known to provide TBG esters with a preference for the α-configuration [348, 349]. As a second trial, we tried this coupling, instead of acyl halide which is mentioned in the literature, we used Boc-ALA-PFP ester as we could not synthesize Boc-ALA acid chlroide. Though this method using the metallation of TBG by n-butyl lithium gave α-anomer of 1′-*O*-(Boc-ALA)-2′, 3′, 4′, 6′-tetra-*O*-benzyl glucose as a major product (α: β = 2:1), the ratio is still not sufficient enough to isolate α-anomer product by crystallization or by column chromatography (scheme 73).

The third attempt was trying the same coupling as that of second trial but using NaH instead of n-butyl lithium. However, this method also gave similar α: β ratio (α: β = 2.7:1) as that of n-butyl lithium (scheme 73). Later, we synthesized azidolevulinic acid chloride from azidolevulinic acid though it could not be isolated by distillation because of its thermal instability [267]. As a fourth trial, when we tried this metallation of TBG by n-butyl lithium followed by acylation with azidolevulinic acid chloride, we obtained 1′-*O*-(azidolevulinic)-2′, 3′, 4′, 6′-tetra-*O*-benzyl glucose with α-anomer as a major product (α: β = 5.6:1) (scheme 73). We did not proceed with this coupling because of the poor yield (34%) probably owing to the not so stable azidolevulinic acid chloride.

As a fifth trial, we tried the coupling of TBG and Boc-ALA-PFP ester in the presence of pyridine to obtain major α anomer product similar to the method done by Schmidt *et al*, Nishikawa *et al* and Bols *et al* for obtaining major α anomer product [352, 356, 365]. This attempt was not successful as the acylation reaction is very slow and yielded β anomer as major product (scheme 73) which can be expected as the above suggested literature utilized reactive acyl halides.

Sixth attempt was Mitsunobu reaction as followed by Lubineau *et al* [351] which provided mixture of anomers (α: β = 1:1) (scheme 73). Lubineau *et al* showed that ratio of α to β anomer ratio depends on the acidity of acyl group and α to β anomer ratio decreases with the increase of the pKa of the acid component under the conditions of Mitsunobu reaction. Their data indicates that starting from a mixture of α to β of 7:3 in the case of the most acidic compound (pKa=3.4), the less acidic compound (pKa= 4.8) gave exclusively the β anomer.



31 **5** **34**

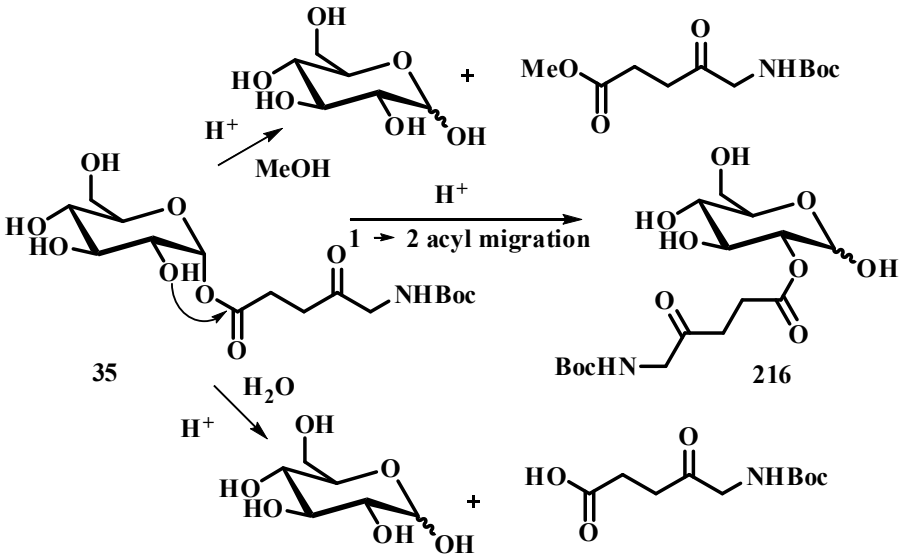
Reagents and conditions: (i) DCC, Catalytic DMAP, 0^0C to rt, 5 h.

Scheme 74: Synthesis of α –anomer of 1′-*O*-(Boc-ALA)-TBG using DCC & catalytic DMAP

Finally, the synthesis of the α anomer product (α: β = 7:1) was best carried out with the yield of 91% by coupling of TBG with Boc-ALA in the presence of DCC and a catalytic amount of DMAP [351, 353] (scheme 74). We found that when we carried out the coupling of TBG with Boc-ALA in the presence of only DCC and not DMAP, the yields were very low (29%) which can be ascribed to a lower degree of activation of the carboxylic acid [363]. We also found that when we carried out the coupling of TBG with Boc-ALA in the presence of DCC and equimolar DMAP, we obtained β anomer of 1′-O-(Boc-ALA)- 2′, 3′, 4′, 6′-tetra-O-benzyl glucose in major quantity.

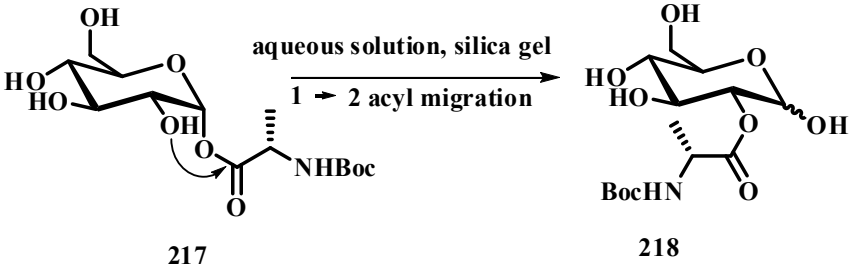
5.3.2.1.2.3. Benzyl deprotection

The next step was to deprotect the benzyl groups attached to the hydroxyl groups by catalytic hydrogenation (scheme 78). We experienced difficulties in isolating the pure 1′-O- (Boc-ALA)- α -glucose **35**. After doing the column chromatography using eluent 8:2 CH_2Cl_2 : CH_3OH and during the evaporation of solvent, there appeared two minor TLC spots besides the product spot. One spot corresponded to Boc-ALA methyl ester (confirmed by ^{1}H-NMR after isolating the spot) and another spot was unknown. The formation of Boc-ALA methyl ester can be explained from the fact that the product in the presence of little acidity and methanol (used in eluent mixture) was undergoing transesterfication (scheme 75).

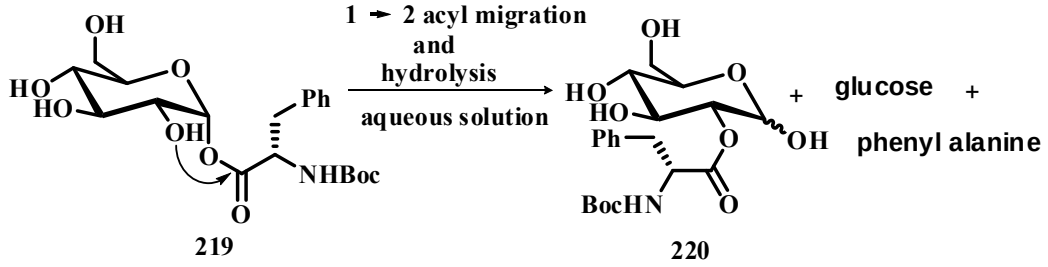


Scheme 75: Side reactions during work-up of benzyl group deprotection step

In acyl glucoses and glucosides, each oxygen atom of a glucose unit is a site of migration origin; an acyl group migrates, in most cases, away from O-1 towards O-6 [366]. The O-acyl migration from C1 to C2 in glucose derivatives catalyzed by acid or base has been extensively studied before (scheme 76 and scheme 77) [361, 366-368]. From these literature reports we could say that the second impurity spot might correspond to C2 acyl substituted isomer **216** (scheme 75). It has been reported earlier similar compounds such as 1-*O*-(Boc-alanine)-α –glucose **217** undergo rearrangement rapidly in silica gel to give 2-*O*-(Boc-alanine)-α- glucose **218** (scheme 76) [361, 368]. It was also proved that N-acetyl protected amino acid underwent less or no acyl migration compared to N-Boc protected aminoacid. The difference between the acyl and alkoxycarbonyl residues as protecting groups of the amino function was studied by Determann *et al*, who found that the nitrogen atom in urethane-type protecting group retains its basic character in contrast to that of normal amides. This suggests that the Boc group induces a higher positive charge on the ester carbonyl carbon thereby facilitating nucleophilic attack by the adjacent hydroxyl group.



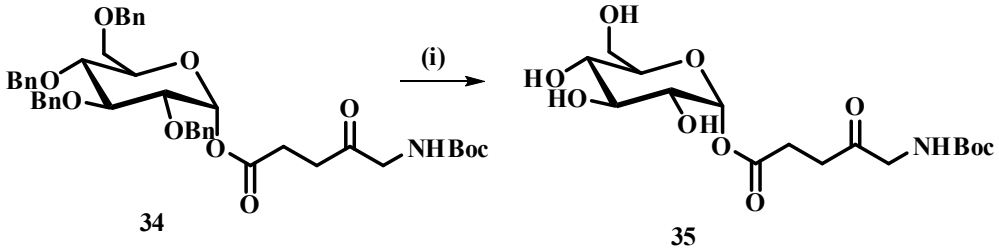
Scheme 76: Literature example of acyl migration: 1-*O*-(Boc-L-alanine)-α –glucose to 2-*O*-(Boc-L-alanine)-α- glucose.



Scheme 77: Literature example of acyl migration and hydrolysis with 1-O-(Boc-phenly alanine)-α-glucose

The catalytic hydrogenation provided 1´-O- (Boc-ALA) glucose **35** as highly hygroscopic foam. So besides the difficulties of 1 → 2 acyl migrations and transesterification in the presence of methanol, we also encountered the problem of hydrolysis (Scheme 75) which led to glucose and Boc-ALA [368]. The competitive reactions 1 → 2 acyl migrations, transesterification and hydrolysis which give impurities while isolating the product are strongly affected by temperature, acidity and the nature of acyl group. Keglevic *et al* and others studied the relative rates of acyl migration and hydrolysis and they also found that the anomeric configuration also affects acyl migration [272, 369, 370]. They observed that under similar conditions α. anomer experienced less acyl migration and hydrolysis [368]. When performing catalytic hydrogenolysis of 2,3,4,6-tetra-O-benzyl-1-O-(N-nenzyloxybarbonyltripeptidyl)-D-glucose, Valentekovic *et al* [355] experienced that in the absence of acid like TFA or oxalic acid, the acyl migration is more pronounced. They added TFA or oxalic acid in the catalytic hydrogenation to isolate the product as TFA or oxalate salt.

There were also reports of literature mentioning facile cleavage of esters occurring during benzyl deprotection [319] . To overcome this problem, researchers preferred the substituted benzyl groups such as p-methoxybenzyl (PMB) protecting groups instead of simple benzyl groups. These substituted benzyl groups were very easy to deprotect under oxidative conditions without affecting the ester group.

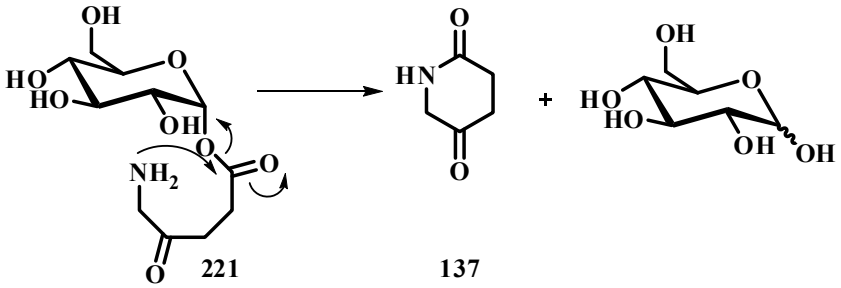


Reagents and conditions: (i) H_2 / Pd, rt, 10 h.

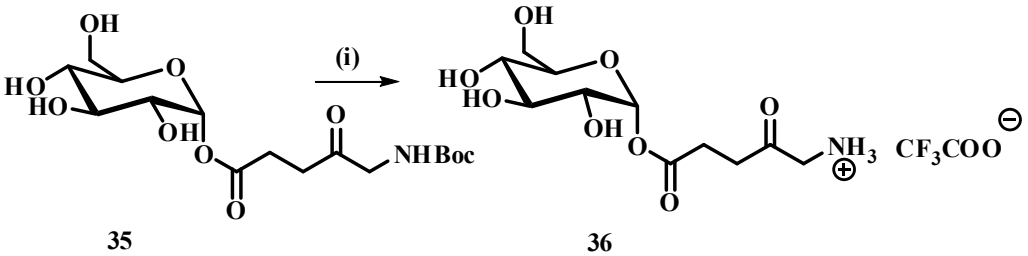
Scheme 78: Benzyl deprotection using catalytic hydrogenation

We were able to overcome all these side reactions while isolating the product by neutralizing the silica gel before doing column chromatography, neutralizing the eluents used for column chromatography, evaporating the column fractions below 30^0C. Thus we obtained pure 1´-*O*- (Boc-ALA)- α –glucose **35.** Before obtaining 1´-*O*- (Boc-ALA)- α – glucose **35**, we carried out trials using the corresponding benzyl protected β anomer and we obtained 1´-*O*- (Boc-ALA)- β –glucose.

5.3.2.1.2.4. Boc deprotection



Scheme 79: Lactam formation during 'Boc' deprotection



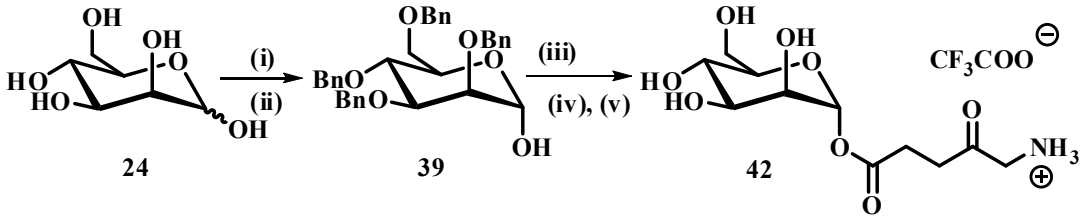
Reagents and conditions: (i) CF_3COOH, CH_2Cl_2, 0-10^{0}C, 0.5 h.

Scheme 80: Synthesis of 1′-O-(ALA) glucose by deprotecting 'Boc'

Though the deprotection of "Boc" (scheme 80) appeared simple initially, we encountered difficulties of ester cleavage (hydrolysis) to give glucose and Boc-ALA or ester cleavage to give glucose and lactam-type derivatives. It is reported in literature that glcosyl esters of amino acids can undergo intramolecular aminolysis leading to glucose and piperazine-type derivatives though it is more pronounced in β. anomers than α. anomers [360, 371] (scheme 79). The possibility of lactam-type **185** derivatives formed due to the intramolecular attack of amino group on the carbonyl carbon of a ALA ester has been reported before [108]. The product 1′-*O*-(ALA) α -glucose **36** (scheme 80) being highly hygroscopic and containing traces of trifluoro acetic acid (TFA) led to hydrolysis of ester bond easily. This problem of hydrolysis was overcome by removing the traces of TFA by co-distillation with dry ether, triturating the product with dry ether and then doing a precipitation using methanol and ether. The intramolecular aminolysis was avoided by adding excess of TFA by which the product is in the form of ammonium trifluoroacetate salt.

Thus we synthesized in a stepwise manner by protection and deprotection, 1′-*O*-(ALA) α -glucose **36.** There are reports in the literature that 1-*O*-(phenyl alanine)-α –glucose undergo not only rearrangement in aqueous solutionto give 2-*O*-(phenyl alanine)-α-glucose, but also hydrolyze rapidly in aqueous solution. We decided to examine the stability of 1′-*O*-(ALA) α -glucose **36** in aqueous solution as for the biological evaluation, the compound is normally dissolved in water which then is diluted with buffer medium. We dissolved 1′-*O*-(ALA) α -glucose **36** in D_2O (which normally has H_2O as well) and checked ^{1}H-NMR and ^{13}C-NMR for every 3h. We checked 3 times (9h totally) and found that the compound is quite stable. Peaks due to glucose did not appear at all, which meant clearly that the compound is not hydrolyzing rapidly.

5.3.2.2. Synthesis of 1′-O-(ALA) α-mannose



24 39 42

Reagents and conditions: (i) NaH, BnBr, TBAI, rt, 8 h; (ii) CH_3COOH, 6N HCl, 60^0C, 4 h; (iii) DCC, Catalytic DMAP, 0^0C to rt, 5 h; (iv) H_2 / Pd, rt, 10 h; (v) CF_3COOH, CH_2Cl_2, 0-10^0C, 0.5 h.

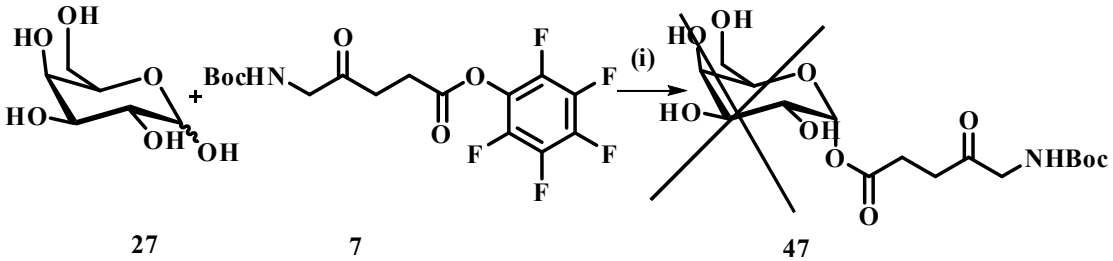
Scheme 81: Synthesis of 1′-O-(ALA) mannose

Once the synthesis of 1′-*O*-(ALA) α-glucose was made in a standardized manner, the same protection-deprotection and coupling methods were followed for mannose also. The scheme starts with synthesizing 1,2,3,4,6 - Penta-*O*- benzyl mannose (major β anomer) **38** from mannose **24** (scheme 81). Then, selective deprotection at C1 provided 2, 3, 4, 6-tetra-*O*-benzyl mannose (TBM) α- anomer **39** being major [346]. Later the coupling of TBM with Boc-ALA in the presence of DCC and a catalytic amount of DMAP (scheme 54) provided 1′-*O*-(Boc-ALA)- 2′, 3′, 4′, 6′-tetra-*O*-benzyl mannose, α- anomer product **40** (α : β = 5:1) being major. We also experienced that the coupling when performed with equimolar DMAP formed α-anomer product as major while in the case of glucose, this coupling formed β-anomer product as major. The α-anomer product of 1′-*O*-(Boc-ALA)-2′, 3′, 4′, 6′-tetra-*O*-benzyl mannose was isolated by column chromatography and then it was subjected to catalytic hydrogenation (scheme 81) to deprotect benzyl groups taking all the necessary precautions to avoid side reactions and impurities. This afforded pure 1′-*O*- (Boc-ALA) - α-mannose **41** which on subsequent "Boc" deprotection (scheme 81) yielded pure 1′-*O*-(ALA) α-mannose **42** as trifluoroacetate salt.

Thus we synthesized pure 1′-*O*-(ALA) α-mannose **42.**

5.3.2.3. Synthesis of 1′-O-(ALA) β-galactose

5.3.2.3.1. Attempted trials using unprotected galactose



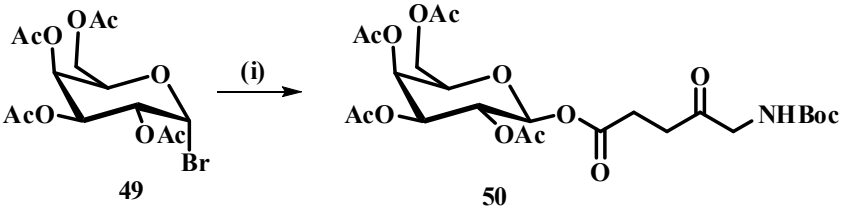
27 7 47

Reagents and conditions: (i) catalytic NaH, rt, 12h.

Scheme 82: Failed reaction using unprotected galactose for synthesizing 1′-*O*-(Boc-ALA) galactose

Initially,we attempted to synthesize anomerically pure 1′-*O*-(Boc-ALA) β-galactose **47** from unprotected galactose and Boc-ALA-PFP ester using catalytic amount of base which were not successful (scheme 82) [341, 342]. As mentioned in 4.3.2.1.1, these transformations were reported for β-glucose.

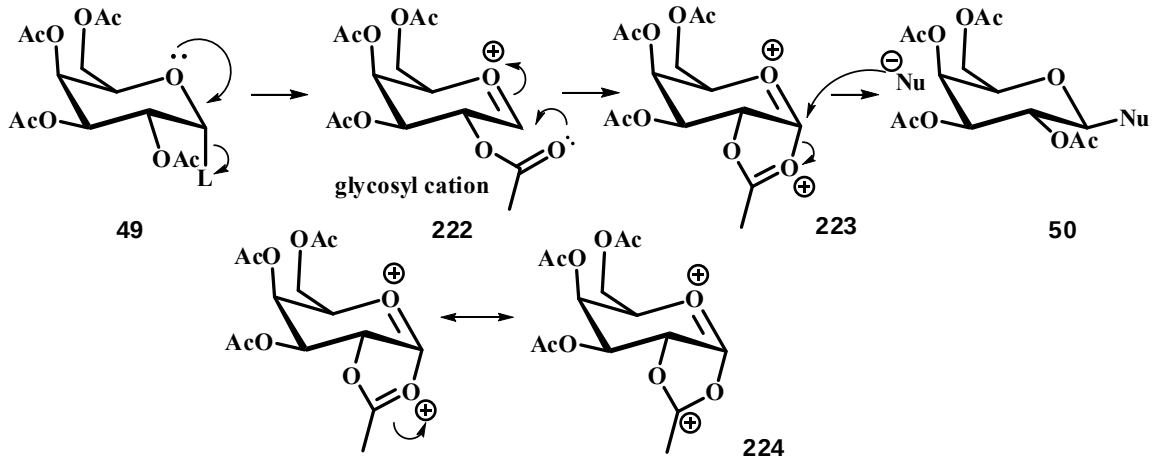
5.3.2.3.2. Koenigs-Knorr type reaction



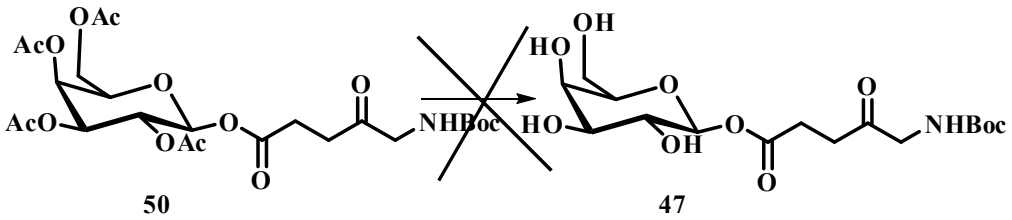
49 50

Reagents and conditions: (i) **5**, Cs_2CO_3, 0^0C to rt, 1 h.
Scheme 83: Synthesis of 2′,3′, 4′,5′ -tetracetyl 1′-*O*-(Boc-ALA)-Koenigs-Knorr reaction

In order to obtain the acyl (ALA) substitution at C1 of galactose in β configuration , we then applied Koenigs-Knorr type reaction [359, 372, 373]. We carried out this reaction using Cs_2CO_3 instead of Ag_2CO_3 as reported earlier [374]. This method provided 1′-*O*-(Boc-ALA) 2′,3′,4′,6′-*O*- tetra acetyl galactose (only β anomer) **50** from 2,3,4,6-*O*-tetra acetyl galactosyl bromide (acetobromo galactose) **49** which is commercially available and Boc-ALA (scheme 84). β-stereoselectivity in the glycosylation reaction was ensured by neighbouring-group participation of acetyl groups at O-2 in the glycosyl donors (scheme 55). According to the literature [375, 376], the glycosylation reaction proceeds by activation of the glycosyl donor by the silver salt (Ag_2CO_3), leading to the irreversible formation of a glycosyl oxocarbenium ion **222** [377]. This ion due to neighbouring-group participation is in equilibrium with the corresponding carbocationic species **224** [378, 379]. Nucelophilic attack of Boc-ALA on the carbocationic species can then result in the formation of desired product **50**.



Scheme 84: Koenigs-Knorr reaction- mechanism for β stereoselectivity



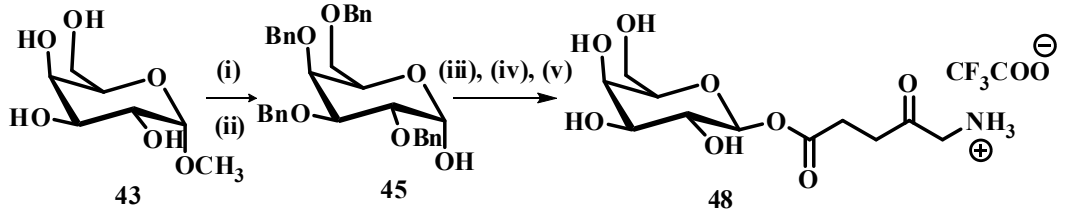
Scheme 85: Failed attempts to deprotect selectively acetyl groups

Various attempts (using NaOMe, K_2CO_3, BaO) to deprotect acetyl groups preferentially in the presence of ester at C1 to obtain 1´-*O*- (Boc-ALA) -β-galactose **47** were not successful (scheme 85) despite that such transformations have been reported in the literature before [380-384]. So we abandoned this route and we thought of performing the same Koenigs-Knorr type reaction with the starting material galactosyl bromide containing monochloroacetyl group instead of acetyl group for protecting the hydroxyl groups. This was the route followed by Shimizu *et al* when they encountered difficulties in deprotecting acetyl group preferentially in the presence of a glycosyl ester [351, 385].

One way of circumventing this problem is to start with 2,3,4,6-*O*-tetra benzyl galactosyl bromide which is not commercially available. But there are reports [386] that this compound is highly prone to decomposition and should be used immediately. Monochloroacetyl groups are known to be more labile than acetyl group during hydrolysis [387] and can be cleaved under neutral conditions (hydrazine dithiocarbonate) [388] or under slightly basic conditions (thiourea) [389]. Though we synthesized the starting material 1,2,3,4,6-penta chloroacetyl galactose, we abandoned this route as the benzyl protection methodology explained here below gave better results.

5.3.2.3.3. Benzyl Protection-deprotection

We performed benzyl protection methodology which starts with synthesizing α- methyl 2,3,4,6-Penta-*O*- benzyl galactoside **44** (major β anomer) from α- methyl galactoside **43** (scheme 86). α- methyl galactoside was taken as starting material instead of galactose unlike the case of 1´-*O*-(ALA) α-glucose and 1´-*O*-(ALA) α-mannose where glucose and mannose were the starting materials, because Decoster *et al* found that 1,2,3,4,6- Penta-*O*- benzyl galactose on selective deprotection at C1 provides the product 2,3,4,6-*O*-tetrabenzyl galactose of which more than 80% exists in furanose form. The selective deprotection at C1 of benzyl protected methyl galatoside **44** provided 2, 3, 4, 6-tetra-*O*-benzyl galactose (TBGa) α- anomer **45** in pyranose form being major (scheme 86) [346, 390].



48

Reagents and conditions: (i) NaH, BnBr, TBAI, rt, 8 h; (ii) CH_3COOH, 6N HCl, 60^0C, 5 h; (iii) EDCI, DMAP, 0^0C to rt, 7 h; (iv) H_2 / Pd, rt, 10 h; (v) CF_3COOH, CH_2Cl_2, 0-10^0C, 0.5 h.

Scheme 86: Synthesis of 1´-*O*-(ALA) galactose

The carbodiimide coupling of 2, 3, 4, 6-tetra-*O*-benzyl galactose (TBGa) with Boc-ALA in the presence of EDCI and DMAP [103], provided 1′-*O*-(Boc-ALA)- 2′, 3′, 4′, 6′-tetra-*O*-benzyl β-galactose **46**, β anomer product (β: α = 6.6:1) being major. The β anomer product was isolated by column chromatography and then it was subjected to catalytic hydrogenation to deprotect benzyl groups taking all the necessary precautions to avoid side reactions and impurities. This afforded pure 1′-*O*- (Boc-ALA) -β-galactose **47** which on subsequent "Boc" deprotection yielded pure 1′-*O*-(ALA) β-galacose as trifluoroacetate salt **48**.Thus we synthesized 1′-*O*-(ALA) β-galacose.

5.3.2.4. Evaluation of PpIX production by cells by second generation ALA-sugar conjugates- 1′-O- (ALA) α- glucose, 1′-O-(ALA) α-mannose and 1′-O-(ALA) β-galactose

5.3.2.4.1. Efficiency- PpIX generation

PpIX production was evaluated for all the regiospecific and anomerically pure 1′-*O*-(ALA) α-glucose, 1′-*O*- (ALA) α-mannose and 1′-*O*- (ALA) β-galactose by measuring increase in fluorescence intensity in living cells at different times by incubating cells in medium containing ALA conjugate at concentrations from 0.1mM to 2 mM. Cells used for experiments were A549 lung carcinoma cells, HCEC human immortalized brain endothelial cells, breast MCF7 carcinoma and SW480 colon carcinoma cells.

1′-*O*- (ALA) α-glucose, 1′-*O*- (ALA) α-mannose and 1′-*O*- (ALA) β-galactose induced strong PpIX production in all cell lines (the graphs of increase in PpIX level by certain selected cell lines are shown here). The increase in PpIX level from 0h to 3 h (figure 12-15) shows that in all cell lines except MCF7, 1′-*O*- (ALA) α-glucose, 1′-*O*-(ALA) α-mannose and 1′-*O*- (ALA) β-galactose produce higher or equivalent level of PpIX than that induced by standard ALA at any point of time.

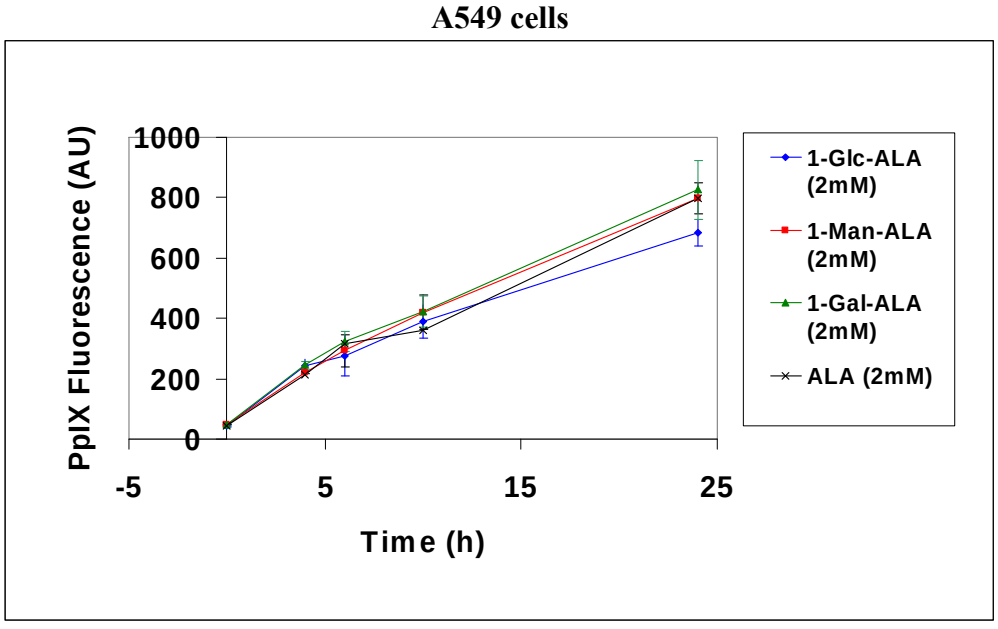


Figure 12: Increase in PpIX level due to 1′-*O*-(ALA) sugars and ALA in A549 cells.

HCEC cells

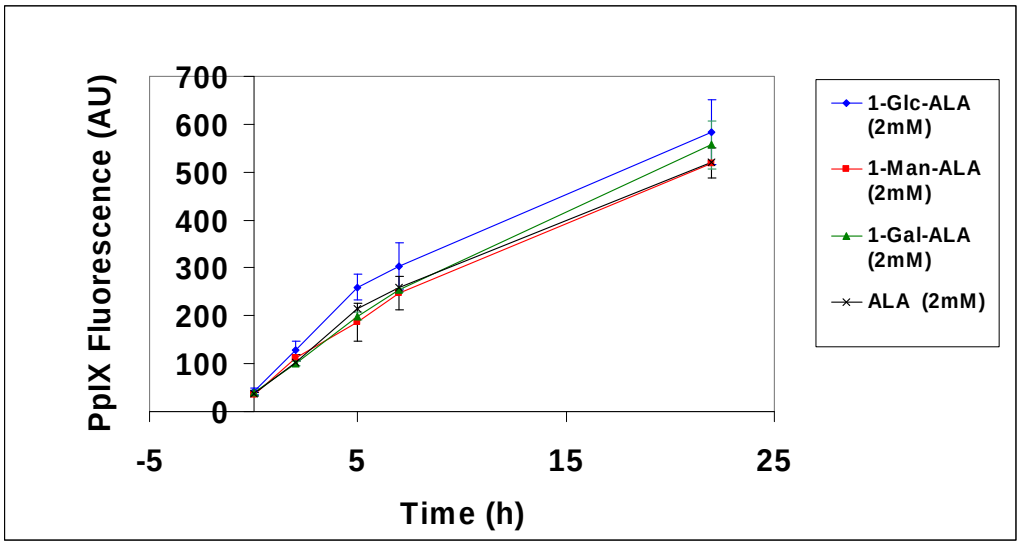


Figure 13: Increase in PpIX level due to 1´-*O*-(ALA) sugars and ALA in HCEC cells.

MCF 7 cells

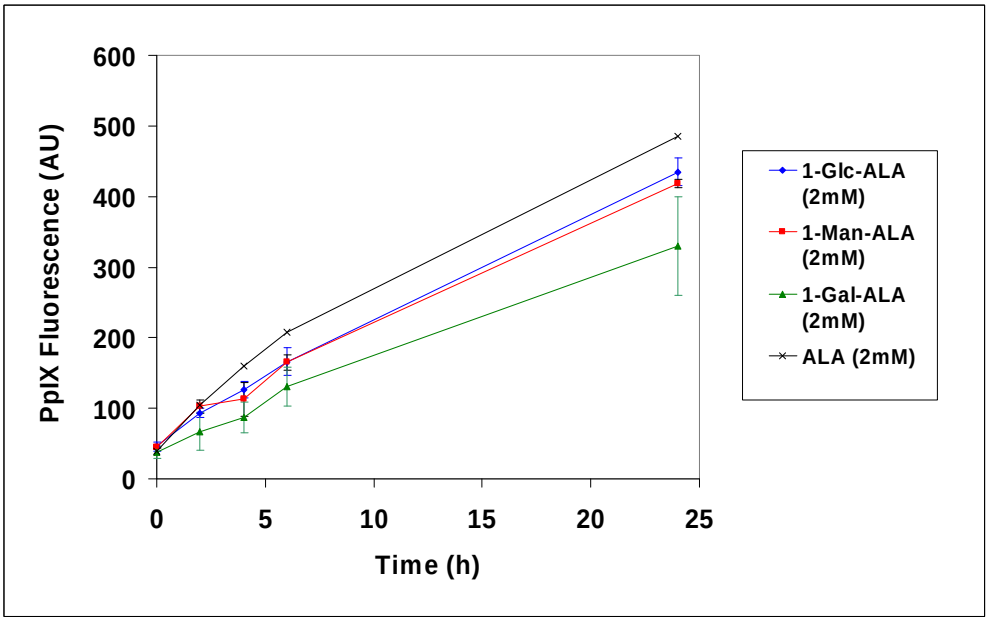


Figure 14: Increase in PpIX level due to 1´-*O*-(ALA) sugars and ALA in MCF7 cells.

SW 480

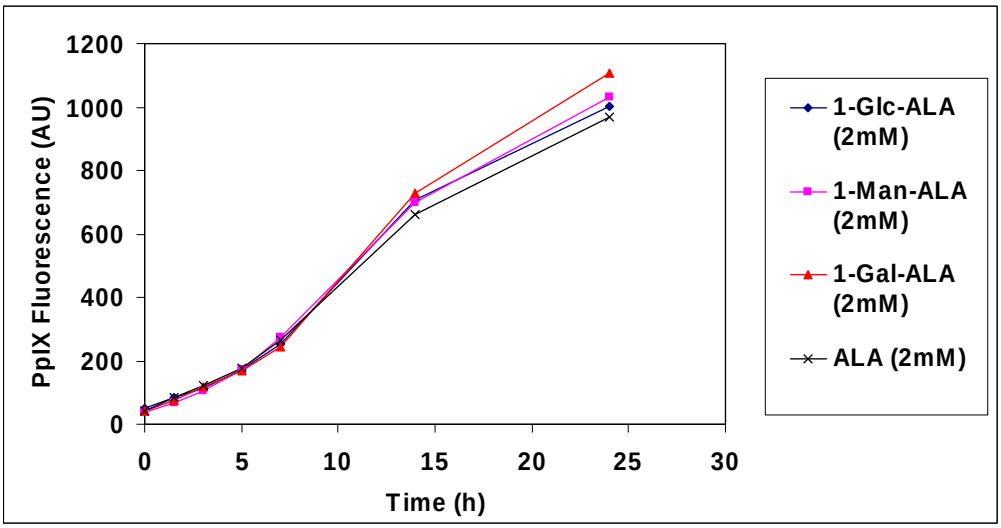


Figure 15: Increase in PpIX level due to 1′-*O*-(ALA) sugars and ALA in SW480 cells.

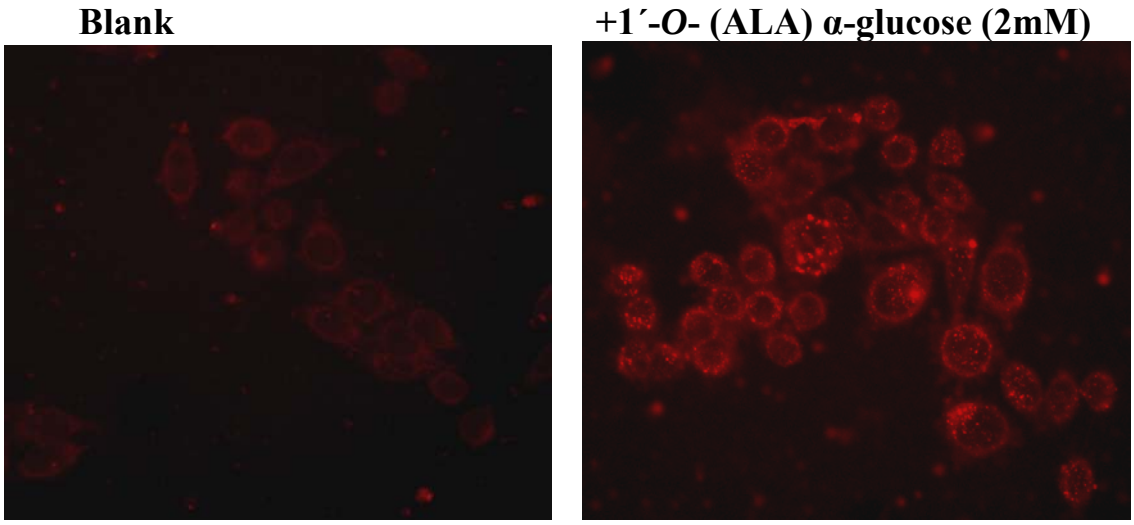


Figure 16: Fluorescence microscopy of SW480 cells exposed to 1'-O-ALA-α-glucose (2mM)

The fluorescence photographs of cells are shown here above in the figure 16. The cells without any derivative (blank) are shown in the left hand side and cells exposed to 1′-*O*-(ALA) α-glucose are shown in the right hand side. As can be seen clearly, 1′-*O*- (ALA) α-glucose induced sufficient PpIX generation which illuminates as red fluorescent spots inside the cells when exposed to 409nm wavelengh light.

5.3.2.4.2. Cytotoxicity

The alamar blue assay showed that all the 1′-*O*-(ALA) sugar conjugates are not cytotoxic. The data of this assay is explained in chapter 4.3.4 where the alamar assay of all the ALA glycoconjugates was described together.

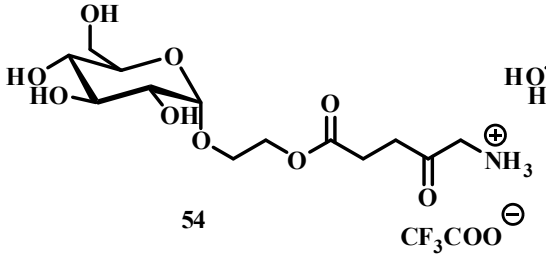
5.3.2.4.3. Selectivity

The PpIX generation due to 1′-*O*- (ALA) sugar conjugates were compared with ALA in fibroblast cells (VS 59). The PpIX generated by 1′-(ALA) sugar conjugates was in the same level as that of ALA. This means the selectivity of 1′-*O*- (ALA) sugar conjugates towards cancer cells is not better than ALA. The PpIX level is shown in chapter 4.3.4 where the PpIX generation due to all the ALA glycoconjugates in fibroblast cells is shown.

5.3.3. Synthesis of 1-*O*-(ALA)- 2-*O*-(glycosyl) ethylene glycol (Third generation ALA-glycoconjugates)

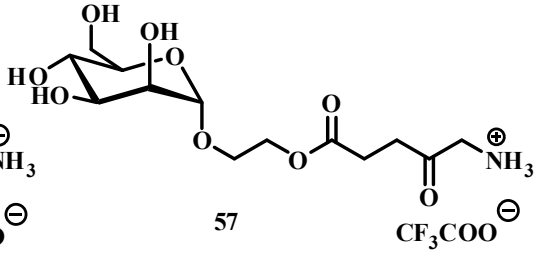
As advanced generation (third generation) of our ALA glycoconjugates to target our glycosidases enzymes namely α-glucosidase, α-mannosidase and β galactosidase, we decided to have the ALA coupled with carbohydrates via a linker. We chose ethylene glycol as linker as ALA-ethylene glycol esters induce not only high levels of PpIX, but also found to be non- toxic over a wide rangeof concnentrations compared to hexyl ALA ester, a well known ALA derivative known in the literature [102, 103].

We decided to couple ALA with the C1 hydroxyl group of glucose, mannose and galactose via an ethylene glycol linker (scheme 87).



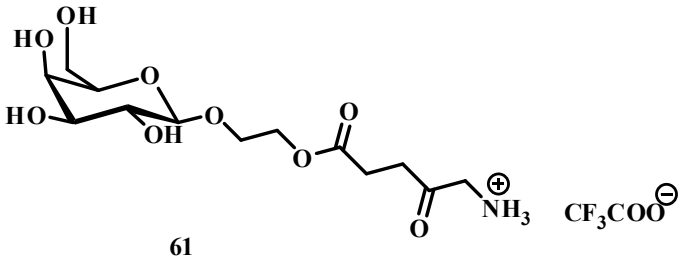
54

1-*O*-(ALA)-2-*O*-(a-glucopyranosyl) ethylene glycol



57

1-*O*-(ALA)-2-*O*-(a-mannopyranosyl) ethylene glycol



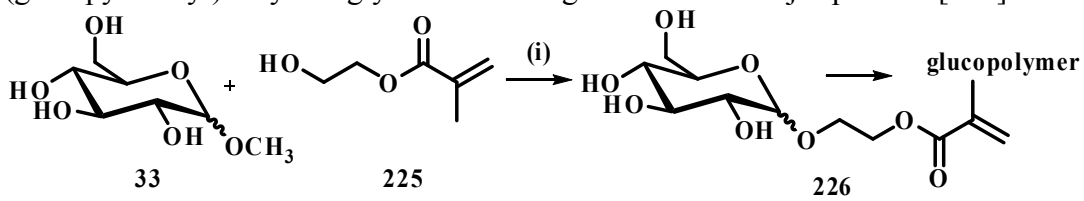
61

1-*O*-(ALA)-2-*O*-(b-galactopyranosyl) ethylene glycol

Scheme 87: 1-*O*-(ALA)-2-O-(glycosyl) ethylene glycol (Third generation ALA-glyvcoconjugates)

5.3.3.1. Synthesis of 1-*O*-(ALA)-2-*O*-(α-glucopyranosyl) ethylene glycol and 1-*O*-(ALA)-2-*O*-(α-mannopyranosyl) ethylene glycol

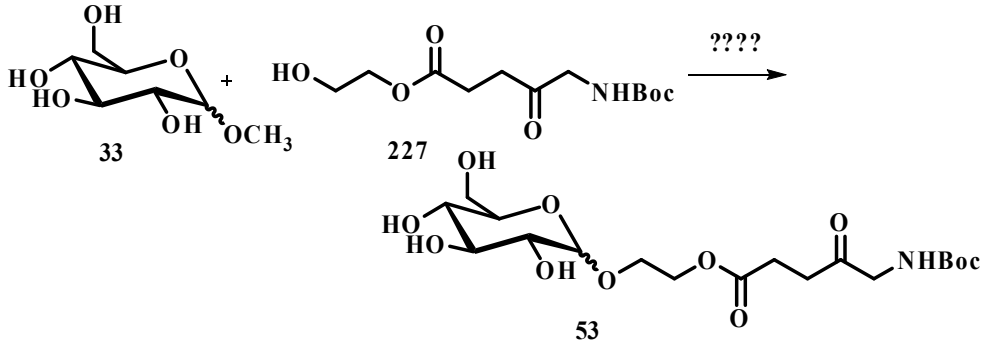
In the field of 'glycopolymers' (carbohydrate based polymers), glycosylated methacrylate derivatives had been reported as monomers which were further polymerized. The synthesis of methacrylate derivatives of glucose and galactose (acid moiety connected to C1 of glucose via an ethylene glycol linker) by glycosylation of 2-hydroxyethyl metharylate (HEMA) **189** have been reported earlier [382, 391-393]. Kitazawa *et al* synthesized (scheme 88) 2-hydroxyethyl methacrylate **225** and then reacted this with methyl glucoside **33** as glycosyl donor in the presence of a heteropoly acid (phosphomolybdic acid) in a catalytic quantity to afford 1- *O*- methacryl- 2-*O*-(glucopyranosyl) ethylene glycol **226** having α anomer as major product [391].



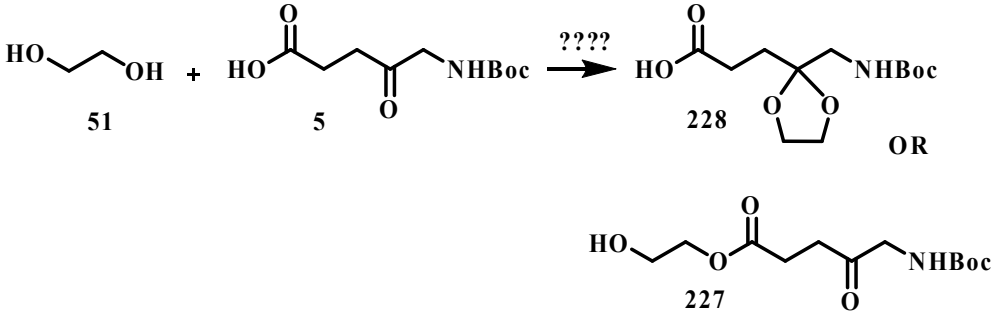
Reagents and conditions: (i) catalytic phosphomolybdic acid, 2,4-dinitrochlorobenzene 112^0C, 3 h

Scheme 88: Reported scheme for the synthesis of a glycomonomer with ethylene glycol as linker between glucose and acrylate ester [391]

Similarly, the third generation ALA glyconjugate 1-*O*-(ALA)-2-*O*-(α-glucopyranosyl) ethylene glycol **54** can be synthesized (scheme 89) by first synthesizing mono Boc-ALA ester of ethylene glycol (2-hydroxyethyl ALA ester) **227** and then reacting this with methyl glucoside **33**.



Scheme 89: Possible reaction scheme by coupling ethylene glycol and Boc-ALA first



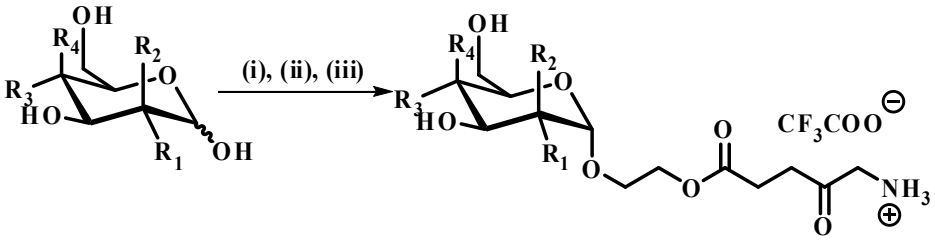
Scheme 90: Reaction of ethylene glycol with Boc-ALA

The synthesis of mono Boc-ALA ester of ethylene glycol **227** has to go through protection of keto group of Boc-ALA as under the acidic conditions, ethylene glycol will add on the keto group first to form a cyclic ketal **228** (scheme 90) [394]. This makes the synthetic route longer by one step to deprotect the cyclic ketal. Also, we envisaged that this route could give rise to problems like ester cleavage etc under the harsh conditions of temperature (120^0C) and heteropoly acid (phosphomolybdic acid).

So, we decided to synthesize first 1-*O*-(α-D-glucopyranosyl) ethylene glycol **52** by Fischer method in order to obtain α anomer as major product and then perform the acylation regioselectively on the primary hydroxyl group of ethylene glycol using the enzyme *Candida antarctica* lipase (Novozyme 435) along with Boc-ALA-PFP ester [395].

5.3.3.1.1. Fischer glycosidation

We synthesized 1-*O*-(α-D-glucopyranosyl) ethylene glycol **52** by a modification (scheme 64) of the method described by Mowery [396-399]. Dowex 50WX 8-200 resin, ethylene glycol and glucose were stirred for 72 h at 70-80^0C. The resin was filtered off, excess ethylene glycol which was added as solvent was evaporated off and the residue was subjected to column chromatography providing 1-*O*-(α-D-glucopyranosyl) ethylene glycol **52** (scheme 91). In the case of 1-*O*-(α-D-mannopyranosyl) ethylene glycol **55** (scheme 91), mannose was taken instead of glucose.



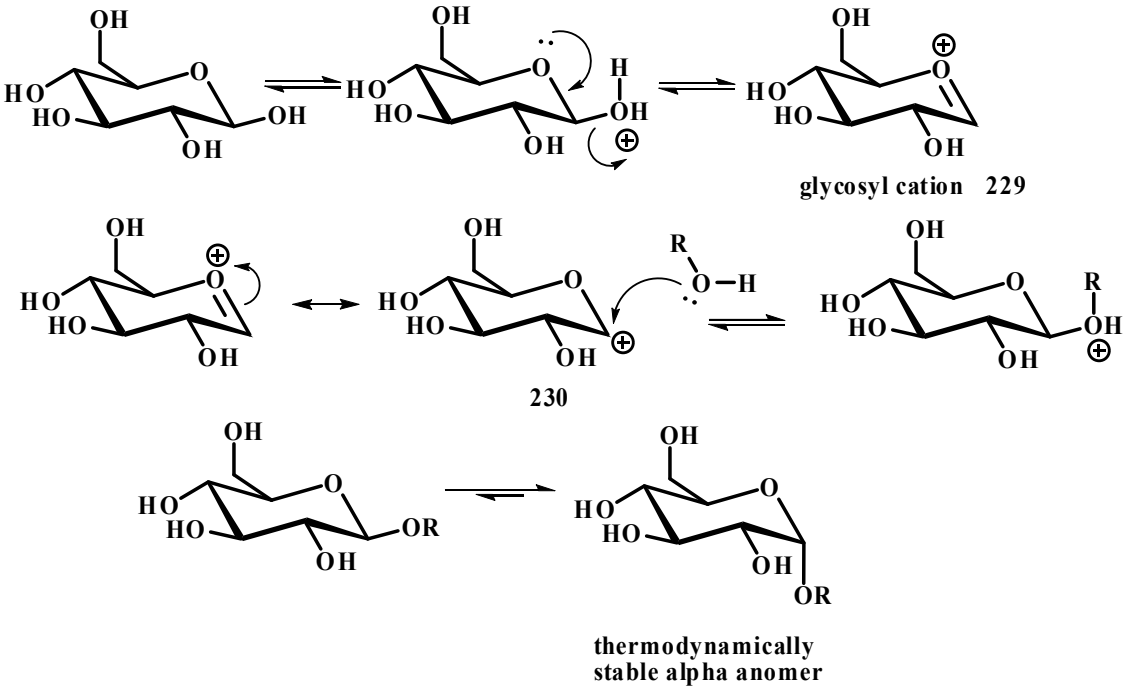
13; R_1=OH, R_2=H, R_3=OH, R_4=H **54; R_1=OH, R_2=H, R_3=OH, R_4=H**

24; R_1=H, R_2=OH, R_3=OH, R_4=H **57; R_1=H, R_2=OH, R_3=OH, R_4=H**

Reagents and conditions: (i) Dowex 50WX 8-200, ethylene glycol, 70-80^0C, 72 h; (ii) **10b**, Candida antarctica (Novozyme 435), 50^0C, 0.5 h; (iii) CF_3COOH, CH_2Cl_2, 0-10^0C, 0.5 h.

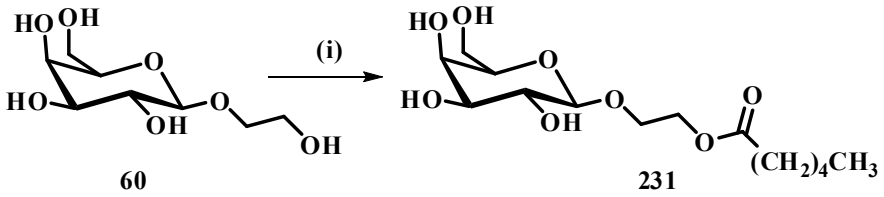
Scheme 91: Synthesis of 1-*O*-(α-D-glycopyranosyl) ethylene glycol **54**

Fischer glycosidation is acid catalyzed and the reaction gives rise to the formation of a cyclic carbocation intermediate called a glycosyl cation **229**. In a S_N1 process, the glycosyl cation is then attacked by the alcohol in the second step to yield the product (scheme 92).



Scheme 92: Fischer glycosidation mechanism

5.3.3.1.2. Regioselective enzymatic esterification



Reagents and conditions [395]: (i) 2,2,2-trifluoroethyl hexanoate, *Candida antarctica* (Novozyme 435), 45^0C, 0.5 h

Scheme 93: Reported example of regioselective enzymatic esterification preferentially on the hydroxyl group of ethylene glycol moiety by Colombo *et al* [395].

Colombo *et al* (scheme 66) [395, 400, 401] had reported that the enzyme *Candida antarctica* lipase (Novozyme 435) can acylate preferentially at the glycerol/glycol moiety in the presence of primary and secondary hydroxyl groups in a sugar (scheme 93). They had also observed that this selectivity towards primary hydroxyl group of ethylene glycol decreases with increase in chain length of the acyl moiety.

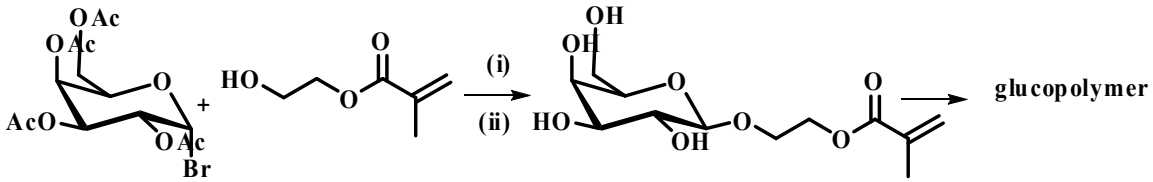
Following the method of Colombo *et al*, we acylated regioselectively (scheme 91) the primary hydroxyl group of ethylene glycol in 1-*O*-(α-D-glucopyranosyl) ethylene glycol **52** using the activated ester Boc-ALA-PFP ester and the enzyme Novozyme 435. Thus we obtained 1-*O*-(Boc-ALA)-2-*O*-(α-glucopyranosyl) ethylene glycol **53** with a moderate yield (48%). Colombo *et al* carried out this regioselective esterification towards glycol moiety with trichloroethyl ester as an activated ester (3eq) and emplyoying 7.5 equivalents of the enzyme *Candida antarctica* lipase (Novozyme 435) (1.0 g with the

activity of 10 units / mg solid). We performed the reaction with 3eq of Boc-ALA-PFP ester as an activated ester. We had to use higher equivalents of enzyme (10 units / mg solid) minimum of 15 equivalents to achieve good regioselectivity in the case of glucose although lesser equivalents of enzyme were sufficient for the case of mannose. With less equivalents of enzyme, the selectivity of acylation towards primary hydroxyl group of ethylene glycol decreases as acylation on C6 primary hydroxyl group competes. The same mode of procedure also worked well for synthesizing 1-*O*-(Boc-ALA)-2-*O*-(α-mannopyraoosyl) ethylene glycol **56** (scheme 94).

5.3.3.1.3. 'Boc' deprotection

Later, the 'Boc' group in both 1-*O*-(Boc-ALA)-2-*O*-(α -glucopyranosyl) ethylene glycol **53** and 1-*O*-(Boc-ALA)-2-*O*-(α -mannpyranosyl) ethylene glycol **56** were deprotected following similar procedure (scheme 91) as we did for 1′-*O*-(ALA) glucose in order to avoid hydrolysis of the ester. Thus we obtained pure 1-*O*-(ALA)-2-*O*-(α -glucopyranosyl) ethylene glycol **54** and 1-*O*-(ALA)-2-*O*-(α -mannopyranosyl) ethylene glycol **57.** These compounds were tested in various cancer cell lines.

5.3.3.2. Synthesis of 1-O-(ALA)-2-O-(β-galactopyranosyl) ethylene glycol



49 225 232

Reagents and conditions: (i) AgOTf, -40^{0}C, 48h; (ii) $NaOCH_3$; rt, 40min.
Scheme 94: Reported scheme by Ambrosi *et al* for synthesis of a β-galactomonomer with ethylene glycol as linker between galactose and acrylate ester [382].

As mentioned in 4.3.3.1, in the field of glycopolymers, there are reports in the literature where 2-hydroxyethyl methacrylate 225 (HEMA) is reacted with 2,3,4,6-*O*-tetra acetyl galactosyl bromide (acetobromo galactose) **49** as glycosyl donor in the presence of silver or mercury salts to afford 1-*O*-methacryl- 2-*O*-(2′,3′,4′,6′-*O*-tetra acetyl-β-galactopyranosyl) ethylene glycol having β anomer as the only product [382, 392, 393]. The acetyl groups of this compound was later deprotected preferentially in the presence of acrylate ester to afford 1-*O*-methacryl- 2-*O*-(β-galactopyranosyl) ethylene glycol having β anomer **232** as the only product [382]. The authors polymerized this glycomonomer to have glycopolymers.

In a similar manner, we thought that 1-*O*-(ALA)-2-*O*-(β -galactopyranosyl) ethylene glycol **62** can be synthesized by first synthesizing mono ALA ester of ethylene glycol (2-hydroxyethyl ALA ester) (scheme 94) and then reacting this 2,3,4,6-*O*-tetra acetyl

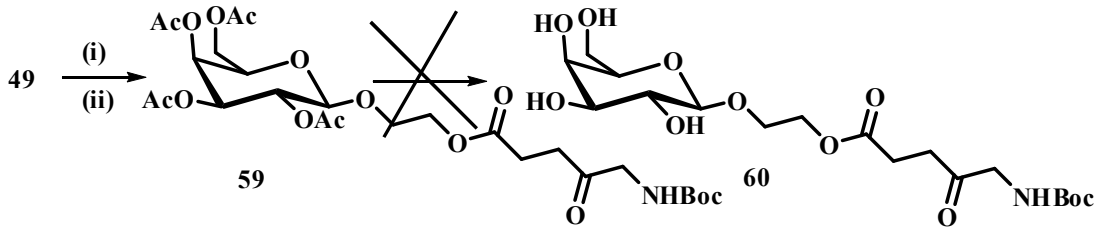
galactosyl bromide (acetobromo galactose) in the presence of silver or mercury salts. Although this route could provide only β anomer, we envisaged that the synthesis of mono Boc-ALA ester of ethylene glycol might be long (3 steps) as ethylene glycol can add to the keto group of Boc-ALA first before getting esterified (scheme 94). So we decided to follow Koenigs-Knorr type reaction to have β stereoslectivity as we needed β substituted product.

5.3.3.2.1. Koenigs-Knorr reaction

We planned to synthesize first 1-*O*-(2′, 3′, 4′, 6′tetraacetyl-β-D-galactopyranosyl) ethylene glycol **58** by Koenigs-Knorr type reaction [359, 372, 373] from 2,3,4,6-*O*-tetra acetyl galactosyl bromide (acetobromo galactose) **49** in order to obtain β anomer as major product. Now, we had two choices; As a first choice, we could couple Boc-ALA with the only free hydroxyl group of the glycol moiety to obtain **59** and then deprotect all the acetyl groups selectively to obtain 1-*O*-(Boc-ALA)-2-*O*-(β -galactopyranosyl) ethylene glycol **61** (Scheme 95). As a second choice, we could deprotect all the acetyl groups of 1-*O*-(2′,3′,4′,6′-tetraacetyl-β-D-galactopyranosyl) ethylene glycol **58** to obtain 1-*O*-(β-D-galactopyranosyl) ethylene glycol **60.** Then carrying out acylation selectively on the primary hydroxyl group of ethylene glycol using the enzyme *Candida antarctica* lipase (Novozyme 435) along with Boc-ALA-PFP ester [395] to obtain **61,** a method reported by Colombo *et al* and also accomplished in the case of glucose and mannose derivatives by us.

We synthesized first 1-*O*-(2′, 3′, 4′, 6′tetraacetyl-β-D-galactopyranosyl) ethylene glycol **58** (only β anomer product) from 2,3,4,6-*O*-tetra acetyl galactosyl bromide (acetobromo galactose) **49** and ethylene glycol by Koenigs-Knorr type reaction (scheme 95). β-stereoselectivity in the glycosylation reaction was ensured by neighbouring-group participation of acetyl groups at O-2 in the glycosyl donors.

5.3.3.2.2. Coupling with Boc-ALA and Selective removal of acetyl group-attempts

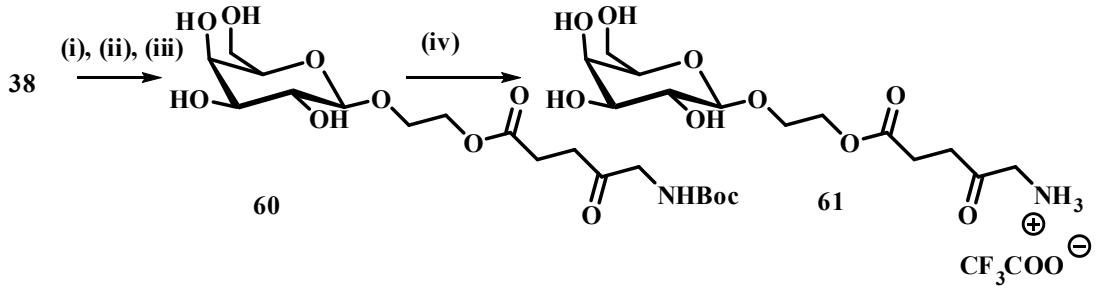


Reagents and conditions: (i) Ag_2CO_3, ethylene glycol, rt, 4 h; (ii) **5**, EDC, DMAP; rt, 7 h.
Scheme 95: Koneigs-Knorr type reaction and attempted trials to deprotect acetyl groups preferentially in the presence of ALA ester.

In our first trial, we coupled this product with Boc-ALA using carbodimide coupling to obtain **59**; the reaction is straight forward as there is only one free hydroxyl group which is from the glycol moiety as the hydroxyl groups of galactose are protected by acetyl groups (scheme 95). Though we were not able to deprotect acetyl group preferentially in the case of 1′-*O*-(Boc-ALA) 2′,3′,4′,6′-*O*- tetra acetyl galactose **50** (scheme 85; chapter

5.3.2.3.2), we tried in this case again. Many attempts (using NaOMe, K_2CO_3, BaO) to deprotect the acetyl groups of **59** preferentially in the presence of ALA ester attached to glycol moiety were not successful [380-384] (scheme 95).

5.3.3.2.3. Regioselective enzymatic esterification



Reagents and conditions: (i) Ag_2CO_3, ethylene glycol, rt, 4 h; (ii) NaOMe, rt, 2 h; (iii) Candida antarctica (Novozyme 435), 50^0C, 0.5 h; (iv) CF_3COOH, CH_2Cl_2, 0-10^0C, 0.5 h.

Scheme 96: Koneigs-Knorr type reaction, enzymatic regioselective esterification and 'Boc' deprotection.

In our second trial (scheme 96), we deprotected the acetyl groups from 1-O-(2′, 3′, 4′, 6′tetraacetyl-β-D-galactopyranosyl) ethylene glycol **58** by using sodium methoxide to afford 1-O-(β-D-galactopyranosyl) ethylene glycol **59a**. Then as in the case of glucose and mannose, we attempted to acylate regioselectively the primary hydroxyl group of ethylene glycol moiety in 1-*O*-(β-D-galactopyranosyl) ethylene glycol using the activated ester Boc-ALA-PFP ester and the enzyme Novozyme 435 (method of Colombo *et al*) [395]. We were successful to obtain 1-*O*-(Boc-ALA)-2-*O*-(β -galactopyranosyl) ethylene glycol **61** in moderate yield (40%) though we had to use more equivalent of the enzyme (20eq) than what we had used in the case of mannose and glucose (15eq) to achieve good regioselectivity.

Like in the case of 6′-*O*-(Boc-ALA) sugars, we found that the ease of regioselectivity and efficiencies of the formation was in the following order: Mannose > glucose > galactose which was same as reported by Boyer *et al* for 6-*O*-phenylalanine-carbohydrate conjugates [318].

Deprotection of 'Boc' from 1-*O*-(Boc-ALA)-2-*O*-(β -galactopyranosyl) ethylene glycol **61** using TFA led to our target compound 1-*O*-(ALA)-2-*O*-(β -galactopyranosyl) ethylene glycol **62** which was tested in various cancer cell lines.

5.3.3.3. Evaluation of PpIX production by cells by third generation ALA-sugar conjugates - 1-O-(ALA)-2-O-(α-glucopyranosyl) ethylene

glycol and 1-O-(ALA)-2-O-(α-mannopyranosyl) ethylene glycol and 1-O-(ALA)-2-O-(β-galactopyranosyl) ethylene glycol

5.3.3.3.1. Efficiency- PpIX generation

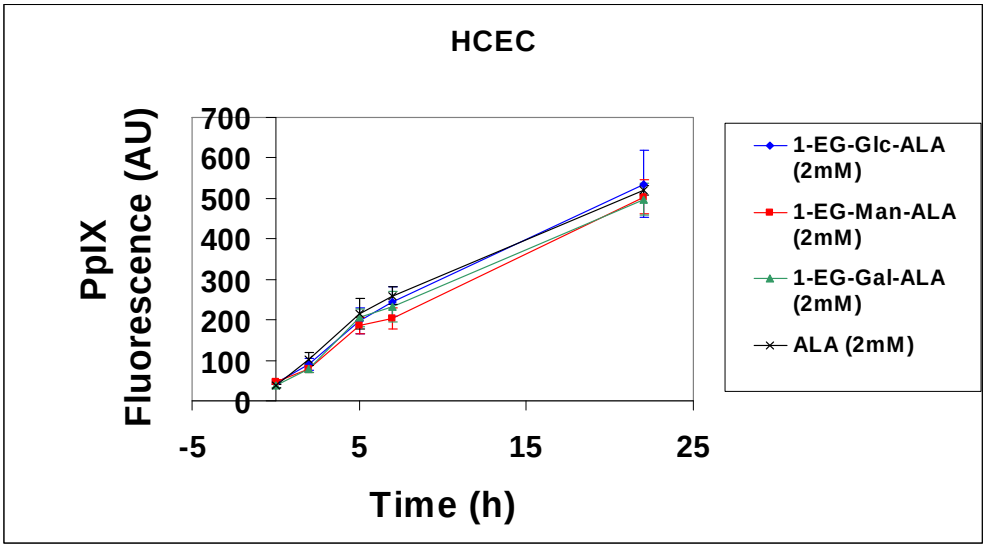


Figure 17: Increase in PpIX level due to 1´-*O*-EG-(ALA) sugars and ALA in HCEC cells

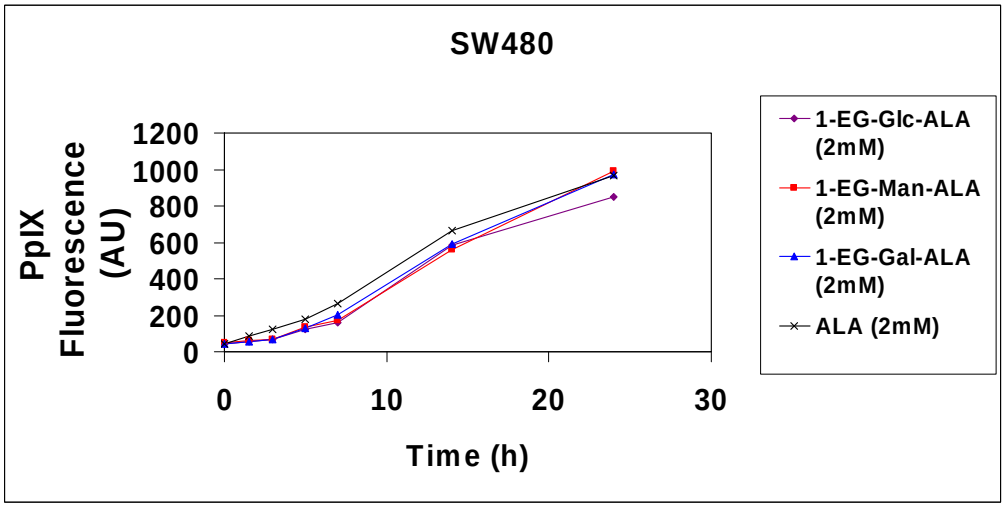


Figure 18: Increase in PpIX level due to 1´-*O*-EG-(ALA) sugars and ALA in SW480 cells

PpIX production was evaluated for the regiospecific and anomerically pure 1-*O*-(ALA)-2-*O*-(α-glucopyranosyl) ethylene glycol **54** and 1-*O*-(ALA)-2-*O*-(α-mannopyranosyl) ethylene glycol **57** and 1-*O*-(ALA)-2-*O*-(β-galactopyranosyl) ethylene glycol **62** by measuring increase in fluorescence intensity in living cells at different times by incubating cells in medium containing ALA conjugate at concentrations from 0.1mM to 2 mM. Cells used for experiments were HCEC human immortalized brain endothelial cells, were A549 lung carcinoma, SW480 colon carcinoma and breast T47D carcinoma cells.

1-*O*-(ALA)-2-*O*-(α-glucopyranosyl) ethylene glycol **54**,1-*O*-(ALA)-2-*O*-(α-mannopyranosyl) ethylene glycol **57** and 1-*O*-(ALA)-2-*O*-(β-galactopyranosyl) ethylene glycol **62** generate PpIX in all cell lines tested (the graphs of increase in PpIX level by certain selected cell lines are shown here). But, the increase in PpIX level from 0h to 3 h (figure 17 and 18) shows that in cell lines HCEC and SW 480, these conjugates with ethylene glycol linker produce PpIX comparable to that of ALA. In cell lines A549 and T47D, these conjugates produce PpIX lesser than that induced by standard ALA. This could be because these conjugates are not effectively internalized into the cells. Thus, the efficiency to generate PpIX induction by these ethylene glycol linker based conjugates is inferior to that of 6´-*O*-(ALA) sugars and 1´-*O*-(ALA) sugars.

5.3.3.3.2. Cytotoxicity

The alamar blue assay and MTT assay showed that all the ethylene glycol linker based ALA sugar conjugates (**54, 57 and 62**) were not cytotoxic. The data of this assay is explained in chapter 4.3.4 where the alamar assay of all the ALA glycoconjugates was described.

5.3.3.3.3. Selectivity

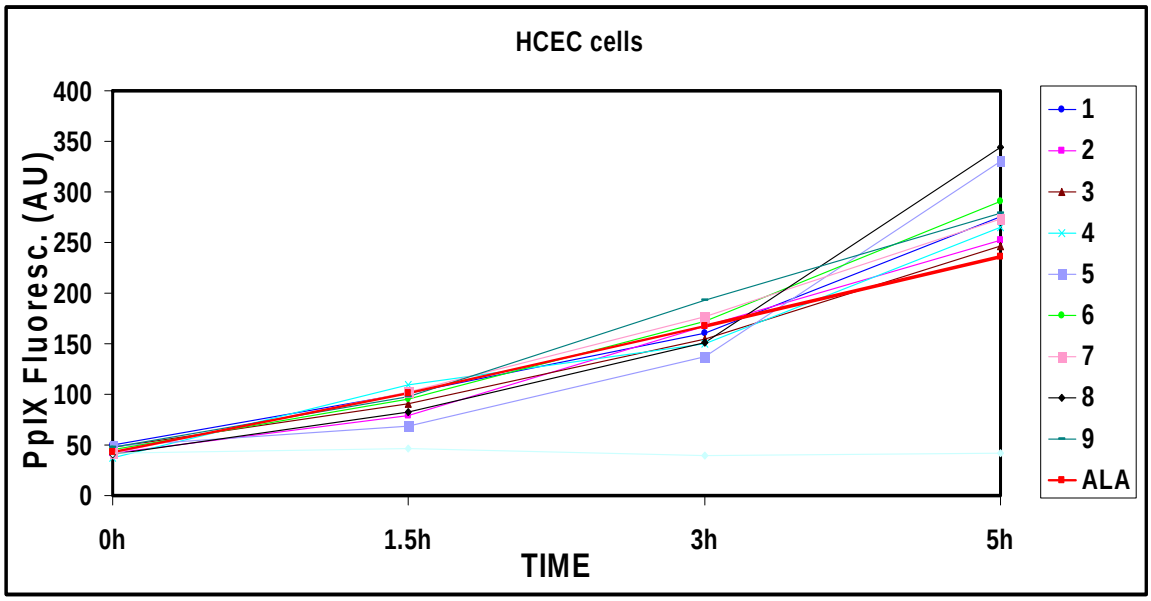
The ethylene glycol linker based ALA sugar conjugates (**54, 57 and 62**) were comparable to ALA on targeting cancer cells. PpIX induction due to ALA and third generation conjugates (**54, 57 and 62**) in three different types of normal fibroblast cells SS 77, GS78 and GE 80 were checked (not shown in figure). In these cells, ALA generated PpIX where as the conjugates (**54, 57 and 62**) generated lower level of PpIX than that of ALA. Further evaluation of the conjugates (**54, 57 and 62**) in normal fibroblast cells is mentioned in chapter 4.3.4.

5.3.4. Evaluation of PpIX production by first (6´-O-(ALA) sugars), second (1´-O-(ALA) sugars) and third (1´-O-(EG-ALA) sugars) generation ALA-sugar conjugates

5.3.4.1. Efficiency- PpIX generation

In summary, we synthesized three different types of ALA-glycocongugates, ALA being substituted at C6-hydroxyl group of sugars (6´-*O*-(ALA) sugars), ALA being substituted at C1-hydroxyl group of sugars (1´-*O*-(ALA) sugars) and ALA being substituted at C1-hydroxyl group of sugars via ethylene glycol linker (1-*O*-(EG-ALA) sugars).

We evaluated recently all the ALA- glycoside conjugates (nine compounds) together in a same well plate on various cancer cell lines (one among them HCEC cells, is shown here). As can be seen from figure 19, the conjugates with ethylene glycol linker on comparison to the standard ALA, though generate less PpIX in the initial 3h, it produced higher PpIX generation after 5h. This might be due to the slow cross over of the cell wall by these conjugates due to its liphilicity compared to other compounds.



Derivative 1: 1-α-Glc ALA (2mM); Derivative 2: 1-α-EG-ALA Glc (2mM); Derivative 3: 6-Glc ALA (2mM);
Derivative 4:1-α-Man ALA (2mM); Derivative 5: 1-α-EG-ALA Man (2mM); Derivative 6: 6-Man ALA (2mM);
Derivative 7: 1-β-Gal ALA (2mM); Derivative 8: 1-β-EG-ALA Gal (2mM); Derivative 9: 6-Gal ALA(2mM).

Figure 19: Increase in PpIX level due to sugar-ALA conjugates

5.3.4.2. Cytotoxicity tests

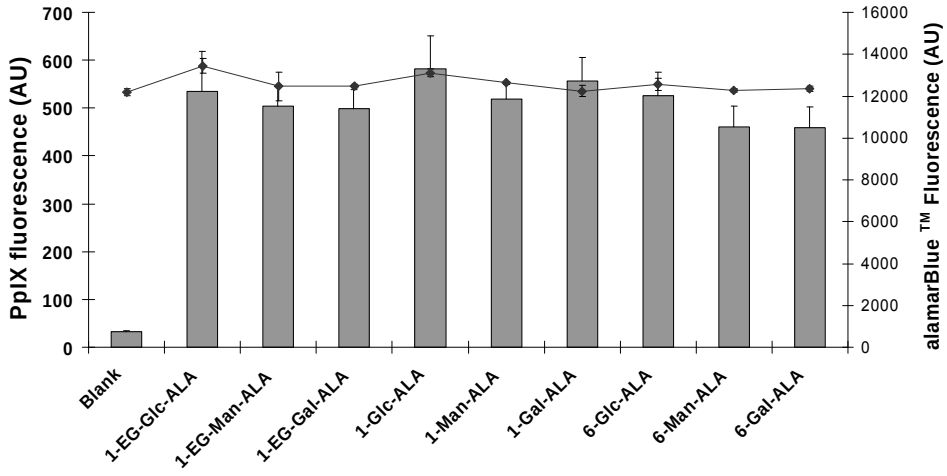


Figure 20: Cell survival measurements by alamar blue assay after 24h exposure.

We also tested the dark cytotoxicity of all these compounds by alamar blue assay. As shown in the figure, cells treated with all the compounds show fluorescence which is due to the reduced form of alamar called resorufin **174** (refer chapter 2.3.2) which is a pink

fluorescent dye. As this reduction is due to cell activity (likely to be by oxygen consumption through metabolism) we could conclude that all of these sugar conjugates showed no cytotoxicity. This is an advantage of these sugar conjugates compared to hexyl ALA ester, an ALA derivative which induces higher PpIX generation than that of ALA, but is cytotoxic.

5.3.4.3. Selectivity

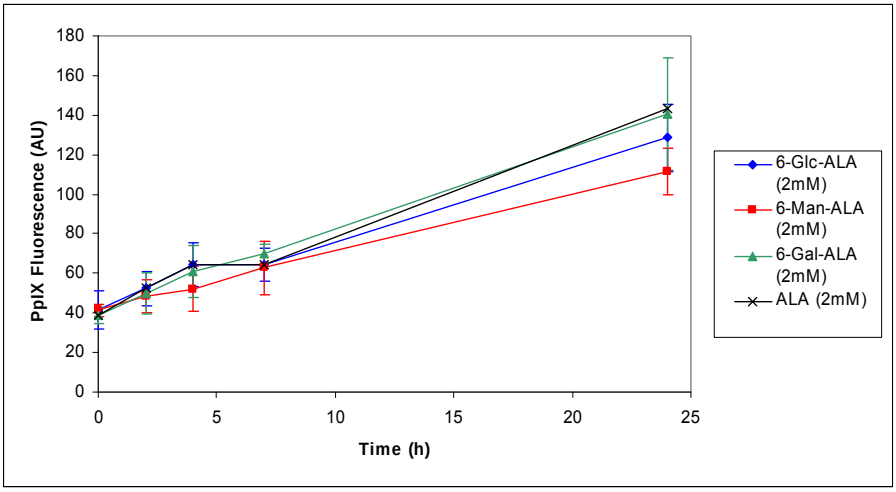


Figure 21: Increase in PpIX level due to 6´-*O*-ALA sugars and ALA in VS 59 fibroblasts

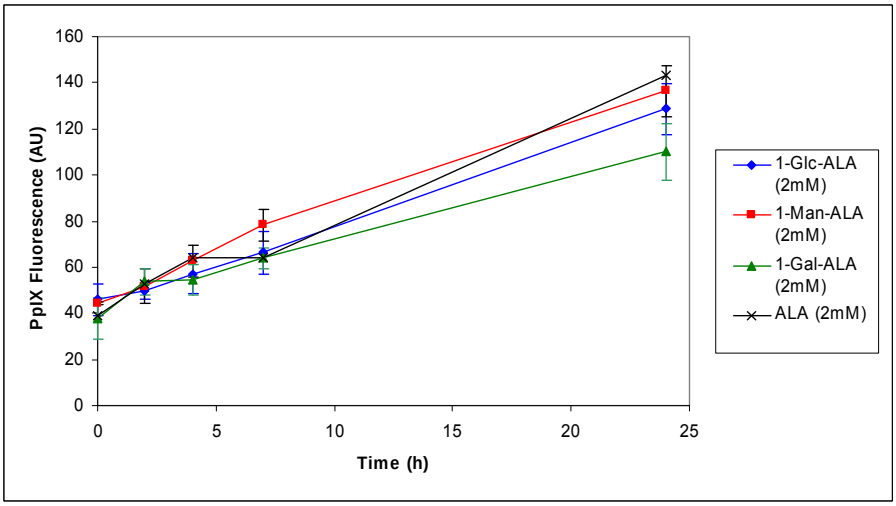


Figure 22: Increase in PpIX level due to 1´-*O*-ALA sugars and ALA in VS 59 fibroblasts

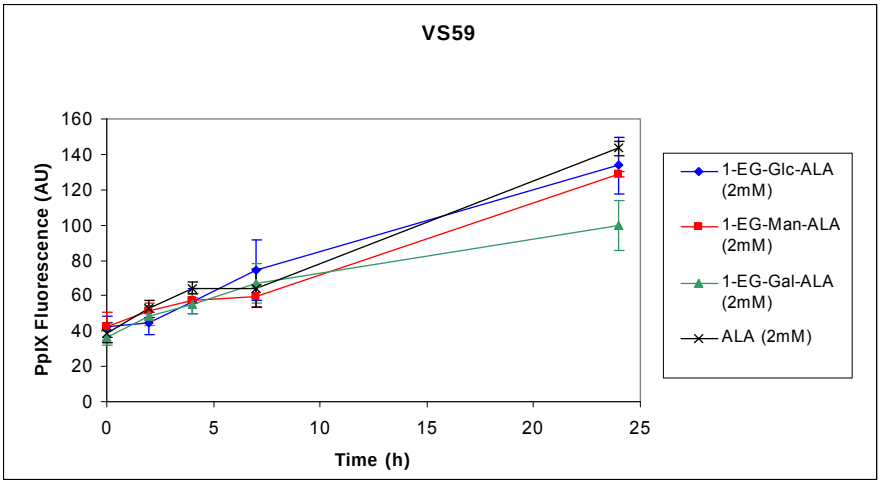


Figure 23: Increase in PpIX level due to 6′-*O*-ALA sugars and ALA in VS 59 fibroblasts

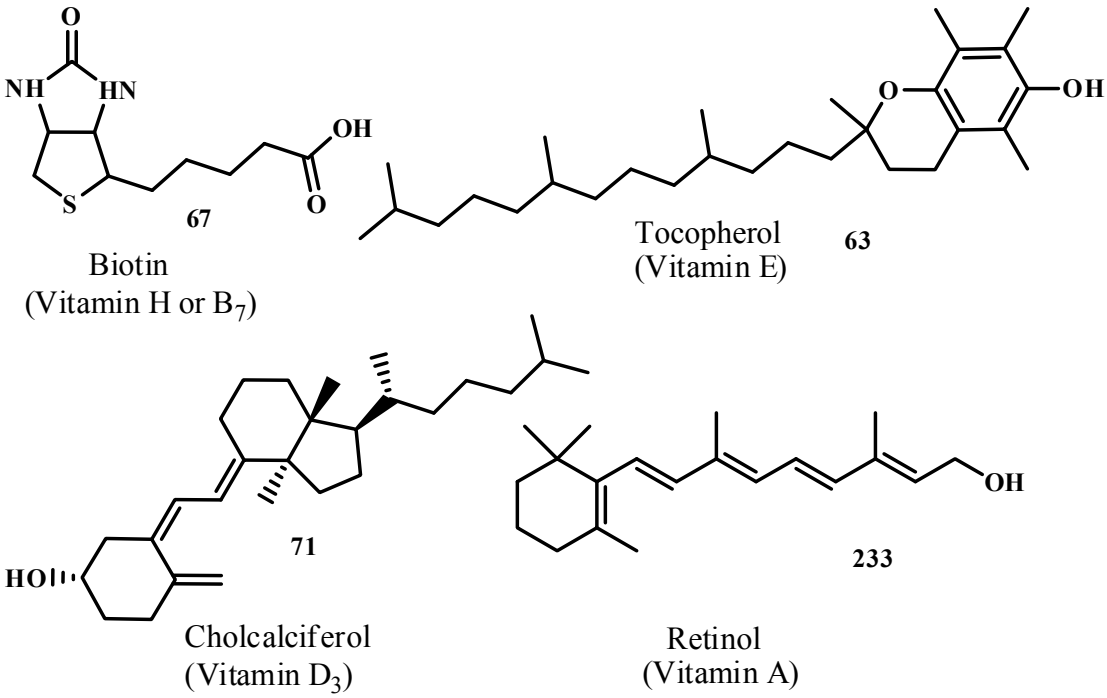
We evaluated the induction of PpIX due to ALA-sugar conjugates and ALA in fibroblast cells (VS 59 cells). As these fibroblasts are normal cells, if there is no PpIX generation due to a derivative, then the compound can be termed as targeted ALA derivatives which selectively get internalized in cancer cells.

We found that all our ALA-sugar conjugates generate PpIX in fibroblasts (VS 59 cells) however at much lower levels than in cancer cells. They induce PpIX level which is comparable to that of induced by ALA. We also observed that galactose derivatives (except 6′-O-(ALA) galactose) generate lower level of PpIX cpmpared to others, espically 1-EG-ALA-galactose **62**, generate PpIX almost half the level than that of ALA.

As concluding remarks for sugar-ALA conjugates with respect to PpIX generation, we can say that the order of efficiency of PpIX generation is as follows:
1′-O-(ALA) sugars > 6′-O-(ALA) sugars > 1-O-(EG-ALA) sugars.

5.4. Synthesis of Vitamin-ALA conjugates

For synthesizing Vitamin-ALA conjugates, we chose three vitamins having hydroxyl functions namely Tocopherol (Vitamin E), Cholcalciferol (Vitamin D_3) and Retinol (Vitamin A); and biotin (Vitamin H or B_7), a vitamin having acid moiety to couple with Boc-ALA as a pseudopeptide.

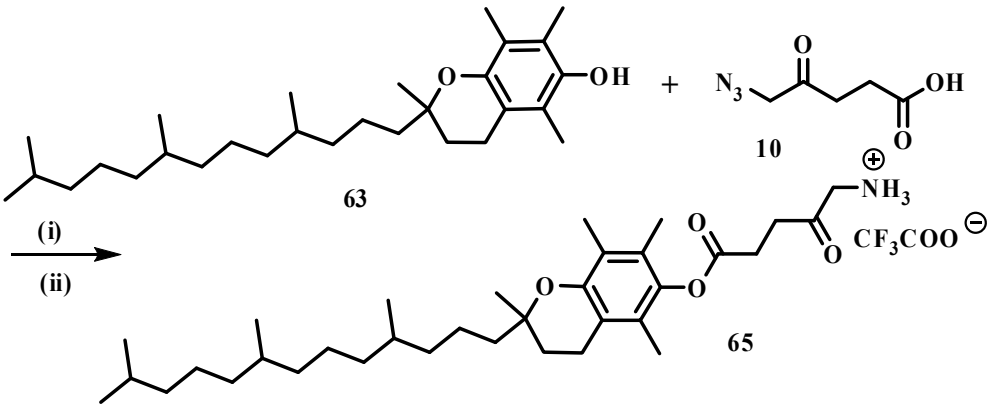


Scheme 97: Structures of vitamins chosen for conjugation with ALA

5.4.1. Synthesis of Tocopherol-ALA (Vitamin E-ALA)

We synthesized tocopherol-ALA conjugate by two ways: First, we coupled tocopherol **67** with azidolevulinic acid **10** and the resulting product was reduced by catalytic hydrogenation in the presence of TFA to afford tocopherol-ALA as trifluoroacetate salt **65**. Second, we coupled tocopherol **67** with Boc-ALA **5** and the resulting product was deprotected by TFA to afford tocopherol-ALA as trifluoroacetate salt **65**.

5.4.1.1. Synthesis of tocopherol-ALA starting from azidolevulinic acid



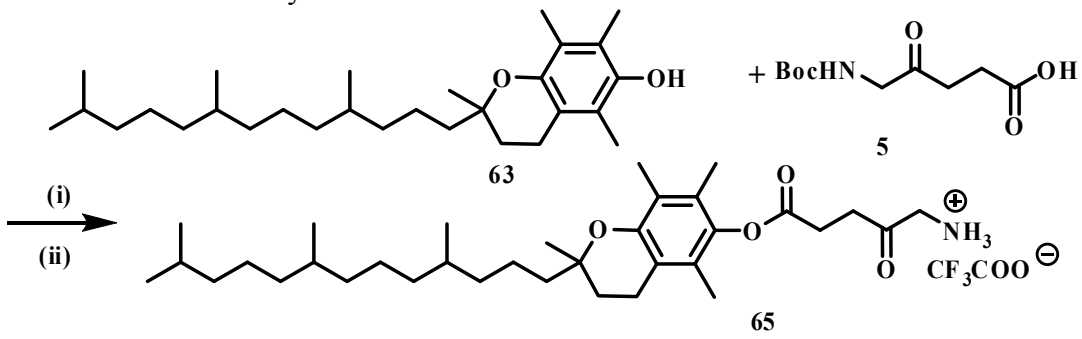
Reagents and conditions: (i) DCC, DMAP, rt, 12h, 83%; (ii) H_2/Pd, rt, TFA, 90%

Scheme 98: Synthesis of tocopherol ester of azidolevulinic acid

The coupling of azidolevulinic acid **10** with tocopherol **63** was mediated by DCC and DMAP. While DCC was added equimolar, DMAP was added in catalytic quantity. The crude after the purification by column chromatography afforded tocopherol ester of azido levulinic acid **66** in 83% yield. The azido group being a masked functionality of amine, the tocopherol-azidolevulinate was reduced by catalytic hydrogenation. The reduction in the presence of TFA proceeded in a straightforward manner with 90% yield and gave tocopherol ester of ALA **65** after purification. We triturated the product with hexane to remove traces of TFA. The solubility of the product was very high in many solvents as the product comprises lipophilic chain.

5.4.1.2. Synthesis of tocopherol-ALA starting from Boc-ALA

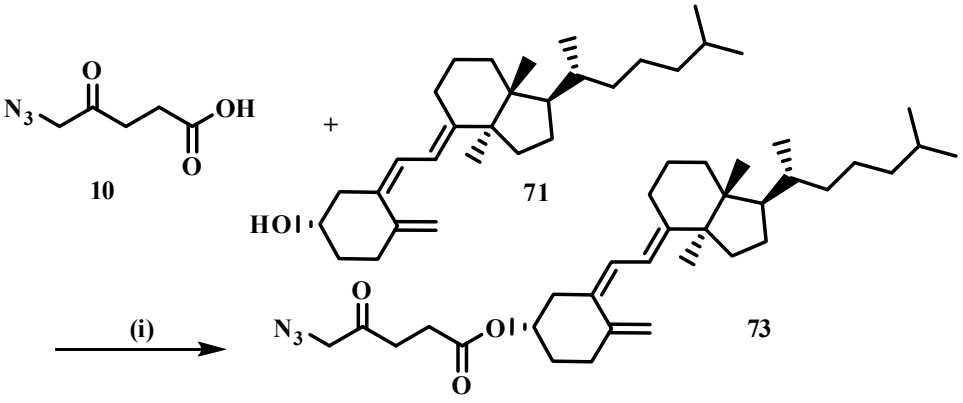
The coupling of Boc-ALA **5** with tocopherol **63** was performed in a similar manner as mentioned above. The DCC/DMAP mediated coupling gave tocopherol-Boc-ALA ester **64** in 78% yield after the purification by column chromatography. The 'Boc' group was deprotected by TFA which after purification to remove traces of TFA afforded tocopherol ester of ALA **65** in 89% yield.



Reagents and conditions: (i) DCC, DMAP, rt, 12h, 78%; (ii) TFA, rt, 30min.
Scheme 99: Synthesis of tocopherol ester of Boc-ALA

5.4.2. Synthesis of cholcalciferol-ALA (Vitamin D_3-ALA)

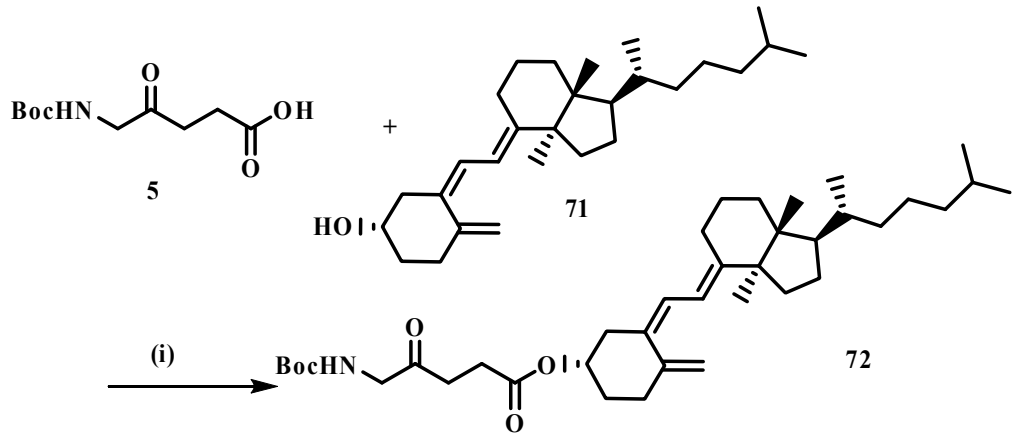
5.4.2.1. Synthesis of cholcalciferol-azidolevulinate



Reagents and conditions: (i) DCC, DMAP, rt, 60h, 30%
Scheme 100: Synthesis of cholcalciferol ester of azidolevulinic acid

The azidolevulinic acid **10** was reacted with cholcalciferol **71**. We employed the same DCC/DMAP mediated coupling as before. The reaction was not completed even after 2 days of maintaining at ambient temperature. This explains the reason for obtaining cholcalciferol ester of azidolevulinic acid **73** in low yield (30%). The unreacted DCC and the DHU was difficult to remove even after the reaction mass was filtered over celite. The low yield can be explained from the fact that acid moiety is not sufficiently activated enough to make coupling. A solution for this could be using azidolevulinic acid chloride or the pentafluorophenyl ester of azidolevulinic acid for coupling with cholcalciferol.

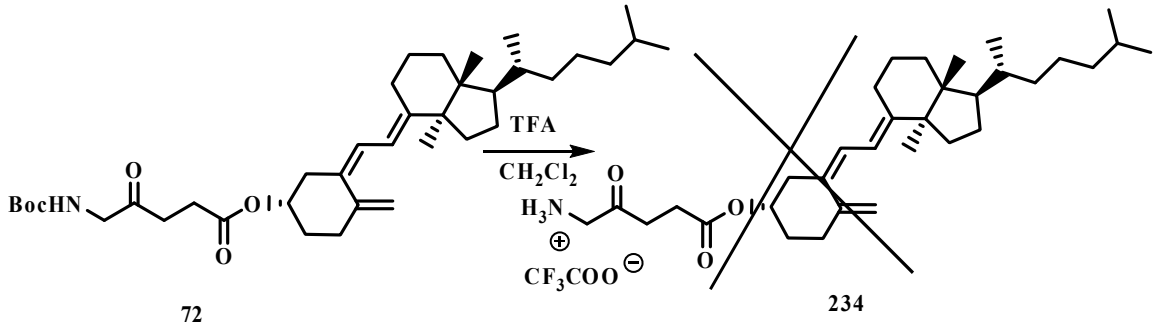
5.4.2.2. Synthesis of cholcalciferol- Boc-ALA



Reagents and conditions: (i) EDCI, DMAP, rt, 60h, 29%
Scheme 101: Synthesis of cholcalciferol ester of Boc-ALA

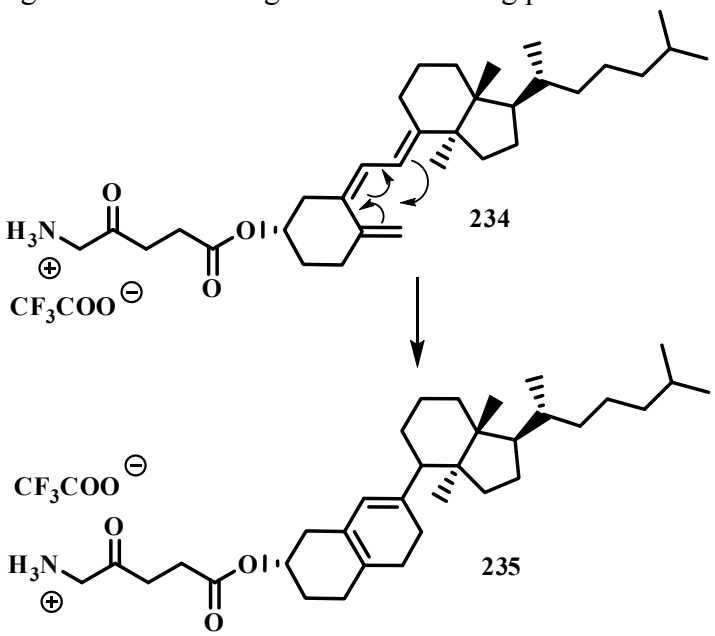
As we had poor reactivity in the coupling of azidolevulinic acid with cholcalciferol and also difficulty in removal of DCC/DHU, for the coupling with Boc-ALA **5** with cholcalciferol **71**, we used EDCI as coupling agent. DMAP was added in a catalytic quantity. Despite the addition of EDCI as coupling agent and maintenance at ambient temperature for 24 h, the reaction was not completed. The crude on purification by column chromatography afforded cholcalciferol ester of Boc-ALA **72** in 29% yield.

5.4.2.3. Synthesis of cholcalciferol-ALA



Scheme 102: Unsuccessful attempt for the deprotection of cholcalciferol-Boc-ALA

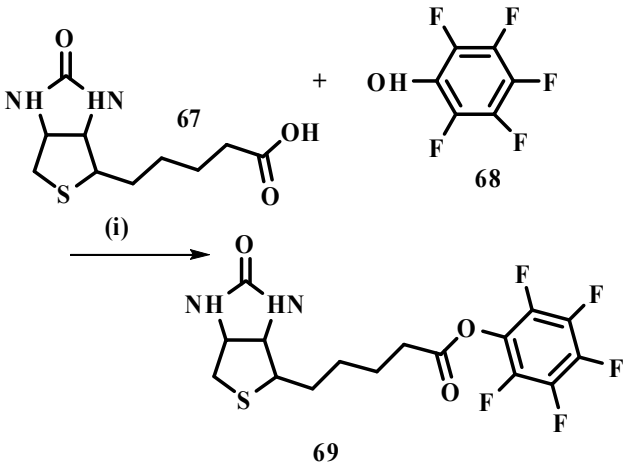
The 'Boc' deprotection of cholciferol ester of Boc-ALA **72** was attempted by TFA. Despite the addition of TFA at low temperature (0^0C) and maintenance at 10^0C, we were surprised to find the reaction got degraded and the mass became dark black in colour. The product after evaporating TFA and triturating with ether was found to compose peaks of ALA but not the relevant peaks of cholcalciferol in ^{1}H-NMR. This showed clearly that the product was degraded either during reaction or during purification.



Scheme 103: Hypothesis for the failed deprotection of 'Boc' in cholcalciferol-Boc-ALA
Our hypothesis is that cholcalciferol having three double bonds in a conjugated fashion, might have cyclized under the condition of 'Boc' deprotection to form **235**.

4.4.3. Synthesis of Biotin-ALA(OMe) pseudopeptide

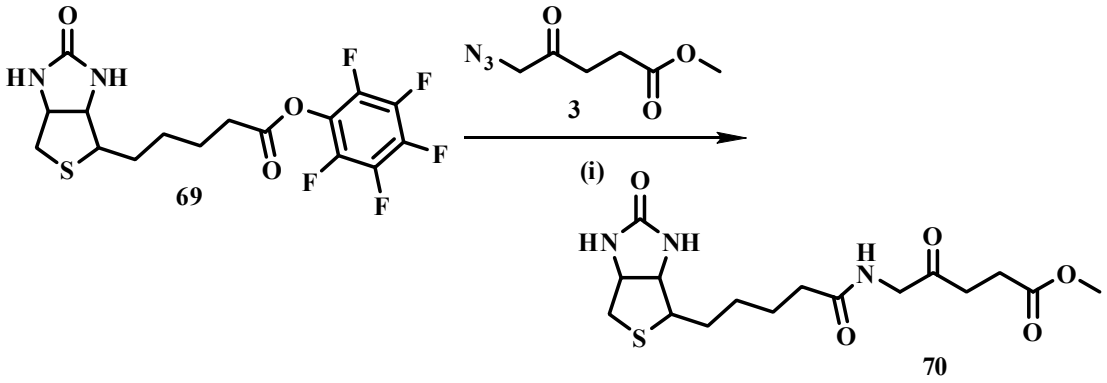
4.4.3.1. Synthesis of Biotin-PFP ester



Reagents and conditions: (i) DCC, DMF, rt, 6h, 76%
Scheme 104: Synthesis of Biotin-PFP ester

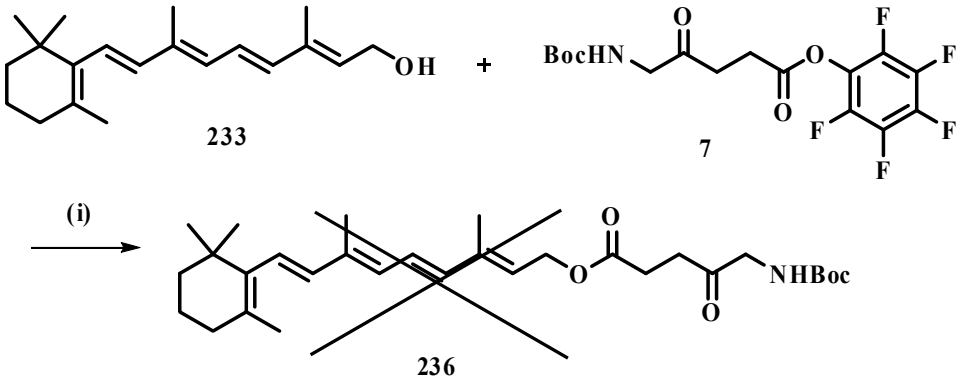
We synthesized Biotin-PFP ester by DCC mediated coupling of biotin with pentafluorophenol. Biotin being polar, we used DMF as solvent to dissolve. Once the reaction was completed, DHU was filtered. The crude product was then purified by crystallization in MeOH as solvent to afford a yield of 76%.

4.4.3.2. Synthesis of Biotin-ALA pseudopeptide



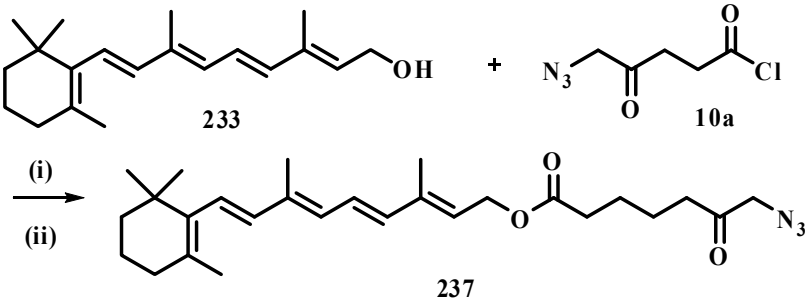
Reagents and conditions: (i) H_2/Pd, ambient temperature, 24h, 56%
Scheme 105: Synthesis of Biotin-ALA pseudopeptide
This reaction is a peptide coupling between azidomethyl levulinate **3** and biotin-PFP ester **69** in the presence of hydrogen atmosphere and catalyst Pd over carbon. Azidomethyl levulinate is a mask of amino methyl levulinate. On reduction by catalytic hydrogenation, the amino methyl levulinate was formed from azidomethyl levulinate. The nitrogen of amine group being a good nucleophile attacks the carbonyl carbon of biotin-PFP ester which is more electropositive than in biotin. Biotin-PFP ester is required to be added on excess otherwise the amino methyl levulinate can condense with another molecule of amino methyl levulinate (intermolecular condensation) to form pyrazine type impurity. The crude on purification by column chromatography yielded biotin-ALA pseudo peptide **70** in 56% yield.

4.4.4. Synthesis of Retinol-ALA (Vitamin A-ALA)



Reagents and conditions: (i) DMAP, rt, 24h.
Scheme 106: Attempted synthesis of retinol ester of Boc-ALA

We attempted the coupling of retinol **233** with Boc-ALA using Boc-ALA-PFP ester **7**. Despite the addition of equimolar DMAP and maintaining at ambient temperature for 24 h, the reaction was not completed. The crude on purification by column chromatography did not provide any fraction which contained the required product **236**.



Reagents and conditions: (i) NaH, THF, rt, 10h; (ii) THF, rt, 6h.
Scheme 107: Synthesis of retinol ester of azidolevulinic acid.

As a second trial, we attempted to deprotonate the hydroxyl proton of retinol and then couple with azidolevulinic acid chloride which was synthesized from azidolevulinic acid. As azidolevulinic acid chloride is thermally stable, we didnot isolate this and we used directly the crude of this reaction for coupling with retinolate of sodium. The crude of the reaction was not purified further due to time constraints.

4.4.5. Evaluation of Vitamin-ALA conjugates

We tested biotin-ALA peptide **70** and tocopherol-ALA ester **65**. Tocopherol-ALA ester was not soluble in the water because of its lipophilic chain. Then, it was dissolved in DMSO, but the product got precipitated when it is diluted with the cell growth medium.
As shown in the figure, biotin-ALA pseudo peptide **70** was not able to generate PpIX. This shows that the peptide derivatives have problems either to be internalized or they are not cleaved to release ALA. The cleavage of peptide might have been not possible due to the inability to recognize the pseudo-peptide.HCEC (human endothelial cells) was used for tests.

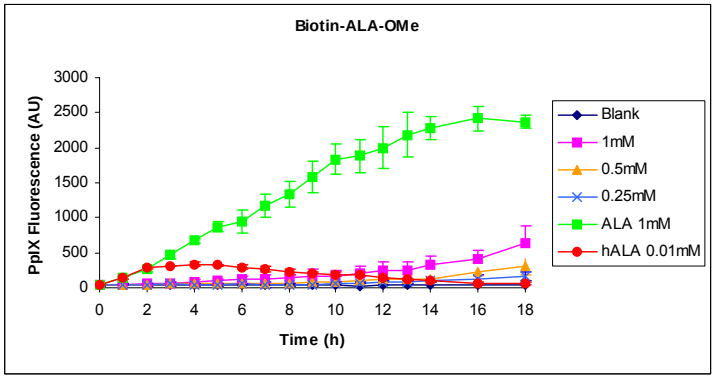


Figure 24: Increase in PpIX level due to biotin-ALA pseudo peptide

5.5. Synthesis of Nucleoside-ALA conjugates

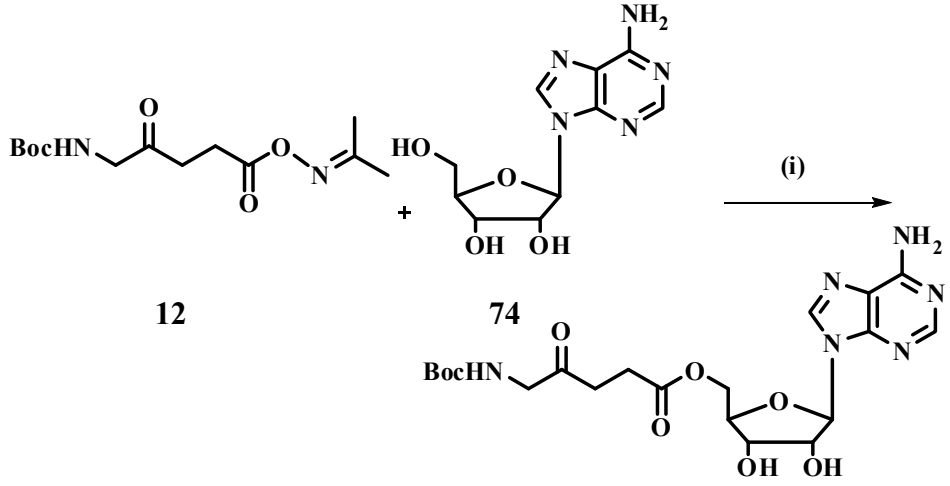
As discussed in chapter 2 (Biological reasons behind Prodrugs ' design), to take advantage of nucleoside receptors for targeted-ALA-PDT, we chose two nucleosides, one from ribonucleoside series (adenosine) and one from deoxyribo nucleoside series (thymidine).

5.5.1. Synthesis of Adenosine-ALA conjugate

Similar to what we performed in sugars, in the case of adenosine, we first wanted to synthesize ALA coupled as ester in the primary hydroxyl group of ribose sugar part of adenosine.

5.5.1.1. Synthesis of 5´-O-(Boc-ALA) adenosine

With the rise in the significance of nucleoside derivatives in the field of medicinal chemistry, especially as antineoplastic and antiviral agents, the development of new and efficient synthetic methodology is also rising. Enzymatic regioselective acylation is one of the methods for achieving high regioselectivity. Moris *et al* reported that selective acylation on 3´-OH of deoxyribonucleosides and exclusive regioselective acylation on 5´-OH of ribonucleosides could be achieved by the enzyme *Candida antarctica* lipase (Novozyme 435) and using oxime esters as activated ester [402].



Reagents and conditions: (i) Lipase from *Candida antarctica*, 60^0C, 24h, 25%.
Scheme 108: Syntesis of 5´-*O*-(Boc-ALA) adenosine.

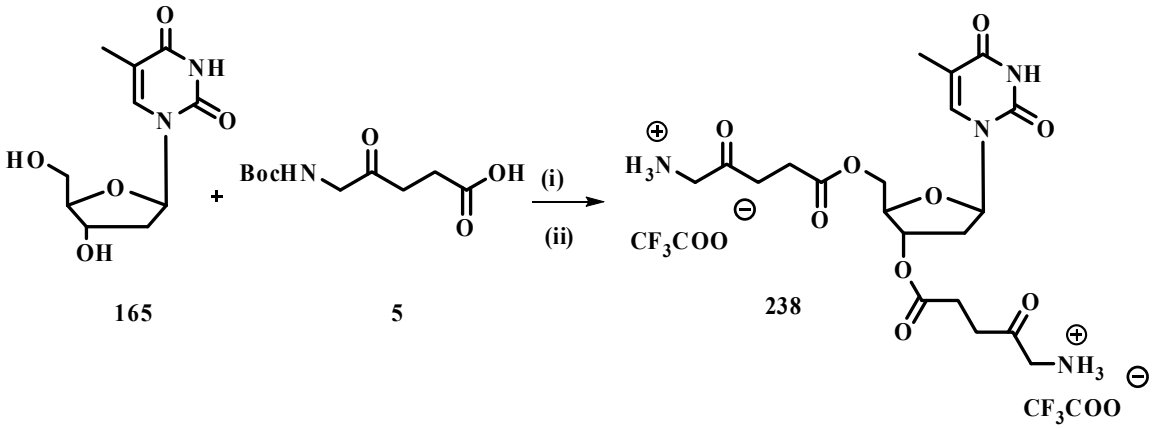
We employed the same protocol as mentioned by Moris *et al*. For an equivalent of adenosine, we used 3 equivalents of Boc-ALA-oxime ester 12 and 2 equivalents of the enzyme *Candida antarctica* lipase (Novozyme 435) (activity 10 units / mg of solid). After maintaining for 24 h at 60^0C, the reaction was worked up and purified (column chromatography) to afford exclusively 5´-*O*-(Boc-ALA) adenosine **75** in 25% yield. The

yield is quite low as compared to what was achieved by Moris *et al* (82% to 92%) when they utilized alkyl chains of length C1 to C6. The activity of the enzyme used by Moris *et al* was not mentioned in the literature [402]. Also, the reaction what we performed has to be optimized. Further work on this including deprotection of 'Boc' has not yet been completed.

5.5.2. Synthesis of Thymidine-ALA conjugate

In the case of thymidine we decided to substitute ALA in both the hydroxyl groups of the ribose sugar part.

5.5.2.1. Synthesis of 3′,5′-O-di (ALA) thymidine



165 5 238

Reagents and conditions: (i) Et_3N, DCC, DMAP, rt, 2h, 90%; (ii) TFA, rt, 30 min, 82%.
Scheme 109: Synthesis of 3′, 5′-di (Boc-ALA)-thymidine.

We synthesized 3′,5′-di (Boc-ALA) thymidine **238** by DCC/DMAP mediated coupling. thymidine **165** dissolved in dioxane was coupled with Boc-ALA using DCC and catalytic DMAP. Boc-ALA was used in excess (4 equivalents for each equivalent of thymidine) and DCC was added equimolar to that of Boc-ALA. The crude on purification by column chromatography yielded 3′,5′-di (Boc-ALA) thymidine 200 in 90% yield. This product on deprotection using TFA yielded 3′,5′-di (ALA) thymidine **238** in 82% yield.
The evaluation of ALA-nucleoside conjugates on cells for PpIX induction has not yet been accomplished.

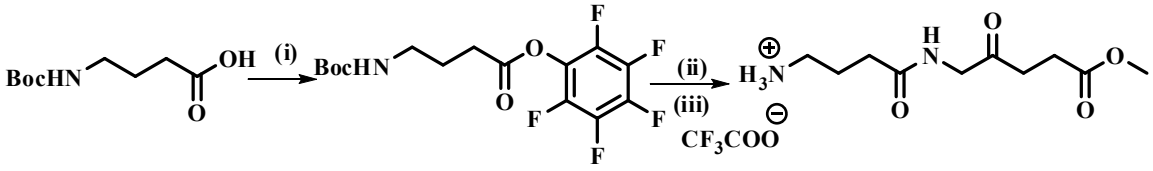
5.6. Synthesis of ALA esters and pseudo peptide

5.6.1. Synthesis of ALA-pseudo peptide (GABA-ALA-(OMe)), (Tyr-ALA-(OMe)) and (ALA-ALA-OMe)

5.6.1.1. GABA-ALA pseudo peptide

Gamma-aminobutyric acid (GABA) is the inhibitory neurotransmitter in the brain. ALA, being structurally similar to GABA, is incorporated into the cells by a carrier protein which is a GABA transporter [208, 209]. In view of this, we planned to synthesize GABA-ALA-OMe. We planned to synthesize Boc-GABA-ALA-OMe **78** in a similar

manner as we accomplished for synthesizing Biotin-ALA (OMe) **70**. Azidomethyl levulinate is a mask of amino methyl levulinate. On reduction by catalytic hydrogenation, the amino methyl levulinate was formed from azidomethyl levulinate. The nitrogen of amine group being a good nucleophile attacks the carbonyl carbon of pentafluorophenyl ester of Boc-GABA (Boc-GABA-PFP ester) which is more electropositive than in Boc-GABA.



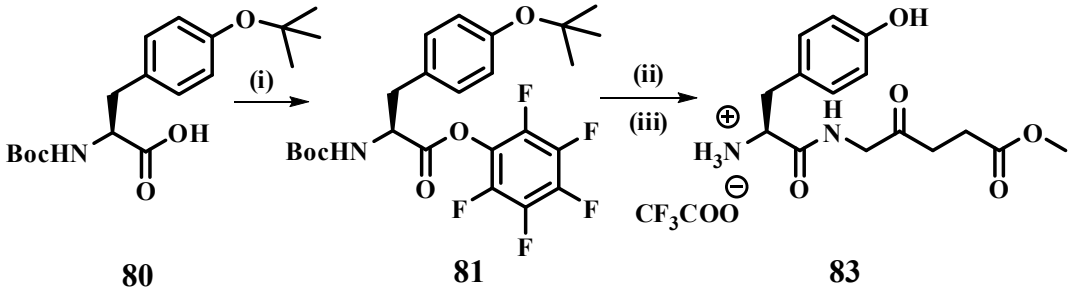
76 **77** **79**

Reagents and conditions: (i) C_6F_5OH, DCC, rt, 2h, 98%; (ii) Azidomethyl levulinate, H_2/Pd, rt, 8h, 87%; (iii) TFA, rt, 30min, 96%.
Scheme 110: Synthesis of GABA-ALA(OMe) pseudopeptide.

We synthesized first pentafluorophenyl ester of Boc-GABA **77** (Boc-GABA-PFP ester) from Boc-GABA **76**. Then we coupled this with azidomethyl levulinate **3** in the presence of hydrogen atmosphere and catalyst Pd over carbon. Boc-GABA-PFP ester is required to be added on excess otherwise the amino methyl levulinate can condense with another molecule of amino methyl levulinate (intermolecular condensation) to form pyrazine type impurity as discussed in chapter 1.3. The product after coupling was purified by column chromatography to afford Boc-GABA-ALA(OMe) **78** in 87% yield. Boc-GABA-ALA(OMe) **78**, then was treated with TFA to deprotect 'Boc' to provide GABA-ALA(OMe) peusdo peptide **79**.

5.6.1.2. Tyr-ALA pseudo peptide

We synthesized Tyr-ALA pseudo peptide **83** in the same manner as we did for synthesizing GABA-ALA(OMe) peusdo peptide **79**.



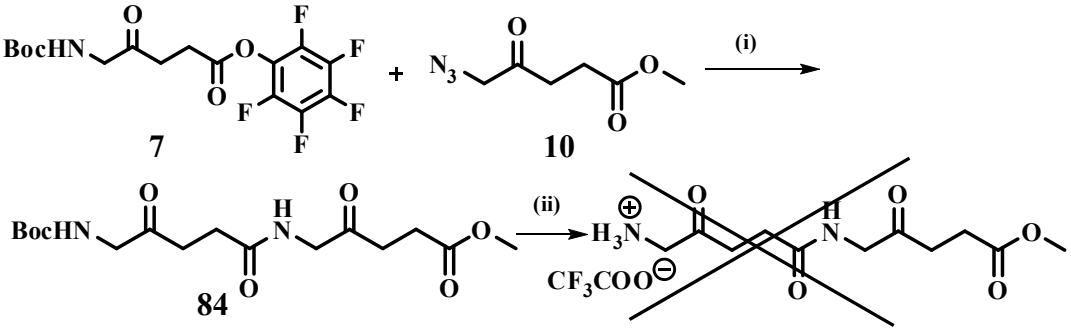
80 **81** **83**

Reagents and conditions: (i) C_6F_5OH, DCC, rt, 2h, 97%; (ii) Azidomethyl levulinate, H_2/Pd, rt, 12h, 90%; (iii) TFA, rt, 30min, 93%.
Scheme 111: Synthesis of tyrosine-ALA pseudo peptide
We synthesized pentafluorophenyl ester of Boc-Tyr-(O-^{t}Bu) **81** (Boc-Tyr-(O-^{t}Bu)-PFP) from Boc-Tyr-(O-^{t}Bu) **80**. This activated ester was coupled with azidomethyl levulinate **3** in the presence of hydrogen atmosphere and catalyst Pd over carbon to give Boc-Tyr-(O-

^{t}Bu)- ALA (OMe) pseudo peptide **82**. The pseudo peptide of Boc-Tyr-(O-^{t}Bu)- ALA (OMe) **82** was then treated with TFA to provide Tyr-(OH)- ALA (OMe) **83**.

5.6.1.3. ALA-ALA(OMe) pseudo peptide

In our desire to synthesize oligopeptides (pentamer, hexamer etc) containing ALA in solution based approach, as a first step, we wanted to synthesize dimer containing two ALA molecules coupled as a peptide.



Reagents and conditions: (i) H_2/Pd, rt, 12h, 18%; (iii) TFA, rt, 30min.

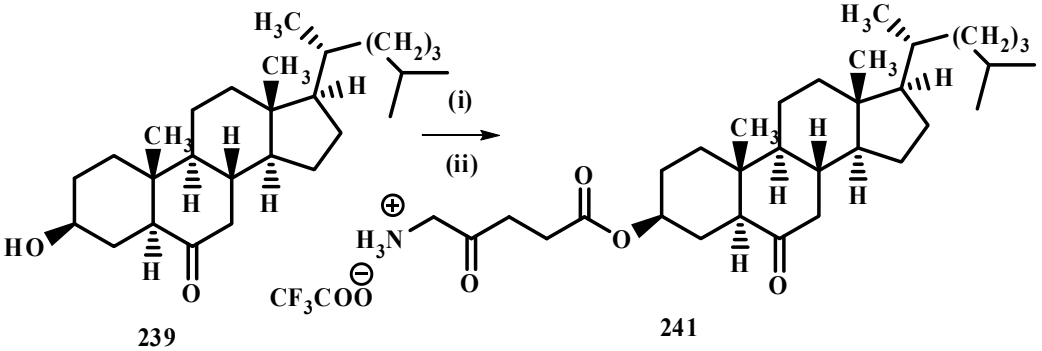
Scheme 112: Synthesis of Boc-ALA-ALA (dimer) and attempted 'Boc' deprotection.

We coupled Boc-ALA-PFP ester with azidomethyl levulinate in the presence of hydrogen atmosphere and catalyst Pd over carbon. The coupled product afer purification by column chromatography provided Boc-ALA-ALA (OMe) in 18% yield. We added Boc-ALA-PFP ester in excess (2 equivalents), otherwise the amino methyl levulinate can condense with another molecule of amino methyl levulinate (intermolecular condensation) to form pyrazine type impurity as discussed in chapter 1.3. Despite this, we obtained a low yield. The reaction was not optimized further to improve yield. Our trials to deprotect 'Boc' group using TFA was unsuccessful and so we did not proceed further.

5.6.2. Synthesis of ALA esters

5.6.2.1. Synthesis of ALA-6-Ketocholestanol ester

As mentioned in the objective chapter, we wanted to synthesize an ester coupling the hydroxyl function of 6-Ketocholestanol with the acid moiety of Boc-ALA.



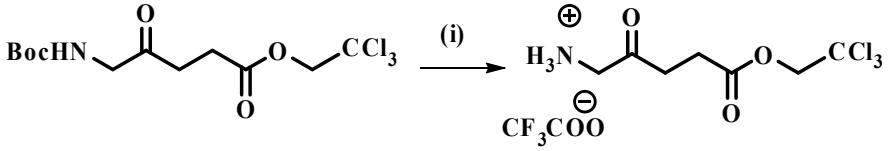
Regants and conditions: (i) EDCI, DMAP, Boc-ALA, rt, 12h, 67%; (ii) TFA, rt, 30mim, 96%.

Scheme 113: Synthesis of 6-Ketocholestanol ester of ALA.

We accomplished the coupling of Boc-ALA and 6-ketocholestanol **239** using EDCI and DMAP. The product after column chromatography afforded 6-ketocholestanol ester of Boc-ALA **240** in 67% yield. This coupled ester was treated with TFA to remove 'Boc'. This crude after purification by precipitation gave 6-ketocholestanol ester of ALA **241.** As the final deprotected compound was still lipophilic, the trituration to remove excess TFA was done using the solvent hexane.

5.6.2.2. Synthesis of Trichloro ethyl ester of ALA

We used tricloroethyl ester of Boc-ALA as an activated ester for various enzymatic regioselective transformations for synthesizing sugar-ALA conjugates. It has been reported by Brunner *et al* [103] that ALA esters containing fluorinated alkyl chains (C5-C7) are efficient in producing PpIX. In view of this report and also as we were using tricloroethyl ester of Boc-ALA for other reactions, we decided to deprotect the 'Boc' group by TFA and evaluate the trichloroethyl ester of ALA trifluoroacetate for its efficiency in generating PpIX.

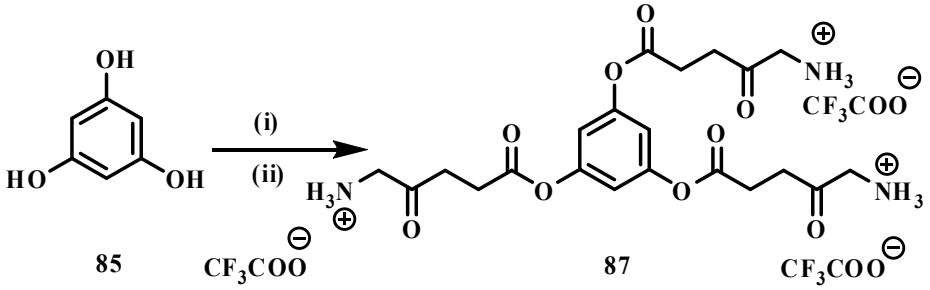


Regants and conditions: (i) TFA, rt, 30mim, 95%.
Scheme 114: Synthesis of trichloroethyl ester of ALA

5.6.3. Synthesis of ALA multisubstituted on poly hydroxyl compounds

5.6.3.1. Synthesis of Tri-(ALA) Phloroglucinol

Phloroglucinol which is a 1,3,5 trihydroxy benzene is commercially available. We planned to synthesize substitution of ALA as ester on all the three hydroxyl groups of phloroglucinol. We accomplished this using Boc-ALA in the presence of DCC and dimethyl amino pyridine salt of p-toulene sulphonic acid (DPTS) [403]. We isolated the tri substituted product **86** from other by-products like di and mono substituted products by column chromatography.

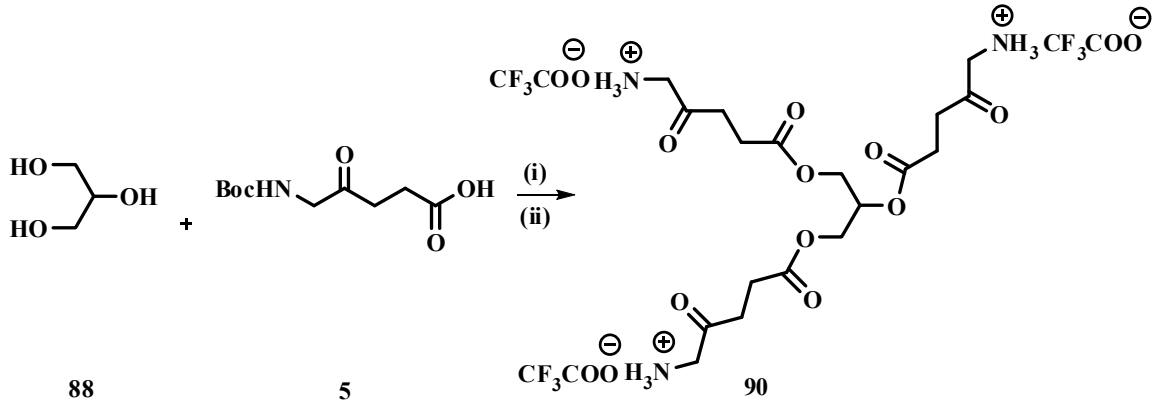


Regants and conditions: (i) DCC, DPTS, Boc-ALA, rt, 24h, 94%; (ii) TFA, rt, 30mim, 90%.
Scheme 115: Synthesis of tri (ALA) phloroglucinol

The tri Boc-ALA substituted phloroglucinol **86** was then treated with TFA to deprotect 'Boc'. The product on purification to remove excess TFA using co-evaporation, trituration etc using ether as solvent provided pure tri(ALA) phloroglucinol **87**.

5.6.3.2. Synthesis of Tri-(ALA) Glycerol

Glycerol and triglycerides being an adjuvant for many formulations, triglycerides containing ALA as ammonium salt can provide a basis for either crossing lipid bilayer of cell wall or stratum corneum of skin (skin applications).



88 **5** **90**

Regants and conditions: (i) DCC, DPTS, Boc-ALA, rt, 24h, 73%; (ii) TFA, rt, 30mim, 89%.

Scheme 116: Synthesis of tri (ALA) glycerol

We carried out the tri ALA substitution on glycerol in a similar manner as we did for phloroglucinol.

5.6.4. Evaluation of ALA esters and ALA-pseudo peptides

PpIX induction in different types of cancer cell lines were evaluated using the following derivatives: ALA pseudo peptides namely GABA-ALA(OMe) **79** and Tyr-ALA(OMe) **83**; ALA esters namely 6-ketocholestanol ester of ALA **241**, tri (ALA) phloroglucinol **87**, tri (ALA) glycerol **90** and trichloroethyl ester of ALA **242**.

5.6.4.1. Evaluation of ALA pseudo peptides

As shown figures 25 and 26 which indicate the increase in level of PpIX level over a period of 3 h, the ALA pseudopeptides did not generate PpIX at all. Although GABA-ALA (OMe) was not able to generate PpIX in many types of cancer cells (the graphs of increase in PpIX level by certain selected cell lines are shown here), it generated very low level of PpIX in breast MCF 7 carcinoma cells. This shows that the peptide derivatives have problems either to be internalized or they are not cleaved to release ALA. The cleavage of peptide might have been not possible due to the inability to recognize the pseudo-peptide.

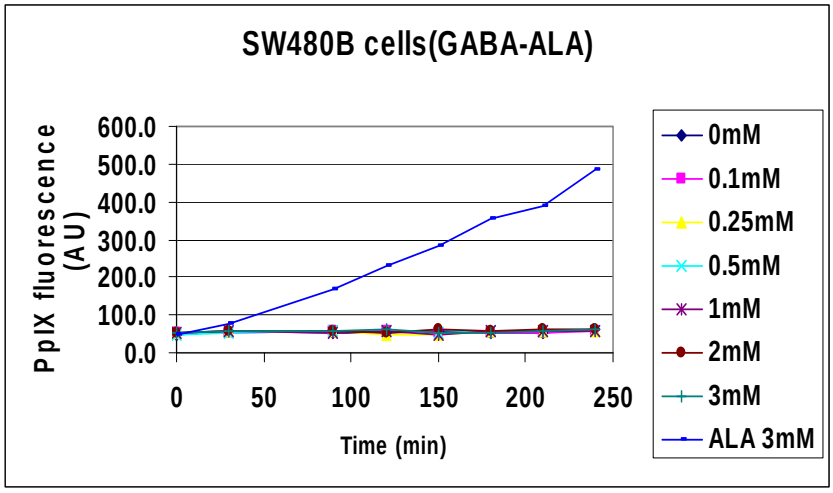


Figure 25: Increase in PpIX level in cells exposed to GABA-ALA **79**

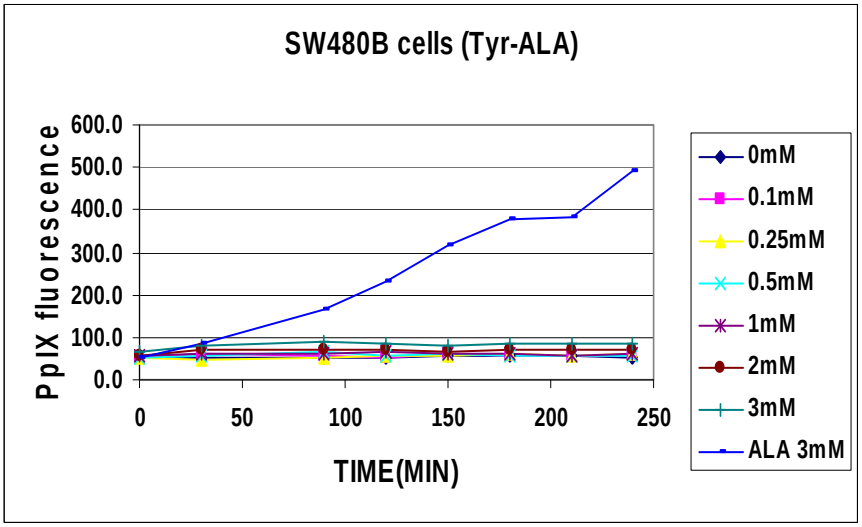


Figure 26: Increase in PpIX level in cells due to tyrosine-ALA **83**

5.6.4.2. Evaluation of ALA esters

5.6.4.2.1. Evaluation of ALA-6-ketocholestanol ester 241

In the case of ALA esters, 6-ketocholestanol ester of ALA did not generate PpIX at all in all types of cancer cell lines (the graphs of increase in PpIX level by certain selected cell lines are shown here; figure 27). As we had the difficulty to dissolve 6-ketocholestanol ester of ALA as it is highly lipophilic, we used DMSO. The compound got precipitated when diluted by growth medium. So the reason for not generating PpIX could be that because of its high lipophilicity, the compound might not have penetrated the cell membrane.

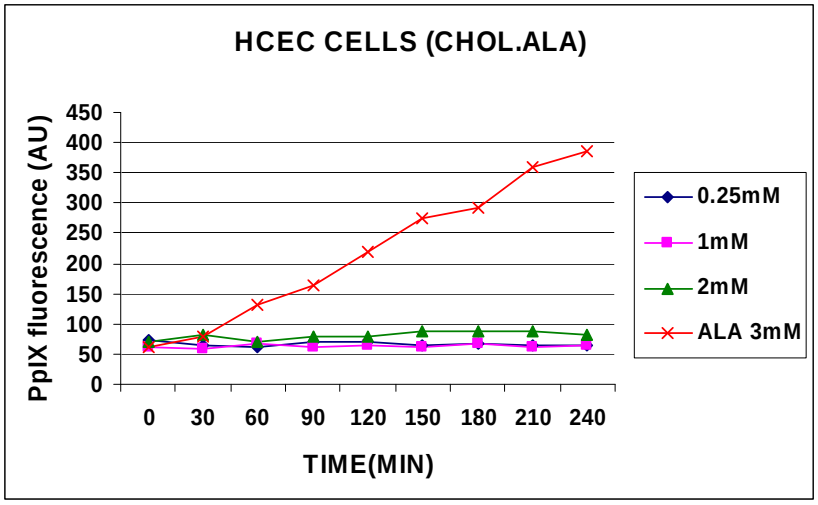


Figure 27: Increase in PpIX level due to 6-ketocholestanol -ALA **241**

5.6.4.2.2. Evaluation of trichloroethyl ester of ALA

Trichloroethyl ester of ALA **242** induced strong PpIX production in all cell lines (the graphs of increase in PpIX level by certain selected cell lines are shown here). As mentioned by Brunner *et al* [103] ALA derivatives when conjugated with functionalities containing electron withdrawing group generates higher production of PpIX as we see in the case of trichloroethyl ester of ALA (TCE-ALA).

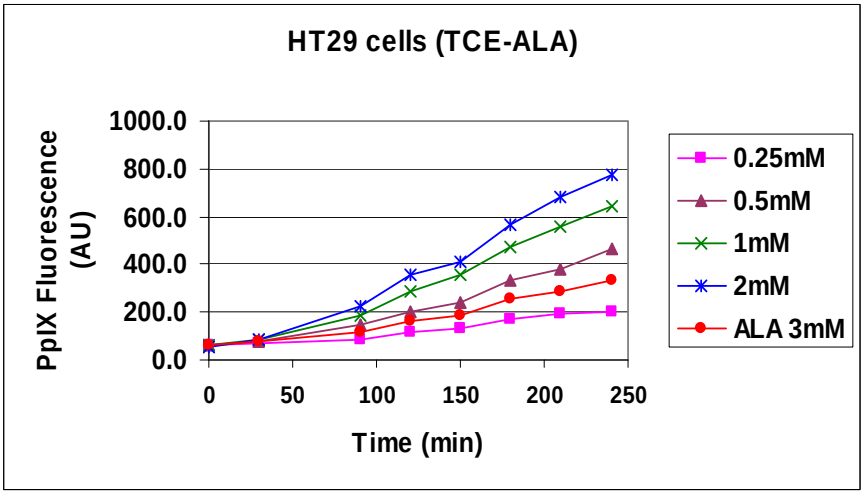


Figure 28: Increase in PpIX level due to trichloroethyl ester of ALA **242** over a period of 6h

5.6.4.2.3. Evaluation of tri-(ALA) phloroglucinol (87) and tri-(ALA) glycerol (90)

In the case of other ALA esters, tri-(ALA) phloroglucinol **87** and tri-(ALA) glycerol **90** were tested only in human endothelial cells (HCEC). Both the derivatives induced strong PpIX production in human endothelial cells. Multi ALA substituted phloroglucinol ester derivative (tri–(ALA) phloroglucinol) induced higher level of PpIX than that induced by multi ALA substituted glycerol ester derivative (tri-(ALA) glycerol). This again can be explained from the fact that in the case of phloroglucinol, the electron rich aromatic ring has better electron withdrawing effect than that of glycerol. But both the derivatives induced PpIX to a comparable level if not higher than that induced by standard ALA.

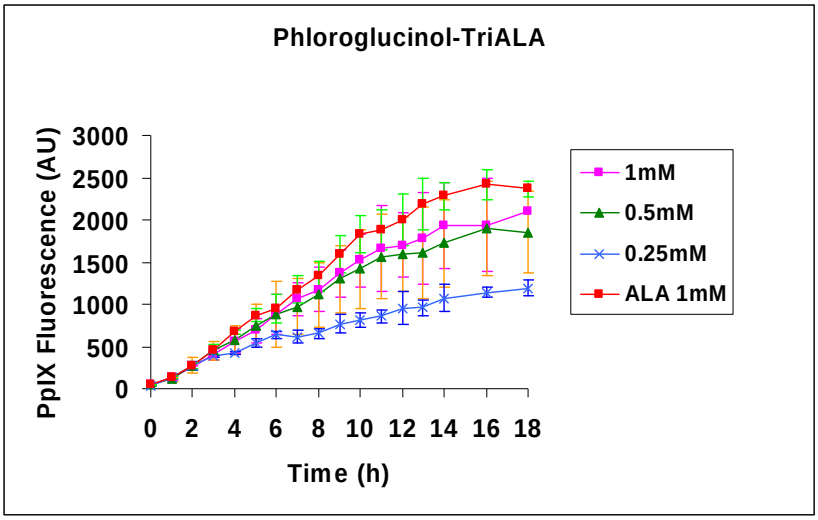


Figure 29: Increase in PpIX level due to tri-(ALA) ester of phloroglucinol 87

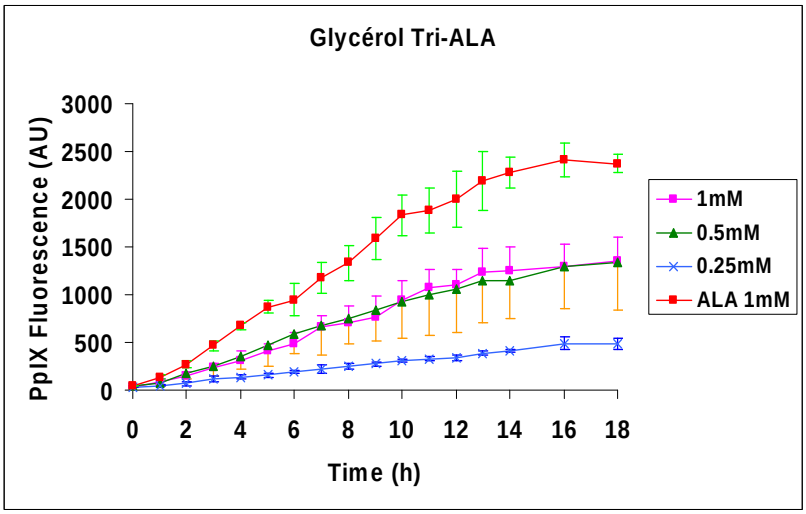
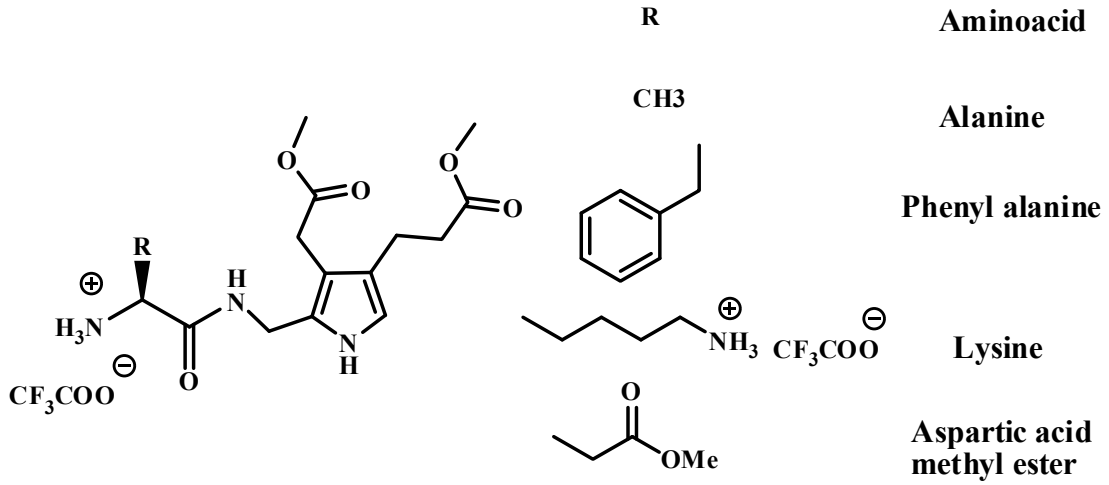


Figure 30: Increase in PpIX level due to Tri-(ALA) ester of glycerol **90**

From the graphs shown here for ALA esters, it can be concluded that the order of efficiency in generating PpIX in the case of ALA esters can be as follows:
Trichloroethyl ester of ALA (TCE-ALA) >Tri(ALA) phloroglucinol > Tri(ALA) glycerol

5.7. Synthesis of PBG derivatives

To the best of our knowledge, not even a single publication is known in the literature where PBG as a precursor for photodynamic therapy or diagnosis had been tested in cancer cell lines though some mention that PBG has the potential for PDT use [404-406]. This is more than surprising given the fact that there are several reports describing synthesis of PBG [215, 218, 404, 405, 407-420] due to its significance in treating lead poisoning [405, 417-419, 421, 422] despite its utility in the studies of biosynthesis [410, 414, 415]. Porphobilinogen being a trialkyl substituted pyrrole, is not stable and highly reactive due to the lack of stabilizing substituents such as an electron withdrawing group or an aromatic ring [215]. Keeping this in mind, we planned to design a stable protected derivative of PBG. The biomimetic synthesis of porphobilinogen has been extensively studied by our group [215-218, 423-428]. This in-house expertise and the requirement for protecting groups in view of cellular mechanisms made us to design PBG where the acid moieties are protected as methyl ester and the amino group linked to acid moieties of different α-amino acids (scheme 117). We chose neutral amino acids (alanine and phenyl alanine), basic amino acid (lysine) and acidic amino acid (aspartic acid). We envisaged that the cellular esterases could cleave the methyl ester moiety and cellular peptidases could cleave the aminoacid-PBG bond thereby release free PBG. This free PBG released in turn can transform into PpIX.

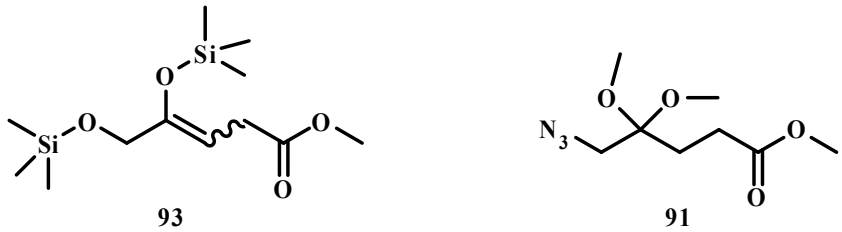


Scheme 117: PBG-amino acid pseudo peptides synthesized

5.7.1. Synthesis of PBG precursors

We synthesized the PBG precursors required for the formation of PBG via a key step Mukaiyama aldol condensation. All the steps in the formation of PBG precursors have been well studied in our group before [216, 217, 428, 429].

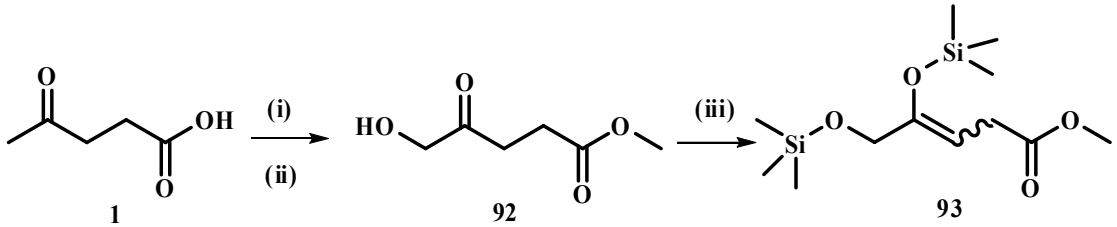
The key step is Mukaiyama condensation which requires silyl enol ether **93** and azido ketal **91**.



Scheme 118: Precursors for Mukaiyama aldol reaction

5.7.1.1. Synthesis of silyl enol ether

The synthesis of enol ether comprises 3 steps and it starts from levulinic acid **1**. Levulinic acid was brominated to obtain 5-bromomethyl levulinate **2** as the major product as described in 5.1.5.2.1. SN2 reaction of 5-bromo methyl levulinate with formic acid under basic conditions (DBU) in benzene yielded 5-formyloxy methyl levulinate. This formyloxy compound was easily hydrolyzed to 5-hydoxy methyl levulinate **92** by passing through a column with basic alumina [430]. The 5-hydoxy methyl levulinate was deprotonated by lithium salt of hexamethyldisilazane to form the enolate and at -78^{0}C was silylated by trimethyl silyl chloride. Methyl iodide was added later for the formation of quaternary amine salt which can be eliminated by precipitation. This gave the regioselective silyl enol ether **93** required for Mukaiyama condensation.

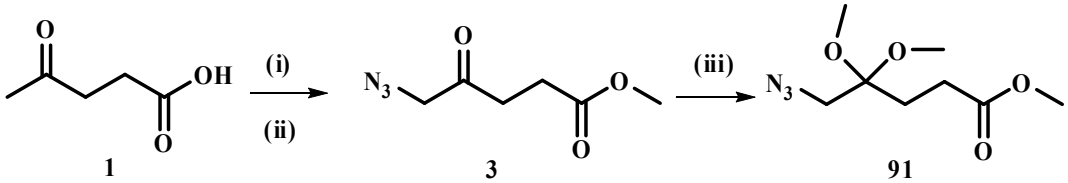


Reagents and conditions: (i) Br_2, MeOH, reflux, 1.5h, 57%; (ii) HCOOH, DBU, neutral alumina, rt, 1h, 69%; (iii) LiHMDS, TMS-Cl, MeI, -78^{0}C, 3h, 63%.

Scheme 119: Synthesis of silyl precursor for Mukaiyama aldol reaction

5.7.1.2. Synthesis of azidoketals

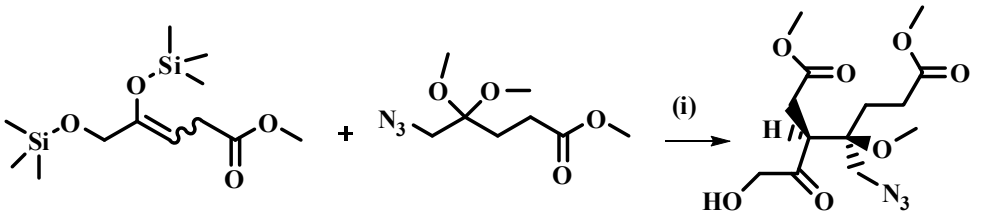
The synthesis of azidoketal comprises 3 steps starting from levulinic acid **1**. Levulinic acid was brominated to obtain 5-bromomethyl levulinate **2** as the major product as described in 5.1.5.2.1. Then with a S_N2 attack by azide nuclophile on 5-bromomethyl levulinate afforded 5-azidomethyl levulinate **3**. This azide **3** on reacting with trimethyl orthoformate in the presence of catalytic p-toluene sulfonic acid gave azidoketal **91.**



Reagents and conditions: (i) Br_2, MeOH, reflux, 1.5h, 57%; (ii) NaN_3, rt, 1h, 95%; (iii) $(H_3CO)_3CH$, p-TsOH, MeOH, reflux, 14h, 89%.

Scheme 120: Synthesis of azido keteal precursor for Mukaiyama aldol reaction

5.7.1.3. Mukaiyama aldol reaction

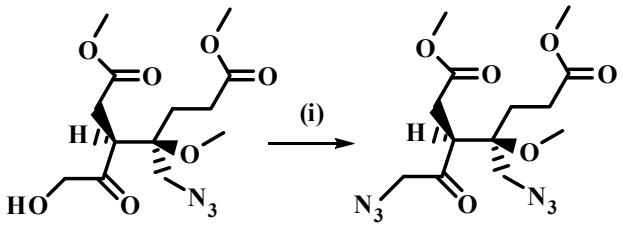


93 **91** **94**

Reagents and conditions: (i) $TiCl_4$, CH_2Cl_2, -78^{0}C to -55^{0}C, 16h, 44%.
Scheme 121: Mukaiyama aldol reaction

The Mukaiyama reaction was carried out between the silyl enol ether **93** and azido ketal **91** in the presence of lewis acid $TiCl_4$ (scheme 121). The silyl enol ether and azido ketal was dissolved in dichloromethane, cooled to -78^{0}C, added $TiCl_4$ and maintained at -55^{0}C for 12h. The reaction is very sensitive to moisture, acidity in the solvent (dichloromethane) etc which otherwise affects the yield. The solvent dichloromethane was passed through basic alumina to remove any acidity. (Distillation over P_2O_5 makes the solvent more acidic, so not advisable). The starting materials were also dried for a long time (6 h) by high vacuum oil pump. The reaction mass was extracted between 2N NaOH and $CHCl_3$. The crude on purification by column chromatography yielded the aldol product **94** with the alcohol functionality free, the desilylation being happened during the basic work-up.

5.7.1.4. Mitsunobu reaction



94 **95**

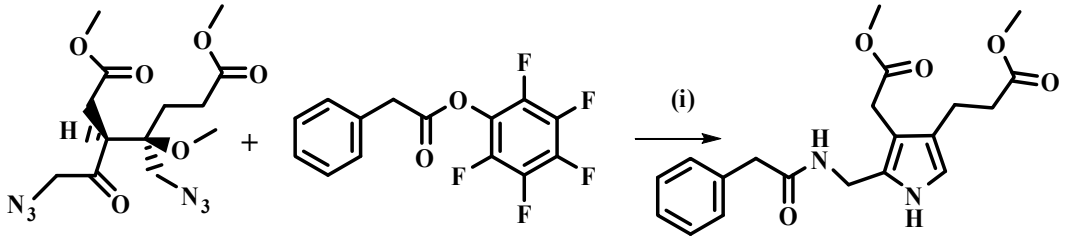
Reagents and conditions: (i) PPh_3, DIAD, HN_3, 10^{0}C, 2h, 93%.
Scheme 122: Mitsunobu reaction

Though the mukaiyama aldol product can be cyclized to form pyrrole ring using catalytic hydrogenation or Staudinger reaction, we decided to introduce a masking group of amine which is azido in the place of hydroxyl group and then cyclize to form PBG. So, the free hydroxyl group of aldol product **94** was converted to azido using hydrazoic acid (HN_3) in the presence of PPh_3 and DIAD (scheme 122). In a solution of PPh3 in benzene at 10^{0}C was added DIAD drop wise. To this solution of quaternary phosphonium salt, was added the solution of aldol product. Finally, we added dropwise the solution of HN_3 in benzene which was freshly prepared and titrated before use. After maintaining at 10^{0}C for 2 h, the solvent was evaporated and subsequently the crude was purified by column

chromatography. We synthesized this Mitsunobu product **95** in sufficient quantity (~5g) and used this for coupling with various activated ester of amino acids. Since this Mitsunobu product is thick yellow oil, we dissolved this solvent to make like a stock solution and used the aliquot of it for further reactions.

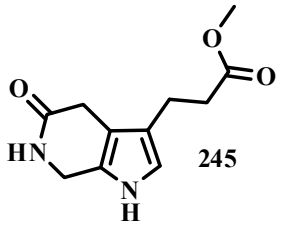
5.7.2. Synthesis of PBG-amino acid peptide

The synthesis of stable derivative of PBG in the form of PBG-phenyl acetamide **244** (scheme 123) had been studied before in the group by C.Soldermann-Pissot [429]. She had coupled the activated ester (pentafluoro phenyl) of phenyl acetic acid **243** with the above mentioned Mitsunobu product **95** under the conditions of catalytic hydrogenation to form PBG-phenyl acetamide **244**.



95 **243** **244**

Reagents and conditions: (i) H_2, Pd/C, MeOH, ambient temperature, 14h, 72%.
Scheme 123: Synthesis of PBG-phenyl acetamide carried out by C.Soldermann-Pissot



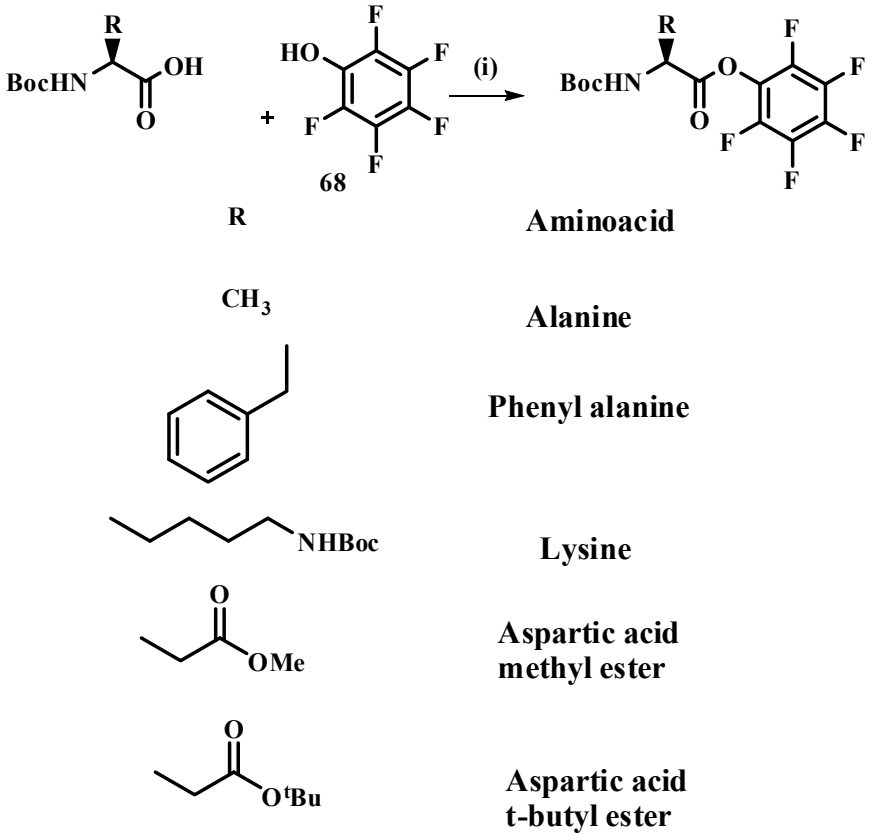
245

Scheme 124: PBG-lactam impuritiy

This reaction was clean without the formation of any impurities like lactam **245** (scheme 124) which could have formed if there is not sufficient pentafluorophenyl (PFP) ester of pheylacetic acid to couple with the Mitsunobu product **95**. She proposed the sequence of reactions as follows:
1) Reduction of azido function in α position of the keto function to amine.
2) Nucleophilic attack of amine on the carbonyl carbon of pentafluoro phenyl ester of phenyl acetic acid and forming amide bond (protection of amine in the form of phenylacetamide)
3) Reduction of azido function in neopentyl position to amine
4) Formation of cyclic enamine.
5) Aromatization leading to the formation of pyrrole.
For synthesizing PBG-aminoacid pseudo peptides, we decided to follow the same methodology which had been used for synthesizing PBG-phenyl acetamide pseudo peptide. So, the first step is to synthesize pentafluorphenyl (PFP) esters of amino acids

and then couple with Mitsunobu product to form pyrrole cyclization and peptide formation together.

5.7.2.1. Synthesis of Pentafluoro phenyl esters of amino acids

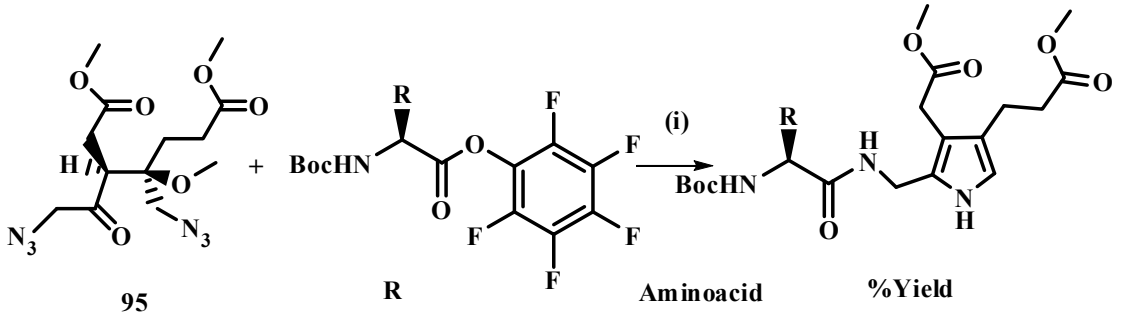


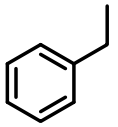
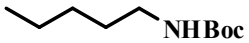
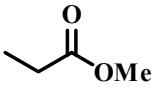
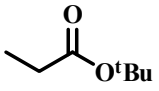
Reagents and conditions: (i) DCC, EtOAc, 10^0C to ambient temperature, 2h, 86% to 90%.

Scheme 125: Synthesis of pentafluorophenyl ester of amino acids

We synthesized pentafluorophenyl (PFP) ester of various protected amino acids (scheme 125) [431-433] namely N-Boc-Alanine; N-Boc-Phenyl alanine; N-Boc,N´-Boc-Lysine and N-Boc-Aspartic acid-OMe. In the case of lysine and aspartic acid we did not isolate the activated ester. In the case of aspartic acid, we also synthesized pentafluorophenyl ester of N-Boc-Aspartic acid-O^{t}Bu.

5.7.2.2. PBG-(Boc-aminoacid) pseudo peptides

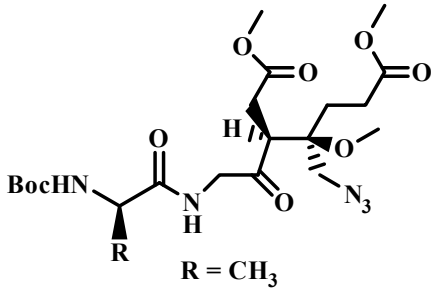


R	Aminoacid	%Yield
CH_3	Alanine	58%
	Phenyl alanine	64%
	Lysine	69%
	Aspartic acid methyl ester	78%
	Aspartic acid t-butyl ester	81%

Reagents and conditions: (i) H_2, Pd/C, MeOH, rt, 14h.

Scheme 126: Synthesis of PBG-(Boc-aminoacid) pseudo peptides

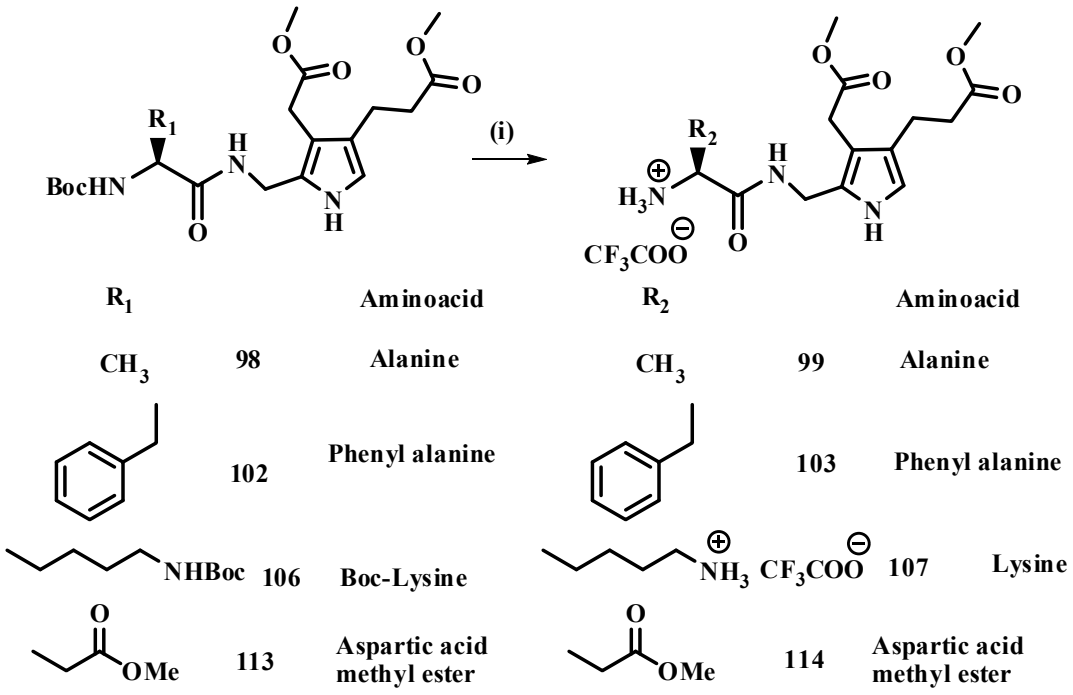
Each of these activated esters of amino acid was coupled with the Mitsunobu product **95** under the conditions of catalytic hydrogenation to provide Boc-amino acid coupled PBG (PBG-Boc-AA) (scheme 126). The PFP ester of amino acid and Mitsunobu product **95** were dissolved in MeOH, 10% Pd over carbon was added as catalyst and then maintained the reaction with hydrogen atmosphere (hydrogen filled in gas bag) at rt for 24h. The reaction was clean, Pd was filtered, solvent evaporated off and the crude was purified by column chromatography to yield PBG-Boc-(aminoacid) pseudo peptide in very good yields (60% to 82%). The addition of two equivalents of PFP ester ensures the rapid protection of amine (formed from azide of alpha to ketone) to amide (peptide) thereby eliminates the chance for the formation of lactam impurity (scheme 124).



Scheme 127: Partially hydrogenated side product during the synthesis of PBG-(Boc-amino acid) pseudo peptides.

We also observed (in the case of alanine) that insufficient maintenance or insufficient hydrogen pressure leads to a product where the amine (formed from azide of alpha to ketone) of Mitsunobu product is coupled with Boc-alanine as a peptide but other azide function in neopentyl position intact without any cyclization (scheme 127). Thus we synthesized PBG- Boc-alanine pseudo peptide, PBG- Boc-phenyl alanine pseudo peptide, and PBG- (Boc), Boc-lysine pseudo peptide. In the case of aspartic acid, we synthesized PBG- Boc-aspartic acid (OMe) pseudo peptide and also PBG- Boc-aspartic acid (O^tBu) pseudo peptide.

5.7.2.3. PBG- (amino acid) pseudo peptides (Deprotection of 'Boc')



Reagents and conditions: (i) TFA, 0^0C to 5^0C, 30 min to 2h, 91% to 94%.
Scheme 128: Boc deprotection of PBG-Boc-amino acid

Though the coupling to form peptide bond between PBG and amino acid worked easily, the deprotection of 'Boc' using TFA was found to be problematic as PBG under highly acidic conditions degraded very fast. We tried different methods of deprotecting 'Boc' using reagents such as trimethyl sily iodide, trimethylsilyl trifluoromethane sulfonate, TBDMS trifluormethane sulfonate, TBAF etc [434-437] which were not successful. We overcame this problem of 'Boc' deprotection by performing the reaction below 0^0C and evaporating TFA below 20^0C. Also, co-evaporation, trituration with dry ether helped to remove traces of TFA. In the case of aspartic acid, repeated attempts [436, 438] to deprotect tert-butyl group alongwith 'Boc' for PBG- Boc-aspartic acid (O^tBu) pseudo peptide, were not successful. So, keeping aside that, we deprotected 'Boc' from PBG-Boc-aspartic acid (OMe) pseudo peptide which we used for biological tests.

Thus, we synthesized PBG- alanine pseudo peptide, PBG- phenyl alanine pseudo peptide, PBG- aspartic acid (OMe) pseudo peptide and PBG- lysine pseudo peptide having the amine of amino acid in the form of salt aminium trifluoroacetate salt. All these four pseudo peptides of PBG were used for biological tests.

5.7.3. Evaluation of PBG-amino acid peptides

PpIX production was evaluated for all the four PBG- amino acid pseudo peptides by measuring increase in fluorescence intensity in living cells at different times by incubating cells in medium containing ALA conjugate at concentrations from 0.1mM to 2 mM. Cells used for experiments were A549 lung carcinoma cells, HCEC human immortalized brain endothelial cells and Human colon HT29 cells.

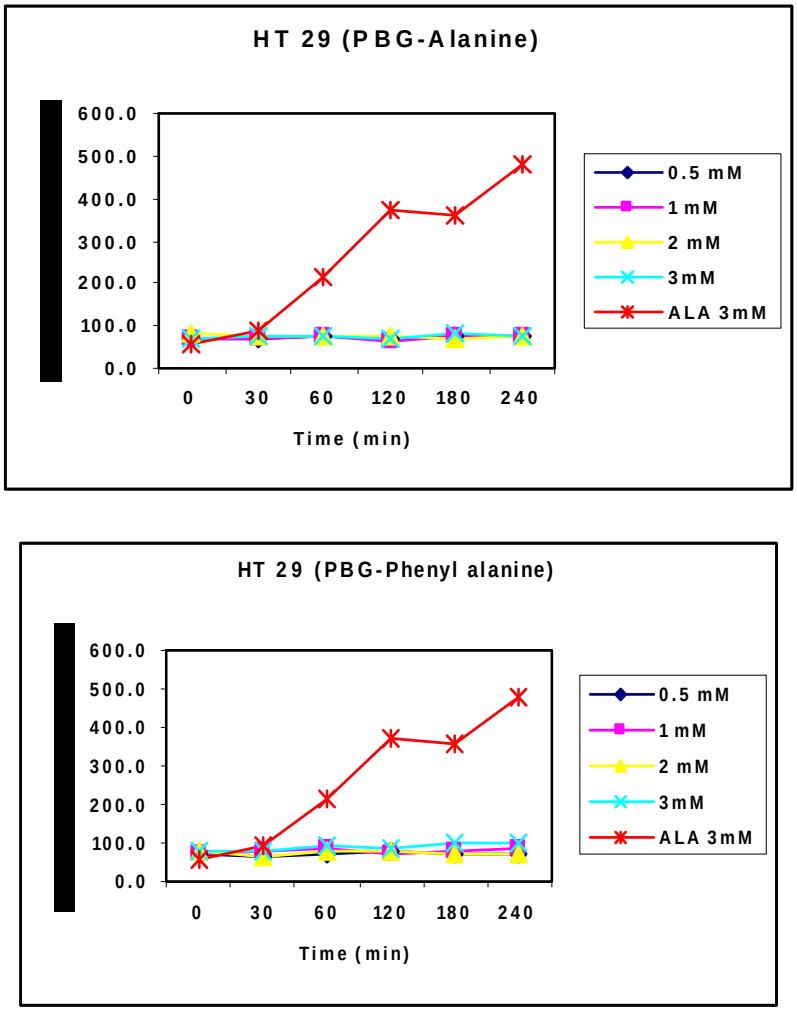


Figure 31: Increase in PpIX level due to PBG-alanine and PBG-phenyl alanine pseudo peptides in HT29 cells.

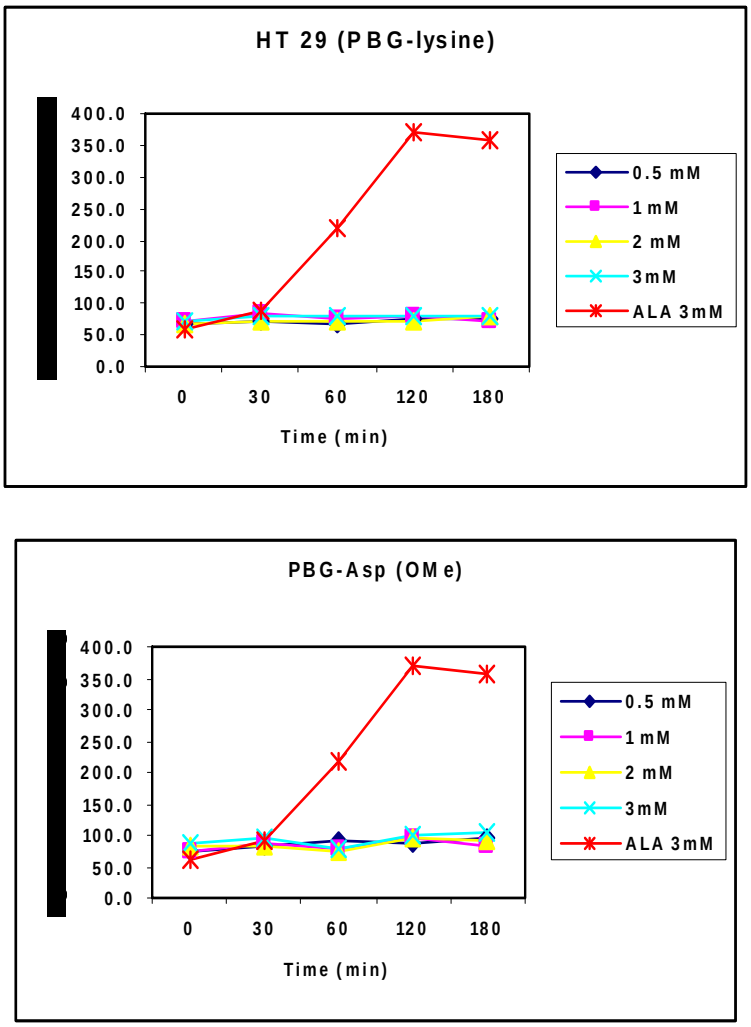


Figure 32: Increase in PpIX level due to PBG-lysine and PBG-aspartic acid methyl ester pseudo peptides in HT29 cells.

The graphs showing increase in fluorescence intensity in HT29 cells are shown here for all the four PBG- amino acid pseudo peptides. As shown in the figures 31 and 32, all the PBG-amino acid pseudo peptides were not able to generate PpIX. The cleavage of peptide might have been not possible due to the inability to recognize the pseudo-peptide.

6. Conclusions

All the ALA bioconjugates synthesized by us are novel and some of them are very efficient in generating protoporphyrin (PpIX). The efficient ALA bioconjugates also has the advantage of being not cytotoxic in the absence of exposure of light.

6.1. Sugar-ALA conjuagtes

All the sugar-ALA conjugates induce PpIX generation, some are more efficient than the standard ALA and some are comparable to that of ALA. The efficiency factor of generating PpIX also varies with respect to the type of cells, as for example in human colon carcinoma HT29 cells, the PpIX induced is comparatively lower than in other types of cells. Sugar-ALA conjugates where ALA is conjugated via ethylene glycol linker (**54**, **57** and **62**) are less efficient than other sugar-ALA conjugates. Some sugar-ALA conjugates are efficient than ALA in generating PpIX while others are comparable to ALA. The main advantage of the sugar-ALA derivatives in comparison to other alkyl ALA esters such as hexyl ALA esters is that they are not cytotoxic in the absence of light exposure.

The major drawback of sugar-ALA conjugates is selectivity. These bioconjugates are not superior to ALA in targeting cancer cells. Having said this, it should also be mentioned that results obtained in cell cultures may not be extrapolated to tumor organ cultures as in the case of hexyl-ALA ester. It was reported that though hexyl-ALA ester generated higher PpIX than ALA in mammary adenocarcinoma cell cultures, in tumor organ cultures of the same line hexyl ALA ester did not increase the rate of tumor porphyrin generation [439]. Keeping this in mind, it may be essential to test sugar-ALA conjugates in tumor organ cultures to find out its efficiency in generating PpIX and also its selectivity towards tumor cells.

6.2. ALA-esters

Although, ALA esters made from tocopherol **65** and 6-ketocholestanol **241** were novel, they have the inherent problem of solubility in the cell culture medium as these compounds have lipophilic chains. These conjugates did not generate higher PpIX than that of ALA which might be due to the difficulty in internalization into the cells because of their lipophilic nature. ALA esters synthesized from phloroglucinol **87** and glycerol **90** contained three ALA molecules. The efficiency of PpIX generation is lesser than that of ALA. This is surprising given the fact that having three ALA molecules, it should generate very high PpIX generation. In between the esters synthesized from phloroglucinol **87** and glycerol **90**, the one having phloroglucinol moiety **87** generated higher PpIX generation than that of the one having glycerol moiety. This could be due to the difference in lipophilicity. Also, it was reported that ALA attached to electron withdrawing group moiety was efficient in generating PpIX [103]. This could be the

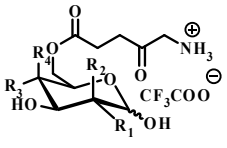
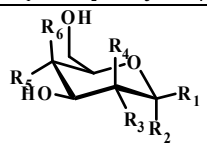
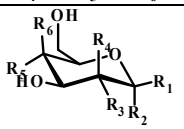
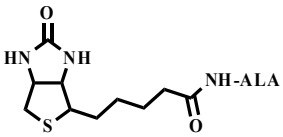
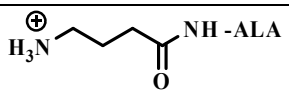
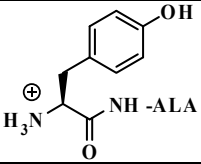
reason for a very high induction of PpIX due to ALA-trichloroethyl ester **242**. The PpIX induction by this ester was superior to ALA.

6.3. ALA-pseudo peptides

All the bioconjugates where the amino group of ALA is protected as amide/peptide moiety (biotin-ALA(OMe) **70**, GABA-ALA (OMe) **79** and tyrosine-ALA (OMe) **83**) did not generate any PpIX. This may not be surprising given the fact that many reported articles mentioned the poor efficiency for ALA when the amino group of ALA is protected [92, 102]. Despite these reports, we synthesized and tested ALA-pseudopeptides because we believed that conjugate moiety attached to ALA, for example biotin could not only aid in targeting cancer cells but also will have unique cellular pathway to transport ALA easily. One of the main reasons for not generating PpIX by these pseudo peptide conjugates could be that these bioconjugates did not release the active ingredient ALA. This might be either due to the non-efficient localization of these conjugates or due to the fact that this unnatural peptide is not recognized by peptidase enzymes for releasing ALA. In a previous study done in our group we found that even in cancer cells which express basic aminopeptidases, ALA conjugated to basic amino acid (lysine) was not efficient in generating PpIX [102]. However, ALA conjugated with N-terminal blocked or N-terminal free dipeptides performed in our group was efficient in generating PpIX [107].

The summary of the ALA bioconjugates synthesized and their evaluation on cell lines is given in a table form as below. The calculated log P of all the conjugates was included as this gives an idea of the lipophilicity of the conjugates. This lipophilicity index is one of the essential things necessary to understand a drug's permeability and absorption as stated in the famous "the rule of 5" by Lipinski [440].

6.4. Summary of Conjugates synthesized and their evaluation

Deriv. No	Structure	Type/ Name	Log P calculated	PpIX compared to ALA	Cytotoxicity	Selectivity and other comments
15, 26, 29	 15; R_1=OH, R_2=H, R_3=OH, R_4=H 26; R_1=H, R_2=OH, R_3=OH, R_4=H 29; R_1=OH, R_2=H, R_3=H, R_4=OH	6′-*O*-(ALA) sugars	-3.71 (unionized)	superior	No	No; 6′-*O*-(ALA) mannose is better than others in selectivity
36, 42, 48	 36; R_1=H, R_2=ALA, R_3=OH, R_4=H, R_5=OH, R_6=H 42; R_1=H, R_2=ALA, R_3=H, R_4=OH, R_5=OH, R_6=H 48; R_1=ALA, R_2=H, R_3=OH, R_4=H, R_5=H, R_6=OH	1′-*O*-(ALA) sugars	-3.71 (unionized)	superior	No	No; 1′-*O*-(ALA) galactose is better than others in selectivity
54, 57, 61	 54; R_1=H, R_2=EG-ALA, R_3=OH, R_4=H, R_5=OH, R_6=H 57; R_1=H, R_2=EG-ALA, R_3=H, R_4=OH, R_5=OH, R_6=H 61; R_1=EG-ALA, R_2=H, R_3=OH, R_4=H, R_5=H, R_6=OH	1′-*O*-(EG-ALA) sugars	-3.87 (unionized)	equal	No	No; 1′-*O*-(EG-ALA) galactose is better than others in selectivity
70		Biotin-ALA peptide	-1.49	No generation	--	----
79		GABA-ALA peptide	-1.59	No generation	---	---
83		Tyr-ALA peptide	-1.5	No generation	---	---

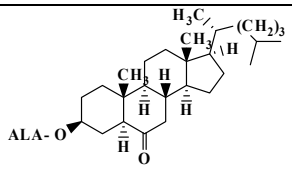
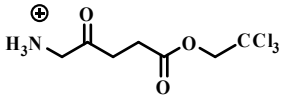
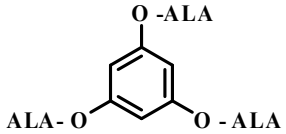
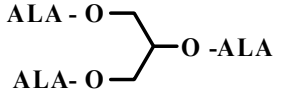
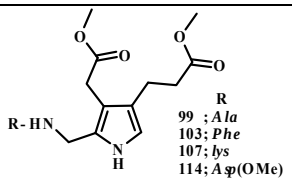
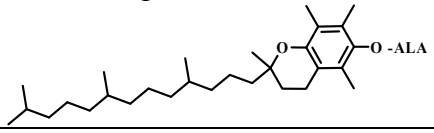
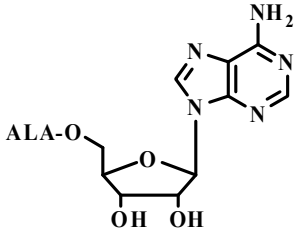
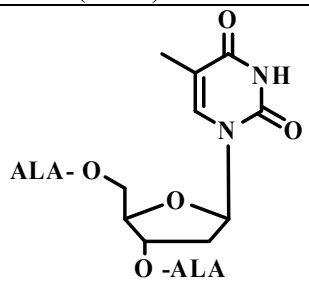
241		6-ketocholestanol - ALA	5.98	No generation	---	Solubilty problem
242		Trichloro ethyl ester of ALA	-0.17	superior	To be tested	To be tested
87		Tri (ALA) phloroglucinol	-3.66	equal	To be tested	To be tested
90		Tri (ALA) glycerol	-4.6	inferior	To be tested	To be tested
99,103, 107, 114		PBG-amino acid peptides	-1.09 0.37 -0.99 -1.05	No generation	---	---
65	Tocopherol-ALA 		9.14	--	--	Solubilty problem
75a	 5′-*O*-(ALA) adenosine		-3.13 (unionized)	To be tested	To be tested	To be tested
238	 3′,5′-*O*- di (ALA) thymidine		-3.78 (unionized)	To be tested	To be tested	To be tested

Table 7: Summary of ALA and PBG conjugates synthesized and their evaluation

7. Perspectives

When ALA conjugates are employed i*n vitro,* it is possible to achieve higher levels of PpIX at lower concentrations than that of ALA. *In vivo*, a higher selectivity for tumoral cells is the main future of ALA based PDT. In this direction, ALA conjugates in future have to be designed not only in the view of higher PpIX generation but also selective affinity towards cancer cells.

Monosaccharide based ALA conjugates (this thesis work) is efficient in transporting ALA inside the cells because of the well defined transport mechanism of sugar which aids ALA transport. But since these conjugates didn't show better selectivity than ALA, it raises the doubt whether monosaccharide alone can provide necessary tool to identify and target cancer cells. In view of this, ALA conjugated to oligosaccharides might be a better option as the cell wall of cancer cells are composed of modified glycans which are mostly oligosaccharides [165].

An important determinant of successful ALA-PDT targeting is the localization of the ALA in cancer tissue. The molecular delivery systems or carrier molecules which have an ability to interact selectively with their targets have to be conjugated with ALA. However there are problems associated with the use of large molecules, such as complicated syntheses, transport barriers, and potential systemic toxicity etc. The research strategy of using monoclonal antibodies (MAbs) (photoimmunotargeting) or low-density lipoproteins (LDLs) to couple with porphyrin based photosensitizers has been reviewed [441, 442]. Tumor targeting with MAbs or LDLs coupled with ALA could be the next step to explore the better selectivity towards cancer cells. The unusual glycans expressed from the cancer cells contains oligosaccharides. The development made in solid phase synthesis of oligosaccharides [158, 159] can help us to synthesize such glycans in sufficient quantities. These synthesized glycans might be used for inducing new antibodies with high affinity which in turn could be coupled with ALA.

The concept of engineering cell surface has been explored [166]. Based on this, the unusual group or moiety in the unusual glycans which are expressed from cancer cells can be exploited for chemical reaction with ALA-reactive conjugate. This could provide selectivity for cancer cells.

For conjugates where there is a basic problem of internalization into the cells, for example PBG, the strategy of coupling with oligoarginine based cell penetrating peptides (CPPs) could be tried because it is reported that such conjugation had increased the efficacy of chlorin based photosensitizers [443].

A selective and efficient ALA-PDT depends on the ALA conjugate which could generate more PpIX (say 10 fold) than that generated by ALA specifically in cancer cells. Besides this, efficient source of light is also necessary for a successful ALA-PDT.

8. Experimental Part- General Methods

8.1. Chromatography

8.1.1. Thin layer chromatography

Thin layer chromatography (TLC) was carried out in TLC plates made out of silica gel 60 F_{254} (fluorescence indicator). The TLC plates are of 5 to 6 cm length, 2.5 cm width and 0.2 mm thickness. After the elution in the eluent mentioned, the plates were dried, looked under UV (254 nm) lamp, marked and then looked for oxidized spots by spraying over aqueous alkaline solution of $KMnO_4$.

8.1.2. Column chromatography

Silica gel $60A^0$, size of 0.04-0.063 mm (230-400 mesh ASTM) (Merck, Darmstadt). Eluent proportions are mentioned in the experimental part. The column chromatography was performed under pressure.

8.1.3. Gas chromatography

Agilent 6850 Series gas chromatography. Column used were HP-5 capillary column. Long 30 m, interior diameter = 0.32 mm, film thickness = 0.25 μm. nature of polymer: polysiloxane (crosslinked 5% Ph, me silixane). Programme: injection at 150°C or 170°C, 2 min, 10°C/min till 290°C; Helium gas: 1.0 mL/minute; Injection temp: 250°C Detector temp (FID): 300°C; ChemStation programme.

8.2. Infrared Spectroscopy (IR)

Perkin Elmer Spectrum One version B FT-IR was used for obtaining IR spectra with the resolution of 2 cm^{-1}. Software used: Spectrum version 5.0.1. The solid substances and thick oil like substances were analyzed by preparing KBr pellet (KBr – Fluka puris p. a). Liquid samples were analyzed as film (sandwich) by applying between two KBr salt plate. The absorption bands between 4000 and 400 cm^{-1} were measured. The intensity of the spectrum was divided into five equal parts for the abbreviations *vs* (the maximum intensity), *s* (intense), *m* (medium intensity), *w* (weak), *br* (broad) and *vw* (very weak).

8.3. Nuclear Magnetic Spectroscopy (NMR)

The NMR spectra were measured using spectrometer Gemini XL-200 of Varian at 298 K where ^{1}H was measured at frequency of 200 and ^{13}C was measured at frequency of 50Hz. Also NMR spectra were measured using spectrometer Bruker Avance- 400 at 298 K where ^{1}H was measured at frequency of 400 and ^{13}C was measured at frequency of 100Hz. The NMR solvents were purchased from Cambridge Isotope Laboratories. The internal standard for the spectra of ^{1}H: TMS (δ= 0.00 ppm) or $CHCl_3$ (δ= 7.26 ppm) or CH_3OH (δ= 3.31 ppm) or $(CH_3)_2SO$ (δ= 2.50 ppm) or D_2O (δ= 4.79 ppm). The internal standard for the spectra of ^{13}C: TMS (δ= 0.00 ppm) or $CHCl_3$ (δ= 77.16 ppm) or CH_3OH (δ= 49.00 ppm) or $(CH_3)_2SO$ (δ= 39.52 ppm). The ^{13}C spectra were measure by decoupling from ^{1}H. The chemical shift is given in ppm in the descending order and the coupling constant J in Hz. The multiplicity of signals were given abbreviations *s* (singlet), *d* (doublet), *t* (triplet), *q* (quartet), *quint* (quintet), *dt* (doublet of triplet), *br* (broad) and *m* (multiplet). The sign '≈' denotes for the average value of J varying from 0.2 to 0.4 Hz. The ratio of α to β is measured by area integration in the case of ^{1}H and by the intensity (height) of peak in the case of ^{13}C. All the products were measured for HETCOR (short range), COSY and DEPT 135 for the accurate interpretation of signals. The numbering of carbons for ALA derivatives were defined from the thesis of Y.Berger [250] .

8.4. Mass Spectroscopy

The mass spectra measurements for ESI (electro-spray ionization) and for APCI (atmospheric pressure chemical ionization) were carried out by an instrument ThermoFinnigan LCQ (San Jose, California, USA). Software used: Tune Plus version 1.2. The values given here are average mass of ion with precision in the range of ±0.1 values. The nature of ion pattern is written in parenthesis with intensity. The ion peaks due to deuterium isotope (probably comes from proton exchange by deuterium as some samples were dissolved in deuteriated solvents) were not considered. The mass corresponding to the highest abundant isotope is written. The high resolution mass spectroscopy (HR-MS) was measured at University of Fribourg (Switzerland) in the group of Professor A. Gossauer by Mr.F.Nydegger. The instrument used was Brucker BioAPEX II daltonics. The ionization used was ESI (electro-spray ionization).

8.5. Glass apparatus

For reactions where argon or nitrogen atmosphere was used, the glass apparatus were dried by keeping in the hot oven at 150°C for at least two hours. The apparatus were removed from hot oven and cooled to ambient temperature with argon or nitrogen atmosphere. For reactions at low temperature, ice bath with salt (0°C to -5°C) and acetone/liquid nitrogen bath (-80°C) were used. For reactions of long maintenance hours

at low temperature (-50°C to 0°C), cryostat was used. For heating reactions, PEG bath (160°C) or oil bath (200°C) were used.

8.6. Karl-Fischer titration

For reactions where the moisture content is very critical such as Mukaiyama reaction, Mitsunobu reaction etc, the purified solvents, reactants and reagents if possible were checked for moisture content using the Karl-Fischer coulometer (Metler Toledo DL 32). For the moisture critical reactions, reactants and solvents were dried till the moisture content is less than 0.05% and then only reactions were carried out.

8.7. Hydrogenation

The hydrogenation reactions were carried out at ambient temperature under magnetic stirring, with hydrogen filled 'gas bag' (Aldrich) whose volume is approximately 1 gallon (3.8 L).

8.8. Solvents

8.8.1. Standard solvents

For the purpose of chromatography and extractions, technical grade solvents were distilled over drying agent.

Solvent	**Abbreviation used**	**Drying agent**
Ethyl acetate	AcOEt	K_2CO_3
Hexane	hexane	$CaCl_2$
Dichloromethane	CH_2Cl_2	$CaCl_2$
Diethyl ether	Et_2O	$CaCl_2$
Methanol	MeOH	CaO
Chloroform	CHCl3	$CaCl_2$

8.8.2. Solvents for reactions- with distillation

Solvent	**Abbreviation used**	**Drying agent**

Hexane	hexane	P_2O_5
Dichloromethane*	CH_2Cl_2	P_2O_5
Diethyl ether **	Et_2O	$LiAlH_4$
Tetrahydrofuran	THF	sodium (benzophenone)
Toluene	toluene	sodium (benzophenone)

* Dichloromethane after distilling over P_2O_5 was passed through basic alumina to remove the acidic traces.

** Only distilled diethyl ether (over $LiAlH_4$) is used for precipitations and triturations

8.8.3. Solvents for reactions- without distillation

Solvent	**Abbreviation used**	**Quality**
Pyridine	pyridine	Fluka, puriss., ≥ 99.5% (with crown cap)
Dimethyl formamide	DMF	Fluka, puriss., ≥ 99.5% (with crown cap)
Methanol	MeOH	Acros, p. a.
Dioxane	dioxane	Fluka, puriss., ≥ 99.5%
Dichloromethane	CH_2Cl_2	Fluka, puriss., ≥ 99.5%
Toluene	toluene	Fluka, puriss., ≥ 99.5%

8.9. Reagents, enzymes and products

8.9.1. Reagents

Reagent	**Abbreviation used**	**Quality**
Acetic acid	CH_3COOH	Acros, p. a.

Acetobromo α-D-galactose	-	Fluka, technical ≥ 90%
Acetone oxime	-	Fluka, puriss., ≥ 99%
Adenosine	-	Acros , ≥ 99%
Alumina neutral	-	Fluka
Alumina basic	-	Fluka
Ammonium chloride	NH_4Cl	Fluka, purum
Ammonium formate	$HCOONH_4$	Acros
Argon	Ar	Carbagas, tech.
Barium oxide	BaO	Fluka, tech. 95%
Benzyl alcohol	BnOH	Fluka, puris, ≥ 99%
Benzyl bromide	BnBr	Fluka, purum
Biotin	-	Fluka
Boc-Alanine	Boc-Ala	Novobiochem ≥ 95%
Boc-γ-aminobutyric acid	Boc-GABA	Novobiochem ≥ 95%
Boc-Aspartic acid –*O*-methyl ester	Boc-Asp-OMe	Novobiochem ≥ 95%
Boc-Aspartic acid –*O*-*tert*-butyl ester. Dicyclohexyl amine salt	Boc-Asp-O^{t}Bu	Novobiochem ≥ 95%
N_α-Boc-N_ε-Boc) lysine –dicyclohexyl amine salt	Boc-Boc-lys.DCHA	Novobiochem ≥ 95%
Boc-Phenyl alnaine	Boc-Phe	Novobiochem ≥ 98%
Bromine	Br_2	Acros p.a
tert-butyl diphenyl silyl chloride	TBDPSCl	Acros, purum

n-butyl lithium	BuLi	~1.6M or ~2.5M in hexans Acros
Celite 535	.-	Fluka (10- 40µm)
Cesium carbonate	Cs_2CO_3	Fluka, puriss., ≥ 99%
Chlorotrimethyl silane	TMSCl	Fluka, puriss., ≥ 99%
Cholcalciferol (+)-Vitamin D3	Vitamin D3	Fluka, puriss., ≥ 99%
1,8-Diazabicyclo undec-7-ene	DBU	Acros 97% +
N, N´-Dicyclohexyl carbodimide	DCC	Fluka, 99% (GC)
Diethyl azodicarboxylate	DEAD	Fluka, technical., ≥ 94%
Diisopropyl azodicarboxylate	DIAD	Fluka, technical., ≥ 94%
4-(N,N´-Dimethyl) amino pyridine	DMAP	Fluka, puriss., ≥ 99%
1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide hydrochloride	EDCI	Acros, 98+%
di-tert-butyl-dicarbonate	Boc_2O	Fluka, purum≥98%
Dowex 50WX resin	Dowex	Acros
Dimethylamino pyridine-p-toluene sulfonate	DPTS	Prepared from DMAP and TsOH
Esterase (Pig liver esterase)	PLE	Sigma-Aldrich, from porcine liver, 24 units per mg of protein
Ethylene glycol	EG	Fluka, purum≥98%
Formic acid	HCOOH	Fluka, puriss., ≥ 99%
D-(+)-Galactose		Fluka, Biochemika, ≥ 99.5%
D-(+)-Glucose		Fluka, Biochemika, ≥ 99.5%

Glycerol	-	Fluka, purum≥98%
Hexamethyl disilazane	HMDS	Fluka, puriss., ≥ 99%
Hydrochloric acid	HCl	32% Fluka
Hydrogen	H_2	Carbagas, tech.
Iodomethane	MeI	Fluka, puriss., ≥ 99%
Imidazole	-	Fluka, puriss., ≥ 99%
Levulinic acid	-	Acros p.a
Lipase from *Candida antarctica*	Novozyme 435 or LCA	Sigma Aldrich, 10 unites per mg protein
Lipase from Porcine pancreatic	PPL	Fluka, 24 units per mg of protein
Lithium aluminium hydride	$LiAlH_4$	Fluka, purum
Lithium bis (trimethylsilyl) amide solution	LiHMDS	Fluka, 1M in hexane or 1M in THF
D-(+)-Mannose		Fluka, Biochemika, ≥ 99.5%
Nitrogen	N_2	Carbagas, tech.
Palladium on activated carbon	Pd/C	Acros 10% unreduced
Palladium over alumina	Pd/Al_2O_3	Acros 5%
Pentafluorphenol	PFP-OH	Acros 98%
Phenyl boronic acid	.	Fluka, purum ≥ 97%
Phloroglucinol		Fluka, puriss., ≥ 99%
Potassium carbonate	K_2CO_3	Siegfreid
Potassium permanganate	$KMnO_4$	Siegfreid
Proteinase *Bacillus subtilis*		Sigma Aldrich, 5 units

		per mg of protein
Retinol		FLUKA, all trans, ≥97,0%(HPLC)
Silvercarbonate	Ag_2CO_3	Fluka, purum ≥ 99%
Sodium azide	NaN_3	Fluka, purum≥99,0%
Sodium hydride	NaH	Fluka ~60% suspension
Sodium hydroxide	NaOH	Fluka , Puriss, p. a
Sodium methoxide	NaOMe	Fluka, purum ≥ 99%
Sodium dihydrogen phosphate	NaH_2PO_4. $2H_2O$	Fluka, purum ≥ 99%
Sodium hydrogen phosphate	Na_2HPO_4. $2H_2O$	Fluka, purum ≥ 99%
Sulfuric acid	H_2SO_4	96% Prochimie
Tetrabutyl ammonium fluoride	TBAF	Fluka, purum ~1M in THF
Tetrabutyl ammonium iodide	TBAI	Fluka, puriss≥99%
Thionyl chloride	$SOCl_2$	Fluka, purum ≥ 98%
Titanium tetrachloride	$TiCl_4$	Fluka, purum ≥ 98% and then distilled over poly vinyl pyridine
Thymidine		Acros 98%
DL-all-rac-(±)-α-tocopherol	Vitamin E	Fluka, Biochemika, ≥97%(HPLC)
p-tolune sulfonic acid monohydrate	*p*-TsOH. H_2O	Fluka, puriss ≥ 98.5%
2,2,2-Trichloro ethanol	TCE-OH	Fluka, purum ≥ 98%
Trifluoroacetic acid	TFA	

Trifluoromethane sulfonic acid	CF_3SO_3H	Fluka, puriss≥99.5%
Trimethyl orthoformate	$(H_3CO)_3HC$	Fluka, puriss≥99%
Triphenyl phosphine	PPh_3	Fluka, puriss≥98.5%

8.9.2. Preparation of HCl gas, HN_3 gas and destruction of NaN_3.

1. Preparation of HCl gas:

In a two necked dry flask, fitted with a gas outlet, a thermometer and an addition funnel was taken 200 ml conc.sulfuric acid (98%). Under stirring, to this was added conc.HCl (32%) drop wise. The flask was cooled by cold water bath outside. The HCl gas liberated from the gas outlet was connected to the reaction flask where HCl gas is required.

2. Preparation of HN_3 gas:

In a two necked dry flask, fitted with a gas outlet, a thermometer and an addition funnel was taken 5 g (77 mmole) sodium azide. This was dissolved in 5 ml deionised water. Under stirring, 30 ml benzene was added. Then, to this reaction mass was added 2.1 ml (3.7 g, 38.5 mmol) of conc.sulfuric acid (98%). drop wise. The flask was cooled by cold water bath outside. The HN_3 gas was dissolved in benzene. From this benzene solution containing HN_3, a sample was taken, diluted with water and titrated against 0.1 N NaOH to determine the concentration of HN_3. Later this benzene solution containing HN_3 was added to the reaction flask where HN_3 is required.

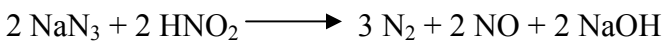
3. Destruction of NaN_3

All the aqueous phases containing HN_3 and NaN_3 were stored separately for treating the solution and then were discarded. The aqueous solution containing NaN_3 comes from the extraction of azido products. The excess solution containing HN_3 was always mixed with 2N NaOH and the benzene layer was decanted.

Treatment: The treatment is performed in a hood. In a three necked flask placed over a magnetic stirrer, was fixed an addition funnel and an outlet for gas. In the aqueous solution containing NaN_3, was added under stirring sodium nitrite solution (7.5 g of $NaNO_2$ in 38 ml water for 5 g of NaN_3) in a drop wise manner. After the addition, 20% H_2SO_4 was added slowly to obtain acidic pH. The nitrogen oxide gas evolved on the

addition sulfuric acid. The completion of destruction is verified by change in color of iodide paper from white to blue (presence of excess nitrite).

Equation 5



$$2\ NaN_3 + 2\ HNO_2 \longrightarrow 3\ N_2 + 2\ NO + 2\ NaOH$$

The aqueous solution was discarded after destruction. The nitrous oxide (NO) evolved gets oxidized to nitric oxide (NO_2) which is dark brown, irritating and toxic.

9. Experimental Part- Synthesis

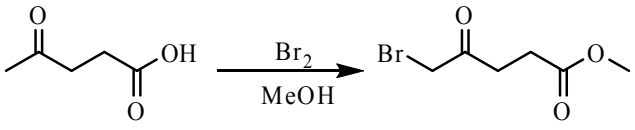
9.1. Synthesis of starting materials

9.1.1. Synthesis of 5-BOC-amino-4-oxo-penatanoic acid (BOC-ALA)

The synthesis of 5- BOC-aminolevulinic acid was carried out by two methods. The first method used levulinic acid as starting material and the second method used 5-aminolevulinic acid hydrochloride as starting material. The first method comprises four steps of which first two steps were carried out many a times by the previous doctorates and students of the group.

9.1.1.1. Synthesis of 5- BOC-amino-4-oxo-pentanoic acid (BOC-ALA) from levulinic acid

9.1.1.1.1. Synthesis of 5-bromo-4-oxo methyl pentanoate (5-bromo methyl levulinate)

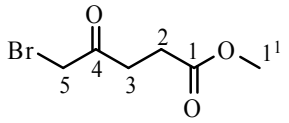


1 $C_5H_8O_3$ [116.12]

2 C_6H_9BrO [209.04]

The assembly set-up consists of a sulfonation flask of 1500 ml capacity, an overhead mechanical stirrer, an addition funnel, a condenser and a thermometer. The outlet of the condenser is connected to a beaker containing 1N NaOH solution for scrubbing the HBr gases which evolved.

A solution of 100 g (861 mmol, 1.0 eq) levulinic acid in 860 ml methanol was charged in the sulfonation flask. This solution was cooled to 0 ^{0}C and then 44ml (861 mmol, 1.0eq) of bromine was added drop wise through addition funnel. The addition of bromine was carried out in such a way that the reaction temperature never exceeds 30 ^{0}C. The addition took approximately 3 hours. At the end of the addition, the solution was dark orange. The reaction mass was brought to room temperature and was kept stirring overnight at room temperature. The resultant colourless solution was refluxed for 90 minutes. The solution was brought to room temperature and then methanol was evaporated off by rotavapor. The yellow residue was dissolved in 400 ml dichloromethane and this solution was extracted with 400 ml of H_2O. The organic phase was further extracted with 200 ml saturated solution of $NaHCO_3$ and then with 3 × 200 ml H_2O. Then the organic phase was dried over MgSO4, filtered by filter paper and then the solvent was evaporated off by rotavapor.The yellow residue of 156 g was subjected to fractional distillation by means of a Widmer column of 40cm under reduced pressure of 4×10^{-2} mbar obtained by high vacuum oil pump.The fractions were changed in accordance with vapour temp and totally 8 fractions were collected keeping the distillation system under vacuum using perkin triangle. One of the fractions contained 81 g of 5-Bromo-4-oxo methyl pentanoate, the required product with 98% purity by ^{1}H-NMR and with 45% yield. (Best yield obtained so far = 57%)



R_f: (Hexane / AcOEt 1: 1): 0.50

Bpt: ~ 80 ^{0}C at pressure = 0.045 mbar

n_D^{20}: 1.4820

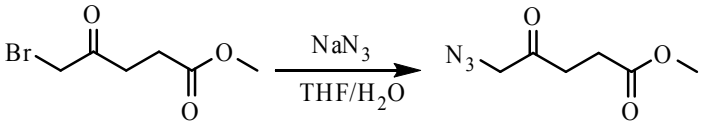
IR (film) : 3000*m*, 2953*m*, 2849*w*, 1734*s*, 1722*s*, 1439*s*, 1411*s*, 1359*s*, 1322*m*, 1207*s*, 1177*s*, 1079*m*, 1025*m*, 988*m*, 862*w*, 768*vw*, 694*w*, 595*vw*, 496*vw*, 464*w*.

^{1}H-NMR (200 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 3.95 (*s*, 2H, $H_2C(5)$); 3.67 (*s*, 3H, $H_3C(1^1)$; 2.95 (*t*, $^3J_{3\text{-}2}$=6.4, 2H, $H_2C(3)$); 2.64 (*t*, $^3J_{2\text{-}3}$= 6.4, 2H, $H_2C(2)$).

^{13}C-NMR (50 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 201.3 (C(4)) ; 173.4 (C(1)) ; 52.6 ($C(1^1)$) ; 35.1 (C(3)) ; 34.7 (C(2)) ; 28.8 (C(5)).

MS (ESI (+)): $[M\ (_{79}Br) + Na]^+$ =231.1, $[M\ (_{81}Br+Na]^+$ =233.0

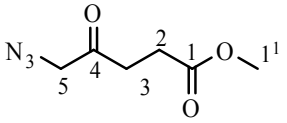
10.1.1.1.2. Synthesis of 5-azido-4-oxo methyl pentanoate (5-azido methyl levulinate)



2 $C_6H_9BrO_3$ [209.04]

3 $C_6H_9N_3O_3$ [171.16]

To a solution of 17.68 g (272 mmol, 2eq) of NaN_3 in 68ml deionised water at 0 ^{0}C and under magnetic stirring, was added drop wise by an addition funnel a solution of 28.43 g (136mmol, 1eq) of 5-Bromo-4-oxo methyl pentanoate in 70ml of THF. The yellow coloured reaction mass was brought to room temperature and then was stirred at that temperature for an hour. Later, the two phases were separated. The aqueous phase was extracted with 3 × 200ml AcOEt. All the organic phases were combined together and then it was washed with 2×200ml H_2O. Then the organic phase was dried over MgSO4, filtered over filter paper, and the solvent was evaporated off by rotavapor. A yellow oil of 22.05 g 5-azido-4-oxo methyl pentanoate was obtained with 95% yield. (Best yield obtained so far = 98%)



R_f: (Hexane / AcOEt 2: 1): 0.50; 0.33 (UV $_{254}$ active, vanillin)

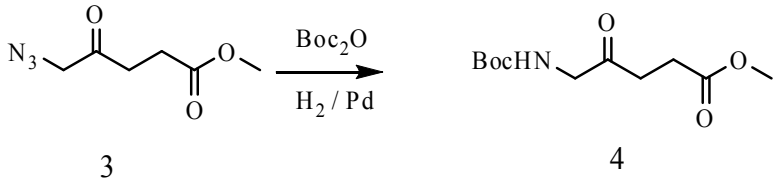
IR (film) : 3445*w*, 3366*w*, 3001*m*, 2956*m*, 2914*m*, 2850*w*, 2543*vw*, 2202*m*, 2106*vs*, 1732*vs*, 1619*w*, 1438*s*, 1417*s*, 1361*s*, 1280*s*, 1210*s*,1172*s*, 1096*s*, 1028*m*, 1008*m*, 988*m*, 921*m*, 885*w*, 843*w*, 799*vw*, 705*vw*, 607*vw*, 556*m*, 489*vw*.

^{1}H-NMR (200 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 4.03 (*s*, 2H, $H_2C(5)$) ; 3.69 (*s*, 3H, $H_3C(1^1)$; 2.76-2.72 (*m*, AA' part of a system AA'BB', 2H, $H_2C(3)$) ; 2.70-2.66 (*m*, BB' part of a system AA'BB', 2H, $H_2C(2)$).

^{13}C-NMR (50 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 202.9 (C(4)) ; 173.0 (C(1)) ; 57.3 (C(5); 51.9 ($C(1^1)$) ; 34.3 (C(3)) ; 27.4 (C(2)).

MS (ESI(+)): m/z relative intensity $[M + Na]^+$ = 194

9.1.1.1.3. Synthesis of 5-BOC-amino-4-oxo methyl pentanoate (5-BOC-amino methyl levulinate)

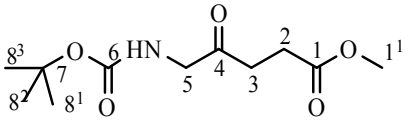


3 4

$C_6H_9N_3O_3$ [171.16]

$C_{11}H_{19}NO_5$ [245.16]

In a hydrogenation autoclave reactor, a solution of 23 g (134.37 mmol, 1eq) of 5-azido-4-oxo methyl pentanoate in 250ml AcOEt was charged. Then 29.63 g (135.76 mmol, 1.01eq) of BOC anhydride was charged followed by 5 g (4.7 mmol, 0.035 eq) of 10% palladium on activated charcoal. The mass was subjected to hydrogenation in ambient temperature with the pressure of 60psi H_2 under mechanical agitation. Once the reaction was completed, (approximately 24hrs; TLC eluent: CH_2Cl_2/AcOEt 95:5) the H_2 pressure was released. The reaction mass was filtered over celite bed to get rid off palladium, the celite bed was washed with 3 × 25ml AcOEt. The solvent in the filtrate was evaporated off by rotovapor and a yellow oily mass was obtained. This yellow crude mass was purified by flash chromatography using gradient eluent system. (from CH_2Cl_2/AcOEt 95:5 to CH_2Cl_2/AcOEt 85:15). The pure fraction of 23.05 g 5-BOC-amino-4-oxo methyl pentanoate was thus obtained as yellow oil. (Yield = 70%; best yield so far = 74%)



R_f: (CH_2Cl_2/ AcOEt 9: 1): 0.25-0.30 (UV $_{254}$ active, $KMnO_4$)

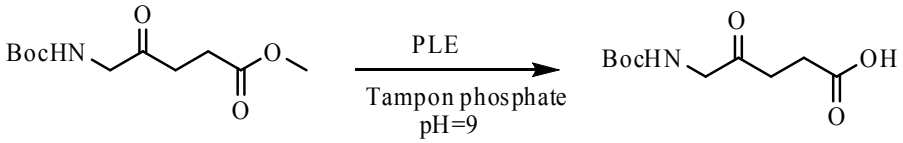
IR (film) : 3387*s*, 2979*s*, 2932*s*, 1735*s*, 1719*s*,1701*s*, 1697*s*, 1523*s*, 1518*s*, 1508*s*, 1500*s*, 1438*s*, 1412*s*, 1392*s*, 1252*s*, 1209*s*,1165*s*, 1098*m*, 1060*m*, 1024*m*, 983*m*, 737*m*.

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 5.24 (*sbr*, 1H, NH) ; 4.03 (*d*, $^3J_{5\text{-}NH}$=3.6 Hz, 2H, $H_2C(5)$) ; 3.64 (*s*, 3H, $H_3C(1^1)$; 2.71-2.68 (*m*, 2H, $H_2C(3)$) ; 2..63-2.59 (*m*, 2H, $H_2C(2)$) ; 1.40 (*s*, 9H, $H_3C(8^1,8^2,8^3)$.

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 204.5 (C(4)) ; 173.0 (C(1)) ; 155.7 (C(6)) ; 79.9 (C(7)) ; 52.0 ($C(1^1)$) ; 50.3 (C(5)) ; 34.4 (C(3)) ; 28.4 ($C(8^1,8^2,8^3)$) ; 27.6 (C(2)).

MS (APCI (+)): m/z relative intensity (15, $[M + Na]^+$) = 269.1

9.1.1.1.4. Synthesis of 5-BOC-amino-4-oxo pentanoic acid (BOC-ALA)



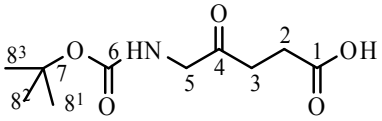
4

$C_{11}H_{19}NO_5$
[245.16]

5

$C_{10}H_{17}NO_5$
[231.16]

In a 1lit Erlenmeyer flask, 500ml 0.1M tampon phosphate buffer solution was charged. (Preparation of 0.1 M tampon phosphate, pH=9.0: 1.58 g NaH_2PO_4 and 5.66 g Na_2HPO_4 was dissolved in 1lit of deionised water.) The pH of the buffer is brought to 9 by 5M NaOH. Then, 22.85 g (93.2 mmol 1eq) of 5-BOC-amino-4-oxo methyl pentanoate ( ) was charged under agitation. Then 300 mg of *pig liver esterase* (PLE) enzyme (24 units per mg of protein; 1unit hydrolyses 1µmol or 0.001mmol of an ester; approximately 7.2 mmol; 0.077eq) was added under agitation. The pH of the reaction mass was continuously maintained at pH 9 using 5N NaOH by means of a Dosimeter connected with a pH meter. The reaction was completed approximately after 4 days (TLC eluent: CH_2Cl_2/AcOEt 90:10).The reaction mass was then extracted with 3 × 200ml AcOEt. The aqueous phase was acidified till pH 2 with 6N HCl. This was followed by extraction of the aqueous phase with 4 × 500ml AcOEt. All the organic phases were combined together and it was washed with 500 ml saturated NaCl solution followed by 500 ml deionised water. The organic phase was then dried over $MgSO_4$ and the solvent was evaporated off by rotavapor. A yellow crude solid product thus obtained was taken for trituration with ether at 0 ^{0}C. The white pure solid thus obtained was filtered and dried. Thus 13.7 g of pure white 5-BOC-amino-4-oxo pentanoic acid was obtained. (Yield= 63.6%; best yield obtained so far = 85%)



R_f: (AcOEt 100%): 0.47 ($KMnO_4$, tailing)

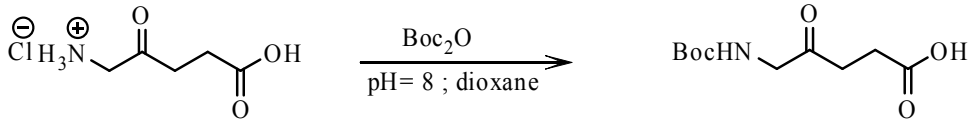
IR (KBr) : 3379*s*, 2986*mbr*, 2919*mbr*, 2765*w*, 2692*w*, 2571*w*, 1707*vs*, 1686*vs*, 1506*s*, 1429*s*, 1407*m*, 1392*m*, 1383*m*, 1371*m*, 1363*m*, 1349*m*, 1295*w*, 1278*m*, 1240*s*, 1209*s*,1170*s*, 1098*w*, 1065*m*, 1039*vw*, 1015*m*, 943*m*, 894*m*, 872*m*, 843*vw*, 803*w*, 787*w*, 759*w*, 741*w*, 653*w*, 575*m*.

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 11.30 (*s*, 1H, COOH); 5.35 (*sbr*, 1H, NH) ; 4.03 (*d*, $^3J_{5\text{-NH}}$= 4.8 Hz, 2H, $H_2C(5)$) ; 2.69-2.62 (*m*, 4H, H_2C(2 and 3)) ; 1.40 (*s*, 9H, $H_3C(8^1,8^2,8^3)$).

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 204.5 (C(4)) ; 177.4 (C(1)) ; 156.0 (C(6)) ; 80.2 (C(7)) ; 50.3 (C(5)) ; 34.2 (C(3)) ; 28.4 ($C(8^1,8^2,8^3)$) ; 27.7 (C(2)).

MS (APCI (-)): m/z relative intensity (95, $[M - H]^-$ = 230.1

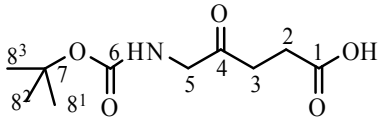
9.1.1.2. Synthesis of 5- BOC-amino-4-oxo-pentanoic acid (BOC-ALA) from 5-aminolevulinic acid hydrochloride (ALA-HCl)



6 — $C_5H_{10}NO_3Cl$ [167.60]

5 — $C_{10}H_{17}NO_5$ [231.16]

In a 1lit Erlenmeyer flask, 11g (65.63 mmol, 1.0eq) was charged and it was dissolved in 170 ml deionised water. The pH of the solution was adjusted to 8 by 1N NaOH. Then 30 g (137.07 mmol, 2.0eq) of BOC anhydride dissolved in 100ml 1, 4-dioxane was added. The pH of the reaction mass was continuously maintained at pH 8 using 1N NaOH by means of a Dosimeter connected with a pH meter. After 18hrs, the reaction was completed. The reaction mass was extracted with 3 × 400ml ether to remove excess BOC anhydride. The aqueous phase was acidified till pH 2 by 10% HCl and then was extracted with 5 × 300ml AcOEt. All the organic phases were combined together, dried over $MgSO_4$ and solvent evaporated off by rotavapor. The remaining pale yellow solid was washed with cold ether and filtered. Thus 7.84 g of pure 5- BOC-amino-4-oxo-pentanoic acid was obtained. (Yield = 52%)



R_f: (AcOEt 100%): 0.47 ($KMnO_4$, tailing)

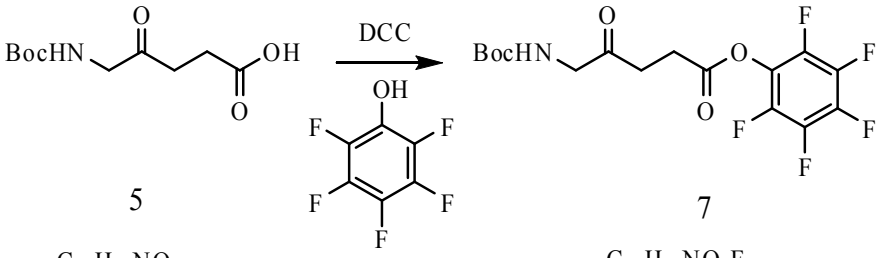
IR (KBr) : 3379*s*, 2986*mbr*, 2919*mbr*, 2765*w*, 2692*w*, 2571*w*, 1707vs, 1686*vs*, 1506*s*, 1429*s*, 1407*m*, 1392*m*, 1383*m*, 1371*m*, 1363*m*, 1349*m*, 1295*w*, 1278*m*, 1240*s*, 1209*s*,1170*s*, 1098*w*, 1065*m*, 1039*vw*, 1015*m*, 943*m*, 894*m*, 872*m*, 843*vw*, 803*w*, 787*w*, 759*w*, 741*w*, 653*w*, 575*m*.

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 11.30 (*s*, 1H, COOH); 5.35 (*sbr*, 1H, NH) ; 4.03 (*d*, $^3J_{5\text{-}NH}$= 4.8 Hz, 2H, $H_2C(5)$) ; 2.69-2.62 (*m*, 4H, $H_2C(2$ and $3)$) ; 1.40 (*s*, 9H, $H_3C(8^1,8^2,8^3)$).

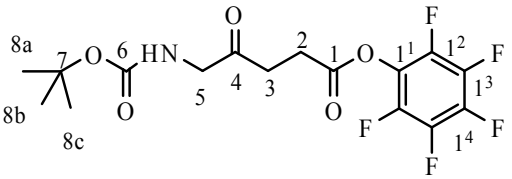
^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 204.5 (C(4)) ; 177.4 (C(1)) ; 156.0 (C(6)) ; 80.2 (C(7)) ; 50.3 (C(5)) ; 34.2 (C(3)) ; 28.4 ($C(8^1,8^2,8^3)$) ; 27.7 (C(2)).

MS (APCI (-)): m/z relative intensity (95, $[M - H]^-$ = 230.1

9.1.2. Synthesis of pentafluorophenyl ester of 5- BOC-amino-4-oxo-pentanoic acid (BOC-ALA-PFP ester)



In a dry flask under argon atmosphere, 3g (12.97 mmol, 1eq) of BOC-ALA was charged. This was dissolved in 50 ml CH_2Cl_2 and the mass was cooled to 0 ^{0}C. Then 2.7g (14.27 mmol, 1.1eq) of pentafluorophenol was added followed by 3g (14.54 mmol, 1.12eq) of DCC. The reaction was brought to room temperature and was kept at room temperature overnight. The reaction mass was then filtered over celite bed to get rid off DCU and the solvent was evaporated off by rotovapor. The thick yellow oily mass obtained was triturated with hexane to remove excess pentafluorphenol and the white solid obtained was filtered off. The white solid was dried which yielded 5.1g of BOC-ALA-PFP ester. (Yield= 98%)



R_f: (CH_2Cl_2/ AcOEt 95: 5): 0.33 (UV $_{254}$ active; $KMnO_4$)

IR (KBr) : 3009*w*, 2982*w*, 2948*w*, 2920*w*, 2669*vw*, 2458*vw*, 1798*m*,1725*s*, 1694*vs*, 1656*w*, 1626*w*, 1527*vs*, 1518*vs*, 1468*w*, 1446*w*, 1426*w*, 1401*w*, 1392*w*, 1370*m*, 1359*w*, 1328*w*, 1271*s*, 1253*m*, 1235*w*, 1170*m*, 1146*m*, 1111*s*, 1042*m*, 1031*m*, 1007*s*, 989*s*, 977*m*, 895*w*, 864*w*, 828*vw*, 781*vw*, 751*vw*, 713*vw*, 666*vw*, 609*w*, 556*w*, 465*vw*.

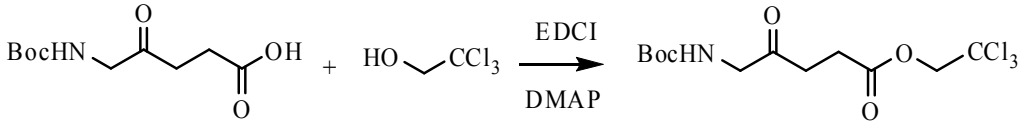
^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 5.25 (*sbr*, 1H, NH) ; 4.06 (*d*, $^3J_{5\text{-}NH}$= 4.9 Hz, 2H, $H_2C(5)$) ; 2.99 (*t*, $^3J_{2\text{-}3}$ = 6.5Hz, 2H, $H_2C(3)$) ; 2.87 (*t*, $^3J_{3\text{-}2}$ = 6.5Hz, 2H, $H_2C(2)$); 1.43 (*s*, 9H, $H_3C(8^a,8^b,8^c)$).

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 203.6 (C(4)) ; 168.8 (C(1)) ; 155.8 (C(6)) ; 142.4 (d × m, $^1J_{C\text{-}F}$= 248Hz, C(1^2) or C(1^3) or C(1^4)); 139.9 (d × m, $^1J_{C\text{-}F}$= 244Hz, C(1^2) or C(1^3) or C(1^4)); 137.5 (d × m, $^1J_{C\text{-}F}$= 240Hz, C(1^2) or C(1^3) or C(1^4)); 125.0 (C1^1); 80.2 (C(7)) ; 50.3 (C(5)) ; 34.2 (C(3)) ; 28.4 (C(8^1,8^2,8^3)) ; 27.0 (C(2)).

^{19}F-NMR (188 MHz, $CDCl_3$, 298K) : δ_F (ppm) = -152.9 (*m*, 2F, (F- C(1^2) and (F- C($1^{2'}$)); -158.2 (*t*, $^3J_{F\text{-}F} \approx$ 21.0Hz, 1F, (F- C(1^4)); -162.6 (*m*, 2F, (F- C(1^3) and (F- C($1^{3'}$)).

MS (APCI (-)): m/z relative intensity (95, $[M - H]^-$ = 230.1

9.1.3. Synthesis of 2, 2, 2-trichloroethyl ester of 5- BOC-amino-4-oxo-pentanoic acid (BOC-ALA-TCE ester)

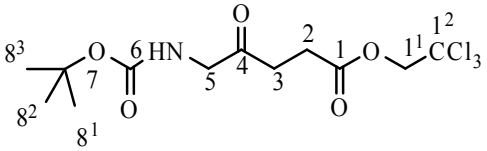


5 $C_{10}H_{17}NO_5$ [231.16]

8 $C_2H_3OCl_3$ [149.4]

9 $C_{12}H_{18}Cl_3NO_5$ [362.63]

In a dry flask under argon atmosphere, 1.267g (8.48 mmol, 2eq) of 2, 2, 2-trichloroethanol was charged and then 80ml CH_2Cl_2 was added. The mass was cooled to 0 ^{0}C, then 0.528 g (4.32 mmol, 1eq) of DMAP was added followed by 0.912 g (4.75 mmol, 1.1eq) of EDCI. Finally, 1g (4.32 mmol, 1eq) of BOC-ALA was charged. The reaction was brought to room temperature and was kept at room temperature overnight. The solvent was evaporated off by rotovapor. The crude mass was dissolved in 100ml AcOEt and extracted with 200ml H_2O.The organic phase is washed with 170 ml cold 1N citric acid solution, followed by 200ml saturated $NaHCO_3$ solution and finally by 200ml saturated NaCl solution. The organic phase is dried over $MgSO_4$ and the solvent was evaporated off by rotavapor. The remaining mass was purified by trituration with hexane to remove excess alcohol and the white solid obtained was filtered off. The white solid was dried which yielded 1.33g of BOC-ALA-TCE ester. (Yield= 85%)



R_f: (Hexane / AcOEt 7: 3): 0.17 (UV $_{254}$ active; $KMnO_4$)

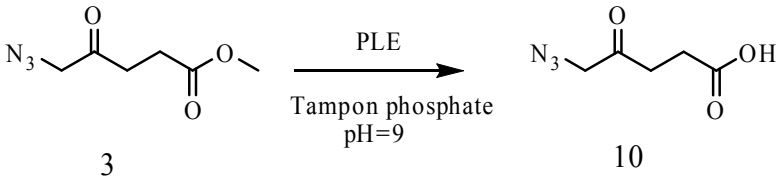
IR (KBr) : 3361*s*, 3000*w*, 2981*w*, 2932*w*, 1759*vs*, 1721*vs*, 1688*vs*, 1528*vs*, 1477*w*, 1452*w*, 1408*m*, 1387*m*, 1369*s*, 1357*m*, 1333*w*, 1283*vs*, 1252*m*, 1195*m*, 1175*s*, 1156*vs*, 1134*vs*, 1084*w*, 1070*w*, 1043*m*, 1029*w*, 1006*vw*, 968*w*, 904*w*, 867*vw*, 831*m*, 797*m*, 768*vw*, 753*vw*, 716*s*, 681*w*, 654*w*, 584*vw*, 571*w*, 487*vw*, 465*vw*.

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 5.24 (*sbr*, 1H, NH) ; 4.70 (*s*, 2H, $H_2C(1^1)$) ; 4.04 (*d*, $^3J_{5\text{-}NH}$= 5.0 Hz, 2H, $H_2C(5)$) ; 2.77 (*s*, 4H, $H_2C(2$ and $3)$) ; 1.41 (*s*, 9H, $H_3C(8^1,8^2,8^3)$.

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 204.0 (C(4)) ; 170.9 (C(1)) ; 155.7 (C(6)) ; 94.8 ($C(1^2)$) ; 80.0 (C(7)) ; 74.2 ($C(1^1)$) ; 50.3 (C(5)) ; 34.2 (C(3)) ; 28.4 ($C(8^1,8^2,8^3)$) ; 27.5 (C(2)).

MS (APCI (+)): m/z relative intensity (95% intensity) $[M + H]^+$ = 363.9 ; (90% intensity) $[M + H]^+$ = 362.0 ; (32% intensity) $[M + H]^+$ = 365.9

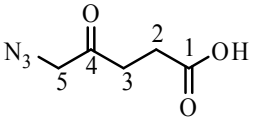
9.1.4. Synthesis of 5-azido-4-oxo pentanoic acid (5-azidolevulinic acid)



3 $C_6H_9N_3O_3$ [171.16]

10 $C_5H_7N_3O_3$ [157.13]

In a 1lit Erlenmeyer flask, 500ml 0.1M tampon phosphate buffer solution was charged. (Preparation of 0.1 M tampon phosphate, pH=9.0: 1.58 g NaH_2PO_4 and 5.66 g Na_2HPO_4 was dissolved in 1lit of deionised water.) The pH of the buffer is brought to 9 by 5M NaOH. Then, 22.05 g (128.8 mmol 1eq) of 5-azido-4-oxo methyl pentanoate ( ) was charged under agitation. Then 300 mg of *pig liver esterase* (PLE) enzyme (24 units per mg of protein; 1unit hydrolyses 1µmol or 0.001mmol of an ester; approximately 7.2 mmol; 0.077eq) was added under agitation. The pH of the reaction mass was continuously maintained at pH 9 using 5N NaOH by means of a Dosimeter connected with a pH meter. The reaction was completed approximately after 4 days (TLC eluent: Hexane / AcOEt 1:1).The reaction mass was then extracted with 3 × 200ml AcOEt. The aqueous phase was acidified till pH 2 with 6N HCl. This was followed by extraction of the aqueous phase with 4 × 500ml AcOEt. All the organic phases were combined together and it was washed with 500 ml saturated NaCl solution followed by 500 ml deionised water. The organic phase was then dried over $MgSO_4$ and the solvent was evaporated off by rotavapor. A yellow crude solid product thus obtained was taken for trituration with ether at 0 ^{0}C. The pale brown pure solid thus obtained was filtered and dried. Thus 17.61 g of pure white 5-azido-4-oxo pentanoic acid was obtained. (Yield= 87%).



R_f: (AcOEt 100%): 0.35; (CH_2Cl_2 / MeOH 4:1) : 0.91 (UV $_{254}$ active, $KMnO_4$)

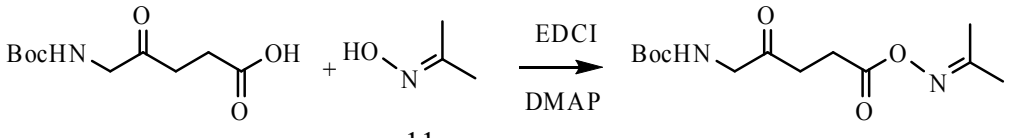
IR (KBr) : 3035*m*, 2914*s*, 2685*m*, 2557*m*, 2221*m*, 2110*vs*, 1720*vs*, 1411*s*, 1399*s*, 1374*s*, 1344*m*, 1288*s*, 1262*s*, 1235*s*, 1224*s*, 1173*m*, 1088*s*, 1046*m*, 1005*w*, 927*s*, 887*m*, 828*w*, 689*w*, 648*w*, 633*m*, 557*m*, 526*w*.

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 9.48 (*s*, 1H, COOH); 4.02 (s, 2H, $H_2C(5)$) ; 2.77-2.69 (*m*, 4H, H_2C(2 and 3)) .

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 203.0 (C(4)) ; 178.4 (C(1)) ; 57.6 (C(5)) ; 34.3 (C(3)) ; 27.7 (C(2)).

MS (APCI (+)): m/z relative intensity (60, $[M - H]^-$ = 156.8

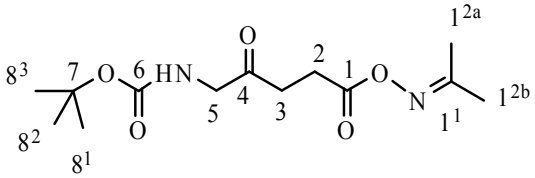
9.1.5. Synthesis of oxime ester of 5- BOC-amino-4-oxo-pentanoic acid (BOC-ALA-oxime ester)



5 11 12

$C_{10}H_{17}NO_5$ [231.16] C_3H_7NO [73.09] $C_{13}H_{22}N_2O_5$ [286.32]

In a dry flask under argon atmosphere, 2.03g (8.80 mmol, 1eq) BOC-ALA of was charged and then 85ml CH_2Cl_2 was added. The mass was cooled to 0 ^{0}C, then 1.18 g (9.68mmol, 1.1eq) of DMAP was added followed by 2.05 g (10.69 mmol, 1.2eq) of EDCI. Finally, 0.643 (8.8 mmol, 1eq) of acetone oxime was charged. The reaction was brought to room temperature and was kept at room temperature overnight. The solvent was evaporated off by rotovapor. The remaining residue was purified by flash chromatography (100% ether) and the pure fraction yielded 2.13 g of BOC-ALA-oxime ester. (Yield= 84.5%)



Batch : VW32, RV 278

R_f: (100% ether): 0.19 (UV $_{254}$ active; $KMnO_4$)

IR (KBr):3353*vs*, 3005*m*, 2978*s*, 2922*m*, 1771*vs*, 1705*vs*, 1651*m*, 1519*vs*, 1457*m*, 1440*m*, 1411*s*, 1397*s*, 1367*vs*, 1287*vs*, 1251*s*, 1164*vs*, 1127*vs*, 1070*s*, 1054*m*, 1944*m*, 1027*m*, 935*m*, 952*w*, 935*w*, 885*vs*, 838*vw*, 804*vw*, 784*m*, 771*w*, 747*w*, 721*vw*, 588*m*, 577*w*, 548*w*, 464*w*.

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 5.24 (*sbr*, 1H, NH); 4.03 (*d*, $^3J_{5\text{-}NH}$= 5.0 Hz, 2H, $H_2C(5)$) ; 2.76-2.69 (*m*, 4H, $H_2C(2$ and $3)$); 1.99 (*s*, 3H, $H_3C(1^{2a})$ or $H_3C(1^{2b})$); 1.96 (*s*, 3H, $H_3C(1^{2a})$ or $H_3C(1^{2b})$); 1.45 (*s*, 9H, $H_3C(8^1,8^2,8^3)$.

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 204.4 (C(4)); 170.4 (C(1)); 164.2 ($C(1^1)$); 155.7 (C(6)); 80.0 (C(7)); 50.3 (C(5)); 34.2 (C(3)); 28.4 ($C(8^1)$, $C(8^2)$, $C(8^3)$); 26.6 (C(2)); 21.9, 17.0 ($C(1^{2a})$, $C(1^{2b})$).

9.2. Synthesis of sugar-ALA derivatives

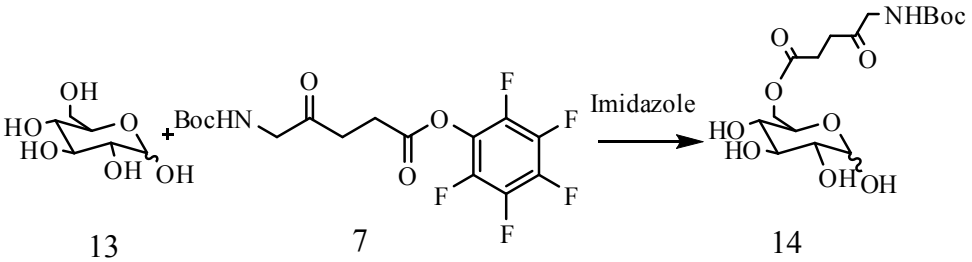
9.2.1. Synthesis of 6´-O-(ALA) -α/β-D-sugars

9.2.1.1. Synthesis of 6´- O-(ALA) -α/β-D-glucose

The synthesis of 6´-O- (ALA) glucose was carried out by two different methods; one by the imidazole catalyzed esterification of glucose and another by the enzymatic transestrification.Also the synthesis of 6´-O- (ALA) glucose was attempted via two different protection & deprotection strategies: one via benzyl protective group and the other via boronate protective group.

9.2.1.1.1. Synthesis of 6´- O-(ALA) -α/β-D-glucose catalyzed by Imidazole:

9.2.1.1.1.1. Synthesis of 6´- O-(BOC-ALA) glucose



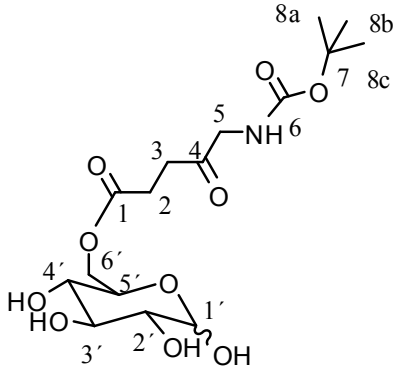
13 7 14

$C_6H_{12}O_6$ [180.16]

$C_{16}H_{16}NO_5F_5$ [397.23]

$C_{16}H_{27}NO_{10}$ [393.39]

1081 mg (6 mmol,3 eq) of glucose was taken in a dry flask under argon atmosphere and was dissolved in 40 ml anhydrous pyridine. The solution was cooled to 0 ^{0}C and 795 mg (2 mmol,1 eq) of Boc-ALA-PFP ester was added to the solution, followed by the addition of 14mg (0.205 mmol, 0.1 eq) of imidazole. The reaction was brought to room temperature. After keeping the reaction for approximately 12hrs at room temperature, the starting material Boc-ALA-PFP ester was consumed totally. (controlled by ^{19}F NMR). The solvent pyridine was evaporated off by rotovapor and the residue was taken for flash chromatography.(gradient eluent from 100% CH_2Cl_2 to 80:20 CH_2Cl_2 : MeOH). The pure fraction of 550 mg was obtained thus as white solid. (Yield = 69%).



Batch: RV84; RV 80

R_f: (CH_2Cl_2 / MeOH 8:2): 0.37; ($KMnO_4$)

IR (KBr) : 3407*vs*, 2978*m*, 2928*m*, 1726*vs*, 1518*s*, 1453*w*, 1392*w*, 1368*s*, 1285*m*, 1253*m*, 1167*vs*, 1055*s*, 902*w*, 869*w*, 776*w*, 560*w*.

^{1}H-NMR (400 MHz, CD_3OD, 298 K): δ_H (ppm) = 5.08 (*d*, $^3J_{1'-2'}$ =3.7Hz, 1H, HC(1′α)); 4.51 (*d*, $^3J_{1'-2'}$ =7.8Hz, 1H, HC(1′β));4.40 (*dd*, $^3J_{5'-6'a}$ =2.1Hz, $^3J_{6'a\ -\ 6'b}$ =11.8Hz ,1H, HC(6′a α or β)) ;4.35 (*dd*, $^3J_{5'-6'a}$ =2.1Hz, $^3J_{6'a\ -\ 6'b}$ =11.8Hz ,1H, HC(6′a α or β)); 4.21 (*dd*, $^3J_{5'-6'b}$ =5.5Hz, $^3J_{6'a\ -\ 6'b}$ =11.8Hz ,1H, HC(6′b α or β)); 4.18 (*dd*, $^3J_{5'-6'b}$ ≈5.5Hz, $^3J_{6'a\ -\ 6'b}$ =11.8Hz ,1H, HC(6′b α or β)); 3.98-3.89 (*m*, 3H, H_2C(5) and HC (5′α));3.68 (*t*, $^3J_{3'-4'}$≈9.3Hz, $^3J_{3'-2'}$ ≈9.3Hz,1H, HC(3′α)) ; 3.52-3.47 (*m*, 1H, HC (5′β)); 3.41-3.28 (*m*, 4H, HC(2′α),HC (3′β),HC(4′α) and HC (4′β)); 3.16 (*dd*, $^3J_{2'-1'}$ = 7.8Hz, $^3J_{2'\ -\ 3'}$ ≈ 9.0Hz ,1H, HC(2′β)) ; 2.81-2.63 (*m*, 4H, H_2C(2 and 3)); 1.46 (*s*, 9H, H_3C(8a, 8b and 8c) and all other peaks due to minor regioisomers.

α/β ratio by ^{1}H-NMR ≈ 1: 1

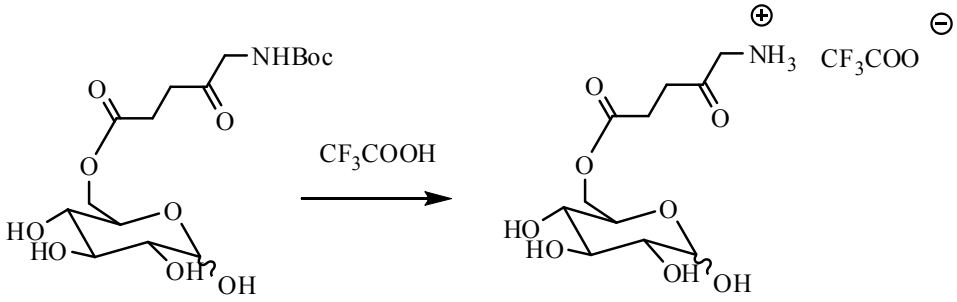
^{13}C-NMR (100 MHz, CD_3OD, 298K) : δ_C (ppm) = 207.4 (C(4)) ; 174.1 (C(1α or β)) ; 174.0 (C(1α or β)); 158.2 (C(6)) ; 98.0 (C(1′β)); 93.7 (C(1′α)); 80.5 (C(7)); 76.0 (C(2′β)); 75.0 (C(5′β)); 74.5(C(3′α)); 77.6, 73.5, 71.6, 71.4 (C(2′α),C(3′β), C(4′α) and C(4′β)) ; 70.4 (C(5′α)); 65.0, 64.9 (C(6′α) and C(6′β)); 50.6 (C(5)); 34.8 (C(3)) ;28.6 (C(8a,8b and8c); 28.5 (C(2)) and all other peaks due to minor regioisomers.

α/β ratio by ^{13}C-NMR ≈ 1 : 1

MS (ESI (+)): m/z relative intensity $[M+ Na]^+$ = 653.5

HR-MS $[M+ Na]^+$ = 416.1528 (calculated = 416.1533)

9.2.1.1.1.1.2. Synthesis of 6′- O-(ALA) α/β-D-glucose



14

$C_{16}H_{27}NO_{10}$

[393.39]

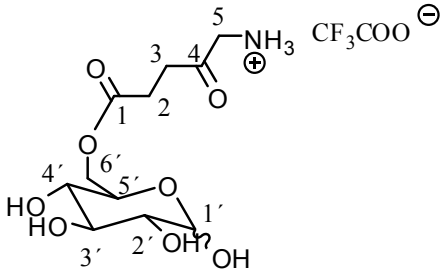
15

$C_{13}H_{20}F_3NO_{10}$

[407.29]

M^+=294.28 ; CF_3COO = 113.01

420 mg (1.067 mmol, 1eq) of 6′-O-(BOC-ALA) glucose was taken and cooled to -10^{0}C. To this, 12.16 g (8ml, 106.7 mmol, 100 eq) of trifluoroacetic acid was added dropwise by syringe. The reaction mass was allowed to come to ambient temperature and maintained at ambient temperature for an hour. Once the BOC group is absent (controlled by ^{1}H-NMR), trifluoroacetic acid was evaporated off. The product dried under high vacuum oil pump yielded 425 mg highly hygroscopic white solid. (Yield=98%).



Batch: RV 72 RV 75 RV 77

IR (KBr) : 3373*vs*, 2926*m*, 1786*m*, 1730*vs*, 1677*vs*, 1512*w*, 1429*m*, 1390*m*, 1317*w*, 1204*vs*, 1137*vs*, 1080*m*, 1054*m*, 913*w*, 839*m*, 800*m*, 772*w*, 723*m*, 632*w*, 597*w*, 559*w*, 519*w*, 500*w*.

^{1}H-NMR (400 MHz, DMSO-d_6, 298 K): δ_H (ppm) 8.09 (*s*, 3H, NH_3); 4.90 (*d*, $^3J_{1'-2'}$ =3.6Hz, 1H, HC(1′α)); 4.30 (*d*, $^3J_{1'-2'}$ =7.7Hz, 1H, HC(1′β));4.28-4.23 (*m*, 2H, HC(6′a α and β)) ; 4.03-3.99 (*m*,5H, HC (6′bα and β) and $H_2C(5)$); 3.79- 3.75 (*m*, 1H, HC(5′α); 3.42 (*t*, $^3J_{3'-4'=}$ 9.2Hz, $^3J_{3'-2'}$ =9.2Hz,1H, HC(3′α)); 3.32 (*m*,1H, HC (5′β)); 3.54 (*dd*, $^3J_{1'-2'}$ =3.6 Hz, $^3J_{3'-2'}$ =9.5Hz,1H, HC(2′α)) ;3.46 (*t,* $J_{3'-4'} \approx$ 9.0Hz, $^3J_{3'-2'}$ ≈9.0Hz, 1H, HC(3′β)); 3.05-3.00 (*m*, 2H HC(4′α) and HC (4′β)); 2.90 (*dd*, $^3J_{2'-1'} \approx$ 7.8Hz, $^3J_{2'-3'} \approx$ 9.0 Hz ,1H, HC(2′β)) ; 2.79-2.75 (*m*, 2H, $H_2C(3)$); 2.60-2.54 (*m*,2H, $H_2C(2)$); 1.51(Boc protected) and all other peaks due to minor regioisomers.

α/β ratio by ^{1}H-NMR ≈ 0.4: 1

^{13}C-NMR (100 MHz, DMSO-d_6,, 298K) : δ_C (ppm) 202.9 (C(4)); 172.2 (C(1)); 158.5 (*q*,$^2J_{C-F} \approx$ 36.0 ($CF_3\underline{C}OO^-$); 115.7 (*q*,$^1J_{C-F}$ = 291.5, $\underline{C}F_3COO^-$); 96.9 (C(1′β)); 92.3 (C(1′α)); 76.4 (C(2′α) or C(3′β)); 74.7 (C(2′β)); 73.5 (C(5′β)); 72.9(C(3′α)); 72.2 (C(2′α) or C(3′β)); 70.6, 70.2 (C(4′α) and C(4′β)) ; 69.2 (C(5′α)); 64.6, 64.5 (C(6′α and 6′β)); 46.8 (C(5)); 34.2(C(3)); 27.5 (C(2)); 27.2 (Boc protected) and all other peaks due to minor regioisomers.

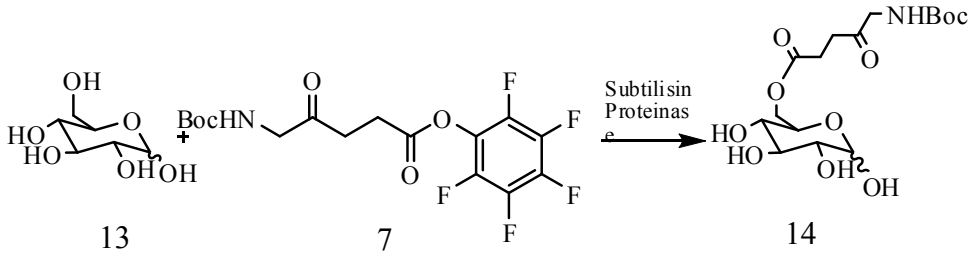
α/β ratio by ^{13}C-NMR ≈ 0.66 : 1

MS (APCI (+)): m/z relative intensity $[M+ Na]^+$ = 416.1

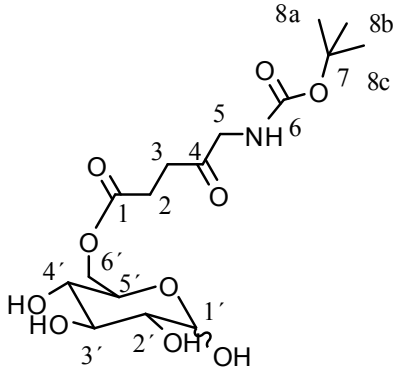
HR-MS $[M- CF_3COO^-]^+$ = 294.1184 (calculated = 294.1189)

9.2.1.1.2. Synthesis of 6′-O- (ALA) α/β-D- glucose catalyzed by enzyme

9.2.1.1.2.1. Synthesis of 6′-O-(BOC-ALA) α/β-D-glucose



360.4 mg (2 mmol, 1eq) of glucose was taken in a dry flask under argon atmosphere and was dissolved in 10 ml anhydrous pyridine. 1000 mg (5 units per mg of protein; 1unit hydrolyses 1µmol or 0.001mmol of an ester; approximately, 5 mmol, 2.5eq) of *subtilisin proteinase* enzyme (before use dried by keeping at vacuum 10^{-2} mbar for 3-4 days) was added and then 2400 mg (6mmol, 3eq) of Boc-ALA-PFP ester was added to the solution. The reaction was heated to 50^0C and maintained at this temperature for 24 hrs (controlled by TLC eluent : AcOEt: MeOH 1:1). The mass was cooled to rt and then the enzyme was filtered over celite bed, the filtered cake was washed with 3× 10ml pyridine. The solvent pyridine was evaporated off by rotovapor and the residue was taken for flash chromatography. (gradient eluent from 100% CH_2Cl_2 to 85:15 CH_2Cl_2 : MeOH). The main fractions were concentrated and the mass was further purified by dissolving the mass in 5ml CH_2Cl_2 and precipitated by 5ml hexane. 375 mg of pure product was obtained as white solid. (Yield = 47.66%).



Batch : RV 313 mix

R_f: (CH_2Cl_2 / MeOH 85:15): 0.28; ($KMnO_4$)

IR (KBr) : 3411*vs*, 2979*m*, 2930*m*,2509*w*, 1724*vs*, 1519*s*, 1424*w*, 1393*m*, 1368*s*, 1282*m*, 1254*m*, 1167*vs*, 1056*s*, 913*w*, 869*w*, 778*w*,734*w*,640*w*, 560*w*.

^{1}H-NMR (400 MHz, CD_3OD, 298 K): δ_H (ppm) = 5.10 (*d*, $^3J_{1'-2'}$ =3.7Hz, 1H, HC(1′α)); 4.48 (*d*, $^3J_{1'-2'}$ =7.8Hz, 1H, HC(1′β));4.38 (*dd*, $^3J_{5'-6'a} \approx$ 2.1Hz, $^3J_{6'a\ -\ 6'b}$ =11.8Hz ,1H, HC(6′a β)) ;4.32 (*dd*, $^3J_{5'-6'a} \approx$ 2.1Hz, $^3J_{6'a\ -\ 6'b}$ =11.8Hz ,1H, HC(6′a α)); 4.21 (*dd*, $^3J_{5'-6'b} \approx$ 5.5Hz, $^3J_{6'a\ -\ 6'b} \approx$ 11.9Hz ,1H, HC(6′b α)); 4.16 (*dd*, $^3J_{5'-6'b} \approx$ 5.5Hz, $^3J_{6'a\ -\ 6'b} \approx$11.8Hz ,1H, HC(6′b β)); 3.97-3.93 (*m*, 3H, $H_2C(5)$ and HC (5′α));3.67 (*t*, $^3J_{3'-4'} \approx$9.3Hz, $^3J_{3'-2'}$ =9.3Hz,1H, HC(3′α)); 3.46 (*ddd*, $^3J_{5'-6'a} \approx$ 2.1Hz, $^3J_{5'-6'b} \approx$ 5.5Hz, $^3J_{5'-4'}$ = 9.5Hz,1H, HC (5′β)); 3.40-3.25 (*m*, 4H, HC(2′α),HC (3′β),HC(4′α) and HC (4′β)); 3.16 (*dd*, $^3J_{2'-1'}$ =

7.8Hz, $^{3}J_{2'-3'} \approx 9.3$Hz ,1H, HC(2′β)) ; 2.75 (*t*, $^{3}J_{3-2} \approx 6.4$Hz, 2H, $H_2C(3)$); 2.64 (*t*, $^{3}J_{2-3} \approx 6.4$Hz, 2H, $H_2C(2)$); 1.43 (s, 9H, H_3C (8a, 8b and 8c)).

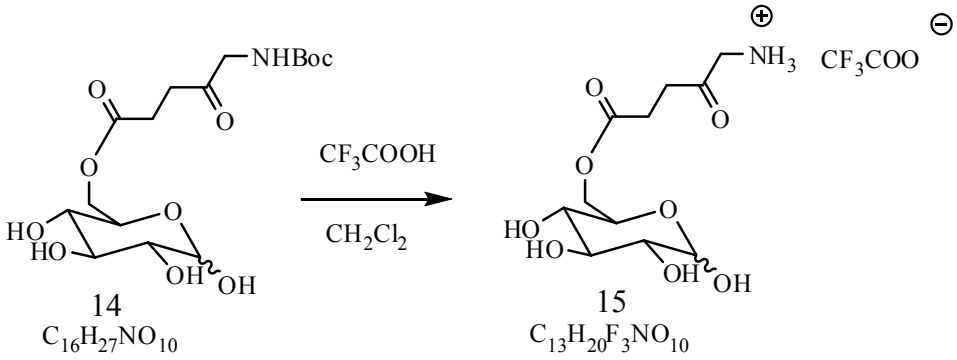
α/β ratio by ^{1}H-NMR ≈ 1.25 : 1

^{13}C-NMR (100 MHz, CD_3OD, 298K) : δ_C (ppm) = 207.0 (C(4)) ; 173.9 (C(1α or β)) ; 173.8 (C(1α or β)); 157.9 (C(6)) ; 97.7 (C(1′β)); 93.5 (C(1′α)); 80.5 (C(7)); 75.7 (C(2′β)); 74.8 (C(5′β)); 74.4(C(3′α)); 77.4, 73.2, 71.4, 71.2 (C(2′α),C(3′β), C(4′α) and C(4′β)) ; 70.2 (C(5′α)); 64.9 (C(6′α)), 64.8 (C(6′β)); 50.6 (C(5)); 34.7 (C(3)) ;28.6 (C(8a,8b and8c); 28.4 (C(2)). ; **α/β ratio by ^{13}C-NMR** ≈ 1.24 : 1

MS (ESI (+)): m/z relative intensity $[M+Na]^+ = 416.1$; $[M+H]^+ = 393.9$

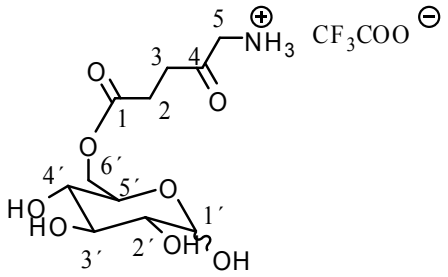
HR-MS $[M+Na]^+ = 416.1524$ (calculated = 416.1533)

9.2.1.1.2.2. Synthesis of 6′- O-(ALA) α/β-D-glucose



M^+=294.28 ; CF_3COO^- = 113.01

480 mg (1.22 mmol, 1eq) of 6′-O-(BOC-ALA) glucose was dissolved in 8ml CH_2Cl_2 and cooled to -10^0C. To this solution, 7g (4.7ml, 61 mmol, 50 eq) of trifluoroacetic acid was added dropwise by syringe. The reaction mass was allowed to come to ambient temperature and maintained at ambient temperature for an hour. Once the deprotection of BOC was completed (controlled by the absence of BOC peak in ^{1}H-NMR), CH_2Cl_2 and trifluoroacetic acid was evaporated off. The product was triturated with dry ether and ether solution was decanted. The trituration with ether and decanting top clear ether solution after centrifuge was repeated 2 times more. Then, the settled solid product was dried under high vacuum oil pump and thereby yielded 457 mg of highly hygroscopic white solid. (Yield=92%).



Batch : RV 320

$[\alpha]_D$ = +24.2 (c=0.49; deionized H_2O)

IR (KBr): 3388*vs*, 2923*m*, 1730*vs*, 1679*vs*, 1512*w*, 1429*m*, 1390*m*, 1317*w*, 1204*vs*, 1137*vs*, 1081*m*, 1056*m*, 913*w*, 839*m*, 800*m*, 772*w*, 723*m*, 632*w*, 597*w*.

^{1}H-NMR (400 MHz, D_2O, 298 K): δ_H (ppm) = 5.21 (*d*, $^3J_{1'-2'}$ =3.7Hz, 1H, HC(1′α)); 4.65 (*d*, $^3J_{1'-2'}$ =7.9Hz, 1H, HC(1′β));4.41 (*dd*, $^3J_{5'-6'a}$ = 2.2 Hz, $^3J_{6'a\ -\ 6'b}$ =12.2Hz ,1H, HC(6′a α or β)) ;4.37 (*dd*, $^3J_{5'-6'a} \approx$ 2.2Hz, $^3J_{6'a\ -\ 6'b} \approx$12.2Hz ,1H, HC(6′a α or β)); 4.34 - 4.25 (*m*, $^3J_{5'-6'b} \approx$ 5.3Hz, $^3J_{6'a\ -\ 6'b} \approx$ 12.2Hz ,2H, HC(6′b α and 6′b β)); 4.13 (*s*, 2H, $H_2C(5)$); 4.03-3.99 (*m*,1H, HC (5′α)); 3.71 (*t*, $^3J_{3'-4'=}$ 9.4Hz, $^3J_{3'-2'}$ =9.4Hz,1H, HC(3′α)); 3.65 (*ddd*, $^3J_{5'-6'a} \approx$ 2.2Hz, $^3J_{5'-6'b} \approx$ 5.3Hz, $^3J_{5'-4'}$ = 9.5Hz,1H, HC (5′β)); 3.54 (*dd*, $^3J_{1'-2'}$ =3.7Hz, $^3J_{3'-2'}$ =9.4Hz,1H, HC(2′α)) ;3.46 (*m,* $J_{3'-4'} \approx$ 9.4Hz, $^3J_{3'-2'} \approx$9.4Hz, $J_{5'-4'} \approx$ 9.4Hz, 3H, HC(3′β), HC(4′α) and HC (4′β)); 3.16 (*dd*, $^3J_{2'-1'} \approx$ 7.8Hz, $^3J_{2'\ -\ 3'} \approx$ 9.4 Hz ,1H, HC(2′β)) ; 2.75 (*t*, $^3J_{3-2}$ =6.0Hz, 2H, $H_2C(3)$); 2.64 (*t*, $^3J_{2-3} \approx$6.4, 2H, $H_2C(2)$).

α/β ratio by ^{1}H-NMR ≈ 0.71 : 1

^{13}C-NMR (100 MHz, D_2O, 298K) : δ_C (ppm) = 204.0 (C(4α and β)) ; 174.6 (C(1α or β)) ; 174.5 (C(1α or β)); 116.7 (*q*, $^1J_{C-F}$ = 292.2, **C**F_3COO^-); 96.1 (C(1′β)); 92.3 (C(1′α)); 74.1 (C(2′β)); 73.5 (C(5′β)); 72.7(C(3′α)); 75.6, 71.5, 69.7, 69.6 (C(2′α),C(3′β), C(4′α) and C(4′β)) ; 69.3 (C(5′α)); 63.8(C(6′α and 6′β)); 47.2 (C(5)); 34.3(C(3)); 27.5 (C(2)).

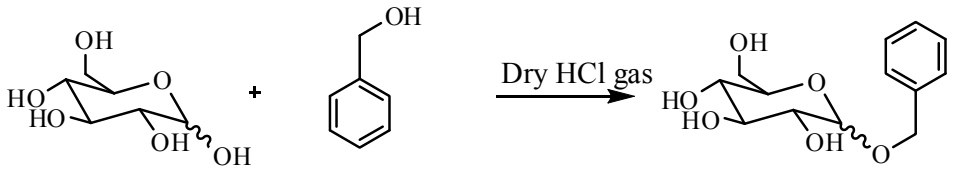
α/β ratio by ^{13}C-NMR ≈ 0.76 : 1

MS (ESI (+)): m/z relative intensity $[M\text{-}CF_3COO^-]^+$ = 293.9;

HR-MS $[M\text{-}\ CF_3COO^-]^+$ = 294.1181 (calculated = 294.1189)

9.2.1.1.3. Synthesis of 6′-O- (ALA) α/β-D- glucose by protection & deprotection method (using benzyl protective group):

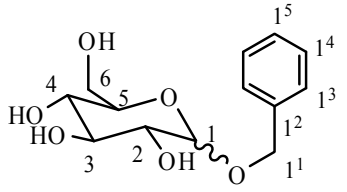
9.2.1.1.3.1. Synthesis of Benzyl- α/β-D-glucose



13 16 17

$C_6H_{12}O_6$ [180.16] C_7H_8O [108.14] $C_{13}H_{18}O_6$ [270.28]

A solution of 73.08 g (70 ml, 674 mmol, 12.15eq) dry benzyl alcohol containing 2% of HCl (dry HCl gas was generated and passed through benzyl alcohol) was heated to 100^0C and treated portionwise with 10 g (55.49 mmol, 1eq) of dry D-glucose and the mixture was maintained at that temparetaure for 3hrs. The solution was neutralized by adding solid 5 g Na_2CO_3, filtered over celite bed and evaporated to dryness at 80^0C and 0.01 mbar. The residue was purified by flash chromatography (silica gel- 70 times of the residue mass; CH_2Cl_2 : MeOH 90:10) to afford 8.68 g benzyl α/β-D-glucose. (Yield= 57.9%).



Batch: RV128, RV302

R_f: (CH_2Cl_2 / MeOH 90:10): 0.14 ($KMnO_4$)

^{1}H-NMR (400 MHz, CD_3OD, 298 K): δ_H (ppm) = 7.44-7.26 (*m*, 5H, Haromatic); 4.96 – 4.92 (*m*, 2H, HC(1^1a β) and HC(1α)); 4.78 (*d*, $^2J_{1^1a\text{-}1^1a'}$=11.8Hz, 1H, HC(1^1a α)); 4.68 (*d*, $^2J_{1^1a\text{-}1^1a'}$=11.8Hz, 1H, HC(1^1a′ β)); 4.57 (*d*, $^2J_{1^1a\text{-}1^1a'}$=11.8Hz, 1H, HC(1^1a′ α)); 4.39 (*d*, $^3J_{1\text{-}2}$ =7.7Hz, 1H, HC(1β)); 3.93 (*dd*, $^3J_{5\text{-}6} \approx$ 2.0Hz, $^3J_{6-6'}$ =11.9Hz ,1H, HC(6 β)) ;3.83 (*dd*, $^3J_{5\text{-}6} \approx$ 2.0Hz, $^3J_{6-6'} \approx$11.9Hz ,1H, HC(6 α)); 3.77- 3.64 (*m*, 5H, HC(6′ α), HC(6′ β), HC(5α), HC(3 α) and HC(3 β)); 3.47 (*dd*, $^3J_{1\text{-}2} \approx$2.3Hz, $^3J_{3\text{-}2}$ =8.8Hz 1H, HC(2α)); 3.45-3.29 (*m*, 4H, HC(2β), HC(4α), HC(4β) and HC(5β)).

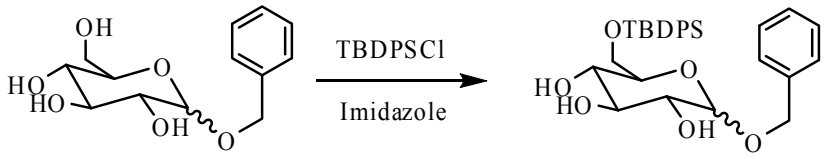
<u>α</u>/β ratio by ^{1}H-NMR could not be determined because of peaks overlap.

^{13}C-NMR (100 MHz, CD_3OD, 298K) : δ_C (ppm) = 138.8 (($C1^2$)); 129.2-128.6 (($C1^3$,$C1^4$, $C1^5$));103.1 (C(1β)); 99.1 (C(1α)); 77.8 (C(5β)); 73.4 (C(2α)); 77.7, 74.9, 73.7, 71.6, 71.4 ((C(2β), C(3α), C(3β), C(4α), C(4β) and C(5α)); 70.1 ($C(1^1)$); 62.6 and 62.5 (C(6α) and C(6β))

α/β ratio by ^{13}C-NMR ≈ 1.5 : 1

MS (ESI (+)): m/z relative intensity $[2\times M]^-$ = 539.1

9.2.1.1.3.2. Synthesis of Benzyl 6-tert-Butyldiphenylsilyl- α/β-D-glucose

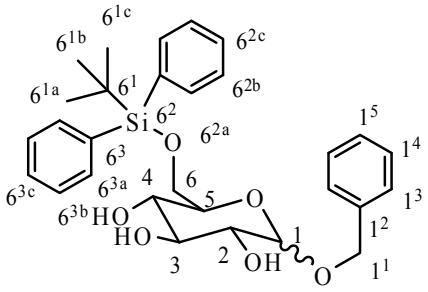


17 18

$C_{13}H_{18}O_6$ [270.28]

$C_{29}H_{36}O_6Si$ [508.67]

To a solution of 2.43 g (9 mmol, 1eq) benzyl α/β glucose and 1.35 g (19.8 mmol, 2.2eq) imidazole in 30 ml DMF at -30^{0}C, was added drop wise by syringe, a solution of 2.72 g (9.9 mmol, 1.1eq) *tert*-butyldiphenylsilyl chloride in 20 ml DMF. After 3.5 h, the reaction was poured into aqueous $NaHCO_3$ solution and ether. The aqueous layer was further extracted with ether. The combined organic layer were dried over $MgSO_4$, filtered and evaporated. The residue on flash chromatography (CH_2Cl_2 : MeOH 95:5) afforded 4.12 g benzyl 6-*tert*-Butyldiphenylsilyl- α/β-D-glucose. (Yield = 90%).



Batch: RV158, RV159, VW35

R_f: (CH_2Cl_2 / MeOH 90:10): 0.48 (UV active; $KMnO_4$)

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.73-7.19 (*m*, 15H, Haromatic); 4.87 (*d*, $^3J_{1\text{-}2}$ =3.5Hz, 1H, HC(1α)); 4.85 (*d*, $^2J_{1^1a\text{-}1^1a'}$≈11.0Hz,1H, HC(1^1a β) ; 4.70 (*d*, $^2J_{1^1a\text{-}1^1a'}$=11.8Hz, 1H, HC(1^1a α)); 4.55 (*d*, $^2J_{1^1a\text{-}1^1a'}$=11.6Hz, 1H, HC(1^1a′ β)); 4.46 (*d*, $^2J_{1^1a\text{-}1^1a'}$=11.8Hz, 1H, HC(1^1a′ α)); 4.33 (*d*, $^3J_{1\text{-}2}$ =7.7Hz, 1H, HC(1β)); 3.98 (*dd*, $^3J_{5\text{-}6}$ ≈ 3.0Hz, $^3J_{6\,-\,6'}$ =11.0Hz ,1H, HC(6 β)) ;3.91 (*dd*, $^3J_{5\text{-}6}$ ≈ 3.0Hz, $^3J_{6\,-\,6'}$ ≈11.0Hz ,1H, HC(6 α)); 3.87- 3.34 (*m*, 10H, HC(6′ α), HC(6′ β), HC(5α), HC(5β), HC(4α), HC(4β),HC(3α), HC(3 β), HC(2α) and HC(2β));
1.05, 1.04 (*s*, 9H, $H_3C(6^{1a}, 6^{1b},6^{1c})$.

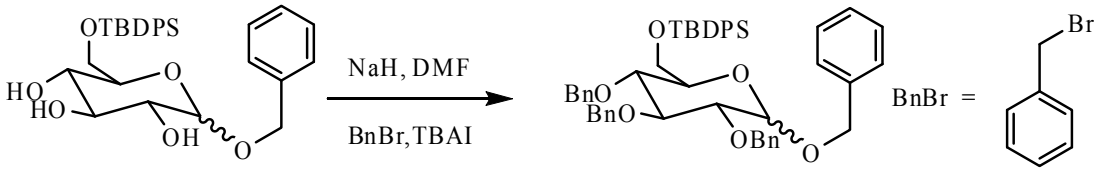
α/β ratio by ^{1}H-NMR could not be determined because of peaks overlap.

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 137.2,137.1,133.4,133.3 ($(C1^2)$, $C(6^2)$,$C(6^3)$ of α and β); 135.6-134.8,129.6-127.6 (($C(1^3)$,$C(1^4)$, $C(1^5)$, $C(6^{2a})$, $C(6^{2b})$, $C(6^{2c})$,$C(6^{3a})$, $C(6^{3b})$, $C(6^{3c})$);101.2 (C(1β)); 97.1 (C(1α)); 70.4, 68.8 (($C(1^1)$) of α and β); 64.0, 64.1 (C(6α) and C(6β)); 76.7, 75.9, 74.6, 73.6, 72.1, 72.0, 71.0, 70.9, 77.8 (C(5β), C(5α), C(4α), C(4β), C(3α), C(3β),C(2α) and (C(2β)); 26.8-26.6 ($C(6^{1a}, 6^{1b},6^{1c})$).

α/β ratio by ^{13}C-NMR ≈ 1.4 : 1

MS (ESI (+)): m/z relative intensity $[2\times M+Na]^+$ = 1038.7

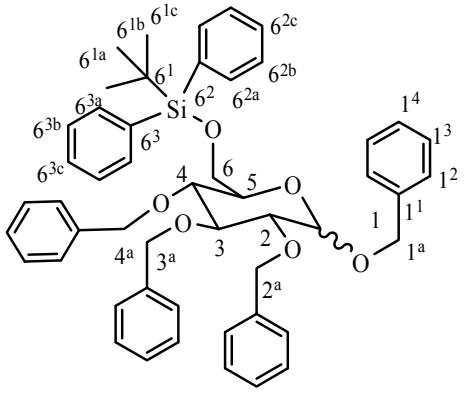
9.2.1.1.3.3. Synthesis of Benzyl 6-tert-Butyldiphenylsilyl- 2,3,4-tri-O-benzyl α/β-D-glucose



18 19

$C_{29}H_{36}O_6Si$	$C_{50}H_{54}O_6Si$	C_7H_7Br
[508.67]	[779.04]	[171.04]

To a solution of 390 mg (0.77 mmol, 1eq) benzyl 6-*tert*-Butyldiphenylsilyl- α/β-D-glucose in 5ml DMF at 0^0C, was added 110mg (2.68 mmol, 3.5eq) of NaH (60% dispersion in mineral oil).The reaction was allowed to reach ambient temperature and kept at that temperature for 1h.Then 440 mg (0.3ml, 2.57 mmol, 3.3eq) of Benzyl bromide was added drop wise via syringe.After 7h, the reaction mass was poured into aqueous $NaHCO_3$ solution and EtOAc.The aqueous layer was further extracted with EtOAc, and the combined organics were dried, filtered and evaporated. The residue on flash chromatography (Hexane: Ether 90:10) afforded 400 mg of pure Benzyl 6-*tert*-Butyldiphenylsilyl- 2,3,4-tri-O-benzyl α/β-D-glucose.(Yield= 66.9%)



Batch: RV160, RV171, VW36

R_f: (Hexane: Ether 90:10): 0.19(UV active; $KMnO_4$)

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 8.13-7.49 (*m*, 30H, Haromatic); 5.35-4.85 (*m*, 18H, 4×$\underline{H_2}$CPh (α and β), HC(1α) and HC(1β)); 4.44 ((*t*, $^3J_{3'\text{-}4'}$≈9.2Hz, $^3J_{3'\text{-}2'}$=9.1 Hz,1H, HC(3′α)); 4.29 (*m*, 2H, H_2C(6 β)); 4.20-4.19 (*m*, 2H, H_2C(6 α)); 4.13-4.10 (*m*, 2H, HC(5α) and HC(4β)); 4.03-3.98 (*m*, 2H, HC(3 β) and HC(4α)); 3.93-3.88 (*m*, 2H,

HC(2α) and HC(2β)); 3.69-3.66 (*m*, 1H, HC(5β); 1.42-1.39 (3×s, 9H, $H_3C(6^{1a}, 6^{1b}, 6^{1c})$. All peaks considered without solvent reference as CDCl3 peak overlaps with aromatic peaks.

α/β ratio by ^{1}H-NMR could not be determined because of peaks overlap.

^{13}C-NMR (100MHz,$CDCl_3$,298K): δ_C(ppm) = 138.8-137.1,133.6-133.1 (($C1^1$), ($C2^1$), ($C3^1$), ($C4^1$), $C(6^2)$, $C(6^3)$ of α and β); 135.9-135.5,129.6-127.6 (($C1^2$),$C(1^3)$, $C(1^4)$, $C(2^2)$, $C(2^3)$, $C(2^4)$, $C(3^2)$, $C(3^3)$, $C(3^4)$, $C(4^2)$, $C(4^3)$, $C(4^4)$, $C(6^{2a})$, $C(6^{2b})$, $C(6^{2c})$,$C(6^{3a})$, $C(6^{3b})$, $C(6^{3c})$); 102.2 (C(1β)); 94.7 (C(1α)); 82.3 (C(3α)); 75.7 (C(5β)); 62.8, 62.7 (C(6α) and C(6β)); 84.7, 82.6, 80.3, 77.8, 77.7, 71.8 (C(5α), C(4α), C(4β), C(3β), C(2α) and (C(2β)); 75.9, 75.8, 75.2, 75.1, 74.9, 72.9, 70.6, 68.5 ($4\times H_2\underline{C}$Ph (α and β)); 26.8 ($C(6^{1a}, 6^{1b}, 6^{1c})$); 19.3 ($C(6^1)$.

α/β ratio by ^{13}C-NMR ≈ 1.4 : 1

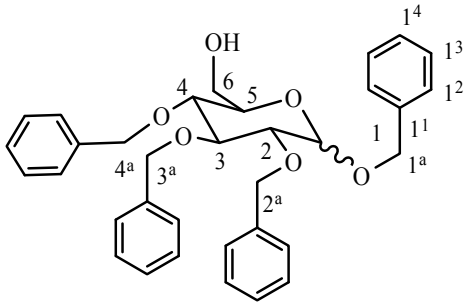
MS (ESI (+)): m/z relative intensity $[M-Ph]^+$ = 702.5

9.2.1.1.3.4. Synthesis of Benzyl-2,3,4-tri-O-benzyl α/β-D-glucose

19
$C_{50}H_{54}O_6Si$
[779.04]

20
$C_{34}H_{36}O_6$
[540.64]

To a solution of 400 mg (0.51 mmol, 1eq) Benzyl 6-*tert*-Butyldiphenylsilyl- 2,3,4-tri-O-benzyl α/β-D-glucose in 5ml THF at 0^0C, was added drop wise a solution of 0.6 ml (0.564 mmol, 1.1eq) of 1M TBAF in THF. The reaction was allowed to reach ambient temperature and kept at that temperature for 9-10h. Then, the reaction mass was poured into aqueous $NaHCO_3$ solution and EtOAc. The aqueous layer was further extracted with EtOAc, and the combined organics were dried, filtered and evaporated. The residue on flash chromatography (Hexane: EtOAc 70:30) afforded 204 mg of pure Benzyl-2,3,4-tri-O-benzyl α/β-D-glucose.(Yield= 73.5%)



Batch: RV169, VW37

R_f: (Hexane: EtOAc 70:30): 0.19 for β anomer and 0.14 for α anomer (UV active; $KMnO_4$)

IR (KBr) : 3368*m*, 3088*vw*, 3063*w*, 3029*m*, 2955*w*, 2918*m*, 2868*m*, 2346*vw*, 2056*vw*, 1945*vw*, 1869*vw*, 1803*vw*, 1706*vw*, 1606*vw*, 1586*vw*, 1497*m*, 1453*s*, 1399*w*, 1360*m*, 1327*w*, 1308*vw*, 1261*vw*, 1236*w*, 1211*m*, 1161*vw*, 1083*vs*, 1070*vs*, 1042*vw*,1027*vs*, 991*s*, 909*w*, 831*vw*, 730*vs*, 694*vs*, 641*w*, 618*w*, 604*vw*, 558*w*, 531*vw*, 499*w*, 461*w*.

α- anomer:

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.39-7.27 (*m*, 20H, Haromatic); 5.01 (*d*, $^2J_{1a\text{-}1a'}$=10.8Hz, 1H, HC(1^a)); 4.88 (*d*, $^2J_{2a\text{-}2a'}$=11.0Hz, 1H, HC(2^a)); 4.84 (*d*, $^2J_{1a\text{-}1a'}$=10.8Hz, 1H, HC($1^{a'}$)); 4.79 (*d*, $^3J_{1\text{-}2}$ =3.5Hz, 1H, HC(1α)); 4.67 (2×*d*, $^2J_{3a\text{-}3a'}$=12.1Hz, 1H, HC(3^a)), $^2J_{4a\text{-}4a'}$=12.1Hz, 1H, HC(4^a)); 4.64 (*d*, $^2J_{2a\text{-}2a'}$=11.3Hz, 1H, HC($2^{a'}$)); 4.55 (*d*, ^2J≈12.1Hz, 1H, HC($4^{a'}$) or HC($3^{a'}$)); 4.53 (*d*, ^2J≈12.1Hz, 1H, HC($4^{a'}$) or HC($3^{a'}$)); 4.07 (*t*, $^3J_{3\text{-}2}$ = 9.2Hz, $^3J_{3\text{-}4}$ = 9.2Hz, 1H, HC(3α)); 3.72-3.65 (*m*,3H, HC(5α) and H_2C(6α)); 3.54 (*t*, $^3J_{4\text{-}3}$ ≈ 9.2Hz, $^3J_{4\text{-}5}$ ≈ 9.2Hz, 1H, HC(4α)); 3.50 (*dd*, $^3J_{2\text{-}1}$ ≈ 3.6Hz, $^3J_{2\text{ -}3}$ ≈ 9.5Hz ,1H, HC(2α)); 1.26, 0.88 (traces of hexane).

<u>α</u>/β ratio by ^{1}H-NMR : Only α anomer

^{13}C-NMR (100MHz,$CDCl_3$,298K): δ_C(ppm) = 138.7-137.0 (($C1^1$), ($C2^1$), ($C3^1$) and ($C4^1$)); 128.4-127.6 (($C1^2$),C(1^3), C(1^4), C(2^2), C(2^3), C(2^4), C(3^2), C(3^3), C(3^4), C(4^2), C(4^3) and C(4^4)); 95.5 (C(1α)); 81.9 (C(3α)); 79.9 (C(2α)); 77.3 (C(4α)); 70.9 (C(5α)); 61.7 (C(6α)); 75.7, 75.0, 73.0, 69.2 (4×H_2<u>C</u>Ph); 29.7, 14.1 (traces of hexane).

α/β ratio by ^{13}C-NMR : Only α anomer

β- anomer:

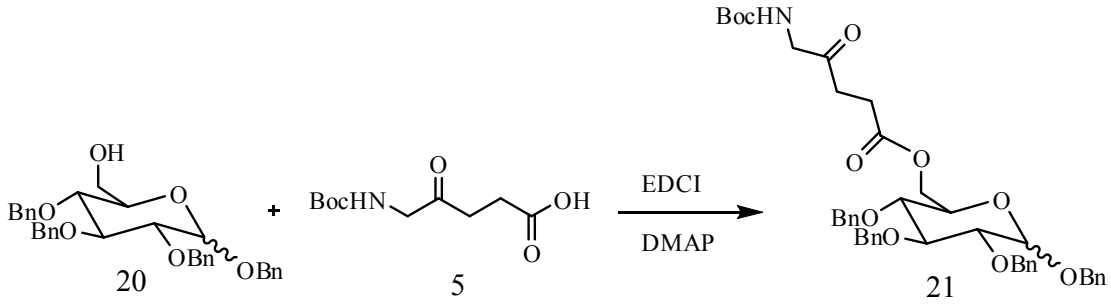
^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.38-7.26 (*m*, 20H, Haromatic); 4.95 (*d*, $^2J_{1a\text{-}1a'}$=11.0Hz, 1H, HC(1^a)); 4.93 (*d*, $^2J_{2a\text{-}2a'}$=10.8Hz, 1H, HC(2^a)); 4.92 (*d*, $^2J_{3a\text{-}3a'}$=12.3Hz, 1H, HC(3^a)); 4.86 (*d*, $^2J_{4a\text{-}4a'}$=10.9Hz, 1H, HC(4^a)); 4.80 (*d*, $^2J_{1a\text{-}1a'}$ ≈11.0Hz, 1H, HC($1^{a'}$)); 4.73 (*d*, $^2J_{2a\text{-}2a'}$=10.8Hz, 1H, HC($2^{a'}$)); 4.69 (*d*, $^2J_{3a\text{-}3a'}$≈12.0Hz , 1H, HC($3^{a'}$)); 4.64 (*d*, $^2J_{4a\text{-}4a'}$=10.9Hz, 1H, HC($4^{a'}$)); 4.57 (*d*, $^3J_{1\text{-}2}$ =7.8Hz, 1H, HC(1β)); 3.87 (*dd*, $^3J_{5\text{-}6a}$ ≈ 2.4Hz, $^3J_{6a\text{ -}6a'}$ =11.8Hz ,1H, HC(6a β)); 3.72-3.60 (*m*,1H, HC(6b β)); 3.67 (*t*, $^3J_{3\text{-}2}$ = 9.0Hz, $^3J_{3\text{-}4}$ = 9.0Hz, 1H, HC(3β)); 3.57 (*t*, $^3J_{4\text{-}3}$ ≈ 9.0Hz, $^3J_{4\text{-}5}$ ≈ 9.5Hz, 1H,HC(4β)); 3.48 (*dd*, $^3J_{2\text{-}1}$ ≈ 7.8Hz, $^3J_{2\text{ -}3}$ ≈ 9.0Hz ,1H, HC(2β)); 3.36 (*ddd*, $^3J_{5\text{-}6a}$ ≈ 2.8Hz, $^3J_{5\text{-}6b}$ ≈ 4.5Hz, $^3J_{5\text{-}4}$ = 9.5Hz,1H, HC (5β)); 1.25, 0.86 (traces of hexane).

<u>α</u>/β ratio by ^{1}H-NMR : Only β anomer

^{13}C-NMR (100MHz,$CDCl_3$,298K): δ_C(ppm) = 138.6-137.2 (($C1^1$), ($C2^1$), ($C3^1$) and ($C4^1$)); 128.9-128.1 (($C1^2$),C(1^3), C(1^4), C(2^2), C(2^3), C(2^4), C(3^2), C(3^3), C(3^4), C(4^2), C(4^3) and C(4^4)); 103.2 (C(1β)); 84.9 (C(3β)); 82.8 (C(2β)); 78.0 (C(4β)); 75.5 (C(5β)); 62.4 (C(6α)); 76.2, 75.5, 75.4, 72.1 (4×H_2<u>C</u>Ph); 29.8, 14.6 (traces of hexane).

α/β ratio by ^{13}C-NMR : Only β anomer

9.2.1.1.3.5. Synthesis of Benzyl 6′-O-(BOC-ALA) - 2′,3′,4′-tri-O-benzyl α/β-D-glucose

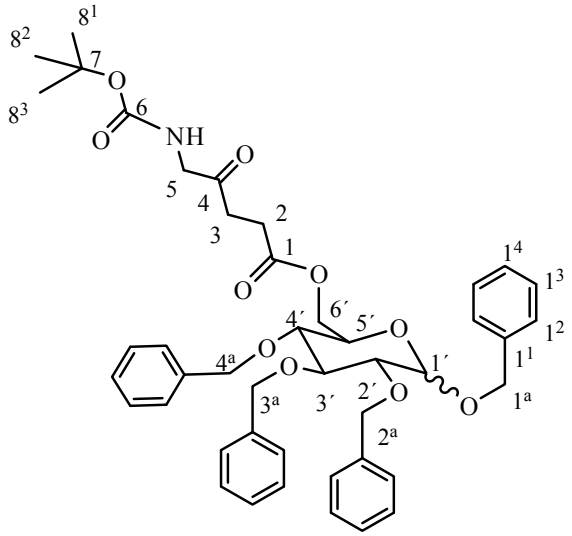


$C_{34}H_{36}O_6$ [540.64]

$C_{10}H_{17}NO_5$ [231.16]

$C_{44}H_{51}NO_{10}$ [753.87]

161 mg (0.298 mmol, 1eq) of Benzyl-2,3,4-tri-O-benzyl α/β-D-glucose was taken in a dry flask under argon atmosphere and was dissolved in 6.7 ml CH_2Cl_2. The solution was cooled to 0^0C, added 40 mg (0.327 mmol, 1.1eq) of DMAP, then 69.4 mg (0.362mmol, 1.2eq) of EDCI and finally 75.7 mg (0.327mmol, 1.1eq) of Boc-ALA acid. The reaction was warmed to ambient temperature and maintained at that temperature for 8 hrs (controlled by TLC, eluent 7:3 Hexane: EtOAc 2 times). The mixture was concentrated and the residue was subjected to flash chromatography (silica gel- 60times of the residue mass) which further afforded 167 mg pure Benzyl 6′-O-(BOC-ALA) - 2′,3′,4′-tri-O-benzyl α/β-D-glucose (Yield= 74%)



Batch: VW33, VW34.

R_f: (Hexane: EtOAc 70:30, 2 times): 0.18 (UV active; $KMnO_4$)

IR (KBr) : 3425*m*, 3088*vw*, 3063*w*, 3031*m*, 3005*vw*, 2976*w*, 2925*s*, 2872*m*, 2315*vw*, 1955*vw*, 1877*vw*, 1807*vw*, 1733*vs*, 1713*vs*, 1624*vw*, 1605*vw*, 1586*vw*, 1497*s*, 1455*s*,

1392*m*, 1366*s*, 1332*vw*, 1308*vw*, 1267*vw*, 1250*m*, 1207*w*, 1164*vs*, 1071*vs*, 1028*vs*, 912*m*, 873*w*, 822*w*, 737*vs*, 699*vs*, 677*vw*, 611*w*, 559*w*, 531*vw*, 464*w*.

^{1}H-NMR (200 MHz, $CDCl_3$, 298 K): δ_H (ppm) = = 7.33-7.16 (*m*, 20H, Haromatic); 5.15 (*sbr*, 1H, HN); 4.94 (*d*, ^{2}J=10.8Hz, 1H, $\underline{H}_2$CPh); 4.87 (*d*, ^{2}J=10.8Hz, 1H, $\underline{H}_2$CPh); 4.86 (*d*, ^{2}J=10.8Hz, 1H, $\underline{H}_2$CPh); 4.83 (*d*, ^{2}J=12.5Hz, 1H, $\underline{H}_2$CPh); 4.78 (*d*, ^{2}J=11.0Hz, 1H, $\underline{H}_2$CPh); 4.76 (*d*, ^{2}J=10.6Hz, 1H, $\underline{H}_2$CPh); 4.71 (*d*, ^{2}J=10.0Hz, 1H, $\underline{H}_2$CPh); 4.62 (*d*, $^{3}J_{1\text{-}2}$=3.5Hz, 1H, HC(1′α)); 4.60 (*d*, ^{2}J=11.9Hz, 1H, $\underline{H}_2$CPh); 4.58 (*d*, ^{2}J=11.5Hz, 1H, $\underline{H}_2$CPh); 4.49 (*d*, ^{2}J=11.0Hz, 1H, $\underline{H}_2$CPh); 4.47 (*d*, ^{2}J=12.5Hz, 1H, $\underline{H}_2$CPh); 4.46 (*d*, ^{2}J=12.4Hz, 1H, $\underline{H}_2$CPh); 4.43 (*d*, $^{3}J_{1\text{-}2}$= 7.8Hz, 1H, HC(1′β)); 4.26 (*dd*, $^{3}J_{5'\text{-}6'a} \approx$ 2.0Hz, $^{3}J_{6'a\text{ - }6'b}$ =11.7Hz ,1H, HC(6′a α or β)); 4.20 (*dd*, $^{3}J_{5'\text{-}6'b} \approx$ 4.5Hz, $^{3}J_{6'a\text{ - }6'b}$ =11.7Hz ,1H, HC(6′b α or β)); 4.07 (*dd*, $^{3}J_{5'\text{-}6'a} \approx$ 2.0Hz, $^{3}J_{6'a\text{ - }6'b}$ =11.8Hz ,1H, HC(6′a α or β)); 4.00-3.95 (*m*, 3H, HC(6′b α or β) and $H_2C(5)$); 3.78 (*ddd*, $^{3}J_{5'\text{-}6'a} \approx$ 2.0Hz, $^{3}J_{5'\text{-}6'b} \approx$ 4.0Hz, $^{3}J_{5'\text{-}4'}$ = 10.0Hz,1H, HC (5′α)); 3.58 (*t*, $^{3}J_{3'\text{-}4'} \approx$9.3Hz, $^{3}J_{3'\text{-}2'}$ =9.3Hz,1H, HC(3′α));)); 3.45 (*dd*, $^{3}J_{2'\text{-}1'} \approx$ 3.7Hz, $^{3}J_{2'\text{ - }3'} \approx$ 9.6Hz ,1H, HC(2′α)); 3.44-3.37 (*m*, 4H, HC(2′β),HC (3′β),HC(4′α) and HC (4′β)); 2.65-2.53 (*m*, 4H, $H_2C(3)$ and $H_2C(2)$); 1.35 (*s*, 9H, H_3C (8^1, 8^2 and 8^3)); 1.18, 0.82-0.76 peaks due to traces of solvent and impurity.

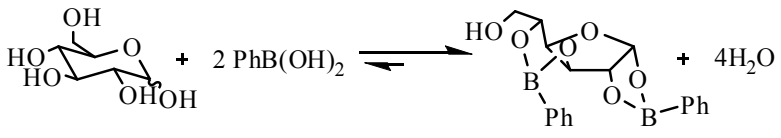
α/β ratio by ^{1}H-NMR : could not be determined because of peaks overlap.

^{13}C-NMR (50MHz,$CDCl_3$,298K): δ_C(ppm) = 204.1,204.0 (C(4) of α and β); 172.2, 172.1 (C(1) of α and β); 155.6 (C(6)) ; 138.7-137.0 (($C1^1$), ($C2^1$), ($C3^1$) and ($C4^1$) of α and β); 128.5 - 127.0 (($C1^2$,$C1^3$,$C1^4$, $C2^2$,$C2^3$,$C2^4$, $C3^2$,$C3^3$,$C3^4$, $C4^2$,$C4^3$,$C4^4$,) of α and β); 102.4 (C(1′β)); 95.5 (C(1′α)); 84.6, 82.2, 82.0, 80.0, 77.4, 77.3, 72.8, 68.8 (C(5′α), C(5′β), C(4′α), C(4′β), C(3′α)), C(3′β), (C(2′α) and (C(2′β)); 63.3 (C(6′α) and C(6′β)); 75.8, 75.7, 75.1, 75.0, 74.9, 73.0, 71.2, 69.3 ($C(2^a)$, $C(3^a)$, $C(4^a)$ and $C(1^a)$ of α and β anomer); 50.3 (C(5)); 34.2 (C(3)); 28.3 ($C(8^1,8^2$ and 8^3); 27.8 (C(2)).

α/β ratio by ^{13}C-NMR ≈1.44 : 1

9.2.1.1.4. Synthesis of 6′-O- (ALA) α/β-D- glucose by protection & deprotection method (using boronate protective group):

9.2.1.1.4.1. Synthesis of α-D-glucofuranose 1,2:3,5-bis phenyl boronate



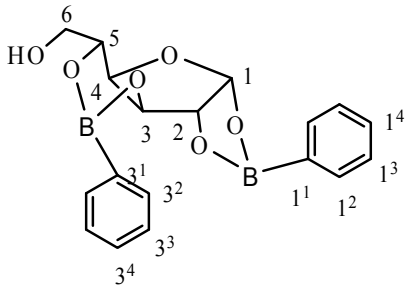
13 — $C_6H_{12}O_6$ [180.16]

22 — $C_6H_7BO_2$ [121.93]

23 — $C_{18}H_{18}B_2O_6$ [351.95]

To a mixture of 968 mg (5.37 mmol, 1eq) of D-glucose and 40 ml toluene, was added 1310 mg (10.74 mmol, 2eq) of phenyl boronic acid. The reaction was refluxed with Dean-Stark apparatus to distil off the toluene-water azeotrope. The reaction was kept

under reflux (approximately for 12 h) till the water collection is stopped and the disappearance pf the precipitate. The toluene was later evaporated completely. To the white residue was added 20 ml toluene, heated 70^0C to dissolve the residue completely, cooled slowly to crystallise pure α-D-glucofuranose 1,2:3,5-bis phenyl boronate and filtered which on after drying afforded 840 mg.(Yield=44.4%)



Batch: RV115, RV124, RV127

IR (KBr): 3482*vs*, 3077*vw*, 3054*vw*, 3043*vw*, 3025*vw*, 2996*vw*, 2947*w*, 2933*w*, 2907*w*, 2877*vw*, 1973*vw*, 1912*vw*, 1836*vw*, 1602*s*, 1574*vw*, 1498*w*, 1459*vw*, 1446*s*, 1416m, 1403w, 1373w, 1355vs, 1345vs, 1330s, 1305vs, 1286*m*, 1258*m*, 1226*w*, 1209*m*, 1163*s*, 1099*s*, 1082*m*, 1055*w*, 1045*w*, 1035*w*, 1025*m*, 1007*s*, 997*m*, 965*w*, 947*vw*, 933*vw*, 874*w*, 864*w*, 851*w*, 830*m*, 802*w*, 761*s*, 746*m*, 708*vw*, 701*s*, 666*w*, 658*vw*, 642*m*, 622*w*, 568*w*, 527*w*, 505*w*.

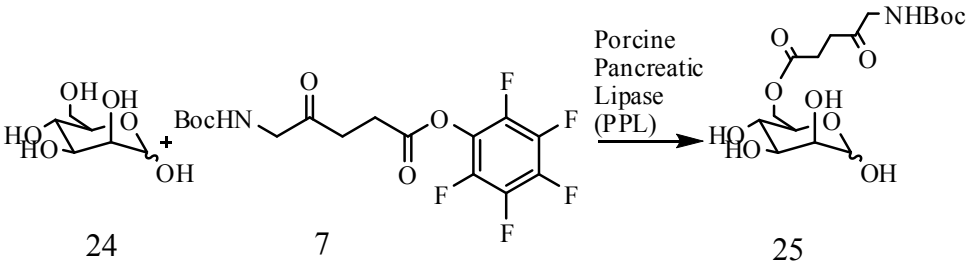
^{1}H-NMR (400 MHz, CD_2Cl_2, 298 K): δ_H (ppm) = 7.71-7.25 (*m*, 10H, Haromatic); 6.17 (*d*, $^3J_{1\text{-}2}$ =4.2Hz, 1H, HC(1)); 4.98 (*d*, $^3J_{1\text{-}2}$ ≈4.2Hz, 1H, HC(2)); 4.72 (*d*, $^3J_{3\text{-}4}$ =2.6Hz, 1H, HC(3)); 4.45 (*t*, $^3J_{5\text{-}6a}$ = 3.6Hz, $^3J_{5\text{-}6b}$ = 3.6Hz, 1H, HC(5)); 4.32 (*d*, $^3J_{4\text{-}3}$ ≈2.6Hz, 1H, HC(4)); 3.88 (*dd*, $^3J_{6a\text{-}5}$ ≈ 4.0Hz, $^3J_{6a\text{-}6b}$ =11.6Hz ,1H, HC(6a)); 3.74 (*dd*, $^3J_{6b\text{-}5}$ =3.6Hz, $^3J_{6a\text{-}6b}$ =11.6Hz ,1H, HC(6b));

^{13}C-NMR (100MHz,CD_2Cl_2,298K): δ_C(ppm) = 136.7-124.0 ((C1^1), (C1^2),C(1^3), C(1^4), (C3^1) , C(3^2), C(3^3), C(3^4)); 104.7 (C(1)); 86.5 (C(2)); 75.4 (C(4)); 74.4 (C(3)); 71.3 (C(5)); 63.9 (C(6));

MS (ESI (+)): m/z relative intensity $[M\text{-}H]^+$ = 351.4

9.2.1.2. Synthesis of 6´- O-(ALA) -α/β-D-mannose

9.1.2.1. Synthesis of 6´-O-(BOC-ALA) α/β-D-mannose

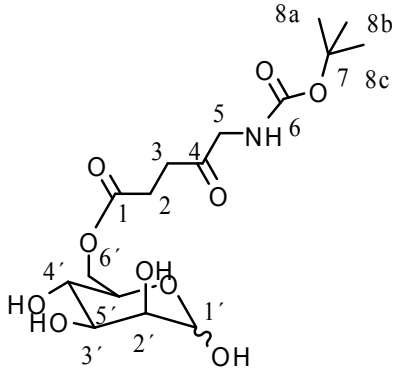


24 $C_6H_{12}O_6$ [180.16]

7 $C_{16}H_{16}NO_5F_5$ [397.23]

25 $C_{16}H_{27}NO_{10}$ [393.39]

360.4 mg (2 mmol, 1eq) of mannose was taken in a dry flask under argon atmosphere and was dissolved in 6 ml anhydrous pyridine. 650 mg (24 units per mg of protein; 1unit hydrolyses 1μmol or 0.001mmol of an ester; approximately, 15.6 mmol, 7.8eq) of *porcine pancreatic lipase* (PPL) enzyme (before use dried by keeping at vacuum 10^{-2} mbar for 3-4 days) was added and then 1600 mg (4mmol, 2eq) of Boc-ALA-PFP ester was added to the solution. The reaction was heated to 50^0C and maintained at this temperature for approximately 48 hrs (controlled by TLC eluent : AcOEt: MeOH 1:1). The mass was cooled to room temperature and then the enzyme was filtered over celite bed, the filtered cake was washed with 3× 10ml pyridine. The solvent pyridine was evaporated off by rotovapor .The residue was extracted with 100ml toluene, centrifuged to remove the unreacted Boc-ALA-PFP ester and Pentafluoro phenol which got dissolved in the toluene and separated as a clear top supernatant solution. The settled residue after the centrifuge was taken for flash chromatography. (gradient eluent from 100% CH_2Cl_2 to 85:15 CH_2Cl_2 : MeOH). The main fractions were concentrated to give 539 mg of pure product was obtained as white solid. (Yield = 68.5%).



Batch : VW 56 and VW4048

R_f: (CH_2Cl_2 / MeOH 85:15): 0.24; ($KMnO_4$)

IR (KBr) : 3420*vs*, 2978*m*, 2931*m*,2509*w*, 1717*vs*, 1520*s*, 1419*w*, 1393*m*, 1368*s*, 1283*m*, 1253*m*, 1167*vs*, 1059*s*, 868*w*, 806*w*,781*w*,674*w*,600*w*, 499*w*.

^{1}H-NMR (400 MHz, CD_3OD, 298 K): δ_H (ppm) = 5.10 (*d*, $^3J_{1'-2'}$ ≈1.7Hz, 1H, HC(1′α)); 4.48 (*d*, $^3J_{1'-2'}$ =1.0Hz, 1H, HC(1′β));4.32 (*dd*, $^3J_{5'-6'a}$ ≈ 2.1Hz, $^3J_{6'a\ -\ 6'b}$ =11.8Hz ,1H, HC(6′a α)) ;4.25 (*dd*, $^3J_{5'-6'b}$ ≈ 5.4Hz, $^3J_{6'a\ -\ 6'b}$ =11.8Hz ,1H, HC(6′b α));3.95-3.88 (*m*, 3H, $H_2C(5)$ and HC (5′α));3.82 (*dd*, $^3J_{2'-1'}$ =1.7Hz, $^3J_{3'-2'}$ =3.4Hz,1H, HC(2′α)); 3.76 (*dd*,

$^{3}J_{2'-3'} \approx 3.4$Hz, $^{3}J_{3'-4'} \approx 9.7$Hz,1H, HC (3′α)); 3.57 (*t*, $^{3}J_{4'-3'} \approx 9.7$Hz, $^{3}J_{4'-5'} \approx 9.7$Hz 1H, HC(4′α));2.75-2.63 (*m*, 4H ,$H_2C(3)$ and $H_2C(2)$); 1.42 (*s*, 9H, H_3C (8a, 8b and 8c)).

α/β ratio by ^{1}H-NMR ≈ 5.2 : 1

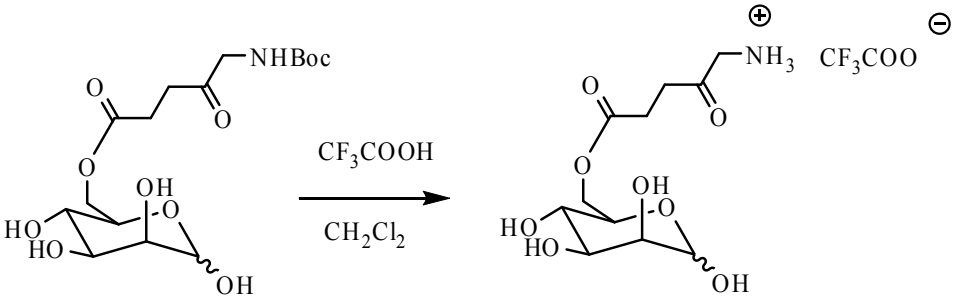
^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 207.0 (C(4)); 173.6 (C(1)); 157.4 (C(6)) ; 95.0 (C(1′α)); 94.8 (C(1′β)); 80.4 (C(7)); 71.8 (C(2′α)); 71.5 (C(3′α)); 70.6 (C(5′α)); 68.0 C(4′α)); 64.7 (C(6′α)),49.6 (C(5)); 34.5 (C(3)) ;28.5 (C(8a,8b and8c); 28.3 (C(2)).

α/β ratio by ^{13}C-NMR ≈ 3.8 : 1

MS (ESI (+)): m/z relative intensity $[M+Na]^+ = 416.2$; $[M+H]^+ = 393.9$; $[M-^{t}Bu + Na]^+ = 360.4$; $[M\text{-Boc} + Na]^+ = 316.1$; $[M\text{-Boc-}(CONH_2) + Na]^+ = 256.0$

HR-MS $[M+Na]^+ = 416.1523$ (calculated = 416.1533)

9.1.2.2. Synthesis of 6′-O-(ALA) α/β-D-mannose



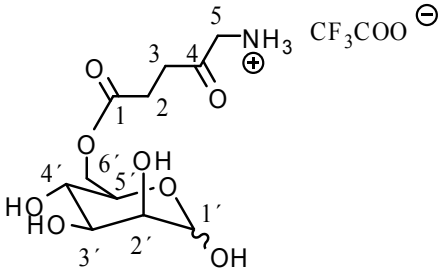
25 26

$C_{16}H_{27}NO_{10}$ [393.39]

$C_{13}H_{20}F_3NO_{10}$ [407.29]

$M^+ = 294.28$; $CF_3COO^- = 113.01$

315 mg (0.8 mmol, 1eq) of 6′-O-(BOC-ALA) mannose was dissolved in 20ml CH_2Cl_2 and cooled to -10^0C. To this solution, 4.58g (3ml, 40 mmol, 50 eq) of trifluoroacetic acid was added dropwise by syringe. The reaction mass was allowed to come to ambient temperature and maintained at ambient temperature for 30 minutes. Once the deprotection of BOC was completed (controlled by the absence of BOC peak in ^{1}H-NMR), CH_2Cl_2 and trifluoroacetic acid was evaporated off. The product was triturated with dry ether and ether solution was decanted. The trituration with ether and decanting top clear ether solution after centrifuge was repeated 2 times more. Then, the settled solid product was dried under high vacuum oil pump and thereby yielded 297 mg of highly hygroscopic white solid. (Yield=91%).



Batch: RV 345

$[\alpha]_D$ = +14.4 (c=0.21; deionized H_2O)

IR (KBr): 3388*vs*, 2928*m*, 1788*w*, 1731*vs*, 1678*vs*, 1517*w*, 1429*m*, 1391*m*, 1327*w*, 1204*vs*, 1138*vs*, 1068*m*, 913*w*, 839*m*, 800*m*, 776*w*, 723*m*, 598*w*, 520*w*,500*w*.

^{1}H-NMR (400 MHz, D_2O, 298 K): δ_H (ppm) = 5.06 (*d*, $^3J_{1'-2'}$ =1.7Hz, 1H, HC(1´α)); 4.82 (*d*, $^3J_{1'-2'}$ =1.0Hz, 1H, HC(1´β));4.32-4.18 (*m*, 4H, H_2C (6`α) and $H_2C$ (6`β)); 4.05 (*s*, 2H, H_2C (5)); 3.89 (*ddd*, $^3J_{5'-6'a} \approx$ 2.6 Hz, $^3J_{5'-6'b} \approx$ 4.7Hz, $^3J_{5'-4'}$ = 9.9Hz ,1H, HC (5`α)); 3.85 (*dd*, $^3J_{2'-1'}$ =1.7Hz, $^3J_{3'-2'} \approx$3.4Hz,1H, HC(2´α)); 3.75 (*dd*, $^3J_{2'-3'a} \approx$ 3.4Hz, $^3J_{3'-4'} \approx$9.8 Hz,1H, HC (3´α)); 3.60 (*t*, $^3J_{4'-3'} \approx$ 9.8Hz, $^3J_{4'-5'}$ = 9.8Hz 1H, HC(4´α)); 3.57-3.44 (*m*,1H, HC (5`β) and peaks due to ether solvent)); 2.85 (*t*, $^3J_{2-3}$ = 6.5Hz, 2H, H_2C(3)) ; 2.69 (*t*, $^3J_{3-2}$ = 6.5Hz, 2H, H_2C(2)) ; 1.07-1.15 (peaks due to ether solvent).

α/β ratio by ^{1}H-NMR ≈ 3.67 : 1

^{13}C-NMR (100 MHz, D_2O, 298K) : δ_C (ppm) = 204.4 (C(4)) ; 174.9 (C(1α or β)) ; 174.8 (C(1α or β)); 163.5 (*q*,$^2J_{C-F}$ = 35.5, $CF_3\underline{C}OO^-$); 116.7 (*q*,$^1J_{C-F} \approx$ 291.0, $\underline{C}F_3COO^-$); 94.6 (C(1´α)); 94.2 (C(1´β)); 70.9, 70.4, 67.0 (C(2´α),C(3´α), and C(5´α)); 73.8,71.4,66.7(C(2`β), C(3`β) and C(5`β)); 66.4(C(4´α and 4`β)); 64.2(C(6´α and 6´β)); 47.4 (C(5)); 34.6(C(3)); 27.7 (C(2)).

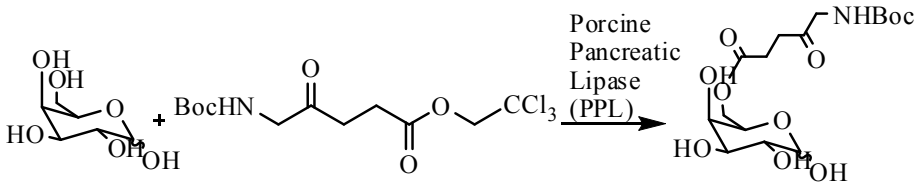
α/β ratio by ^{13}C-NMR ≈ 2.4 : 1

MS (ESI (+)): m/z relative intensity $[M\text{-}CF_3COO^-]^+$ = 294.0

HR-MS $[M\text{-}CF_3COO^-]^+$ = 294.1184 (calculated = 294.1189)

9.1.3. Synthesis of 6´- O-(ALA) -α/β-D-galactose

9.1.3.1. Synthesis of 6´-O-(BOC-ALA) α/β-D-galactose

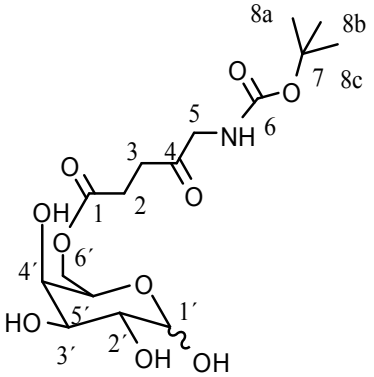


27 $C_6H_{12}O_6$ [180.16]

9 $C_{16}H_{16}NO_5F_5$ [362.56]

28 $C_{16}H_{27}NO_{10}$ [393.39]

360.4 mg (2 mmol, 1eq) of galactose was taken in a dry flask under argon atmosphere and was dissolved in 16 ml anhydrous pyridine. 1900 mg (20 units per mg of protein; 1unit hydrolyses 1μmol or 0.001mmol of an ester; approximately, 38 mmol, 19.0eq) of *porcine pancreatic lipase* (PPL) enzyme (before use dried by keeping at vacuum 10^{-2} mbar for 3-4 days) was added and then 4680 mg (12.8mmol, 6.4eq) of Boc-ALA-TCE ester was added to the solution. The reaction was heated to 50^0C and maintained at this temperature for approximately 60hrs (controlled by TLC eluent : AcOEt: MeOH 1:1). The mass was cooled to room temperature and then the enzyme was filtered over celite bed, the filtered cake was washed with 3× 10ml pyridine. The solvent pyridine was evaporated off by rotovapor .The residue was extracted with 100ml toluene, centrifuged to remove the unreacted Boc-ALA-TCE ester and Trichloro ethanol which got dissolved in the toluene and separated as a clear top supernatant solution. The settled residue after the centrifuge was taken for flash chromatography. (gradient eluent from 100% CH_2Cl_2 to 85:15 CH_2Cl_2 : MeOH). The main fractions were concentrated and the mass was further purified by dissolving the mass in 8ml CH_2Cl_2 and precipitated by 8ml hexane. 348 mg of pure product was obtained as white solid. (Yield = 44.2%).



Batch :RV337

R_f: (CH_2Cl_2 / MeOH 85:15): 0.29 ($KMnO_4$)

IR (KBr): 3414*vs*, 2981*m*, 2931*m*,2347*w*,1736*vs*,1715*vs*,1684*vs*, 1536*s*, 1416*w*, 1393*w*, 1368*m*,1323*w*,1301*m*,1253*m*,1200*m*,1174*s*,1149*s*,1105*m*,1091*m*,1074*s*,1030*s*,997*w*,921*w* , 859*w*,800*m*,692*m*,645*w*,568*w*,468*w*.

^{1}H-NMR (400 MHz, CD_3OD, 298 K): δ_H (ppm) = 5.16 (*d*, $^3J_{1'-2'}$ =3.5Hz, 1H, HC(1′α)); 4.42 (*d*, $^3J_{1'-2'}$ =7.2Hz, 1H, HC(1′β)); 4.26-4.16 (*m*, 5H, H_2C(6′α and β) and HC(5′α));

3.95-3.93 (*m*, 3H, $H_2C(5)$ and HC (4′ α)); 3.86 (*m*,1H, HC(3′α)); 3.75 (*dd*, $^3J_{2'\text{-}1'}$ ≈3.5Hz, $^3J_{3'\text{-}2'}$ =9.8Hz,1H, HC(2′α)); 3.72-3.69 (*m*,1H, HC(5′β)); 3.47 (*dd*, $^3J_{2'\text{-}1'}$ ≈7Hz, $^3J_{3'\text{-}2'}$ =9.8Hz,1H, HC(2′β)); 2.77-2.63 (*m*, 4H ,$H_2C(3)$ and $H_2C(2)$); 1.43 (*s*, 9H, H_3C (8a, 8b and 8c)).

α/β ratio by ^{1}H-NMR ≈ 2.8 : 1

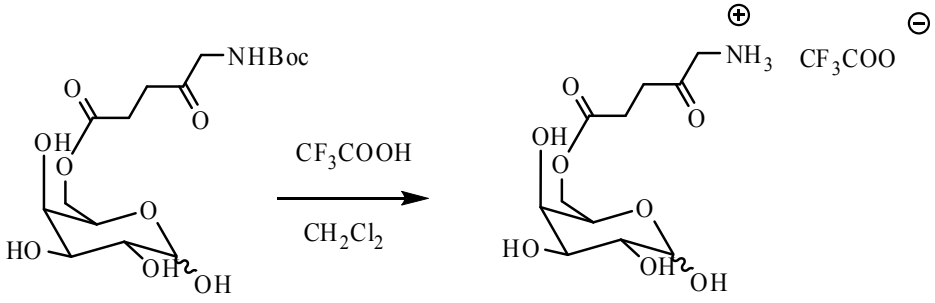
^{13}C-NMR (100 MHz, CD_3OD, 298K) : δ_C (ppm) = 207.2 (C(4)); 173.8 (C(1)); 158.0 (C(6)) ; 98.2 (C(1′β)); 93.7 (C(1′α)); 80.5 (C(7)); 70.6 (C(2′α)); 70.5, 69.9 (C(3′α) and C(4′α)); 68.7 (C(5′α)); 64.9 (C(6′α)), 74.3,73.4,73.2,64.6 (peaks due to β anomer); 50.5 (C(5)); 34.7 (C(3)) ;28.6 (C(8a,8b and8c); 28.4 (C(2)).

α/β ratio by ^{13}C-NMR ≈ 3.4 : 1

MS (ESI (+)): m/z relative intensity $[M+ Na]^+$ = 416.1

HR-MS $[M+ Na]^+$ = 416.1526 (calculated = 416.1533)

9.1.3.2. Synthesis of 6′- O-(ALA) α/β-D-galactose



28

$C_{16}H_{27}NO_{10}$

[393.39]

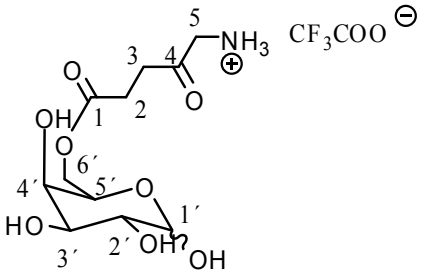
29

$C_{13}H_{20}F_3NO_{10}$

[407.29]

M^+=294.28 ; CF_3COO^- = 113.01

280 mg (0.71 mmol, 1eq) of 6′-O-(BOC-ALA) galactose was dissolved in 20ml CH_2Cl_2 and cooled to -10^{0}C. To this solution, 4.07g (2.75ml, 35.55 mmol, 50 eq) of trifluoroacetic acid was added dropwise by syringe. The reaction mass was allowed to come to ambient temperature and maintained at ambient temperature for 30 minutes. Once the deprotection of BOC was completed (controlled by the absence of BOC peak in ^{1}H-NMR), CH_2Cl_2 and trifluoroacetic acid was evaporated off. The product was triturated with dry ether and ether solution was decanted. The trituration with ether and decanting top clear ether solution after centrifuge was repeated 2 times more. Then, the settled solid product was dried under high vacuum oil pump and thereby yielded 270 mg of highly hygroscopic white solid. (Yield=93.2%).



Batch: RV 347

$[\alpha]_D$ = -48.5 (c=0.47; deionized H_2O)

IR (KBr): 3392*vs*, 2929*m*, 2027*w*,1731*vs*, 1678*vs*, 1512*w*, 1430*m*, 1384*m*, 1317*w*, 1205*vs*, 1138*vs*, 1073*m*,1038*w*, 917*w*, 872*w*,839*m*, 800*m*, 723*m*, 598*w*, 520*w*,500*w*.

^{1}H-NMR (400 MHz, D_2O, 298 K): δ_H (ppm) = 5.23 (*d*, $^3J_{1'-2'}$ =3.7Hz, 1H, HC(1′α)); 4.55 (*d*, $^3J_{1'-2'}$ =7.9Hz, 1H, HC(1′β));4.48-4.11 (*m*,5H, H_2C (6`α),$H_2C$ (6`β) and HC (5′α); 4.10 (*s*, 2H, H_2C (5)); 3.97 (*m*, 1H, HC (3`α)); 3.92 (*dd*, $^3J_{4'-5'}$ =0.9Hz, $^3J_{4'-3'}$ =3.5Hz,1H, HC(4′β)); 3.88 (*ddd*, $^3J_{5'-4'} \approx$ 0.9Hz, $^3J_{5'-6'a}$ = 4.3Hz, $^3J_{5'-6'b}$ = 7.8Hz ,1H, HC (5`β)); 3.84-3.83 (*m*, 1H, HC(4′α)); 3.80(*dd*, $^3J_{2'-1'} \approx$3.7Hz, $^3J_{3'-2'}$ =11Hz,1H, HC(2′α)); 3.62 (*dd*, $^3J_{3'-4'}$ =3.5Hz, $^3J_{3'-2'} \approx$10 Hz,1H, HC (3′β)); 3.55-3.48 (*q*,2H, peaks due to ether solvent);3.46 (*dd*, $^3J_{2'-1'}$ =7.9Hz, $^3J_{3'-2'}$ =10Hz,1H, HC(2′β)); 2.95 (*t*, $^3J_{2-3} \approx$6.5Hz, 2H, H_2C(3)) ; 2.72 (*t*, $^3J_{3-2} \approx$ 6.5Hz, 2H, H_2C(2)) ; 1.07-1.15 (peaks due to ether solvent).

α/β ratio by ^{1}H-NMR ≈ 0.60: 1

^{13}C-NMR (100 MHz, D_2O, 298K) : δ_C (ppm) = 204.4 (C(4)) ; 174.8 (C(1α or β)) ; 174.7 (C(1α or β)); 163.3 (*q*,$^2J_{C-F}$ = 35.0, $CF_3\underline{C}OO^-$); 116.6 (*q*,$^1J_{C-F} \approx$ 291.0, $\underline{C}F_3COO^-$); 96.8 (C(1′β)); 92.7 (C(1′α));72.9 (C(3′β));72.7 (C(4′β));72.0(C(2′β));69.6,69.2,69.0,68.5,68.4 (C(3′α),C(5′β),C(2′α),C(5′α) and C(4′α));64.7,64.5(C(6′α and 6′β)); 47.4 (C(5)); 34.6(C(3)); 27.7 (C(2)).

α/β ratio by ^{13}C-NMR ≈ 0.47 : 1

MS (ESI (+)): m/z relative intensity $[M\text{-}CF_3COO^-]^+$ = 294.0; $[M\text{-}CF_3COO^-+ H]^+$=295.0; $[M\text{-}CF_3COO^-\text{-}NH_3]^+$=276.1

HR-MS $[M\text{-}CF_3COO^-]^+$ = 294.1181 (calculated = 294.1189)

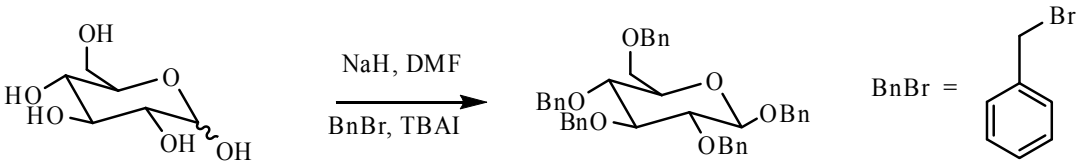
9.2.2. Synthesis of 1′-O-(ALA)-sugars

9.2.2.1. Synthesis of 1′- O-(ALA) α/β-D-glucose

The synthesis of 1´-O-(ALA)-α/β-D-glucose was carried out using Boc-ALA-acid and benzyl protection &deprotection methodology. This method employed DMAP & EDCI coupling method to obtain β anomer and catalytic DMAP & DCC coupling method to obtain α anomer. Also the synthesis of 1´-O-(ALA)-α-D-glucose was attempted using activated ester (Boc-ALA-PFPester) and azido levulinic acid chloride and deprotonation by BuLi or NaH.

9.2.2.1.1. Synthesis of 1´- O-(ALA) α/β-D-glucose using Boc-ALA acid

9.2.2.1.1.1. Synthesis of 1,2,3,4,6-Penta-O-benzyl α/β-D-glucose (penta benzyl glucose)

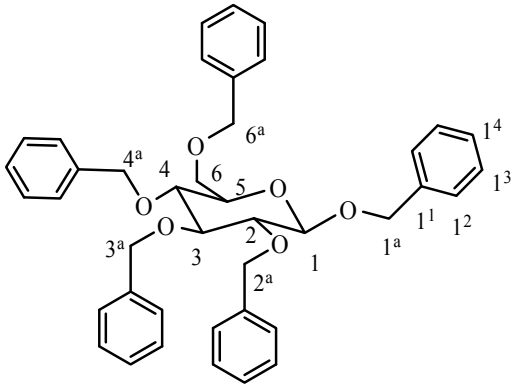


13 — $C_6H_{12}O_6$ [180.16]

30 — $C_{41}H_{42}O_6$ [630.77]

C_7H_7Br [171.04]

541 mg (3.0 mmol, 1eq) of glucose was taken in a dry flask under argon atmosphere and was dissolved in 20 ml anhydrous DMF. The mass was cooled to -20^{0}C and added 730 mg (30.0 mmol, 10eq, 2eq per –OH) sodium hydrid (purity=99%) in portionwise. The reaction mass was warmed to ambient temperature and kept at that temperature for 2hrs. Then the mass was cooled to 5^{0}C, added 55.5 mg (0.15 mmol, 0.05eq) of tetra butyl ammonium iodide (TBAI) and then 5.13 g (3.6 ml, 30.0mmol, 10eq, 2 eq per –OH) of Benzyl bromide was added drop wise by syringe. The reaction mass was maintained at ambient temperature for 48hrs.Later, the mass was cooled to 5^{0}C and quenched with 20ml methanol. The crude product was extracted with 200ml EtOAc and 200ml water. The aqueous layer is extracted with 3×200ml EtOAc. The organic layer was combined and washed with 200ml brine. The organic layer was dried over $MgSO_4$ and the solvent was evaporated off till 60^{0}C /40 mbar to remove DMF. The crude residue was subjected to flash chromatography (silica gel- 50 times of the residue mass) using gradient eluent from 100% hexane to 80:20 Hexane: Ether. The pure fractions were collected and concentrated to afford 1.4g pure 1,2,3,4,6-Penta-O-benzyl α/β-D-glucose **(**penta benzyl glucose). (Yield=74%).



Batch: RV81, RV137, RV229, RV248, VW24

R_f: (Hexane / Ether 80:20): 0.36 (UV $_{254}$ active; $KMnO_4$)

IR (KBr) : 3435*w*, 3088*w*, 3064*w*, 3031*m*, 2914*m* , 2868*m*, 2809*w*, 1956*w*, 1874*w*, 1808*w*, 1636*w*, 1605*w*, 1587*w*,1496*m*, 1453*m*, 1402*w*, 1363*m*, 1314*w*, 1277*w*, 1236*w*, 1215*m*, 1197*w*, 1157*w*, 1120*s*, 1074*vs*, 1041*m*, 1028*s*, 1002*m*, 949*m*, 909*m*, 831*w*, 753*s*, 736*s*, 698*vs*, 679*w*, 630*w*, 610*w*, 587*w*, 576*w*, 558*w*, 532*w*, 508*w*, 500*w*, 459*w*.

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.44-7.21 (*m*, 25H, Haromatic); 5.01(*d*, $^2J_{1a\text{-}1a'}$=11.9Hz, 1H, HC(1^a)); 4.99 (*d*, $^2J_{2a\text{-}2a'}$=10.9Hz, 1H, HC(2^a)); 4.96 (*d*, $^2J_{3a\text{-}3a'}$=10.9Hz, 1H, HC(3^a)); 4.89 (*d*, $^3J_{1\text{-}2}$=3.6Hz, 1H, HC(1α)); 4.86 (*d*, $^2J_{4a\text{-}4a'}$=10.8Hz, 1H, HC(4^a)); 4.82 (*d*, $^2J_{3a\text{-}3a'}$=10.9Hz, 1H, HC($3^{a'}$)); 4.76 (*d*, $^2J_{2a\text{-}2a'}$=10.9Hz, 1H, HC($2^{a'}$)); 4.71 (*d*, $^2J_{1a\text{-}1a'}$ ≈11.9Hz, 1H, HC($1^{a'}$)); 4.67 (*d*, $^2J_{6a\text{-}6a'}$=12.2Hz, 1H, HC(6^a)); 4.64 (*d*, $^2J_{6a\text{-}6a'}$ ≈12.2Hz, 1H, HC($6^{a'}$); 4.61 (*d*, $^2J_{4a\text{-}4a'}$ ≈10.8Hz, 1H, HC($4^{a'}$)); 4.55 (*d*, $^3J_{1\text{-}2}$ =7.9Hz, 1H, HC(1β)); 3.80 (*dd*, $^3J_{5\text{-}6}$ ≈ 2.0Hz, $^3J_{6\,-\,6'}$ ≈11.0Hz ,1H, HC(6β)) ;3.74 (*dd*, $^3J_{5\text{-}6a}$ ≈ 5.0Hz, $^3J_{6\,-\,6'}$ =11.8Hz ,1H, HC(6′β)); 3.73-3.50 (*m*, 4H, HC(2β), HC(3β), HC(4β) and HC(5β)).

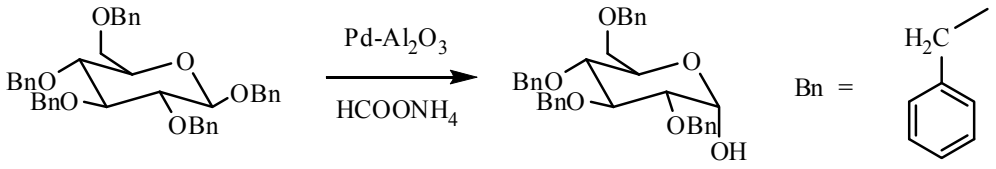
α/β ratio by ^{1}H-NMR could not be determined because of peaks overlap.

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 138.8-137.2 (($C1^1$), ($C2^1$), ($C3^1$), ($C4^1$) and ($C6^1$) of α and β); 128.4-127.5 (($C1^2$,$C1^3$,$C1^4$, $C2^2$,$C2^3$,$C2^4$, $C3^2$,$C3^3$,$C3^4$, $C4^2$,$C4^3$,$C4^4$, $C6^2$,$C6^3$,$C6^4$) of α and β);102.6 (C(1β)); 95.7 (C(1α)); 84.7, 82.3, 77.9, 74.9 (C(2β), C(3β), C(4β), C(5β)); 75.6, 75.0, 74.8, 73.5, 71.1 ($C(1^a)$, $C(2^a)$, $C(3^a)$, $C(4^a)$, $C(6^a)$); 68.9 (C(6β))

α/β ratio by ^{13}C-NMR ≈ 0.21 : 1

MS (ESI (+)): m/z relative intensity $[M+ Na]^+$ = 653.5

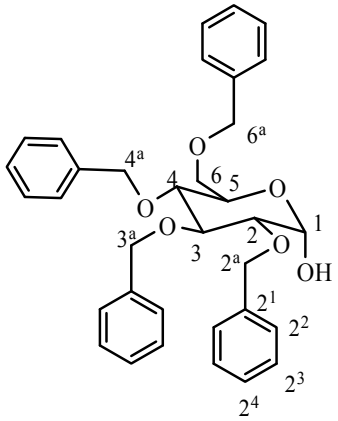
9.2.2.1.1.2. Synthesis of 2,3,4,6-tetra-O-benzyl α/β-D glucose from 1,2,3,4,6-penta-O-benzyl α/β-D-glucose by catalytic hydrogenolysis



30
$C_{41}H_{42}O_6$
[630.77]

31
$C_{34}H_{36}O_6$
[540.64]

630.7mg (1.0 mmol, 1eq) of 1,2,3,4,6-Penta-O-benzyl α/β-D-glucose was dissolved in 50ml methanol at ambient temperature. Then, 986mg (15.6 mmol, 15.6 eq) of ammonium formate was added followed by the addition of 2000mg (0.94 mmol, 0.94 eq) of 5% Palladium over alumina. The reaction mixture was maintained at ambient temperature for 10hrs. Once the starting material is absent (controlled by TLC Hexane: Ether = 80:20), the palladium is filtered over celite and the solvent evaporated off. The crude is subjected to flash chromatography (silica gel- 50 times of the residue mass; eluent =9:1 CH_2Cl_2: EtOAc) which afforded 329mg of pure 2,3,4,6-tetrabenzyl glucose. (Yield= 60.8%)



Batch: RV96, RV137.

R_f: (CH_2Cl_2: EtOAc 9:1): 0.37 (UV $_{254}$ active; $KMnO_4$)

IR (KBr) : 3367*s*, 3107*w*, 3088*w*, 3063*m*, 3030*s*, 2916*s*, 2861*s*, 2759*w*, 1947*w*, 1870*w*, 1806*w*, 1724*w*, 1604*w*, 1586*w*,1496*m*, 1470*w*,1453*s*, 1400*m*, 1358*s*, 1326*w*, 1311*w*, 1275*w*, 1260*w*, 1213*m*, 1147*vs*, 1088*vs*, 1073*vs*, 1047*vs*, 1028*vs*, 1002*m*, 936*w*, 905*w*, 858*w*, 837*w*, 782*w*, 744*vs*, 695*vs*, 627*w*, 609*w*, 597*w*, 576*w*, 547*w*, 560*w*, 528*w*, 500*w*, 463*w*.

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.40-7.16 (*m*, 20H, Haromatic); 5.27 (*d*, $^3J_{1\text{-}2}$=3.5Hz, 1H, HC(1α)); 5.01- 4.50 (*m*, $\underline{H_2}$CPh (α and β) and HC(1β)); 4.10-4.07 (*m*, 1H, HC(5α)); 4.03 (*t*, $^3J_{3\text{-}2}$= 9.3Hz, 1H, HC(3α)); 3.76-3.56 (*m*, 8H, H_2C(6α), H_2C(6β), HC(4α),HC(4β)),HC(5β), HC(3β)); 3.62 (*dd*, $^3J_{1\text{-}2}$=3.6Hz, $^3J_{2\text{-}3}$=9.5Hz, 1H, HC(2α)); 3.45 (*dd*, $^3J_{1\text{-}2}$=7.8Hz, $^3J_{2\text{-}3}$=9.5Hz, 1H, HC(2β))

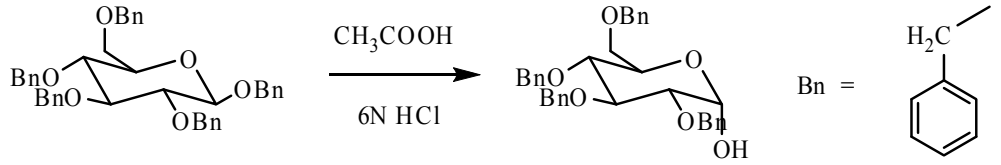
α/β ratio by ^{1}H-NMR could not be determined because of peaks overlap. (α anomer= major)

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 139.1-138.2 (($C2^1$), ($C3^1$), ($C4^1$) and ($C6^1$) of α and β);128.9-128.0 (($C2^2$,$C2^3$,$C2^4$, $C3^2$,$C3^3$,$C3^4$, $C4^2$,$C4^3$,$C4^4$, $C6^2$,$C6^3$,$C6^4$) of α and β; 97.9 (C(1β)); 91.7 (C(1α)); 83.5(C(2β)); 82.2 (C(3α)); 80.4(C(2α)); 70.7(C(5α)); 69.3(C(6β)); 69.0 (C(6α)); 85.0, 78.2, 78.1, 75.0 (C(3β), C(4β), C(5β) and C(4α)); 76.2, 76.1,75.4, 75.2, 75.0, 73.9, 73.6 (C(2^a), C(3^a), C(4^a) and C(6^a) of both α and β).

α/β ratio by ^{13}C-NMR ≈ 2.6 : 1

MS (ESI (+)): m/z relative intensity $[M+ Na]^+$ = 563.3

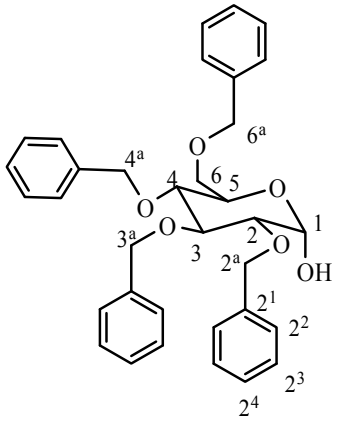
9.2.2.1.1.3. Synthesis of 2,3,4,6-tetra-O-benzyl α/β-D glucose from 1,2,3,4,6-penta-O-benzyl α/β-D-glucose by acidic hydrolysis



30
$C_{41}H_{42}O_6$
[630.77]

31
$C_{34}H_{36}O_6$
[540.64]

5500 mg (8.72 mmol, 1eq) of 1,2,3,4,6-Penta-O-benzyl α/β-D-glucose was taken in a dry flask and was dissolved in 110 ml acetic acid. Then 16.5 ml of 6N HCl was added. The solution is heated to 60^0C. The mass was maintained at 60^0C and was taken for workup once the starting material was absent by TLC (approximately 4 to 6hrs, TLC eluent = Hexane: EtOAc 8:2). The mixture was diluted with 400ml water, extracted with 3×250ml CH_2Cl_2. The organic layer was washed with 300ml saturated $NaHCO_3$ aqueous solution and then with 300ml water. The organic layer was dried over $MgSO_4$, concentrated and the residue was subjected to flash chromatography which further afforded 2.74 g pure 2,3,4,6-tetra-O-benzyl α/β-D glucose (major α anomer; Yield= 58.1%)



Batch: RV232, RV252, VW27

R_f: (CH_2Cl_2: EtOAc 9:1): 0.34 (UV $_{254}$ active; $KMnO_4$)

IR (KBr) : 3435*s*, 3065*w*, 3031*w*, 2919*m*, 2863*m*, 1629*m*, 1497*m*, 1453*s*, 1400*m*, 1363*m*, 1327*w*, 1259*w*, 1209*m*, 1147*vs*, 1088*vs*, 1073*vs*, 1028*vs*, 1001*m*, 858*w*, 837*w*, 745*vs*, 696*vs*, 608*w*, 500*w*, 462*w*.

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.37-7.16 (*m*, 20H, Haromatic); 5.26 (*d*, $^3J_{1\text{-}2}$=3.5Hz, 1H, HC(1α)); 4.99- 4.50 (*m*, $\underline{H_2}$CPh (α and β) and HC(1β)); 4.07 (*ddd*, 1H, $^3J_{5'\text{-}6'a} \approx$ 2.0 Hz, $^3J_{5'\text{-}6'b} \approx$ 4.0Hz, $^3J_{5'\text{-}4'}$ =10.0Hz,1H, HC(5α)); 4.00 (*t*, $^3J_{3\text{-}2}$= 9.3Hz, 1H, HC(3α)); 3.75-3.65 (*m*, 8H, H_2C(6α), H_2C(6β), HC(4α).HC(4β)), HC(5β) and HC(3β)););3.62 (*dd*, $^3J_{1\text{-}2}$=3.6Hz,$^3J_{2\text{-}3}$=9.5Hz, 1H, HC(2α)); 3.43 (*dd*, $^3J_{1\text{-}2}$=7.7Hz, $^3J_{2\text{-}3}$=9.5Hz, 1H, HC(2β)).

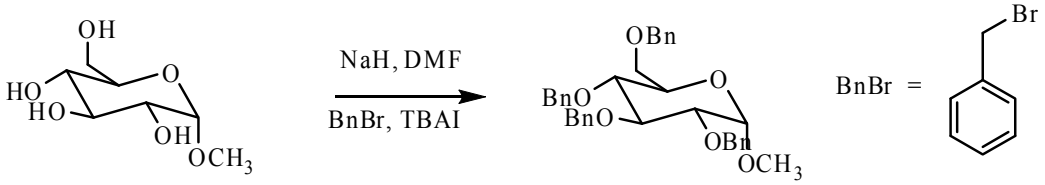
α/β ratio by ^{1}H-NMR could not be determined because of peaks overlap. (α anomer= major)

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 138.6-137.7 (($C2^1$), ($C3^1$), ($C4^1$) and ($C6^1$) of α and β);128.5-127.6 (($C2^2$,$C2^3$,$C2^4$, $C3^2$,$C3^3$,$C3^4$, $C4^2$,$C4^3$,$C4^4$, $C6^2$,$C6^3$,$C6^4$) of α and β; 97.4 (C(1β)); 91.3 (C(1α)); 83.1(C(2β)); 81.7 (C(3α)); 80.0(C(2α)); 70.3(C(5α)); 68.8(C(6β)); 68.50 (C(6α)); 84.5, 77.7, 77.6, 74.7 (C(3β), C(4α), C(4β) and C(5β)); 75.7, 75.6, 75.0, 74.7, 73.5, 73.4, 73.2 (C(2^a), C(3^a), C(4^a) and C(6^a) of both α and β).

α/β ratio by ^{13}C-NMR ≈ 2.7 : 1

MS (ESI (+)): m/z relative intensity $[M+ Na]^+$ = 563.3

9.2.2.1.1.4. Synthesis of α-Methyl 2,3,4,6-tetra-O-benzyl glucoside



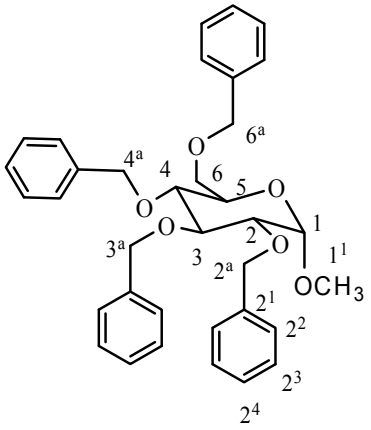
32 — $C_7H_{14}O_6$ — [194.18]

33 — $C_{35}H_{38}O_6$ — [554.67]

C_7H_7Br — [171.04]

3890 mg (20.0 mmol, 1eq) of α-methyl glucoside was taken in a dry flask under argon atmosphere and was dissolved in 100 ml anhydrous DMF. The mass was cooled to -10^{0}C and added 7000 mg (160.0 mmol, 8eq, 2eq per –OH) sodium hydrid (55% dispersion in mineral oil) in portionwise. The reaction mass was warmed to ambient temperature and kept at that temperature for 2hrs. Then the mass was cooled to 5^{0}C, added 370 mg (1.0 mmol, 0.05eq) of tetra butyl ammonium iodide (TBAI) and then 20.5 g (14.2 ml,120.0mmol, 6eq, 1.5 eq per –OH) of Benzyl bromide was added drop wise by syringe. The reaction mass was maintained at ambient temperature for 24hrs.Later, the mass was cooled to 5^{0}C and quenched with 40ml methanol. The crude product was extracted with

300ml EtOAc and 400ml water. The aqueous layer is extracted with 3×200ml EtOAc. The organic layer was combined and washed with 300ml brine. The organic layer was dried over $MgSO_4$ and the solvent was evaporated off till 60^0C /40 mbar to remove DMF. The crude residue was subjected to flash chromatography (silica gel- 40 times of the residue mass) using 80:20 Hexane:EtOAc eluent. The pure fractions were collected and concentrated to afford 10.1g pure of α-Methyl 2,3,4,6-tetra-O-benzyl glucoside. (Yield=91%).



Batch: RV103, RV142, RV148

R_f: (Hexane: EtOAc 8:2): 0.26 (UV $_{254}$ active; $KMnO_4$)

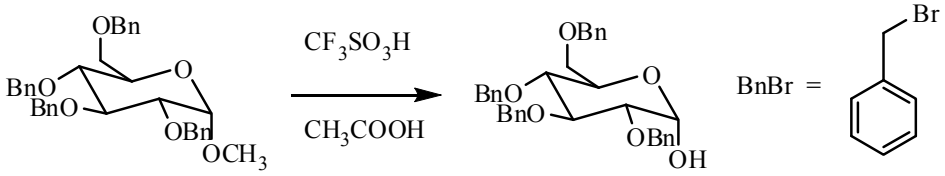
^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.49-7.26 (*m*, 25H, Haromatic); 5.12 (*d*, $^2J_{2a-2a'}$=10.9Hz, 1H, HC(2^a)); 4.97 (*d*, $^2J_{3a-3a'}$=10.8Hz, 1H, HC(3^a)); 4.96 (*d*, $^2J_{2a-2a'}$=10.9Hz, 1H, HC($2^{a'}$)); 4.91 (*d*, $^2J_{4a-4a'}$=12.1Hz, 1H, HC(4^a)); 4.78 (*d*, $^2J_{4a-4a'}$ ≈12.1Hz, 1H, HC($4^{a'}$)); 4.77 (*d*, $^3J_{1-2}$ ≈3.5Hz, 1H, HC(1α)); 4.72 (*d*, $^2J_{6a-6a'}$=12.1Hz, 1H, HC(6^a)); 4.61 (*d*, $^2J_{3a-3a'}$=10.8Hz, 1H, HC($3^{a'}$)); 4.59 (*d*, $^2J_{6a-6a'}$ =12.1Hz, 1H, HC($6^{a'}$); 4.14 (*t*, $^3J_{3-2}$ ≈ 10Hz, $^3J_{3-4}$ ≈ 10Hz, 1H, HC(3α)); 3.90-3.75 (*m*,3H, HC(4α),HC(5α) and HC(6α)); 3.70 (*dd*, $^3J_{2-1}$ ≈ 3.5Hz, $^3J_{2-3}$ ≈10.0Hz ,1H, HC(2α)); 3.50 (*s*,3H, $H_3C(1^1)$.

α/β ratio by ^{1}H-NMR = Only α anomer.

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 139.3-138.5(($C2^1$), ($C3^1$), ($C4^1$)and ($C6^1$)); 128.9-128.1 (($C2^2$,$C2^3$,$C2^4$, $C3^2$,$C3^3$,$C3^4$, $C4^2$,$C4^3$,$C4^4$, $C6^2$,$C6^3$,$C6^4$); 98.7(C(1α)); 82.7 (C(3α)); 80.4(C(2α)); 78.2,70.6 (C(5α) and C(4α)); 69.0(C(6α)); 76.2, 75.5, 74.0, 73.9(C(2^a), C(3^a), C(4^a) and C(6^a); 55.7 (C(1^1)).; **α/β ratio by ^{13}C-NMR** = Only α anomer.

MS (ESI (+)): m/z relative intensity $[M+ Na]^+$ = 577.5

9.2.2.1.1.5. Synthesis of 2,3,4,6-tetra-O-benzyl α/β-D glucose from α-methyl 2,3,4,6-tetra-O-benzyl glucoside

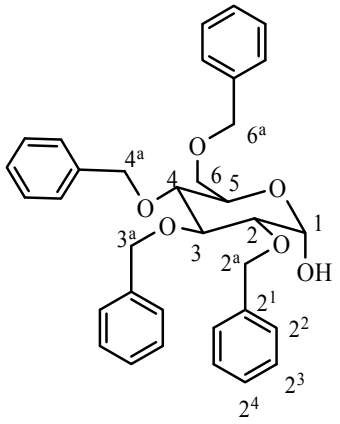


33
$C_{35}H_{38}O_6$
[554.67]

31
$C_{34}H_{36}O_6$
[540.64]

C_7H_7Br
[171.04]

4458 mg (8.03 mmol, 1eq) of α-Methyl 2,3,4,6-Tetra-O-benzyl glucoside was taken in a dry flask under argon atmosphere and was dissolved in 80 ml acetic acid. The solution is heated to 80^0C and then 16ml (32 mmol, 4eq) of 2M aqueous trifluoromethanesulfonic acid was added drop wise. The mass was maintained at 80^0C for 7hrs, cooled and diluted with 150ml CH_2Cl_2. The mixture was washed with 2×100ml saturated $NaHCO_3$ aqueous solution and then with 2×200ml water. The organic layer was dried over $MgSO_4$, concentrated and the residue was taken for crystallisation with minimum quantity of EtOAc which afforded 1.44 g pure 2,3,4,6-Tetra-O-benzyl α/β-D glucose. The filtrate after crystallisation was concentrated and was subjected to flash chromatography which further afforded 1.1 g pure 2,3,4,6-tetra-O-benzyl α/β-D glucose. (Yield= 58.5%)



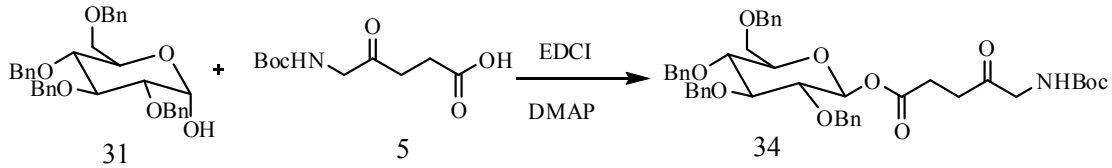
Batch: RV110, RV144, RV149

R_f: (CH_2Cl_2: EtOAc 9:1): 0.38 (UV $_{254}$ active; $KMnO_4$)

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.40-7.16 (*m*, 20H, Haromatic); 5.27 (*d*, $^3J_{1\text{-}2}$=3.5Hz, 1H, HC(1α)); 5.01- 4.50 (*m*, $\underline{H_2}$CPh (α and β) and HC(1β)); 4.10-4.07 (*m*, 1H, HC(5α)); 4.03 (*t*, $^3J_{3\text{-}2}$= 9.3Hz, 1H, HC(3α)); 3.76-3.56 (*m*, 8H, H_2C(6α), H_2C(6β), HC(4α),HC(4β)),HC(5β), HC(3β)); 3.62 (*dd*, $^3J_{1\text{-}2}$=3.6Hz, $^3J_{2\text{-}3}$=9.5Hz, 1H, HC(2α));

α/β ratio by ^{1}H-NMR could not be determined because of peaks overlap.(α anomer= major)

9.2.2.1.1.6. Synthesis of 2′,3′,4′,6′-tetra-O- benzyl 1′-O-(BOC-ALA) β-D-glucose



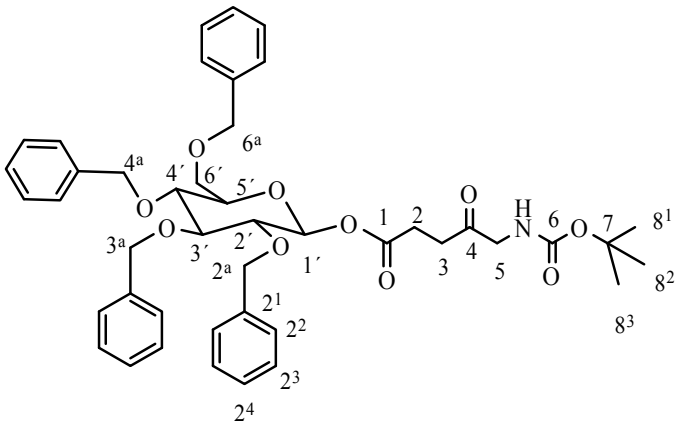
31 5 34

$C_{34}H_{36}O_6$ [540.64]

$C_{10}H_{17}NO_5$ [231.16]

$C_{44}H_{51}NO_{10}$ [753.87]

2440 mg (4.51 mmol, 1eq) of 2,3,4,6-Tetra- O-benzyl glucose (α anomer major ≈ 70%) was taken in a dry flask under argon atmosphere and was dissolved in 50 ml CH_2Cl_2. The solution was cooled to 0^0C, added 607 mg (4.97 mmol, 1.1eq) of DMAP, then 1052 mg (5.49 mmol, 1.2eq) of EDCI and finally 1150 mg (4.97 mmol, 1.1eq) of Boc-ALA acid. The reaction was warmed to ambient temperature and maintained at that temperature for 5 hrs (controlled by TLC, eluent 7:3 Hexane: EtOAc 2 times). The mixture was then diluted with 300ml water, extracted with 2×250ml EtOAc. The organic layer was washed with 300ml 1M citric acid aqueous solution followed by 300ml $NaHCO_3$ aqueous solution and then with 300ml brine. The organic layer was dried over $MgSO_4$, concentrated and the residue was subjected to flash chromatography (silica gel- 70times of the residue mass) which further afforded 3.0 g pure 2′,3′,4′,6′-tetra-O- benzyl 1′-O-(BOC-ALA) α / β-D-glucose (major β anomer; Yield= 88.1%)



Batch: RV 119, RV130, RV138, RV152, VW39

R_f: (Hexane / EtOAc 70:30, 2 times): 0.19 (UV $_{254}$ active; $KMnO_4$)

IR (KBr) : 3426*w*, 3089*w*, 3063*m*, 3031*m*, 2977*s*, 2923*s*, 2869*s*, 2315*w*, 1954*w*, 1876*w*, 1809*w*, 1755*vs*, 1738*vs*, 1713*vs*, 1605*w*, 1586*w*, 1497*s*, 1454*s*, 1392*w*, 1366*s*, 1309*w*, 1250*m*, 1159*s*, 1075*vs*, 1028*m*, 910*w*, 873*w*, 780*w*, 737*s*, 698*s*, 603*w*, 560*w*, 463*w*.

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.38-7.15 (*m*, 20H, Haromatic); 6.35 (*d*, $^3J_{1-2}$=3.5Hz, 1H, HC(1′α)); 5.64 (*d*, $^3J_{1-2}$=8.0Hz, 1H, HC(1′β)); 5.22 (s, 1H, HN); 4.99-4.49 (*m*, $\underline{H_2}$CPh (α and β)) ; 4.05 (*d*, $^3J_{5\text{-}NH}$=4.6Hz, $H_2C(5)$); 3.95 (*t*, $^3J_{3-2}$= 9.3Hz, 1H, HC(3′α)); 3.89 (*m*, 1H, HC(5′α)); 3.79-3.69 (*m*, 6H, HC(3′β), HC(4′α),HC(4′β) , HC(5′β)), $H_2C(6'\alpha)$ and $H_2C(6'\beta)$) ; 3.69-3.66 (*m*, 1H, HC(2′α)); 3.64-3.57 (*m*, 1H,

HC(2′β)); 2.77-2.60 (*m*, 4H ,$H_2C(3)$ and $H_2C(2)$); 1.48 (*s*, 9H, H_3C (8a, 8b and 8c)) ; 4.80, 4.17-4.11, 2.0, 1.29-1.26 (EtOAc solvent impurity).

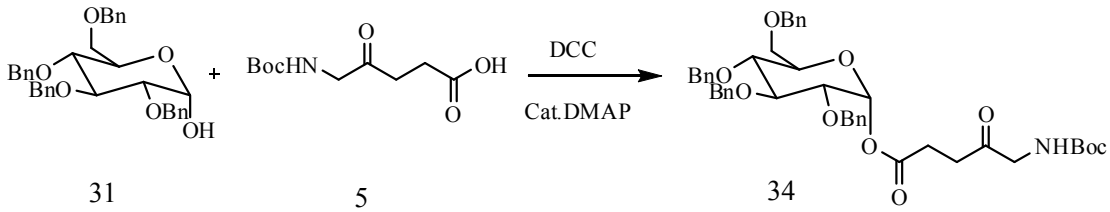
α/β ratio by ^{1}H-NMR = 0.28 : 1

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 204.2 (C(4)); 171.6 (C(1)); 156.1 (C(6)) ; 139.0-138.0 (($C2^1$), ($C3^1$), ($C4^1$) and ($C6^1$) of α and β); 128.9-128.1 (($C2^2$,$C2^3$,$C2^4$, $C3^2$,$C3^3$,$C3^4$, $C4^2$,$C4^3$,$C4^4$, $C6^2$,$C6^3$,$C6^4$) of α and β); 94.77 (C(1′β)); 90.9 (C(1′α)); 82.0 (C(3′α)); 81.4 (C(2′β)); 80.3 (C(7)); 79.2 (C(2′α)); 73.3(C(5′α)); 68.5 (C(6′β) and C(6′α)); 85.2, 77.6, 77.2, 76.0 (C(3′β), C(4′α) C(4′β) and C(5′β)); 76.1, 75.7, 75.4, 74.0, 73.6 ($C(2^a)$, $C(3^a)$, $C(4^a)$ and $C(6^a)$ of both α and β); 50.7 (C(5)); 34.6 (C(3) of α) ;34.4 (C(3) of β) ;28.7 (C(8a,8b and8c); 28.3 (C(2)) and other peaks due to traces of EtOAc solvent.; **α/β ratio by ^{13}C-NMR** ≈ 0.4 : 1

MS (ESI (+)): m/z relative intensity $[M+ Na]^+$ = 776.3

HR-MS $[M+ Na]^+$ = 776.3400 (Calculated =776.3410)

9.2.2.1.1.7. Synthesis of 2′,3′,4′,6′-tetra-O- benzyl 1′-O-(BOC-ALA) α -D-glucose using cat.DMAP & DCC

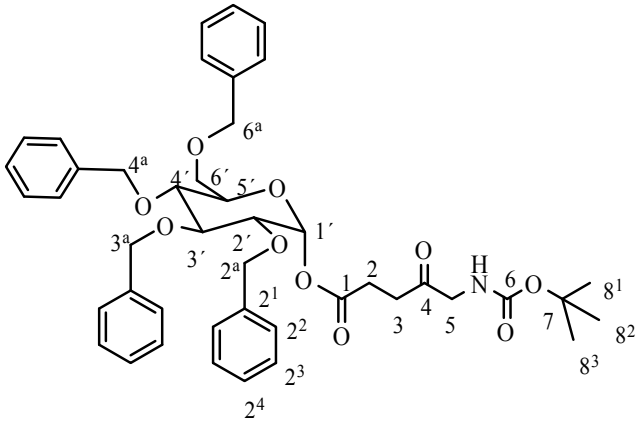


31 $C_{34}H_{36}O_6$ [540.64]

5 $C_{10}H_{17}NO_5$ [231.16]

34 $C_{44}H_{51}NO_{10}$ [753.87]

1520 mg (2.81 mmol, 1eq) of 2,3,4,6-Tetra- O-benzyl glucose (α anomer major ≈ 73%) was taken in a dry flask under argon atmosphere and was dissolved in 50 ml CH_2Cl_2. The solution was cooled to 0^0C, added 1050 mg (4.54 mmol, 1.6eq) of Boc-ALA acid followed by 930 mg (4.50 mmol, 1.6eq) of DCC and finally 10 mg (0.082 mmol, 0.03 eq) of DMAP in a catalytic amount. The reaction was warmed to ambient temperature and maintained at that temperature for 5 hrs (controlled by TLC, eluent 7:3 Hexane: EtOAc 2 times). The mixture was filtered over celite bed and the filtrate was concentrated .The residue was subjected to flash chromatography (silica gel- 70times of the residue mass) which afforded 1931 mg pure 2′,3′,4′,6′-Tetra-O- benzyl 1′-O-(BOC-ALA) α -D-glucose (major α anomer; Yield= 91.1%)



Batch: RV 268, RV269, VW 28

R_f: (Hexane / EtOAc 70:30, 2 times): 0.19 (UV $_{254}$ active; $KMnO_4$)

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.39-7.19 (*m*, 20H, Haromatic); 6.39 (*d*, $^3J_{1\text{-}2}$=3.5Hz, 1H, HC(1´α)); 5.68 (*d*, $^3J_{1\text{-}2}$=8.0Hz, 1H, HC(1´β)); 5.30 (s, 1H, HN); 5.01 (*d*, $^2J_{2a\text{-}2a'}$=10.9Hz, 1H, HC(2^a)); 4.90 (*d*, $^2J_{3a\text{-}3a'}$=10.7Hz, 1H, HC(3^a)); 4.89 (*d*, $^2J_{2a\text{-}2a'}$=10.9Hz, 1H, HC($2^{a'}$)); 4.72 (*d*, $^2J_{4a\text{-}4a'}$=11.5Hz, 1H, HC(4^a)); 4.67 (*d*, $^2J_{4a\text{-}4a'}$ =11.5Hz, 1H, HC($4^{a'}$)); 4.65 (*d*, $^2J_{6a\text{-}6a'}$=12.1Hz, 1H, HC(6^a)) ; 4.57 (*d*, $^2J_{3a\text{-}3a'}$=10.7Hz, 1H, HC($3^{a'}$)); 4.52 (*d*, $^2J_{6a\text{-}6a'}$ =12.1Hz, 1H, HC($6^{a'}$) and other small peaks in this region due to $\underline{H_2}$CPh of β anomer); 4.05 (*d*, $^3J_{5\text{-}NH} \approx$ 4.0Hz, 2H, $H_2C(5)$); 3.99 (*t*, $^3J_{3\text{-}2}$= 9.3Hz, 1H, HC(3´α)); 3.93 (*m*, 1H, HC(5´α)); 3.83 - 3.69 (*m*, 6H, HC(3´β), HC(4´α),HC(4´β) , HC(5´β)), $H_2C(6'\alpha)$, $H_2C(6'\beta)$, HC(2´α)); 3.68-3.61 (*m*, 1H, HC(2´β)); 2.76 - 2.67 (*m*, 4H ,$H_2C(3)$ and $H_2C(2)$); 1.49 (*s*, 9H, H_3C (8a, 8b and 8c)) ; 3.53 – 3.42, 1.50 (peaks due to traces of impurity).

α/β ratio by ^{1}H-NMR = 6.9 : 1

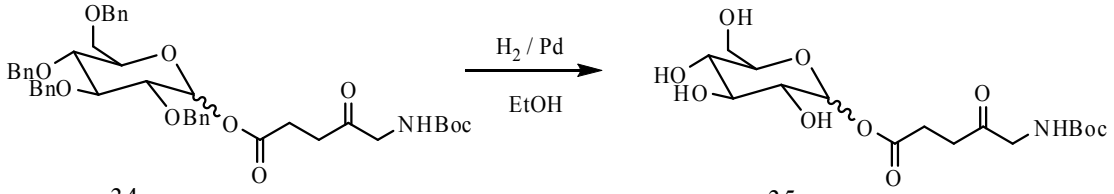
^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 203.9 (C(4)); 171.0 (C(1)); 155.6 (C(6)) ; 138.5-137.5 ((C2^1), (C3^1), (C4^1) and (C6^1) of α and β); 128.4 - 127.6 ((C2^2,C2^3,C2^4, C3^2,C3^3,C3^4, C4^2,C4^3,C4^4, C6^2,C6^3,C6^4) of α and β); 94.3 (C(1´β)); 90.4 (C(1´α)); 81.6 (C(3´α)); 80.8 (C(2´β)); 79.7 (C(7)); 78.7 (C(2´α)); 72.8 (C(5´α)); 68.0 (C(6´α) and C(6´β)); 84.7, 77.1, 76.8, 75.5 (C(3´β), C(4´α) C(4´β) and C(5´β)); 75.6, 75.2, 73.4, 73.1 (C(2^a), C(3^a), C(4^a) and C(6^a) of α anomer); 50.2 (C(5)); 34.1 (C(3)); 28.3 (C(8a,8b and8c); 27.8 (C(2)).

α/β ratio by ^{13}C-NMR ≈ 4.2 : 1

MS (ESI (+)): m/z relative intensity $[M+Na]^+$ = 776.4

HR-MS $[M+ Na]^+$ = 776.3407 (calculated =776.3410)

9.2.2.1.1.8. Synthesis of 1´- O-(BOC-ALA) α / β-D-glucose



34

$C_{44}H_{51}NO_{10}$

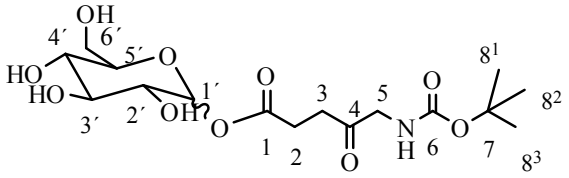
[753.87]

35

$C_{16}H_{27}NO_{10}$

[393.39]

3000 mg (3.98 mmol, 1eq) of 2′,3′,4′,6′-tetra-O- benzyl 1′-O-(BOC-ALA) α /β -D-glucose (to obtain β anomer product as major, starting material having major β anomer was taken and to obtain α-anomer product as major, starting material having major α anomer was taken) was taken in a dry flask under argon atmosphere and was dissolved in 100 ml CH_2Cl_2 and then 50 ml EtOH was added. To this solution was added 4234 mg (3.98 mmol, 1.0eq) of 10% Pd over activated carbon. The reaction mass was agitated under H_2 pressure of 60psi for 12 hrs. Later, the mixture was filtered over celite bed and the filtrate was concentrated .The residue was subjected to flash chromatography (silica gel- 40times of the residue mass) which afforded 1513 mg pure 1-O-(BOC-ALA) α/β -D-glucose (major β anomer; Yield= 96.7%). In the case of α- anomer, the residue after flash chromatography furnished 97% yield composing approximately 65-70% α- anomer which was further subjected to flash chromatography to afford 1′-O-(BOC-ALA) α -D-glucose (~90% α- anomer) with 50% yield.



Batch: RV 120, RV146, RV154 (β anomer); RV 272, RV 288 (α anomer)

R_f: (CH_2Cl_2 / MeOH 8:2): = 0.37 ($KMnO_4$)

Major β- anomer:

IR (KBr) : 3398*s*, 2924*vs*, 2854*m*, 2508*vw*, 2046*vw*, 1730*vs*, 1688*vs*, 1517*m*, 1433*s*, 1368*s*, 1255*s*, 1167*vs*, 1082*s*, 1041*s*, 992*s*, 897*w*, 780*w*, 736*w*, 581*vw*, 464*vw*.

^{1}H-NMR (400 MHz, $CDCl_3$ and CD_3OD, 298 K): δ_H (ppm) = 6.78 (*sbr*, N<u>H</u>); 6.10 (*d*, $^3J_{1'-2'}$=3.5Hz, 1H, HC(1′α)); 5.47 (*d*, $^3J_{1'-2'}$=8.0Hz, 1H, HC(1′β)); 3.94 (*s*, $H_2C(5)$); 3.84 (*dd*, $^3J_{6'a-5'}$ ≈2.0Hz, $^3J_{6'a-6'b}$ ≈12.0Hz 1H, HC(6′a β)); 3.78 (*dd*, $^3J_{6'a-5'}$ ≈2.5Hz, $^3J_{6'a-6'b}$ ≈12.0Hz 1H, HC(6′a α)); 3.73-3.62 (*m*, 4H, HC(6′bα),HC(6′bβ),HC(5′α) and HC(3′α)); 3.58 (*dd*,$^3J_{1'-2'}$≈3.5Hz,$^3J_{2'-3'}$=9.5Hz,1H,HC(2′α));3.43-3.32 (*m*, 5H, HC(2′β), HC(3′β), HC(4′β), HC(5′β) and HC(4′α))); 2.82 - 2.70 (*m*, 4H ,$H_2C(3)$ and $H_2C(2)$); 1.46 (*s*, 9H, H_3C (8a, 8b and 8c)) ; 5.51($C\underline{H_2}Cl_2$)

α/β ratio by ^{1}H-NMR ≈ 0.30 : 1

^{13}C-NMR (100 MHz, $CDCl_3$ and CD_3OD, 298K) : δ_C (ppm) = 207.2 (C(4)); 173.2 (C(1α)); 173.1 (C(1β); 158.4 (C(6)) ; 95.8 (C(1′β)); 93.7 (C(1′α)); 80.6 (C(7)); 78.7, 77.8, 73.9,70.9 (C(2′β), C(3′β), C(4′β) and C(5′β)); 72.2 (C(2′α)); 75.9, 74.8,70.9 ((C(3′α)), C(4′α) and (C(5′α)); 62.2 (C(6′β)); 62.1 (C(6′α); 54.8 ($\underline{C}H_2Cl_2$); 50.7 (C(5)); 34.9 (C(3 of α)); 34.7 (C(3 of β));28.7 (C(8a,8b and8c) and (C(2)).

α/β ratio by ^{13}C-NMR ≈ 0.30 : 1

MS (ESI (+)): m/z relative intensity $[M+ Na]^+$ = 416.1

HR-MS $[M+ Na]^+$ = 416.1528 (calculated = 416.1533)

Major α- anomer:

IR (KBr) : 3410vs, 2978m, 2925m, 1730vs, 1698vs, 1518m, 1393s, 1368s, 1254s, 1167vs, 1085s, 1049m, 1025m, 992m, 896w, 781w, 582vw, 464vw.

^{1}H-NMR (400 MHz, $CDCl_3$ and CD_3OD, 298 K): δ_H (ppm) = 6.68 (*sbr*, N$\underline{H}$); 6.08 (*d*, $^3J_{1′-2′}$=3.5Hz, 1H, HC(1′α)); 5.45 (*d*, $^3J_{1′-2′}$=8.0Hz, 1H, HC(1′β)); 3.93 (*s*, $H_2C(5)$); 3.78 (*dd*, $^3J_{6′a-5′}$ ≈2.5Hz, $^3J_{6′a-6′b}$ ≈12.0Hz 1H, HC(6′α)); 3.73-3.61 (*m*, 3H, HC(6′bα),HC(5′α) and HC(3′α)); 3.55 (*dd*, $^3J_{1′-2′}$ ≈3.5Hz, $^3J_{2′-3′}$ =9.7Hz 1H, HC(2′α)); 3.42 (*t*, $^3J_{3′-4′}$ ≈9.5Hz, $^3J_{3′-4′}$ ≈9.5Hz 1H, HC(4′α)); 2.81 - 2.68 (*m*, 4H ,$H_2C(3)$ and $H_2C(2)$); 1.43 (*s*, 9H, H_3C (8a, 8b and 8c)) ; 5.23-4.81, 4.73-3.97, 1.39-1.23 (peaks due to impurity).

α/β ratio by ^{1}H-NMR ≈ 7 :1

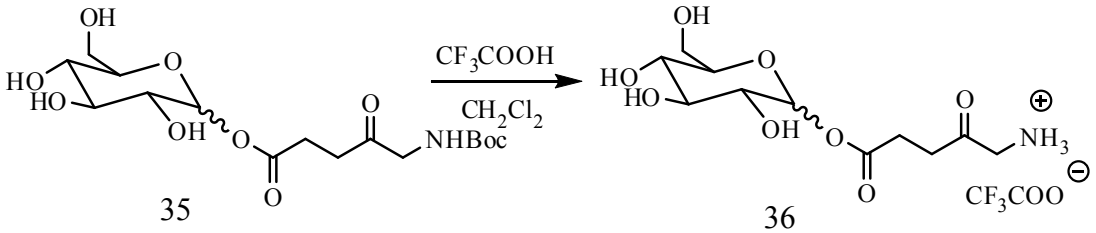
^{13}C-NMR (100 MHz, $CDCl_3$ and CD_3OD, 298K) : δ_C (ppm) = 206.8 (C(4)); 172.9 (C(1)); 157.9 (C(6)) ; 93.5 (C(1′α)); 80.5 (C(7)); 71.8 (C(2′α)); 75.4, 74.4, 70.5 ((C(3′α)), C(4′α) and (C(5′α)); 61.9 (C(6′α); 50.5 (C(5)); 34.7 (C(3)); 28.6 (C(8a,8b and8c) ; 28.5 (C(2)).

α/β ratio by ^{13}C-NMR ≈ 7.7 : 1

MS (ESI (+)): m/z relative intensity $[M+ Na]^+$ = 416.1

HR-MS $[M+ Na]^+$ = 416.1529 (calculated = 416.1533)

9.2.2.1.1.9. Synthesis of 1′- O-(ALA) α / β-D-glucose:



35

$C_{16}H_{27}NO_{10}$

[393.39]

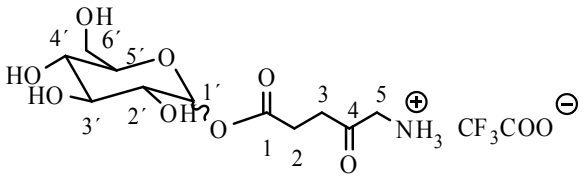
36

$C_{13}H_{20}F_3NO_{10}$

[407.29]

M^+=294.28 ; CF_3COO^- = 113.01

1240 mg (3.15 mmol, 1eq) of 1´-O-(BOC-ALA) α / β- glucose was dissolved in 50ml CH_2Cl_2 and cooled to -10^{0}C. To this solution, 28.7g (19.3ml, 252mmol, 80 eq) of trifluoroacetic acid was added dropwise by syringe. The reaction mass was maintained at 0-5^{0}C for an hour. Once the deprotection of BOC was completed (controlled by the absence of BOC peak in ^{1}H-NMR), CH_2Cl_2 and trifluoroacetic acid was evaporated off. The product was triturated with dry ether and the ether solution was decanted. The trituration with ether and decanting top clear ether solution after centrifuge was repeated 2 times more. Then, the settled solid product was purified by dissolving in 4ml MeOH and precipitated by adding 20ml ether. The mass was centrifuged, the settled solid on drying yielded 818 mg of highly hygroscopic white solid. (Yield=64 % for β anomer and 73% for α anomer).



Batch: RV 125, 146, RV155, RV167 (β anomer); RV 277, RV 293 (α anomer)

Major β- anomer:

IR (KBr) : 3054*s*, 2922*s*, 2681*m*, 2630*w*, 2046*vw*, 1738*vs*, 1688*vs*, 1635*w*, 1577*m*, 1540*w*, 1453*m*, 1433*s*, 1407*m*, 1392*m*, 1381*vw*, 1373*vw*, 1362*w*, 1343*w*, 1276*w*, 1255*s*, 1207*vs* 1181*vs*, 1135*vs*, 1096*w*, 1041*s*, 1000*w*, 966*w*, 944*m*, 907*vw*, 871*w*, 839*s*, 797*s*, 723*vs*, 670*vw*, 604*w*, 560*w*, 519*w*, 500*w*, 462*vw*.

^{1}H-NMR (400 MHz, D_2O, 298 K): δ_H (ppm) = 6.06 (*d*, $^3J_{1'-2'}$=3.5Hz, 1H, HC(1´α)); 5.51 (*d*, $^3J_{1'-2'}$=8.0Hz, 1H, HC(1´β)); 4.10 (*s*, $H_2C(5)$); 3.85 (*dd*, $^3J_{6'a\ -5'}$ ≈2.0Hz, $^3J_{6'a\ -6'b}$ ≈12.0Hz 1H, HC(6´a β)); 3.81- 3.73 (*m*,2H,6´a α and 6´b α); 3.68 (*dd*, $^3J_{6'b\ -5'}$ ≈ 5.4Hz, $^3J_{6'a\ -6'b}$ ≈12.0Hz 1H, HC(6´b β)); 3.56-3.49 (*m*, 2H, HC(3´β) and HC(5´β)); 3.46-3.37 ((*m*, 2H, HC(2´β) and HC(4´β)); 2.85 - 2.68 (*m*, 4H ,$H_2C(3)$ and $H_2C(2)$); other peaks due to ether solvent.

α/β ratio by ^{1}H-NMR ≈ 0.1 : 1

^{13}C-NMR (100 MHz, D_2O, 298K) : δ_C (ppm) = 204.3 (C(4 of α); 203.9 (C(4) of β) ; 173.9 (C(1) of α); 173.6 (C(1) of β) ; 163.1 (*q*,$^2J_{C-F}$ ≈ 36.0, $CF_3\underline{C}OO^-$); 116.6 (*q*,$^1J_{C-F}$ =291.0, $\underline{C}F_3COO^-$); 94.5 (C(1′β)); 92.8 (C(1′α)); 77.1 (C(5′β)); 75.7 (C(3′β)); 72.2 (C(2′β)); 69.4 (C(4′β)); 60.7 (C(6′β)); 47.3 (C(5)); 34.3 (C(3)); 27.7 (C(2)) and peaks due to ether solvent.

α/β ratio by ^{13}C-NMR ≈ 0.15 : 1

MS (ESI (+)): m/z relative intensity $[M\text{-}CF_3COO^-]^+$ = 294.1; $[M\text{-}H+Na]^+$ = 316.2;

HR-MS $[M\text{-}CF_3COO^-]^+$ = 294.1185 (calculated = 294.1189)

Major α- anomer:

$[\alpha]_D$ = +128.1 (c=0.24; deionized H_2O)

IR (KBr) : 3379*vs*, 2929*s*, 2345*vw*, 1789*s*,1734*vs*, 1678*vs*, 1516*m*, 1428*s*, 1384*s*, 1356*m*, 1204*vs* 1190*vs*, 1139*vs*, 1083*s*, 1050*s*, 1029*s*, 992*m*, 957*w*, 905*w*, 839*m*, 801*m*, 775*w*, 723*m*, 598*w*, 552*w*, 521*w*, 464*vw*.

^{1}H-NMR (400 MHz, D_2O, 298 K): δ_H (ppm) = 5.95 (*d*, $^3J_{1'-2'}$=3.5Hz, 1H, HC(1′α)); 5.38 (*d*, $^3J_{1'-2'}$=8.0Hz, 1H, HC(1′β)); 3.97 (*s*, $H_2C(5)$); 3.66 – 3.50 (*m*, 5H, 2′α, 3′α, 5′α, 6′a α and 6′b α); 3.33 (*t*, $^3J_{4'-3'} \approx 9.2$Hz, $^3J_{4'-5'} \approx 9.2$Hz 1H, HC(4′α); 2.81 - 2.65 (*m*, 4H ,$H_2C(3)$ and $H_2C(2)$); other small peaks due to β anomer and ether solvent .

α/β ratio by ^{1}H-NMR ≈ 13 :1

^{13}C-NMR (100 MHz, D_2O, 298K) : δ_C (ppm) = 203.9 (C(4); 173.9 (C(1) ; 163.0 (*q*,$^2J_{C-F}$ ≈ 36.0, $CF_3\underline{C}OO^-$); 116.4 (*q*,$^1J_{C-F}$ ≈ 291.0, $\underline{C}F_3COO^-$); 94.4 (C(1′β)); 92.7 (C(1′α)); 74.2 (C(5′α)); 73.0 (C(3′α)); 70.3 (C(2′α)); 69.0 (C(4′α)); 60.4 (C(6′α)); 47.2 (C(5)); 34.3 (C(3)); 27.6 (C(2)) and other small peaks due to β anomer and ether solvent

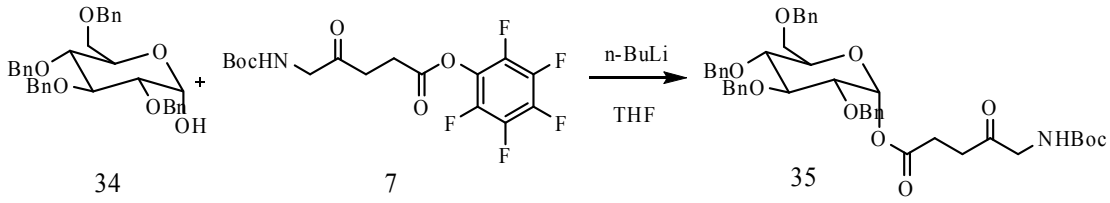
α/β ratio by ^{13}C-NMR ≈ 13 : 1

MS (ESI (+)): m/z relative intensity $[M\text{-}CF_3COO^-]^+$ = 293.9; $[M\text{-}CF_3COO^-+H+Na]^+$ = 316.1; $[M\text{-}CF_3COO^-+H_2O]^+$ = 276.0;

HR-MS $[M\text{-}CF_3COO^-]^+$ = 294.11910 (calculated = 294.1189)

9.2.2.1.2. Synthesis of 1′- O-(ALA) α-D-glucose (using activated ester or azidolevulinic acid)

9.2.2.1.2.1. Synthesis of 2′,3′,4′,6′-tetra-O- benzyl 1′-O-(BOC-ALA) α -D-glucose using BuLi and act.ester

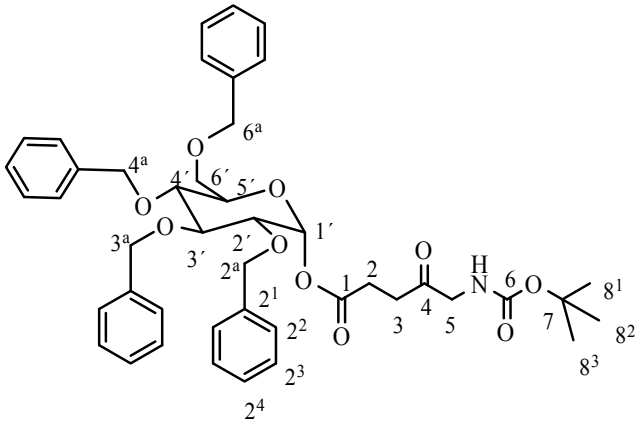


34 $C_{34}H_{36}O_6$ [540.64]

7 $C_{16}H_{16}NO_5F_5$ [397.23]

35 $C_{44}H_{51}NO_{10}$ [753.87]

541 mg (1.0 mmol, 1eq) of 2,3,4,6-tetra- O-benzyl glucose (α anomer major ≈ 73%) was taken in a dry flask under argon atmosphere and was dissolved in 15 ml THF. The solution was cooled to -20^{0}C, added 0.45 ml (1.05 mmol, 1,05eq) of 2.5M n-BuLi solution in hexane, and followed by 440 mg (1.1 mmol, 1.1eq) of Boc-ALA-PFP ester. The reaction was allowed to reach ambient temperature and maintained at that temperature for 1 hr (controlled by TLC, eluent 7:3 Hexane: EtOAc 2 times). The reaction was quenched by adding 20 ml satd.NH_4Cl solution, extracted with 4 × 50 ml CH_2Cl_2 . The organic layer was dried over $MgSO_4$ and was concentrated .The residue was subjected to flash chromatography (silica gel- 80times of the residue mass) which afforded 230 mg pure 2′,3′,4′,6′-Tetra-O- benzyl 1′-O-(BOC-ALA) α -D-glucose (major α anomer; Yield= 30.5%)



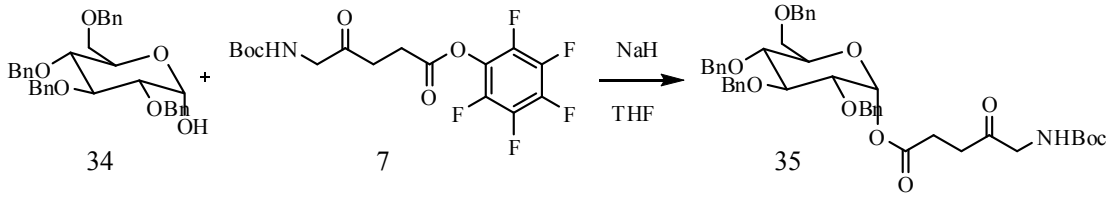
Batch: RV 257

R_f: (Hexane / EtOAc 70:30, 2 times): 0.19 (UV $_{254}$ active; $KMnO_4$)

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.39-7.18 (*m*, 20H, Haromatic); 6.37 (*d*, $^3J_{1\text{-}2}$=3.5Hz, 1H, HC(1′α)); 5.66 (*d*, $^3J_{1\text{-}2}$=8.0Hz, 1H, HC(1′β)); 5.26 (*sbr*, 1H, HN); 5.00 (*d*, $^2J_{2a\text{-}2a'}$=10.9Hz, 1H, HC(2^a)); 4.90 (*d*, $^2J_{3a\text{-}3a'}$=10.6Hz, 1H, HC(3^a)); 4.88 (*d*, $^2J_{2a\text{-}2a'}$=10.9Hz, 1H, HC(2$^{a'}$)); 4.72 (*d*, $^2J_{4a\text{-}4a'}$=11.5Hz, 1H, HC(4^a)); 4.67 (*d*, $^2J_{4a\text{-}4a'}$ =11.5Hz, 1H, HC(4$^{a'}$)); 4.65 (*d*, $^2J_{6a\text{-}6a'}$=12.1Hz, 1H, HC(6^a)); 4.55 (*d*, $^2J_{3a\text{-}3a'}$=10.7Hz, 1H, HC(3$^{a'}$)); 4.51 (*d*, $^2J_{6a\text{-}6a'}$ =12.1Hz, 1H, HC(6$^{a'}$) and other small peaks in this region due to $\underline{H_2}$CPh of β anomer); 4.05 (*d*, $^3J_{5\text{-}NH}$ ≈ 4.6Hz, 2H, $H_2C(5)$); 3.99 (*t*, $^3J_{3\text{-}2}$= 9.3Hz, 1H, HC(3′α)); 3.92 (*m*, 1H, HC(5′α)); 3.83 - 3.69 (*m*, 6H, HC(3′β), HC(4′α),HC(4′β) , HC(5′β)), $H_2C(6'\alpha)$, $H_2C(6'\beta)$, HC(2′α)); 3.68-3.61 (*m*, 1H, HC(2′β)); 2.77 - 2.69 (*m*, 4H ,$H_2C(3)$ and $H_2C(2)$); 1.49 (*s*, 9H, H_3C (8a, 8b and 8c)).

α/β ratio by ^{1}H-NMR = 2.14 : 1

9.2.2.1.2.2. Synthesis of 2′,3′,4′,6′-tetra-O- benzyl 1′-O-(BOC-ALA) α -D-glucose using NaH and act.ester



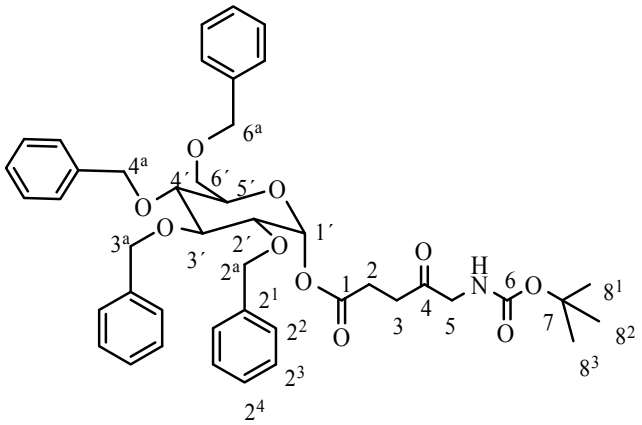
34 7 35

$C_{34}H_{36}O_6$ [540.64]

$C_{16}H_{16}NO_5F_5$ [397.23]

$C_{44}H_{51}NO_{10}$ [753.87]

271 mg (0.5 mmol, 1eq) of 2,3,4,6-tetra- O-benzyl glucose (α anomer major ≈ 73%) was taken in a dry flask under argon atmosphere and was dissolved in 10 ml THF. The solution was cooled to -20^{0}C, added 21 mg (0.525 mmol, 1,05eq) of sodium hydride (60% dispersion in mineral oil). The reaction mixture was brought to 10^{0}C and kept at 10^{0}C for an hour. The mixture was again cooled to -20^{0}C, added 220 mg (0.55 mmol, 1.1eq) of Boc-ALA-PFP ester. The reaction was allowed to reach 0^{0}C (starting material controlled by TLC, eluent 7:3 Hexane: EtOAc 2 times). The reaction was quenched by adding 1 ml MeOH and was concentrated .The residue was subjected to flash chromatography (silica gel- 80times of the residue mass) which afforded 100 mg pure 2′,3′,4′,6′-Tetra-O- benzyl 1′-O-(BOC-ALA) α -D-glucose (major α anomer; Yield= 26.5%)



Batch: RV 258

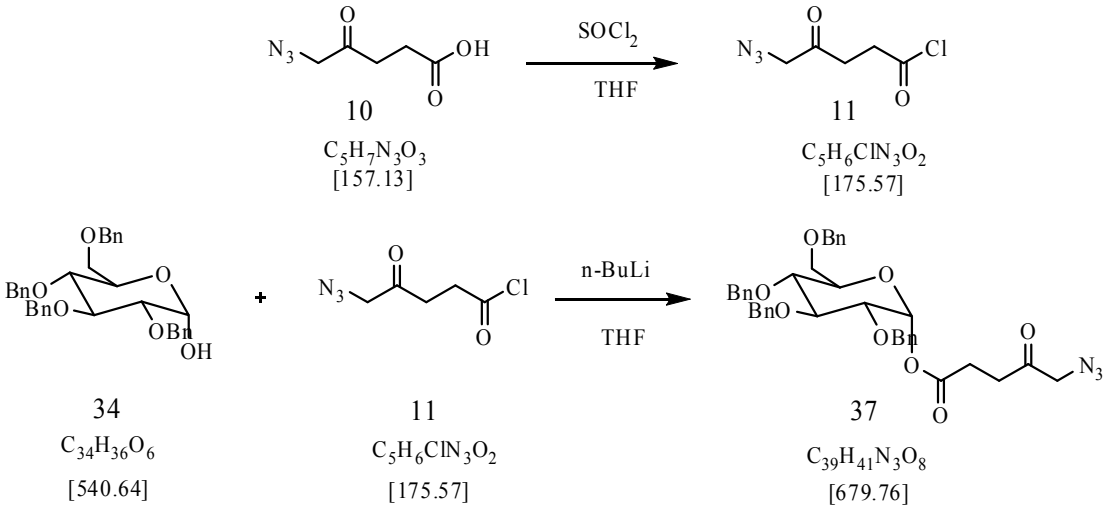
R$_f$: (Hexane / EtOAc 70:30, 2 times): 0.19 (UV $_{254}$ active; $KMnO_4$)

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.35-7.14 (*m*, 20H, Haromatic); 6.37 (*d*, $^3J_{1\text{-}2}$=3.5Hz, 1H, HC(1′α)); 5.66 (*d*, $^3J_{1\text{-}2}$=8.0Hz, 1H, HC(1′β)); 4.96 (*m,* 8H, $\underline{H_2}$CPh of α and β anomer); 4.07 (*d*, $^3J_{5\text{-}NH} \approx$ 4.6Hz, 2H, H_2C(5)); 3.93 (*t*, $^3J_{3\text{-}2}$= 9.3Hz, 1H, HC(3′α)); 3.92 (*m*, 1H, HC(5′α)); 3.83 - 3.69 (*m*, 6H, HC(3′β), HC(4′α),HC(4′β) , HC(5′β)),

$H_2C(6'\alpha)$, $H_2C(6'\beta)$, $HC(2'\alpha)$); 3.68-3.61 (*m*, 1H, $HC(2'\beta)$); 2.78 - 2.69 (*m*, 4H ,$H_2C(3)$ and $H_2C(2)$); 1.46 (*s*, 9H, H_3C (8a, 8b and 8c)); other peaks due to Boc-ALA-Ome impurity.

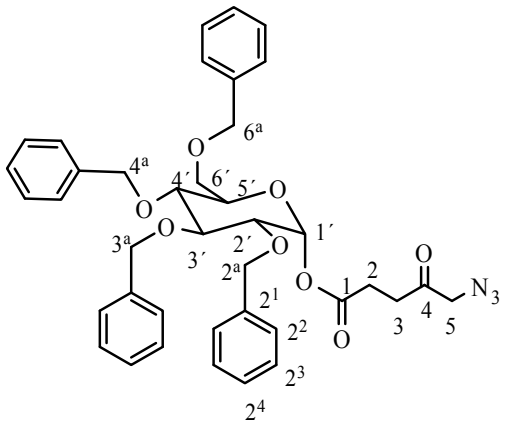
α/β ratio by ^{1}H-NMR = 2.76 : 1

9.2.2.1.2.3. Synthesis of 2′,3′,4′,6′-tetra-O- benzyl 1′-O-(Azidolevulinic) α -D-glucose using BuLi and act.ester



600 mg (3.82 mmol, 1eq) of azidolevulinic acid was taken in a dry flask under argon atmosphere and was dissolved in 10 ml THF. Cooled the solution to 0^0C, added 0.8 ml (11.5 mmol, 3eq) of $SOCl_2$ drop wise via syringe. The mixture was brought to ambient temperature and kept for 2h. Then the solvent THF and excess $SOCl_2$ were evaporated to get azidolevulinic acid chloride. (90% yield assumed 3.44 mmol).

620 mg (1.145 mmol, 1eq) of 2,3,4,6-tetra- O-benzyl glucose (α anomer major ≈ 73%) was taken in a dry flask under argon atmosphere and was dissolved in 15 ml THF. The solution was cooled to -20^0C, added 0.5 ml (1.26 mmol, 1.1eq) of 2.5M n-BuLi solution in hexane, and followed by the above freshly prepared azidolevulinic acid chloride (3.44 mmol, 3eq). The reaction was allowed to reach ambient temperature and maintained at that temperature for 1 hr (controlled by TLC, eluent 7:3 Hexane: EtOAc 2 times). The reaction was quenched by adding 10 ml MeOH and was concentrated .The residue was subjected to flash chromatography (silica gel- 80times of the residue mass) which afforded 267 mg pure 2′,3′,4′,6′-Tetra-O- benzyl 1′-O-(Azidolevulinic) α -D-glucose (major α anomer; Yield= 34.3%)



Batch: RV 260, RV263

R_f: (CH_2Cl_2 / EtOAc 95:5): 0.37 (UV $_{254}$ active; $KMnO_4$)

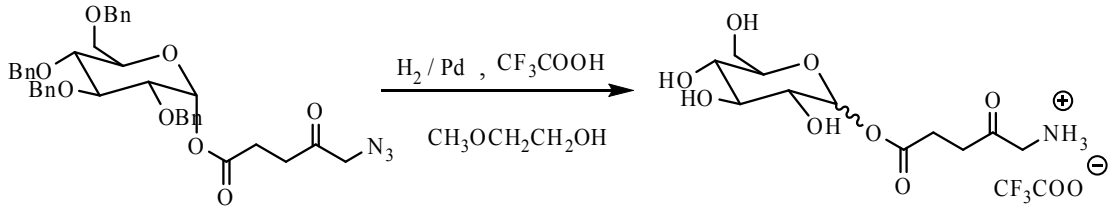
^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.38-7.18 (*m*, 20H, Haromatic); 6.35 (*d*, $^3J_{1\text{-}2}$=3.5Hz, 1H, HC(1´α)); 5.66 (*d*, $^3J_{1\text{-}2}$=8.0Hz, 1H, HC(1´β)); 5.00 (*d*, $^2J_{2a\text{-}2a'}$=10.9Hz, 1H, HC(2^a)); 4.88 (*d*, $^2J_{3a\text{-}3a'}$=10.7Hz, 1H, HC(3^a)); 4.87 (*d*, $^2J_{2a\text{-}2a'}$=10.9Hz, 1H, HC($2^{a'}$)); 4.71 (*d*, $^2J_{4a\text{-}4a'}$=11.5Hz, 1H, HC(4^a)); 4.67 (*d*, $^2J_{4a\text{-}4a'}$ =11.5Hz, 1H, HC($4^{a'}$)); 4.64 (*d*, $^2J_{6a\text{-}6a'}$=12.1Hz, 1H, HC(6^a)); 4.55 (*d*, $^2J_{3a\text{-}3a'}$=10.7Hz, 1H, HC($3^{a'}$)); 4.50 (*d*, $^2J_{6a\text{-}6a'}$ =12.1Hz, 1H, HC($6^{a'}$) and other small peaks in this region due to $\underline{H_2}$CPh of β anomer); 4.00 (*s*, 2H, $H_2C(5)$); 3.99-3.91 (*m*, 2H, HC(3´α) and HC(5´α)); 3.82 - 3.69 (*m*, 6H, HC(3´β), HC(4´α),HC(4´β) , HC(5´β)), $H_2C(6'\alpha)$, $H_2C(6'\beta)$, HC(2´α)); 3.67-3.62 (*m*, 1H, HC(2´β)); 2.78 - 2.67 (*m*, 4H ,$H_2C(3)$ and $H_2C(2)$); 4.14, 3.99, 1.28 (peaks due to solvent and impurity).

α/β ratio by ^{1}H-NMR = 5.6 : 1

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 203.0 (C(4)); 171.0 (C(1)); 138.5-137.5 (($C2^1$), ($C3^1$), ($C4^1$) and ($C6^1$) of α and β); 128.4 - 127.6 (($C2^2$,$C2^3$,$C2^4$, $C3^2$,$C3^3$,$C3^4$, $C4^2$,$C4^3$,$C4^4$, $C6^2$,$C6^3$,$C6^4$) of α and β); 94.3 (C(1´β)); 90.5 (C(1´α)); 81.6 (C(3´α)); 78.7 (C(2´β)); 76.7 (C(2´α)); 72.8 (C(5´α)); 68.0 (C(6´α) and C(6´β)); 75.6, 75.2, 73.4, 73.1 (C(2^a), C(3^a), C(4^a) and C(6^a) of α anomer); 57.3 (C(5)); 34.3 (C(3)); 27.8 (C(2)); 202.5, 172.2, 57.2, 60.8, 14.0 (peaks due to solvent and impurity).

α/β ratio by ^{13}C-NMR ≈ 4.3 : 1

9.2.2.1.2.4. Synthesis of 1´- O-(ALA) α -D-glucose



37

$C_{39}H_{41}N_3O_8$

[679.76]

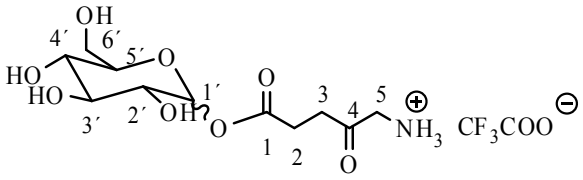
36

$C_{13}H_{20}F_3NO_{10}$

[407.29]

M^+=294.28 ; CF_3COO^- = 113.01

485 mg (0.713 mmol, 1eq) of 2′,3′,4′,6′-tetra-O- benzyl 1′-O-(Azidolevulinic) α -D-glucose was taken in a dry flask under argon atmosphere and was dissolved in 40 ml methoxy ethanol. To this solution was added 760 mg (0.713mmol, 1.0eq) of 10% Pd over activated carbon, followed by 250 mg (0.2 ml, 2.14 mmol, 3eq) of trifluoroacetic acid . The reaction mass was agitated under H_2 pressure of 60psi for 12 hrs. Later, the mixture was filtered over celite bed and the filtrate was concentrated. The product was triturated with dry ether and the ether solution was decanted. The trituration with ether and decanting top clear ether solution after centrifuge was repeated 2 times more. Then, the settled solid product on drying yielded 280 mg of highly hygroscopic white solid. (Yield=96.5 %).



Batch: RV 267

^{1}H-NMR (400 MHz, D_2O, 298 K): δ_H (ppm) = 5.92 (*d*, $^3J_{1'-2'}$=3.5Hz, 1H, HC(1′α)); 5.38 (*d*, $^3J_{1'-2'}$=8.0Hz, 1H, HC(1′β)); 3.96 (*s*, $H_2C(5)$); 3.72 – 3.44 (*m*, 5H, 2′α, 3′α, 5′α, 6′a α and 6′b α); 3.32 (*t*, $^3J_{4'-3'} \approx 9.2$Hz, $^3J_{4'-5'} \approx 9.2$Hz 1H, HC(4′α); 2.81 - 2.68 (*m*, 4H ,$H_2C(3)$ and $H_2C(2)$); 3.96, 3.54, 3.45, 3.27,2.74, 2.61 due to solvent and impurity.

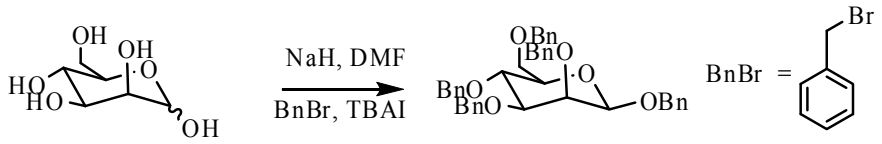
α/β ratio by ^{1}H-NMR ≈ 4 :1

^{13}C-NMR (100 MHz, D_2O, 298K) : δ_C (ppm) = 204.0 (C(4); 174.0 (C(1) ; 163.4 (*q*,$^2J_{C-F}$ ≈ 35.0, $CF_3\underline{C}OO^-$); 116.7 (*q*,$^1J_{C-F}$ ≈ 291.0, $\underline{C}F_3COO^-$); 94.5 (C(1′β)); 92.8 (C(1′α)); 74.3 (C(5′α)); 73.1 (C(3′α)); 70.4 (C(2′α)); 69.1 (C(4′α)); 60.5 (C(6′α)); 57.8 (C(5)); 34.5 (C(3)); 27.7 (C(2)); 204.3, 175.3, 73.4, 58.3, 60.5, 47.4, 34.6, 27.9, 17.2 (peaks due to solvent and impurity).

α/β ratio by ^{13}C-NMR ≈ 4 :1

9.2.2.2. Synthesis of 1´- O-(ALA) -α-D-mannose

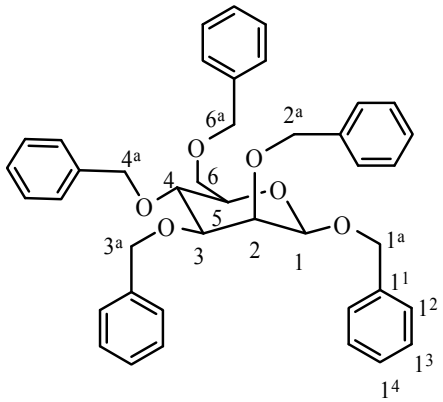
9.2.2.2.1. Synthesis of 1,2,3,4,6-Penta- O-benzyl β-mannose (penta benzyl mannose)



24 38

$C_6H_{12}O_6$ [180.16] $C_{41}H_{42}O_6$ [630.77] C_7H_7Br [171.04]

2162 mg (12.0 mmol, 1eq) of mannose was taken in a dry flask under argon atmosphere and was dissolved in 80 ml anhydrous DMF. The mass was cooled to -20^{0}C and added 4800 mg (120.0 mmol, 10eq, 2eq per –OH) sodium hydride (60% dispersion in mineral oil) in portionwise. The reaction mass was warmed to ambient temperature and kept at that temperature for 2hrs. Then the mass was cooled to 5^{0}C, added 222 mg (0.6 mmol, 0.05eq) of tetra butyl ammonium iodide (TBAI) and then 20.52 g (14.2 ml, 120.0mmol, 10eq, 2 eq per –OH) of Benzyl bromide was added drop wise by syringe. The reaction mass was maintained at ambient temperature for 48hrs.Later, the mass was cooled to 5^{0}C and quenched with 40ml methanol. The crude product was extracted with 200ml EtOAc and 200ml water. The aqueous layer is extracted with 3×200ml EtOAc. The organic layer was combined and washed with 200ml brine. The organic layer was dried over $MgSO_4$ and the solvent was evaporated off till 60^{0}C /40 mbar to remove DMF. The crude residue was subjected to flash chromatography (silica gel- 50 times of the residue mass) using gradient eluent from 100% hexane to 70:30 Hexane: Ether. The pure fractions were collected and concentrated to afford 3.91g pure 1,2,3,4,6-Penta- O-benzyl β-mannose (only β anomer). (Yield=52%).



Batch: RV219, VW20, RV273

R_f: (Hexane / Ether 70:30): 0.21 (UV $_{254}$ active; $KMnO_4$)

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.49-7.20 (*m*, 25H, Haromatic); 5.04 (*d*, $^2J_{1a\text{-}1a'}$=12.5Hz, 1H, HC(1^a)); 5.03 (*d*, $^2J_{2a\text{-}2a'}$=12.0Hz, 1H, HC(2^a)); 4.94 (*d*, $^2J_{3a\text{-}3a'}$=10.8Hz, 1H, HC(3^a)); 4.92 (*d*, $^2J_{1a\text{-}1a'}$ ≈11.9Hz, 1H, HC(1$^{a'}$)); 4.69 (*d*, $^2J_{4a\text{-}4a'}$=12.1Hz,

1H, HC(4^a)); 4.63 (*d*, $^2J_{2a\text{-}2a'}$ ≈12.0Hz, 1H, HC($2^{a'}$)); 4.62 (*d*, $^2J_{4a\text{-}4a'}$ =12.1Hz, 1H, HC($4^{a'}$)); 4.57 (*d*, $^2J_{3a\text{-}3a'}$=10.8Hz, 1H, HC($3^{a'}$)); 4.53 (*d*, $^2J_{6a\text{-}6a'}$=11.9Hz, 1H, HC(6^a)); 4.46 (*d*, $^2J_{6a\text{-}6a'}$ ≈11.9Hz, 1H, HC($6^{a'}$); 4.70-4.45 (*m*, 1H, HC(1β)); 3.95-3.90 (*m*,1H, HC(2β)); 3.93 (*t*, $^3J_{3\text{-}4}$=9.8Hz, $^3J_{4\text{–}5}$ ≈ 9.8Hz,1H, HC(4β)); 3.87 (*dd*, $^3J_{5\text{-}6}$ ≈ 2.0Hz, $^3J_{6\text{–}6'}$ ≈11.0Hz ,1H, HC(6β)); 3.81 (*dd*, $^3J_{5\text{-}6}$ ≈ 6.0 Hz, $^3J_{6\text{–}6'}$ =11.0Hz ,1H, HC(6′β)); 3.52 (*dd*, $^3J_{2\text{-}3}$ ≈ 3.0Hz, $^3J_{3\text{-}4}$ ≈9.8Hz ,1H, HC(3β)); 3.49 (*ddd*, $^3J_{5\text{-}6}$ ≈ 2.0Hz, $^3J_{5\text{-}6'}$ ≈6.0Hz , $^3J_{5\text{-}4}$ ≈9.8Hz 1H, HC(5β)).

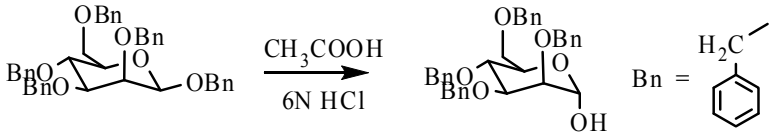
α/β ratio by ^{1}H-NMR could not be determined because of peaks overlap.

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 138.8-137.6 (($C1^1$), ($C2^1$), ($C3^1$), ($C4^1$) and ($C6^1$) α and β); 128.4-127.5 (($C1^2$,$C1^3$,$C1^4$, $C2^2$,$C2^3$,$C2^4$, $C3^2$,$C3^3$,$C3^4$, $C4^2$,$C4^3$,$C4^4$, $C6^2$,$C6^3$,$C6^4$) of α and β);100.4 (C(1β)); 82.5, 76.1, 75.1, 73.9 (C(2β), C(3β), C(4β), C(5β)); 75.2, 74.0, 73.6, 71.6, 70.9 (C(1^a), C(2^a), C(3^a), C(4^a), C(6^a)); 69.8 (C(6β))

α/β ratio by ^{13}C-NMR Only β anomer

MS (ESI (+)): m/z relative intensity $[M+ Na]^+$ = 653.6

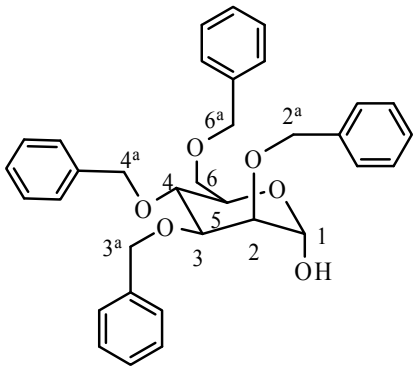
9.2.2.2.2. Synthesis of 2,3,4,6-tetra-O-benzyl α-D mannose



38
$C_{41}H_{42}O_6$
[630.77]

39
$C_{34}H_{36}O_6$
[540.64]

850 mg (1.35 mmol, 1eq) of 1,2,3,4,6-Penta- O-benzyl β-mannose was taken in a dry flask and was dissolved in 20 ml acetic acid. Then 3ml of 6N HCl was added. The solution is heated to 60^0C. The mass was maintained at 60^0C and was taken for workup once the starting material was absent by TLC (approximately 4 to 6hrs, TLC eluent = Hexane: EtOAc 8:2). The mixture was diluted with 50ml water, extracted with 3×75ml CH_2Cl_2. The organic layer was washed with 100ml saturated $NaHCO_3$ aqueous solution and then with 100ml water. The organic layer was dried over $MgSO_4$, concentrated and the residue was subjected to flash chromatography (silica gel- 60 times of the residue mass) which further afforded 517 mg pure 2,3,4,6-tetra-O-benzyl α -D mannose (major α anomer; Yield= 70.8%)



Batch: RV226, VW23, VW44

R_f: (Hexane : EtOAc 6:4): 0.28 (UV $_{254}$ active; $KMnO_4$)

IR (KBr) : 3400*s*, 3087*m*, 3063*m*, 3030*s*, 3006*w*, 2918*s*, 2866*s*, 1952*vw*, 1877*vw*, 1811*vw*, 1737*w*, 1605*w*, 1586*vw*, 1546*vw*, 1496*s*, 1453*vs*, 1393*m*, 1364*s*, 1265*m*, 1244*m*, 1208*s*, 1095*vs*, 1027*vs*, 910*m*, 843*m*, 820*w*, 797*w*, 736*w*, 697*w*, 607*m*, 528*vw*, 486*vw*, 464*vw*.

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.43-7.21 (*m*, 20H, Haromatic); 5.28(*d*, $^3J_{1\text{-}2} \approx 2.0$Hz, 1H, HC(1α)); 4.93 (*d*, $^2J_{2a\text{-}2a'}$=10.9Hz, 1H, HC(2^a)); 4.78 (*d*, $^2J_{3a\text{-}3a'}$=12.5Hz, 1H, HC(3^a)); 4.74 (*d*, $^2J_{3a\text{-}3a'}$≈12.5Hz, 1H, HC($3^{a'}$)); 4.67 (*d*, $J_{1\text{-}2}$ =1.1Hz 1H, HC(1β)); 4.61 (*d*, $^2J_{4a\text{-}4a'}$=12.1Hz, 1H, HC(4^a)); 4.59 (*d*, $^2J_{2a\text{-}2a'}$ ≈10.9Hz, 1H, HC($2^{a'}$)); 4.58 (*d*, $^2J_{6a\text{-}6a'}$=11.5Hz, 1H, HC(6^a)); 4.56 (*d*, $^2J_{4a\text{-}4a'}$ =12.1Hz, 1H, HC($4^{a'}$)); 4.55 (*d*, $^2J_{6a\text{-}6a'}$ =11.5 Hz, 1H, HC($6^{a'}$); 4.13-4.08 (*m*,1H, HC(5α)); 4.02 (*dd*, $^3J_{2\text{-}3} \approx 3.0$Hz, $^3J_{3\text{-}4}$ ≈9.8Hz ,1H, HC(3α)); 3.90 (*t*, $^3J_{3\text{-}4}$=9.8Hz, $^3J_{4\text{–}5} \approx 9.8$Hz,1H, HC(4α)); 3.83(*dd*, $^3J_{1\text{-}2} \approx 2.0$Hz, $^3J_{2\text{-}3}$ ≈3.0Hz ,1H, HC(2α)); 3.76 (*dd*, $^3J_{5\text{-}6} \approx 2.0$Hz, $^3J_{6\text{–}6'}$ ≈11.0Hz ,1H, HC(6α)); 3.71 (*dd*, $^3J_{5\text{-}6}$ ≈ 6.5 Hz, $^3J_{6\text{–}6'}$ ≈11.0Hz ,1H, HC(6′β)); 3.60 (*dd*, $^3J_{2\text{-}3} \approx 3.0$Hz, $^3J_{3\text{-}4}$ ≈9.8Hz ,1H, HC(3β)); 4.65 (*s*, impurity); 4.19-4.13*q*, 2.08*s*, 1.3*t* (peaks due to EtOAc solvent)

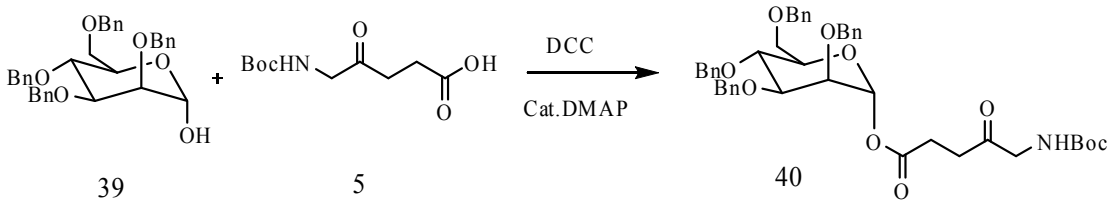
α/β ratio by ^{1}H-NMR could not be determined because of peaks overlap. (α anomer= major)

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 138.5-137.9 (($C2^1$), ($C3^1$), ($C4^1$) and ($C6^1$) α and β); 128.7-127.0 (($C2^2$,$C2^3$,$C2^4$, $C3^2$,$C3^3$,$C3^4$, $C4^2$,$C4^3$,$C4^4$, $C6^2$,$C6^3$,$C6^4$) of α and β); 94.0 (C(1β)); 92.6(C(1α)); 83.0(C(3β)); 79.8(C(3α)); 75.3(C(4α)); 75.00(C(2α)); 71.3(C(5α)); 75.1, 73.3, 72.6, 72.1 (($C(2^a)$, $C(3^a)$, $C(4^a)$, $C(6^a)$); 69.7 (C(6α)); 171.3, 60.5, 21.0, 14.2 (peaks due to EtOAc solvent)

α/β ratio by ^{13}C-NMR ≈ 5.2 : 1

MS (ESI (+)): m/z relative intensity $[M+ Na]^+ = 563.3$; $[M+ H]^+ = 541.1$

9.2.2.2.3. Synthesis of 2′,3′,4′,6′-tetra-O- benzyl 1′-O-(BOC-ALA) α -D-mannose

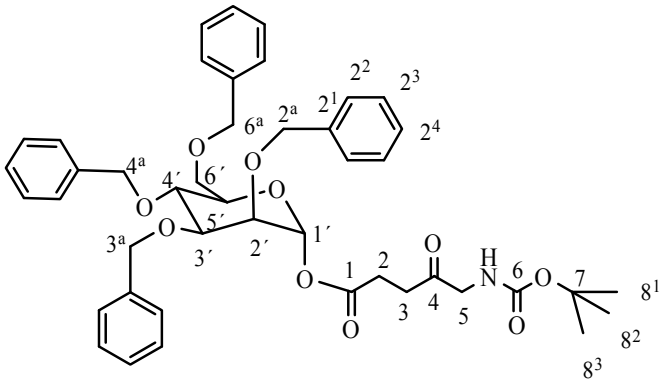


39 $C_{34}H_{36}O_6$ [540.64]

5 $C_{10}H_{17}NO_5$ [231.16]

40 $C_{44}H_{51}NO_{10}$ [753.87]

2000 mg (3.70 mmol, 1eq) of 2,3,4,6-tetra- O-benzyl α-mannose (α anomer major ≈ 85%) was taken in a dry flask under argon atmosphere and was dissolved in 50 ml CH_2Cl_2. The solution was cooled to 0^0C, added 1370 mg (5.93 mmol, 1.6eq) of Boc-ALA acid followed by 1225 mg (5.93 mmol, 1.6eq) of DCC and finally 10 mg (0.082 mmol, 0.02 eq) of DMAP in a catalytic amount. The reaction was warmed to ambient temperature and maintained at that temperature for 5 hrs (controlled by TLC, eluent 7:3 Hexane: EtOAc 2 times). The mixture was filtered over celite bed and the filtrate was concentrated .The residue was subjected to flash chromatography (silica gel- 70times of the residue mass) which afforded 2292 mg pure 2′,3′,4′,6′-tetra-O- benzyl 1′-O-(BOC-ALA) α -D-mannose (major α anomer; Yield= 82.2%)



Batch: RV 270, VW 25, RV231

R_f: (Hexane / EtOAc 70:30, 2 times): 0.15 (UV $_{254}$ active; $KMnO_4$)

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.41-7.15 (*m*, 20H, Haromatic); 6.22 (*d*, $^3J_{1\text{-}2}$=1.9Hz, 1H, HC(1′α)); 5.60 (*m*, 1H, HC(1′β)); 5.18 (s, 1H, HN); 4.90 (*d*, $^2J_{2a\text{-}2a'}$=10.7Hz, 1H, HC(2^a)); 4.78 (*d*, $^2J_{3a\text{-}3a'}$=12.3Hz, 1H, HC(3^a)); 4.73 (*d*, $^2J_{3a\text{-}3a'}$=12.3Hz, 1H, HC($3^{a'}$)); 4.67 (*d*, $^2J_{4a\text{-}4a'}$=12.1Hz, 1H, HC(4^a)); 4.63 (*d*, $^2J_{6a\text{-}6a'}$=12.0Hz, 1H, HC(6^a)); 4.59 (*d*, $^2J_{6a\text{-}6a'}$ =12.0Hz, 1H, HC($6^{a'}$); 4.55(*d*, $^2J_{2a\text{-}2a'}$≈10.7Hz, 1H, HC($2^{a'}$)); 4.54 (*d*, $^2J_{4a\text{-}4a'}$ ≈12.1Hz, 1H, HC($4^{a'}$));and other small peaks in this region due to $\underline{H_2}$CPh of β anomer); 4.08 ((*t*, $^3J_{3\text{-}4}$= 9.6Hz, 1H, HC(4′α)); 4.03 (*d*, $^3J_{5\text{-}NH}$ =5.0Hz, $H_2C(5)$); 3.87 (*d*, $^3J_{2\text{-}3}$≈3Hz, $^3J_{3\text{-}4}$=9.4Hz, 1H, HC(3′α); 3.89-3.84 (*m*, 1H, HC(5′α); 3.80 - 3.76 (*m*, 2H, HC(2′α) and HC(6′α); 2.79 - 2.57 (*m*, 4H ,$H_2C(3)$ and $H_2C(2)$); 1.45 (*s*, 9H, H_3C (8a, 8b and 8c)); 4.16-4.10*q*, 2.05*s*, 1.27*t* (peaks due to EtOAc solvent).

α/β ratio by ^{1}H-NMR ≈ 5 : 1

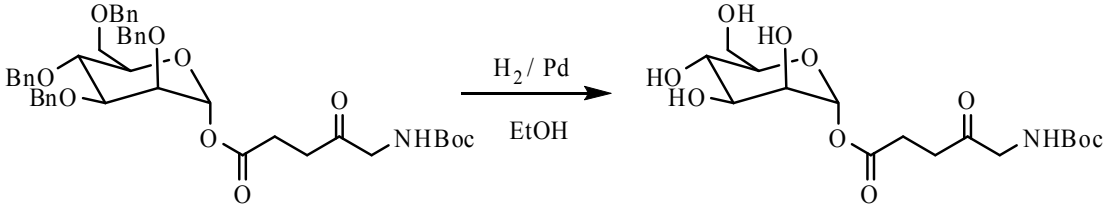
^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 204.1 (C(4)); 171.1 (C(1β)); 170.7 (C(1α)); 155.7 (C(6)) ; 138.4-137.9 ((C2^1), (C3^1), (C4^1) and (C6^1) of α and β); 128.5 - 127.6 ((C2^2,C2^3,C2^4, C3^2,C3^3,C3^4, C4^2,C4^3,C4^4, C6^2,C6^3,C6^4) of α and β); 93.6 (C(1′β)); 92.4 (C(1′α)); 80.0 (C(7)); 79.2 ((C(3′α) or (C(5′α)); 74.5 ((C(3′α) or (C(5′α)); 74.2 (C(4′α)); 73.4 (C(2′α)); 68.9 (C(6′α)); 75.4, 73.5, 72.6, 72.1 (C(2^a), C(3^a), C(4^a) and C(6^a) of α anomer); 50.3 (C(5)); 34.2 (C(3)); 28.4 (C(8a,8b and8c); 27.8 (C(2)).

α/β ratio by ^{13}C-NMR ≈ 7.5 : 1

MS (ESI (+)): m/z relative intensity $[M+ Na]^+$ = 776.3

HR-MS $[M+ Na]^+$ = 776.3409 (calculated =776.3410)

9.2.2.2.4. Synthesis of 1-O-(BOC-ALA) α-D-mannose



40

$C_{44}H_{51}NO_{10}$

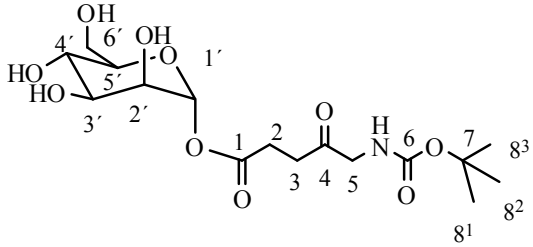
[753.87]

41

$C_{16}H_{27}NO_{10}$

[393.39]

2240 mg (2.97 mmol, 1eq) of 2′,3′,4′,6′-tetra-O- benzyl 1′-O-(BOC-ALA) α -D-mannose was taken in a dry flask under argon atmosphere and was dissolved in 60 ml CH_2Cl_2 and then 20 ml EtOH was added. To this solution was added 3160 mg (2.97 mmol, 1.0eq) of 10% Pd over activated carbon. The reaction mass was agitated under H_2 pressure of 60psi for 12 hrs. Later, the mixture was filtered over celite bed and the filtrate was concentrated .The residue was subjected to flash chromatography (silica gel-40times of the residue mass) which afforded 1000 mg pure 1-O-(BOC-ALA) α -D-glucose (major α anomer; Yield= 85.6%).



Batch: RV 274

R_f: (CH_2Cl_2 / MeOH 8:2): = 0.38 ($KMnO_4$)

IR (KBr) : 3435vs, 2992*vw*, 2978*vw*, 2950*vw*, 2933*m*, 2942*vw*, 2536*w*, 2495*w*, 1731*vs*, 1682*vs*, 1518*vs*, 1461*vw*, 1423*w*, 1408*vw*, 1392*w*, 1368*s*, 1349*w*, 1335*vw*, 1248*s*, 1183*m*, 1139*s*, 1116*vw*, 1097*m*, 1075*w*, 1055*m*, 1043*m*, 1025*vw*, 1014*vw*, 990*m*, 973*s*, 950*vw*, 899*w*, 870*w*, 828*w*, 815*vw*, 800*m*, 781*vw*, 767*vw*, 684*vw*, 667*vw*, 649*vw*, 585*w*, 566*vw*, 538*w*, 501*vw*, 460*vw*.

^{1}H-NMR (400 MHz, $CDCl_3$ and CD_3OD, 298 K): δ_H (ppm) = 5.99 (*d*, $^3J_{1'-2'}$=1.7Hz, 1H, HC(1′α)); 4.75 (*d*, $^3J_{1'-2'}$=1.6Hz, 1H, HC(1′β)); 3.92 (*s*, $H_2C(5)$); 3.78 (*dd*, $^3J_{6'a-5'}$ ≈2.5Hz, $^3J_{6'a-6'b}$ ≈12.0Hz 1H, HC(6′a α)); 3.76-3.60 (*m*, 5H, HC(6′b α), HC(5′α), (4′α), HC(3′α)) and HC(2′α)); 2.81 - 2.63 (*m*, 4H ,$H_2C(3)$ and $H_2C(2)$); 1.44 (*s*, 9H, H_3C (8a, 8b and 8c)).

α/β ratio by ^{1}H-NMR = 8:1

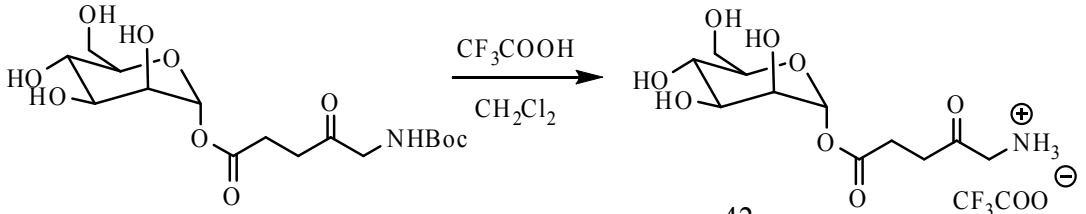
^{13}C-NMR (100 MHz, $CDCl_3$ and CD_3OD, 298K) : δ_C (ppm) = 207.1 (C(4)); 172.3 (C(1)); 158.1 (C(6)) ; 101.0 (C(1′β); 95.3 (C(1′α)); 80.5 (C(7));76.6, 71.9, 70.6, 67.6(C(2α), (C(3′α)), C(4′α) and (C(5′α)); 62.3 (C(6′α); 50.5 (C(5)); 34.7 (C(3)); 28.6 (C(8a,8b and8c) ; 28.5 (C(2)).

α/β ratio by ^{13}C-NMR ≈ 16:1

MS (ESI (+)): m/z relative intensity $[M+ Na]^+$ = 416.1; $[M-H+H_2O]^-$ =428.1

HR-MS $[M+ Na]^+$ = 416.1524 (calculated = 416.1533)

9.2.2.2.5. Synthesis of 1-O-(ALA) α-D-mannose



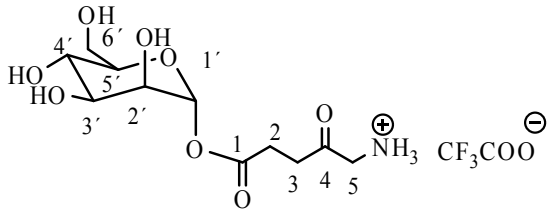
41
$C_{16}H_{27}NO_{10}$
[393.39]

42
$C_{13}H_{20}F_3NO_{10}$
[407.29]

M^+=294.28 ; CF_3COO^- = 113.01

850 mg (2.16 mmol, 1eq) of 1′-O-(BOC-ALA) mannose was dissolved in 30ml CH_2Cl_2 and cooled to -10^{0}C. To this solution, 12.38 g (8.3ml, 108mmol, 50 eq) of trifluoroacetic acid was added dropwise by syringe. The reaction mass was maintained at 0-5^{0}C for 2h. Once the deprotection of BOC was completed (controlled by the absence of BOC peak in ^{1}H-NMR), CH_2Cl_2 and trifluoroacetic acid was evaporated off. The product was co-evaporated with dry ether, triturated with dry ether and the ether solution was decanted.

The trituration with ether and decanting top clear ether solution after centrifuge was repeated 2 times more. Then, the settled solid on drying yielded 795 mg of highly hygroscopic white solid. (Yield=90.3 %).



Batch: RV 276

$[\alpha]_D$ = +228.7 (c=0.12; deionized H_2O)

IR (KBr) : 3392*vs*, 2943*w*, 2527*vw*, 1731*m*, 1677*vs*, 1516*vw*, 1428*m*, 1203*vs*, 1137*vs*, 1068*m*, 960*vw*, 840*w*, 801*m*, 723*m*, 600*vw*, 518*vw*.

^{1}H-NMR (400 MHz, D_2O, 298 K): δ_H (ppm) = 5.77 (*d*, $^3J_{1'-2'}$=1.8Hz, 1H, HC(1′α)); 4.66 (*d*, $^3J_{1'-2'}$=1.5Hz, 1H, HC(1′β)); 3.92 (*s*, $H_2C(5)$); 3.78 (*dd*, $^3J_{1'-2'}$ ≈1.8Hz, $^3J_{2'-3'}$ =3.3Hz 1H, HC(2′α)); 3.71 (*dd*, $^3J_{1'-2'}$ ≈1.8Hz, $^3J_{2'-3'}$ =3.4Hz 1H, HC(2′β)); 3.67-3.59 (*m*, 2H, HC(3α) and HC(6′a α)); 3.57-3.49 (*m*, 3H, HC(6′b α), HC(5′α) and HC(4′α)); 2.74 (*t*, $^3J_{2-3}$ ≈6.5Hz, 2H, $H_2C(3)$) ; 2.57 (*t*, $^3J_{3-2}$ ≈ 6.5Hz, 2H, $H_2C(2)$) ; 1.03- 0.94 (peaks due to traces impurity).

α/β ratio by ^{1}H-NMR ≈ 14:1

^{13}C-NMR (100 MHz, D_2O, 298K) : δ_C (ppm) = 203.9 (C(4)) ; 173.1 (C(1)) ; 162.7 (*q*,$^2J_{C-F}$ ≈ 36.0, $CF_3\underline{C}OO^-$); 116.3 (*q*,$^1J_{C-F}$ ≈ 290.0, $\underline{C}F_3COO^-$); 99.6 (C(1′β)); 94.2 (C(1′α)); 69.1 (C(2′α)); 60.8(C(6′α)); 75.1, 70.4, 66.3 (C(3′α), C(5′α) and C(4′α)); 47.2 (C(5)); 34.3 (C(3)); 27.5 (C(2)).

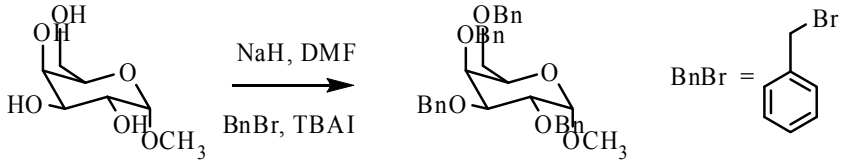
α/β ratio by ^{13}C-NMR ≈ 15 : 1

MS (ESI (+)): m/z relative intensity $[M\text{-}CF_3COO^-]^+$ = 294.1; $[M\text{- }CF_3COO^-\text{+H+Na}]^+$ = 316.2.

HR-MS $[M\text{- }CF_3COO^-]^+$ = 294.1184 (calculated = 294.1189)

9.2.2.3. Synthesis of 1′- O-(ALA) -β-D-galactose

9.2.2.3.1. Synthesis of α-methyl 2,3,4,6-tetra-O-benzyl galactoside

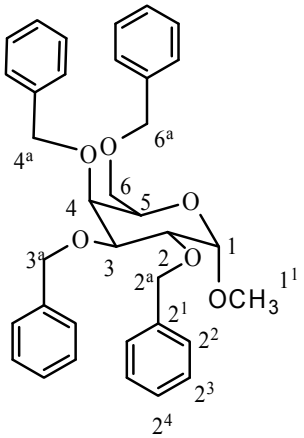


43 $C_7H_{14}O_6$ [194.18]

44 $C_{35}H_{38}O_6$ [554.67]

C_7H_7Br [171.04]

2500 (12.87mmol, 1eq) of α-methyl galactoside was taken in a dry flask under argon atmosphere and was dissolved in 140 ml anhydrous DMF. The mass was cooled to -10^{0}C and added 3370 mg (77.2 mmol, 6eq, 1.5eq per –OH) sodium hydride (55% dispersion in mineral oil) in portion wise. The reaction mass was warmed to ambient temperature and kept at that temperature for 2hrs. Then the mass was cooled to 5^{0}C, added 240 mg (0.65 mmol, 0.05eq) of tetra butyl ammonium iodide (TBAI) and then 13.2g (9.2 ml, 77.2 mmol, 6eq, 1.5eq per –OH) of Benzyl bromide was added drop wise by syringe. The reaction mass was maintained at ambient temperature for 24hrs.Later, the mass was cooled to 5^{0}C and quenched with 40ml methanol. The crude product was extracted with 300ml EtOAc and 400ml water. The aqueous layer is extracted with 3×200ml EtOAc. The organic layer was combined and washed with 300ml brine. The organic layer was dried over $MgSO_4$ and the solvent was evaporated off till 60^{0}C /40 mbar to remove DMF. The crude residue was subjected to flash chromatography (silica gel- 50 times of the residue mass) using 70:30 Hexane:EtOAc eluent. The pure fractions were collected and concentrated to afford 6.35g pure of α-methyl 2,3,4,6-tetra-O-benzyl galactoside. (Yield=89%).



Batch: RV271, RV287, RV298, VW 51

R_f: (Hexane: EtOAc 7:3): 0.33 (UV $_{254}$ active; $KMnO_4$)

IR (KBr) : 3445*w*, 3087*vw*, 3063*w*, 3030*m*, 2917*s*, 2867*m*, 1951*vw*, 1874*vw*, 1809*vw*, 1736*w*, 1677*w*, 1606*vw*, 1586*vw*, 1496*m*, 1454*s*, 1351*m*, 1308*vw*, 1241*w*, 1194*m*, 1152*vw*, 1133*w*, 1099*vs*, 1051*vs*, 1028*w*, 911*w*, 839*vw*, 736*vs*, 697*vs*, 607*w*, 547*w*, 464*vw*.

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.38-7.19 (*m*, 25H, Haromatic); 4.94 (*d*, $^2J_{2a\text{-}2a'}$=11.5Hz, 1H, HC(2^a)); 4.82 (*d*, $^2J_{3a\text{-}3a'}$=11.7Hz, 1H, HC(3^a)); 4.79 (*d*, $^2J_{4a\text{-}}$

$_{4a'}$=11.6Hz, 1H, HC(4^{a})); 4.70 (*d*, $^{2}J_{3a-3a'}$≈11.7Hz, 1H, HC(3$^{a'}$)); 4.68 (*d*, $^{3}J_{1-2}$≈3.8Hz, 1H, HC(1α)); 4.65 (*d*, $^{2}J_{4a-4a'}$≈11.61Hz, 1H, HC(4$^{a'}$)); 4.56 (*d*, $^{2}J_{2a-2a'}$=11.5Hz, 1H, HC(2$^{a'}$)); 4.45 (*d*, $^{2}J_{6a-6a'}$=11.8Hz, 1H, HC(6^{a})); 4.36 (*d*, $^{2}J_{6a-6a'}$=11.8Hz, 1H, HC(6$^{a'}$); 4.04 (*dd*, $^{3}J_{2-1}$ ≈ 3.8Hz, $^{3}J_{2-3}$ ≈10.0Hz ,1H, HC(2α));4.03-3.92 (*m*,2H, HC(3α) and HC(4α));3.90 (*t*, $^{3}J_{5-6}$≈6.4Hz, $^{3}J_{5-6'}$≈6.4Hz, $^{3}J_{5-4}$≈1.0Hz, 1H, HC(5α)); 3.52 (*d*, $^{2}J_{5-6}$=6.4Hz, $^{3}J_{5-6'}$=6.4Hz, 2H, H_2C(6α)); 3.34 (*s*,3H, $H_3C(1^{1})$.

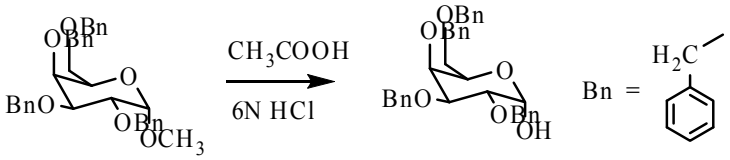
α/β ratio by ^{1}H-NMR = Only α anomer.

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 138.7-137.9 (($C2^{1}$), ($C3^{1}$), ($C4^{1}$)and ($C6^{1}$)); 128.3-127.4 (($C2^{2}$,$C2^{3}$,$C2^{4}$, $C3^{2}$,$C3^{3}$,$C3^{4}$, $C4^{2}$,$C4^{3}$,$C4^{4}$, $C6^{2}$,$C6^{3}$,$C6^{4}$); 98.7(C(1α)); 79.0, 75.1 (C(3α) and C(4α)); 76.4(C(2α)); 69.1 (C(5α); 69.0(C(6α)); 74.7, 73.5, 73.4, 73.2(C(2^{a}), C(3^{a}), C(4^{a}) and C(6^{a}); 55.3 ($C(1^{1})$).

α/β ratio by ^{13}C-NMR = Only α anomer.

MS (ESI (+)): m/z relative intensity $[M+ Na]^{+}$ = 577.5

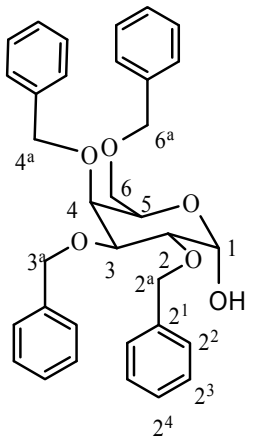
9.2.2.3.2. Synthesis of 2,3,4,6-tetra-O-benzyl α- galactose



44
$C_{35}H_{38}O_6$
[554.67]

45
$C_{34}H_{36}O_6$
[540.64]

4700 mg (8.47 mmol, 1eq) of α-methyl 2,3,4,6-tetra-O-benzyl galactoside was taken in a dry flask and was dissolved in 100 ml acetic acid. Then 15 ml of 6N HCl was added. The solution is heated to 60^{0}C. The mass was maintained at 60^{0}C and was taken for workup once the starting material was absent by TLC (approximately 5 to 6hrs, TLC eluent = Hexane: EtOAc 8:2). The mixture was diluted with 150ml water, extracted with 3×300ml CH_2Cl_2. The organic layer was washed with 250ml saturated $NaHCO_3$ aqueous solution and then with 250ml water. The organic layer was dried over $MgSO_4$, concentrated and the residue was subjected to flash chromatography (silica gel- 80 times of the residue mass) which further afforded 2.1 g pure 2,3,4,6-tetra-O-benzyl α-D galactose (major α anomer; Yield= 45.7%)



Batch: RV275, RV 280, RV 301, VW 26, VW 53

R_f: (Hexane: EtOAc 7:3): 0.33 (UV $_{254}$ active; $KMnO_4$)

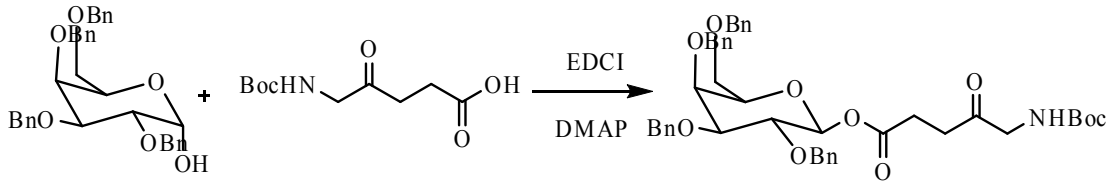
^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.49-7.23 (*m*, 20H, Haromatic); 5.37 (*d*, $^3J_{1\text{-}2}$=3.5Hz, 1H, HC(1α)); 5.03 (*d*, $^2J_{HC\text{-}HC}$ = 11.6Hz, $\underline{H_2}$CPh); 5.02 (*d*, $^2J_{HC\text{-}HC}$ = 11.5Hz, $\underline{H_2}$CPh); 4.89 (*d*, $^2J_{HC\text{-}HC}$ = 11.7Hz, $\underline{H_2}$CPh); 4.88 (*d*, $^2J_{HC\text{-}HC}$ = 11.0Hz, $\underline{H_2}$CPh); 4.87 (*d*, $^2J_{HC\text{-}HC}$ = 11.8Hz, $\underline{H_2}$CPh); 4.83 (*d*, $^2J_{HC\text{-}HC}$ = 11.8Hz, $\underline{H_2}$CPh); 4.81 (*d*, $^2J_{HC\text{-}HC}$ = 13.0Hz, $\underline{H_2}$CPh); 4.78 (*d*, $^2J_{HC\text{-}HC}$ = 11.8Hz, $\underline{H_2}$CPh); 4.72 (*d*, $^3J_{1\text{-}2}$=7.6Hz, 1H, HC(1β)); 4.68 (*d*, $^2J_{HC\text{-}HC}$ = 11.6Hz, $\underline{H_2}$CPh); 4.66 (*d*, $^2J_{HC\text{-}HC}$ = 11.5Hz, $\underline{H_2}$CPh); 4.56 (*d*, $^2J_{HC\text{-}HC}$ = 11.9Hz, $\underline{H_2}$CPh); 4.54 (*d*, $^2J_{HC\text{-}HC}$ = 11.8Hz, $\underline{H_2}$CPh); 4.47 (*d*, $^2J_{HC\text{-}HC}$ = 11.9Hz, $\underline{H_2}$CPh); 4.46 (*d*, $^2J_{HC\text{-}HC}$ = 11.9Hz, $\underline{H_2}$CPh); 4.26 (*t*, $^3J_{5\text{-}6a}$ =6.3 Hz, $^3J_{5\text{-}6b}$ = 6.3Hz, $^3J_{5\text{-}4}$ =1.0Hz,1H, HC(5α)); 4.12 (*dd*, $^3J_{1\text{-}2}$≈3.6Hz,$^3J_{2\text{-}3}$=9.6Hz, 1H, HC(2α)); 3.87 (*dd*, $^3J_{1\text{-}2}$=7.6Hz,$^3J_{2\text{-}3}$=9.6Hz, 1H, HC(2β)); 4.08-3.51 (*m*, 9H, H_2C(6α), H_2C(6β), HC(4α), HC(4β)), HC(5β), HC(3α)) and HC(3β); 2.13, 1.66,1.43, 0.95 peaks due to traces of solvent and impurity.

α/β ratio by ^{1}H-NMR = 2.2 : 1.0

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 138.6-137.7 (($C2^1$), ($C3^1$), ($C4^1$) and ($C6^1$) of α and β); 128.8-127.4 (($C2^2$,$C2^3$,$C2^4$, $C3^2$,$C3^3$,$C3^4$, $C4^2$,$C4^3$,$C4^4$, $C6^2$,$C6^3$,$C6^4$) of α and β; 98.3 (C(1β)); 92.3 (C(1α)); 79.2, 77.0, 75.3, 69.8 (C(2α), (C(3α), C(4α) and C(5α)); 82.6, 81.2, 74.1, 71.7 ((C(2β)), C(3β), C(4β) and C(5β)); 75.1, 73.9, 73.8, 73.4 ($\underline{C}H_2$Ph of α anomer); 75.5, 75.0, 73.9, 73.4 ($\underline{C}H_2$Ph of βanomer); 69.6 (C(6α)); 69.4 (C(6β)).

α/β ratio by ^{13}C-NMR = 2.5:1

9.2.2.3.3. Synthesis of 2´,3´,4´,6´-tetra-O- benzyl 1´-O-(BOC-ALA) β -D-galactose

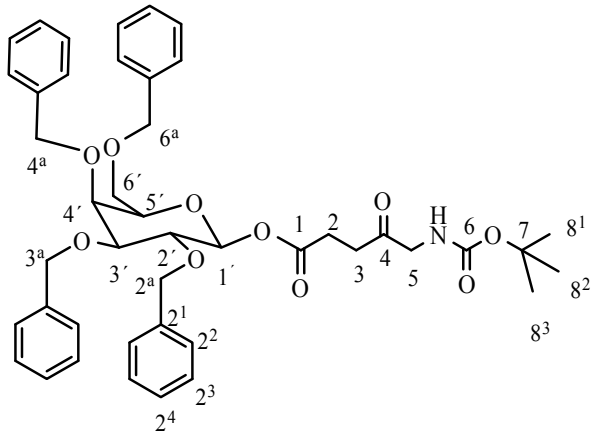


45 $C_{34}H_{36}O_6$ [540.64]

5 $C_{10}H_{17}NO_5$ [231.16]

46 $C_{44}H_{51}NO_{10}$ [753.87]

1976 mg (3.65 mmol, 1eq) of 2,3,4,6-tetra- O-benzyl galactose (α anomer major ≈ 70%) was taken in a dry flask under argon atmosphere and was dissolved in 80 ml CH_2Cl_2. The solution was cooled to 0^0C, added 491 mg (4.02 mmol, 1.1eq) of DMAP, then 851 mg (4.44 mmol, 1.21eq) of EDCI and finally 929 mg (4.02 mmol, 1.1eq) of Boc-ALA acid. The reaction was warmed to ambient temperature and maintained at that temperature for 7-8 hrs (controlled by TLC, eluent 7:3 Hexane: EtOAc 2 times). Once the reaction was complete, the mixture was concentrated and the residue was subjected to flash chromatography (silica gel- 80times of the residue mass) which afforded 2.66g pure 2′,3′,4′,6′-tetra-O- benzyl 1′-O-(BOC-ALA) α / β-D-galactose (major β anomer; Yield= 96.5 %)



Batch: RV 279, VW30

R_f: (Hexane / EtOAc 70:30, 2 times): 0.15 (UV $_{254}$ active; $KMnO_4$)

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) =7.38-7.24 (*m*, 20H, Haromatic); 6.33 (*d*, $^3J_{1\text{-}2}$=3.5Hz, 1H, HC(1′α)); 5.57 (*d*, $^3J_{1\text{-}2}$=8.0Hz, 1H, HC(1′β)); 5.19 (s, 1H, HN); 4.93 (*d*, $^2J_{2a\text{-}2a'}$=11.5Hz, 1H, HC(2^a)); 4.83 (*d*, $^2J_{3a\text{-}3a'}$=11.3Hz, 1H, HC(3^a)); 4.81 (*d*, $^2J_{4a\text{-}4a'}$=11.7Hz, 1H, HC(4^a)); 4.75 (*d*, $^2J_{3a\text{-}3a'}$=11.3Hz, 1H, HC($3^{a'}$)); 4.74 (*d*, $^2J_{4a\text{-}4a'}$ ≈11.7Hz, 1H, HC($4^{a'}$)); 4.61 (*d*, $^2J_{2a\text{-}2a'}$=11.5Hz, 1H, HC($2^{a'}$)); 4.43 (*d*, $^2J_{6a\text{-}6a'}$=11.7Hz, 1H, HC(6^a)); 4.38 (*d*, $^2J_{6a\text{-}6a'}$ =11.7Hz, 1H, HC($6^{a'}$) and other small peaks in this region due to $\underline{H_2}$CPh of α anomer); 4.00 (*d*, $^3J_{5\text{-}NH}$ ≈ 4.7Hz, $H_2C(5)$); 3.95 (*dd*, $^3J_{2'\text{-}1'}$=8.0Hz, $^3J_{2'\text{-}3'}$≈10.0Hz, 1H, HC(2′β)); 3.59 (*dd*, $^3J_{3'\text{-}4'}$≈2.0Hz, $^3J_{3'\text{-}2'}$≈10.0Hz, 1H, HC(3′β)); 3.68 - 3.51 (*m*, 3H, HC(4′β),HC(5′β)) and $H_2C(6'\beta)$; 2.70 - 2.58 (*m*, 4H ,$H_2C(3)$ and $H_2C(2)$); 1.44 (*s*, 9H, H_3C (8^1, 8^2 and 8^3)); 4.13-4.08, 2.03 1.25 (peaks due to EtOAc).

α/β ratio by ^{1}H-NMR = 0.15 : 1.0

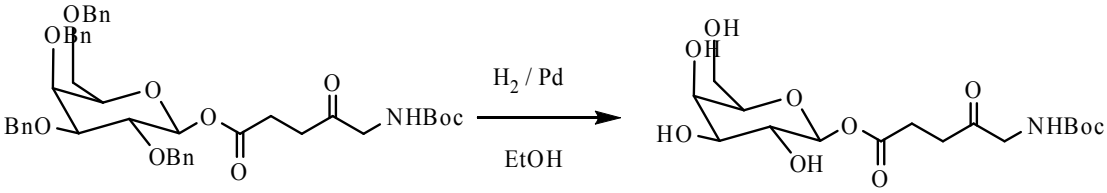
^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 203.7 (C(4)); 171.0 (C(1)); 155.5 (C(6)) ; 138.3-137.6 ((C2^1), (C3^1), (C4^1) and (C6^1) of α and β); 128.3 - 127.3 ((C2^2,C2^3,C2^4, C3^2,C3^3,C3^4, C4^2,C4^3,C4^4, C6^2,C6^3,C6^4) of α and β); 94.5 (C(1′β)); 91.1 (C(1′α)); 82.2, 77.9, 74.0, 72.9 (C(2′β),C(3′β), C(4′β) and C(5′β)); 67.8 C(6′β));75.2, 74.6, 73.4, 72.7 (C(2^a), C(3^a), C(4^a) and C(6^a)); 50.1 (C(5)); 33.8 (C(3)); 28.2 (C(8a,8b and8c); 27.7 (C(2)).

α/β ratio by ^{13}C-NMR ≈ 0.15 : 1.0

MS (ESI (+)): m/z relative intensity $[M+Na]^+$ = 776.4

HR-MS $[M+ Na]^+$ = 776.3406 (calculated =776.3410)

9.2.2.3.4. Synthesis of 1′-O-(BOC-ALA) β- galactose



46

$C_{44}H_{51}NO_{10}$

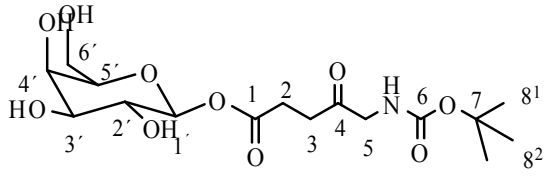
[753.87]

47

$C_{16}H_{27}NO_{10}$

[393.39]

2300 mg (3.05 mmol, 1eq) of 2′,3′,4′,6′-tetra-O- benzyl 1′-O-(BOC-ALA)-β -D-galactose was taken in a dry flask under argon atmosphere and was dissolved in 60 ml CH_2Cl_2 and then 30 ml EtOH was added. To this solution was added 3250 mg (3.98 mmol, 1.0eq) of 10% Pd over activated carbon. The reaction mass was agitated under H_2 pressure of 60psi for 12 hrs. Later, the mixture was filtered over celite bed and the filtrate was concentrated .The residue was subjected to flash chromatography (silica gel-70times of the residue mass) which afforded 890 mg pure 1-O-(BOC-ALA)-β -D-galactose (major β anomer; Yield= 74.2%).



Batch: RV 286

R_f: (CH_2Cl_2 / MeOH 8:2): = 0.21 ($KMnO_4$)

IR (KBr) : 3411*vss*, 2977*w*, 2926*m*, 1732*vs*, 1695*vs*, 1520*s*, 1394*vw*, 1368*s*, 1336*vw*, 1253*m*, 1167*s*, 1073*vs*,1058*w*, 873*vw*, 782*w*, 767*w*, 686*w*, 538*vw*, 494*vw*.

^{1}H-NMR (200 MHz, $CDCl_3$ and CD_3OD, 298 K): δ_H (ppm) = 6.12 (*d*, $^3J_{1'-2'}$=3.8Hz, 1H, HC(1′α)); 5.42 (*d*, $^3J_{1'-2'}$=7.8Hz, 1H, HC(1′β)); 3.93 (*s*, $H_2C(5)$); 3.88 (*dd*, $^3J_{4'-5'} \approx 0.8$Hz, $^3J_{4'-3'}$ =2.2Hz, 1H, HC(4′β)); 3.84-3.58 (*m*,4H, HC(2′β), HC(5′β) and $H_2C(6'$ β)); 3.53 (*dd*, $^3J_{3'-4'} \approx 3.3$Hz, $^3J_{3'-2'}$ =10.0Hz, 1H, HC(3′β)); 2.80-2.68 (*m*, 4H, $H_2C(3)$ and $H_2C(2)$); 1.44 (*s*, 9H, H_3C (8^1, 8^2 and 8^3)). (other peaks due to α anomer and ether solvent).

α/β ratio by ^{1}H-NMR = 0.07 : 1

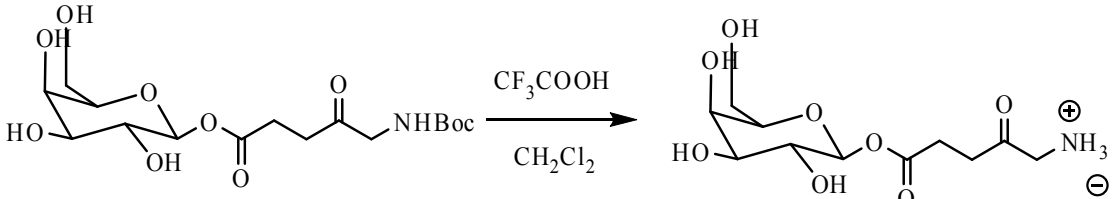
^{13}C-NMR (50 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 207.2 (C(4)); 173.2 (C(1)); 96.4 (C(1′β)); 80.6 (C(7)); 77.5 (C(5′β)); 74.7, 71.2, 70.0 (C(4′β), (C(2′β) and C(3′β)); 62.2 (C(6′β)); 50.7 (C(5)); 34.6 (C(3)); 28.7 (C(8^1, 8^2 and 8^3) and (C(2)).

α/β ratio by ^{13}C-NMR ≈ only β anomer.

MS (ESI (+)): m/z relative intensity $[M+Na]^+$ = 416.1.

HR-MS $[M+ Na]^+$ = 416.1526 (calculated = 416.1533)

9.2.2.3.5. Synthesis of 1-O-(ALA) β- galactose



47

$C_{16}H_{27}NO_{10}$

[393.39]

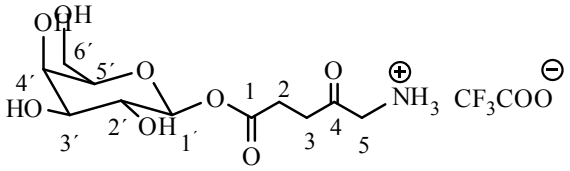
48

$C_{13}H_{20}F_3NO_{10}$

[407.29]

M^+=294.28 ; CF_3COO^- = 113.01

810 mg (2.06 mmol, 1eq) of 1′-O-(BOC-ALA) galactose was dissolved in 30ml CH_2Cl_2 and cooled to -10^{0}C. To this solution, 14.9 g (10ml, 130mmol, 63 eq) of trifluoroacetic acid was added dropwise by syringe. The reaction mass was maintained at 0-5^{0}C for an hour. Once the deprotection of BOC was completed (controlled by the absence of BOC peak in ^{1}H-NMR), CH_2Cl_2 and trifluoroacetic acid was evaporated off. The product was triturated with dry ether and the ether solution was decanted. The trituration with ether and decanting top clear ether solution after centrifuge was repeated 2 times more. Then, the settled solid product was purified by dissolving in 4ml MeOH and precipitated by adding 20ml ether. The mass was centrifuged, the settled solid on drying yielded 334 mg of highly hygroscopic white solid. (Yield=40 %).



Batch: RV 291

$[\alpha]_D$ = +40.9 (c=0.33; deionized H_2O)

IR (KBr) : 3378*vs*, 2933*w*, 1732*vs*, 1678*vs*, 1513*m*, 1428*m*, 1315*w*, 1204*vs*, 1136*vs*, 1071*vs*, 1021*vw*, 955*vw*, 892*w*, 839*m*, 800*m*, 781*w*, 723*s*, 704*vw*, 599*vw*, 530*w*.

^{1}H-NMR (400 MHz, D_2O, 298 K): δ_H (ppm) = 5.99 (*d*, $^3J_{1'-2'}$=3.6Hz, 1H, HC(1´α)); 5.36 (*d*, $^3J_{1'-2'}$=7.6Hz, 1H, HC(1´β)); 3.99 (*s*, $H_2C(5)$); 3.82 (*d*, $^3J_{4'-3'}$ =2.2Hz, 1H, HC(4´β)); 3.67 (*m*,1H, HC(5´β)); 3.60-3.59 (*m*, 4H, HC(2´β),HC(3´β),HC(6´a β) and HC(6´bβ); 2.82 (*t*, $^3J_{2-3}$ ≈6.5Hz, 2H, $H_2C(3)$) ; 2.69 (*t*, $^3J_{3-2}$ ≈ 6.0Hz, 2H, $H_2C(2)$) ; (other peaks due to α anomer and ether solvent).

α/β ratio by ^{1}H-NMR ≈ 0.18 :1

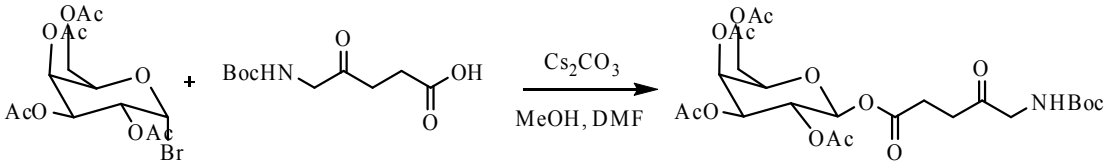
^{13}C-NMR (100 MHz, D_2O, 298K) : δ_C (ppm) = 204.0 (C(4)) ; 173.7 (C(1)) ; 163.3 (*q*,$^2J_{C-F}$ = 35.3, $CF_3\underline{C}OO^-$); 116.6 (*q*,$^1J_{C-F}$ = 291.9, $\underline{C}F_3COO^-$); 95.0 (C(1´β)); 93.0 (C(1´α)); 76.5 (C(5´β)); 68.6(C(4´β)); 72.7, 69.6 (C(2´β) and C(3´β)); 61.1(C(6´β)); 47.3 (C(5)); 34.3 (C(3)); 27.2 (C(2)) and other peaks due to α anomer and ether solvent.

α/β ratio by ^{13}C-NMR ≈ 0.11 : 1

MS (ESI (+)): m/z relative intensity $[M- CF_3COO^-]^+$ = 294.1

HR-MS $[M- CF_3COO^-]^+$ = 294.11869 (calculated = 294.1189)

9.2.2.3.6. Synthesis of 1´-O-(BOC- ALA) - 2´, 3´, 4´, 6´tetraacetyl-β-D-galactopyranose



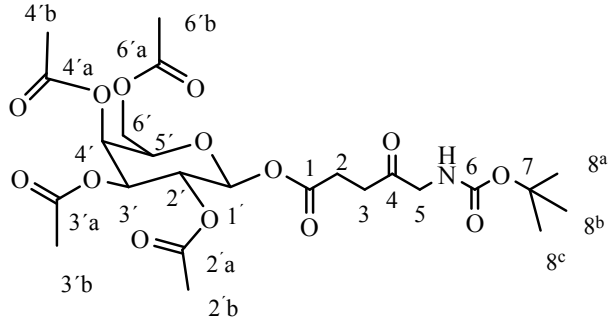
49 5 50

$C_{14}H_{19}BrO_9$ $C_{10}H_{17}NO_5$ $C_{24}H_{35}NO_{14}$

[411.20] [231.16] [561.53]

1387 mg (6 mmol, 1eq) of Boc-ALA acid was taken in a dry flask under argon atmosphere and was dissolved in 25 ml MeOH. The solution was cooled to 0^0C, added 978 mg (3 mmol, 0.5eq) of Cs_2CO_3. The reaction was warmed to ambient temperature and maintained at that temperature for 1h. (controlled by TLC, eluent 100% EtOAc). The

solution then was concentrated to remove methanol, then added 25 ml DMF. The solution was cooled to 0^0C, added 2467 mg (6 mmol, 1.0eq) of 2, 3, 4, 6-β-D-galactopyranosyl bromide (acetobromogalactose). The reaction was mainatained at ambient temperature for an hour, filtered CsBr, distilled off DMF and was subjected to flash chromatography (silica gel- 80times of the residue mass) which afforded 2130 mg pure 1′-O-(BOC- ALA) - 2′, 3′, 4′, 6′tetraacetyl-β-D-galactopyranose (only β anomer; Yield= 63.2 %).



Batch : RV 225, RV 230, RV 236

R_f: (100% ether): 0.29 ($KMnO_4$)

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 5.65 (*d*, $^3J_{1'-2'}$=8.0Hz, 1H, HC(1′β); 5.35 (*d*, $^3J_{4'-3'}$=3.8Hz, 1H, HC(4′β);5.26 (*sbr*, 1H, NH); 5.22 (*dd*, $^3J_{2'-1'}\approx$ 8.0Hz, $^3J_{2'-3'}$=10.7Hz,1H, HC(2′β)); 5.02 (*dd*, $^3J_{3'-4'}$ =3.4Hz, $^3J_{3'-2'}$=10.4Hz, 1H, HC(3′β)); 4.09-4.05 (*m*, 2H, $H_2C(6')$); 4.04-4.01 (*m*, 1H, HC(5′)); 3.96 (*t*, $^3J_1{}^5{}_{-NH}\approx$ 6.0Hz, 2H, $H_2C(1^5)$); 2.70-2.60 (*m*, 4H, $H_2C(1^3)$ and $H_2C(1^2)$); 2.10, 2.02, 1.97, 1.92 (4 × *s*, 12H, H_3C(2′b), H_3C(3′b), H_3C(4′b) and H_3C(6′b); 1.37 (s, 9H, H_3C (1^{8a}, 1^{8b} and 1^{8c})); 4.94 (*q*), 1.28 (*d*) due to impurity.

α/β ratio by ^{1}H-NMR ≈ Only β anomer

^{13}C-NMR (100 MHz, $CDCl_3$ & CD_3OD, 298K) : δ_C (ppm) = 204.1 ($C(1^4)$); 170.3 ($C(1^1)$); 170.1, 170.0, 169.9, 169.8 (C2′a), (C3′a), (C4′a) and (C6′a)); 155.8 ($C(1^6)$); 92.2 ((C(1′β)); 79.8 ($C(1^7)$); 71.6 (C(5′β); 70.6 (C(3′β)); 67.6 (C(2′β)); 66.7 (C(4′β)); 61.0 (C(6′β)); 50.0 ($C(1^5)$); 33.8 ($C(1^3)$) ; 28.3 ($C(1^{8a},1^{8b}$ and1^{8c}); 27.6 ($C(1^2)$); 20.7, 20.6, 20.5, 20.4 ((C2′b), (C3′b), (C4′b) and (C6′b)); 104.0, 18.0 due to impurity.

α/β ratio by DEPT ≈ Only β anomer

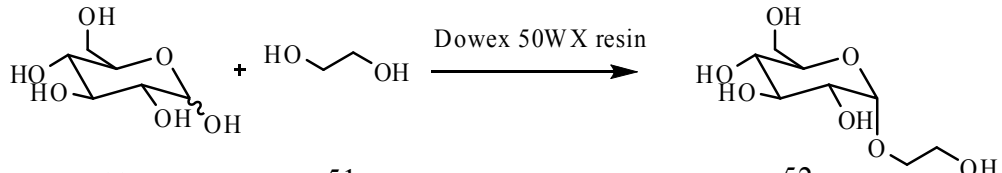
MS (ESI (+)): m/z relative intensity $[M + Na]^+$ = 584.1; $[M + Na\text{-}^tBu]^+$= 528.2.

9.2.3. Synthesis of 1-O-(ALA)-2-O-(glycosyl) ethylene glycol

The synthesis of 1-O-(ALA)-2-O-(α-D-glucopyranosyl) ethylene glycol and 1-O-(ALA)-2-O-(α-D-mannopyranosyl) ethylene glycol were carried out vis Fischer glycosidation to get predominantly α anomer and the synthesis of 1-O-(ALA)-2-O-(β-D-galactopyranosyl) ethylene glycol was carried out vis Koenigs-Knorr synthesis to get prodominantly β anomer.

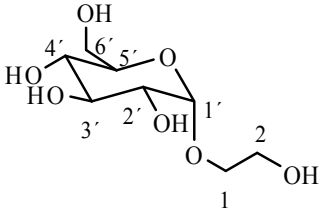
9.2.3.1. Synthesis of 1-O-(ALA)-2-O-(α-D-glucopyranosyl) ethylene glycol

9.2.3.1.1. Synthesis of 1-O-(α-D-glucopyranosyl) ethylene glycol



13 $C_6H_{12}O_6$ [180.16] + 51 $C_2H_6O_2$ [62.07] → 52 $C_8H_{16}O_7$ [224.21]

5700 mg (31.64 mmol, 1eq) D-Glucose was taken in a dry flask under argon atmosphere. To this were added 40g (633 mmol, 20 eq) of ethylene glycol and then 12g of dried Dowex 50WX 8-200 resin. The mixture was then heated to 75^0C and maintained at 70-80^0C for 72h. The resin was filtered over alumina sandwiched between celite bed, washed with ethylene glycol. Ethylene glycol was evaporated off from the filtrate and the crude was subjected to flash chromatography (silica gel- 70times of the residue mass; gradient eluent from 9:1 CH_2Cl_2 / MeOH to 7:3 CH_2Cl_2 / MeOH) which afforded 2570 mg of pure 1-O-(α-D-glucopyranosyl) ethylene glycol. (major α-anomer; yield= 46.2%).



Batch: RV 178, RV 198, RV332

R_f: (CH_2Cl_2 / MeOH 3:1): = 0.23 ($KMnO_4$)

IR (KBr): 3369vs, 2927m, 2148vw, 1643w, 1415w, 1342w, 1261w, 1229w, 1151m, 1075w, 1028vs, 938w, 880w, 752vw, 702vw, 665vw, 604vw, 531vw, 496w, 458vw.

^{1}H-NMR (400 MHz, D_2O, 298 K): δ_H (ppm) = 4.93 (*d*, $^3J_{1'-2'}$ =3.7Hz, 1H, HC(1′α)); 4.47 (*d*, $^3J_{1'-2'}$ =7.9Hz, 1H, HC(1′β)); 3.88-3.58 (*m*,8H, H_2C (6`α),H_2C (1), H_2C (2), HC (5′α) and HC(4′α); 3.54 (*dd*, $^3J_{2'-1'}$ ≈3.7Hz, $^3J_{3'-2'}$ =11Hz,1H, HC(2′α)); 3.39 (*t*, $^3J_{3'-4'}$ =9.5Hz, $^3J_{3'-2'}$ =9.5 Hz,1H, HC (3′α)).

α/β ratio by ^{1}H-NMR ≈ 0.11 : 1

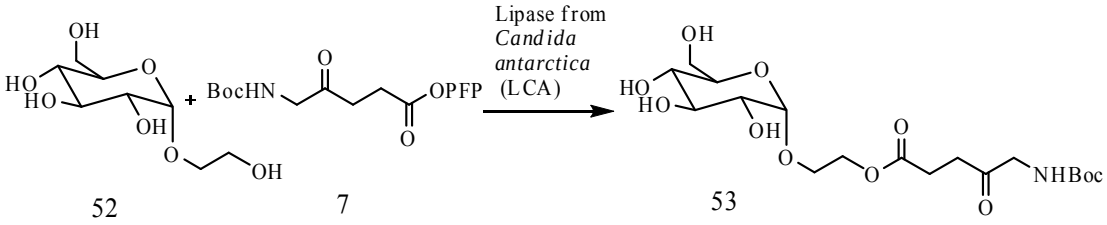
^{13}C-NMR (100 MHz, D_2O, 298K) : δ_C (ppm) = 105.0(C(1′β)); 100.9(C(1′α)); 74.1(C2′α)); 72.2((C(3′α)); 75.7, 74.4, (C(4′α) and (C(5′α)); 71.6, 63.3, 63.2(C(1), C(2) and C(6′α));

α/β ratio by ^{13}C-NMR ≈ 0.11 : 1

MS (ESI (+)): m/z relative intensity [M with OH/OD + Na] $^{+}$ = 252.1

HR-MS [M+ Na] $^{+}$ = 247.0783 (calculated =247.0794)

9.2.3.1.2. Synthesis of 1-O-(BOC- ALA) -2-O-(α-D-glucopyranosyl) ethylene glycol

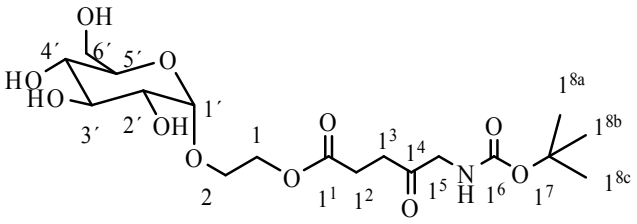


52 $C_8H_{16}O_7$ [224.21]

7 $C_{16}H_{16}F_5NO_5$ [397.29]

53 $C_{18}H_{31}NO_{11}$ [437.44]

225mg (1 mmol, 1eq) of 1-O-(α-D-glucopyranosyl) ethylene glycol.was taken in a dry flask under argon atmosphere and was dissolved in 5 ml anhydrous pyridine. 1500 mg (10 units per mg of protein; 1unit hydrolyses 1μmol or 0.001mmol of an ester; approximately, 15 mmol, 15eq) of lipase from *Candida antarctica* (LCA, Novozyme 435) enzyme (before use dried by keeping at vacuum 10^{-2} mbar for 3-4 days) was added and then 1200 mg (3mmol, 3eq) of Boc-ALA-PFP ester was added to the solution. The reaction was heated to 50^0C and maintained at this temperature for 30min. (controlled by TLC eluent : CH_2Cl_2: MeOH 3:1). The mass was cooled to room temperature and then the enzyme was filtered over celite bed, the filtered cake was washed with 3× 10ml pyridine. The solvent pyridine was evaporated off by rotovapor .The residue was taken for flash chromatography. (silica gel- 90times of the residue mass; gradient eluent from 100% CH_2Cl_2 to 85:15 CH_2Cl_2 : MeOH). The main fractions were concentrated to give 210 mg of pure product 1-O-(BOC- ALA) -2-O-(α-D-glucopyranosyl) ethylene glycol was obtained as white solid. (Yield = 48%).



Batch : RV 206, RV 220

R_f: (CH_2Cl_2 / MeOH 85:15): 0.28; ($KMnO_4$)

IR (KBr) : 3395*vs*, 2977*s*, 2929*s*, 2355*vw*, 1728*vs*, 1709*vs*, 1517*m*, 1453*vw*, 1392*vw*, 1368*m*, 1285*vw*, 1254*w*, 1163*s*, 1076*vw*, 1024*s*, 887*w*, 782*vw*, 763*vw*, 703*vw*, 602*vw*,562*vw*, 530*vw*, 500*w*.

^{1}H-NMR (400 MHz, D_2O, 298 K): δ_H (ppm) = 4.82 (*d*, $^3J_{1'-2'}$=3.7Hz, 1H, HC(1´α)); 4.32-4.24 (*m*, 3H, $H_2C(1)$ and HC(1´β)); 3.94 (*s*, 2H, $H_2C(1^5)$); 3.89 (*ddd*, $^3J_{1b-2a}$≈3.3Hz, $^3J_{1a-2a}$=6.4Hz vicinal, $^3J_{2b-2a}$=11.5Hz geminal, 1H, HC(2a)); 3.79 (*dd*, $^3J_{5'-6'a}$≈2.6Hz, $^3J_{6'a\ -\ 6'b}$ =11.9Hz ,1H, HC(6´a α)) ;3.71 (*dd*, $^3J_{5'-6'b}$ ≈ 5.3Hz, $^3J_{6'a\ -\ 6'b}$ ≈ 11.9Hz ,1H, HC(6´b α)); 3.70-3.66 (*m*, 1H, HC (2b)); 3.63 (*t*, $^3J_{3'-4'}$≈9.3Hz, $^3J_{3'-2'}$ =9.3Hz,1H, HC(3´α)); 3.58 (ddd, $^3J_{5'-6'a}$ ≈ 2.7Hz, $^3J_{5'-6'b}$≈5.0Hz, $^3J_{5'-4'}$≈9.9Hz,1H, HC (5´α)); 3.41 (*dd*, $^3J_{2'-1'}$ = 3.7Hz, $^3J_{2'-3'}$= 9.7Hz ,1H, HC(2´α)); 3.33 (*t*, $^3J_{3'-4'}$=9.2Hz, $^3J_{4'-5'}$ ≈9.3Hz,1H, HC(4´α)); 2.75 (*t*, $^3J_1{}^3{}_{-1}{}^2$ ≈6.5Hz, 2H, $H_2C(1^3)$); 2.64 (*t*, $^3J_1{}^2{}_{-1}{}^3$ ≈6.5Hz, 2H, $H_2C(1^2)$); 1.42 (s, 9H, H_3C (1^{8a}, 1^{8b} and 1^{8c})).

α/β ratio by ^{1}H-NMR ≈ could not be determined because of overlap

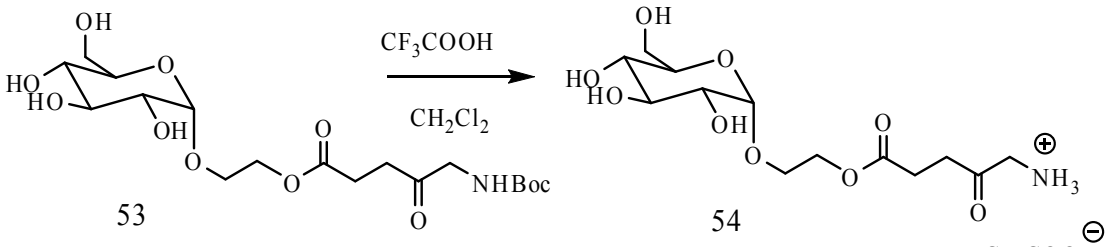
^{13}C-NMR (100 MHz, D_2O, 298K) : δ_C (ppm) = 206.7 ($C(1^4)$); 173.6 ($C(1^1)$); 157.4 ($C(1^6)$); 103.7 ((C(1´β) by DEPT); 99.5 (C(1´α)); 80.4 ($C(1^7)$); 74.3(C(3´α)); 72.8 (C(5´α)); 72.6 (C(2´α)); 70.8 (C(4´α)); 66.3 (C(2)); 64.2(C(1)); 62.0 (C(6´α)); 50.4 ($C(1^5)$); 34.6 ($C(1^3)$) ; 28.5 ($C(1^{8a}$,1^{8b} and1^{8c}); 28.2 ($C(1^2)$).

α/β ratio by ^{13}C-NMR ≈ 9.5 : 1

MS (ESI (+)): m/z relative intensity $[M+ Na]^+$ = 460.2;

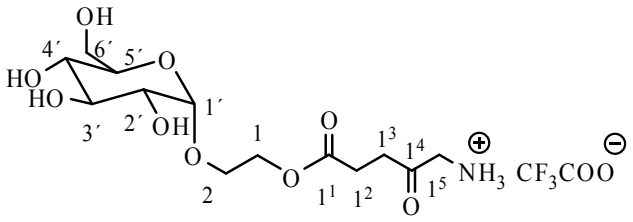
HR-MS $[M+ Na]^+$ = 460.1795 (calculated =460.1795)

9.2.3.1.3. Synthesis of 1-O-(ALA) -2-O-(α-D-glucopyranosyl) ethylene glycol



220 mg (0.50 mmol, 1eq) of 1-O-(BOC- ALA) -2-O-(α-D-glucopyranosyl) ethylene glycol was dissolved in 15ml CH_2Cl_2 and cooled to -10^0C. To this solution, 5.73g (3.85ml, 50mmol, 100 eq) of trifluoroacetic acid was added dropwise by syringe. The

reaction mass was maintained at 0-5^{0}C for an hour. Once the deprotection of BOC was completed (controlled by the absence of BOC peak in ^{1}H-NMR), CH_2Cl_2 and trifluoroacetic acid was evaporated off. The product was co evaporated 2times with distilled ether, then triturated with dry ether and the ether solution was decanted. The trituration with ether and decanting top clear ether solution after centrifuge was repeated 2 times more. Then, the settled solid product was purified by dissolving in 5ml MeOH and precipitated by adding 30ml ether. The mass was centrifuged, the settled solid on drying yielded 149 mg of 1-O-(ALA) -2-O-(α-D-glucopyranoosyl) ethylene glycol which was a highly hygroscopic white solid. (Yield=66%).



Batch: RV 222, RV359

$[\alpha]_D$ = +246.1 (c=0.10; deionized H_2O)

IR (KBr): 3426*vs*, 2924*m*, 1789*m*, 1731*m*, 1679*vs*, 1521*vw*, 1399*m*, 1207*vs*, 1179*vs*, 1051*w*, 838*w*, 802*w*, 723*m*, 519*vw*, 501*vw*.

^{1}H-NMR (400 MHz, D_2O, 298 K): δ_H (ppm) = 4.91 (*d*, $^3J_{1'-2'}$ =3.7Hz, 1H, HC(1′α)); 4.31-4.26 (*m*, 3H, $H_2C(1)$);4.09 (*s*, 2H, $H_2C(1^5)$); 3.92-3.88 (*m,* 1H, HC(2a)); 3.80 (*dd*, $^3J_{5'-6'a}$≈2.0Hz, $^3J_{6'a\ -\ 6'b}$ =12.1Hz ,1H, HC(6′a α)) ;3.72 (*dd*, $^3J_{5'-6'b}$ ≈ 5.3Hz, $^3J_{6'a\ -\ 6'b}$ ≈ 12.2Hz ,1H, HC(6′b α)); 3.70-3.66 (*m*, 2H, HC (2b) and (5′α)); 3.66 (*t*, $^3J_{3'-4'}$≈9.5Hz, $^3J_{3'-2'}$ =9.2Hz,1H, HC(3′α)); 3.51 (*dd*, $^3J_{2'-1'}$ = 3.7Hz, $^3J_{2'\ -\ 3'}$= 9.8Hz ,1H, HC(2′α)); 3.45-3.34 (*m*, 1H, HC(4′α)); 2.89 (*t*, $^3J_1{}^3{}_{-1}{}^2$ ≈6.5Hz, 2H, $H_2C(1^3)$); 2.72 (*t*, $^3J_1{}^2{}_{-1}{}^3$ ≈6.5Hz, 2H, $H_2C(1^2)$);

α/β ratio by ^{1}H-NMR ≈:only α anomer

^{13}C-NMR (100 MHz, D_2O, 298K) : δ_C (ppm) = 204.0 ($C(1^4)$) ; 174.8 ($C(1^3)$) ; 163.1 (*q*,$^2J_{C-F}$ = 35.2, $CF_3\underline{C}OO^-$); 116.5 (*q*,$^1J_{C-F}$ ≈ 291.9, $\underline{C}F_3COO^-$); 98.5 (C(1′α)); 73.1 (C(5′α)); 72.0(C(3′α)); 71.4 (C(2′α)); 69.6 (C(4′α)); 65.8 (C(2)); 64.4(C(1)); 60.6 (C(6′α)); 47.1 ($C(1^5)$); 34.3 ($C(1^3)$) ; 27.5 ($C(1^2)$).

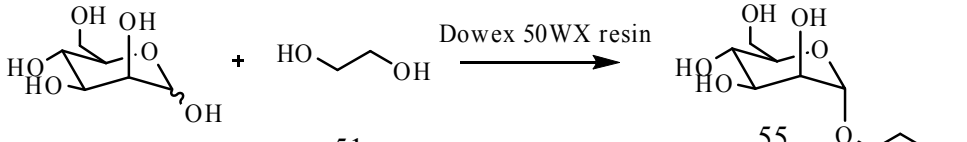
α/β ratio by ^{13}C-NMR : only α anomer

MS (ESI (+)): m/z relative intensity $[M\text{-}CF_3COO^-+H]^+$ = 340.1; $[M\text{-}CF_3COO^-]^+$=339.1.

HR-MS $[M\text{-}\ CF_3COO^-]^+$ = 338.1441 (calculated =338.1451)

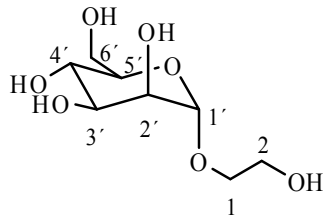
9.2.3.2. Synthesis of 1-O-(ALA)-2-O-(α-D-mannopyranosyl) ethylene glycol

9.2.3.2.1. Synthesis of 1-O-(α-D-mannopyranosyl) ethylene glycol



24 $C_6H_{12}O_6$ [180.16] + 51 $C_2H_6O_2$ [62.07] → 55 $C_8H_{16}O_7$ [224.21]

2850 mg (15.82 mmol, 1eq) D-mannose was taken in a dry flask under argon atmosphere. To this were added 24.5 g (395 mmol, 25 eq) of ethylene glycol and then 6g of dried Dowex 50WX 8-200 resin. The mixture was then heated to 75^0C and maintained at 70-80^0C for 72h. The resin was filtered over alumina sandwiched between celite bed, washed with ethylene glycol. Ethylene glycol was evaporated off from the filtrate and the crude was subjected to flash chromatography (silica gel- 70times of the residue mass; gradient eluent from 9:1 CH_2Cl_2 / MeOH to 7:3 CH_2Cl_2 / MeOH) which afforded 2230 mg of pure 1-O-(α-D-mannopyranosyl) ethylene glycol. (major α-anomer; yield= 62.8%).



Batch: RV 181, RV 333

R_f: (CH_2Cl_2 / MeOH 3:1) = 0.28 ($KMnO_4$)

^{1}H-NMR (400 MHz, D_2O, 298 K): δ_H (ppm) = 4.87 (*d*, $^3J_{1'-2'}$ =1.7Hz, 1H, HC(1′α)); 4.68 (*d*, $^3J_{1'-2'}$ =0.8Hz, 1H, HC(1′β)); 3.96 (*dd*, $^3J_{2'-1'}$ =1.7Hz, $^3J_{3'-2'}$ =3.4Hz,1H, HC(2′α)); 3.87 (*dd*, $^3J_{6'a-5'}$ ≈2.0Hz, $^3J_{6'a-6'b}$ =11.4Hz,1H, HC(6′α a));3.84-3.71 (*m*,5H, HC(6`α b),$H_2$C(2), HC(1a), and HC(3′α); 3.65 -3.57 (*m*,3H, HC(5`α), HC(4′α) and HC(1b);

α/β ratio by ^{1}H-NMR ≈ 8 : 1

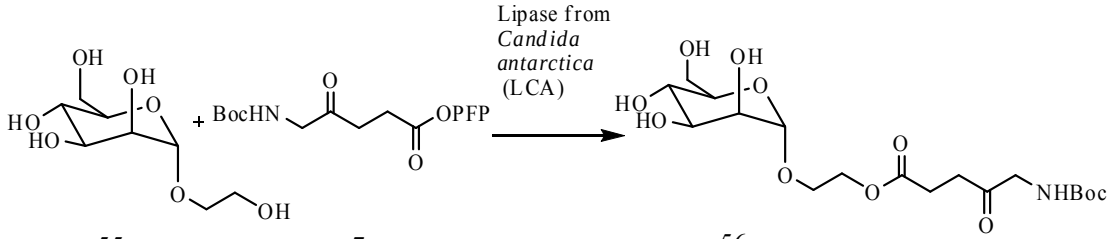
^{13}C-NMR (100 MHz, D_2O, 298K) : δ_C (ppm) = 100.7(C(1′β)); 100.6(C(1′α)); 73.4(C(5′α); 71.2((C(3′α)); 70.7((C2′α)); 69.2(C(1)); 67.5(C(4′α); 61.7(C(6′α)); 61.2 ((C(2)).

α/β ratio by ^{13}C-NMR ≈ 10 : 1

MS (ESI (+)): m/z relative intensity [M with OH/OD + Na] $^{+}$ = 252.1

HR-MS [M+ Na] $^{+}$ = 247.0786 (calculated =247.0794)

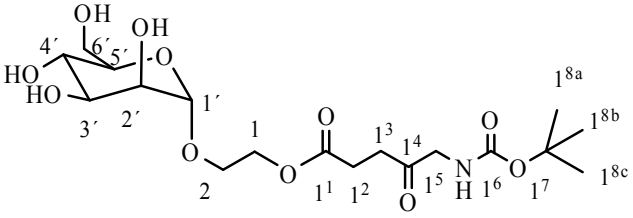
9.2.3.2.2. Synthesis of 1-O-(BOC- ALA) -2-O-(α-D-mannopyranosyl) ethylene glycol



55 7 56

$C_8H_{16}O_7$ [224.21] $C_{16}H_{16}F_5NO_5$ [397.29] $C_{18}H_{31}NO_{11}$ [437.44]

540 mg (2.4 mmol, 1eq) of 2′-O-(α-D-mannopyranosyl) ethylene glycol.was taken in a dry flask under argon atmosphere and was dissolved in 13 ml anhydrous pyridine. 3600 mg (10 units per mg of protein; 1unit hydrolyses 1µmol or 0.001mmol of an ester; approximately, 36 mmol, 15eq) of lipase from *Candida antarctica* (LCA, Novozyme 435) enzyme (before use, dried by keeping at vacuum 10^{-2} mbar for 3-4 days) was added and then 2850 mg (7.18 mmol, 3eq) of Boc-ALA-PFP ester was added to the solution. The reaction was maintained at ambient temperature and maintained for 30min. (controlled by TLC eluent : CH_2Cl_2: MeOH 3:1). The mass was filtered over celite bed, the filtered cake was washed with 3× 10ml pyridine. The solvent pyridine was evaporated off by rotovapor .The residue was triturated with 2×50 ml dry 1:1 toluene : hexane and the top clear supernatant solution was removed, thesettled sticky residue was taken for flash chromatography. (silica gel- 100times of the residue mass; gradient eluent from 100% CH_2Cl_2 to 85:15 CH_2Cl_2 : MeOH). The main fractions were concentrated to give 440 mg of pure product 1-O-(BOC- ALA) -2-O-(α-D-mannopyranosyl) ethylene glycol was obtained as white solid. (Yield = 42%).



Batch : RV 186, RV207, RV 338

R_f: (CH_2Cl_2 / MeOH 85:15): 0.20 ($KMnO_4$)

IR (KBr) : 3390*vs*, 2924*vs*, 1728*vs*, 1694*vs*, 1516*m*, 1384*w*, 1368*m*, 1252*w*, 1203*w*, 1175*s*, 1137*s*, 1063*s*, 977*m*, 915*w*, 878*w*, 804*w*, 723*w*, 680*w*, 579*vw*, 513*vw*.

^{1}H-NMR (400 MHz, $CDCl_3$ & CD_3OD, 298 K): δ_H (ppm) = 4.78 (*d*, $^{3}J_{1'-2'}$=1.6Hz, 1H, HC(1′α)); 4.26 (*ddd*, $^{3}J_{1a-2b}$≈3.1 Hz, $^{3}J_{1a-2a}$≈6.3 Hz vicinal, $^{3}J_{1a-1a'}$=11.9Hz geminal, 1H, HC(1a)); 4.00 (*ddd*, $^{3}J_{1b-2a}$≈3.1Hz, $^{3}J_{1b-2b}$=6.4Hz vicinal, $^{3}J_{1b-1b'}$=11.9Hz geminal, 1H, HC(1b)); 3.92 (*s*, 2H, $H_2C(1^5)$); 3.87 (*ddd*, $^{3}J_{1b-2a}$≈3.1Hz, $^{3}J_{1a-2a}$≈6.3Hz vicinal, $^{3}J_{2b-2a}$=11.5Hz geminal, 1H, HC(2a)); 3.82 (*dd*, $^{3}J_{5'-6'a}$≈2.5Hz, $^{3}J_{6'a\ -\ 6'b}$ =11.5Hz ,1H, HC(6′a α)); 3.84- 3.80 (*m*, 1H, HC(2′α)); 3.75- 3.60 (*m*, 1H, HC(2b)); 3.73 (*dd*, $^{3}J_{5'-6'b}$ ≈ 5.3Hz, $^{3}J_{6'a\ -\ 6'b}$ ≈ 11.5Hz ,1H, HC(6′bα); 3.69 (*dd*, $^{3}J_{3'-2'}$≈3.3Hz, $^{3}J_{3'-4'}$ =9.2Hz,1H, HC(3′α)); 3.63 (*t*, $^{3}J_{3'-4'}$=9.5Hz, $^{3}J_{4'-5'}$ ≈9.5Hz,1H, HC(4′α); 3.54 (*ddd*, $^{3}J_{5'-6'a}$ ≈ 2.5Hz, $^{3}J_{5'-6'b}$≈5.3Hz, $^{3}J_{5'-4'}$≈9.3Hz,1H, HC (5′α)); 2.76 (*t*, $^{3}J_{1}{}^{3}{}_{-1}{}^{2}$ ≈6.5Hz, 2H, $H_2C(1^3)$); 2.62 (*t*, $^{3}J_{1}{}^{2}{}_{-1}{}^{3}$ ≈6.5Hz, 2H, $H_2C(1^2)$); 1.43 (s, 9H, H_3C (1^{8a}, 1^{8b} and 1^{8c})).

α/β ratio by ^{1}H-NMR ≈ could not be determined because of overlap

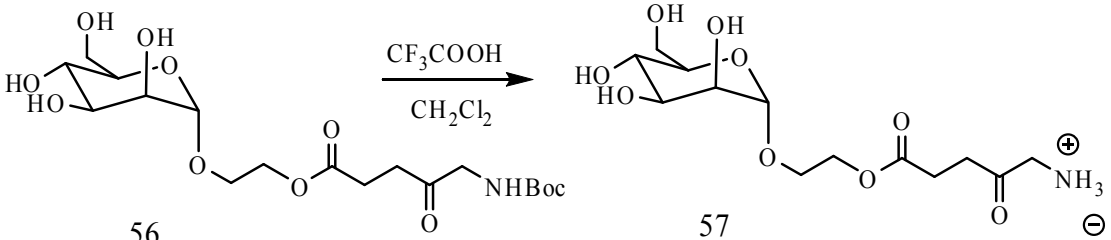
^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 207.0 ($C(1^4)$); 173.8 ($C(1^1)$); 157.9 ($C(1^6)$); 101.4 ((C(1′β) by DEPT); 101.1 (C(1′α)); 80.5 ($C(1^7)$); 74.1 (C(5′α)); 72.1(C(3′α)); 71.5 (C(2′α)); 68.1 (C(4′α)); 65.9 (C(2)); 64.4(C(1)); 62.5 (C(6′α)); 50.5 ($C(1^5)$); 34.7 ($C(1^3)$) ; 28.6 ($C(1^{8a}$,1^{8b} and1^{8c}); 28.4 ($C(1^2)$).

α/β ratio by DEPT ≈ 14 : 1

MS (ESI (+)): m/z relative intensity $[M+ Na]^+$ = 460.1;

HR-MS $[M+ Na]^+$ = 460.1790 (calculated =460.1795)

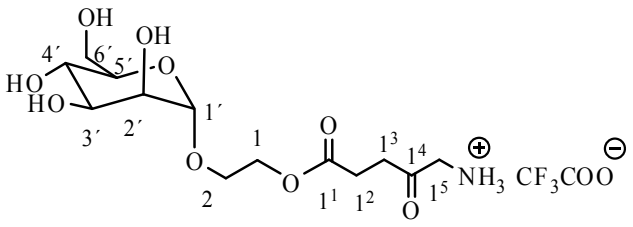
9.2.3.2.3. Synthesis of 1-O-(ALA) -2-O-(α-D-mannopyranosyl) ethylene glycol



M^+=338.33 ; CF_3COO^- = 113.01

400 mg (0.91 mmol, 1eq) of 1-O-(BOC- ALA) -2-O-(α-D-mannopyranosyl) ethylene glycol was dissolved in 20ml CH_2Cl_2 and cooled to -10^{0}C. To this solution, 5.24g (3.5ml, 45.72 mmol, 50eq) of trifluoroacetic acid was added dropwise by syringe. The reaction mass was maintained at 0-5^{0}C for an hour. Once the deprotection of BOC was completed (controlled by the absence of BOC peak in ^{1}H-NMR), CH_2Cl_2 and trifluoroacetic acid was evaporated off. The product was co evaporated 2times with distilled ether, then triturated with dry ether and the ether solution was decanted. The trituration with ether

and decanting top clear ether solution after centrifuge was repeated 2 times more. Then, the settled solid product was purified by dissolving in 7ml MeOH and precipitated by adding 35ml ether. The mass was centrifuged, the settled solid on drying yielded 309 mg of 1-O-(ALA) -2-O-(α-D-mannopyranosyl) ethylene glycol which was a highly hygroscopic white solid. (Yield=74.6%).



Batch: RV 202, RV223, RV348

$[\alpha]_D$ = +85.7 (c=0.31; deionized H_2O)

IR (KBr): 3394*s*, 2929*s*, 1789*s*, 1732*vs*, 1678*vs*, 1516*w*, 1428*vw*, 1389*vw*, 1355*vw*, 1319*vw*, 1205*vs*, 1180*vs*, 1136*vs*, 1092*w*, 1064*w*, 975*w*, 884*vw*, 839*m*, 801*m*, 774*w*, 723*m*, 680*w*, 600*vw*, 519*vw*, 500*w*.

^{1}H-NMR (400 MHz, D_2O, 298 K): δ_H (ppm) = 4.83 (*d*, $^3J_{1'-2'}$=1.7Hz, 1H, HC(1′α)); 4.29 (*ddd*, $^3J_{1a-2b}$≈2.7 Hz, $^3J_{1a-2a}$≈6.3 Hz vicinal, $^3J_{1a-1a'}$=12.3Hz geminal, 1H, HC(1a)); 4.22 (*ddd*, $^3J_{1b-2a}$≈2.7Hz, $^3J_{1b-2b}$≈6.3Hz vicinal, $^3J_{1b-1b'}$=12.3Hz geminal, 1H, HC(1b)); 4.06 (*s*, 2H, $H_2C(1^5)$); 3.90-3.85 (*m*, 1H, HC(2a)); 3.88 (*dd*, $^3J_{1'-2'}$≈1.7Hz, $^3J_{3'-2'}$=3.3Hz, 1H, HC(2′α)); 3.85-3.67 (*m*, 2H, HC(2b) and HC(6′bα); 3.82 (*dd*, $^3J_{5'-6'a}$≈2.0 Hz, $^3J_{6'a\ -\ 6'b}$ =11.6Hz ,1H, HC(6′a α)); 3.71 (*dd*, $^3J_{3'-2'}$≈3.7Hz, $^3J_{3'-4'}$ =9.1Hz,1H, HC(3′α)); 3.63 (*m*, 2H, HC (5′α) and HC(4′α); 2.87 (*t*, $^3J_1{}^3{}_{-1}{}^2$ ≈6.0Hz, 2H, $H_2C(1^3)$); 2.69 (*t*, $^3J_1{}^2{}_{-1}{}^3$ ≈6.0Hz, 2H, $H_2C(1^2)$);

α/β ratio by ^{1}H-NMR ≈: 12.5:1

^{13}C-NMR (100 MHz, D_2O, 298K) : δ_C (ppm) = 204.6 ($C(1^4)$) ; 175.5 ($C(1^3)$) ; 163.8 (*q*,$^2J_{C-F}$ = 36.2, $CF_3\underline{C}OO^-$); 117.3 (*q*,$^1J_{C-F}$ ≈ 292, $\underline{C}F_3COO^-$); 100.9 (($C(1'\beta)$ by DEPT); 100.7 (C(1′α)); 73.8 (C(5′α)); 71.5(C(3′α)); 70.9 (C(2′α)); 67.7 (C(4′α)); 66.1 (C(2)); 65.0(C(1)); 61.9 (C(6′α)); 47.9 ($C(1^5)$); 35.2 ($C(1^3)$) ; 28.3 ($C(1^2)$).

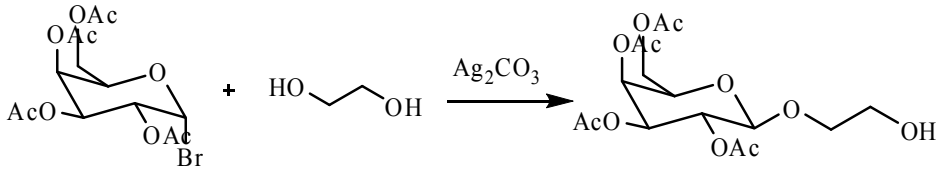
α/β ratio by DEPT ≈ 14 : 1

MS (ESI (+)): m/z relative intensity $[M\text{-}CF_3COO^-]^+$ = 338.1; $[M\text{-}CF_3COO^-\text{-}H+Na]^+$=360.1;

HR-MS $[M\text{-}\ CF_3COO^-]^+$ = 338.1440 (calculated =338.1451)

9.2.3.3. Synthesis of 1-O-(ALA)-2-O-(α-D-galactopyranosyl) ethylene glycol

9.2.3.3.1. Synthesis of 1-O-(2′, 3′, 4′, 6′tetraacetyl-β-D-galactopyranosyl) ethylene glycol

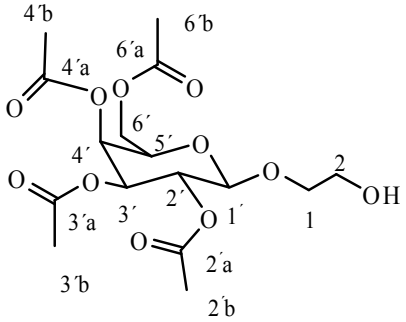


49 $C_{14}H_{19}BrO_9$ [411.20]

51 $C_2H_6O_2$ [62.07]

58 $C_{16}H_{24}O_{11}$ [392.35]

To 13.14 g (215 mmol, 22 eq) of ethylene glycol were added 4 g (9.73 mmol, 1 eq) of 2, 3, 4, 6-β-D-galactopyranosyl bromide (acetobromogalactose) and 4.8 g (17.4 mmol, 1.8 eq) of silver carbonate. The mixture was stirred until the CO_2 evolution ceased, 50ml toluene was added and stirred and precipitated silver salts were filtered. The filtrate was separated into two layers, a toluene and an ethylene glycol layer. The latter was repeatedly extracted with 3× 50 ml toluene. The combined toluene layer was evaporated under vacuum. The residue was crystallised with 10 ml ether to give pure 2.66g 1-O-(2′, 3′, 4′, 6′ tetraacetyl-β-D-galactopyranosyl) ethylene glycol. (Yield= 70%). Alternatively, the residue can also be purified by flash chromatography. (Eluent EtOAc : Hexane 3:1).



Batch: RV 177, RV200, RV327

R_f: (EtOAc / Hexane 3:1) = 0.21 ($KMnO_4$)

IR (KBr): 3580*w*, 3477*vw*, 2981*vw*, 2956*vw*, 2885*vw*, 1750*vs*, 1639*vw*, 1486*vw*, 1429*w*, 1368*m*, 1338*vw*, 1315*w*, 1254*s*, 1232*s*,1219*s*, 1166*vw*, 1156*w*, 1128*m*, 1102*vw*, 1084*w*, 1067*m*, 1041*s*, 1022*m*, 969*vw*, 953*w*, 927*w*, 917*vw*, 899*w*, 875*vw*, 855*w*, 733*w*, 716*w*, 650*vw*, 629*vw*, 587*w*, 540*w*, 502*vw*, 493*vw*, 475*vw*.

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 5.29 (*d*, $^3J_{4'-3'}$=3.8Hz, 1H, HC(4′β); 5.10 (*dd*, $^3J_{2'-1'}$=8.0Hz, $^3J_{2'-3'}$=10.4Hz,1H, HC(2′β)); 4.94 (*dd*, $^3J_{3'-4'}$ =3.4Hz, $^3J_{3'-2'}$=10.4Hz, 1H, HC(3′β)); 4.44 (*d*, $^3J_{1'-2'}$=8.0Hz, 1H, HC(1′β); 4.05 (*m*, 2H, H_2C(6′)); 3.89 (*t*,$^3J_{5'-6'a}$ ≈ 6.6Hz, $^3J_{5'-6'b}$≈6.6Hz,1H, HC (5′)); 3.77 (*ddd*, $^3J_{1a-2b}$≈4.0Hz, $^3J_{1a-2a}$≈5.4Hz

vicinal, $^{3}J_{1a\text{-}1b}$=11.0Hz geminal, 1H, HC(1a)); 3.72 (*ddd*, $^{3}J_{1b\text{-}2a}$≈3.5Hz, $^{3}J_{1b\text{-}2b}$≈5.4Hz vicinal, $^{3}J_{1b\text{-}1a}$=11.0Hz geminal, 1H, HC(1b)); 3.87 (*m*, 2H, $H_2C(2)$); 2.71 (*d*, $^{3}J_{2\text{-}OH}$ ≈ 5.7Hz, 1H, O<u>H</u>); 2.06, 1.97, 1.96, 1.88 (4 × *s*, 12H, $H_3C(2'b)$, $H_3C(3'b)$, $H_3C(4'b)$ and $H_3C(6'b)$.

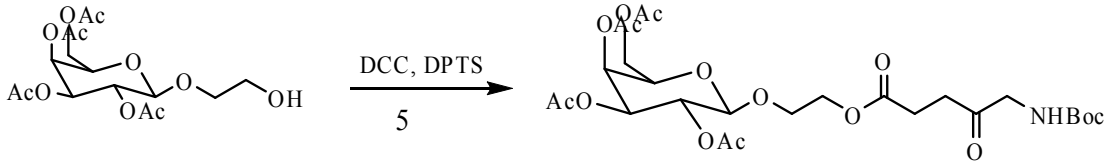
α/β ratio by ^{1}H-NMR : Only β anomer

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 170.3, 170.1, 169.9, 169.5 (C2′a), (C3′a), (C4′a) and (C6′a)); 101.7(C(1′β)); 72.7((C(1)); 70.7, 70.6(C(5′) andC(3′)); 68.8((C2′)); 66.9(C(4′); 61.7, 61.4 (C(6′) and (C(2)); 20.6, 20.5, 20.4 ((C2′b), (C3′b), (C4′b) and (C6′b)).

α/β ratio by ^{13}C-NMR: Only β anomer

MS (ESI (+)): m/z relative intensity $[M + Na]^{+}$ = 415.1

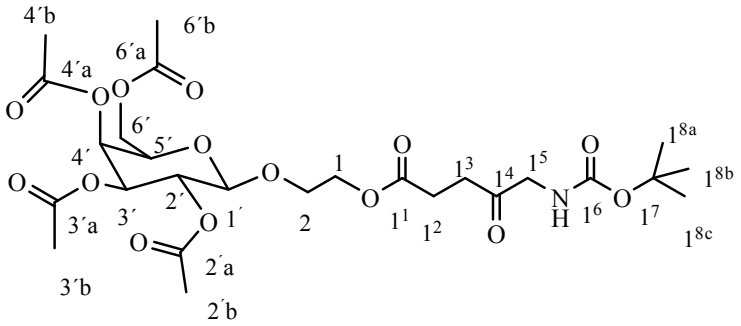
9.2.3.3.2. Synthesis of 1-O-(BOC- ALA) -2-O-(2′, 3′, 4′, 6′tetraacetyl-β-D-galactopyranosyl) ethylene glycol



58
$C_{16}H_{24}O_{11}$
[392.35]

59
$C_{26}H_{39}NO_{15}$
[605.59]

In a dry flask under argon atmosphere, 196 mg (0.5 mmol, 1.0eq) of 1-O-(2′, 3′, 4′, 6′ tetraacetyl-β-D-galactopyranosyl) ethylene glycol was dissolved in 10ml CH_2Cl_2, followed by addition of 40 mg (0.13 mmol, 0.27eq) of DPTS and 10 ml CH_2Cl_2. Then, 124 mg (0.6 mmol, 1.2eq) of DCC was added and finally 139 mg (0.6 mmol, 1.2eq) of Boc-ALA was added. The reaction was kept at room temperature for overnight. The reaction mixture was filtered over celite bed and the solvent was evaporated off by rotovapor. The crude mass was purified by flash chromatography (3:1 ether: hexane; silica gel mass- 60 times the crude mass) which yielded 251 mg of 1-O-(BOC- ALA) -2-O-(2′, 3′, 4′, 6′tetraacetyl-β-D-galactopyranosyl) ethylene glycol. (Yield= 82.9%)



Batch : RV 194

R_f: (ether/ hexane 3:1): 0.33 ($KMnO_4$)

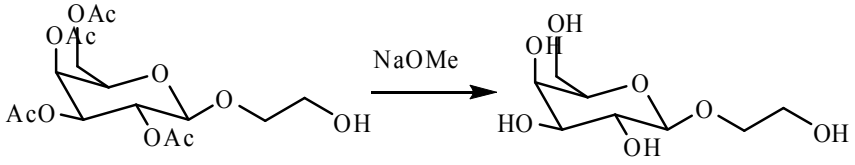
^{1}H-NMR (400 MHz, $CDCl_3$ & CD_3OD, 298 K): δ_H (ppm) = 5.31 (*dd*, $^3J_{4'-5'}$=1.0Hz, $^3J_{4'-3'}$=3.8Hz, 1H, HC(4′β);5.25 (*sbr*, 1H, NH); 5.17 (*dd*, $^3J_{2'-1'}$=8.0Hz, $^3J_{2'-3'}$=10.5Hz,1H, HC(2′β)); 5.00 (*dd*, $^3J_{3'-4'}$ =3.4Hz, $^3J_{3'-2'}$=10.4Hz, 1H, HC(3′β)); 4.51 (*d*, $^3J_{1'-2'}$=8.0Hz, 1H, HC(1′β); 4.20-4.18 (*m*, 2H, $H_2C(1)$); 4.15-4.10 (*m*, 2H, $H_2C(6')$); 4.03 (d, $^3J_{1^5-NH}$=5Hz , 2H, $H_2C(1^5)$); 3.96 (*ddd*, $^3J_{2a-1b}$≈4.0Hz, $^3J_{2a-1b}$≈6.0Hz vicinal, $^3J_{2a-2b}$=11.0Hz geminal, 1H, HC(2a)); 3.91 (*triplotide*, $^3J_{5'-4'}$ ≈1.0Hz, $^3J_{5'-6'a}$ ≈ 6.6Hz, $^3J_{5'-6'b}$≈6.6Hz,1H, HC (5′)); 3.74 (*ddd*, $^3J_{2b-1a}$≈4.5Hz, $^3J_{2b-1b}$≈6.0Hz vicinal, $^3J_{2b-2a}$=11.0Hz geminal, 1H, HC(2b)); 2.71-2.61 (*m*, 4H, $H_2C(1^3)$ and $H_2C(1^2)$); 2.12, 2.04, 2.02, 1.95 (4 × *s*, 12H, $H_3C(2'b)$, $H_3C(3'b)$, $H_3C(4'b)$ and $H_3C(6'b)$; 1.41 (s, 9H, H_3C (1^{8a}, 1^{8b} and 1^{8c})).

α/β ratio by ^{1}H-NMR ≈ Only β anomer

^{13}C-NMR (100 MHz, $CDCl_3$ & CD_3OD, 298K) : δ_C (ppm) = 204.4 ($C(1^4)$); 172.3 ($C(1^1)$); 170.5, 170.3, 170.2, 169.5 (C2′a), (C3′a), (C4′a) and (C6′a)); 155.7 ($C(1^6)$); 101.4 ((C(1′β)); 80.0 ($C(1^7)$); 70.9, 70.8 (C(5′β) and (C(3′β)); 68.7 (C(2′β)); 67.4 (C(2)); 67.0 (C(4′β)); 63.7 (C(1)); 61.3 (C(6′β)); 50.3 ($C(1^5)$); 34.3 ($C(1^3)$) ; 28.4 ($C(1^{8a}$,1^{8b} and1^{8c}); 27.7 ($C(1^2)$); 20.8, 20.7, 20.6, 20.5 ((C2′b), (C3′b), (C4′b) and (C6′b)).

α/β ratio by DEPT ≈ Only β anomer

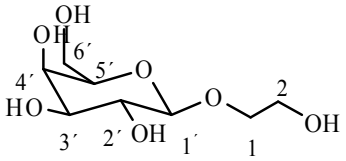
9.2.3.3.3. Synthesis of 1-O-(β-D-galactopyranosyl) ethylene glycol



58 $C_{16}H_{24}O_{11}$ [392.35]

59a $C_8H_{16}O_7$ [224.21]

2500mg (6.37 mmol, 1eq) of 1-O-(2′, 3′, 4′, 6′ tetraacetyl-β-D-galactopyranosyl) ethylene glycol was dissolved in 20 ml MeOH. 0.625 ml (0.0625 mmol, 0.01eq) of 0.1N sodium methoxide solution in MeOH was added slowly at ambient temperature and maintained at ambient temperature till the starting material was absent. (2-3hrs; TLC eluent : 3:1 CH_2Cl_2 :MeOH). The reaction mass was then neutralised by adding dried dowex resin, filtered over basic alumina sandwiched between celite bed. The filtrate is concentrated and the residue was crystallised using ether as solvent. 1.29 g of 1-O-(β-D-galactopyranosyl) ethylene glycol was obtained. (Yield = 90.3%)



Batch: RV 193, RV201, RV211, RV 336, RV355

R_f: (CH_2Cl_2 / MeOH 3:1) = 0.24 ($KMnO_4$)

IR (KBr): 3407*vs*, 3338*vs*, 2971*vw*, 2917*m*, 2893*m*, 2867*m*, 2657*vw*, 2157*vw*, 2041*vw*, 1635*vw*, 1471*vw*, 1482*vw*, 1457*w*, 1449*w*, 1406*w*, 1372*w*, 1343*vw*, 1288*m*, 1241*w*, 1225*w*, 1164*m*, 1146*m*, 1130*m*, 1118*m*, 1078*vs*, 1054*w*, 1031*s*, 1019*s*, 1000*w*, 945*m*, 907*m*, 889*m*, 835*w*, 780*m*, 706*m*, 632*w*, 604*w*, 548*vw*, 502*w*, 470*vw*.

^{1}H-NMR (400 MHz, D_2O, 298 K): δ_H (ppm) = 4.43 (*d*, $^3J_{1'-2'}$ =7.8Hz, 1H, HC(1′β)); 4.05-3.98 (*m,* 1H, HC(1a); 3.93 (*dd*, 1H, HC(4′β); 3.83-3.73 (*m*, $H_2C(6`)$, $H_2C(2)$ and HC(1b); 3.71 ((*dd*, $^3J_{6'a-5'}$ ≈4.2Hz, $^3J_{5'-6'b}$ =7.9Hz,1H, HC(5′β)); 3.67 (*dd*, $^3J_{3'-4'}$ =3.4Hz, $^3J_{3'-2'}$ =9.9Hz,1H, HC(3′β)); 3.56 (*dd*, $^3J_{2'-1'}$ =7.8Hz, $^3J_{3'-2'}$ =9.9Hz,1H, HC(2′β));

α/β ratio by ^{1}H-NMR ≈ Only β anomer

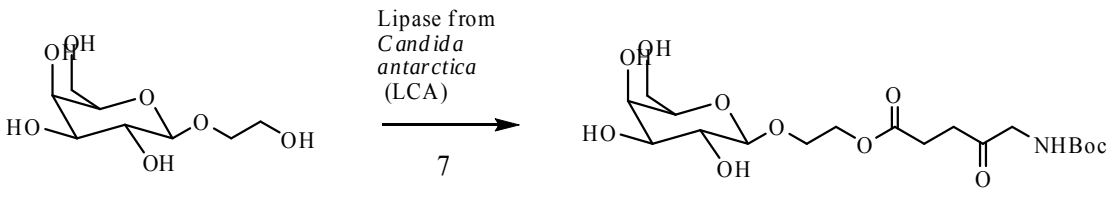
^{13}C-NMR (100 MHz, $CDCl_3$, 298K): δ_C (ppm) = 103.4 (C(1′β)); 75.5 (C(5′β); 73.1((C(3′β)); 71.5 (C(1)); 71.2((C2′β)); 69.0(C(4′β); 61.4 , 61.1 (C(6′β) and ((C(2)).

α/β ratio by ^{13}C-NMR ≈ Only β anomer

MS (ESI (+)): m/z relative intensity [M with OH/OD + Na] $^+$ = 247.1

HR-MS [M+ Na] $^+$ = 247.0783 (calculated =247.0794)

9.2.3.3.4. Synthesis of 1-O-(BOC- ALA) -2-O-(β-D-galactopyranosyl) ethylene glycol



59a

$C_8H_{16}O_7$

[224.21]

7

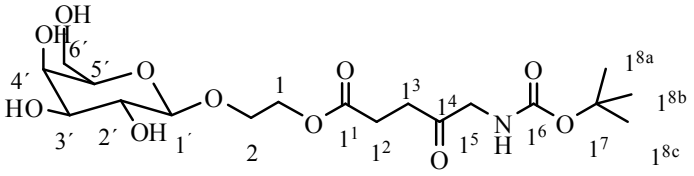
60

$C_{18}H_{31}NO_{11}$

[437.44]

115 mg (0.51 mmol, 1eq) of 2′-O-(α-D-galactopyranosyl) ethylene glycol.was taken in a dry flask under argon atmosphere and was dissolved in 3.5 ml anhydrous pyridine. 1000 mg (10 units per mg of protein; 1unit hydrolyses 1μmol or 0.001mmol of an ester; approximately, 10 mmol, 20eq) of lipase from *Candida antarctica* (LCA, Novozyme 435) enzyme (before use, dried by keeping at vacuum 10^{-2} mbar for 3-4 days) was added and then 1000 mg (2.5 mmol, 5eq) of Boc-ALA-PFP ester was added to the solution. The

reaction was maintained at ambient temperature and maintained for 10min. (controlled by TLC eluent : CH_2Cl_2: MeOH 3:1). The mass was filtered over celite bed, the filtered cake was washed with 3× 10ml pyridine. The solvent pyridine was evaporated off by rotovapor .The residue was triturated with 2×50 ml dry 1:1 toluene : hexane and the top clear supernatant solution was removed, thesettled sticky residue was taken for flash chromatography. (silica gel- 100times of the residue mass; gradient eluent from 100% CH_2Cl_2 to 90:10 CH_2Cl_2 : MeOH). The main fractions were concentrated to give 108 mg of pure product 1-O-(BOC- ALA) -2-O-(α-D-galactopyranosyl) ethylene glycol was obtained as white solid. (Yield = 48%).



Batch : RV 227, RV 344, RV356

R_f: (CH_2Cl_2 / MeOH 85:15): 0.23 ($KMnO_4$)

IR (KBr) : 3405*vs*, 2977*m*, 2930*m*, 1732*vs*, 1709*vs*, 1520*m*, 1393*w*, 1368*m*, 1286*w*, 1253*w*, 1165*s*, 1076*s*, 921*w*, 891*w*, 878*w*, 780*m*, 702*w*, 600*vw*, 548*vw*, 500*vw*.

^{1}H-NMR (400 MHz, $CDCl_3$ & CD_3OD, 298 K): δ_H (ppm) = 4.25-4.22 (*m*, 3H, $H_2C(1)$ and HC(1′β)); 4.03 (*ddd*, $^3J_{1b\text{-}2a}$≈4.0Hz, $^3J_{1a\text{-}2a}$≈6.0Hz vicinal, $^3J_{2b\text{-}2a}$=11.1Hz geminal, 1H, HC(2a)); 3.91 (*s*, 2H, $H_2C(1^5)$); 3.82 (*d*, $^3J_{3'\text{-}4'}$≈3.0Hz, 1H, HC(4′β); 3.79-3.65 (*m*, 3H, $H_2C(6')$ and HC(2b)); 3.56- 3.47 (*m*, 2H, HC (5′β) and HC(2′β)); 3.45 (*dd*, $^3J_{3'\text{-}2'}$≈3.0Hz, $^3J_{3'\text{-}4'}$ =9.2Hz,1H, HC(3′β)); 2.75 (*t*, $^3J_1{}^3{}_{\text{-}1}{}^2$ ≈6.5Hz, 2H, $H_2C(1^3)$); 2.61 (*t*, $^3J_1{}^2{}_{\text{-}1}{}^3$ ≈6.5Hz, 2H, $H_2C(1^2)$); 1.43 (s, 9H, H_3C (1^{8a}, 1^{8b} and 1^{8c})).

α/β ratio by ^{1}H-NMR ≈ Only β anomer

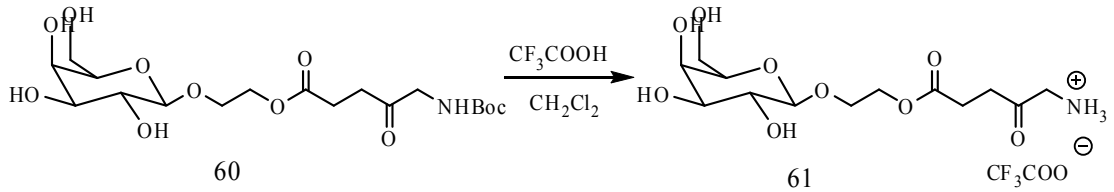
^{13}C-NMR (100 MHz, $CDCl_3$ & CD_3OD, 298K) : δ_C (ppm) = 205.9 ($C(1^4)$); 174.0 ($C(1^1)$); 157.0 ($C(1^6)$); 104.7 (($C(1'\beta)$); 80.0 ($C(1^7)$); 76.5 (C(5′β)); 74.6(C(3′β)); 72.2 (C(2′β)); 70.0 (C(4′β)); 68.2 (C(2)); 64.8 (C(1)); 62.3 (C(6′β)); 50.7 ($C(1^5)$); 34.8 ($C(1^3)$) ; 28.7 ($C(1^{8a}$,1^{8b} and1^{8c}); 28.6 ($C(1^2)$).

α/β ratio by DEPT ≈ Only β anomer

MS (ESI (+)): m/z relative intensity $[M+Na]^+$ = 460.3.

HR-MS $[M+ Na]^+$ = 460.1791 (calculated =460.1795)

9.2.3.3.5. Synthesis of 1-O-(ALA) -2-O-(β-D-galactopyranosyl) ethylene glycol



60

$C_{18}H_{31}NO_{11}$

[437.44]

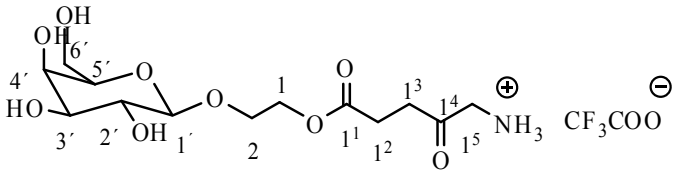
61

$C_{15}H_{24}F_3NO_{11}$

[451.34]

M^+=338.33 ; CF_3COO^- = 113.01

170 mg (0.39 mmol, 1eq) of 1-O-(BOC- ALA) -2-O-(β-D-galactopyranosyl) ethylene glycol was dissolved in 15ml CH_2Cl_2 and cooled to -10^{0}C. To this solution, 4.5g (3.0ml, 39.0mmol, 100eq) of trifluoroacetic acid was added dropwise by syringe. The reaction mass was maintained at 0-5^{0}C for an hour. Once the deprotection of BOC was completed (controlled by the absence of BOC peak in ^{1}H-NMR), CH_2Cl_2 and trifluoroacetic acid was evaporated off. The product was co evaporated 2times with distilled ether, then triturated with dry ether and the ether solution was decanted. The trituration with ether and decanting top clear ether solution after centrifuge was repeated 2 times more. Then, the settled solid product was purified by dissolving in 2ml MeOH and precipitated by adding 15ml ether. The mass was centrifuged, the settled solid on drying yielded 100 mg of 1-O-(ALA) -2-O-(α-D-mannopyranosyl) ethylene glycol which was a highly hygroscopic white solid. (Yield=56.8%).



Batch: RV 228, RV204, RV350, RV358

$[\alpha]_D$ = -55.9 (c=0.5; deionized H_2O)

IR (KBr): 3407*s*, 2924*s*, 2854*w*, 1787*s*, 1733*vs*, 1677*vs*, 1513*vw*, 1431*w*, 1393*w*, 1205*vs*, 1182*vs*, 1135*vs*, 1078*s*, 1063*w*, 839*m*, 800*m*, 777*w*, 723*m*, 706*w*, 665*vw*, 598*vw*, 519*vw*.

^{1}H-NMR (400 MHz, D_2O, 298 K): δ_H (ppm) = 4.43 (*d*, $^3J_{1'-2'}$ = 7.8Hz 1H, HC(1′β)); 4.31(*t*, $^3J_{1-2}$≈4.5Hz $H_2C(1)$); 4.13 (*s*, 2H, $H_2C(1^5)$); 4.12-4.09 (*m*, 1H, HC(2a)); 3.92 (*ddd*, $^3J_{1b-2a}$≈4.0Hz, $^3J_{1a-2a}$≈5.0Hz vicinal, $^3J_{2b-2a}$=12Hz geminal, 1H, HC(2b)); 3.91 (*t*, $^3J_{3'-4'}$≈3.0Hz, 1H, HC(4′β)); 3.78-3.74 (*m*, 2H, $H_2C(6')$); 3.69 (*ddd*, $^3J_{5'-4'}$ ≈1.0Hz, $^3J_{5'-6'a}$≈3.5Hz, $^3J_{5'-6'b}$≈ overlap, 1H, HC (5′β)); 3.65 (*dd*, $^3J_{3'-4'}$ ≈3.5Hz, $^3J_{3'-2'}$ ≈10.0Hz, 1H, HC(3′β)); 3.51 (*dd*, $^3J_{2'-1'}$=7.8Hz, $^3J_{2'-3'}$ ≈10.0Hz,1H, HC(2′β)); 2.93 (*t*, $^3J_1{}^3{}_{-1}{}^2$ ≈6.5Hz, 2H, $H_2C(1^3)$); 2.75 (*t*, $^3J_1{}^2{}_{-1}{}^3$ ≈6.5Hz, 2H, $H_2C(1^2)$).

α/β ratio by ^{1}H-NMR ≈ Only β anomer

^{13}C-NMR (100 MHz, D_2O, 298K) : δ_C (ppm) = 204.4 ($C(1^4)$) ; 175.0 ($C(1^3)$) ; ~165 ($CF_3\underline{C}OO^-$); 115.3 ($\underline{C}F_3COO^-$); 103.3 (($C(1'\beta)$); 75.5 (C(5′)); 73.0 (C(3′)); 71.0

(C(2´)); 68.9 (C(4´)); 68.0 (C(2)); 64.7 (C(1)); 61.3 (C(6´)); 47.4 (C(1^5)); 34.8 (C(1^3)) ; 27.8 (C(1^2)).

α/β ratio by ^{13}C-NMR ≈ Only β anomer

MS (ESI (+)): m/z relative intensity [M-CF_3COO^-] $^+$ = 338.1

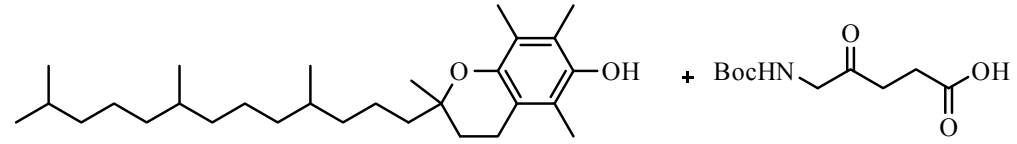
HR-MS [M- CF_3COO^-] $^+$ = 338.1445 (calculated =338.1451)

9.3. Synthesis of vitamin-ALA derivatives

9.3.1. Synthesis of (±)-α-tocopherol-ALA ester

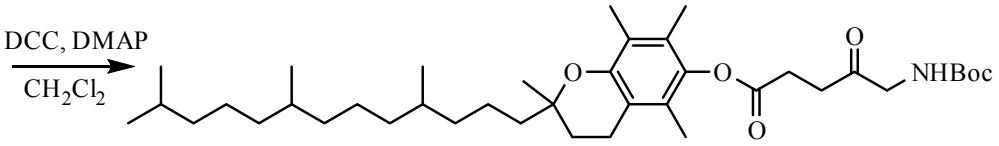
The synthesis of (±)-α-tocopherol-ALA ester was carried out using Boc-ALA acid and also using azidolevulinic acid.

9.3.1.1. Synthesis of (±)-α-tocopherol-Boc-ALA ester



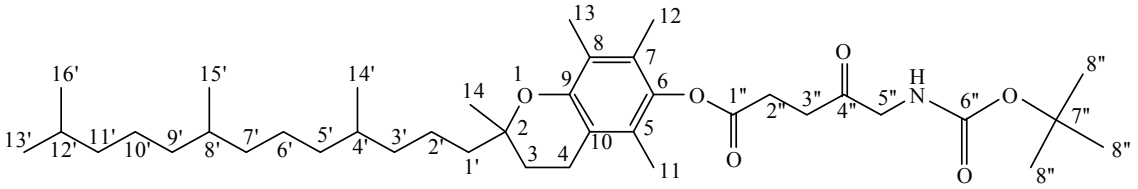
62
$C_{29}H_{50}O_2$
[430.71]

5
$C_{10}H_{17}NO_5$
[231.25]



63
$C_{39}H_{65}NO_6$
[643.94]

In a dry flask under argon atmosphere, 2150 mg (5.0 mmol, 1eq) of (±)-α-tocopherol was charged and then 25ml CH_2Cl_2 was added. The mass was cooled to 0 ^{0}C, 61.8 mg (0.5 mmol, 0.1eq) of DMAP was added followed by 1281 mg (6.2 mmol, 1.2eq) of DCC. Finally, 1308 mg (5.66 mmol, 1.1eq) of BOC-ALA was charged. The reaction was brought to room temperature and was kept at room temperature overnight. Once the reaction was completed (Hex/AcOEt 8:2), the mixture was filtered through celite bed.The solvent was evaporated off by rotovapor. The crude mass was purified by flash chromatography (Hex/AcOEt 8:2, silica gel- 60 times the crude mass) which yielded 2524 mg of (±)-α-tocopherol-Boc-ALA ester. (Yield= 78.4%).



R_f : 0.2 (Hex/AcOEt 8:2)

IR (film) : 3433*wb*; 2927*s*; 2868*m*; 2253*w*; 1740*s*; 1714*s*; 1502*m*; 1462*m*; 1413*m*; 1367*s*; 1247*s*; 1154*s*; 1108*m*; 1049*w*; 910*w*; 874*vw*; 781*vw*; 734*m*; 648*vw*

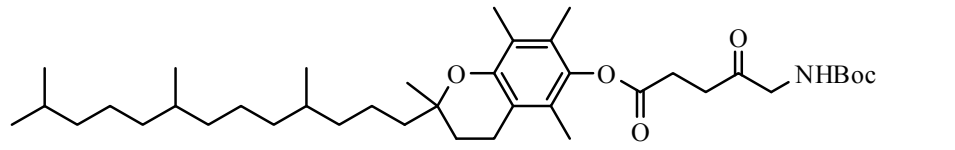
^{1}H-NMR (400 MHz, $CDCl_3$, 300K, δ (ppm)) : 5.25 (s, 1H, NH) ; 4.08 (d, $^3J_{5'',-NH}$=4.9, CH_2(5'') ; 2.96 (t, 2H, $^3J_{2'',-3''}$=5.9, $^3J_{2'',-3''}$=6.7, CH_2(2'')); 2.83 (t, 2H, $^3J_{3'',-2''}$=5.9, $^3J_{3'',-2''}$=6.6, CH_2(3'')); 2.58 (t, 2H, $^3J_{4-3}$=6.5, $^3J_{4-3}$=6.8, CH_2(4)); 2.08 (s, 3H, CH_3(12)); 2.00 (s, 3H, CH_3(11)); 1.96 (s, 3H, CH_3(13)); 1.84-1.72 (m, 2H, CH_2(3)); 1.60-1.47 (m, 1H, CH(12')); 1.44 (s, 9H, 3xCH_3(8'') ; 1.43-1.32 (m, 2H, CH(4',8')); 1.28-1.24 (m, CH_2(2', 6' and 10'), CH_2(3' or 5' or 7' or 9')) ; 1,23 (s, 3H, CH_3(14)) ; 1,21-1,03 (m, CH_2(1' and 11'), CH_2(3' or 5' or 7' or 9')) ; 0,87-0,83 (3d, 12H, ^{3}J=6,5, ^{3}J=5,7, ^{3}J=6,5, CH_3(13', 14', 15', 16'))

^{13}C-NMR (100 MHz, $CDCl_3$, 300K, δ (ppm)) : 204.3 C(4''); 171.4 C(1''); 155.7 C(6'') ; 149.5 C(10) ; 140.5 C(9) ; 126.7 C(7) ; 125.0 C(8) ; 123.1 C(6) ; 117.5 C(5) ; 79.9 C(7'') ; 75.1 C(2) ; 50.4 C(5'') ; 39.4 C(1',11') ; 37.6 C(3' or 5' or 7' or 9') ; 37.5 C(3' or 5' or 7' or 9') ; 37.5 C(3' or 5' or 7' or 9') ; 37.4 C(3' or 5' or 7' or 9') ; 34.3 C(3'') ; 32.9 C(8') ; 32.8 C(4') ; 31.1 C(3) ; 28.4 3xC(8'') ; 28.1 C(12') ; 27.6 C(2'') ; 24.9 C(10') ; 24.5 C(6') ; 23.8 C(14)***** ; 22.8 C(13' or 16') ; 22.7 C(13' or 16') ; 21.1 C(2')***** ; 20.7 C(4) ; 19.8 C(14' or 15') ; 19.8 C(14' or 15') ; 13.0 C(11) ; 12.2 C(13) ; 11.9 C(12) (***** peaks visible by DEPT)

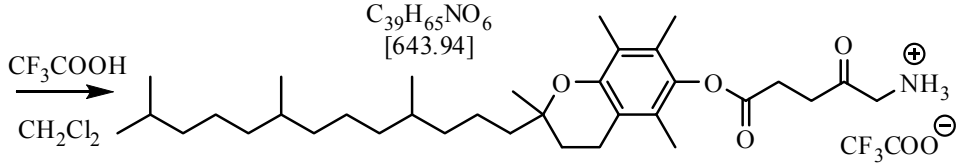
APCI ((+), solution in ethyl acetate diluted in acetone + CH_3COONa (10% in H_2O)): 544,3 $(Toc\text{-}ALA\text{-}NH_3)^+$; 666,4 $(M+Na)^+$

APCI ((+), solution in ethyl acetate diluted in acetone): 544,3 $(Toc\text{-}ALA\text{-}NH_3)^+$;

9.3.1.2. Synthesis of (±)-α-tocopherol-ALA

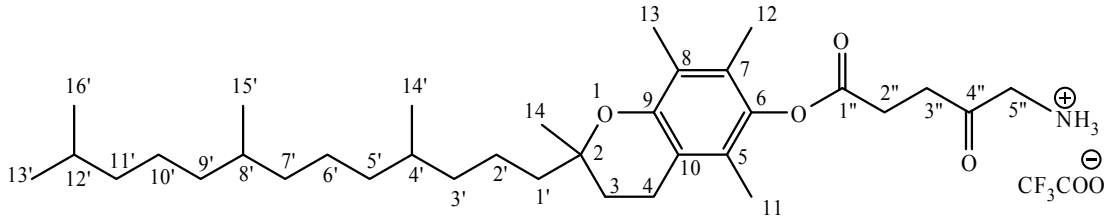


63
$C_{39}H_{65}NO_6$
[643.94]



64
$C_{36}H_{58}F_3NO_6$
[657.84]

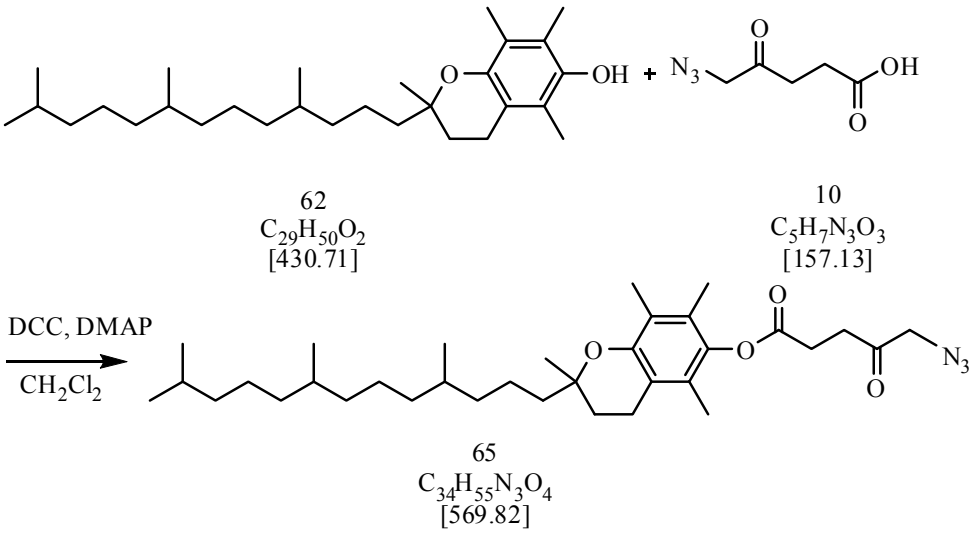
522 mg (0.811 mmol, 1eq) of (±)-α-Tocopherol-Boc-ALA ester was dissolved in 2.5 ml CH_2Cl_2 and cooled to -10^0C. To this solution, 4.6 g (3.1 ml, 40.48 mmol, 50eq) of trifluoroacetic acid was added dropwise by syringe. The reaction mass was maintained at $0\text{-}5^0$C for an hour. Once the deprotection of BOC was completed (controlled by the absence of BOC peak in ^{1}H-NMR), CH_2Cl_2 and trifluoroacetic acid was evaporated off. The product was co evaporated 2times with distilled ether, then the solid on drying yielded 472 mg of (±)-α-tocopherol-ALA which was an oil of yellow colour. (Yield=89%).



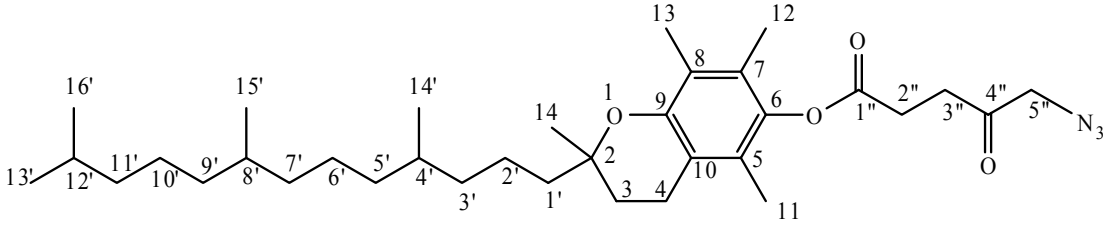
^{1}H-NMR (400 MHz, $CDCl_3$, 300K, δ (ppm)) : 7.54 (sb, 3H, NH_3) ; 3.85 (sb, 2H, CH_2(5'')) ; 2.94 (s, 2H, CH_2(2'')); 2.77 (s, 2H, CH_2(3'')); 2.59 (s, 2H, CH_2(4)); 2.09 (s, 3H, CH_3(12)); 1.98 (s, 3H, CH_3(11)); 1.93 (s, 3H, CH_3(13)); 1.77 (sb, 2H, CH_2(3)); 1.60-1.50 (sept, 1H, ^{3}J=6.6, CH(12')); 1.45-1.40 (m, 2H, CH(4',8')); 1.34-1.26 (m, CH_2(2', 6' and 10'), CH_2(3' or 5' or 7' or 9'), CH_3(14)) ; 1.19-0.98 (m, CH_2(1' and 11'), CH_2(3' or 5' or 7' or 9')) ; 0.90-0.86 (3d, 12H, ^{3}J=6.6, ^{3}J=5.9, ^{3}J=6.6, CH_3(13', 14', 15', 16'))

^{13}C-NMR (100 MHz, $CDCl_3$, 300K, δ (ppm)) : 201.4 C(4''); 172.7 C(1''); 149.8 C(10) ; 140.4 C(9) ; 126.8 C(7) ; 125.2 C(8) ; 123.4 C(6) ; 118.0 C(5) ; 75.5 C(2) ; 47.5 C(5'') ; 39.5 C(1',11') ; 37.6 C(3' or 5' or 7' or 9') ; 37.5* C(3' or 5' or 7' or 9') ; 37.5 C(3' or 5' or 7' or 9') ; 37.4 C(3' or 5' or 7' or 9') ; 34.1 C(3'') ; 32.9 C(8') ; 32.9 C(4') ; 31.1 C(3) ; 28.1 C(12') ; 27.3 C(2'') ; 25.0 C(10') ; 24.6 C(6') ; 24.5* C(14) ; 22.8 C(13' or 16') ; 22.7 C(13' or 16') ; 21.2 C(2') ; 20.6 C(4) ; 19.9 C(14' or 15') ; 19.8 C(14' or 15') ; 12.8 C(11) ; 11.9 C(13) ; 11.8 C(12)
(* peaks visible by DEPT)

9.3.1.3. Synthesis of (±)-α-tocopherol-azido levulinate ester



In a dry flask under argon atmosphere, 431 mg (1.0 mmol, 1eq) of (±)-α-tocopherol was charged and then 20ml CH_2Cl_2 was added. The mass was cooled to 0 ^{0}C, 12.3 mg (0.1 mmol, 0.1eq) of DMAP was added followed by 227 mg (1.1 mmol, 1.1eq) of DCC. Finally, 173 mg (1.1 mmol, 1.1eq) of azido levulinic acid was charged. The reaction was brought to room temperature and was kept at room temperature overnight. Once the reaction was completed (Hex/AcOEt 8:2), the mixture was filtered through celite bed.The solvent was evaporated off by rotovapor. The crude mass was purified by flash chromatography (Hex/AcOEt 8:2, silica gel- 60 times the crude mass) which yielded 471 mg of (±)-α-tocopherol-azido levulinate ester. (Yield= 82.6%).



R_f : 0.6 (100% AcOEt) ; 0,24 (Hex/AcOEt 8:2)

IR (film) : 2926*s* ; 2103*s*; 1748*s*; 1576*w*; 1462*m*; 1414*m*; 1377*m*; 1280*m*; 1244*m*; 1187*m*; 1153*s*; 1092*m*; 923*w*; 735*w*; 554*w*

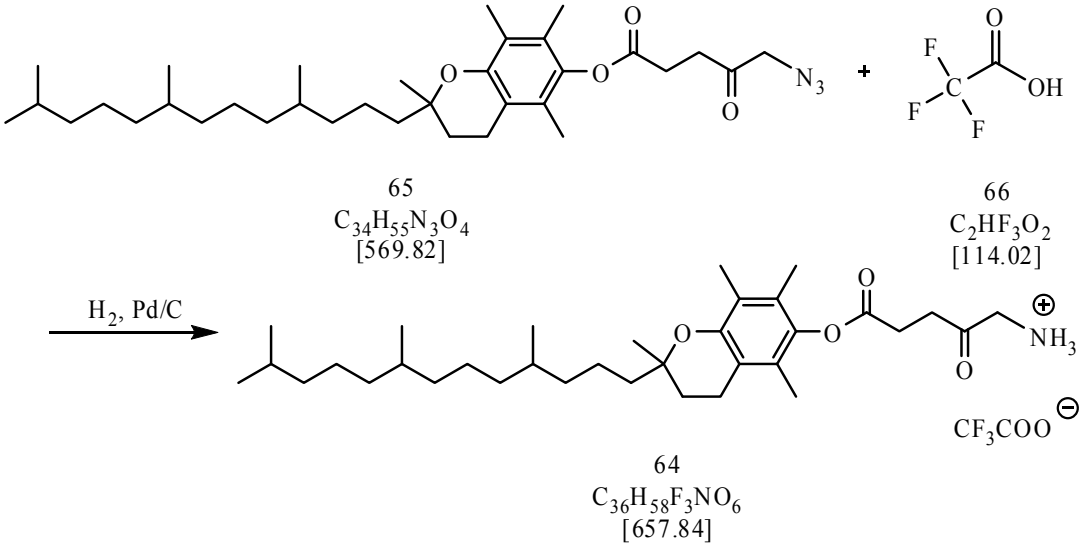
^{1}H-NMR (400 MHz, $CDCl_3$, 300K, δ (ppm)) : 4.05 (s, 2H, CH_2(5'')); 3.01-2.98 (m, 2H, AA' of a system AA'BB', CH_2(2'')) ; 2.85-2.81 (m, 2H, BB' of a system AA'BB', CH_2(3'')) ; 2.59 (t, 2H, $^3J_{4\text{-}3}$=6.7, CH_2(4)) ; 2.09(s, 3H, CH_3(12)) ; 2.01 (s, 3H, CH_3(11)) ; 1.97 (s, 3H, CH_3(13)) ; 1.84-1.73 (m, 2H, CH_2(3)) ; 1.63-1.32 (m, CH(4', 8', 12')) ; 1.28-1.25 (m, CH_2(1' or 2' or 3' or 5' or 6' or 7' or 9' or 10' or 11')) ; 1.24 (s, 3H, CH_3(14)) ; 1.21-1.03 (m, CH_2(1' or 2' or 3' or 5' or 6' or 7' or 9' or 10' or 11')) ; 0.88-0.84 (3d, 12H, ^{3}J=6.48, ^{3}J=5.80, ^{3}J=6.56, CH_3(13', 14', 15', 16'))

^{13}C-NMR (100 MHz, $CDCl_3$, 300K, δ (ppm)) : 203.0 (C4'') ; 171.3 (C1'') ; 149.6 (C10) ; 140.5 (C9) ; 126.7 (C7) ; 125.0 (C8) ; 123.2 (C6) ; 117.5 (C5) ; 75.2 (C2) ; 57.7 (C5'') ; 39.5 (C1'+11'); 37.6 (C3'or 5' or 7' or 9') ; 37.6 (C3'or 5' or 7' or 9') ; 37.5

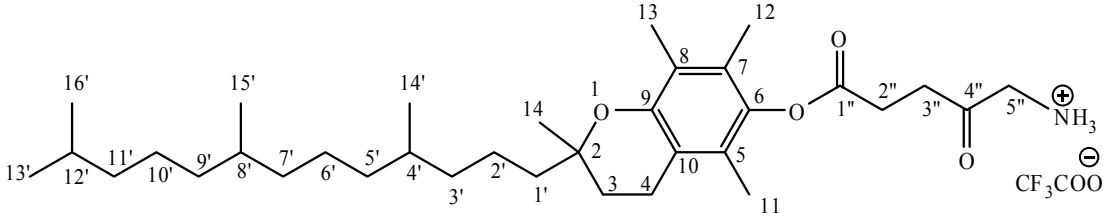
(C3'or 5' or 7' or 9') ; 37.4 (C3'or 5' or 7' or 9') ; 34.5 (C3'') ; 32.9 (C8') ; 32.8 (C4') ; 31.1* (C3) ; 28.1 (C12') ; 27.7 (C2'') ; 24.9 (C10') ; 24.5 (C6') ; 24.2* (C14) ; 22.8 (C16' or C13') ; 22.7 (C16' or C13') ; 21.1 (C2') ; 20.7 (C4) ; 19.9 (C14' or C15') ; 19.8 (C14' or C15') ; 13.0 (C11) ; 12.2 (C13) ; 11.9 (C12)
(* peaks visible by DEPT)

ESI (mode positive, acetone) : 153,3 (major peak). Probably, the product had decomposed as it is very sensitive to light and air.

9.3.1.4. Synthesis of (±)-α-tocopherol-ALA



471 mg (0.827 mmol, 1eq) of (±)-α-Tocopherol-Azido levulinate ester taken in a dry flask was dissolved in 35 ml isoprpanol and added dropwise by syringe 0.15 ml (1.96 mmol, 2.4eq) of trifluoroacetic acid. To this solution, 52.8 mg (0.05 mmol, 0.06eq) of Pd over activated carbon. The mass was subjected to hydrogenation in ambient temperature with the pressure of 60psi H_2 under mechanical agitation. Once the reaction was completed (Hex/AcOEt 8:2), the Pd was filtered over celite bed and then the solvent was evaporated off. The product was co evaporated 2times with distilled ether, then the solid on drying yielded 488 mg of (±)-α-tocopherol-ALA which was an oil of yellow colour. (Yield=90%).



IR (film) : 2928*m*; 2868*m*; 2253*w*; 1737*m*; 1677*m*; 1463*w*; 1378*w*; 1205*m*; 1169*m*; 1108*w*; 909*s*; 840*vw*; 800*vw*; 735*s*; 650*w*

^{1}H-NMR (400 MHz, $CDCl_3$, 300K, δ (ppm)) : 8.03 (sb, 3H, NH_3) ; 3.91 (s, 2H, CH_2(5'')) ; 2.89 (s, 2H, CH_2(2'')); 2.77 (s, 2H, CH_2(3'')); 2.58 (s, 2H, CH_2(4)); 2.09 (s, 3H, CH_3(12)); 1.97 (s, 3H, CH_3(11)); 1.93 (s, 3H, CH_3(13)); 1.77-1.74 (mb, 2H, CH_2(3)); 1.60-1.50 (sept, 1H, ^{3}J=6,6, CH(12')); 1.46-1.35 (m, 2H, CH(4',8')); 1.31-1.24 (m, CH_2(2', 6' and 10'), CH_2(3' or 5' or 7' or 9'), CH_3(14)) ; 1.19-1.04 (m, CH_2(1' and 11'), CH_2(3' or 5' or 7' or 9')) ; 0.90-0.86 (3d, 12H, ^{3}J=6.6, ^{3}J=6.6, ^{3}J=5.9, CH_3(13', 14', 15', 16'))

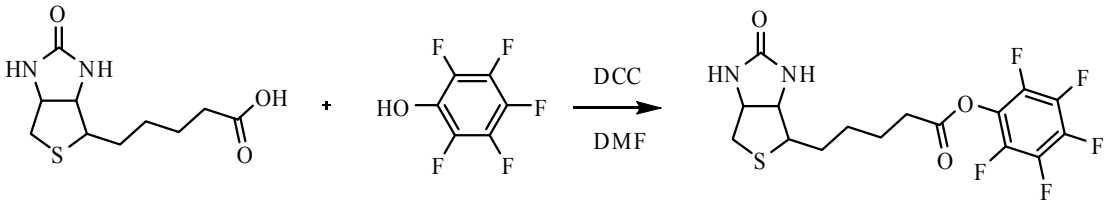
^{13}C-NMR (100 MHz, $CDCl_3$, 300K, δ (ppm)) : 201.8 C(4''); 171.8 C(1''); 162.0-161.0 ($\underline{C}OOCF_3$) ; 149.6 C(10) ; 140.5 C(9) ; 126.8 C(7) ; 125.2 C(8) ; 123.1 C(6) ; 117.7 C(5) ; 114.8 ($\underline{C}F_3$) ; 75.3 C(2) ; 47.4 C(5'') ; 39.5 C(1',11') ; 37.7 C(3' or 5' or 7' or 9') ; 37.6 C(3' or 5' or 7' or 9') ; 37.5 C(3' or 5' or 7' or 9') ; 37.4 C(3' or 5' or 7' or 9') ; 34.2 C(3'') ; 32.9 C(8') ; 32.8 C(4') ; 31.1 C(3) ; 28.1 C(12') ; 27.2 C(2'') ; 24.9 C(10') ; 24.6 C(6') ; 24.4 C(14) ; 22.8 C(13' or 16') ; 22.7 C(13' or 16') ; 21.1 C(2') ; 20.5 C(4) ; 19.8 C(14' or15') ; 19.8 C(14' or 15') ; 12.8 C(11) ; 11.9 C(13) ; 11.8 C(12)

ESI ((+), solution in ethyl acetate diluted in acetone): 544.5 $(M)^{+}$; 584,6 (major peak) ; ((-),solution in ethyl acetate diluted in acetone) : 113 $(CF_3COO)^{-}$

APCI ((+), solution in ethyl acetate diluted in acetone) : 544.5 $(M)^{+}$; 584.5 (major peak)

9.3.2. Synthesis of biotin-ALA peptide

9.3.2.1. Synthesis of biotin PFP ester

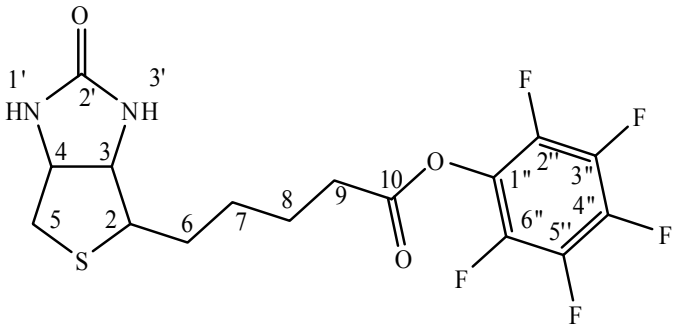


67
$C_{10}H_{16}N_2O_3S$
[244.31]

68
C_6HF_5O
[184,06]

69
$C_{16}H_{15}F_5N_2O_3S$
[410,36]

In a dry flask under argon atmosphere, 244 mg (1 mmol, 1eq) of biotin was charged. This was dissolved in 4ml DMF with heating over hot gun and the mass was cooled to 0 ^{0}C. Then 240 mg (1.3 mmol, 1.3eq) of pentafluorophenol was added followed by 312 mg (1.5 mmol, 1.5eq) of DCC. The reaction was brought to room temperature and was kept at room temperature overnight. The reaction mass was then filtered over celite bed to get rid off DCU and the solvent DMF was evaporated off by distillation. The white solid mass obtained was recrystallised with 20ml methanol. The white solid obtained was filtered off which on drying yielded 311 mg of biotin-PFP ester. (Yield= 75.9%)



R_f : 0,5 (Hex/AcOEt 1:1)

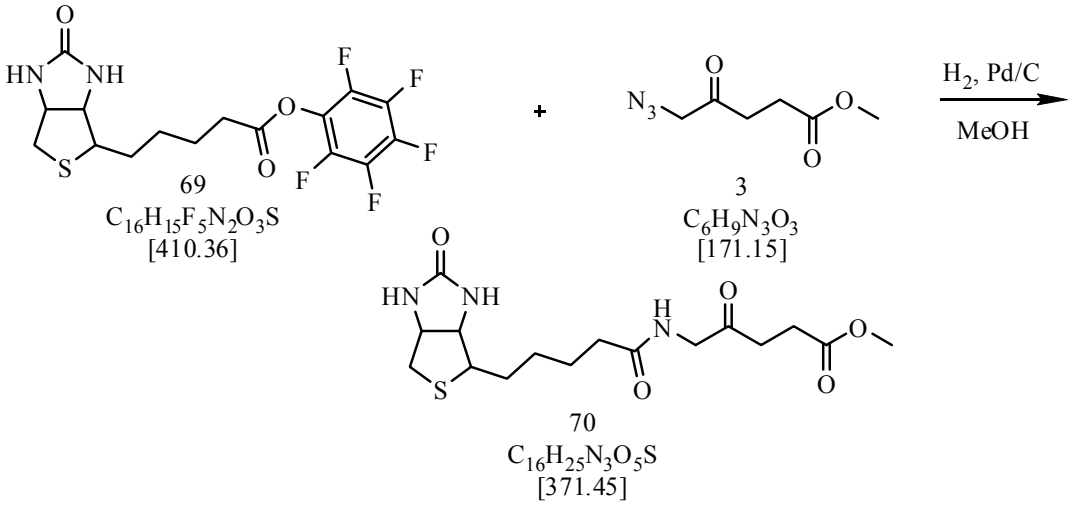
^{19}F-NMR (200 MHz, $CDCl_3$, 300K, δ (ppm)) : -153.1 (d, 2F, $^3J_{ortho-meta}$= 17,3, F_{ortho}-2'' et F_{ortho}-6''); -158.3 (t, 1,4F, ^3J= 21, F_{para}-4''); -162.5 (dd, 2,1F, ^3J=17,3, ^3J=21,9, F_{meta}-3'' et F_{meta}-5'')

^{1}H-NMR (400 MHz, DMSO, 300K, δ (ppm)) : 6.44 (d, 2H, J=3.0, NH(1' and 2')); 4.34 (t, 1H, ^3J=4.9, ^3J=7.2, CH(4)); 4.18-4,15 (m, 1H, CH(3)); 3.16-3.11 (m, 1H, CH(2)); 2.85-2.81 (dd, 1H, $^3J_{H5-H4}$=4,8, $^2J_{H5\alpha-H5\beta}$=12.6, CH_2(5α)); 2.77 (t, 2H, ^3J=7.1, CH_2(9)); 2.60 (d, 1H, $^2J_{H5\alpha-H5\beta}$=12.5, CH_2(5β); 1.69 (s, 2H, CH_2(6 or 7 or 8)); 1.52-1.41 (m, 4H, CH_2(6 or 7 or 8))

^{13}C-NMR (100 MHz, DMSO, 300K, δ (ppm)) : 170.5 C(10); 163.8 C(2'); 163.8 2xC(ar); 142.6 C(ar); 1401 C(ar); 139.6 C(ar); 125.2 C(ar); 61.9 C(3); 60.0 C(4); 56.1 C(2); 40.6 C(9); 33.1 C(5); 28.7 C(6 or 7); 28.6 C(6 or 7); 25.1 C(8)

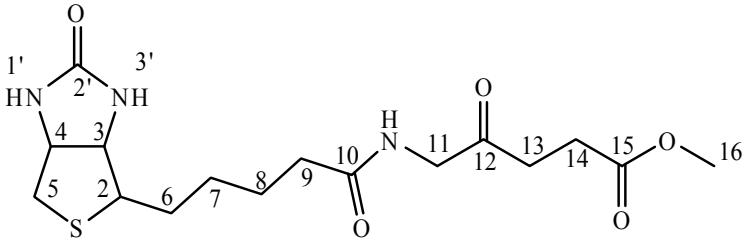
APCI ((+), DMSO diluted in acetone): 410.9 $(M+H)^+$.

9.3.2.2. Synthesis of biotin-ALA (OMe) peptide



To a solution of 205 mg (0.5 mmol, 1.5eq) biotin-PFP ester in 30 ml MeOH, was added 69 mg (0.40 mmol, 1eq) of 5-azido-4-oxo methyl pentanoate (5-azido methyl levulinate)

in 10 ml MeOH. To this mixture was added 25 mg (0.02 mmol, 0.06eq) of 10% Palladium over activated carbon. The mass was subjected to hydrogenation in ambient temperature with the pressure of 60psi H_2 under mechanical agitation. Once the reaction was completed, (approximately 24hrs; TLC eluent: Hex/AcOEt 2:1, CH_2Cl_2/MeOH 9:1) the H_2 pressure was released. The reaction mass was filtered over celite bed to get rid off palladium, the celite bed was washed with 3 × 10ml MeOH. The solvent in the filtrate was evaporated off by rotovapor and a yellow oily mass was obtained. This yellow crude mass was purified by flash chromatography.(eluent = CH_2Cl_2/MeOH 9:1). The pure fraction of 104.5 mg biotin-ALA (OMe) peptide was thus obtained. (Yield = 56.4%)



R_f : 0.34 (CH_2Cl_2/MeOH 9:1)

IR (KBr pellet) : 3343*m* ; 2946*m* ; 2925*m* ; 2877*w* ; 2860*w*; 1709*s*; 1632*s*; 1535*m*; 1467*m*; 1437*m*; 1423*m*; 1388*w*; 1357*m*; 1308*m*; 1270*m*; 1251*w*; 1204*m*; 1171*m*; 1134*w*; 1116*m*; 1040*w*; 976*w*; 888*vw*; 869*w*; 828*vw*; 760*vw*; 733*vw*; 697*vw*; 645*w*; 605*w*; 583*w*; 521*w*

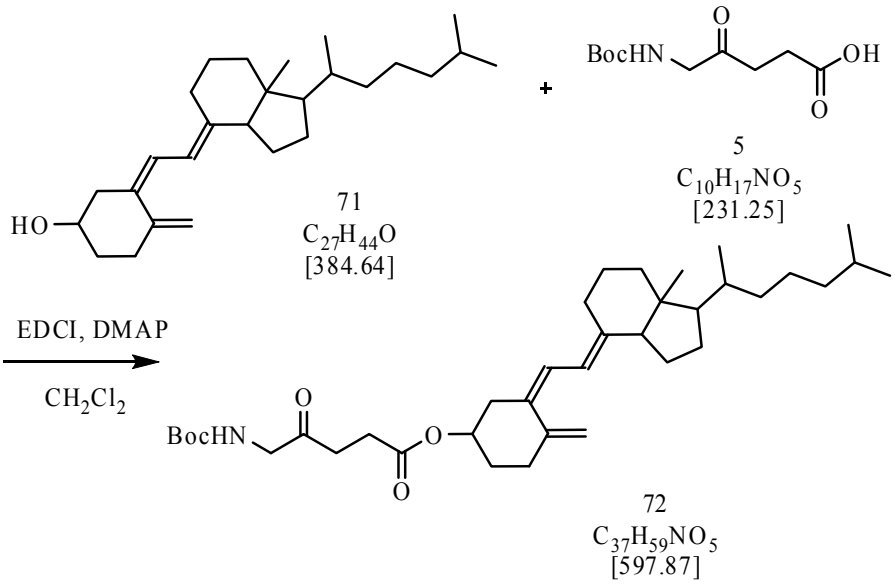
^{1}H-NMR (400 MHz, DMSO, 300K, δ (ppm)) : 8.16 (t, 1H, J=5.5, peptide NH) ; 6.45 (d, 2H, J=28.0, NH(1' et 2')); 4.35-4.32 (dd, 1H, J=5, J=7.3, J=5, CH(4)); 4.16 (t, 1H, J=4.7, J=5.8, CH(3)); 3.95 (d, 2H, J=5.6, CH_2(11)) ; 3.61 (s, 3H, CH_3(16)) ; 3.19-3.10 (m, 1H, CH(2)); 2.87-2.82 (dd, 1H, $^3J_{H5\text{-}H4}$=5, $^2J_{H5\alpha\text{-}H5\beta}$=12.5, CH_2(5α)); 2.71 (t, 2H, J=6.4, J=6.6, CH_2(13)) ; 2.60 (d, 1H, $^2J_{H5\alpha\text{-}H5\beta}$=12.5, CH_2(5β); 2.54-2.46 (m, 2H, CH_2(14)) ; 2.16 (t, 2H, J=7.4, CH_2(9) ; 1.68-1.59 (m, 2H, CH_2(6)); 1.56-1.40 (m, 2H, CH_2(7)) ; 1.39-1.23 (m, 2H, CH_2(8))

^{13}C-NMR (100 MHz, DMSO, 300K, δ (ppm)) : 205.7 C(12) ; 173.5 C(15); 172.7 C(10) ; 162.8 C(2'); 61.1 C(3); 59.3 C(4); 55.5 C(2); 51.5 C(16) ; 48.3 C(11) ; 40.1 C(5); 34.9 C(9) ; 33.9 C(13); 28.2 C(6); 28.1 C(8); 27.2 C(14) ; 25.3 C(7)

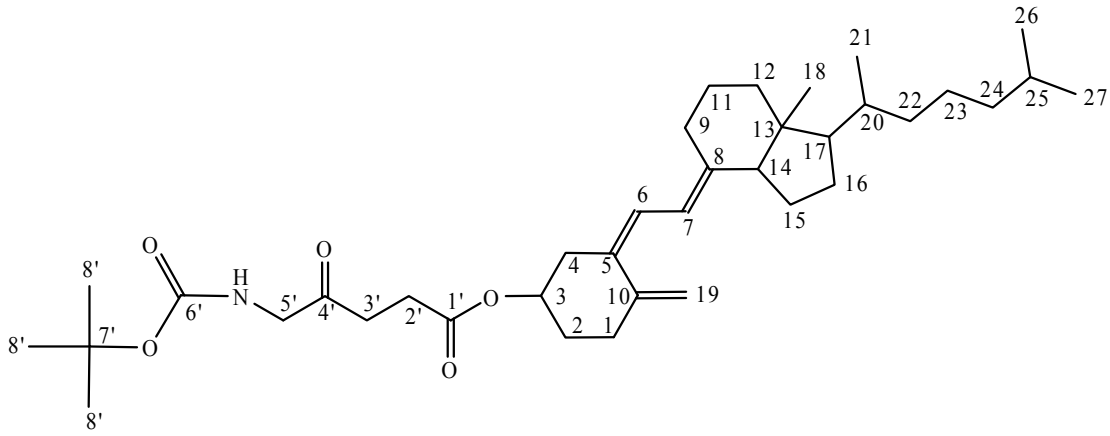
ESI ((+), DMSO diluted in acetone): 372.1 $(M+H)^+$

9.3.3. Synthesis of cholcalciferol –ALA ester (vitamin D_3- ALA)

9.3.3.1. Synthesis of cholcalciferol-Boc-ALA ester (vitamin D_3-Boc-ALA ester)



In a dry flask under argon atmosphere, 614 mg (1.60 mmol, 1eq) of cholcalciferol (vitamin D_3) was charged and then 25ml CH_2Cl_2 was added. The mass was cooled to 0 ^{0}C, 219.5 mg (1.77 mmol, 1.1eq) of DMAP was added followed by 371 mg (1.93 mmol, 1.2eq) of EDCI. Finally, 410 mg (1.77 mmol, 1.1eq) of BOC-ALA was charged. The reaction was brought to room temperature and was kept at room temperature overnight. The reaction was not completed even after 2 days (Hex/AcOEt 7:3), so the solvent was evaporated off by rotovapor. The crude mass was purified by flash chromatography (Hex/AcOEt 7:3, silica gel- 40 times the crude mass) which yielded 278 mg of cholcalciferol-Boc-ALA ester.(vitamin D_3-Boc-ALA ester)(Yield= 29%).



R_f : 0.3 (Hexane/AcOEt 7:3) ; 0.13 (Hexane/AcOEt 8:2)

IR (film) : 3431*bw*; 2950*s*; 2869*m*; 2253*w*; 1719v*s*; 1502*m*; 1440*m*; 1367*s*; 1249*s*; 1170*s*; 1047*m*; 1017*m*; 910*s*; 781*vw*; 734*s*; 648*w*

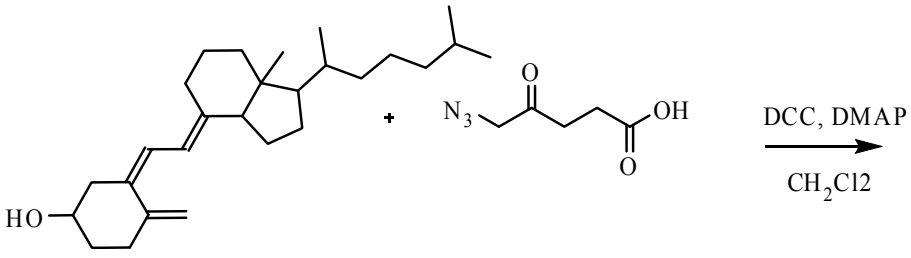
^{1}H-NMR (400 MHz, $CDCl_3$, 300K, δ (ppm)) : 6.16 (d, 1H, $^3J_{6\text{-}7}$= 11.2, CH(6)) ; 5.99 (d, 1H, $^3J_{7\text{-}6}$= 11.3, CH(7)) ; 5.22 (s, NH) ; 5.02 (s, 1H, CH_2(19E)) ; 4.93-4.89 (quin, 1H,

J=3,8, CH(3α)) ; 4.80 (d, 1H, ^{2}J=2.3, CH_2(19Z)); 3.99 (t, 2H, J=3.6, J=4.3, CH_2(5')) ; 2.78 (d, 1H, $^{3}J_{9\text{-}11}$= 12.3, CH_2(9β)) ; 2.66 (d, 2H, J=4.4, CH_2(3')) ; 2.60 (d, 2H, J=5.4, CH_2(2') ; 2.53-2.48 (dd, 1H, J=3.6, J=13.5, CH_2(4α)) ; 2.34-2.29 (m, 2H, CH_2(1β+4β)) ; 2.19-2.12 (m, 1H, CH_2(1α)) ; 1.98-1.93 (m, CH(14), CH_2(12β)) ; 1.88-1.82 (m, CH_2(2α + 16α) ; 1.76-1.68 (m, 1H, CH_2(2β)) ; 1.65-1.61 (m, 2H, CH_2(9α + 11α)) ; 1.57-1.43 (m, CH(25), CH_2(15 + 11β)) ; 1.41 (s, 9H, 3xCH_3(8')) ; 1.32-1.23 (m, 7H, CH(17), CH_2(12α + 16β + 22 + 23(1))) ; 1.10-1.07 (m, CH_2(23(2) + 24)) ; 1.01-0.96 (m, 1H, CH(20)) ; 0.89-0.88 (d, 3H, J=6.4, CH_3(21)) ; 0.84-0.82 (dd, 6H, J=1.8, J=6.6, CH_3(26 + 27)) ; 0.50 (s, 3H, CH_3(18))

^{13}C-NMR (100 MHz, $CDCl_3$, 300K, δ (ppm)) : 204.4 (C4'); 171.8 (C1'); 155.7 C(6') ; 144.5 (C10) ; 142.6 (C8) ; 134.2 (C5) ; 122.6 (C6) ; 117.5 (C7) ; 112.9 (C19) ; 79.8 C(7') ; 72.2 (C3) ; 56.6 (C17) ; 56.4 (C14) ; 50.3 C(5') ; 46.0 (C13) ; 42.0 (C4) ; 40.6 (C12) ; 39.5 (C24) ; 36.2 (C20) ; 34.7 (C22) ; 34.4 (C3') ; 32.1 (C1) ; 31.8 (C2) ; 29.1 (C9) ; 28.4 (C8') ; 28.2 C(2') ; 28.1 (C25) ; 27.7 (C16) ; 23.9 (C23) ; 23.6 (C11) ; 22.9 (C26 or C27) ; 22.6 (C26 or C27) ; 22.3 (C15) ; 18.9 (C21) ; 12.0 (C18)

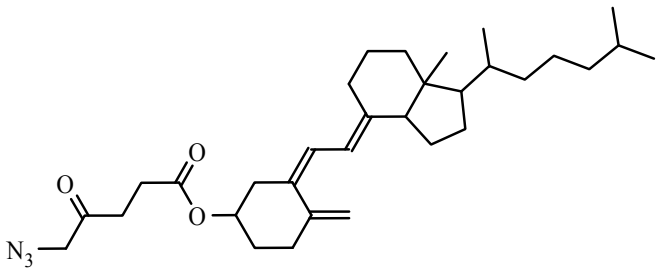
ESI ((+),soultion in $CHCl_3$ dissolved in acetone + CH_3COONa (10% in H_2O)): 620.3 $(M+Na)^+$

9.3.3.2. Synthesis of cholcalciferol -azido levulinate ester



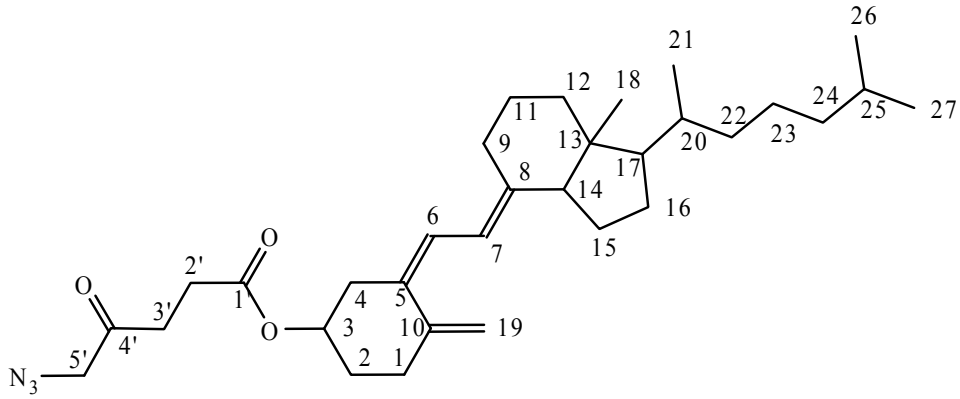
71
$C_{27}H_{44}O$
[384.64]

10
$C_5H_7N_3O_3$
[157.13]



73
$C_{32}H_{49}N_3O_3$
[523.75]

In a dry flask under argon atmosphere, 384 mg (1.0 mmol, 1eq) of cholcalciferol (vitamin D_3) was charged and then 15ml CH_2Cl_2 was added. The mass was cooled to 0 ^{0}C, 16 mg (0.1 mmol, 0.1eq) of DMAP was added followed by 228 mg (1.1 mmol, 1.1eq) of DCC. Finally, 176 mg (1.1 mmol, 1.1eq) of azido levulinic acid was charged. The reaction was brought to room temperature and was kept at room temperature overnight. The reaction was not completed even after 5 days (Hex/AcOEt 8:2), the mixture was filtered through celite bed.The solvent was evaporated off by rotovapor. The crude mass was purified by flash chromatography (CH_2Cl_2/AcOEt 9:1, silica gel- 60 times the crude mass) which yielded 156 mg of cholcalciferol -azido levulinate ester.(vitamin D_3 -azido levulinate ester). (Yield= 29.8%).



R_f : 0.24 (Hexane/AcOEt 8:2) ; 0.28 (Hexane/éther 1:1) ; 0.77 (CH_2Cl_2/AcOEt 9:1)

IR (film) : 3441*bw*; 3079*w*; 2949*s*; 2104*s*; 1808*w*; 1731*s*; 1645*w*; 1467*m*; 1440*m*; 1415*m*; 1379*s*; 1277*s*; 1201*s*; 1094*m*; 1047*m*; 1010*m*; 909*m*; 835*w*; 735*m*; 649*vw*; 554*vw*

^{1}H-NMR (400 MHz, $CDCl_3$, 300K, δ (ppm)) : 6.18 (d, 1H, $^3J_{6\text{-}7}$= 11.21, CH(6)) ; 6,00 (d, 1H, $^3J_{7\text{-}6}$= 11.27, CH(7)) ; 5.04 (s, 1H, CH_2(19E)) ; 4.95-4.91 (m, 1H, CH(3α)) ; 4.81 (s, 1H, CH_2(19Z)) ; 3.98 (s, 2H, CH_2(5')) ; 2.79 (d, 1H, $^3J_{9\text{-}11}$= 12.29, CH_2(9β)) ; 2.67-2.61 (m, 4H, CH_2(3'+2')) ; 2.53 (d, 1H, $^3J_{4\text{-}3}$= 0.03, CH_2(4α)) ; 2.35-2.30 (m, 2H, CH_2(1β+4β)) ; 2.18-2.15 (m, 1H, CH_2(1α)) ; 1.96-1.77 (m, 5H, CH(14), CH_2(2α + 12β + 16β)) ; 1.75-1.68 (m, 1H, CH_2(2β)) ; 1.65-1.62 (m, 2H, CH_2(9α + 11α)) ; 1.52-1.44 (m, 4H, CH(25), CH_2(15 + 11β)) ; 1.33-1.21 (m, 7H, CH(17), CH_2(12α + 16α + 22 + 23)) ; 1.11-1.09 (m, 4H, CH_2(23 + 24)) ; 0.99-0.95 (m, 1H, CH(20)) ; 0.90-0.88 (m, 3H, CH_3(21)) ; 0.85-0.83 (m, 6H, CH_3(26 + 27)) ; 0.51 (s, 3H, CH_3(18))

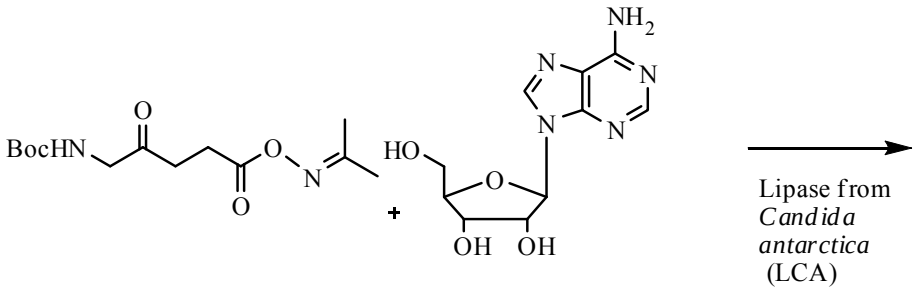
^{13}C-NMR (100 MHz, $CDCl_3$, 300K, δ (ppm)) : 203.1 (C4') ; 171.7 (C1') ; 144.5 (C10) ; 142.7 (C8) ; 134.2 (C5) ; 122.6 (C6) ; 117.5 (C7) ; 112.9 (C19) ; 72.4 (C3) ; 57.5 (C5') ; 56.6 (C17) ; 56.4 (C14) ; 46.0 (C13) ; 42.0 (C4) ; 40.6 (C12) ; 39.5 (C24) ; 36.2 (C20) ; 36.1* (C22) ; 34.5 (C3') ; 32.1 (C1) ; 31.8 (C2) ; 29.1 (C9) ; 28.2 (C2') ; 28.1 (C25) ; 27.7 (C16) ; 23.9 (C23) ; 23.6 (C11) ; 23.0 (C26 or C27) ; 22.6 (C26 or C27) ; 22.3 (C15) ; 18.9 (C21) ; 12.0 (C18)
(***** peaks visible by DEPT)

ESI ((+), acetone + CH_3COONa (10% in H_2O)): 546.4 $(M+Na)^+$; 562.4 $(M+O+Na)^+$

9.4. Synthesis of nucleoside-ALA derivatives

9.4.1. Synthesis of adenosine-ALA derivative

9.4.1.1. Synthesis of 5´- O-(Boc-ALA)-adenosine



12

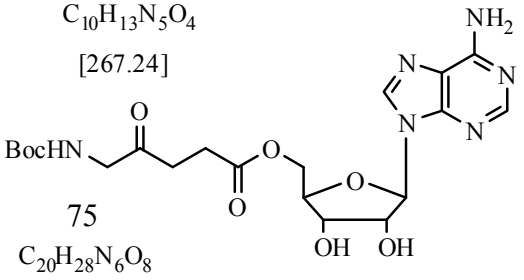
$C_{13}H_{22}N_2O_5$

[286.32]

74

$C_{10}H_{13}N_5O_4$

[267.24]

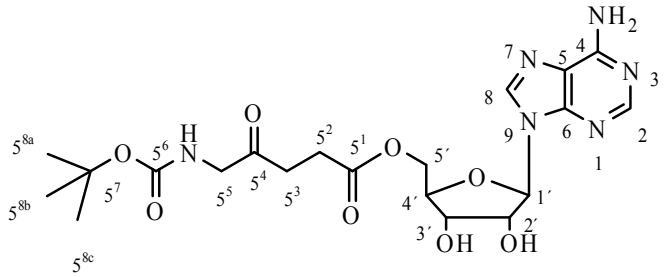


75

$C_{20}H_{28}N_6O_8$

[480.47]

267 mg (1.0 mmol, 1eq) of adenosine.was taken in a dry flask under argon atmosphere and was dissolved in 7 ml anhydrous distilled THF. 200 mg (10 units per mg of protein; 1unit hydrolyses 1µmol or 0.001mmol of an ester; approximately, 2 mmol, 2eq) of lipase from *Candida antarctica* (LCA, Novozyme 435) enzyme (before use, dried by keeping at vacuum 10^{-2} mbar for 3-4 days) was added and then 860 mg (3.0 mmol, 3eq) of Boc-ALA-oxime ester was added to the solution. The reaction was maintained at 60^0C for 24h. (controlled by TLC eluent :ether: MeOH 8:2). The mass was filtered over celite bed, the filtered cake was washed with 3× 10ml THF. The THF was evaporated off by rotovapor .The residue was taken for flash chromatography. (silica gel- 80times of the residue mass; eluent from ether: MeOH 8:2). The main fractions were concentrated to give 117 mg of pure product 5´-O-(BOC- ALA) -adenosine was obtained as white solid. (Yield = 24.8 %).



Batch: RV 284

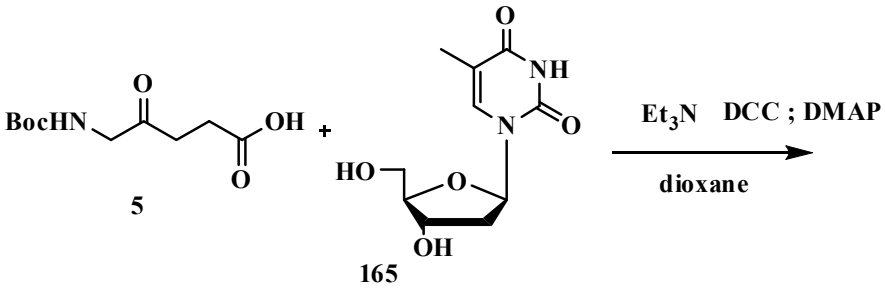
^{1}H-NMR (400 MHz, CD_3OD, 298 K): δ_H (ppm) = 8.29 (*s*, 1H, HC(2)); 8.18 (*s*, 1H, HC(8)); 6.83 (*sbr*, 1H, NH); 6.02 (*d*, $^{3}J_{1'-2'}$ = 4.2 Hz, 1H, HC(1′)); 4.65 (*t*, $^{3}J_{2'-1'} \approx 4.2$ Hz, $^{3}J_{2'-3'} \approx 4.2$ Hz, 1H, HC(2′)); 4.41 (*dd*, $^{3}J_{5'a-4'} \approx 3.3$Hz, $^{3}J_{5'a-5'b} = 12$Hz, 1H, HC(5′a)); 4.38-4.33 (*m*, 2H, HC(3′) and HC(5′b)); 4.30-4.27 (*m*, 1H, HC(4′)); 3.94 (*s*, 2H, $H_2C(5^5)$); 2.76 (*t*, $^{3}J_{2-3} \approx 6.0$Hz, 2H, $H_2C(5^2)$); 2.63 ($^{3}J_{3-2} \approx 6.0$Hz, 2H, $H_2C(5^3)$); 1.40 (*s*, 9H, $H_3C(5^{8a},5^{8b},5^{8c})$; and other peaks due to traces of ether and impuritiy.

^{13}C-NMR (100 MHz, CD_3OD, 298K) : δ_C (ppm) = 206.6 ($C5^4$); 173.4 ($C(5^1)$); 157.4 (5^6); 156.4 (C(4)); 153.2 (C(2)); 149.6 (C(6)); 140.0 (C(8)), 120.0 (C(5)); 89.7(C(1′)); 82.7 (C(4′)); 74.8 (C(2′)); 70.9 (C(3′)); 64.4 (C(5′)); 50.4 ($C(5^5)$; 34.5 ($C(5^3)$); 28.5 ($C(5^{8a},5^{8b},5^{8c})$); 28.1 ($C(5^2)$); and other peaks due to traces of ether and impuritiy.

MS (APCI (-)): m/z relative intensity $[M + Na]^+ = 503.1$; $[M + H]^+ = 481.1$.

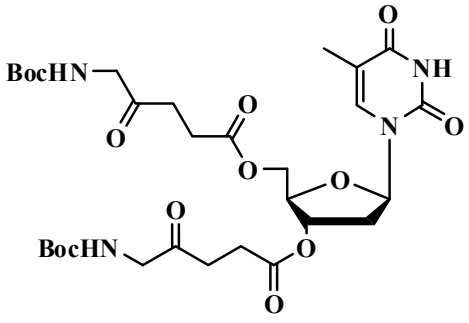
9.4.2. Synthesis of thymidine-ALA derivative

9.4.2.1. Synthesis of 3′, 5′- O-di (Boc-ALA)-thymidine



$C_{10}H_{17}NO_5$ 231.16 g/mol

$C_{10}H_{14}N_2O_5$ 242.23 g/mol

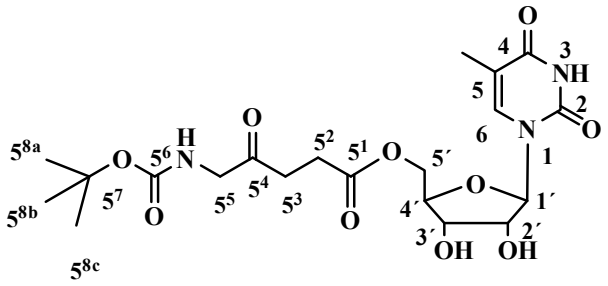


238a

$C_{30}H_{44}N_4O_{13}$

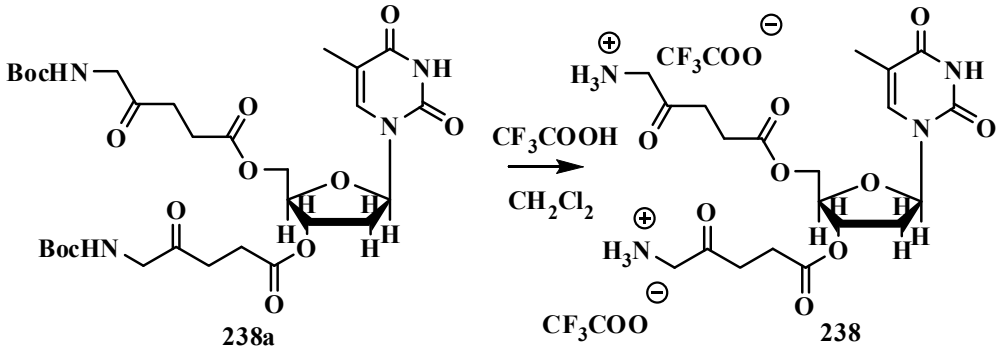
668.69 g/mol

In a dry two necked flask 0.5 g (2.06 mmol) of thymidine and 1.2 g (11.9 mmol) of triethyl amine was dissolved in 20 ml dioxane under nitrogen atmosphere. Then 2.4 g (10.4 mmol) of Boc-ALA **5**, 2.15 g (10.4 mmol) of DCC and 0.02 g (0.16 mmol) of DMAP. The reaction was stirred at ambient temperature for 2h. The reaction mass was then filtered over celite and the solvent was evaporated. The mass was later extracted with 200 ml CH_2Cl_2, followed by extraction with saturated $NaHCO_3$ solution. The organic phase is dried over $MgSO_4$ and solvent evaporated. The 4.3 g crude was purified by column chromatography using 200 g silica gel with AcOEt. The pure final product was obtained with 98% yield.



Analysis in progress

9.4.2.2. Synthesis of 3′, 5′- O-di (ALA)-thymidine



238a

668.69 g/mol

$C_{30}H_{44}N_4O_{13}$

238

470.47 g/mol

$C_{20}H_{30}N_4O_9^{2+}$

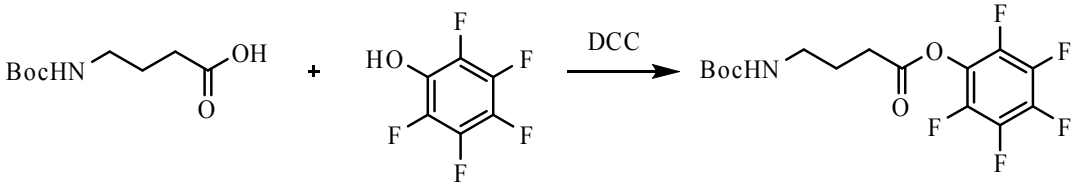
In a dry flask, 0.7 g (1.05 mmol) of 3′, 5′- O-di (Boc-ALA)-thymidine was dissolved in 20 ml of CH_2Cl_2 . The solution is cooled to 0-5°C and 6.0 g (52.61 mmol) of TFA was added slowly. Then, the reaction was stirred for 30 minutes at ambient temperature. After evaporating TFA, the traces of TFA were removed by trituraation and co evaporation with ether. Finally dry product of 0.4 g 3′, 5′- O-di (ALA)-thymidine was obtained with 82% yield.

Analysis in progress

9.5. Synthesis of ALA based peptides and ALA esters (non sugars)

9.5.1. Synthesis of GABA- ALA(OMe) peptide

9.5.1.1. Synthesis of Boc-GABA-PFP ester

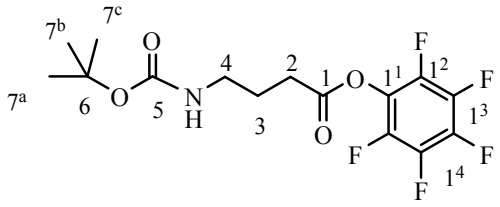


76 $C_9H_{17}NO_4$ [203.23]

68 C_6HF_5O [184.06]

77 $C_{15}H_{16}NO_4F_5$ [369.28]

To a solution of 813 mg (4 mmol, 1eq) Boc-GABA (Boc-γ-aminobutyric acid) in 25 ml EtOAc at -10^0C, was added a solution of 740 mg (4 mmol, 1eq) pentafluoro phenol in 10 ml EtOAc. The reaction mass was brought to ambient temperature and kept at that temperature for 3 h. The mixture was filtered over celite bed and the solvent was evaporated off. The residue on precipitation with ether afforded 1447 mg Boc-GABA-PFP ester. (Yield = 98%)



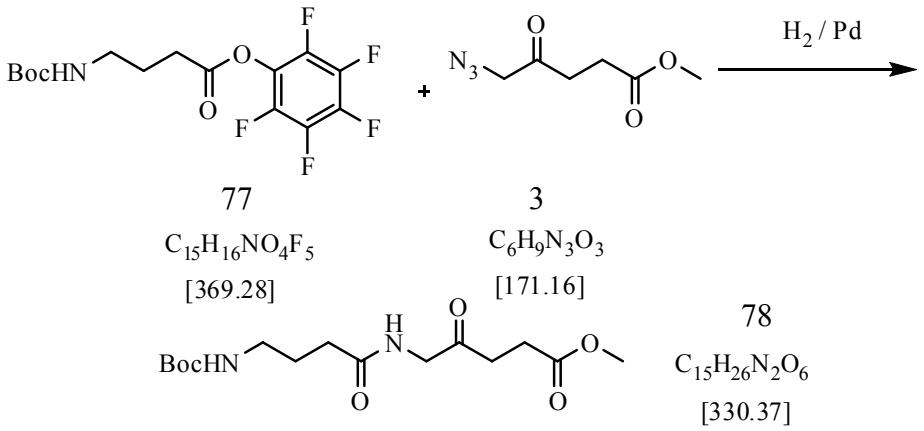
Batch : RV 61

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 4.68 (*sbr*, 1H, NH) ;3.24 (*q*, $^3J_{4\text{-}NH} \approx 6.5$ Hz, $^3J_{4\text{-}3} \approx 6.5$ Hz, 2H, $H_2C(4)$); 2.71 (*t*, $^3J_{2\text{-}3}$ = 7.4Hz, 2H, $H_2C(2)$); 1.95 (*quint*, $^3J_{3\text{-}2} \approx$ 7.1Hz, $^3J_{3\text{-}4} \approx$ 7.1Hz, 2H, $H_2C(3)$); 1.44 (*s*, 9H, $H_3C(7^a,7^b,7^c)$.

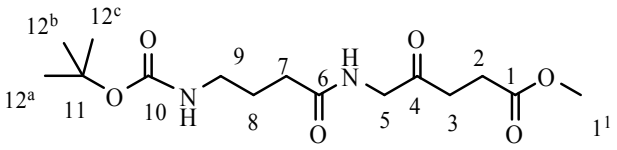
^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 169.5 (C(1)) ; 156.4 (C(5)) ; 142.5, 142.4, 142.4, 142.3, 140.0, 140.0, 139.9, 139.2, 138.5, 138.4, 136.7 and 136.5; 125.0 ($C1^1$), ($C1^2$), ($C1^3$), ($C1^4$) ; 79.9 (C(6)) ; 39.9 (C(4)) ; 30.9 (C(2)) ; 28.7 ($C(7^a,7^b,7^c)$) ; 25.6 (C(3)).

MS (APCI (-)): m/z relative intensity $[M + Na]^+ = 392.0$

9.5.1.2. Synthesis of Boc-GABA-ALA (OMe) peptide



To a solution of 1447 mg (3.92 mmol, 1.5eq) Boc-GABA-PFP ester in 20 ml MeOH, was added 457 mg (2.67 mmol, 1eq) of 5-azido-4-oxo methyl pentanoate (5-azido methyl levulinate) in 10 ml MeOH. To this mixture was added 85 mg (0.08 mmol, 0.03eq) of 10% Palladium over activated carbon. The mass was subjected to hydrogenation in ambient temperature with the pressure of 60psi H_2 under mechanical agitation. Once the reaction was completed, (approximately 24hrs; TLC eluent: EtOAc/ Hexane 3:1) the H_2 pressure was released. The reaction mass was filtered over celite bed to get rid off palladium, the celite bed was washed with 3 × 10ml MeOH. The solvent in the filtrate was evaporated off by rotovapor and a yellow oily mass was obtained. This yellow crude mass was purified by flash chromatography. (eluent = 100% EtOAc). The pure fraction of 770 mg BOC-GABA-ALA (OMe) peptide was thus obtained. (Yield = 87.3%)



Batch: RV 67

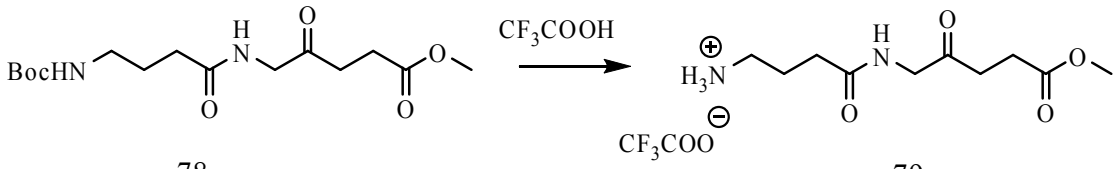
R_f: (100% EtOAc): 0.14 ($KMnO_4$)

^{1}H-NMR (400 MHz, $CDCl_3$ & CD_3OD, 298 K): δ_H (ppm) = 6.80 (*sbr*, 1H, CONH); 4.92 ((*sbr*, 1H, NHBoc); 4.13 (*d*, $^3J_{5\text{-}NH}$=5.0Hz, 2H, $H_2C(5)$); 3.62 (*s*, 3H, $H_3C(1^1)$); 3.12 (*sbr*, 2H, $H_2C(9)$; 2.72-2.68 (*m*, 2H, $H_2C(3)$); 2.61-2.59 (*m*, 2H, $H_2C(2)$); 2.24 (*t*, $^3J_{7\text{-}8}$ = 7.4Hz, 2H, $H_2C(7)$); 1.76 (*quint*, $^3J_{8\text{-}7} \approx 7.0$Hz, $^3J_{8\text{-}9} \approx 7.0$Hz, 2H, $H_2C(8)$); 1.40 (*s*, 9H, $H_3C(12^a,12^b,12^c)$, other peaks due to EtOAc solvent.

^{13}C-NMR (100 MHz, $CDCl_3$ & CD_3OD, 298K) : δ_C (ppm) = 173.3 (C(1)) ; 156.7 (C(10) and C(6)) ; 79.8 (C(11)); 52.2 ($C(1^1)$; 49.5 (C(5)); 40.1 (C(9)); 34.9 (C(3)); 33.5 (C(7)); 28.7 ($C(12^a,12^b,12^c)$); 27.9 (C(2)); 26.5 (C(8))

MS (ESI (+)): m/z relative intensity $[M+ Na]^+ = 353.1$

9.5.1.3. Synthesis of GABA-ALA (OMe) peptide



78

$C_{15}H_{26}N_2O_6$

[330.37]

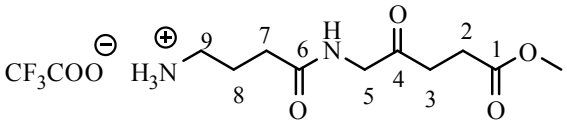
79

$C_{12}H_{19}F_3N_2O_6$

[344.28]

M^+=231.27 ; CF_3COO^- = 113.01

330 mg (1.0 mmol, 1eq) of BOC-GABA-ALA (OMe) peptide was dissolved in 10ml CH_2Cl_2 and cooled to -10^{0}C. To this solution, 14.8g (10ml, 129.0mmol, 129eq) of trifluoroacetic acid was added dropwise by syringe. The reaction mass was maintained at 0-5^{0}C for an hour. Once the deprotection of BOC was completed (controlled by the absence of BOC peak in ^{1}H-NMR), CH_2Cl_2 and trifluoroacetic acid was evaporated off. The product was co evaporated 2times with distilled ether, then triturated with dry ether and the ether solution was decanted. The trituration with ether and decanting top clear ether solution after centrifuge was repeated 2 times more. The settled solid product on drying yielded 330 mg of GABA-ALA (OMe) peptide (Yield=95.8%).



Batch: RV 70, RV87

IR (KBr): 3428*m*, 3280*m*, 3079*m*, 2959*m*, 2925*m*, 2345*vw*, 2115*vw*, 1742*vs*, 1723*vs*, 1688*s*, 1666*vs*, 1639*vs*, 1557*m*, 1480*vw*, 1431*m*,1407*w*, 1385*w*, 1366*w*, 1275*vw*, 1206*vs*, 1179*s*, 1167*m*, 1139*s*, 1086*w*, 1018*w*, 978*w*, 879*vw*, 838*m*, 801*m*,724*s*, 656*vw*, 600*vw*, 553*vw*, 518*vw*, 485*vw*, 467*vw*.

^{1}H-NMR (400 MHz, CD_3OD, 298 K): δ_H (ppm) = 4.09 (*s*, 2H, $H_2C(5)$); 3.64 (*s*, 3H, $H_3C(1^1)$; 3.01 (*t*, $^3J_{9\text{-}8} \approx 7.5$Hz, 2H, $H_2C(9)$; 2.78-2.75 (*m*, 2H, $H_2C(3)$); 2.60-2.57 (*m*, 2H, $H_2C(2)$); 2.43 (*t*, $^3J_{7\text{-}8} = 7.0$Hz, 2H, $H_2C(7)$); 1.95 (*quint*, $^3J_{8\text{-}7} \approx 7.0$Hz, $^3J_{8\text{-}9} \approx 7.0$Hz, 2H, $H_2C(8)$).

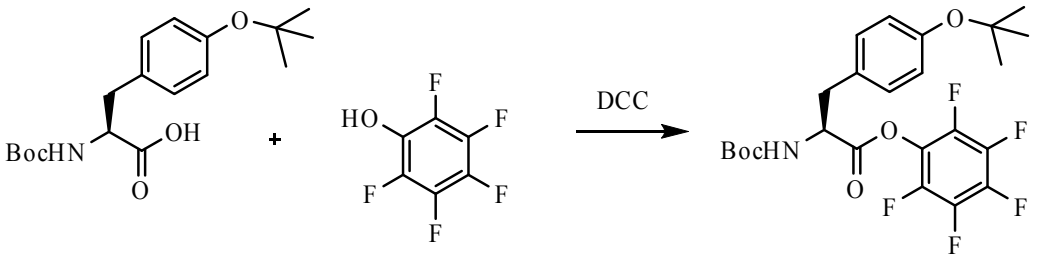
^{13}C-NMR (100 MHz, CD_3OD, 298K) : δ_C (ppm) = 206.3 (C(4)); 174.9 (C(1)); 173.3 (C(6)); 162.9 (*q*,$^2J_{C\text{-}F} \approx 35.9$, $CF_3\underline{C}OO^-$); 118.1 (*q*,$^1J_{C\text{-}F} \approx 290.0$, $\underline{C}F_3COO^-$); 52.2 ($C(1^1)$; 49.7 (C(5)); 40.2 (C(9)); 35.1 (C(3)); 33.3 (C(7)); 28.3 (C(2)); 24.3 (C(8))

MS (ESI (+)): m/z relative intensity $[M\text{-}CF_3COO^-]^+ = 231.1$

CHN: $C_{12}H_{19}F_3N_2O_6$ Calculated: C 41.86 H 5.56 N 8.14;
Observed: C 41.80 H 5.53 N 7.76

9.5.2. Synthesis of Tyr- ALA peptide

9.5.2.1. Synthesis of Boc-Tyr (O^{t}Bu)-PFP ester

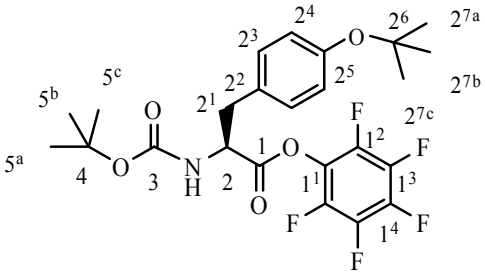


80 — $C_{18}H_{27}NO_5$ [337.41]

68 — C_6HF_5O [184.06]

81 — $C_{24}H_{26}NO_5F_5$ [503.46]

To a solution of 1000 mg (2.96 mmol, 1eq) Boc-tyrosine (O^{t}Bu) (Boc-TyrO^{t}Bu) in 25 ml EtOAc at -10^{0}C, was added a solution of 546 mg (2.96 mmol, 1eq) pentafluoro phenol in 10 ml EtOAc. The reaction mass was brought to ambient temperature and kept at that temperature for 3 h. The mixture was filtered over celite bed and the solvent was evaporated off. The residue on precipitation with ether afforded 1471 mg Boc-Tyr(O^{t}Bu)-PFP ester. (Yield = 98.6%)



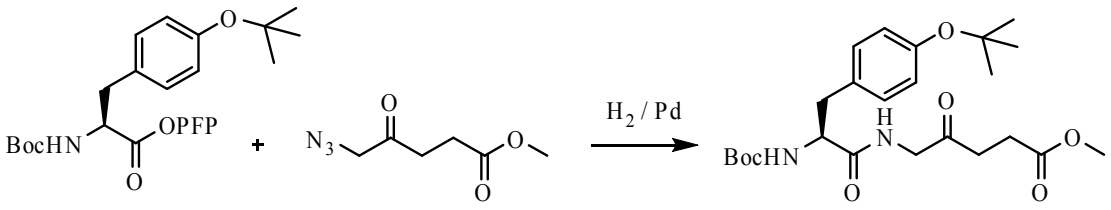
Batch : RV 62

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.14- 7.11 (*m*, 2H, Aromatic C(2^3) and C(2^4)); 6.97- 6.94 (*m*, 2H, Aromatic C($2^{3'}$) and C($2^{4'}$)); 5.00 (*d*, $^3J_{2\text{-}NH}$ =8.0Hz, 1H, NH); 4.88 (*d*, $^3J_{2\text{-}2^1}$ =6.0Hz, 1H, HC(2)); 3.25 (*dd*, $^3J_{2\text{-}2^{1a}} \approx$ 6.0Hz, $^3J_{2^{1a}\text{-}2^{1b}}$ =14.0Hz, 2H, HC(2^{1a})); 3.14 (*dd*, $^3J_{2\text{-}2^{1b}} \approx$ 6.5Hz, $^3J_{2^{1a}\text{-}2^{1b}}$ =14.0Hz, 2H, HC(2^{1b}))); 1.43 and 1.34 (2× *s*, 18H, $H_3C(5^a,5^b,5^c)$ and $H_3C(2^{7a},2^{7b},2^{7c})$.

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 168.8 (C(1)); 155.3 (C(3)); 142.7, 142.6, 141.3, 140.1, 139.5, 138.8, 137.0 and 130.0 (C1^1), (C1^2), (C1^3), (C1^4), C(2^2) and C(2^5); 130.1,124.7 (C(2^3), C($2^{3'}$), C(2^4) and C($2^{4'}$)); 80.9 and 78.9 (C(4) and C(2^6)); 54.8 (C(2)); 37.6 (C(2^1)) ; 29.2 and 28.6 (C(5^a,5^b,5^c) and C(2^{7a},2^{7b},2^{7c})).

MS (ESI): m/z relative intensity $[M + Na]^+$ = 526.1

9.5.2.2. Synthesis of Boc-Tyr(O^{t}Bu)-ALA (OMe) peptide

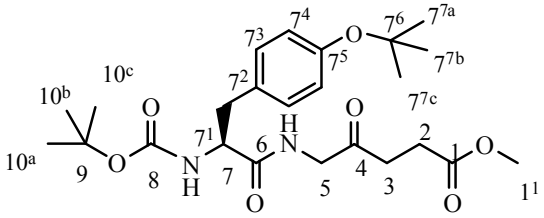


81 $C_{24}H_{26}NO_5F_5$ [503.46]

10 $C_6H_9N_3O_3$ [171.16]

82 $C_{24}H_{36}N_2O_7$ [464.55]

To a solution of 1471 mg (2.92 mmol, 1.5eq) Boc-Tyr(O^{t}Bu)-PFP ester in 20 ml MeOH, was added 338 mg (1.97 mmol, 1eq) of 5-azido-4-oxo methyl pentanoate (5-azido methyl levulinate) in10 ml MeOH. To this mixture was added 65 mg (0.061 mmol, 0.03eq) of 10% Palladium over activated carbon. The mass was subjected to hydrogenation in ambient temperature with the pressure of 60psi H_2 under mechanical agitation. Once the reaction was completed, (approximately 24hrs; TLC eluent: EtOAc/ Hexane 3:1) the H_2 pressure was released. The reaction mass was filtered over celite bed to get rid off palladium, the celite bed was washed with 3 × 10ml MeOH. The solvent in the filtrate was evaporated off by rotovapor and a yellow oily mass was obtained. This yellow crude mass was purified by flash chromatography. (silica gel- 65 times the crude mass; eluent = 3:1 EtOAc: Hexane). The pure fraction of 823 mg Boc-Tyr(O^{t}Bu)-ALA (OMe) peptide was thus obtained. (Yield = 89.9%)



Batch: RV 68

R_f: (3:1 EtOAc: Hexane): 0.37 ($KMnO_4$)

IR (KBr): 3354*vs*, 2986*s*, 2937*m*, 2921*m*, 2873*vw*, 2854*vw*, 1741*vs*, 1719*vs*, 1694*vs*, 1650*vs*, 1610*w*, 1578*vw*, 1513*vs*, 1476*vw*, 1462*w*, 1437*s*, 1402*m*, 1390*m*, 1367*vs*, 1334*w*,1312*w*, 1291*w*, 1250*s*, 1237*s*, 1205*s*, 1165*vs*, 1126*w*, 1105*m*, 1078*m*, 1017*m*, 1050*m*, 968*m*, 941*vw*, 926*w*, 904*m*, 868*vw*, 840*m*, 804*vw*, 794*vw*, 779*w*, 755*w*, 725*w*, 701*vw*, 657*m*, 638*vw*, 620*m*, 568*m*, 544*w*, 503*vw*, 478*vw*, 460*vw*.

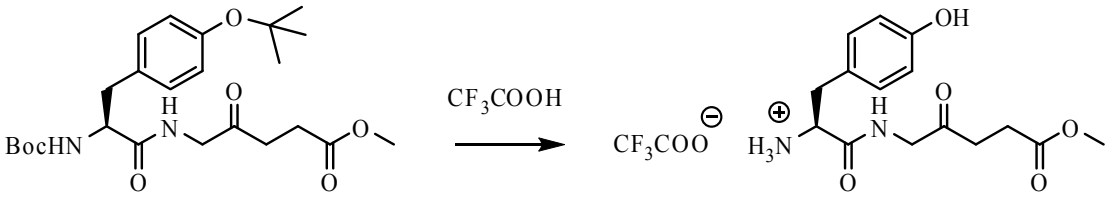
^{1}H-NMR (400 MHz, $CDCl_3$ & CD_3OD, 298 K): δ_H (ppm) = 7.06 (*d*, $^3J_7{}^3{}_{-7}{}^4$ =8.3Hz, 2H, HC(7^4) and HC($7^{4'}$)); 6.88 (*d*, $^3J_7{}^3{}_{-7}{}^4 \approx$ 8.3Hz, 2H, HC(7^3) and HC($7^{3'}$)); 6.71 (*sbr*, 1H, CONH); 5.08 ((*sbr*, 1H, NHBoc); 4.36 (*m*, 1H, HC(7)); 4.15 (*dd*, $^3J_{5a\text{-}NH} \approx$ 5.0Hz, $^3J_{5a\text{-}5b}$ =19.4Hz 1H, HC(5a)); 4.05 (*dd*, $^3J_{5b\text{-}NH} \approx$ 5.0Hz, $^3J_{5a\text{-}5b}$ =19.4Hz 1H, HC(5b)); 3.64 (*s*, 3H, $H_3C(1^1)$; 3.06 (*dd*, $^3J_7{}^{1a}{}_{-7} \approx$ 6.5Hz, $^3J_7{}^{1a}{}_{-7}{}^{1b}$ =14.0Hz 1H, HC(7^1a)); 2.97-2.95 (*m*, 1H,

HC(7^1b)); 2.69-2.66 (*m*, 2H, $H_2C(3)$); 2.61-2.59 (*m*, 2H, $H_2C(2)$); 1.37 and 1.30 (2×*s*, 18H, $H_3C(7^a,7^b,7^c)$ and $H_3C(10^a,10^b,10^c)$).

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 203.7 (C(4)); 173.1 (C(1)); 171.9 (C(6)); 154.6 (C(8)); 131.8, 130.0, 124.7 (aromatic C(7^2), C(7^5), C(7^3), C(7^4), C($7^{3'}$) and C($7^{4'}$)); 80.5 and 78.7 (C(9) and C(7^6)); 56.1 (C(7)); 52.3 (C(1^1); 49.4 (C(5)); 38.2 (C(7^1)); 34.8 (C(3)); 29.2, 28.6 (C(10^a,10^b,10^c) and C(7^{7a},7^{7b},7^{7c})); 27.9 (C(2).

MS (ESI (+)): m/z relative intensity $[M+ Na]^+ = 487.1$

9.5.2.3. Synthesis of Tyr- ALA(OMe) peptide



82

$C_{24}H_{36}N_2O_7$

[464.55]

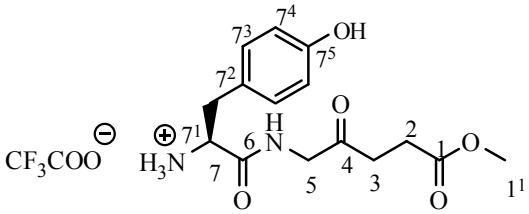
83

$C_{17}H_{21}F_3N_2O_7$

[422.35]

M^+=309.34 ; CF_3COO^- = 113.01

465 mg (1.0 mmol, 1eq) of Boc-Tyr(O^{t}Bu)-ALA (OMe) peptide was dissolved in 10ml CH_2Cl_2 and cooled to -10^{0}C. To this solution, 14.8g (10ml, 129.0mmol, 129eq) of trifluoroacetic acid was added dropwise by syringe. The reaction mass was maintained at 0-5^{0}C for an hour. Once the deprotection of BOC was completed (controlled by the absence of BOC peak in ^{1}H-NMR), CH_2Cl_2 and trifluoroacetic acid was evaporated off. The product was co evaporated 2times with distilled ether, then triturated with dry ether and the ether solution was decanted. The trituration with ether and decanting top clear ether solution after centrifuge was repeated 2 times more. The settled solid product on drying yielded 392 mg of Tyr- ALA(OMe) peptide (Yield=92.8%).



Batch: RV71, RV 86

^{1}H-NMR (400 MHz, DMSO-d_6, 298 K): δ_H (ppm) = 8.81 (*d*, $^3J_{NH\text{-}5} \approx$ 5.3Hz, 1H, CONH); 8.16 (*sbr*, 3H, NH_3^+); 7.12-6.91 (*m*, 2H, HC(7^4) and HC($7^{4'}$)); 6.72-6.60 (*m*, 2H, HC(7^3) and HC($7^{3'}$)); 4.07 (*d*, $^3J_{5\text{-}NH} \approx$ 5.3Hz, 2H, $H_2C(5)$); 4.03 (*m*, 1H, HC(7)); 3.57 (*s*, 3H, $H_3C(1^1)$; 3.02 (*dd*, $^3J_{7}{}^{1a}{}_{\text{-}7} \approx$ 5.8Hz, $^3J_{7}{}^{1a}{}_{\text{-}7}{}^{1b}$ =14.0Hz 1H, HC(7^1a)); 2.87 (*dd*, $^3J_{7}{}^{1a}{}_{\text{-}7} \approx$

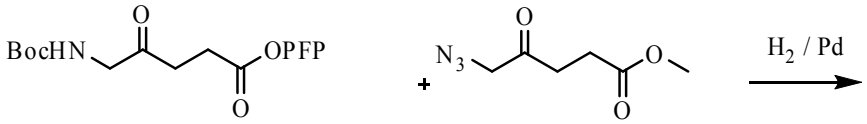
7.8Hz, $^{3}J_{7^{1a}\text{-}7^{1b}}$ =14.0Hz 1H, HC(7^{1}b)); 2.69-2.60 (*m*, 2H, $H_2C(3)$); 2.59-2.48 (*m*, 2H, $H_2C(2)$); 3.81, 3.60, 3.55-3.50, 3.17 peaks due to impurity.

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 204.9 (C(4)); 172.8 (C(1)); 159.3-155.0 ($CF_3\underline{C}OO^-$); 130.9 - 124.9 (aromatic C(7^2), C(7^5), C(7^3), C(7^4), C($7^{3'}$) and C($7^{4'}$)); 120.5-114.7 ($\underline{C}F_3COO^-$); 53.8 (C(7)); 51.5 (C(1^1); 48.5 (C(5)); 36.5 (C(7^1)); 34.1 (C(3)); 27.3 (C(2); 168.7, 51.4, 29.8-28.0 peaks due to impurity.

MS (ESI (+)): m/z relative intensity [M-CF_3COO^-+ with OH/OD] $^+$ = 312.0

9.5.3. Synthesis of ALA- ALA(OMe) peptide

9.5.3.1. Synthesis of Boc-ALA-ALA (OMe) peptide



7

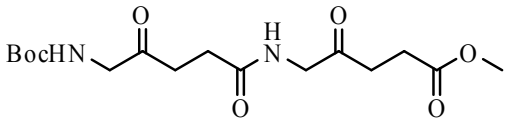
$C_{16}H_{16}NO_5F_5$

[397.23]

10

$C_6H_9N_3O_3$

[171.16]

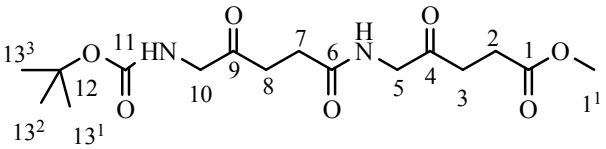


84

$C_{16}H_{26}N_2O_7$

[358.38]

To a solution of 1589 mg (4.0 mmol, 2.0eq) Boc-ALA-PFP ester in 40 ml AcOEt, was added 342 mg (2.0 mmol, 1eq) of 5-azido-4-oxo methyl pentanoate (5-azido methyl levulinate) in 10 ml MeOH. To this mixture was added 64 mg (0.06 mmol, 0.03eq) of 10% Palladium over activated carbon. The mass was subjected to hydrogenation in ambient temperature with the pressure of 60psi H_2 under mechanical agitation. Once the reaction was completed, (approximately 24hrs; TLC eluent: EtOAc/ Hexane 3:1) the H_2 pressure was released. The reaction mass was filtered over celite bed to get rid off palladium, the celite bed was washed with 3 × 10ml MeOH. The solvent in the filtrate was evaporated off by rotovapor and a yellow oily mass was obtained. This yellow crude mass was purified by flash chromatography. (gradient eluent = 100% CH_2Cl_2 to 95:5 CH_2Cl_2 :MeOH). The pure fraction of 62 mg BOC-ALA-ALA (OMe) peptide was thus obtained. (Yield = 17.3%)



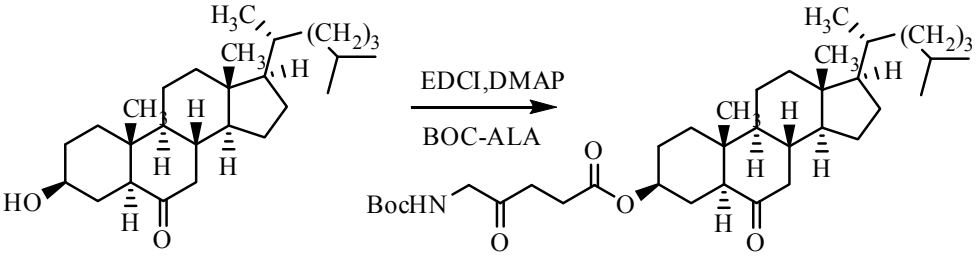
R_f: (95:5 CH_2Cl_2 :MeOH): 0.25 ($KMnO_4$)

^{1}H-NMR (400 MHz, CD_3OD, 298 K): δ_H (ppm) = 4.16 (*s*, 2H, $H_2C(10)$); 3.66 (*s*, 3H, $H_3C(1^1)$;3.35 and 3.02 (2× *d*, AB system, ^2J =14.5Hz, 2H, $H_2C(5)$); 2.89 (*ddd*, A of ABX_2 system, $^2J_{3a\text{-}3b}$=18.2Hz, $^3J_{3a\text{-}2a}$=7.7Hz, $^3J_{3a\text{-}2b}$=6.0Hz, 1H, HC(3a)); 2.77 (*ddd*, B of ABX_2 system, $^2J_{3b\text{-}3a}$=18.2Hz, $^3J_{3b\text{-}2a}$=6.6Hz, $^3J_{3b\text{-}2b}$=5.7Hz, 1H, HC(3b)); 2.66 (*ddd*, A of ABX_2 system, $^2J_{2a\text{-}2b}$=17.2Hz, $^3J_{2a\text{-}3a}$=7.7Hz, $^3J_{2a\text{-}3b}$=5.6Hz, 1H, HC(2a)); 2.59 (*ddd*, B of ABX_2 system, $^2J_{2b\text{-}2a}$=17.2Hz, $^3J_{2b\text{-}3a}$=6.0Hz, $^3J_{2b\text{-}3b}$=5.7Hz, 1H, HC(2b)); 2.51-2.34 (*m*, 3H, $H_2C(8)$ and HC(7a)); 2.03-1.95 (*m*, 1H, HC(7b)); 1.44 (*s*, 9H, $H_3C(13^1,13^2,13^3)$).

^{13}C-NMR (100 MHz, $CDCl_3$ & CD_3OD, 298K) : δ_C (ppm) = 207.0 (C(4) and C(9)); 177.3 (C(1)); 174.8 (C(6)); 158.4 (C(11); 80.6 (C(12)); 52.3 ($C(1^1)$; 48.7 (C(10)); 46.6 (C(5)); 35.4 (C(3)); 32.1 (C(7)); 30.4 (C(8)); 28.7 ($C(13^1,13^2,13^3)$); 28.6 (C(2)).

9.5.4. Synthesis of 6-ketocholestanol ester of ALA (6KC-ALA)

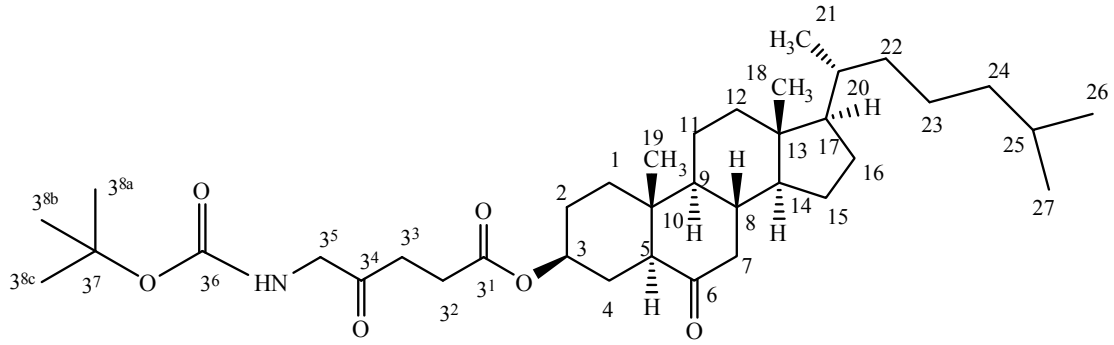
9.5.4.1. Synthesis of 6-ketocholestanol ester of Boc-ALA



239
$C_{27}H_{46}O_2$
[402.65]

240
$C_{37}H_{61}NO_6$
[615.88]

In a dry flask under argon atmosphere, 725 mg (1.8 mmol, 1.2eq) of 6-ketocholestanol was charged and then 20ml CH_2Cl_2 was added. The mass was cooled to 0 ^{0}C, then 185 mg (1.5 mmol, 1eq) of DMAP was added followed by 316.3 mg (1.65 mmol, 1.1eq) of EDCI. Finally, 350 mg (1.5 mmol, 1eq) of BOC-ALA was charged. The reaction was brought to room temperature and was kept at room temperature overnight. The solvent was evaporated off by rotovapor. The crude mass was dissolved in 50ml AcOEt and extracted with 75 ml H_2O.The organic phase is washed with 75 ml cold 1N citric acid solution, followed by 100ml saturated $NaHCO_3$ solution and finally by 100ml saturated NaCl solution. The organic phase is dried over $MgSO_4$ and the solvent was evaporated off by rotavapor. The remaining mass was purified by flash chromatography (1:1 EtOAc: Hexane) which yielded 620 mg of 6-ketocholestanol ester of Boc-ALA. (Yield= 67.1%)



Batch: RV 157, RV 162

R_f: (1:1 EtOAc: Hexane): 0.35 ($KMnO_4$)

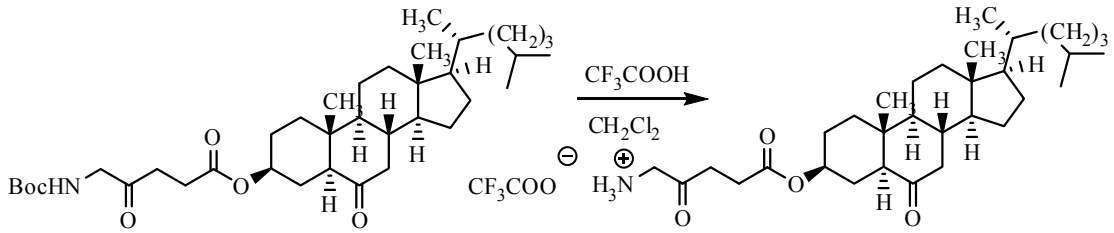
IR (KBr): 3428m, 2951s, 2870m, 2851m, 1745s, 1731s, 1713vs, 1703vs, 1496s, 1470m, 1443w, 1414w, 1382w, 1366s, 1302w,1282w, 1251w, 1226w, 1199m, 1169s, 1100w, 1073w, 1005w, 994w, 957w, 925vw, 887vw, 856vw, 780vw, 745vw, 640vw, 520vw.

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 5.22 (*sbr*, 1H, NH); 4.6 (*sbr*,1H, HC(3)); 4.1 (*s*, 2H, $H_2C(3^5)$); 2.69-2.66 (*m*, 2H, $H_2C(3^3)$; 2.60-2.53 (*s*, 2H, $H_2C(3^2)$; 2.27 (*dd*, $^3J_{5\text{-}4a}$=13.1Hz, $^3J_{5\text{-}4b}$=4.4Hz, 1H, HC(5)); 2.22 (*dd*, $^2J_{7a\text{-}7b}$=12.5Hz, $^3J_{7a\text{-}8}$≈2.3Hz, 1H, HC(7a)); 2.01-1.98 (*m*, 1H, HC(7b)); 1.92 (*t*, $^3J_{4\text{-}3}$, $^3J_{4\text{-}5}$ ≈ 12.8Hz, 2H, $H_2C(4)$; 1.88-0.93 (*m*, ≈36H); 0.87 (*d*, $^3J_{21\text{-}20}$ =6.5Hz, 3H, $H_3C(21)$); 0.81 (*d*, $^3J_{26\text{-}25}$ =6.6Hz, 3H, $H_3C(26)$); 0.80 (*d*, $^3J_{27\text{-}25}$ =6.6Hz, 3H, $H_3C(27)$); 0.73 (*s*, 3H, $H_3C(19)$); 0.62 (*s*, 3H, $H_3C(18)$).

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 210.5 (C(6)); 204.7 ($C(3^4)$); 172.2 ($C(3^1)$); 156.0 ($C(3^6)$); 80.0 $C(3^7)$); 73.7 (C(3)); 50.6 ($C(3^5)$; 34.7 ($C(3^3)$); 28.5 ($C(3^2)$; 28.3 ($C(3^{8a},3^{8b},3^{8c})$; 23.2, 23.0, 19.0, 13.3, 12.3 (C(27), C(26), C(21), C(19), C(18)).

MS (ESI (+)): m/z relative intensity $[2\times M+ Na]^+$ = 1252.8

9.5.4.2. Synthesis of 6-ketocholestanol ester of ALA



240

$C_{37}H_{61}NO_6$

[615.88]

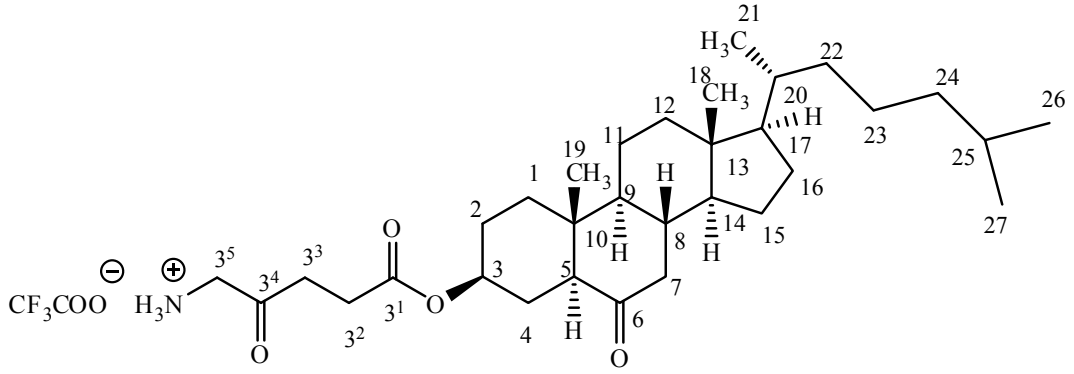
241

$C_{34}H_{54}F_3NO_6$

[629.79]

M^+=516.77 ; $CF_3COO^\ominus$ = 113.01

500 mg (0.81 mmol, 1eq) of 6-ketocholestanol ester of Boc-ALA was dissolved in 20ml CH_2Cl_2 and cooled to -10^0C. To this solution, 9.2 g (6.2ml, 81.2mmol, 100eq) of trifluoroacetic acid was added dropwise by syringe. The reaction mass was maintained at $0\text{-}5^0$C for an hour. Once the deprotection of BOC was completed (controlled by the absence of BOC peak in ^{1}H-NMR), CH_2Cl_2 and trifluoroacetic acid was evaporated off. The product was co evaporated 2times with distilled ether, then the solid on drying yielded 490 mg of 6-ketocholestanol ester of ALA which was a highly hygroscopic white solid. (Yield=95.9%).



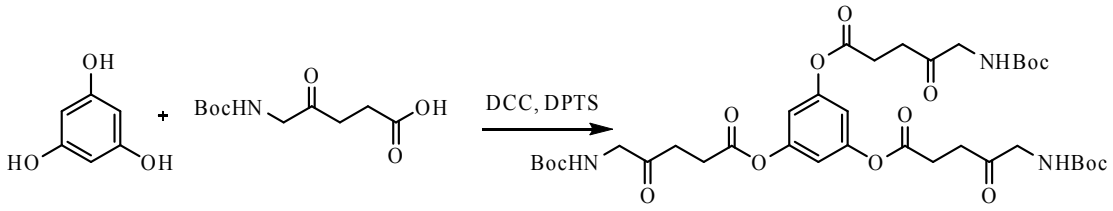
Batch: RV 163

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.98 (*sbr*, 3H, NH); 4.6 (*sbr*,1H, HC(3)); 4.1 (*s*, 2H, $H_2C(3^5)$); 2.77 (*s*, 2H, $H_2C(3^3)$; 2.59 (*s*, 2H, $H_2C(3^2)$; 2.27-2.24 (*m*, 2H); 2.04-1.98 (*m*, 1H)); 1.95 (*t*, $^3J_{4\text{-}3}$, $^3J_{4\text{-}5} \approx 12.8$Hz, 2H, $H_2C(4)$; 1.83-0.96 (*m*, ≈27H); 0.90 (*d*, $^3J_{21\text{-}20} = 6.3$Hz, 3H, $H_3C(21)$); 0.84 (*d*, $^3J_{26\text{-}25} = 6.5$Hz, 3H, $H_3C(26)$); 0.84 (*d*, $^3J_{27\text{-}25} = 6.5$Hz, 3H, $H_3C(27)$); 0.73 (*s*, 3H, $H_3C(19)$); 0.64 (*s*, 3H, $H_3C(18)$).

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 212.4 (C(6)); 202.4 ($C(3^4)$); 172.3 ($C(3^1)$); 161.0 (*q*, $^2J_{C\text{-}F} \approx 35.0$, $CF_3\underline{C}OO^-$); 115.6 (*q*, $^1J_{C\text{-}F} \approx 291.0$, $\underline{C}F_3COO^-$); 73.8 (C(3)); 47.7 ($C(3^5)$); 34.4 ($C(3^3)$); 28.1 ($C(3^2)$; 23.2, 23.0, 19.0, 13.3, 12.3 (C(27), C(26), C(21), C(19), C(18)).

9.5.5. Synthesis of ALA substituted phloroglucinol

9.5.5.1. Synthesis of tri Boc-ALA substituted phloroglucinol



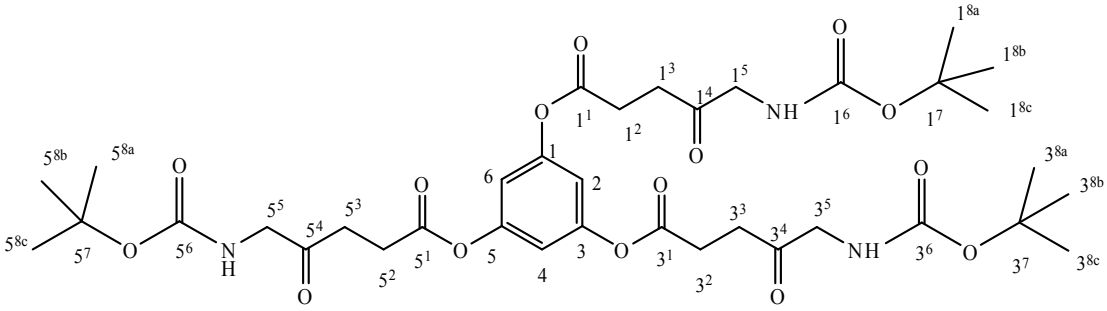
85 $C_6H_6O_3$ [126.11]

5 $C_{10}H_{17}NO_5$ [231.16]

86 $C_{36}H_{51}N_3O_{15}$ [765.80]

In a dry flask under argon atmosphere, 380 mg (3.0 mmol, 1.0eq) of phloroglucinol was dissolved in 75ml EtOAc, followed by addition of 540 mg (1.8 mmol, 0.6eq, 0.2eq per -

OH) of DPTS and 20 ml CH_2Cl_2. Then, 2228 mg (10.8 mmol, 3.6eq, 1.2eq per –OH) of DCC was added and finally 2500 mg (10.8 mmol, 3.6eq, 1.2eq per –OH) of Boc-ALA was added. The reaction was kept at room temperature for 72h. The reaction mixture was filtered over celite bed and the solvent was evaporated off by rotovapor. The crude mass was purified by flash chromatography (100% ether; silica gel mass- 60 times the crude mass) which yielded 2.14 g of tri Boc-ALA substituted phloroglucinol. (Yield= 93.4%)



Batch: RV 175, RV179

R_f: (3:1 EtOAc: Hexane): 0.37 ($KMnO_4$)

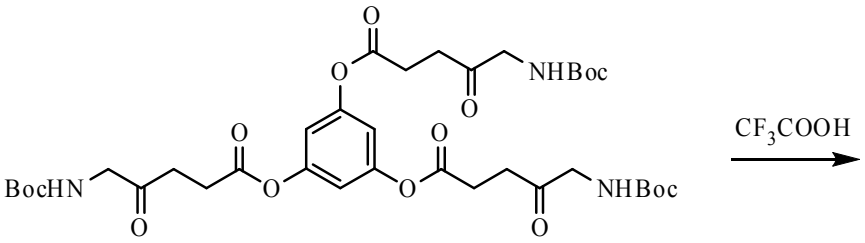
IR (KBr): 3443*w*, 3360*m*, 3087*vw*, 3006*w*, 2977*m*, 2931*w*, 1768*vs*, 1745*s*, 1720*vs*, 1702*vs*, 1603*m*, 1551*m*, 1521*s*, 1456*m*, 1407*m*, 1391*m*, 1367*s*, 1340*w*, 1283s, 1250*s*, 1212*m*, 1146*vs*, 1091*m*, 1060*m*, 1017*m*, 993*vw*, 980*vw*, 968*w*, 915*w*, 896*vw*, 876*w*, 779*vw*, 764*vw*, 752*vw*, 723*vw*, 724*vw*, 691*vw*, 666*vw*, 647*vw*, 567*vw*, 518*vw*, 509*vw*, 464*vw*.

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 6.76 (*s*, 3H, HC(2), HC(4) and HC(6)); 5.33 (*sbr*, 3H, 3 × N<u>H</u>); 3.99 (*d*, $^3J_1{}^5{}_{\text{-NH}} \approx 5.0$Hz, $^3J_3{}^5{}_{\text{-NH}} \approx 5.0$Hz, $^3J_5{}^5{}_{\text{-NH}} \approx 5.0$Hz, 6H, $H_2C(1^5)$, $H_2C(3^5)$and $H_2C(5^5)$); 2.81- 2.74 (*m*, 12H, $H_2C(1^2)$, $H_2C(1^3)$, $H_2C(3^2)$, $H_2C(3^3)$, $H_2C(5^2)$ and $H_2C(5^3)$); 1.40 (*s*, 27H, $H_3C(1^{8a},1^{8b},1^{8c})$, $H_3C(3^{8a},3^{8b},3^{8c})$ and $H_3C(5^{8a},5^{8b},5^{8c})$.

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 204.3 ($C(1^4)$, $C(3^4)$, $C(5^4)$); 170.5 ($C(1^1)$, $C(3^1)$, $C(5^1)$)); 155.7 ($C(1^6)$, $C(3^6)$, $C(5^6)$); 151.0 (aromatic C(1), C(3), C(5)); 112.9 (aromatic C(2), C(4), C(6)); 80.3 ($C(1^7)$, $C(3^7)$, $C(5^7)$); 50.2 ($C(1^5)$, $C(3^5)$, $C(5^5)$); 34.1($C(1^3)$, $C(3^3)$, $C(5^3)$); 28.3 ($C(1^{8a},1^{8b},1^{8c})$, $C(3^{8a},3^{8b},3^{8c})$ and $C(5^{8a},5^{8b},5^{8c})$); 27.8 ($C(1^2)$, $C(3^2)$, $C(5^2)$);

HR-MS $[M+ Na]^+ = 788.3214$ (Calculated =788.3218)

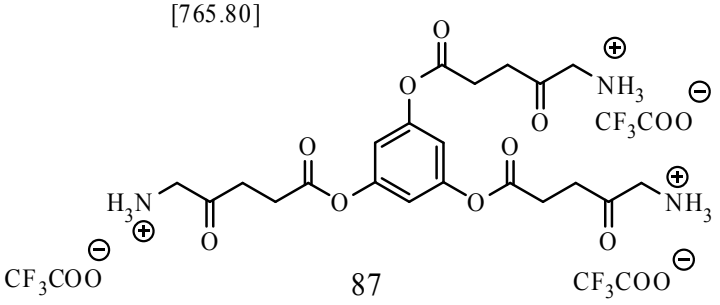
9.5.5.2. Synthesis of tri ALA substituted phloroglucinol



86

$C_{36}H_{51}N_3O_{15}$

[765.80]



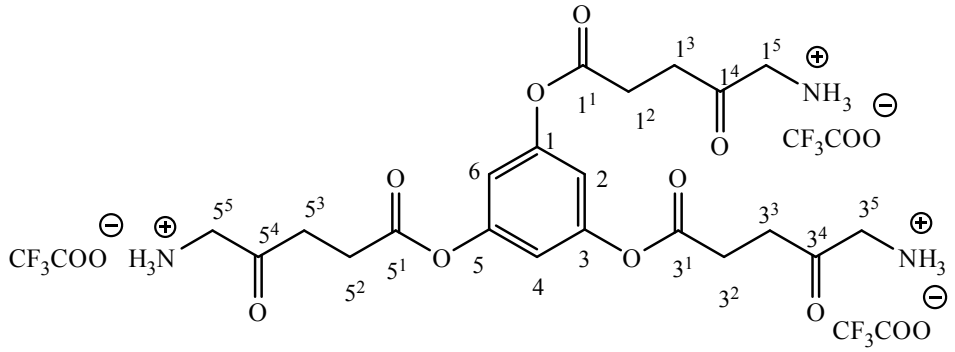
87

$C_{27}H_{30}F_9N_3O_{15}$

[807.52]

M^{3+}=468.47 ; CF_3COO^- = 113.01

765.8 mg (1.0 mmol, 1eq) tri Boc-ALA substituted phloroglucinol was dissolved in 10ml CH_2Cl_2 and cooled to -10^{0}C. To this solution, 14.8g (10ml, 129.0mmol, 129eq) of trifluoroacetic acid was added dropwise by syringe. The reaction mass was maintained at 0-5^{0}C for an hour. Once the deprotection of BOC was completed (controlled by the absence of BOC peak in ^{1}H-NMR), CH_2Cl_2 and trifluoroacetic acid was evaporated off. The product was co evaporated 2times with distilled ether, then triturated with dry ether and the ether solution was decanted. The trituration with ether and decanting top clear ether solution after centrifuge was repeated 2 times more. The settled solid product on drying yielded 728 mg of tri ALA substituted phloroglucinol (Yield=90.1%).



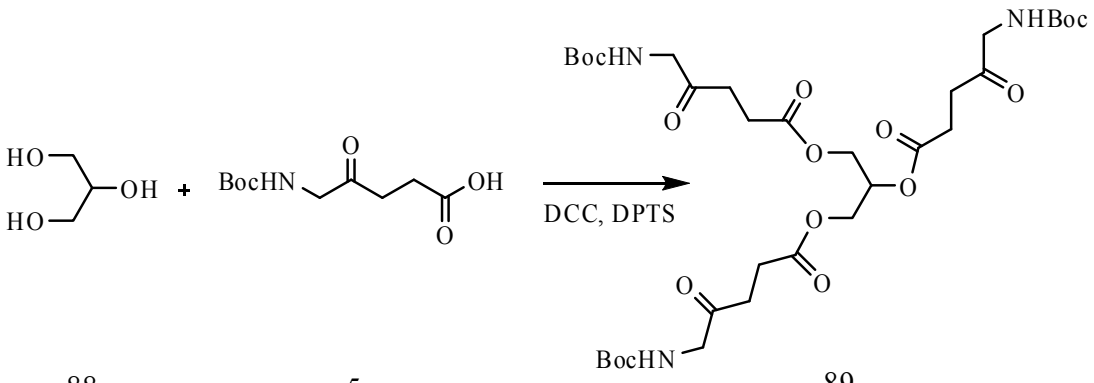
Batch: RV 182

^{1}H-NMR (400 MHz, D_2O, 298 K): δ_H (ppm) = 6.87 (*s*, 3H, HC(2), HC(4) and HC(6)); 4.06 (*s*, 6H, $H_2C(1^5)$, $H_2C(3^5)$and $H_2C(5^5)$); 2.96- 2.88 (*m*, 12H, $H_2C(1^2)$, $H_2C(1^3)$, $H_2C(3^2)$, $H_2C(3^3)$, $H_2C(5^2)$ and $H_2C(5^3)$).

^{13}C-NMR (100 MHz, D_2O, 298K) : δ_C (ppm) = 203.9 ($C(1^4)$, $C(3^4)$, $C(5^4)$); 173.4 ($C(1^1)$, $C(3^1)$, $C(5^1)$)); 163.0 (*q*,$^2J_{C\text{-}F} \approx$ 35.0, $CF_3\underline{C}OO^-$); 151.2 (aromatic C(1), C(3), C(5)); 116.5 (*q*,$^1J_{C\text{-}F} \approx$ 291.0, $\underline{C}F_3COO^-$); 114.1 (aromatic C(2), C(4), C(6)); 47.3 ($C(1^5)$, $C(3^5)$, $C(5^5)$); 34.5($C(1^3)$, $C(3^3)$, $C(5^3)$); 27.7 ($C(1^2)$, $C(3^2)$, $C(5^2)$).

9.5.6. Synthesis of ALA substituted glycerol

9.5.6.1. Synthesis of tri-Boc-ALA substituted glycerol

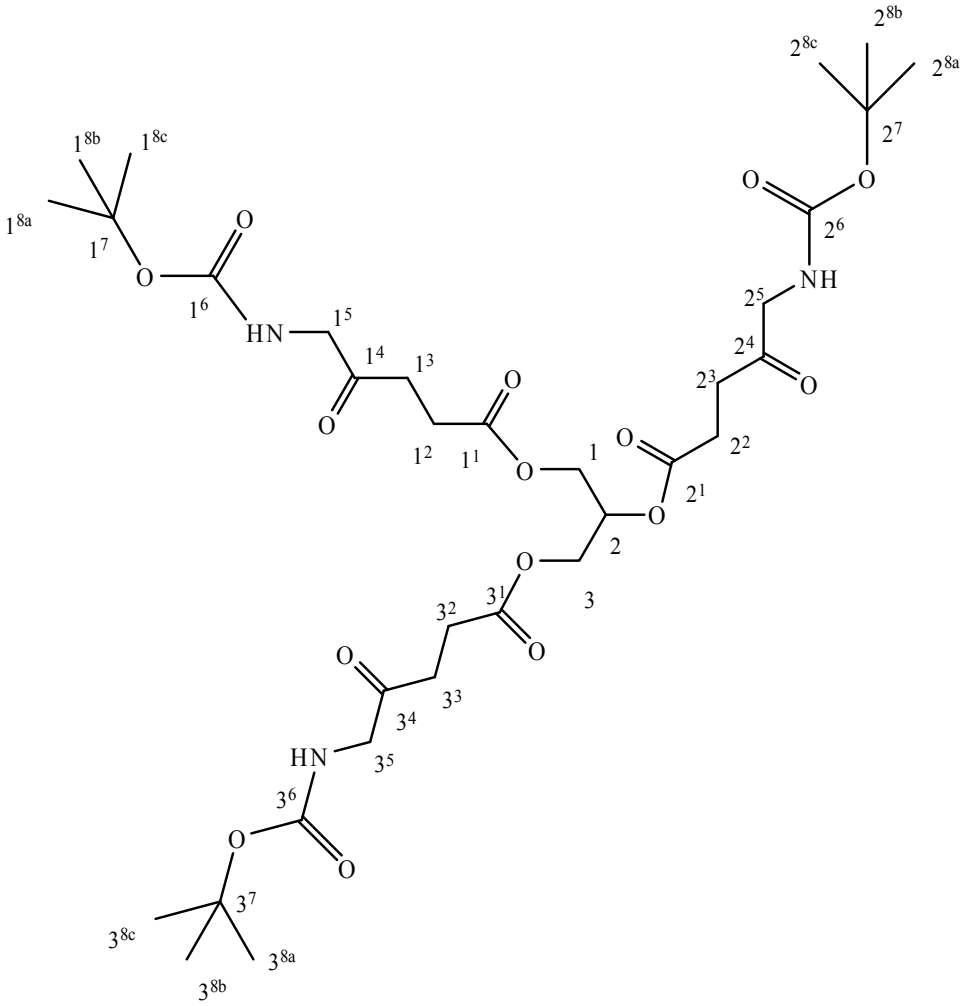


88 $C_3H_8O_3$ [92.09]

5 $C_{10}H_{17}NO_5$ [231.16]

89 $C_{33}H_{53}N_3O_{15}$ [731.78]

In a dry flask under argon atmosphere, 319 mg (3.46 mmol, 1.0eq) of glycerol was dissolved in 25ml CH_2Cl_2, followed by addition of 1250 mg (4.24 mmol, 1.2eq, 0.4eq per -OH) of DPTS. Then, the mixture was cooled to 0^0C, 2600 mg (12.5 mmol, 3.6eq, 1.2eq per –OH) of DCC was added and finally 2884 mg (12.5 mmol, 3.6eq, 1.2eq per –OH) of Boc-ALA was added. The reaction was kept at room temperature for 72h. The reaction mixture was filtered over celite bed and the solvent was evaporated off by rotovapor. The crude mass was purified by flash chromatography (100% EtOAc; silica gel mass- 70 times the crude mass) which yielded 1850 mg of tri Boc-ALA substituted glycerol. (Yield= 73%).



Batch: RV 217.

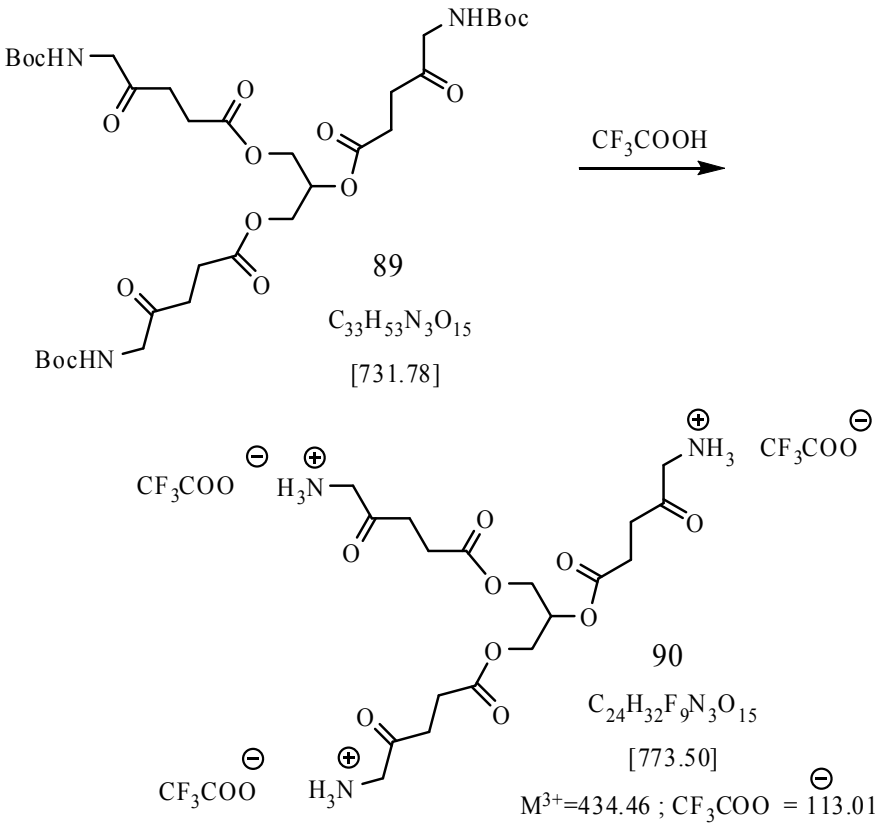
R_f: (3:1 EtOAc: Hexane): 0.37 ($KMnO_4$)

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 5.42, 5.36 (2× *sbr*, 3H, 3 × N<u>H</u>); 5.18 (*ddd*, $^3J_{1a\text{-}2}\approx4.0$Hz, $^3J_{3a\text{-}2}\approx4.0$Hz, $^3J_{1b\text{-}2}\approx6.0$Hz, $^3J_{3b\text{-}2}\approx6.0$Hz, ≈10.2Hz ,1H, HC(2)); 4.20 (*dd*, $^3J_{1a\text{-}2}\approx4.0$Hz, $^3J_{3a\text{-}2}\approx4.0$Hz (vicinal), $^3J_{1b\text{-}1a}=12.0$Hz, $^3J_{3b\text{-}3a}=12.0$Hz (geminal)); 4.11 (*dd*, $^3J_{1b\text{-}2}\approx6.0$Hz, $^3J_{3b\text{-}2}\approx6.0$Hz (vicinal), $^3J_{1b\text{-}1a}=12.0$Hz, $^3J_{3b\text{-}3a}=12.0$Hz (geminal)); 3.99 (*d*, $^3J_1{}^5{}_{\text{-NH}}\approx5.0$Hz, $^3J_2{}^5{}_{\text{-NH}}\approx5.0$Hz, $^3J_3{}^5{}_{\text{-NH}}\approx5.0$Hz, 6H, $H_2C(1^5)$, $H_2C(2^5)$and $H_2C(3^5)$); 2.70- 2.58 (*m*, 12H, $H_2C(1^2)$, $H_2C(1^3)$, $H_2C(2^2)$, $H_2C(2^3)$, $H_2C(3^2)$ and $H_2C(3^3)$); 1.38 (*s*, 27H, $H_3C(1^{8a},1^{8b},1^{8c})$, $H_3C(2^{8a},2^{8b},2^{8c})$ and $H_3C(3^{8a},3^{8b},3^{8c})$.

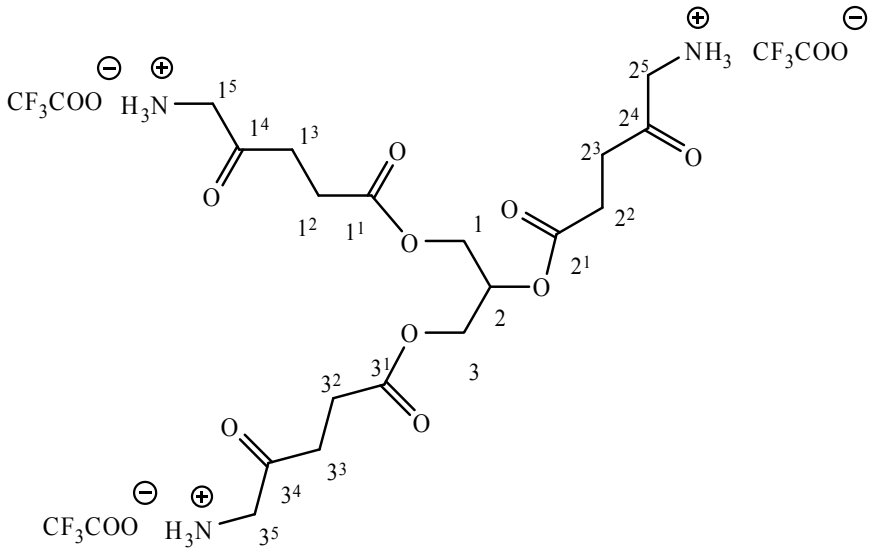
^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 204.5 ($C(1^4)$, $C(2^4)$, $C(3^4)$); 172.0 ($C(1^1)$, $C(2^1)$, $C(3^1)$)); 155.6 ($C(1^6)$, $C(2^6)$, $C(3^6)$)); 79.8 ($C(1^7)$, $C(2^7)$, $C(3^7)$)); 69.2 (C(2)); 62.3 (C(1) and C(3)); 50.2 ($C(1^5)$, $C(2^5)$, $C(3^5)$)); 34.2($C(1^3)$, $C(2^3)$, $C(3^3)$)); 28.3 ($C(1^{8a},1^{8b},1^{8c})$, $C(2^{8a},2^{8b},2^{8c})$ and $C(3^{8a},3^{8b},3^{8c})$); 27.5 ($C(1^2)$, $C(2^2)$, $C(3^2)$).

MS (ESI (+)): m/z relative intensity $[M+Na]^+ = 754.3$

9.5.6.2. Synthesis of tri ALA substituted glycerol



732 mg (1.0 mmol, 1eq) tri Boc-ALA substituted glycerol was dissolved in 10ml CH_2Cl_2 and cooled to -10^{0}C. To this solution, 14.8g (10ml, 129.0mmol, 129eq) of trifluoroacetic acid was added dropwise by syringe. The reaction mass was maintained at 0-5^{0}C for an hour. Once the deprotection of BOC was completed (controlled by the absence of BOC peak in ^{1}H-NMR), CH_2Cl_2 and trifluoroacetic acid was evaporated off. The product was co evaporated 2times with distilled ether, then triturated with dry ether and the ether solution was decanted. The trituration with ether and decanting top clear ether solution after centrifuge was repeated 2 times more. The settled solid product on drying yielded 687 mg of tri ALA substituted glycerol (Yield=89%).



Batch: RV 299

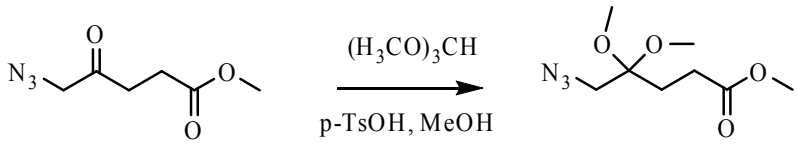
^{1}H-NMR (400 MHz, D_2O, 298 K): δ_H (ppm) = 5.34- 5.29 (*m*, 1H, HC(2)); 4.35 (*dd*, $^{3}J_{1a-2}$≈4.0Hz, $^{3}J_{3a-2}$≈4.0Hz (vicinal), $^{3}J_{1b-1a}$=12.0Hz, $^{3}J_{3b-3a}$ =12.0Hz (geminal)); 4.29 (*dd*, $^{3}J_{1b-2}$≈6.0Hz, $^{3}J_{3b-2}$≈6.0Hz (vicinal), $^{3}J_{1b-1a}$=12.0Hz, $^{3}J_{3b-3a}$ =12.0Hz (geminal)); 4.13 (*s*, 6H, $H_2C(1^5)$, $H_2C(2^5)$and $H_2C(3^5)$); 2.95- 2.72 (*m*, 12H, $H_2C(1^2)$, $H_2C(1^3)$, $H_2C(2^2)$, $H_2C(2^3)$, $H_2C(3^2)$ and $H_2C(3^3)$); 1.38 (*s*, 27H, $H_3C(1^{8a},1^{8b},1^{8c})$, $H_3C(2^{8a},2^{8b},2^{8c})$ and $H_3C(3^{8a},3^{8b},3^{8c})$ and other peaks due to solvent ether.

^{13}C-NMR (100 MHz, D_2O, 298K) : δ_C (ppm) = 204.1, 204.0 ($C(1^4)$, $C(2^4)$, $C(3^4)$); 174.5, 174.1 ($C(1^1)$, $C(2^1)$, $C(3^1)$)); 163.2 (*q*,$^{2}J_{C-F}$ ≈ 35.0, $CF_3\underline{C}OO^-$); 116.7 (*q*,$^{1}J_{C-F}$ ≈ 292.0, $\underline{C}F_3COO^-$); 70.3 (C(2)); 63.1 (C(1) and C(3)); 47.3 ($C(1^5)$, $C(2^5)$, $C(3^5)$); 34.5 ($C(1^3)$, $C(2^3)$, $C(3^3)$); 27.5 ($C(1^2)$, $C(2^2)$, $C(3^2)$);

9.6. Synthesis of porphobilinogen based peptides

9.6.1. Synthesis of precursors for porphobilinogen

9.6.1.1. Synthesis of 5-azido-4,4-dimethoxy methyl pentanoate



3

$C_6H_9N_3O_3$

[171.16]

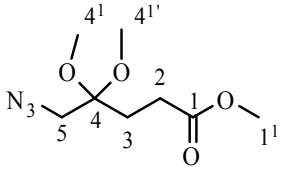
91

$C_8H_{15}N_3O_4$

[217.23]

In a dry flask fitted with a reflux condenser, under argon atmosphere, 7.5 g (43.82 mmol, 1.0eq) of 5-azido methyl levulinate was dissolved in 60ml MeOH, followed by addition

of 23.25 g (24ml, 219 mmol, 5eq) of trimethyl orthoformate drop wise and finally added 0.33 g (1.75 mmol, 0.04eq) of p-toluene sulphonic acid monohydrate. Then, the mixture was heated to reflux and kept at reflux for 14h. The mass was later cooled; added 1.44 g $NaHCO_3$, stirred for 15min, filtered over paper and the solvent was evaporated. The oily residue was purified by distilling the product using oil vacuum pump (65^0C at 0.07 mbar).8.55 g of pure 5-azido-4,4-dimethoxy methyl pentanoate (5- azido-4,4-dimethoxy levulinate)was obtained. (Yield= 89%).

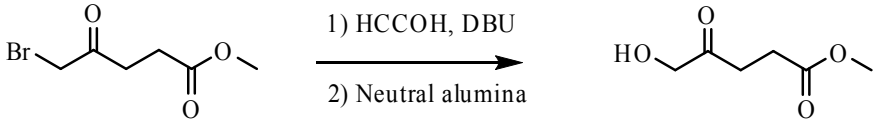


Batch: Step02B #01, Step02B #02

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 3.62 (*s*, 3H, $H_3C(1^1)$; 3.21 (*s*, 2H, $H_2C(5)$) ; 3.17 (*s*, 6H, $H_2C(4^1)$ and $H_2C(4^{1'})$) ; 2.28-2.00 (*m*, AA' part of a system AA'BB', 4H, $H_2C(3)$ and $H_2C(2)$) .

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 173.6 (C(1)) ; 101.7 (C(4)); 52.0 ($C(4^1)$ and $C(4^{1'})$); 51.3 ($C(1^1)$) ; 48.7 (C(5)); 29.0 (C(3)) ; 28.0 (C(2)).

9.6.1.2. Synthesis of 5-hydroxy -4-oxo methyl pentanoate



2

$C_6H_9BrO_3$

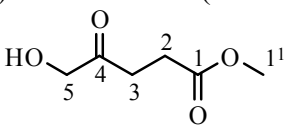
[209.04]

92

$C_6H_{10}O_4$

[146.14]

In a dry flask under argon atmosphere, 5.75 g (27.5 mmol, 1.0eq) of 5-bromo methyl levulinate was dissolved in 37ml benzene, followed by addition of 1.52 g (1.1ml, 33mmol, 1.2eq) of formic acid drop wise. The mixture was cooled 10^0C and added 5.22 g (34.8mmol, 1.25eq) of DBU drop wise in 1h by using an addition funnel. Then, the mixture was brought to ambient temperature, maintained at that temperature for 1h. Later 50 ml of water was added and extracted with 5×50 ml CH_2Cl_2. The combined organic layer was extracted with 140 ml 0.1N HCl and then washed with 150 ml water. The organic layer was dried over $MgSO_4$ and the solvent was evaporated. The residue of 4.12 g of 5.formyloxy methyl levulinate was taken for hydrolysis on a column prepared using 240 g neutral alumina. The column was run using 98:2 CH_2Cl_2: MeOH solvent mixture and then using 1:1 CH_2Cl_2: MeOH solvent mixture. The solvent from pure fractions were evaporated and the residue was taken for distillation using ball tube glass oven (*Kugelrohr*), (100- 120^0C at 0.07 mbar). 2.81 g of pure 5-hydroxy-4-oxo-methyl pentanoate (5-hydroxy-levulinate) was obtained. (Yield= 69.7%).



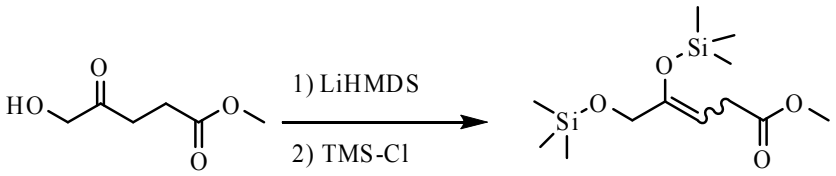
Batch: Step02 #01, Step02 #02

R_f: (1:1 EtOAc: Hexane): 0.39 ($KMnO_4$)

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 4.19 (*s*, 2H, $H_2C(5)$) ; 3.56 (*s*, 3H, $H_3C(1^1)$; 2.64-2.56 (*m*, AA' part of a system AA'BB', 4H, $H_2C(3)$ and $H_2C(2)$) .

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 208.8 (C(4)); 173.2 (C(1)); 68.5 (C(5)); 52.2 ($C(1^1)$); 33.1 (C(3)) ; 27.7 (C(2)).

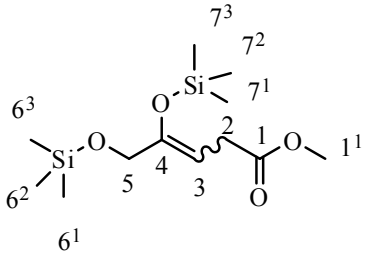
9.6.1.3. Synthesis of 4,5-bis-trimethylsilyloxy- methyl pent-3-enoate



92
$C_6H_{10}O_4$
[146.14]

93
$C_{12}H_{26}O_4Si_2$
[290.51]

In a dry flask under argon atmosphere, 30.3ml (30.3 mmol, 2.2 eq) of 1M LiHMDS solution in THF was taken and cooled to -78^{0}C, added by syringea solution of 2 g (13.68 mmol, 1.0eq) of 5-hydroxy-4-oxo-methyl pentanoate dissolved in 30ml THF. This organge-yellow solution was stirred at -78^{0}C for 2 h, then added drop wise 3.3 g (3.85 ml, 30.3mmol, 2.2eq) of trimethylchlorosilane. The reaction mixture was brought to ambient temperature, evaporated solvent. The residue was dissolved in 150 ml pentane, filtered over celite bed and evaporated solvent. The yellow oily residue was taken for distillation using ball tube glass oven (*Kugelrohr*), (130- 140^{0}C at 0.02 mbar). 2.52 g of pure 4,5-bis-trimethylsilyloxy- methyl pent-3-enoate was obtained. (Yield= 62.9%).



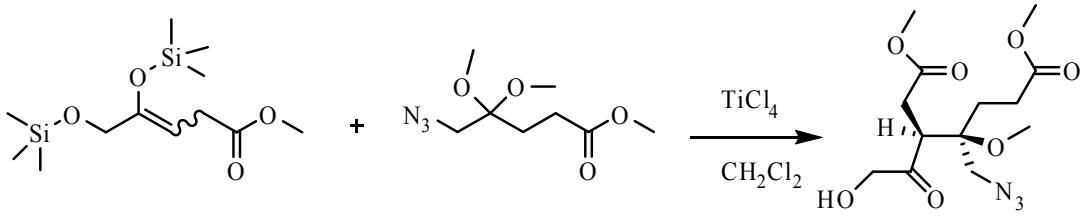
Batch: Step03 #01,#02,#03,#04 and #05,

R_f: (1:1 EtOAc: Hexane): 0.72 ($KMnO_4$)

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 4.92 (*tt*, ^{3}J=7.0Hz, ^{4}J=1.0Hz, 1H, HC(3)) ; 3.92 ((*d*, ^{4}J=1.0Hz, 2H, $H_2C(5)$); 3.63 (*s*, 3H, $H_3C(1^1)$; 3.04 ((*d*, ^{3}J=7.0Hz, 2H, $H_2C(2)$); 0.16 and 0.10 (2×*s*, 18H, H_3C (6^1, 6^2, 6^3, 7^1, 7^2 and 7^3).

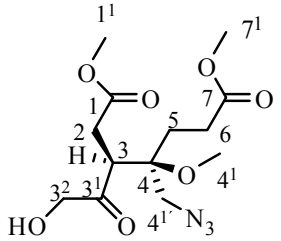
^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 172.9 (C(1)); 151.8 (C(4)); 100.7 (C(3)); 64.2 (C(5)); 51.9 (C(1^1)); 31.0 (C(2)); 1.0 and -0.20 (C (6^1, 6^2, 6^3, 7^1, 7^2 and 7^3).

9.6.1.4. Synthesis of 4-azidomethyl-3-(2-hydroxy acetyl)-4-methoxy- dimethyl heptanedioate



93	91	94
$C_{12}H_{26}O_4Si_2$	$C_8H_{15}N_3O_4$	$C_{13}H_{21}N_3O_7$
[290.51]	[217.23]	[331.3]

In a dry flask under argon atmosphere, 2.3 g (7.92 mmol, 1.0eq) of 4,5-bis-trimethylsilyloxy- methyl pent-3-enoate was dissolved in 16ml CH_2Cl_2 (basic alox treated) , cooled to -78^{0}C and added drop wise of 2.07 g (9.53 mmol, 1.2eq) of -azido-4,4-dimethoxy methyl pentanoate in 16ml CH_2Cl_2. To the mixture was then added 7.6 g (40.2mmol, 5eq) freshly distilled $TiCl_4$. The mixture was maintained at -55 ^{0}C for 16h. Then, the mixture was brought to ambient temperature; 75 ml of 2N NaOH was added and extracted with 5×100 ml $CHCl_3$. The combined organic layer was extracted with 300 ml satd.NH_4Cl solution, the organic layer was dried over $MgSO_4$ and the solvent was evaporated. The residue was taken for column chromatography (silica gel -100 times the crude mass) using eluent gradient from 100% hexane to 1:1 hexane:EtOAc. Pure fractions afforded 1.16 g of 4-azidomethyl-3-(2-hydroxy acetyl)-4-methoxy- dimethyl heptanedioate. (Yield= 44.1%).



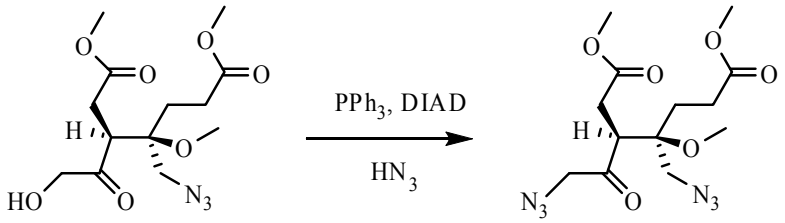
Batch: Step04 #08 to #13 and #16 to #19.

R_f: (1:1 EtOAc: Hexane): 0.30 ($KMnO_4$)

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 4.47 (*dd*, ^3J =5.4Hz, ^2J=19.1Hz, 1H, HC(3^2a)); 4.34 (*dd*, ^3J =3.7Hz, ^2J=19.1Hz, 1H, HC(3^2b)); 3.69 (*s*, 3H, $H_3C(7^1)$; 3.65 (*s*, 3H, $H_3C(1^1)$; 3.52 and 3.35 (2×*d*, AB system, ^2J=13.3Hz, 1H each, $H_2C(4^{1'})$); 3.31 (*dd*, $^3J_{3\text{-}2a}$ =2.5Hz, $^3J_{3\text{-}2b}$ =12.0Hz, 1H, HC(3)); 3.24 (*s*, 3H, $H_3C(4^1)$; 2.98 (*dd*, $^3J_{2a\text{-}3}$ =12.0Hz, $^2J_{2a\text{-}2b}$=17.3Hz, 1H, HC(2a)); 2.45 (*dd*, $^3J_{2b\text{-}3}$ =2.5Hz, $^2J_{2b\text{-}2a}$ =17.3Hz, 1H, HC(2b)); 2.37 (*ddd*, $^2J_{6a\text{-}6b}$ ≈16.0Hz, $^3J_{6\text{-}5b}$ ≈10.0Hz, $^3J_{6\text{-}5a}$ ≈6.0Hz, 2H, HC (6a) and HC(6b)); 2.13 (*ddd*, $^2J_{5a\text{-}5b}$ ≈15.5Hz, $^3J_{5a\text{-}6b}$ ≈9.6Hz, $^3J_{5a\text{-}6a}$ ≈6.0Hz, 1H, HC (5a)); 1.81(*ddd*, $^2J_{5b\text{-}5a}$ ≈15. 6Hz, $^3J_{5b\text{-}6a}$ ≈9.7Hz, $^3J_{5b\text{-}6b}$ ≈6.0Hz, 1H, HC (5b)).

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 211.9 (C(3^1); 173.6 (C(7); 172.6 (C(1)); 79.0 (C(4)); 71.1 (C(3^2)); 54.0 (C($4^{1'}$)); 52.6(C(1^1));. 52.4(C(7^1)); 50.7(C(4^1));. 47.7 (C(3)); 32.4 (C(2)); 27.6 (C(6)); 26.1(C(5)).

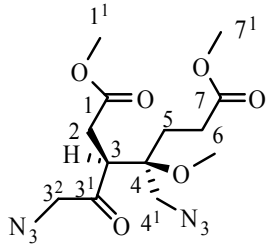
9.6.1.5. Synthesis of 3-(2-azido-acetyl)-4-azidomethyl--4-methoxy- dimethyl heptanedioate



94
$C_{13}H_{21}N_3O_7$
[331.3]

95
$C_{13}H_{20}N_6O_6$
[356.3]

In a dry flask under argon atmosphere, 885 mg (3.37 mmol, 1.12 eq) was dissolved in 35 ml benzene, cooled to 5-10^{0}C, added drop wise 680 mg (3.36 mmol, 1.11eq) of DIAD, added then drop wise 1000 mg (3.02 mmol, 1.0eq) of 4-azidomethyl-3-(2-hydroxy acetyl)-4-methoxy- dimethyl heptanedioate dissolved in 15ml benzene. Finally added drop wise 3ml (0.2 g, 6.88% w/v, 4.8 mmol, 1.6eq) of HN_3 solution in benzene. (HN_3 generated by adding 1ml of 98%H_2SO_4 to 2.27 g NaN_3 in 3 ml water). The mixture was maintained at 10 ^{0}C for 2h-3h. Then, the mixture was brought to ambient temperature and the solvent was evaporated. The residue was taken for column chromatography (silica gel - 50 times the crude mass) using eluent gradient from 95:5 CH_2Cl_2: EtOAc to 80:20 CH_2Cl_2: EtOAc. Pure fractions afforded 1.0 g of 3-(2-azido-acetyl)-4-azidomethyl--4-methoxy- dimethyl heptanedioate. (Yield= 93.0%).



Batch: Step05 #01 to #09.

R_f: (8:2 CH_2Cl_2: EtOAc): 0.57 ($KMnO_4$)

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 4.22 (*d*, ^{2}J=18.3Hz, 1H, HC(3^2a)); 4.14 (*d*, ^{2}J=18.3Hz, 1H, HC(3^2b)); 3.68 (*s*, 3H, $H_3C(7^1)$; 3.65 (*s*, 3H, $H_3C(1^1)$; 3.55 and 3.36 (2×*d*, AB system, ^{2}J=13.2Hz, 1H each, $H_2C(4^{1'})$); 3.24 (*dd*, $^3J_{3\text{-}2a}$ =2.5Hz, $^3J_{3\text{-}2b}$ =12.0Hz, 1H, HC(3)); 3.23 (*s*, 3H, $H_3C(4^1)$; 2.98 (*dd*, $^3J_{2a\text{-}3}$ =12.2Hz, $^2J_{2a\text{-}2b}$ =19.0Hz, 1H, HC(2a)); 2.42 (*dd*, $^3J_{2b\text{-}3}$ =2.5Hz, $^2J_{2b\text{-}2a}$ =17.3Hz, 1H, HC(2b)); 2.50-2.23 (*m*, 2H, H_2C (6)); 2.14 (*ddd*, $^2J_{5a\text{-}5b}$ ≈15.5Hz, $^3J_{5a\text{-}6b}$ ≈9.5Hz, $^3J_{5a\text{-}6a}$ ≈6.0Hz, 1H, HC (5a)); 1.79 (*ddd*, $^2J_{5b\text{-}5a}$ ≈15.

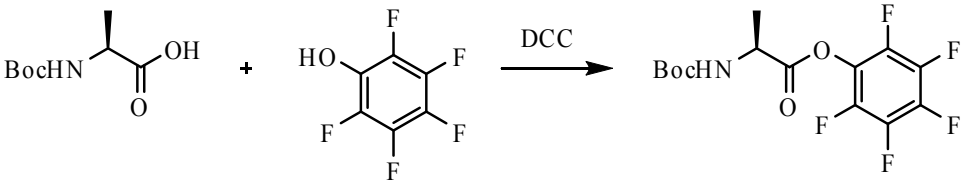
6Hz, $^{3}J_{5b\ -6a}$ ≈9.7Hz, $^{3}J_{5b\ -6b}$ ≈6.0Hz, 1H, HC (5b)); 4.36, 1.29-1.25 (minor peaks due to impurity).

^{13}C-NMR (100 MHz, $CDCl_3$, 298K): δ_C (ppm) == 206.2 (C(3^1); 173.2 (C(7); 172.4 (C(1)); 79.0 (C(4)); 60.2 (C(3^2)); 53.4 (C(4^1)); 52.3 (C(1^1)); 52.1(C(7^1)); 50.4 (C(4^1)); 48.7 (C(3)); 32.4 (C(2)); 27.3 (C(6)); 25.6 (C(5)); 208.5,173.7,79.5,68.9,52.3,50.2,28.6,28.1,27.7,22.5,14.6 (minor peaks due to traces of impuriy).

MS (ESI (+)): m/z relative intensity $[M + Na]^+$ = 379.1

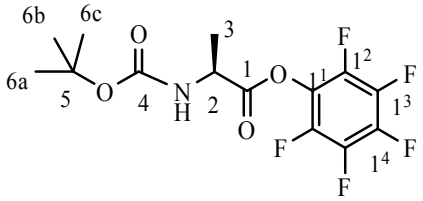
9.6.2. Synthesis of porphobilinogen-Ala-peptide

9.6.2.1. Synthesis of Boc-Ala -PFP ester



96	68	97
$C_8H_{15}NO_4$	C_6HF_5O	$C_{14}H_{14}NO_4F_5$
[189.21]	[184.06]	[355.08]

In a dry flask under argon atmosphere, 2.25 g (11.89 mmol, 1eq) of BOC-*Ala* was charged. This was dissolved in 50 ml dioxane and the mass was cooled to 0 ^{0}C. Then 2.2g (11.90 mmol, 1.0eq) of pentafluorophenol was added followed by 2.5 g (12.12 mmol, 1.02eq) of DCC. The reaction was brought to room temperature and was kept at room temperature overnight. The reaction mass was then filtered over celite bed to get rid off DCU and the solvent was evaporated off by rotovapor. The thick yellow oily mass obtained was triturated with hexane to remove excess pentafluorphenol and the white solid obtained was filtered off. The white solid was dried which yielded 3.76g of BOC-*Ala*-PFP ester. (Yield= 89.2%)



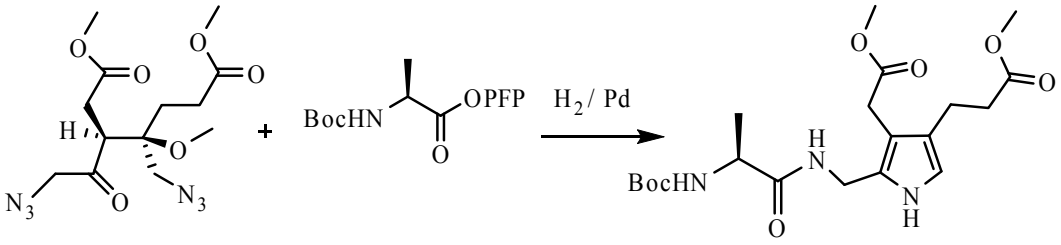
Batch: Step06B Ala #01 to #04 .

R_f: (8:2 Hexane: EtOAc): 0.53 (UV active; $KMnO_4$)

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 5.01 (*sbr*, 1H, N<u>H</u>); 4.66 (*m*, $^{3}J_{2\ -3}$ ≈7Hz, 1H, HC(2)); 1.58 (*d*, $^{3}J_{3-2}$=7.0 Hz, 3H, H_3C(3)); 1.46 (*s*, 9H, H_3C(6a, 6b, 6c).

[13]C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 170.1 (C(1); 155.3 (C(4)); 80.5 (C(5)); 49.5 (C(2)); 28.6 (C(6a,6b,6c)); 18.6 (C(3)).

9.6.2.2. Synthesis of porphobilinogen-Boc-Ala -peptide

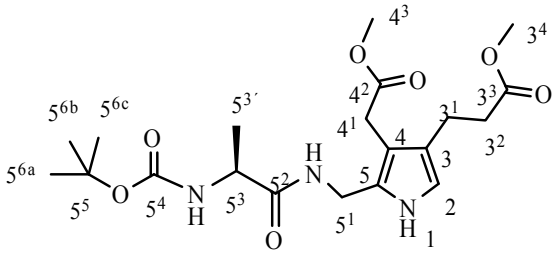


95 $C_{13}H_{20}N_6O_6$ [356.3]

97 $C_{14}H_{14}NO_4F_5$ [355.08]

98 $C_{20}H_{31}N_3O_7$ [425.47]

In a hydrogenation autoclave reactor, added 20ml MeOH followed by 60mg (0.0564 mmol, 0.04 eq) of 10% palladium on activated charcoal. This mixture was prehydrogenated with hydrogen for 10 min, later added 1.1 g (3.09 mmol, 2.3eq) of BOC-*Ala*-PFP ester followed by a solution of 0.476 g (1.337 mmol, 1eq) 3-(2-azido-acetyl)-4-azidomethyl--4-methoxy- dimethyl heptanedioate in 20ml MeOH. The mass was subjected to hydrogenation in ambient temperature with the pressure of 60psi H_2 under mechanical agitation. Once the reaction was completed, (approximately 24hrs; TLC eluent: hexane/AcOEt 1:1) the H_2 pressure was released. The reaction mass was filtered over celite bed to get rid off palladium, the celite bed was washed with 3 × 25ml MeOH. The solvent in the filtrate was evaporated off by rotovapor and a yellow oily mass was obtained. This yellow crude mass was purified by flash chromatography (silica gel- 70 times the curude mass) using gradient eluent system. (from hexane/AcOEt 4:6 to 100% AcOEt). The pure fraction of 332 mg porphobilinogen-Boc-*Ala* -peptide was thus obtained. (Yield = 58%)



Batch: Step06 Ala #01 to #05.

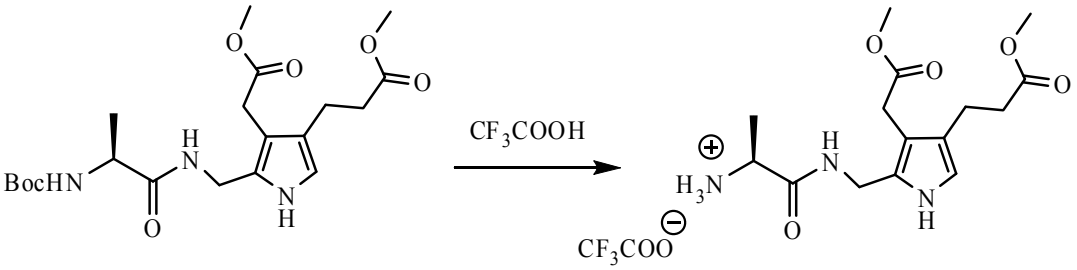
R_f: (3:1 EtOAc: Hexane): 0.35 (UV $_{254,}$ $KMnO_4$ and violet Ehlrich)

[1]H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 8.95 (*sbr*, 1H, N<u>H</u> pyrrole); 7.06 ((*sbr*, 1H, N<u>H</u>-$H_2C(5^1)$); 6.43 (*d*, $^3J_{2\text{-}NH}$ =2.6Hz, 1H, HC(2)); 5.20 (*d*, $^3J_{NH\text{-}5^3}$ =7.1Hz, 1H, N<u>H</u>-$HC(5^3)$); 4.32-4.13 (*m*, 3H, $H_2C(5^1)$ and $HC(5^3)$); 3.67 (*s*, 3H, $H_3C(3^4)$ or $H_3C(4^3)$; 3.65 (*s*, 3H, $H_3C(3^4)$ or $H_3C(4^3)$; 3.42 (*s*, 3H, $H_2C(4^1)$); 2.74-2.70 (*m*, 2H, $HC(3^1)$); 2.54-2.50 (*m*, 2H, $HC(3^2)$); 1.44-1.30 (*m*, 12H, $H_3C(5^{6a}, 5^{6b}, 5^{6c})$ and $H_3C(5^{3'})$).

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 173.7 ($C(3^3)$); 173.5 ($C(4^2)$); 173.2 ($C(5^2)$); 155.4 ($C(5^4)$); 126.7 (C(5)); 121.1 (C(3)); 114.5 (C(2)); 111.4 (C(4)); 80.0 ($C(5^5)$); 52.1 ($C4^3$)); 51.5 ($C(3^4)$); 50.2 ($C(5^3)$); 35.3 ($C5^1$)); 31.9 ($C(3^2)$); 29.8 ($C(4^1)$); 28.2 ($C(5^{6a}$,5^{6b} and 5^{6c})); 20.4 ($C(3^1)$); 18.5 ($C(5^{3'})$).

MS (ESI (+)): m/z relative intensity $[M + Na]^+ = 448.1$

9.6.2.3. Synthesis of porphobilinogen-Ala -peptide



98

$C_{20}H_{31}N_3O_7$

[425.47]

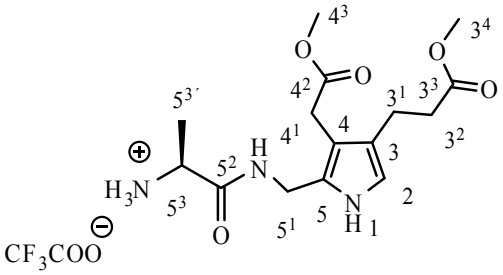
99

$C_{17}H_{24}F_3N_3O_7$

[439.38]

M^+=326.36 ; CF_3COO^- = 113.01

84 mg (0.19 mmol, 1eq) of porphobilinogen-Boc-*Ala* -peptide was dissolved in 5ml CH_2Cl_2 (Basic alox treated) and cooled to -10^{0}C. To this solution, 2.2g (1.5ml, 19.3mmol, 100eq) of trifluoroacetic acid was added dropwise by syringe. The reaction mass was maintained at 5-10^{0}C for an hour. Once the deprotection of BOC was completed (controlled by the absence of BOC peak in ^{1}H-NMR), CH_2Cl_2 and trifluoroacetic acid was evaporated off. The product was dried by high vacuum oil pimp for 2-3 days and yielded 83 mg of porphobilinogen-*Ala* -peptide which was a highly hygroscopic and viscous semisolid. (Yield=98%).



Batch: Step 07 #04 to #09.

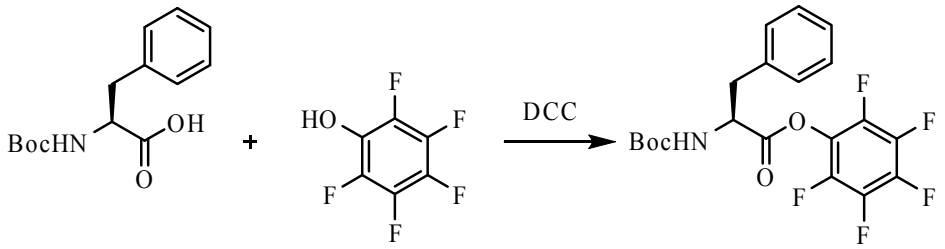
^{1}H-NMR (400 MHz, DMSO-d_6, 298 K): δ_H (ppm) = 10.47 ((*sbr*, 1H, NH pyrrole); 8.51 ((*t*, $^3J_{NH\text{-}5^1}$ =5.0 Hz, 1H, NH-$H_2C(5^1)$); 8.08 (*sbr*, 3H, NH_3^+)); 6.44 (*d*, $^3J_{2\text{-}NH}$ =2.6Hz, 1H, HC(2)); 4.22-4.19 (*m*, 2H, $H_2C(5^1)$); 3.78-3.75 (*m*, 1H, $HC(5^3)$); 3.58 (*s*, 3H, $H_3C(3^4)$ or $H_3C(4^3)$; 3.57 (*s*, 3H, $H_3C(3^4)$ or $H_3C(4^3)$; 3.44 (*d*, $^3J_{4^{1a}\text{-}4^{1b}}$ =16.0 Hz, 1H, $HC(4^{1a})$; 3.40 (*d*, $^3J_{4^{1b}\text{-}4^{1a}}$ =16.0 Hz, 1H, $HC(4^{1b})$;2.59-2.54 (*m*, 2H, $HC(3^1)$); 2.49-2.46 (*m*, 2H, $HC(3^2)$); 1.31 (*d*, 3H, $H_3C(5^{3'})$).

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 173.2 (C(3^3); 172.2 (C(4^2)); 169.0 (C(5^2)); 159.1(*q*,$^2J_{C\text{-}F}$ = 36.2, $CF_3\underline{C}OO^-$); 125.4 (C(5)); 120.8 (C(3)); 115.9 (*q*,$^1J_{C\text{-}F}$ ≈ 291.9, $\underline{C}F_3COO^-$); 114.1 (C(2)); 111.5 (C(4)); 51.6 (C4^3)); 51.3 (C(3^4)); 48.2 (C(5^3)); 34.4 (C5^1)); 34.3 (C(3^2)); 29.4 (C(4^1)); 20.3 (C(3^1)); 17.2 (C($5^{3\prime}$)).

MS (ESI (+)):m/z relative intensity [M-CF_3COO^-] $^+$ = 326

9.6.3. Synthesis of porphobilinogen-Phe-peptide

9.6.3.1. Synthesis of Boc-Phe -PFP ester

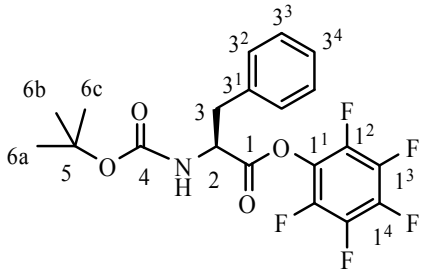


100 — $C_{14}H_{19}NO_4$ — [265.30]

68 — C_6HF_5O — [184.06]

101 — $C_{20}H_{18}NO_4F_5$ — [431.35]

In a dry flask under argon atmosphere, 1.33 g (5 mmol, 1eq) of BOC-*Phe* was charged. This was dissolved in 25 ml dioxane and the mass was cooled to 0 ^{0}C. Then 0.92g (5 mmol, 1.0eq) of pentafluorophenol was added followed by 1.12 g (5.43 mmol, 1.08eq) of DCC. The reaction was brought to room temperature and was kept at room temperature overnight. The reaction mass was then filtered over celite bed to get rid off DCU and the solvent was evaporated off by rotovapor. The thick yellow oily mass obtained was triturated with hexane to remove excess pentafluorphenol and the white solid obtained was filtered off. The white solid was dried which yielded 1.85 g of BOC-*Phe*-PFP ester. (Yield= 85.8 %)



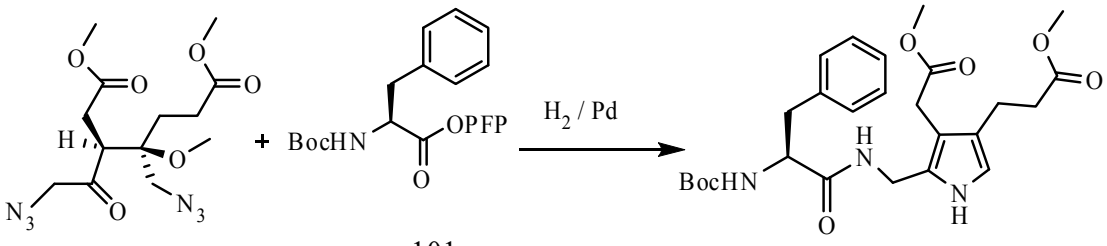
Batch: Step06B Phe #01

R_f: (8:2 Hexane: EtOAc): 0.7 (UV active; $KMnO_4$)

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 7.39-7.30 (*m*, 5H, aromatic); 5.00 (*d*, $^3J_{NH\text{-}2}$ =8Hz, 1H, N<u>H</u>); 4.94 (*q*, $^3J_{2\text{-}3}$ ≈7Hz, $^3J_{2\text{-}NH}$ ≈7Hz 1H, HC(2)); 3.34 (*dd*, $^3J_{3a\text{-}2}$=5.7 Hz, $^3J_{3a\text{-}3b}$=14.0 Hz, 1H, H_2C(3a)); 3.23 (*dd*, $^3J_{3b\text{-}2}$= 6.6 Hz, $^3J_{3b\text{-}3a}$=14.0 Hz, 1H, H_2C(3b)); 1.46 (*s*, 9H, H_3C(6a, 6b, 6c).

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 168.8 (C(1); 155.3 (C(4)); 142.8-137.0 ($C1^1$, $C1^2$, $C1^3$, $C1^4$, $C1^{2'}$, $C1^{3'}$); 135.3 ($C(C3^1)$; 129.7-127.8 ($C3^2$, $C3^3$, $C3^4$, $C3^{2'}$, $C3^{3'}$); 81.0 (C(5)); 54.8 (C(2)); 38.2 (C(3)); 28.6 (C(6a,6b,6c)).

9.6.3.2. Synthesis of porphobilinogen-Boc-Phe -peptide

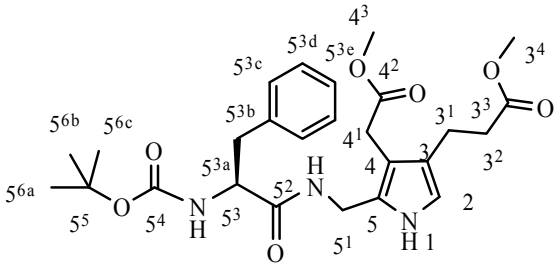


95 $C_{13}H_{20}N_6O_6$ [356.3]

101 $C_{20}H_{18}NO_4F_5$ [431.35]

102 $C_{26}H_{35}N_3O_7$ [501.57]

In a hydrogenation autoclave reactor, added 40ml MeOH followed by 55 mg (0.0517 mmol, 0.04 eq) of 10% palladium on activated charcoal. This mixture was prehydrogenated with hydrogen for 10 min, later added 1.21 g (2.8 mmol, 2.1eq) of BOC-*Phe*-PFP ester followed by a solution of 0.476 g (1.337 mmol, 1eq) 3-(2-azido-acetyl)-4-azidomethyl--4-methoxy- dimethyl heptanedioate in 20ml MeOH. The mass was subjected to hydrogenation in ambient temperature with the pressure of 60psi H_2 under mechanical agitation. Once the reaction was completed, (approximately 24hrs; TLC eluent: hexane/AcOEt 1:1) the H_2 pressure was released. The reaction mass was filtered over celite bed to get rid off palladium, the celite bed was washed with 3 × 25ml MeOH. The solvent in the filtrate was evaporated off by rotovapor and a yellow oily mass was obtained. This yellow crude mass was purified by flash chromatography (silica gel- 70 times the curude mass) using gradient eluent system. (from hexane/AcOEt 4:6 to 100% AcOEt). The pure fraction of 425 mg porphobilinogen-Boc-*Phe* -peptide was thus obtained. (Yield = 63.4%)



Batch: Step06 Phe #01 and #02.

R_f: (3:1 EtOAc: Hexane): 0.46 (UV $_{254,}$ $KMnO_4$ and violet Ehlrich)

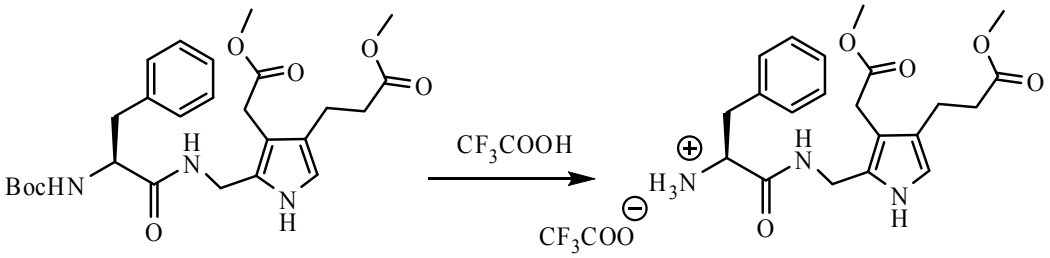
^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 8.60 (*sbr*, 1H, N<u>H</u> pyrrole); 7.30-7.08 (*m*, 5H, aromatic); 6.72 ((*sbr*, 1H, N<u>H</u>-$H_2C(5^1)$); 6.45 (*d*, $^3J_{2\text{-}NH}$ =2.4Hz, 1H, HC(2)); 5.10 (*sbr*, 1H, N<u>H</u>-$HC(5^3)$); 4.36-4.32 (*m*, 1H, $HC(5^3)$); 4.29 (*dd*, ^4J =2.4Hz, $^3J_5{}^1{}_{\text{-NH}}$ =5.6Hz,

$H_2C(5^1)$); 3.69 (*s*, 3H, $H_3C(3^4)$ or $H_3C(4^3)$; 3.64 (*s*, 3H, $H_3C(3^4)$ or $H_3C(4^3)$; 3.38 (*s*, 3H, $H_2C(4^1)$); 3.06 (*d*, $^3J_{5^3a\text{-}5^3}$ =6.6Hz, 2H, $HC(5^{3a})$); 2.78-2.74 (*m*, 2H, $HC(3^1)$); 2.59-2.55 (*m*, 2H, $HC(3^2)$); 1.40 (*s*, H, $H_3C(5^{6a}, 5^{6b}, 5^{6c})$.

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 174.1 ($C(3^3)$; 173.4 ($C(4^2)$); 172.4 ($C(5^2)$); 155.7 ($C(5^4)$); 136.8 ($C(5^{3b})$); 129.7-127.0 (aromatic); 127.2 (C(5)); 121.6 (C(3)); 114.8 (C(2)); 111.8 (C(4)); 80.5 ($C(5^5)$); 56.2 ($C(5^3)$);52.5 ($C4^3$)); 51.9 ($C(3^4)$); 39.1 ($C(5^{3a})$); 35.3 ($C5^1$) and ($C(3^2)$); 30.2 ($C(4^1)$); 28.6 ($C(5^{6a}$,5^{6b} and 5^{6c})); 20.9 ($C(3^1)$).

MS (ESI (+)): m/z relative intensity $[M + Na]^+$ = 524.2

9.6.3.3. Synthesis of porphobilinogen-Phe -peptide



102

$C_{26}H_{35}N_3O_7$

[501.57]

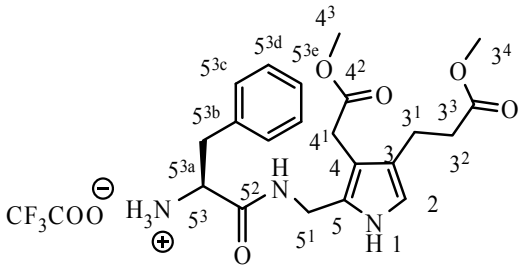
103

$C_{23}H_{28}F_3N_3O_7$

[515.48]

M^+=402.46 ; CF_3COO^- = 113.01

103 mg (0.20 mmol, 1eq) of Porphobilinogen-Boc-*Phe* -peptide was dissolved in 5ml CH_2Cl_2 (Basic alox treated) and cooled to -10^{0}C. To this solution, 2.3g (1.5ml, 20 mmol, 100eq) of trifluoroacetic acid was added dropwise by syringe. The reaction mass was maintained at 5-10^{0}C for an hour. Once the deprotection of BOC was completed (controlled by the absence of BOC peak in ^{1}H-NMR), CH_2Cl_2 and trifluoroacetic acid was evaporated off. The product was dried by high vacuum oil pimp for 2-3 days and yielded 96 mg of Porphobilinogen-*Ala* -peptide which was a highly hygroscopic and viscous semisolid. (Yield=93%).



Batch: Step 07 Phe #05.

^{1}H-NMR (400 MHz, DMSO-d_6, 298 K): δ_H (ppm) = 10.40 ((*d*, $^3J_{NH\text{-}2}$ =2.0Hz, 1H, N<u>H</u> pyrrole); 8.58 ((*t*, $^3J_{NH\text{-}5^1}$ =5.0 Hz, 1H, N<u>H</u>-$H_2C(5^1)$); 8.08 (*d*, $^3J_{NH\text{-}5^3}$ =3.4 Hz, 3H, NH_3^+)); 7.30-7.18 (*m*, 5H, aromatic); 6.44 (*d*, $^3J_{2\text{-}NH}$ =2.6Hz, 1H, HC(2)); 4.17 (*d*, $^3J_{5^1\text{-}NH}$ =5.0 Hz, 2H, $H_2C(5^1)$); 3.96-3.95 (*mbr*, 1H, $HC(5^3)$); 3.58 (*s*, 3H, $H_3C(3^4)$ or $H_3C(4^3)$; 3.56 (*s*, 3H,

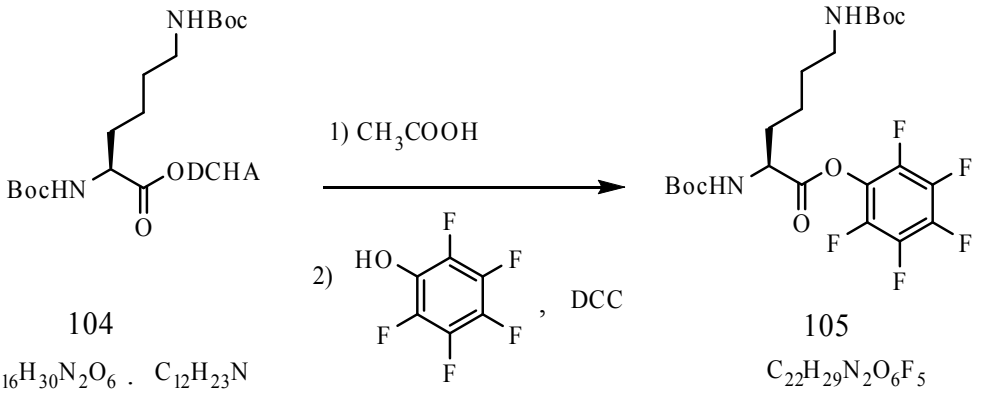
$H_3C(3^4)$ or $H_3C(4^3)$; 3.38 (*d*, $^3J_4{}^{1a}{}_{-4}{}^{1b}$ =16.0 Hz, 1H, HC(4^{1a}); 3.34 (*d*, $^3J_4{}^{1b}{}_{-4}{}^{1a}$ =16.0 Hz, 1H, HC(4^{1b}); 3.04 (*dd*, $^3J_5{}^{3a}{}_{-5}{}^3$ ≈6.0Hz, $^2J_5{}^{3a}{}_{-5}{}^{3a'}$ =14.0Hz ,1H, HC(5^{3a})); 2.92 (*dd*, $^3J_5{}^{3a}{}_{-5}{}^3$ ≈6.0Hz, $^2J_5{}^{3a}{}_{-5}{}^{3a'}$ =14.0Hz ,1H, HC($5^{3a'}$)) ; 2.58-2.52 (*m*, 2H, $H_2C(3^1)$); 2.51-2.48 (*m*, 2H, $H_2C(3^2)$); 1.52, 1.15-1.12 (minor peaks due to traces of impurity).

^{13}C-NMR (100 MHz, DMSO-d_6, 298K) : δ_C (ppm) = 173.1 ($C(3^3)$; 172.2 ($C(4^2)$); 167.6 ($C(5^2)$); 158.4(*q*, $^2J_{C-F}$ = 36.2, $CF_3\underline{C}OO^-$); 135.0 ($C(5^{3b})$); 129.5 ($C(5^{3c})$ and ($5^{3c'}$)); 128.6 ($C(5^{3d})$ and ($5^{3d'}$)); 127.2 ($C(5^{3e})$); 125.1 (C(5)); 120.8 (C(3)); 115.8 (*q*, $^1J_{C-F}$ ≈ 292, $\underline{C}F_3COO^-$); 114.1 (C(2)); 111.6 (C(4)); 53.5 ($C(5^3)$); 51.5 ($C4^3$)); 51.2 ($C(3^4)$); 37.1 ($C(5^{3a})$); 34.4 ($C5^1$)); 34.3 ($C(3^2)$); 29.4 ($C(4^1)$); 20.3 ($C(3^1)$).

MS (ESI (+)): m/z relative intensity $[M\text{-}CF_3COO^-]^+$ = 401.9

9.6.4. Synthesis of porphobilinogen-Lys-peptide

9.6.4.1. Synthesis of Boc-Boc-lys -PFP ester



104

$C_{16}H_{30}N_2O_6$. $C_{12}H_{23}N$

[527.70]

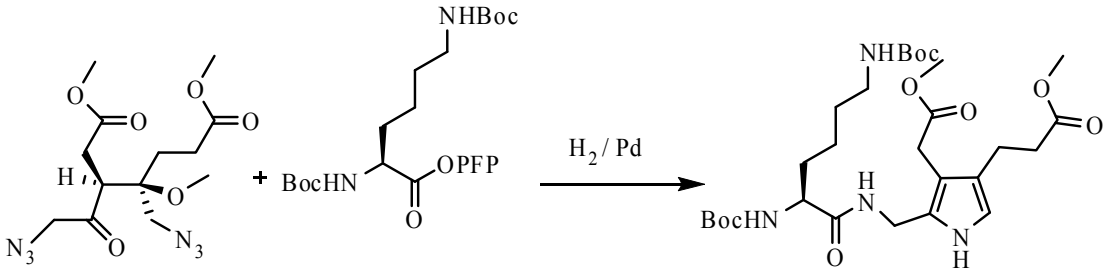
105

$C_{22}H_{29}N_2O_6F_5$

[512.47]

In a dry flask under argon atmosphere, 1.45 g (2.75 mmol, 1eq) of Boc-Boc-*lys*-DCHA salt was charged. This was dissolved in 40 ml EtOAc, the mass was cooled to 0 ^{0}C, added 40 ml water and neutralised with 0.7 g (11.66 mmol, 4.25eq) acetic acid to obtain Boc-Boc-*lys*-OH free acid. The organic layer was washed with 3×50 ml water and the organic layer concentrated to give 1.2 g Boc-Boc-*lys*-OH free acid. This was dissolved in 30 ml EtOAc and cooled to 0 ^{0}C.Then 0.49g (2.66 mmol, 1.0eq) of pentafluorophenol was added followed by 0.63 g (3.05 mmol, 1.1eq) of DCC. The reaction was brought to room temperature and was kept at room temperature overnight. The reaction mass was then filtered over celite bed to get rid off DCU and the solvent was evaporated off by rotovapor. 1.4 g of Boc-Boc-*lys*-PFP ester was obtained (Yield= 99%). This thick oily mass was taken directly for next step.

9.6.4.2. Synthesis of porphobilinogen- Boc-Boc-lys -peptide

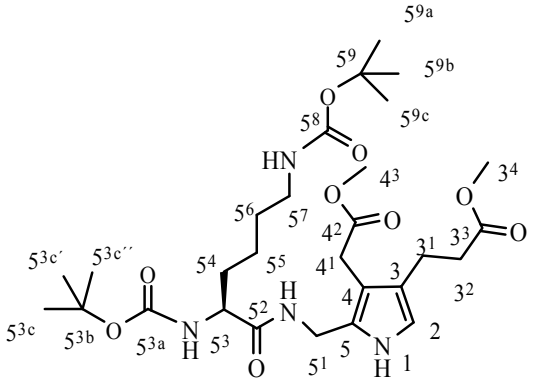


95 105 106

$C_{13}H_{20}N_6O_6$ $C_{22}H_{29}N_2O_6F_5$ $C_{28}H_{46}N_4O_9$

[356.3] [512.47] [582.68]

In a hydrogenation autoclave reactor, added 20ml MeOH followed by 50 mg (0.047 mmol, 0.04 eq) of 10% palladium on activated charcoal. This mixture was prehydrogenated with hydrogen for 10 min, later added 1.4 g (2.73 mmol, 2.2eq) of Boc-Boc-*lys*-PFP ester followed by a solution of 0.44 g (1.234 mmol, 1eq) 3-(2-azido-acetyl)-4-azidomethyl--4-methoxy- dimethyl heptanedioate in 20ml MeOH. The mass was subjected to hydrogenation in ambient temperature with the pressure of 60psi H_2 under mechanical agitation. Once the reaction was completed, (approximately 24hrs; TLC eluent: hexane/AcOEt 1:1) the H_2 pressure was released. The reaction mass was filtered over celite bed to get rid off palladium, the celite bed was washed with 3 × 25ml MeOH. The solvent in the filtrate was evaporated off by rotovapor and a yellow oily mass was obtained. This yellow crude mass was purified by flash chromatography (silica gel- 70 times the curude mass) using gradient eluent system. (from hexane/AcOEt 4:6 to 100% AcOEt). The pure fraction of 494 mg porphobilinogen- Boc-Boc-*lys* -peptide was thus obtained. (Yield = 68.7%)



Batch: Step06 lys #01 to #03.

R_f: (3:1 EtOAc: Hexane): 0.36 (UV $_{254,}$ $KMnO_4$ and violet Ehlrich)

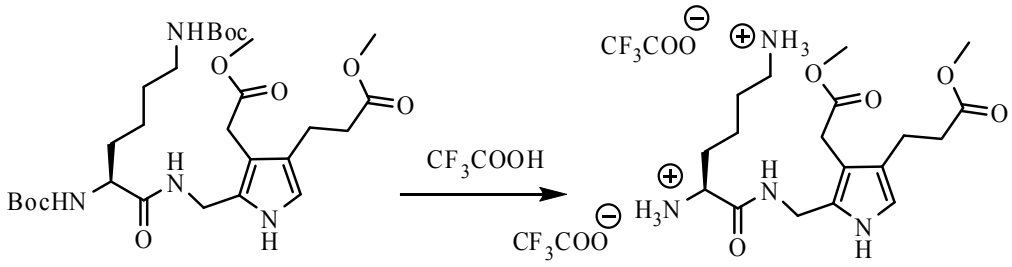
^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 8.98 (*sbr*, 1H, N<u>H</u> pyrrole); 7.03 ((*sbr*, 1H, N<u>H</u>-$H_2C(5^1)$); 6.43 (*d*, $^3J_{2\text{-NH}}$ =2.5Hz, 1H, HC(2)); 5.28 (*sbr*, 1H, N<u>H</u>-$HC(5^3)$); 4.71 (*t*, $^3J_{NH\text{-}5^7}$ =6.0 Hz, 1H, N<u>H</u>-$H_2C(5^7)$); 4.31 (*d*, $^3J_{5^1\text{-NH}}$ =5.7Hz, $H_2C(5^1)$); 4.04-4.03 (*mbr*, 1H, $HC(5^3)$); 3.66 (*s*, 3H, $H_3C(3^4)$ or $H_3C(4^3)$); 3.64 (*s*, 3H, $H_3C(3^4)$ or $H_3C(4^3)$); 3.42 (*s*, 2H, $H_2C(4^1)$); 3.06-3.03 (*m*, $^3J_{5^7\text{-NH}}$ =6.0 Hz, 2H, $H_2C(5^7)$); 2.73-2.69 (*m*, 2H, $H_2C(3^1)$); 2.54-2.49 (*m*, 2H, $H_2C(3^2)$); 1.92-1.89 (*m*, 1H, $HC(5^{4a})$); 1.70-1.64 (*m*, 1H,

HC(5^{5a}));1.44-1.26 (*m*, 21H, $H_3C(5^{9a}, 5^{9b}, 5^{9c})$, $H_3C(5^{3c}, 5^{3c'}, 5^{3c''})$, $HC(5^{5b})$ and $H_2C(5^{6})$); 1.18-1.05 (*m*, 1H, $HC(5^{4b})$); 1.78-1.70 and 1.62- 1.50 peaks due to impurity.

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 173.7 ($C(3^3)$; 173.2 ($C(4^2)$); 171.1 ($C(5^2)$);156.1 ($C(5^8)$); 155.7 ($C(5^{3a})$); 126.7 (C(5)); 121.1 (C(3)); 114.5 (C(2)); 111.4 (C(4)); 80.0 ($C(5^9)$); 79.1 ($C(5^{3b})$); 54.5 ($C(5^3)$); 52.1 ($C4^3$)); 51.5 ($C(3^4)$); 39.8 ($C(5^7)$); 34.9 ($C5^1$) and ($C(3^2)$); 33.9 ($C(5^4)$); 29.8 ($C(4^1)$); 29.5 ($C(5^6)$); 28.4 ($C(5^{9a}, 5^{9b}, 5^{9c})$, $C(5^{3c}, 5^{3c'}, 5^{3c''})$); 24.9 ($C(5^5)$); 20.5 ($C(3^1)$); 32.1, 25.6, 22.4 peaks due to impurity.

MS (ESI (+)): m/z relative intensity $[M + Na]^+ = 605.4$; $[M + K]^+ = 621.2$

9.6.4.3. Synthesis of porphobilinogen- lys -peptide



106

$C_{28}H_{46}N_4O_9$

[582.68]

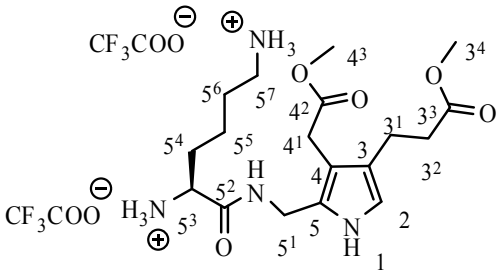
107

$C_{25}H_{39}F_3N_4O_9$

[610.50]

M^+=384.47 ; CF_3COO^- = 113.01

70 mg (0.12 mmol, 1eq) of porphobilinogen- Boc-Boc-*lys* -peptide was dissolved in 7ml CH_2Cl_2 (Basic alox treated) and cooled to -10^0C. To this solution, 1.33 g (0.9ml, 11.9 mmol, 100eq) of trifluoroacetic acid was added dropwise by syringe. The reaction mass was maintained at $5\text{-}10^0$C for an hour. Once the deprotection of BOC was completed (controlled by the absence of BOC peak in ^{1}H-NMR), CH_2Cl_2 and trifluoroacetic acid was evaporated off. The product was dried by high vacuum oil pimp for 2-3 days and yielded 65 mg of porphobilinogen- *lys* -peptide which was a highly hygroscopic and viscous semisolid (Yield=90.8%).



Batch: Step 07 lys #02 to #06.

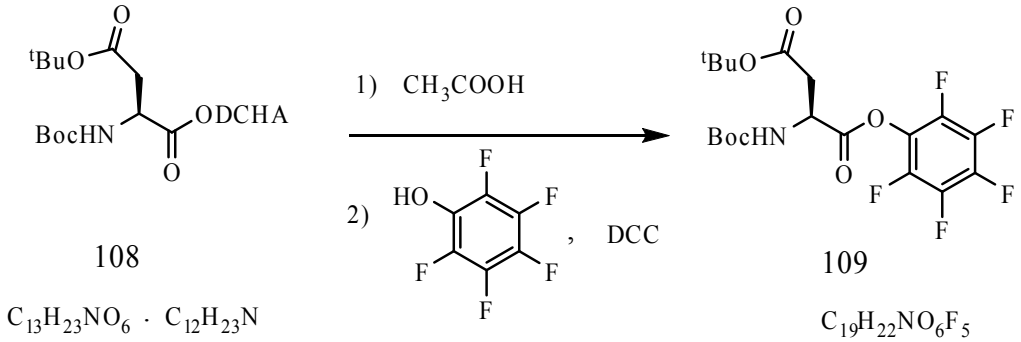
^{1}H-NMR (400 MHz, DMSO-d_6, 298 K): δ_H (ppm) = 10.46 ((*d*, $^{3}J_{NH\text{-}2}$ =2.3Hz, 1H, N<u>H</u> pyrrole); 8.62 ((*t*, $^{3}J_{NH\text{-}5^1}$ =5.0 Hz, 1H, N<u>H</u>-$H_2C(5^1)$); 8.08 (*s*, 3H, N<u>H_3</u>$^{+}$- $HC(5^3)$); 7.81 (*s*, 3H, N<u>H_3</u>$^{+}$- $H_2C(5^7)$); 6.44 (*d*, $^{3}J_{2\text{-}NH}$ =2.6 Hz, 1H, HC(2)); 4.21 (*d*, $^{3}J_{5^1\text{-}NH}$ =5.0 Hz, 2H, $H_2C(5^1)$); 3.69 (*mbr*, 1H, $HC(5^3)$); 3.58 (*s*, 3H, $H_3C(3^4)$ or $H_3C(4^3)$; 3.56 (*s*, 3H, $H_3C(3^4)$ or $H_3C(4^3)$; 3.44 (*d*, $^{3}J_{4^{1a}\text{-}4^{1b}}$ =16.0 Hz, 1H, $HC(4^{1a})$; 3.34 (*d*, $^{3}J_{4^{1b}\text{-}4^{1a}}$ =16.0 Hz, 1H, $HC(4^{1b})$; 2.73 (*m*, 2H, $H_2C(5^7)$); 2.58-2.53 (*m*, 2H, $H_2C(3^1)$); 2.50-2.46 (*m*, 2H, $H_2C(3^2)$); 1.72-1.64 (*m*, 2H, $H_2C(5^4)$); 1.53-1.46 (*m*, 2H, $H_2C(5^6)$); 1.31-1.23 (*m*, 2H, $H_2C(5^5)$); 1.38 due to traces of "Boc" unprotected product.

^{13}C-NMR (100 MHz, DMSO-d_6, 298K) : δ_C (ppm) = 173.2 ($C(3^3)$; 172.3 ($C(4^2)$); 168.2 ($C(5^2)$); 158.5 (*q*,$^{2}J_{C\text{-}F} \approx$ 35.0, CF_3<u>C</u>OO^-); 125.4 (C(5)); 120.8 (C(3)); 116.2 (*q*,$^{1}J_{C\text{-}F} \approx$ 293, <u>C</u>F_3COO^-); 114.2 (C(2)); 111.5 (C(4)); 52.1 ($C(5^3)$); 51.6 ($C4^3$)); 51.3 ($C(3^4)$); 38.6 ($C(5^7)$); 34.4 ($C5^1$)); 34.3 ($C(3^2)$); 30.6 ($C5^4$)); 29.4 ($C(4^1)$); 26.4 ($C5^6$)); 21.3 ($C5^5$)); 20.3 ($C(3^1)$); 79.8, 78.3, 28.3, 49.6, 34.2, 31.5 due to traces of "Boc" unprotected product.

MS (ESI (+)): m/z relative intensity $[M\text{-}CF_3COO^-]^+$ =383.2

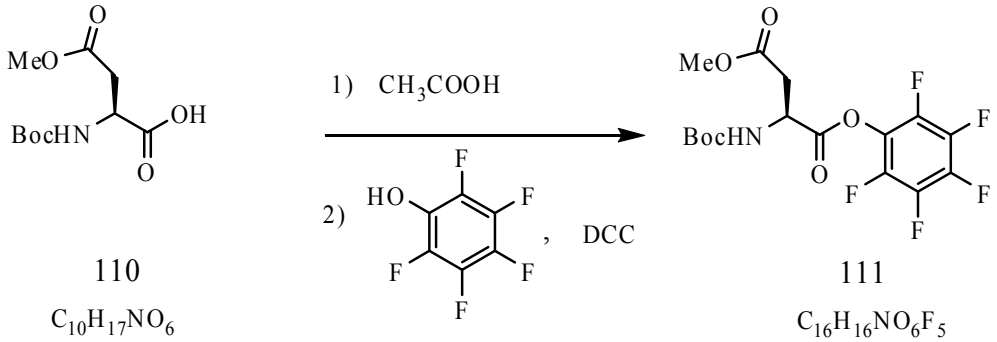
9.6.5. Synthesis of porphobilinogen-Asp-peptide

9.6.5.1. Synthesis of Boc-Asp-(O^{t}Bu) -PFP ester



In a dry flask under argon atmosphere, 1.42 g (3.02 mmol, 1eq) of Boc-*Asp*-(O^{t}Bu)-DCHA salt was charged. This was dissolved in 40 ml EtOAc, the mass was cooled to 0 ^{0}C, added 40 ml water and neutralised with 1.5 g (24 mmol, 8eq) acetic acid to obtain Boc-*Asp* (O^{t}Bu)-OH free acid. The organic layer was washed with 3×50 ml water and the organic layer concentrated to give Boc-*Asp* (O^{t}Bu)-OH free acid. This was dissolved in 20 ml EtOAc and cooled to 0 ^{0}C. Then 0.58g (3.15 mmol, 1.04eq) of pentafluorophenol was added followed by 0.68 g (3.29 mmol, 1.1eq) of DCC. The reaction was brought to room temperature and was kept at room temperature overnight. The reaction mass was then filtered over celite bed to get rid off DCU and the solvent was evaporated off by rotovapor. 1.36 g of Boc-*Asp*-(O^{t}Bu)-PFP ester was obtained (Yield= 99%). This thick oily mass was taken directly for next step.

9.6.5.2. Synthesis of Boc-Asp-(OMe) -PFP ester



110

$C_{10}H_{17}NO_6$

[247.24]

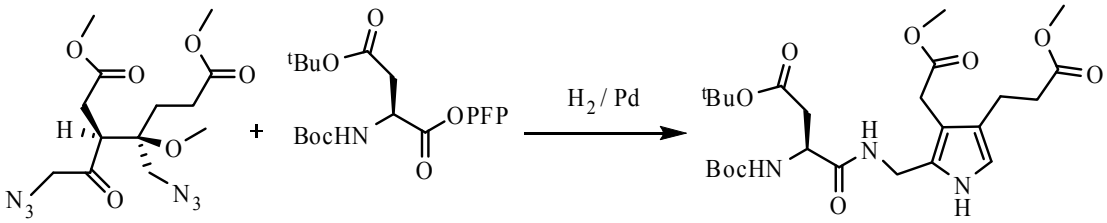
111

$C_{16}H_{16}NO_6F_5$

[413.29]

In a dry flask under argon atmosphere, 0.65 g (2.63 mmol, 1eq) of Boc-*Asp*-(O^{t}Bu)-DCHA salt was charged. This was dissolved in 20 ml EtOAc, the mass was cooled to 0 ^{0}C, then 0.48g (2.63 mmol, 1.0eq) of pentafluorophenol in 10 ml EtOAc was added followed by 0.61 g (2.95 mmol, 1.1eq) of DCC. The reaction was brought to room temperature and was kept at room temperature overnight. The reaction mass was then filtered over celite bed to get rid off DCU and the solvent was evaporated off by rotovapor. 1.07 g of Boc-*Asp*-(OMe)-PFP ester was obtained (Yield= 98%). This thick oily mass was taken directly for next step.

9.6.5.3. Synthesis of porphobilinogen- Boc-Asp-(O^{t}Bu) -peptide



95

$C_{13}H_{20}N_6O_6$

[356.3]

109

$C_{19}H_{22}NO_6F_5$

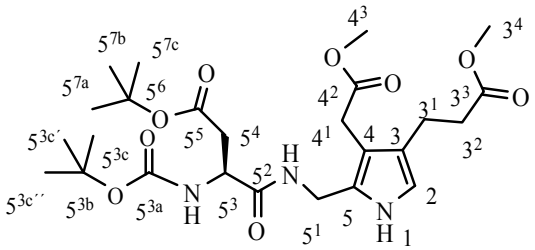
[455.37]

112

$C_{25}H_{39}N_3O_9$

[525.59]

In a hydrogenation autoclave reactor, added 30ml MeOH followed by 60 mg (0.05 mmol, 0.04 eq) of 10% palladium on activated charcoal. This mixture was prehydrogenated with hydrogen for 10 min, later added 1.36 g (2.98 mmol, 2.2eq) of Boc-*Asp*-(O^{t}Bu) -PFP ester followed by a solution of 0.484 g (1.358 mmol, 1eq) 3-(2-azido-acetyl)-4-azidomethyl--4-methoxy- dimethyl heptanedioate in 20ml MeOH. The mass was subjected to hydrogenation in ambient temperature with the pressure of 60psi H_2 under mechanical agitation. Once the reaction was completed, (approximately 24hrs; TLC eluent: hexane/AcOEt 1:1) the H_2 pressure was released. The reaction mass was filtered over celite bed to get rid off palladium, the celite bed was washed with 3 × 25ml MeOH. The solvent in the filtrate was evaporated off by rotovapor and a yellow oily mass was obtained. This yellow crude mass was purified by flash chromatography (silica gel- 70 times the curude mass) using gradient eluent system. (from hexane/AcOEt 4:6 to 100% AcOEt). The pure fraction of 578 mg porphobilinogen- Boc-*Asp*-(O^{t}Bu) -peptide was thus obtained. (Yield = 80.9%)



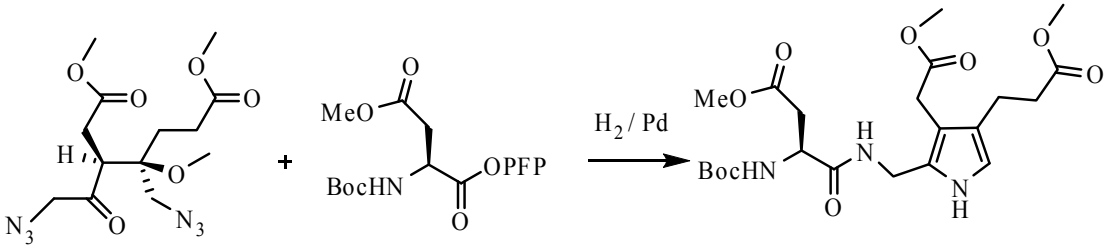
Batch: Step06 Asp(O^{t}Bu) #01 and #02.

R_f: (3:1 EtOAc: Hexane): 0.35 (UV $_{254,}$ $KMnO_4$ and violet Ehlrich)

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 8.75 (*sbr*, 1H, NH pyrrole); 7.08 ((*dbr*, $^3J_{NH\text{-}5^1}$ =9.0Hz,1H, NH-$H_2C(5^1)$); 6.42 (*d*, $^3J_{2\text{-}NH}$ =2.3Hz, 1H, HC(2)); 5.56 (*dbr*, $^3J_{NH\text{-}5^3}$ =8.0Hz, 1H, NH-$HC(5^3)$); 4.40 (*dd*, $^3J_{5^{1a}\text{-}NH}$ =6.4Hz, $^2J_{5^{1a}\text{-}5^{1b}}$ =15.3Hz, $HC(5^{1a})$); 4.27 (*dd*, $^3J_{5^{1b}\text{-}NH}$ =5.6Hz, $^2J_{5^{1a}\text{-}5^{1b}}$ =15.3Hz, $HC(5^{1b})$); 4.42- 4.38 (*mbr*, 1H, $HC(5^3)$); 3.66 (*s*, 3H, $H_3C(3^4)$ or $H_3C(4^3)$; 3.65 (*s*, 3H, $H_3C(3^4)$ or $H_3C(4^3)$; 3.46-3.41 (*m*, 2H, $H_2C(4^1)$); 2.91- 2.80 (*m*, 1H, $HC(5^{4a})$); 2.74-2.71 (*m*, 2H, $H_2C(3^1)$); 2.60 (*dd*, $^3J_{5^{4a}\text{-}5^3}\approx$5.5Hz, $^2J_{5^{4a}\text{-}5^{4b}}$ =16.0Hz, 1H, $HC(5^{4b})$); 2.55-2.51 (*m*, 2H, $H_2C(3^2)$); 1.44-1.40 (*m*, 18H, $H_3C(5^{7a}, 5^{7b}, 5^{7c})$ and $H_3C(5^{3c}, 5^{3c'}, 5^{3c''})$); other minor peaks due to impurities and solvent EtOAc.

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 173.9 ($C(3^3)$; 172.9 ($C(4^2)$); 172.0 ($C(5^2)$); 171.3 ($C(5^5)$);155.5 ($C(5^{3a})$); 127.1 (C(5)); 121.2 (C(3)); 114.5 (C(2)); 111.3 (C(4)); 81.9 ($C(5^{3b})$); 80.5 ($C(5^6)$); 52.1 ($C(3^4)$); 51.6 ($C4^3$)); 50.8 ($C(5^3)$); 37.6 ($C(5^4)$); 35.1 ($C5^1$); 35.0 ($C(3^2)$); 30.0 ($C(4^1)$); 28.3 and 28.1 ($C(5^{7a}, 5^{7b}, 5^{7c})$ and $C(5^{3c}, 5^{3c'}, 5^{3c''})$); 20.6 ($C(3^1)$); other minor peaks due to solvent and impurity.

9.6.5.4. Synthesis of porphobilinogen- Boc-Asp-(OMe) -peptide



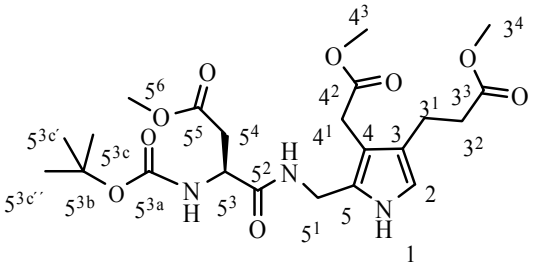
95
$C_{13}H_{20}N_6O_6$
[356.3]

111
$C_{16}H_{16}NO_6F_5$
[413.29]

113
$C_{22}H_{33}N_3O_9$
[483.51]

In a hydrogenation autoclave reactor, added 30ml MeOH followed by 60 mg (0.05 mmol, 0.04 eq) of 10% palladium on activated charcoal. This mixture was prehydrogenated with hydrogen for 10 min, later added 1.07 g (2.59 mmol, 2.2eq) of Boc-*Asp*-(OMe) -PFP ester followed by a solution of 0.44 g (1.234 mmol, 1eq) 3-(2-azido-acetyl)-4-azidomethyl--4-methoxy- dimethyl heptanedioate in 20ml MeOH. The mass was subjected to hydrogenation in ambient temperature with the pressure of 60psi H_2 under mechanical agitation. Once the reaction was completed, (approximately 24hrs; TLC

eluent: hexane/AcOEt 1:1) the H_2 pressure was released. The reaction mass was filtered over celite bed to get rid off palladium, the celite bed was washed with 3 × 25ml MeOH. The solvent in the filtrate was evaporated off by rotovapor and a yellow oily mass was obtained. This yellow crude mass was purified by flash chromatography (silica gel- 70 times the curude mass) using gradient eluent system. (from hexane/AcOEt 4:6 to 100% AcOEt). The pure fraction of 468 mg porphobilinogen- Boc-*Asp*-(OMe) -peptide was thus obtained. (Yield = 78.4%)



Batch: Step06 Asp(OMe) #01to #03.

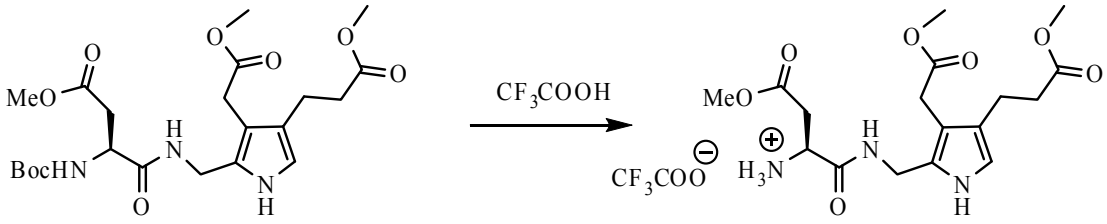
R_f: (3:1 EtOAc: Hexane): 0.35 (UV $_{254,}$ $KMnO_4$ and violet Ehlrich)

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ_H (ppm) = 8.82 (*sbr*, 1H, N<u>H</u> pyrrole); 7.18 ((*sbr*, 1H, N<u>H</u>-$H_2C(5^1)$); 6.43 (*d*, $^3J_{2\text{-}NH}$ =2.5Hz, 1H, HC(2)); 5.63 (*dbr*, $^3J_{NH\text{-}5^3}$ =8.7Hz, 1H, N<u>H</u>-$HC(5^3)$); 4.37 (*dd*, $^3J_{5^{1a}\text{-}NH}$ =6.2Hz, $^2J_{5^{1a}\text{-}5^{1b}}$ =15.2Hz, $HC(5^{1a})$); 4.29 (*dd*, $^3J_{5^{1b}\text{-}NH}$ =5.6Hz, $^2J_{5^{1a}\text{-}5^{1b}}$ =15.2Hz, $HC(5^{1b})$); 4.48 (*mbr*, 1H, $HC(5^3)$); 3.66 (*s*, 3H, $H_3C(3^4)$ or $H_3C(4^3)$ or $H_3C(5^6)$; 3.65 (*s*, 3H, $H_3C(3^4)$ or $H_3C(4^3)$ or $H_3C(5^6)$; 3.62 (*s*, 3H, $H_3C(3^4)$ or $H_3C(4^3)$ or $H_3C(5^6)$; 3.42 (*s*, 2H, $H_2C(4^1)$); 2.74-2.65 (*m*, 2H, $H_2C(3^1)$); 2.54-2.51 (*m*, 2H, $H_2C(3^2)$); 1.90 (*dd*, $^3J_{5^{4a}\text{-}5^3}\approx$3.5Hz, $^2J_{5^{4a}\text{-}5^{4b}}$ =12.5Hz ,1H, $HC(5^{4a})$); 1.40 (*s*, 9H, $H_3C(5^{3c}, 5^{3c'}, 5^{3c''})$); 1.14- 1.06 (*m*, 1H, $HC(5^{4b})$); other minor peaks (3.01-2.94, 1.69-1.55, 1.35-1.26) due to impurities.

^{13}C-NMR (100 MHz, $CDCl_3$, 298K) : δ_C (ppm) = 173.7 ($C(3^3)$; 172.9 ($C(4^2)$); 172.1 ($C(5^2)$); 171.5 ($C(5^5)$); 155.4 ($C(5^{3a})$); 126.7 (C(5)); 121.1 (C(3)); 114.5 (C(2)); 111.4 (C(4)); 80.4 ($C(5^{3b})$); 52.0 ($C(3^4)$ or ($C4^3$)); 51.9 ($C(3^4)$ or ($C4^3$)); 51.5 ($C(5^6)$); 50.7 ($C(5^3)$); 34.9 ($C5^1$); 34.8 ($C(3^2)$); 33.8 ($C(5^4)$); 29.7 ($C(4^1)$); 28.2 ($C(5^{3c}, 5^{3c'}, 5^{3c''})$); 20.4 ($C(3^1)$); other minor peaks (52.5;49.0;36.1;25.5;24.8) due to solvent and impurity.

MS (ESI (+)): m/z relative intensity $[M + Na]^+$ = 506.2; $[M + K]^+$ = 522.1

9.6.5.5. Synthesis of porphobilinogen-Asp (OMe)-peptide



113

$C_{22}H_{33}N_3O_9$

[483.51]

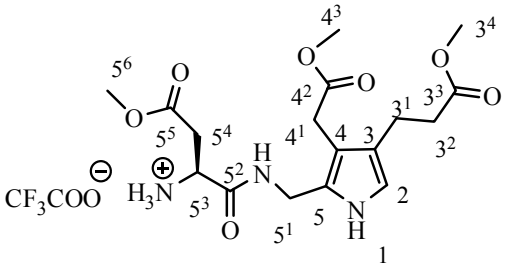
114

$C_{19}H_{26}F_3N_3O_9$

[497.42]

M^+=384.40 ; $CF_3COO^{\ominus}$ = 113.01

108 mg (0.22 mmol, 1eq) of porphobilinogen- Boc-*Asp*-(OMe) -peptide was dissolved in 5ml CH_2Cl_2 (Basic alox treated) and cooled to -10^{0}C. To this solution, 2.48 g (1.7ml, 21.7 mmol, 98eq) of trifluoroacetic acid was added dropwise by syringe. The reaction mass was maintained at 5-10^{0}C for an hour. Once the deprotection of BOC was completed (controlled by the absence of BOC peak in ^{1}H-NMR), CH_2Cl_2 and trifluoroacetic acid was evaporated off. The product was dried by high vacuum oil pimp for 2-3 days and yielded 102 mg of porphobilinogen- *lys* -peptide which was a highly hygroscopic and viscous semisolid. (Yield=92.6%)



Batch: Step 07Asp (OMe) #03 and #05.

^{1}H-NMR (400 MHz, DMSO-d_6, 298 K): δ_H (ppm) = 10.45 ((*d*, $^3J_{NH\text{-}2}$ =2.0Hz, 1H, N<u>H</u> pyrrole); 8.62 ((*t*, $^3J_{NH\text{-}5^1}$ ≈5.0 Hz, 1H, N<u>H</u>-$H_2C(5^1)$); 8.25 (*s*, 3H, N<u>H$_3^+$</u>- $HC(5^3)$); 6.44 (*d*, $^3J_{2\text{-}NH}$ =2.6 Hz, 1H, HC(2)); 4.23 (*dd*, $^3J_5{}^{1a}{}_{\text{-}NH}$ ≈5.0 Hz, $^2J_5{}^{1a}{}_{\text{-}5}{}^{1b}$ ≈14.8 Hz 1H, $HC(5^{1a})$); 4.18 (*dd*, $^3J_5{}^{1b}{}_{\text{-}NH}$ ≈5.0 Hz, $^2J_5{}^{1b}{}_{\text{-}5}{}^{1a}$ ≈14.8 Hz 1H, $HC(5^{1b})$); 4.06 (*mbr*, 1H, $HC(5^3)$); 3.64 (*s*, 3H, $H_3C(5^6)$); 3.58 (*s*, 3H, $H_3C(3^4)$ or $H_3C(4^3)$); 3.57 (*s*, 3H, $H_3C(3^4)$ or $H_3C(4^3)$); 3.41 (*s*, 2H, $H_2C(4^1)$; 2.90 (*dd*, $^3J_5{}^{4a}{}_{\text{-}5}{}^3$ ≈4.3 Hz, $^2J_5{}^{4a}{}_{\text{-}5}{}^{4b}$ ≈17.2 Hz 1H, $HC(5^{4a})$); 2.78 (*dd*, $^3J_5{}^{4b}{}_{\text{-}5}{}^3$ ≈8.3 Hz, $^2J_5{}^{4b}{}_{\text{-}5}{}^{4a}$ ≈17.2 Hz 1H, $HC(5^{4b})$); 2.59-2.54 (*m*, 2H, $H_2C(3^1)$); 2.50-2.46 (*m*, 2H, $H_2C(3^2)$); 2.09, 1.54, 1.24 and 1.11 peaks due to traces of impurity.

^{13}C-NMR (100 MHz, DMSO-d_6, 298K) : δ_C (ppm) = 173.1 ($C(3^3)$; 172.2 ($C(4^2)$); 169.6 ($C(5^2)$ or $C(5^5)$); 166.8 ($C(5^2)$ or $C(5^5)$); 158.2 (*q*,$^2J_{C\text{-}F}$ ≈ 34.0, $CF_3\underline{C}OO^-$); 125.1 (C(5)); 120.8 (C(3)); 116.2 (*q*,$^1J_{C\text{-}F}$ ≈ 295, $\underline{C}F_3COO^-$); 114.1 (C(2)); 111.5 (C(4)); 52.1, 51.5, 51.2 ($C(3^4)$,($C4^3$) and $C(5^6)$); 48.9 ($C(5^3)$); 35.4 ($C(5^4)$); 34.4 ($C5^1$)); 34.3 ($C(3^2)$); 29.3 ($C(4^1)$); 20.3 ($C(3^1)$);

MS (ESI (+)): m/z relative intensity $[M\text{-}CF_3COO^-]^+$ =384.0

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