

UNIVERSITÉ DE NEUCHÂTEL
FACULTÉ DES SCIENCES
INSTITUT DE CHIMIE

**Investigation of Second Coordination Sphere Interactions
Between Biotinylated Coordination Complexes and
(Strept)Avidin: CD Spectroscopy as a Powerful
Tool for Stability Constant Determinations**

Thèse présentée à la Faculté des Sciences de l'Université de Neuchâtel par

Andreas Loosli

Chimiste diplômé de l'Université de Berne

pour l'obtention du titre de Docteur ès Sciences



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Novembre 2004

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IMPRIMATUR POUR LA THESE

Etude des interactions de seconde sphère de coordination entre des complexes de coordination biotinylés et la (strept)avidine: la spectroscopie CD comme technique puissante pour la détermination des constantes de stabilité

M. Andreas LOOSLI

UNIVERSITE DE NEUCHÂTEL

FACULTE DES SCIENCES

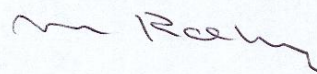
La Faculté des sciences de l'Université de Neuchâtel, sur le rapport des membres du jury

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et C.W. Schlaepfer (Fribourg)

autorise l'impression de la présente thèse.

Neuchâtel, le 25 novembre 2004

La doyenne:



Prof. M. Rahier

A ma famille

Per aspera ad astra.

Lucius Annaeus Seneca, 3 BC – AD 65

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CHAPTER 1: INTRODUCTION

1.1. General Considerations

A wide variety of physiological processes in nature are the reflection of ligand interactions with macromolecules, especially with proteins. Among such interactions, the most common are those between enzymes and their substrates and with other molecules that influence activity (e.g. coenzymes). In addition, there are interactions between hormones and hormone receptors, between small molecules and proteins involved in the active transport of the small molecules, between ions and both nucleic acids and proteins, and so forth.

The present work focuses on the interaction between proteins and small molecules. Macromolecules (e.g. proteins) can interact with an heterogeneous array of surrounding species. Their affinities range from weak interactions with different solutes to extremely tight binding of specific ligands.

One of the strongest binding affinity between a ligand and a protein is the interaction between biotin and (strept)avidin (hereafter biotin $\rightleftharpoons$ (strept)avidin where (strept)avidin refers to either avidin or streptavidin). This non-covalent interaction is the strongest known in nature.¹⁻³ The estimated association constants K_a are 10^{13} to 10^{14} M⁻¹ for biotin $\rightleftharpoons$ streptavidin^{3,4} and 10^{15} M⁻¹ for biotin $\rightleftharpoons$ avidin.^{1,3,5} Hypotheses about the source of this very high stability and the mechanism of binding have been proposed based on structural studies,⁶⁻⁹ minimized fragments procedure,¹⁰ chemical modifications of amino acids,^{1,11-14} single point mutations¹⁵⁻

²⁰ or a combination of several of these.²¹⁻²⁹ There is an ongoing debate as to whether the binding of biotin to the tetrameric (strept)avidin occurs with cooperativity³⁰⁻³⁴ or not.^{1,35-39}

Though the natural, unmodified biotin-(strept)avidin system has been known for four decades and is beginning to be well understood, very few studies exist on the kinetic and thermodynamic properties of biotin modified by metal complexes. Metals have been incorporated into this protein-ligand system mainly for medical and targeting applications (e.g. cancer pretargeting).⁴⁰⁻⁴⁴ Examples of such studies include the following metals: astatine,⁴⁵ cobalt,⁴⁶⁻⁴⁸ copper,⁴⁹ dysprosium,⁵⁰ europium,⁵¹ gadolinium,⁵² holmium,⁵⁰ indium,^{41,49,53-56} iron,^{46,57,58} manganese,⁴⁶ nickel,⁵⁹ rhenium,⁶⁰⁻⁶² ruthenium,⁶³ samarium,^{64,65} technetium^{64,66-68} and yttrium.⁶⁹⁻⁷²

1.2. Biotin and (Strept)Avidin

1.2.1. Biotin

Biotin is the water-soluble vitamin H and has a molecular weight M_r of 244 daltons.⁷³ The structure of biotin is shown in **Figure 1-1**.

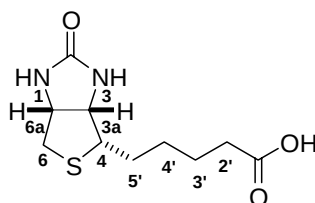


Figure 1-1. Structure of (+)-biotin (*d*-biotin).

Biotin has three asymmetric centers (C-3a, C-4 and C-6a) and consists of two rings, a thiophan (=tetrahydrothiophene) ring and an imidazolidone (=ureido) ring. The rings are fused in the *cis*-configuration. The valeric acid side chain at C-4 is also *cis* related to the imidazolidone ring.

In mammalian cells, biotin is involved in the metabolism of fatty acids, amino acids and carbohydrates. Biotin is used as a coenzyme for the removal of carbon dioxide from oxaloacetate, succinate, malate and aspartate. It is also used in biosynthesis of citrulline, aspartate and unsaturated fatty acids and in other reactions involving the transfer of carbon dioxide.^{74,75}

Biotin deficiencies are not frequently found in humans. One possible cause of biotin deficiency is the consumption of raw egg white. Raw egg white contains the protein avidin, which binds biotin and prevents its absorption into the body.

Food sources of biotin are yeast, eggs, chicken, liver, salmon, mushrooms, corn, rice, cauliflower, chick-peas, soybeans, barley, wheat, nuts and chocolate.

1.2.2. Avidin and Streptavidin

1.2.2.1. Properties of Avidin

Avidin is a glycosylated (at Asn-17)¹ and positively charged protein at neutral pH, found in the egg white and oviducts of many bird species, in the egg jelly of frogs and also in the egg white of others amphibia and reptiles, at a maximum concentration of about 0.05% of the total protein weight. The protein was discovered during investigations on water-soluble vitamins, when it was found that it could induce nutritional deficiency in rats, due to the formation of a highly stable, non-covalent complex with the vitamin biotin.⁷⁶ Avidin is secreted as a highly

stable homo-tetrameric protein, which, in hen egg-white, is composed of 4 x 128 amino-acid residue chains ($M_r = 62400$ daltons).^{1,3} The isoelectric point of the glycoprotein is 10.4 and the activity is 13-14 units (unit definition: 1 unit binds 1 μ g of *d*-biotin per mg of dry weight of avidin at pH 8.9). Avidin, native or modified, is highly soluble in water or saline solution at physiological pH (7.5-8.0): 50mg of avidin per mL of solution. Moreover, it is very stable against heat, pH changes and chaotropic reagents. Avidin is stable between pH = 2 and 13 as long as the concentration of guanidinium hydrochloride is smaller than 3*M*. Denaturation outside of these limits causes monomer formation, which is fully reversible if the pH changes again into the pH range of 2 to 13 by keeping the concentration of guanidinium hydrochloride smaller than 3*M*. An avidin solution is stable for weeks at 4°C. Avidin binds up to four molecules of vitamin H, in a non-covalent interaction, which is extremely tight. The affinity ($K_a \cong 10^{15} \text{ M}^{-1}$) for biotin is about five orders of magnitude higher than that of typical antigen-antibody complexes (**Table 1-1**).^{1,3,77}

Due to such a strong affinity for biotin, avidin has been proposed to act as an antibiotic protein inhibiting bacterial growth. Together with two other families of small-ligand binding proteins, the lipocalins and the fatty-acid binding proteins, avidin belongs to the “calycins” protein structural superfamily.⁷⁸

1.2.2.2. Properties of Streptavidin

In the early 1960s, scientists at Merck isolated, from soil samples, a series of bacterial cultures, which produced an interesting form of antibiotic. This was found in gram-negative bacteria and could be inhibited by vitamin H (biotin) to give an antibiotic complex. Upon further study, the researchers were surprised to discover that the macromolecular component of this antibiotic complex was structurally and functionally related to avidin. Because of this

similarity, the bacterial strain (a strain of *Streptomyces lavendulae*), which produced this new antibiotic was called *Streptomyces avidinii* and therefore the produced protein was termed streptavidin.⁷⁹ Streptavidin is a tetrameric protein composed of four identical chains ($M_r = 65700$ daltons). The monomeric subunits of streptavidin are synthesized as 183 amino-acid prepeptides. During secretion by *Streptomyces avidinii*, a 24 amino-acid leader sequence (signal peptide) is cleaved from these polypeptides resulting in newly secreted monomers of 159 amino acids.^{79,80} Upon longer incubations in culture these monomers are progressively cleaved to “core” subunits containing 125-127 amino acids.^{81,82}

Preparations of streptavidin are, as for avidin, relatively stable over a wide pH range and extremely heat stable, requiring up to 20 minutes at 100°C in 0.2% sodium dodecyl sulphate (SDS) to dissociate the subunits.⁸³ Strong chaotropic agents such as 6 M urea have been reported to dissociate the streptavidin tetramer into dimers.³³ These dimers appear to be stable in urea without the appearance of monomers.

1.2.2.3. Structure of Avidin and Streptavidin

Comparison of the primary structure of the two proteins shows moderate sequence homology (30% identity, 41% similarity).⁶ The respective values within the six homologous segments are 64% (identity) and 74% (similarity), respectively, and 7% (identity) and 17% (similarity), respectively, outside of these segments (**Figure 1-2**).⁶

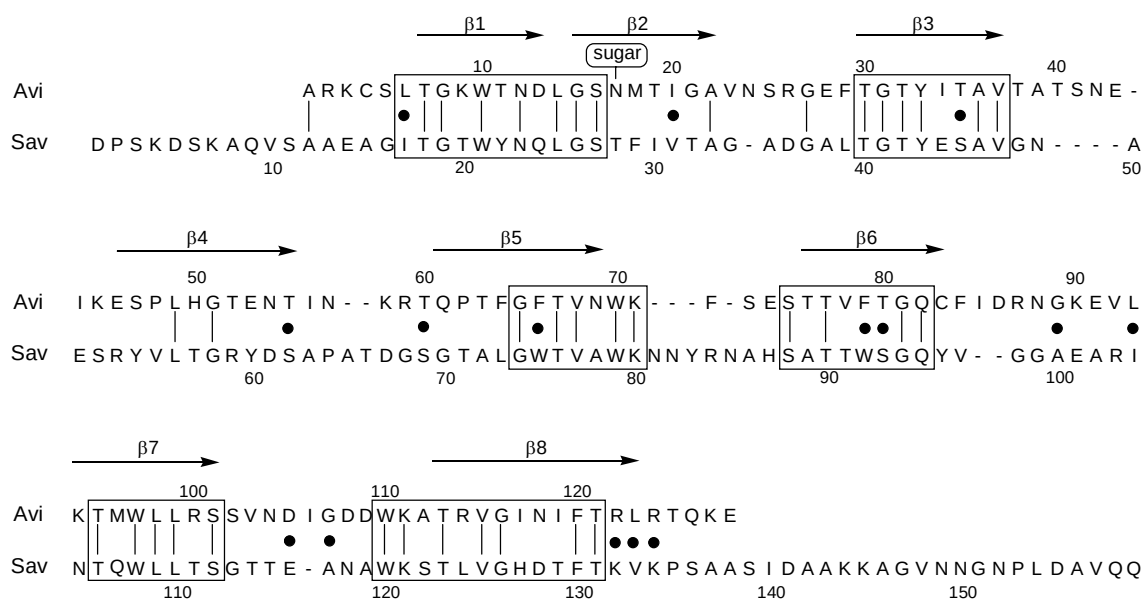


Figure 1-2. Alignment of primary structures of avidin and streptavidin. Conserved residues are linked by vertical lines. Similar residues (A, G; S, T; D, E; V, L, I, M; F, W, Y) are linked by dots. The six homologous segments are enclosed in boxes. The positions of the respective β -strands are marked by arrows. The position of every tenth amino residue is shown above (avidin) and below (streptavidin), respectively, in each line.^{1,2,6}

Streptavidin also resembles avidin in its secondary structure, predominantly β strands and loops.⁸⁰ Each subunit (monomer) is constructed of eight antiparallel β -strands, which form the β -barrel. **Figure 1-3** represents a monomer of tetrameric avidin and streptavidin, respectively. Avidin and streptavidin have similar tertiary fold and quaternary assemblies and contain four closely related biotin-binding sites.^{15,19} The most striking differences in their tertiary fold lie in the size and conformation of the six extended hairpin loops that connect the strands.

The tetrameric arrangement of avidin and streptavidin can be divided into a dimer of dimers (1-2 as well as 3-4 where 1, 2, 3 and 4 are the monomers) with a 222 (D_2) symmetry.

There are three regions of monomer-monomer interaction in (strept)avidin:

- interaction 1-2: occurs between monomers 1 and 2 (interactions within a dimer where monomers 1 and 2 are related by a crystallographic two-fold axis)
- interaction 1-3: occurs between monomers 1 and 3 (interactions between two different dimers)
- interaction 1-4: occurs between monomers 1 and 4 (interactions between two different dimers)

These interactions contribute to the rigidity of the quaternary structure and also provide part of the framework for the tight binding of biotin. Comparison of avidin and streptavidin revealed interactions 1-2 and 1-4 to be remarkably similar. In both proteins, interaction 1-4 makes the largest contribution to the tetrameric structure and can actually be considered to serve as a tetrameric glue in both cases. Most of the contacts in interaction 1-4 involve van der Waals forces, but there are also prominent hydrogen-bonding interactions involving polar residues and water molecules. Interaction 1-2 is formed by linking monomers 1 and 2 through hydrogen-bond interactions between the respective N-terminal portions of the β 8-strands of each monomer, which, in consequence, form a short antiparallel β -sheet.

There are, however, some intriguing differences between avidin and streptavidin with respect to interaction 1-3. In avidin, this particular monomer-monomer interaction is characterized solely by van der Waals forces, whereas in streptavidin polar amino-acid residues predominate.

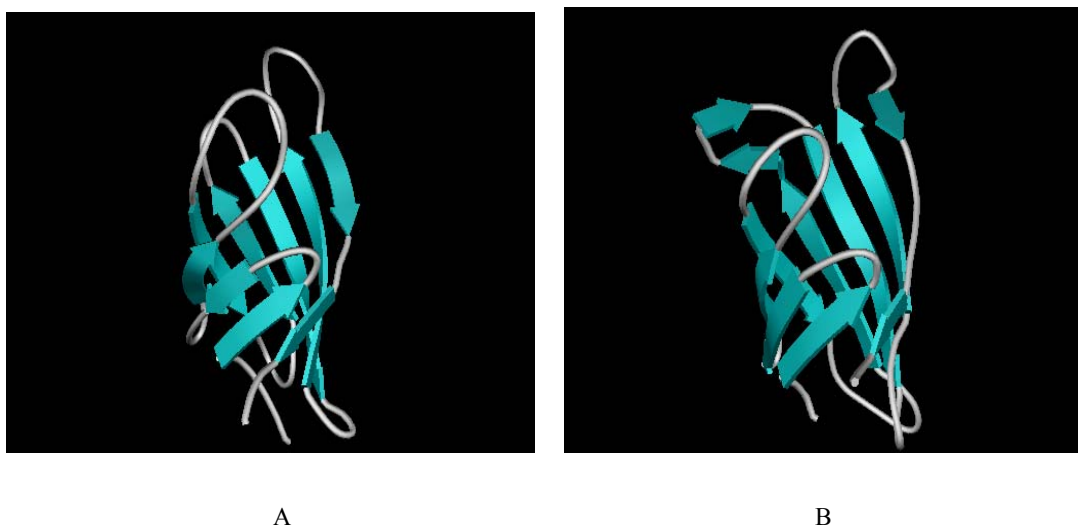


Figure 1-3. Schematic structure of a monomer of avidin (A) as well as streptavidin (B). Arrows indicate the eight anti-parallel β -sheets, which form the β -barrel.

Differences between avidin and streptavidin are the following:

- Compared to avidin, which is glycosylated at Asn-17,¹ streptavidin contains no carbohydrate.
- Streptavidin has a slightly acidic isoelectric point ($pI = 6.4$),^{3,4} which minimizes non-specific adsorption to nucleic acids and negatively charged cell membranes. In contrast, avidin displays $pI = 10.4$.
- Streptavidin is devoid of sulphur-containing amino-acid residues, whereas avidin is a disulphide-bridged protein and contains two additional methionine residues.
- Streptavidin has six tryptophan (Trp), six tyrosine (Tyr) and two phenylalanine (Phe) residues, whereas avidin has four Trp, one Tyr and seven Phe residues.

1.3. Biotin-(Strept)Avidin System

1.3.1. Various Biotin-Binding Proteins

Avidin, the biotin-binding protein of egg white of birds, and streptavidin, the bacterial analogue, have a very high affinity for biotin, with a K_a of about 10^{13} to 10^{14} M^{-1} for biotin-streptavidin^{3,4} and about 10^{15} M^{-1} for biotin-avidin,^{1,3,5} the strongest non-covalent interactions known between a protein and a low molecular weight ligand. **Table 1-1** presents an overview of different ligand-protein affinities.

Affinity Interaction	Affinity Constant (K_a , M^{-1})
biotin-avidin	1.7×10^{15}
biotin-streptavidin	2.5×10^{13}
inhibitor-protease	10^{10} - 10^{13}
ligand-receptor	10^9 - 10^{12}
antigen-antibody	10^7 - 10^{11}
ligand-transport protein	10^6 - 10^8
sugar-lectin	10^3 - 10^6
substrate-enzyme	10^3 - 10^5

Table 1-1. Hierarchy of ligand-protein affinity interactions.²

In addition to (strept)avidin with an exceptionally strong affinity for biotin, there are other biotin-binding proteins like the egg yolk protein, the biotinidase, the biotin carboxyl carrier protein and the biotin holocarboxylase synthetase. The two enzymes biotinidase and biotin holocarboxylase synthetase may eventually be extensively exploited in biotin-avidin technology as a replacement for avidin. Biotinidase could be used for biotinylation of binders or as an analytical tool for releasing the biotin moiety from biotinylated material. The other

enzyme biotin holocarboxylase synthetase, which exists as a monomer in the native state and has an affinity constant K_a of about 10^7 M^{-1} , could be applied for biotinylation of other proteins (in addition to the biotin carboxylase). This enzyme is known to be specific for a conserved sequence in all biotin-requiring apoenzymes, in which the lysine residue of Ala-Met-Lys-Met is biotinylated. This tetrapeptide can then be incorporated into any protein (e.g. at the C terminus) and the holocarboxylase synthetase can be used to incorporate biotin at this specific site.^{84,85}

Nevertheless, the question of the biological function of biotin-binding proteins is frequently raised and up to now there is no clear answer. A storage function is unlikely in view of the occurrence of these proteins in an essentially biotin-free state. A general catalytic function does not seem to be probable in view of the restricted distribution of the protein. Moreover, the catalytically active region of the biotin molecule is the part, which interacts most strongly with the avidin so that it is unlikely to be available for catalysis. Therefore, as already mentioned before, an antibacterial function is the most plausible one.

1.3.2. Structure of Biotin-(Strept)Avidin

The binding of biotin-(strept)avidin was investigated by differential scanning calorimetry^{37,86-88} and a number of spectroscopic techniques, including UV absorption,^{1,14} circular dichroism (CD),¹ vibrational circular dichroism (VCD)⁸⁹ and fluorescence spectroscopy.^{1,37,90} The UV absorption and fluorescence data indicate that the Trp residues within the binding site undergo a change to a more hydrophobic environment upon addition of biotin, whereas the CD data indicate a small change in the secondary structure when biotin is added. These systems were also studied by IR^{37,91} and Raman spectroscopy.^{92,93} IR studies of avidin revealed secondary structural changes with the addition of biotin and evidence for the

lengthening of the biotin ureido carbonyl group upon complex formation. IR studies of streptavidin revealed increased protein thermostability and conformational changes with biotin binding.

Knowledge of the three-dimensional structures of both avidin^{6,94-97} and streptavidin^{7,82} allows to compare their binding properties to biotin in more detail. X-ray data have revealed the importance of several amino-acid residues in the biotin binding site. The amino-acid residues in the binding of biotin-(strept)avidin can be divided into two groups, one group of amino-acid residues with hydrophobic interactions and another group of amino-acid residues with hydrophilic interactions.

1.3.2.1. Hydrophobic Interactions of Biotin-(Strept)Avidin

In the biotin binding site of avidin, the amino-acid residues Trp-70, Phe-72, Phe-79 and Trp-97 from one monomer, and Trp-110, which is provided by the adjacent symmetry-related monomer, are involved in the hydrophobic interactions. In streptavidin, the amino-acid residues Trp-79, Trp-92 and Trp-108 from one monomer and Trp-120 from the adjacent monomer are responsible for the hydrophobic interaction in the biotin binding site. The hydrophobic interactions of biotin-(strept)avidin are schematically depicted in **Figure 1-4**. The amino-acid residues involved in the hydrophobic biotin-(strept)avidin interactions are listed in **Table 1-2**.



Figure 1-4. Hydrophobic amino-acid residues in the biotin binding site of avidin (on the left) as well as streptavidin (on the right). For avidin (streptavidin), these are Trp-70, Phe-72, Phe-79 and Trp-97 (Trp-79, Trp-92 and Trp-108) from one monomer and Trp-110 (Trp-120), which is provided by the adjacent symmetry-related monomer.

1.3.2.2. Hydrophilic Interactions of Biotin-(Strept)Avidin

For avidin, the hydrophilic amino-acid residues within the binding site are Asn-12, Ser-16 and Tyr-33, which form with their side chains hydrogen bonds to the ureido oxygen of biotin. In addition, each of the two ureido nitrogen participates in a single hydrogen-bond interaction with Thr-35 and Asn-118 while the biotin sulphur interacts with Thr-77. The two carboxylate oxygen of the valeryl moiety of biotin form five hydrogen bonds. One carboxylate oxygen interacts with the main chain N $\cdots$ H of Ala-39 and Thr-40 as well as with the side chain of Thr-38 while the other forms hydrogen bonds with the side chains of Ser-73 and Ser-75.

The network of hydrogen bonds in biotin-streptavidin is similar to that one of biotin-avidin. The ureido ring oxygen forms three hydrogen bonds with the side chains of the hydrophilic amino-acid residues Asn-23, Ser-27 and Tyr-43. Each of the ring nitrogens forms one hydrogen bond with Ser-45 and Asp-128, respectively, while the biotin sulphur forms a hydrogen bond with Thr-90. The carboxylate oxygens of the biotin valeryl moiety form only

two hydrogen bonds, e.g. one with the main chain N...H of Asn-49 (substituted for Ala-39 in avidin) and the other with the side chain of Ser-88 (the equivalent of Ser-75 in avidin).

The first coordination sphere hydrogen-bonding network involved in the biotin-(strept)avidin binding is shown as a schematic representation in **Figure 1-5**. The amino-acid residues involved in the hydrophilic biotin-(strept)avidin interactions are listed in **Table 1-2**.

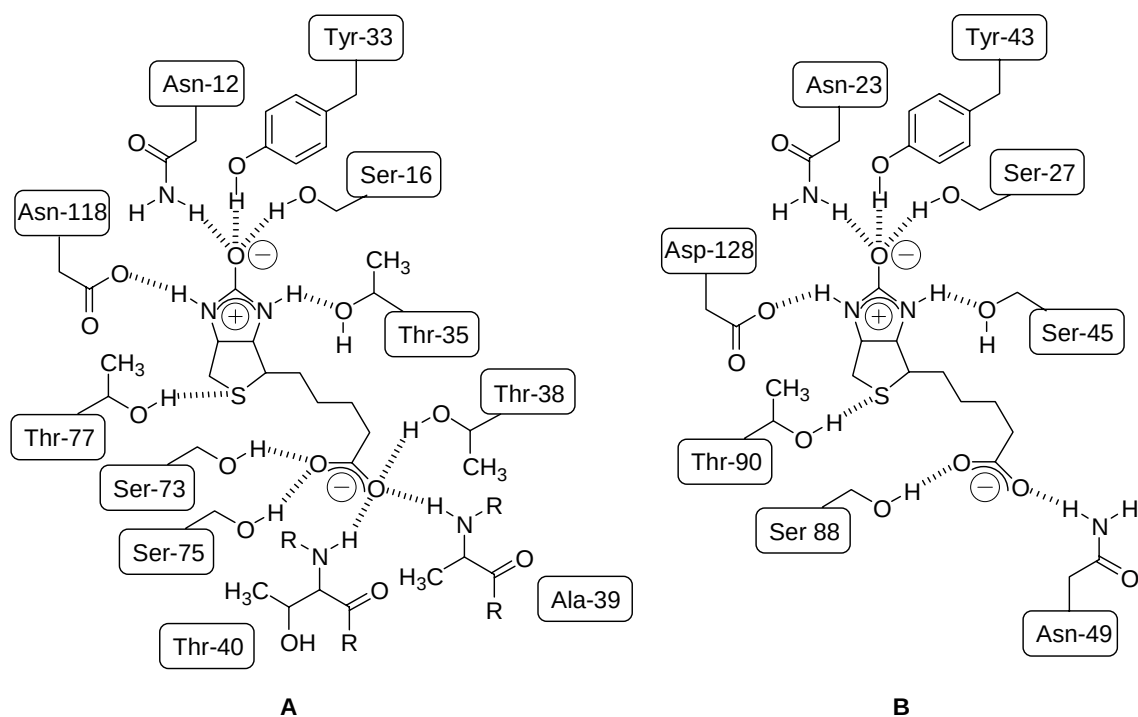


Figure 1-5. Schematic representation of the first coordination sphere hydrogen-bonding network of biotin-avidin (**A**) and biotin-streptavidin (**B**), respectively. R indicates the continuation of the peptide backbone in the case of avidin.^{8,98}

Interaction	Avidin	Streptavidin
H-bond	Asn-12	Asn-23
H-bond	Ser-16	Ser-27
H-bond	Tyr-33	Tyr-43
H-bond ^a	Thr-35	Ser-45
H-bond ^a	Asn-118	Asp-128
H-bond ^b	Thr-77	Thr-90
H-bond ^c	Ala-39	Asn-49
H-bond ^c	Ser-75	Ser-88
H-bond ^c	Thr-38	—
H-bond ^c	Thr-40	—
H-bond ^c	Ser-73	—
hydrophobic	Trp-70	Trp-79
hydrophobic	Phe-79	Trp-92
hydrophobic	Trp-97	Trp-108
hydrophobic	Trp-110 ^d	Trp-120 ^d
hydrophobic	Phe-72	—

Table 1-2. Amino-acid residues involved in the binding of biotin–avidin and biotin–streptavidin, respectively.

^a Interaction with a nitrogen atom of the ureido moiety of the biotin molecule.

^b Interaction with the sulphur atom of the tetrahydrothiophene moiety of the biotin molecule.

^c Interaction with the valeryl chain of the biotin molecule.

^d Amino-acid residue from an adjacent monomer.

There are two major differences between the binding sites of avidin and streptavidin. The first is an additional aromatic group (Phe-72) in the avidin binding pocket, e.g. it contains five aromatic amino-acid residues, whereas the streptavidin pocket contains only four. The second major difference is the hairpin loop connecting strands $\beta 3$ and $\beta 4$ (the L3,4 loop), which is discussed below.

1.3.2.3. Hydrogen-Bonding Pattern in the L3,4 Loop

In avidin, the L3,4 loop is three residues larger than the corresponding L3,4 loop of streptavidin (residues 36-44 in avidin vs 45-50 in streptavidin). In the apoproteins, the L3,4 loop in both avidin and streptavidin has an open or disordered conformation, respectively. Upon binding biotin, the loop closes in a lid-like manner, thus burying the ligand almost completely. The difference in length of the L3,4 loop is the reason for the different hydrogen-bonding network with the valeryl carboxylate group mentioned above. These two factors, an additional aromatic amino-acid residue (Phe-72) and three extra hydrogen bonds (provided by residues of the L3,4 loop), may explain why the binding of biotin $\rightleftharpoons$ avidin is reportedly tighter than biotin $\rightleftharpoons$ streptavidin.^{9,99} A comparison of the hydrogen-bonding network between the valeryl carboxylate and either avidin or streptavidin region is shown in **Figure 1-6**.

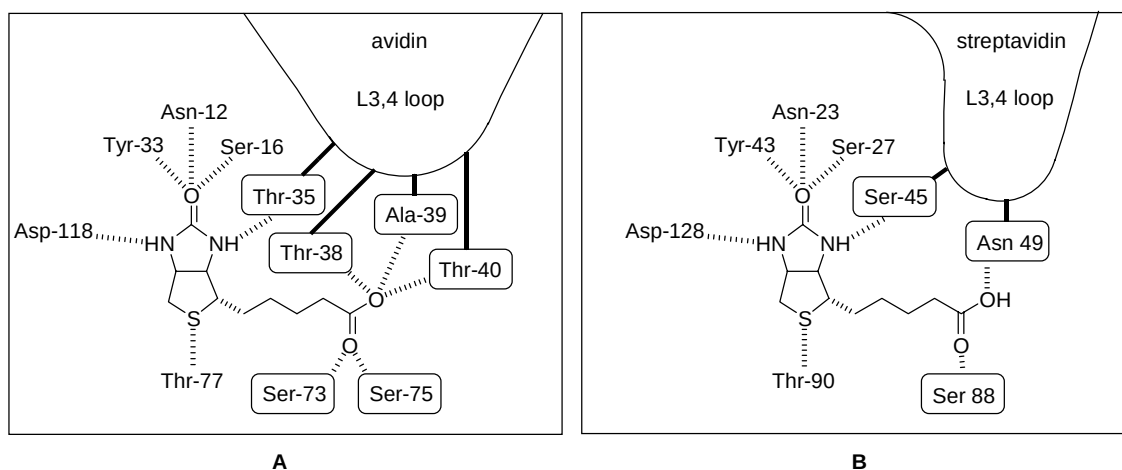


Figure 1-6. Schematic representation of the hydrogen-bonding network in the L3,4 loop region between avidin (A) or streptavidin (B) and the carboxylate group of biotin. In avidin, one of the carboxylate oxygens forms three hydrogen-bonding interactions with residues of the L3,4 loop, whereas in streptavidin, where the L3,4 loop is three residues shorter, only one hydrogen-bonding interaction is formed with the biotin carboxylate.⁹

1.3.2.4. Importance of Water Molecules in (Strept)Avidin

The importance of water molecules within the binding site of (strept)avidin is not negligible. In the absence of biotin, both avidin and streptavidin contain several “ordered” molecules of water, which form a well defined structure within the biotin-binding site. It was postulated that the bound water was necessary to maintain the shape of the binding site prior to interaction between the protein and the ligand. The involvement of water in the interaction of both avidin and streptavidin may suggest a general role for water in protein-protein and other biological interactions as well. Upon binding biotin, five of the “ordered” molecules of water vacate the binding site.

1.3.3. Biotin-(Strept)Avidin Binding Energetics and Dissociation Kinetics

Stayton and co-workers^{16,17,21,23,24,28,100} have investigated the biotin-streptavidin binding energetics by site-directed mutagenesis, biophysical and computational studies. They have shown that the high affinity energetics in the biotin-streptavidin system is an excellent model system for studying how proteins balance enthalpic and entropic components to generate an impressive overall free energy for ligand binding and how streptavidin builds a large activation barrier to dissociation by managing these ones. The large activation barrier of $\Delta G^\ddagger = 102.1 \text{ kJ/mol}$ (responsible for the high affinity constant K_a of biotin-streptavidin, where $\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger$) for native streptavidin is built through a large activation enthalpy $\Delta H^\ddagger$ of 133.9 kJ/mol while the activation entropy is favourable with a $T\Delta S^\ddagger = 31.8 \text{ kJ/mol}$ (all parameters at 298 K).^{16,100} The large and positive activation enthalpy indicates a significant loss of binding contacts between protein and ligand in the transition state and the presence of endothermic conformational alterations. The positive activation entropy change, associated with the formation of the transition state, is also consistent with significant ligand-protein conformational alterations in the transition state, resulting therefore in a gain in conformational entropy of the system. The importance of the tryptophan residues in biotin-streptavidin, thus regulating the dissociation rate k_{off} of biotin-streptavidin, was investigated in detail by Stayton and co-workers.^{15,16} They have probed the structural and energetic contributions of the Trp residues at the positions 79, 108 and 120 with conservative alterations of tryptophan side chains to phenylalanine.¹⁵ A complete structural analysis of all these mutants has also been reported.^{22,24} A general finding is that the Trp side chains clearly optimize the balance between enthalpic benefits and entropic costs in either the ground state or the transition state. This is related to the common phenomenon of enthalpy/entropy compensation, which can be viewed in a descriptive sense as the energetic balance between

favourable solvent release and bonding interactions (H bonds, van der Waals interactions, hydrophobic interactions) *vs* the unfavourable conformational immobilization of the protein and the ligand. These results also have provided insight into the biotin dissociation pathway and into how ligands exit protein binding pockets. The same results should be also valid for the biotin-avidin system.

There are other studies on the association constant K_a and the dissociation rate constants k_{off} of biotin-(strept)avidin, biotin-streptavidin analogues and biotin-streptavidin mutants.^{3,5,16,17,101,102} Investigations of the association constant K_a of the biotin-(strept)avidin complex were performed especially by Green, which gave the generally accepted K_a values of 10^{13} to 10^{14} M⁻¹ for biotin-streptavidin and 10^{15} M⁻¹ for biotin-avidin.^{1,3-5} The obtained K_a values for biotin-streptavidin analogues¹⁰³ and for biotin-streptavidin mutants¹⁹ were significantly lower than for the biotin-streptavidin system.

1.3.4. Thermostability of Biotin-(Strept)Avidin

Biotin-depleted avidin is fairly thermostable,¹⁰⁴ with a temperature T_m (T_m : concentration of folded and unfolded protein are equal, folded $\rightarrow$ unfolded form transition) of 85°C in the pH range 7 to 9, almost irrespective of the buffer medium and/or of the ionic strength. Binding of biotin increases substantially the thermal stability of the complex ($T_m = 132^\circ\text{C}$) and the enthalpy of denaturation.⁸⁶ Moreover, the biotin-avidin complex is so stable that biotin cannot be released from the binding site, even when subjected to a variety of drastic conditions such as high concentrations of denaturing agents at room temperature, e.g. 6M guanidinium hydrochloride, 3M guanidinium thiocyanate, 8M urea or 10% SDS. Under combined treatment with guanidinium hydrochloride at low pH (1.5) or upon heating ($>$

70°C) in the presence of denaturing agents or detergents, the protein is denatured and biotin is dislodged from the disrupted binding site.

There is a similar effect in the thermostability of streptavidin. Biotin increases the midpoint temperature T_m of thermally induced denaturation of streptavidin from 75°C in apoprotein to 112°C at full ligand saturation. Fidelio and co-workers³⁷ have shown by IR studies that biotin binding leads to a more structured protein, which is in good agreement with X-ray results that showed that two loops (L3,4: residues 45-50 and L4,5: residues 63-69), that are disordered in the unliganded streptavidin, are ordered in the presence of biotin.⁷ Also fluorescence-quenching experiments are in agreement with the higher structural order observed after biotin binding, since a drastic decrease of the aqueous quencher accessibility to the tryptophan residues is found for liganded as compared to apostreptavidin.³⁷

1.3.5. (Non-)Cooperativity of Biotin-(Strept)Avidin

The association constant K_a for the binding of biotin to (strept)avidin is among the highest for any known non-covalent interaction. Even with this huge K_a it has been suggested that the binding of the four biotin ligands is positively cooperative.³⁰⁻³⁴ Usually the term cooperativity in binding is used to the increased or decreased affinity of a protein with multiple ligand binding sites in the presence of increasing number of bound ligands. The ratio K_{i+1}/K_i is larger (positive cooperativity) or smaller (negative cooperativity) than the statistical value, $\{i(n-1)\} / \{(i+1) \cdot (n-i+1)\}$ where n is the number of equivalent sites. Both positive (e.g. for a macromolecule with four ligands: $1/4K_1 < 2/3K_2 < 3/2K_3 < 4K_4$) and negative cooperativity (e.g. for a macromolecule with four ligands: $1/4K_1 > 2/3K_2 > 3/2K_3 > 4K_4$) are found in Nature.^{37,96,105} A binding event is defined as positively cooperative with respect to another interaction (or a set of interactions) when its affinity is increased in the presence of the

previous interaction (or set of interactions). Conversely, a binding event is defined as negatively cooperative when its affinity is decreased in the presence of another interaction (or set of interactions). A schematic illustration of two different types of biotin-(strept)avidin interactions is depicted in **Figure 1-7**.

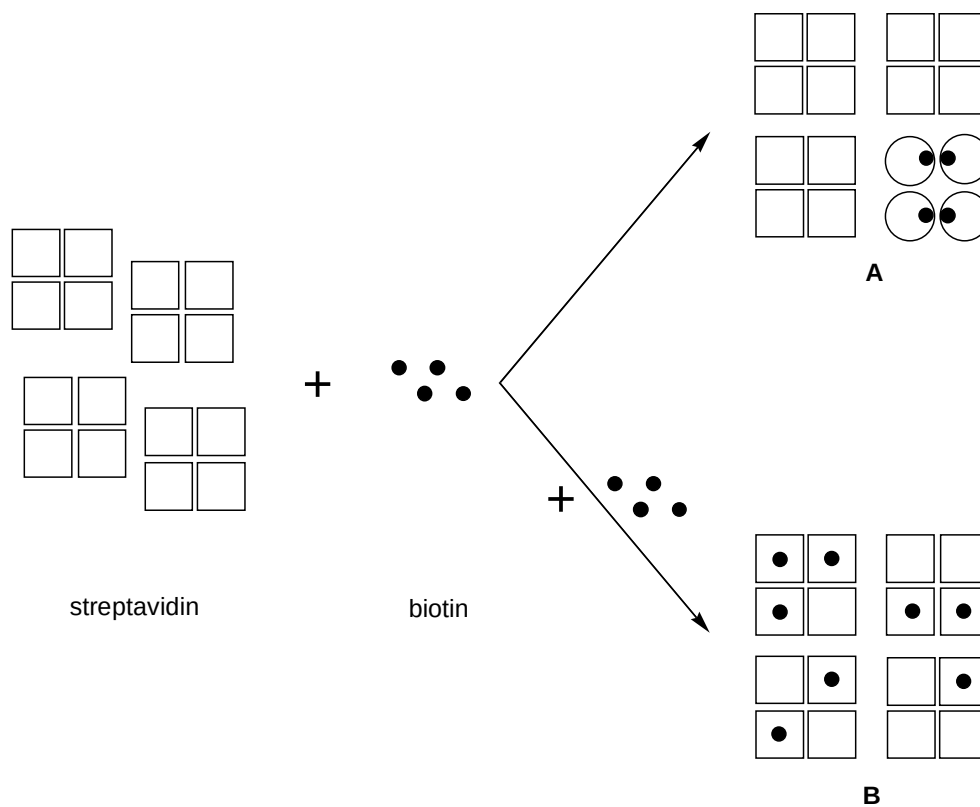


Figure 1-7. Schematic illustration of two models for biotin-(strept)avidin interaction: **(A) cooperativity:** structural changes induced by cooperative binding ($\Delta H > 0$: binding cooperativity; $\Delta S > 0$: structural cooperativity), **(B) non-cooperativity.**

The negatively cooperative binding of a ligand to a protein is expected, if the binding to the protein is coupled with an additional cost in enthalpy and a benefit in entropy. The positively cooperative binding, in contrary, is due to a better packing in the protein system and benefits in enthalpy and costs in entropy. The typical textbook example for positively cooperative binding is oxygen binding to tetrameric hemoglobin. It is very difficult to imagine (and experimentally difficult to prove) whether the cooperativity that seems to occur with biotin binding to (strept)avidin works in a similar way than that observed for the interaction of oxygen with hemoglobin, even when the quaternary structural changes seen in (strept)avidin are consistent with the possibility of cooperativity.

A model in which a first ligand influences the affinity of the remaining ligands, giving an overall macroscopic K_a of 10^{13} to 10^{14} M^{-1} for biotin-streptavidin^{3,4} and 10^{15} M^{-1} for biotin-avidin,^{1,3,5,37} is not in agreement with the calorimetric data.³⁷ Furthermore, evidence for non-cooperativity in biotin binding has been reported in a fairly convincing way by Kurzban and collaborators.³⁹ Thereby, streptavidin tetramers have been separated according to their biotin content by anion exchange chromatography. Biotin-free and biotin-saturated streptavidin were coincubated. Streptavidin at intermediate ligation levels, *i.e.* with one, two or three molecules of bound biotin, accumulates over time. A steady-state distribution of ligation levels is reached after 2 days. When biotin was allowed to redistribute starting from homogeneous populations containing two molecules of biotin per tetramer, a similar steady-state distribution of ligation levels was observed, thereby demonstrating an equilibrium distribution. Quantification of this equilibrium indicates that biotin-streptavidin binding is non-cooperative.

On the other hand, it has been shown that the streptavidin structure tightens extensively upon biotin binding.¹⁰⁵ In analogy to the binding of O_2 to hemoglobine, these structural changes

might induce positive cooperativity in the binding of biotin. Based on this fact, it seems that biotin binding is occurring with structural changes without affecting the binding affinity of the overall process.³⁷

A method to distinguish whether binding occurs with cooperativity is the so-called Scatchard plot.

1.3.5.1. Scatchard Plot

The Scatchard plot is given by **equation 1.1**:

$$\frac{v}{[L]_{\text{free}}} = \frac{n}{K_d} - \frac{1}{K_d} v \quad (1.1)$$

where n = number of binding sites

$[L]_{\text{free}}$ = free ligand concentration

K_d = dissociation constant

$$\text{and } v = \frac{[L]_{\text{bound}}}{[P]_{\text{tot}}} = \frac{[\text{ligand bound}]}{[\text{protein total}]}$$

The relationship between v and $[L]_{\text{free}}$ for the simple case of **identical independent sites** is represented in **Figure 1-8**. This plot is linear with an ordinate intercept of n/K_d , an abscissa intercept of n and a slope of $-1/K_d$. A straight line indicates non-cooperativity.¹⁰⁶

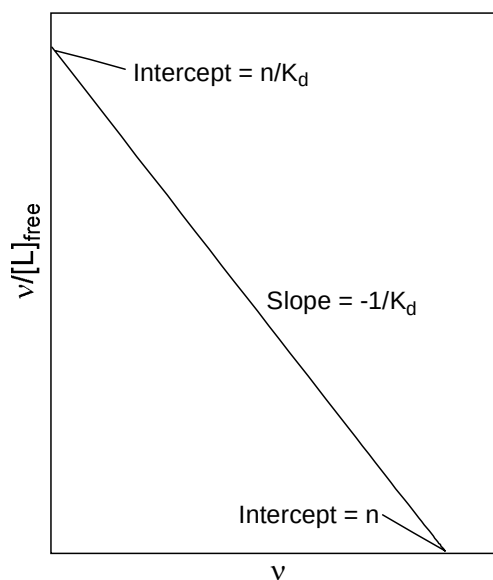


Figure 1-8. Scatchard plot for a system with identical independent sites.

In many cases, a Scatchard plot of $v/[L]_{\text{free}}$ vs v proves to be curved rather than linear. A plot for a system with **two classes of independent sites** is shown in **Figure 1-9**. This plot is biphasic with an ordinate intercept of $n_1/K_{d1} + n_2/K_{d2}$ and an abscissa intercept of $n_1 + n_2$.¹⁰⁶

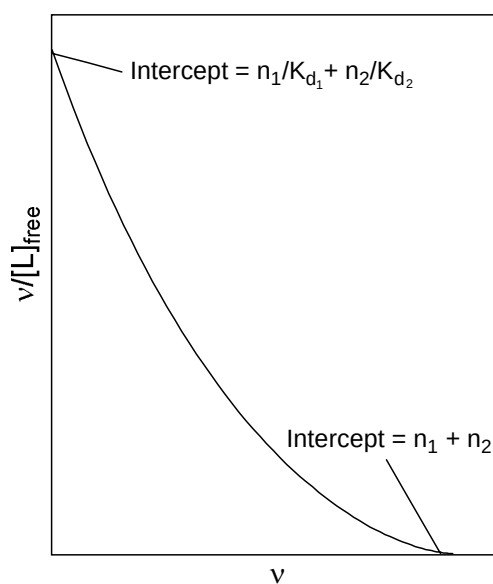


Figure 1-9. Scatchard plot for a system with two classes of independent sites.

The assumption of separate classes of sites is not the only way to account for curved Scatchard plots. In the case of cooperativity, the Scatchard plot is also curved. A concave downward curve represents positive cooperativity, whereas a concave upward curve is for negative cooperativity (anticooperativity) (**Chapter 3**).

1.3.5.2. Hill Plot

Another kind of plot applied as aid in the study of cooperative binding is the so-called Hill plot. There are different possibilities to represent Hill plots:¹⁰⁶⁻¹⁰⁸

- a) $\ln[L]_{\text{free}}$ vs $\ln[(n/v)-1]$
- b) $\log[v/(n-v)]$ vs $\log[L]_{\text{free}}$
- c) $\ln[\gamma/(1-\gamma)]$ vs $\ln[L]_{\text{free}}$

where n is the number of binding sites, $[L]_{\text{free}}$ the free ligand concentration, v equals to $[L]_{\text{bound}}/[P]_{\text{tot}}$ (= [ligand bound]/[protein total]) and γ is the fractional saturation ($\gamma = v/n$).

Using Hill plots, where non-cooperativity is evidenced by a straight line, cooperativity manifests itself as two lines of unit slope connected by an S-shaped curve. The value of the slope in the central region of the curve is the Hill coefficient α_H . Values of $\alpha_H > 1$ and $\alpha_H < 1$ are diagnostic for positive cooperativity and negative cooperativity, respectively. Since α_H can vary between 0 and n (number of binding sites, for (strept)avidin $n = 4$), it provides a quantitative measure of cooperativity. When $\alpha_H = n$, the system behaves as perfectly cooperative (for (strept)avidin this would be 4), whereas $\alpha_H = 1$ indicates non-cooperativity (**Chapter 3**).

Cooperativity, which is the basis of enzyme control and many other vital biological processes, has been rigorously defined in the case of multiple intermolecular binding of a monovalent ligand to a polyvalent macromolecule.^{106,109-112} Cooperativity was also observed in the field of supramolecular chemistry, where multiple intramolecular interactions play a key role.¹⁰⁷

1.4. HABAC(Strept)Avidin System

Green suggested that the biotin binding sites of (strept)avidin were in part non-polar.¹⁴ Thus, he tested by spectroscopic methods the interaction of (strept)avidin with several organic aromatic dyes, which differ structurally from biotin. The most striking effects were shown by the dye HABA (2-(4'-hydroxyazoybenzene)benzoic acid) that has been used for the estimation of serum albumin and studied in detail by Baxter.¹¹³ Upon binding to (strept)avidin, the colour of HABA changes from orange to red. The orange colour is restored upon replacement of the dye from the avidin complex by biotin. HABA binds with a considerably lower affinity to streptavidin than to avidin (K_a of $1 \times 10^4 \text{ M}^{-1}$ for streptavidin vs K_a of $1.7 \times 10^5 \text{ M}^{-1}$ for avidin).³ Moreover, HABA is quantitatively displaced when biotin binds, suggesting that both ligands share common binding sites. The three-dimensional structures of the HABACavidin complex as well as of the HABACstreptavidin complex have been described.^{8,98}

The general fold of the HABAC(strept)avidin structure is very similar to the biotinC(strept)avidin complex.^{6,7,82} HABA is a planar molecule, but when bound to (strept)avidin, HABA loses its planarity and exists in a twisted (along the N-N bond) conformer (hydrazone tautomer), which is depicted in **Figure 1-10**.

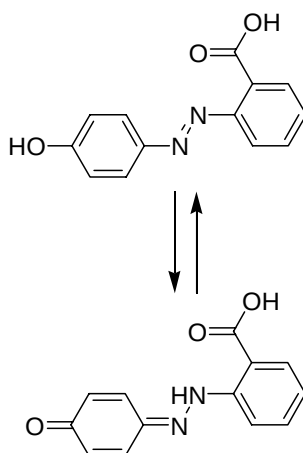


Figure 1-10. The possible tautomers of HABA. The structure shown at the top represents the free dye, and the structure below is formed and stabilized in the HABA-(strept)avidin complex.⁹⁸

As a result, the nitrogen atom nearest the benzoic acid ring changes its geometry from a planar sp^2 to a tetrahedral sp^3 . Therefore, the HABA molecule loses the extended conjugation of its π -electrons and consists of two planar entities with a slight kink between them.⁹⁸

When comparing the interactions of HABA with avidin and its bacterial analogue streptavidin, one can observe similarities and differences in their interactions and rationalize the observed variations in the association constants K_a .⁹⁸ The similarities in the binding properties of HABA by the two proteins are manifested by the interactions of the oxygens in the benzoate ring, which are analogous.

In avidin, one of these oxygens interacts via three hydrogen bonds with Asn-12, Ser-16 and Tyr-33, thus forming an oxyanion (the position of this oxygen atom corresponds to that of the ureido oxygen of biotin). The other carboxylate oxygen of HABA forms hydrogen bonds with Ser-16 and Thr-35 of avidin and an intramolecular hydrogen bond with one of the HABA nitrogens (**Figure 1-11** and **Table 1-3**).

In the case of streptavidin, hydrogen bonds are formed between one of the oxygen atoms of the benzoate group and Asn-23, Ser-27 and Tyr-43 (the position of this oxygen atom corresponds to that of the ureido oxygen of biotin). The other oxygen atom of the benzoate group builds hydrogen bonds with Ser-27 and Ser-45 of the protein and via intramolecular interaction with the adjacent nitrogen (**Figure 1-12** and **Table 1-3**).

The hydrophobic interactions within both proteins are similar. In avidin, the benzoate ring exhibits hydrophobic interactions with three aromatic amino-acid residues, *i. e.* Phe-79, Trp-97 and Trp-110 from the adjacent monomer. In streptavidin, hydrophobic interactions involve amino-acid residues Trp-92 and Trp-108 as well as Trp-120 from the adjacent monomer. Moreover, the hydroxyphenyl ring of the ligand is stacked with the indole ring of Trp-70 (in avidin) or Trp-79 (in streptavidin), thus forming a charge-transfer complex, where the tryptophan is acting as donor and the hydroxyphenyl ring of HABA as acceptor (UV/Vis and CD induced signal with $\lambda_{\text{max}} = 506\text{nm}$).⁹⁸

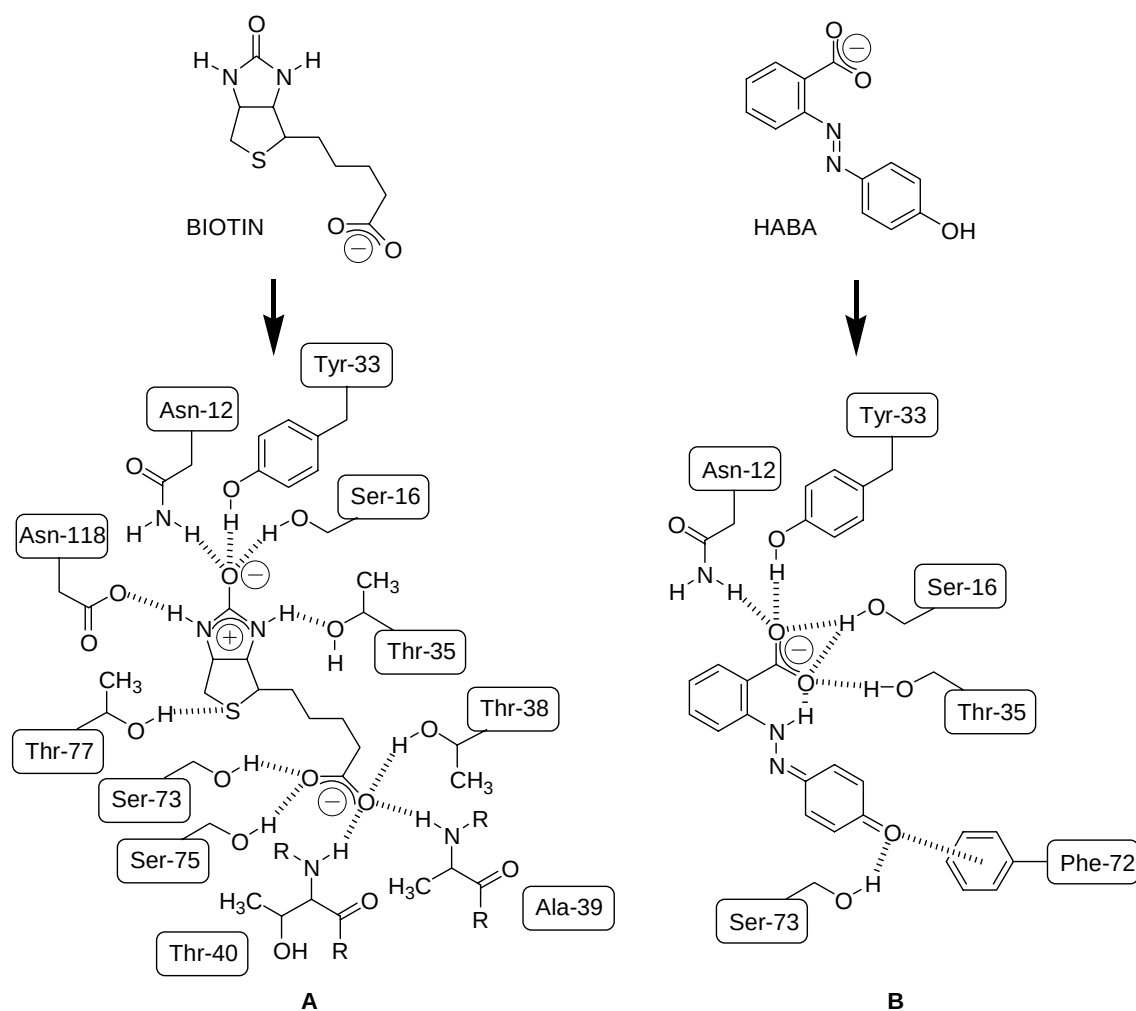


Figure 1-11. Schematic illustration of biotin (**A**) and HABA (**B**) binding to avidin. Biotin is bound as a minor resonance form owing to specific protein interactions that polarize the ureido group and allow strong hydrogen-bond formation, with the amino-acid residues Asn-12, Ser-16 and Tyr-33, in the oxyanion pocket. HABA binds as a hydrazone tautomer, in part because the hydrazone NH proton can stabilize the charge or orientation of the carboxyl group in the hydrophobic environment of the binding site.⁹⁸

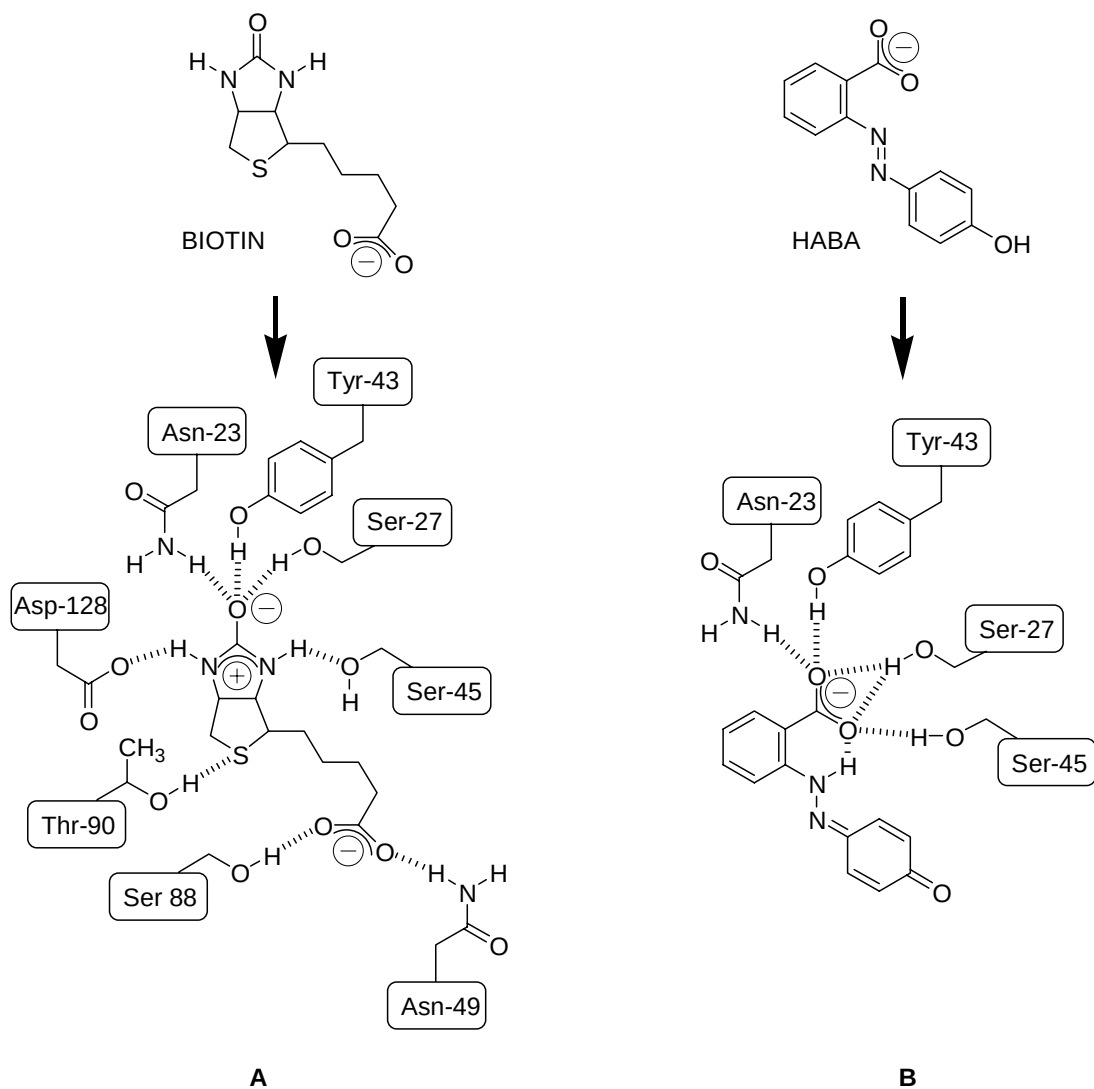


Figure 1-12. Schematic illustration of biotin (**A**) and HABA (**B**) binding to streptavidin. Biotin is bound as a minor resonance form owing to specific protein interactions that polarize the ureido group and allow strong hydrogen-bond formation, with the amino-acid residues Asn-23, Ser-27 and Tyr-43, in the oxyanion pocket. HABA binds as a hydrazone tautomer, in part because the hydrazone NH proton can stabilize the charge or orientation of the carboxyl group in the hydrophobic environment of the binding site.⁸

The key difference of HABA-avidin and HABA-streptavidin lies in two unique interactions between the hydroxyphenyl ring of the dye and avidin, which do not exist in streptavidin. In avidin, the hydroxyphenyl oxygen of HABA forms an hydrogen bond with Ser-73 and interacts with π -electron plane of the phenyl ring of the Phe-72. The amino-acid residues involved in the HABA-avidin and the corresponding HABA-streptavidin system are listed in **Table 1-3**.

Interaction	Avidin	Streptavidin
H-bond ^a	Asn-12	Asn-23
H-bond ^{a,b}	Ser-16	Ser-27
H-bond ^a	Tyr-33	Tyr-43
H-bond ^b	Thr-35	Ser-45
H-bond	Ser-73	—
hydrophobic	Trp-70 ^c	Trp-79 ^c
hydrophobic	Phe-79	Trp-92
hydrophobic	Trp-97	Trp-108
hydrophobic	Trp-110 ^d	Trp-120 ^d
aromatic oxygen ^e	Phe-72	—

Table 1-3. Amino-acid residues involved in the binding of HABA-avidin and HABA-streptavidin, respectively.

^a Interaction with a carboxylate oxygen atom.

^b Interaction with the other carboxylate oxygen atom.

^c Involved in the charge-transfer between (strept)avidin and HABA.

^d Amino-acid residue from an adjacent monomer.

^e The aromatic ring of Phe-72 interacts with the hydroxyphenyl oxygen of the HABA molecule.

As mentioned above, HABA occupies the biotin binding site in (strept)avidin and displaces five of the “ordered” bound water molecules within the biotin binding site. In modified HABA dyes, six of the “ordered” water molecules can be displaced. As a consequence, such modified HABA dyes show a 165fold greater affinity towards streptavidin.¹¹⁴ Many of the characteristic interactions of biotin⇌(strept)avidin and HABA⇌(strept)avidin complexes are equivalent as the position of the benzoate oxygen atoms of HABA, which forms the hydrogen network with Asn-12, Ser-16 and Tyr-33 in avidin (Asn-23, Ser-27 and Tyr-43 in streptavidin) corresponding to the position of the ureido oxygen of biotin (**Figure 1-11** and **Figure 1-12**). Moreover, the involvement of the Phe-72 and Ser-73 amino-acid residues in the binding of HABA by avidin is highly reminiscent of their role in the binding of biotin, which is also considered to reflect the differences in affinity observed for forming the biotin⇌avidin and biotin⇌streptavidin complexes. But the consequences of such a difference in actual terms are much more significant for the low-affinity interaction with HABA compared to the high-affinity interaction with biotin.

Because biotin as well as HABA associate non-covalently at the same binding site of (strept)avidin, the HABA⇌(strept)avidin system can be used for the estimation of the degree of biotinylation of molecules by its displacement by the biotin moiety. Thereby, the displacement is accompanied by a change in colour from red to orange, thus allowing to assess, by the disappearance of the absorption maximum at $\lambda_{\text{max}} \sim 500\text{nm}$ and appearance of an absorption maximum at $\lambda_{\text{max}} \sim 350\text{nm}$ at the same time, the amount of biotin that has bound to (strept)avidin.¹¹⁵

The HABA⇌(strept)avidin was already used in e.g. structure-aided drug design,¹¹⁶ as a nonradioactive, chromogenic label system for detection, localization, isolation and

purification in bioanalytical applications¹¹⁷ and for showing the principle of signal transduction cascades.¹¹⁸

1.5. Applications of the Biotin-(Strept)Avidin Technology

The biotin-(strept)avidin technology is nowadays used in many research fields, especially in affinity chromatography, cytochemistry, immunoassays, diagnostics, biosensors and for targeted drug or metal isotopes delivery.^{2,3,41,119,120} **Figure 1-13** shows various applications of the biotin-(strept)avidin technology.

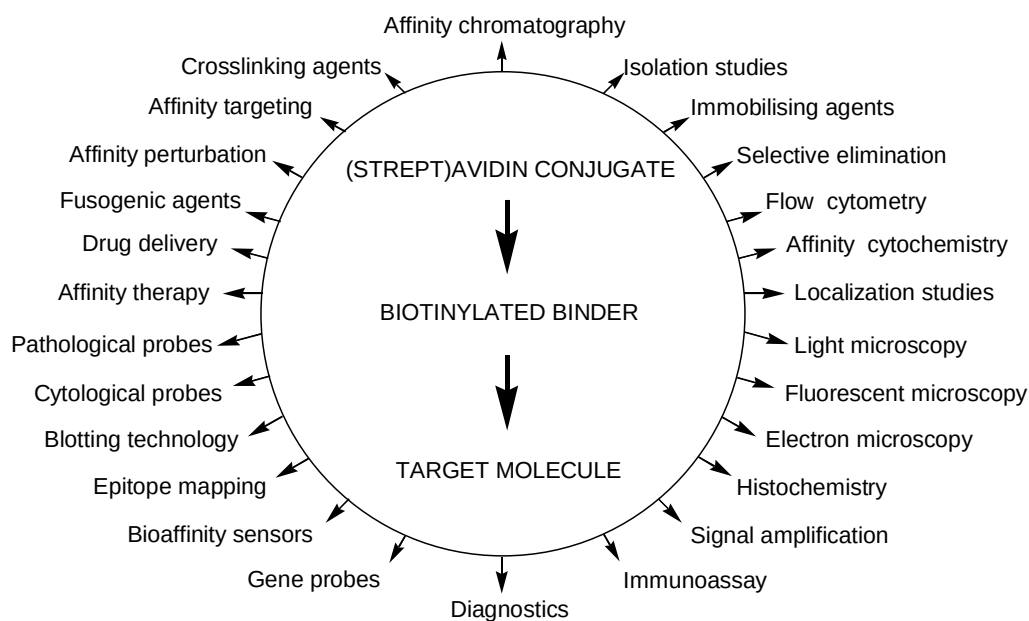


Figure 1-13. Applications of the biotin-(strept)avidin system. Sequential treatment of a target molecule with a biotinylated binder and a (strept)avidin probe has been used for a plethora of applications, many of which are listed.^{2,119,120}

Its general approach is outlined schematically in **Figure 1-14A**. An alternative mode is also available, as shown in **Figure 1-14B**. Having four biotin binding sites, avidin can be applied in native (unconjugated) form, together with a biotinylated probe. The two can be applied either sequentially in stepwise manner or in a single step as preformed complexes.

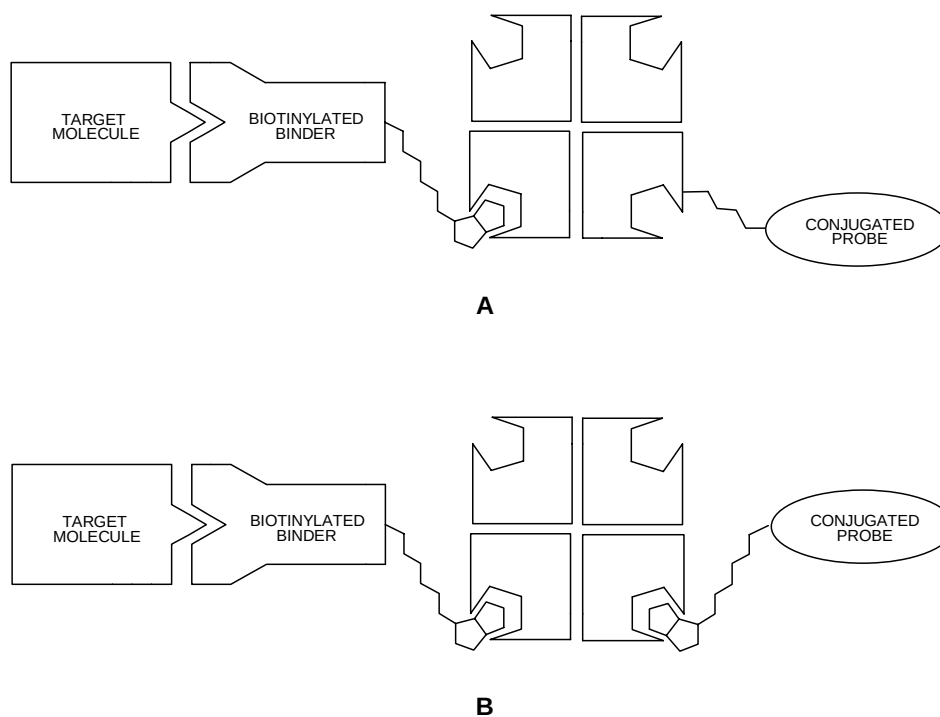


Figure 1-14 . The general approach in biotin-(strept)avidin technology. A target molecule in an experimental system is labelled with a biotinylated binder molecule. The biotinylated binder is in turn labelled with a (strept)avidin-conjugated probe (scheme **A**). Alternatively, the biotinylated binder can be labelled with free (strept)avidin followed by a biotinylated probe (scheme **B**).^{119,120}

A list of various biologically active pairs, target and binder molecules, is presented in **Table 1-4** and diverse types of (strept)avidin-conjugated or biotinylated probes is given in **Table 1-5**.

Target	Binder
antigens	antibodies
antibodies	antigens
lectins	glycoconjugates
glycoconjugates	lectins
enzymes	substrates, cofactors, inhibitors, etc.
receptors	hormones, effectors, toxins, etc.
transport proteins	vitamins, amino acids, sugars, etc.
hydrophobic sites	lipids, fatty acids
membranes	liposomes
nucleic acids, genes	DNA/RNA probes
phages, viruses, bacteria, subcellular organelles, cells, tissues, whole organisms	all of the above

Table 1-4. Target-binder pairs for application in biotin-(strept)avidin technology.^{119,120}

Probes (Conjugates)
enzymes
radiolabels
fluorescent agents
chemiluminescent agents
chromophores
heavy metals
colloidal gold
ferritin, hemocyanine
phages
macromolecular carriers
liposomes
solid supports

Table 1-5. Commonly used (strept)avidin-containing probes.^{119,120}

1.6. CD Spectroscopy as a Powerful Tool

Circular Dichroism (CD) is nowadays more widely used to study biological systems than non-biological systems. This despite the fact that the molecules concerned are usually very large and therefore detailed structural analysis of their CD data is not yet possible.

The CD of biological macromolecules is most commonly used in one of two ways:

- Investigation of changes in the conformation of the macromolecules itself.
- Investigation of their interactions with small molecules, especially achiral ones whose **induced CD** is due solely to their interaction with the chiral macromolecule.

There are many advantages of CD as a probe for biological molecules:

- CD experiments are simple and quick to perform.
- CD is uniquely sensitive to the dissymmetry (chirality) of a system.
- CD experiments are performed in the solution phase. This is important for biological systems because the crystallization process can change the structure of a molecule.
- CD experiments require significantly lower concentrations than are appropriate for NMR. This means that CD do not only require less sample but it can be used for systems, where the concentrations required for NMR may change the properties of the system.
- The intrinsic timescale of a CD measurement is defined by the energy of the photons used (10^{-15} s for the absorption of a UV photon). It is thus much shorter than that for NMR, which uses radio waves (10^{-9} s). This difference may be of relevance for ligand-macromolecule interaction studies if the ligand has a particularly short residence time in a given orientation.
- In general, both NMR and crystallography give detailed structural information only for small proteins (molecular weight < 20000 daltons) and nucleic acids (~ 24 bases), whereas CD can be used for any size of molecule.

As proteins are chiral macromolecules (all amino-acid residues except glycine are chiral), CD spectra show, in the region between 190nm and 300nm, CD signals. The region between 250nm and 300nm (near UV) is described as the aromatic region, though transitions of disulphide bonds (cysteines) also contribute to the total CD intensity in this region. The region below 250nm (far UV) is dominated by transitions of the peptide backbone of the protein, but transitions from some side chains also contribute to this region. The side chains of

phenylalanine, tyrosine, tryptophan and histidine have transitions in the region between 250nm and 300nm. Since each subunit of avidin (streptavidin) contains four (six) tryptophan residues and one (six) tyrosine residues, taking into account that the tryptophan residues have by far the most intense transitions in this region, the CD spectra (UV spectra) are dominated by the contribution of the tryptophan residues, hence the CD band (UV absorbance) between 250 and 300nm comes mostly from the tryptophan residues.^{121,122}

1.7. Goal of this Work

Green and co-workers¹ have shown that modifications at the valeryl side chain have much less influence towards the binding properties between biotinylated conjugates and (strept)avidin than modifying the imidazolidone ring of biotin.

Based on this, an application field of the biotin-(strept)avidin technology was developed in the past three years in our group using modified biotin-(strept)avidin systems for:

- a) enantioselective hydrogenation catalysis (**Chapter 2**)
- b) photosystem II analogues (**Chapter 3**)

In a), incorporation of biotinylated achiral transition metal catalysts [Biot-spacer-M]⁺ (M = transition metal) in (strept)avidin ensures that enantioselection is induced by the second coordination sphere provided by (strept)avidin. In b), it was expected that incorporation of a prototypical biotinylated coordination complex [Biot-spacer-M]²⁺ (M = transition metal) in a (strept)avidin microenvironment would influence its excited state lifetime properties.

Ultimately, it may be interesting to exploit a donor/acceptor system in a protein environment, similar to the photosynthetic reaction center.

Goals of this work are the following:

- 1) Determine thermodynamic data, which lays the basis for the work on artificial metalloenzymes (**Chapter 2**)
- 2) Study the effect of the second coordination sphere on the photophysical properties of a prototypical coordination complex $[\text{Biot-spacer-M}]^{2+} \subset (\text{strept})\text{avidin}$, including thermodynamic and kinetic data (**Chapter 3**)
- 3) Investigate the chiral discrimination properties of (strept)avidin towards a biotinylated enantiopure probe $[\text{Biot-spacer-M}]^{2+}$ (**Chapter 3**)
- 4) Study the cooperativity in the $[\text{Biot-spacer-M}]^{2+} \subset (\text{strept})\text{avidin}$ system (**Chapter 3**)

In order to determine the incorporation of biotinylated organometallic species in (strept)avidin, we rely on a direct and an indirect titration method by using CD spectroscopy. The direct method on the one hand was in the case of $[\text{Biot-spacer-M}]^{2+}$ directly based on the appearance of an induced CD signal upon addition of the biotinylated metal complex $[\text{Biot-spacer-M}]^{2+}$ to (strept)avidin. The indirect method on the other hand consists of a displacement reaction with HABA (following the disappearance of its induced CD band). The derived average stability constant (the same for all four binding sites) is the product of the stability constant K_{HABA} and the exchange constant between HABA and $[\text{Biot-spacer-M}]^+$ or $[\text{Biot-spacer-M}]^{2+}$. The spectrophotometric data were fitted by applying different programs and models. All of them based on the law of mass action, proposed first in 1864 by the two Norwegian chemists Cato Guldberg and Peter Waage. Thereby, the programs and models

used the balance equations of the components and calculated the stability constants of the absorbing species.

The exchange procedure mentioned above is a commonly used method either for the determination of the degree of biotinylation (number of binding sites) of (strept)avidin or other biotin-binding molecules^{30,52,115,117,123} or for a rough estimation of affinity constants,^{58,63,77} though there are doubts about the exactitude of this method. On one hand, Zenobi and co-workers¹²⁴ proposed a method based on matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry. In this study, it was found that for biotinylated proteins the label ratios obtained by the HABA-avidin method were much smaller than those determined by MALDI mass spectrometry. On the other hand, Bachas and co-workers¹²⁵ developed a method, where the detection of the extent of the protein biotinylation based on a fluorescence binding assay. That one was determined by complete acid hydrolysis or by enzymatic digestion using proteinase K to release biotin from the biotinylated proteins, followed by sensitive detection of biotin using a coupled HPLC-binding assay system. It was found that this method determines the amount of liberated biotin after hydrolysis and suffers therefore not from steric hindrance problems associated with the ability of biotin on a protein surface to displace HABA from (strept)avidin. But this is especially relevant for highly biotinylated proteins and not for (strept)avidin.

Dimidium facti, qui coepit, habet.

Quintus Horatius Flaccus, 65 BC – 8 BC

CHAPTER 2:

RESULTS AND DISCUSSION

The $[\text{Rh}(\text{COD})(\text{Biot-NP}_2)]^+ \subset (\text{Strept})\text{Avidin}$ System

2.1. General Considerations

As suggested by Hofmann and co-workers,¹²⁶ the affinity of N⁶-iminobiotinyllysineamide for avidin is significantly lower than that of neutral iminobiotinylated amino acids. This unusual behaviour was traced to the repulsion between the positively charged avidin host at neutral pH and the positively charged iminobiotinylated guest. It was therefore suspected that the charge of the biotinylated cationic achiral catalyst precursor may significantly influence the affinity of $[\text{Biot-spacer-M}]^+ \subset (\text{strept})\text{avidin}$, depending on the isoelectric point of the host protein ($\text{strept})\text{avidin}$ ($\text{pI}_{\text{avidin}} = 10.4$ vs $\text{pI}_{\text{streptavidin}} = 6.4$).

Moreover, to the best of our knowledge, most of the thermodynamic data was compiled for biotinylated systems incorporating long spacers between biotin and the biotinylated probe. It was suspected that incorporation of a large biotinylated cationic organometallic moiety with only a short spacer within $(\text{strept})\text{avidin}$ may significantly affect the affinity of such $[\text{Biot-spacer-M}]^+ \subset (\text{strept})\text{avidin}$ supramolecular assemblies. For the reasons mentioned above, the thermodynamic data of the $[\text{Rh}(\text{COD})(\text{Biot-NP}_2)]^+ \subset (\text{strept})\text{avidin}$ system was investigated. This rhodium-diphosphine moiety lays the basis for the work on artificial metalloenzymes in the group.

2.2. Synthesis of $[\text{Rh}(\text{COD})(\text{Biot-NP}_2)]^+$

The synthesis of $[\text{Rh}(\text{COD})(\text{Biot-NP}_2)]^+$ (COD = cyclooctadiene) was carried out using Whitesides' procedure.¹²⁷ The first step consists in the formation of the activated ester **BiotNHS** (**Figure 2-1**). This reaction was performed in DMF at room temperature to afford the desired **BiotNHS** product in 70% yield.

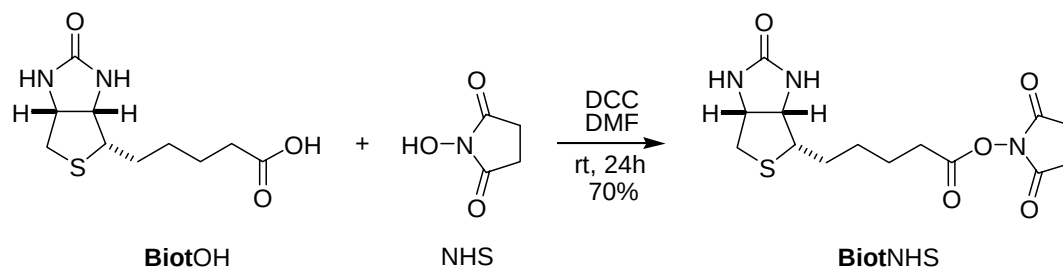


Figure 2-1. Synthesis of the activated ester of biotin **BiotNHS**.

The coupling of the activated ester with bis-(2-diphenylphosphanyl-ethyl)-amide **H-NP₂** was also performed in DMF at room temperature. The diphosphine amide **Biot-NP₂** was isolated by column chromatography as a white foam in 67% yield (**Figure 2-2**).

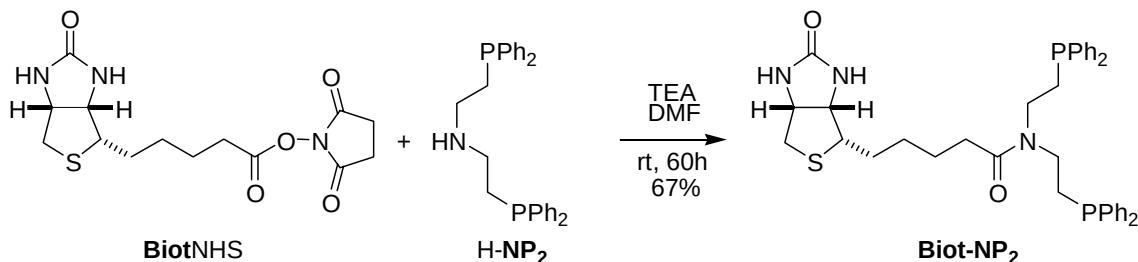


Figure 2-2. Synthesis of the biotinylated ligand **Biot-NP₂**.

The synthesis of the air-sensitive rhodium complex $[\text{Rh}(\text{COD})(\text{Biot-NP}_2)]^+$ was performed *in situ* under a nitrogen atmosphere using a glove box. The metal source was $[\text{Rh}(\text{COD})_2]\text{BF}_4$ (complex was synthesized within the research group). This reaction step was assumed to be quantitative (**Figure 2-3**), and the resulting product was used without purification.

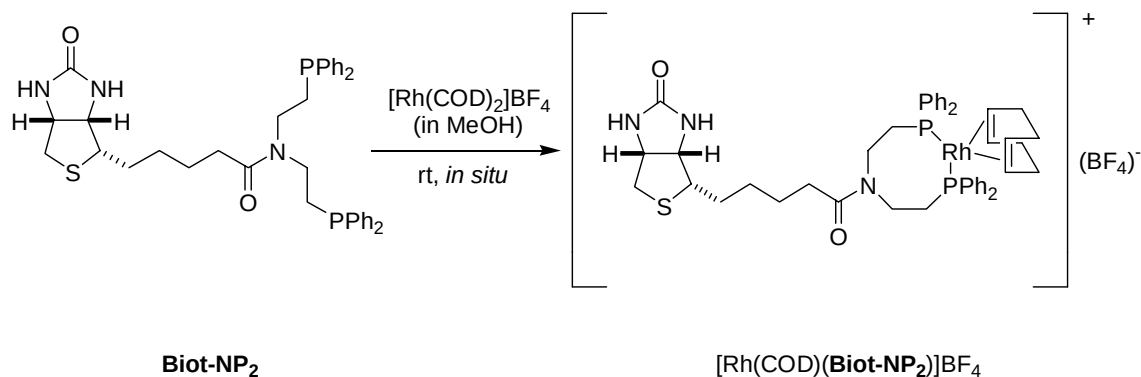


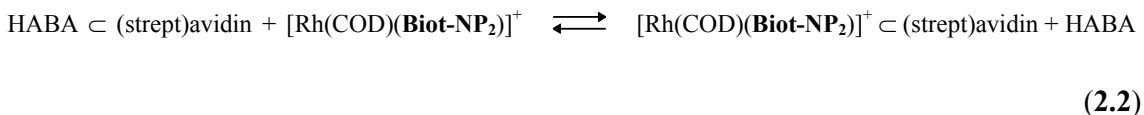
Figure 2-3. Synthesis of the air sensitive rhodium complex $[\text{Rh}(\text{COD})(\text{Biot-NP}_2)]^+$ using $[\text{Rh}(\text{COD})_2]\text{BF}_4$ as metal source.

The ratio of $[\text{Rh}(\text{COD})_2]\text{BF}_4$ to the biotinylated ligand for the formation of the $[\text{Rh}(\text{COD})(\text{Biot-NP}_2)]^+$ complex was 1:1. The volume of MeOH was adjusted to obtain a desired final working concentration of 0.96mM for the following CD measurements.

2.3. Titration Measurement and Determination of

$$K_{[\text{Rh}(\text{COD})(\text{Biot-}\mathbf{NP}_2)]^+ \subset (\text{strept})\text{avidin}}$$

Because $[\text{Rh}(\text{COD})(\text{Biot-}\mathbf{NP}_2)]^+ \subset (\text{strept})\text{avidin}$ does not display an induced CD signal to exploit in the determination of the stability constant, the determination of this association constant was carried out using an exchange procedure with 2-(4'-hydroxyazobenzene)benzoic acid (HABA). To this end, the host proteins wild type avidin (**WT Avi**) and wild type streptavidin (**WT Sav**) was initially loaded with a large excess of HABA in order to ensure nearly complete saturation of the biotin sites (**Equation 2.1**). Both UV/Vis ($\lambda_{\text{max}} = 495\text{nm}$) and CD ($\lambda_{\text{max}} = 506\text{nm}$) spectra of the guest $\subset$ host system HABA $\subset$ (strept)avidin display an absorption or an induced signal coming from a charge transfer between the indole ring of tryptophan (Trp-70 in avidin and Trp-79 in streptavidin, respectively, acting as donor) and the hydroxyphenyl ring (acting as acceptor) of HABA.⁹⁸ Addition of $[\text{Rh}(\text{COD})(\text{Biot-}\mathbf{NP}_2)]^+$ aliquots causes a decrease of both UV/Vis and CD signal of HABA $\subset$ (strept)avidin, allowing the determination of the stability constant K_{exch} for the displacement equilibrium (**Equation 2.2**).



$$K_{\text{HABA}} = \frac{[\text{HABA} \subset (\text{strept})\text{avidin}]}{[\text{HABA}] \cdot [(\text{strept})\text{avidin}]} \quad (2.3)$$

$$K_{\text{exch}} = \frac{[[\text{Rh}(\text{COD})(\text{Biot-}\mathbf{NP}_2)]^+ \subset (\text{strept})\text{avidin}] \cdot [\text{HABA}]}{[\text{HABA} \subset (\text{strept})\text{avidin}] \cdot [[\text{Rh}(\text{COD})(\text{Biot-}\mathbf{NP}_2)]^+]} \quad (2.4)$$

$$K_{[\text{Rh}(\text{COD})(\text{Biot}-\text{NP}_2)]^+ \subset (\text{strept})\text{avidin}} = \frac{[[\text{Rh}(\text{COD})(\text{Biot}-\text{NP}_2)]^+ \subset (\text{strept})\text{avidin}]}{[[\text{Rh}(\text{COD})(\text{Biot}-\text{NP}_2)]^+] \cdot [(\text{strept})\text{avidin}]} = K_{\text{HABA}} \cdot K_{\text{exch}} \quad (2.5)$$

Since the achiral biotinylated rhodium-diphosphine complex is not water-soluble, it is usually dissolved in DMSO (for catalysis) and added to the buffered solution containing the substrate and the host protein. During the CD measurement for the determination of the stability constant of the displacement equilibrium it was observed that the solvent influences significantly the first step of the two-step procedure (**Equation 2.1**). This was manifested by a decrease of the CD band ($\lambda_{\text{max}} = 506\text{nm}$) upon addition of solvent aliquots devoid of $[\text{Rh}(\text{COD})(\text{Biot}-\text{NP}_2)]^+$, which varied depending on the type of solvent used (**Figure 2-4**).

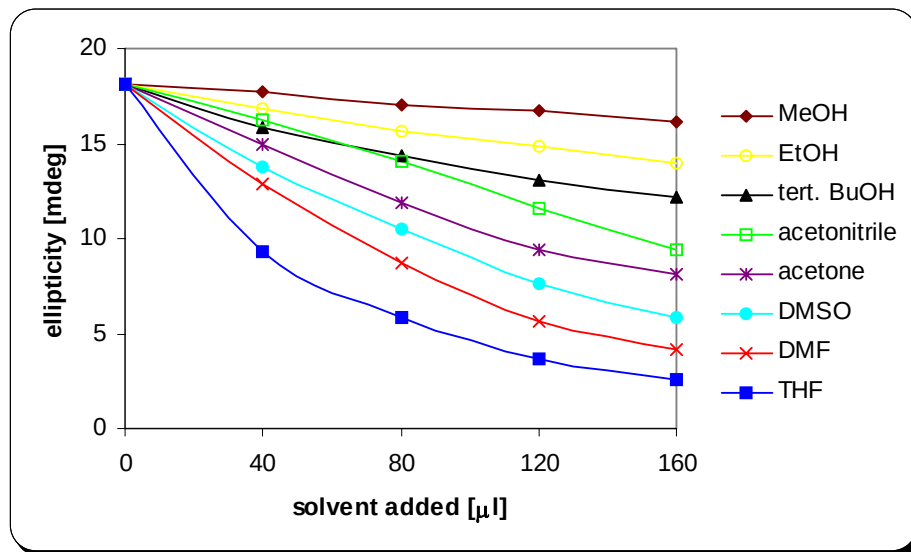


Figure 2-4. Influence of the solvent on the CD band of HABA-protein (here WT Avi) at the absorption maximum ($\lambda_{\text{max}} = 506\text{nm}$). Initial protein concentration: $7.87\mu\text{M}$; initial HABA concentration: $157.38\mu\text{M}$; initial volume: $2400\mu\text{L}$.

Of all solvents tested, DMSO has one of the most significant influence on the CD signal of HABA $\subset$ protein. Green has shown that a number of organic compounds bind weakly to avidin (e.g. THF whose $\log(K_{\text{THF}})$ value is 2.0).^{1,14} It is thus probable that HABA is displaced by e.g. THF, DMF and DMSO. The influence of alcohols was significantly smaller than of e.g. THF, DMF and DMSO. MeOH turned out to be the solvent, which influences least the CD signal and was thus used for dissolving the catalyst precursors for the stability constant determination.

For the determination of the stability constant K_{HABA} two different programs were used. On one hand, the commercial program PSEQUAD¹²⁸ (section 5.4.2.) and on the other hand the so-called “monomer-like” model, which was implemented in Excel by J. Sauser¹²⁹ (section 5.4.1.). It was assumed for both models that the four binding events are independent (non-cooperative) from each other.

The stability constant K_{HABA} (**Equation 2.3**) for WT Avi ($\log(K_{\text{HABA}}) = 5.5$; $R^2 = 0.998$) and WT Sav ($\log(K_{\text{HABA}}) = 3.5$; $R^2 = 0.999$) was determined by following the appearance of the visible band centered at 506nm caused by the HABA $\subset$ (strept)avidin interaction. The $\log(K_{\text{HABA}})$ values of 5.5 for WT Avi and 3.5 for WT Sav are in good agreement with other measurements giving $\log(K_{\text{HABA}})$ values of 5.2 (Green)^{3,115} and 5.3 (Srivastava and co-workers)¹³⁰ for avidin and 4.0 (Green)³ for streptavidin (**Table 2-1**). The calculated and measured curves for the determination of the formation constant K_{HABA} for HABA $\subset$ (strept)avidin are depicted in **Figure 2-5**.

With help of these values, the stability constant $K_{[\text{Rh}(\text{COD})(\text{Biot-}\mathbf{NP}_2)]^+\subset(\text{strept})\text{avidin}}$ was determined according to **Equation 2.5**. Using the known total concentrations $[\text{HABA}]_{\text{total}}$, $[(\text{strept})\text{avidin}]_{\text{total}}$ and $[[\text{Rh}(\text{COD})(\text{Biot-}\mathbf{NP}_2)]^+]_{\text{total}}$, and rearranging **Equation 2.4**, one can

derive a single quadratic equation depending only on $[\text{HABA} \subset (\text{strept})\text{avidin}]$. This latter concentration is proportional to the signal centered at 506nm (**Equation 2.6**).

$$(1 - K_{\text{exch}}) \cdot [\text{HABA} \subset (\text{strept})\text{avidin}]^2 + (K_{\text{exch}} \cdot \{[(\text{strept})\text{avidin}]_{\text{total}} - [[\text{Rh}(\text{COD})(\text{Biot} - \text{NP}_2)]^+]_{\text{total}}\} - [\text{HABA}]_{\text{total}} - [(\text{strept})\text{avidin}]_{\text{total}}) \cdot [\text{HABA} \subset (\text{strept})\text{avidin}] + [\text{HABA}]_{\text{total}} \cdot [(\text{strept})\text{avidin}]_{\text{total}} = 0 \quad (2.6)$$

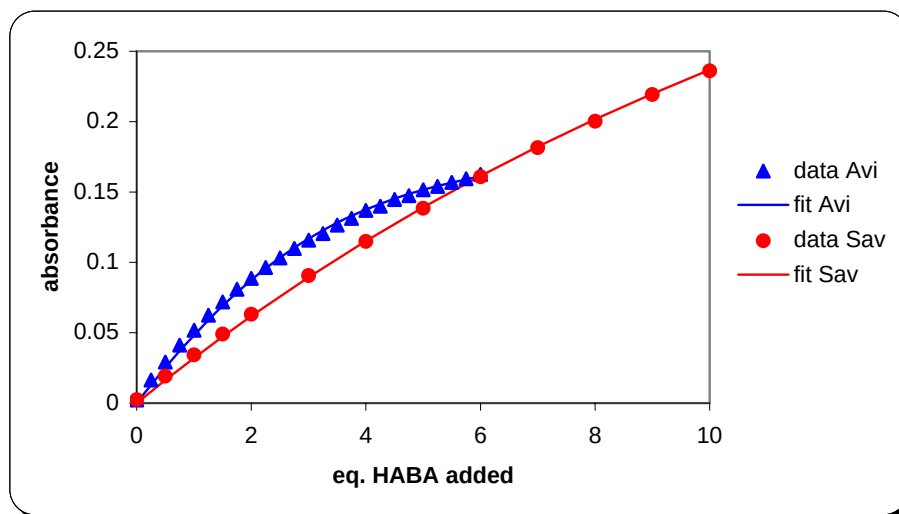


Figure 2-5. Appearance of an absorbance band ($\lambda_{\text{max}} = 506\text{nm}$) upon addition of HABA aliquots (0.96mM) to (strept)avidin allows the determination of K_{HABA} for $\text{HABA} \subset (\text{strept})\text{avidin}$ (data Avi (Sav) = measured points for WT (strept)avidin; fit Avi (Sav) = calculated points for WT (strept)avidin using PSEQUAD or the “monomer-like” model). Initial protein concentration: 8.00 μM ; solvent: phosphate buffer (pH = 7.0, I = 0.3M).

For a given value of the fitting parameter K_{exch} , $[\text{HABA} \subset (\text{strept})\text{avidin}]$, and therefore the corresponding theoretical titration curve, is calculated and a least-squares minimization is performed until the sum on all data points of $(\text{signal}_{\text{calculated}} - \text{signal}_{\text{measured}})^2$ reaches a minimum.

The calculated and the measured curves for the displacement equilibrium are shown in **Figure 2-6**. The obtained $\log(K_{\text{exch}})$ values are 2.2 ($R^2 = 0.997$) for WT Avi and 3.7 ($R^2 = 0.994$) for WT Sav. The quality of the fit for WT Sav is not as good as that for WT Avi. It is probably due to slightly cooperative effects between the four binding sites (**Chapter 3**), hence cooperativity upon binding for WT Sav can not be excluded completely. In contrary, the measured and fitted data points of WT Avi are in good agreement with each other.

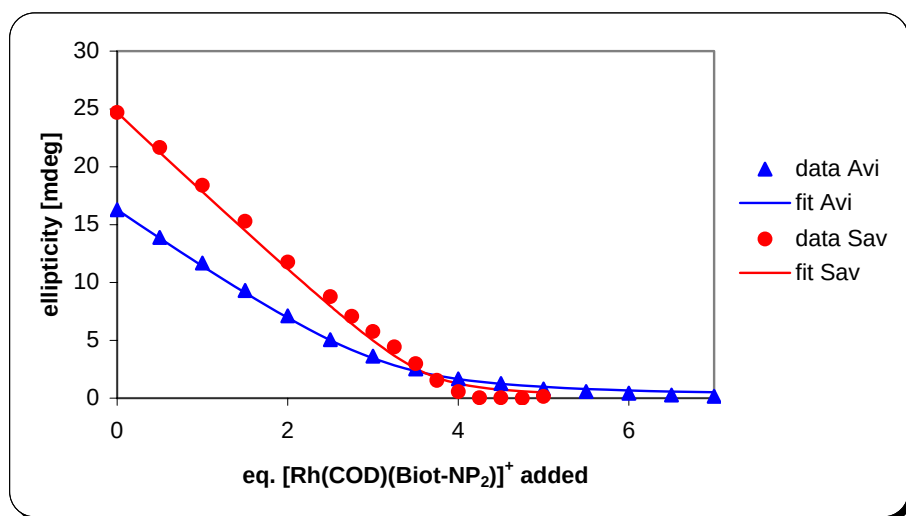


Figure 2-6. Disappearance of a CD band ($\lambda_{\text{max}} = 506\text{nm}$) upon addition of $[\text{Rh}(\text{COD})(\text{Biot-NP}_2)]^+$ aliquots (0.96mM) to $\text{HABA} \subset (\text{strept})\text{avidin}$ allows the determination of the K_{exch} between HABA and $[\text{Rh}(\text{COD})(\text{Biot-NP}_2)]^+$ (data Avi (Sav) = measured points for WT (strept)avidin; fit Avi (Sav) = calculated points for WT (strept)avidin using the two-step procedure with HABA). Initial protein concentration: 7.87 μM for Avi and 7.11 μM for Sav; initial HABA concentration: 157.38 μM (in the case of Avi) and 1.07mM (in the case of Sav); solvent: phosphate buffer (pH = 7.0, $I = 0.3M$).

The determination of the stability constant was also performed for the recombinant glycosylated avidin **r-GAvi** whose isoelectric point is significantly lower than that of avidin ($\text{pI} = 5.4$ for r-GAvi vs $\text{pI} = 10.4$ for WT Avi).¹³¹ Compared to the wild type avidin WT Avi, r-GAvi contains the Lys-3 $\rightarrow$ Glu-3, Lys-9 $\rightarrow$ Asp-9, Arg-122 $\rightarrow$ Ala-122 and Arg-124 $\rightarrow$

Ala-124 mutations¹³² as well as three additional amino-acid residues (Glu-Ala-Glu) at its N-terminus. In this way, it is not only possible to compare the stability constants of WT Avi and WT Sav, but also to study the difference of the formation constant between the WT Avi and the recombinant avidin r-GAvi and to explore the influence of the pI value on the association constant K_a . For the determination of the overall stability constant for $[\text{Rh}(\text{COD})(\text{Biot-}\mathbf{NP}_2)]^+\text{-r-GAvi}$, the same two-step procedure was applied (**Figure 2-7** and **Figure 2-8**).

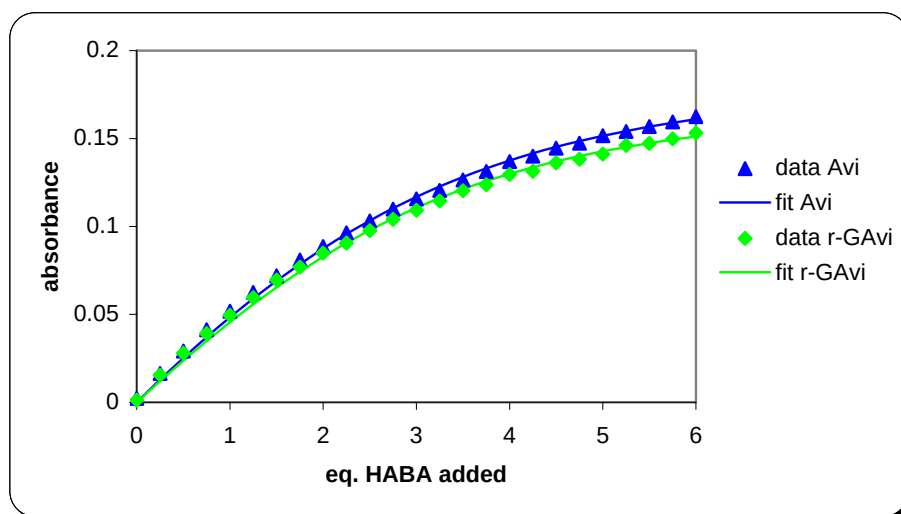


Figure 2-7. Appearance of an absorbance band ($\lambda_{\text{max}} = 506\text{nm}$) upon addition of HABA aliquots (0.96mM) to WT Avi and r-GAvi allows the determination of the K_{HABA} for $\text{HABA} \subset \text{avidin}$ (WT Avi & r-GAvi) (data Avi (r-GAvi) = measured points for WT avidin (r-GAvi); fit Avi (r-GAvi) = calculated points for WT avidin (r-GAvi) using PSEQUAD or the “monomer-like” model). Initial protein concentration: $8.00\mu\text{M}$; solvent: phosphate buffer ($\text{pH} = 7.0$, $I = 0.3M$).

The $\log(K_{\text{HABA}})$ and $\log(K_{\text{exch}})$ values are very similar ($\log(K_{\text{HABA}}) = 5.5$ ($R^2 = 0.998$) for WT Avi vs $\log(K_{\text{HABA}}) = 5.6$ ($R^2 = 0.998$) for r-GAvi and $\log(K_{\text{exch}}) = 2.2$ ($R^2 = 0.997$) for WT Avi vs $\log(K_{\text{exch}}) = 2.1$ ($R^2 = 0.992$) for r-GAvi) though the fit of r-GAvi is much less satisfactory than for WT Avi. The change of pI has maybe an influence on structural changes

but without affecting the affinity of $[\text{Rh}(\text{COD})(\text{Biot-NP}_2)]^+$. An overview of all determined stability constants is presented in **Table 2-1**. All obtained stability constants ($\log(K_a)$ values) are average values (the same for all four binding sites) over three runs.

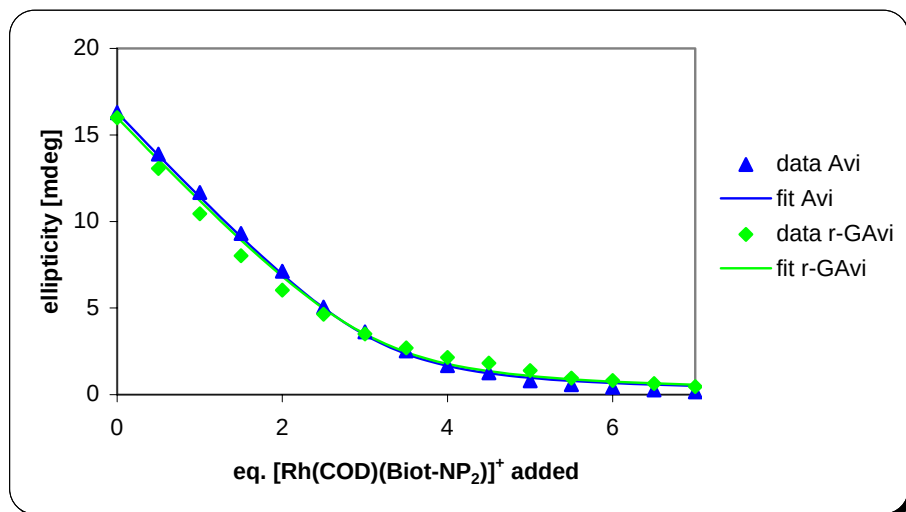


Figure 2-8. Disappearance of a CD band ($\lambda_{\text{max}} = 506\text{nm}$) upon addition of $[\text{Rh}(\text{COD})(\text{Biot-NP}_2)]^+$ aliquots (0.96mM) to HABA-avidin (WT Avi & r-GAvi) allows the determination of the K_{exch} between HABA and $[\text{Rh}(\text{COD})(\text{Biot-NP}_2)]^+$ (data Avi (r-GAvi) = measured points for WT avidin (r-GAvi); fit Avi (r-GAvi) = calculated points for WT avidin (r-GAvi) using the two-step procedure with HABA). Initial protein concentration: $7.87\mu\text{M}$; initial HABA concentration: $157.38\mu\text{M}$; solvent: phosphate buffer ($\text{pH} = 7.0$, $I = 0.3M$).

From these stability constant determinations, which were all performed in phosphate buffer ($\text{pH} = 7.0$, $I = 0.3M$), it can be concluded that the affinity of $[\text{Rh}(\text{COD})(\text{Biot-NP}_2)]^+$ for the considered host proteins is independent of the isoelectric point of the protein and essentially constant: $\log(K_{[\text{Rh}(\text{COD})(\text{Biot-NP}_2)]^+ \subset \text{WT Avi}}) = 7.7$, $\log(K_{[\text{Rh}(\text{COD})(\text{Biot-NP}_2)]^+ \subset \text{WT Sav}}) = 7.2$ and $\log(K_{[\text{Rh}(\text{COD})(\text{Biot-NP}_2)]^+ \subset \text{r-GAvi}}) = 7.7$. Thus, at a given pH, the affinity of a cationic catalyst precursor for a protein does not depend on the isoelectric point of the host protein.

	WT Avi	WT Sav	r-GAvi	Model
$\log(K_{\text{HABA}})$	5.5 ($R^2=0.998$)	3.5 ($R^2=0.999$)	5.6 ($R^2=0.998$)	“monomer-like” model ^c
$\log(K_{\text{HABA}})$	5.4 ($\delta = \pm 0.01$)	3.4 ($\delta = \pm 0.01$)	5.3 ($\delta = \pm 0.02$)	PSEQUAD ^c
$\log(K_{\text{HABA}})$	5.2	4.0	—	literature ^a
$\log(K_{\text{HABA}})$	5.3	—	—	literature ^b
$\log(K_{\text{exch}})$	2.2 ($R^2=0.997$)	3.7 ($R^2=0.994$)	2.1 ($R^2=0.992$)	“monomer-like” model ^c
$\log(K_{[\text{Rh}(\text{COD})(\text{Biot-}\mathbf{NP}_2)]^+})$	7.7	7.2	7.7	“monomer-like” model ^c

Table 2-1. Overview of all determined stability constants ($\log(K_a)$ values) for WT Avi, WT Sav and r-GAvi: $\log(K_{\text{HABA}})$ (“monomer-like” model or PSEQUAD) and $\log(K_{\text{exch}})$ (“monomer-like” model) are obtained from each single step of the two-step procedure and $\log(K_{[\text{Rh}(\text{COD})(\text{Biot-}\mathbf{NP}_2)]^+})$ (WT Avi, WT Sav, r-GAvi) (“monomer-like” model) by taking the sum of the $\log(K_{\text{HABA}})$ and $\log(K_{\text{exch}})$ values.

^a N. M. Green^{3,115} ^b D. K. Srivastava and co-workers¹³⁰

^c T. R. Ward and co-workers, *J. Am. Chem. Soc.* **2004**, 126, 14411-14418

Therefore, all considered host proteins are suitable for the enantioselective hydrogenation of prochiral olefins catalysed by $[\text{Rh}(\text{COD})(\text{Biot-}\mathbf{NP}_2)]^+$. The difference in performance is not caused by a difference in affinity, but, most likely, it is caused by a difference in the host protein’s binding pocket topography.

Quod erat demonstrandum!

Euklid, around 300 BC

CHAPTER 3:

RESULTS AND DISCUSSION

The $[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+} \subset (\text{Strept})\text{Avidin}$ System

3.1. General Considerations

The tris-2,2'-bipyridine complexes of ruthenium(II) ($[\text{Ru}(\text{bpy})_3]^{2+}$) have become one of the most widely studied transition metal species due to their unique photophysical behaviour. It is these properties that have led to their application in supramolecular chemistry, both as a luminophore in molecular sensors and as photoactive center in light induced charge-separated species (e.g. in photosynthesis). In the generation of long-lived charge-separated species, distance, relative orientation and the molecular structure that separates the donor group from the acceptor moiety largely influence the feasibility of intramolecular electron- and energy-transfer processes. Nature uses proteins as second coordination sphere ligands (hosts) to set up, orient and interface molecular components (guests). As a model, we focus on $[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+} \subset (\text{strept})\text{avidin}$.

3.2. Synthesis of $[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$ (*rac*-, Δ - and Λ -Form)

3.2.1. Ligand Synthesis

The general structure of the $[\text{Ru}(\text{bpy})_2(\text{Biot-spacer-bpy})]^{2+}$ complex consists of three moieties, an octahedral tris-chelated ruthenium complex, which is connected through a spacer arm from one of the bipyridine moieties to the biotin. A schematic representation of the structure of $[\text{Ru}(\text{bpy})_2(\text{Biot-spacer-bpy})]^{2+}$ is shown in **Figure 3-1**.

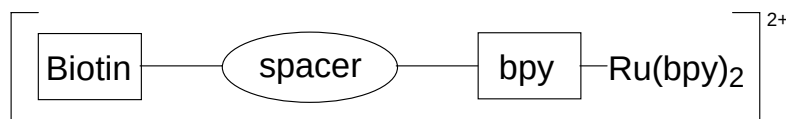


Figure 3-1. Schematic representation of the structure of $[\text{Ru}(\text{bpy})_2(\text{Biot-spacer-bpy})]^{2+}$. The spacer arm can be an aliphatic or an aromatic diamine, which is linked through an amide on both sides.

The synthesis of the $[\text{Ru}(\text{bpy})_2(\text{Biot-spacer-bpy})]^{2+}$ complex was performed in several steps. The **bpyOEt** was synthesized by refluxing freshly distilled ethyl nicotinate and Raney nickel in xylene during five days.^{133,134} The yield of this reaction step was 29% (**Figure 3-2**).

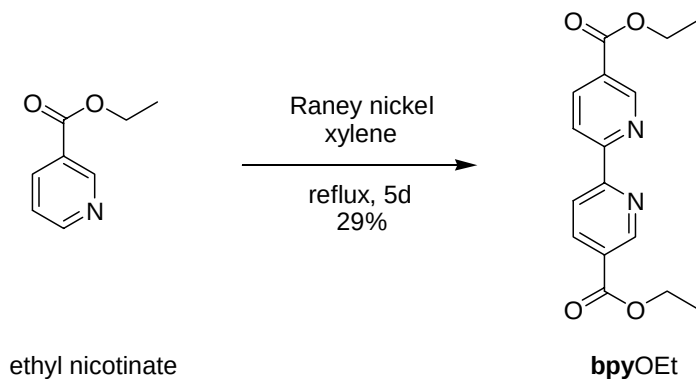


Figure 3-2. Synthesis of **bpyOEt**.

Other possibilities to synthesize **bpyOEt** would be either the use of another catalyst, refluxing freshly distilled ethyl nicotinate and 10% Pd/C under reduced pressure (16mmHg) for six days,¹³⁵ or oxidation of dimethyl-2,2'-bipyridyl-5,5'-bipyridine, following acidification with hydrochloric acid and reflux with ethanol in the presence of concentrated sulphuric acid.¹³⁶

The acid chloride **bpyCl** itself is a good activated species for further coupling, e.g. amide coupling, but the yields of the resulting amide were very variable. Moreover, it is sensitive to water and therefore not very stable for storage over a longer period of time. The activated pentafluorophenylester **bpyOC₆F₅** is a good reagent for peptide coupling (amide coupling). Therefore **bpyOC₆F₅** was prepared via its acid chloride **bpyCl**.¹³⁴ The intermediate product **bpyCl** was directly used without any characterization for the following step. The reaction scheme is shown in **Figure 3-3**.

The activation of the biotin moiety was achieved in the same way as **bpyOC₆F₅**, hence via pentafluorophenylester. The reaction was performed in DMF during 3h at a reaction temperature of about 65-70°C giving the biotinylated pentafluorophenylester **BiotOC₆F₅** (**Figure 3-4**).¹³⁷

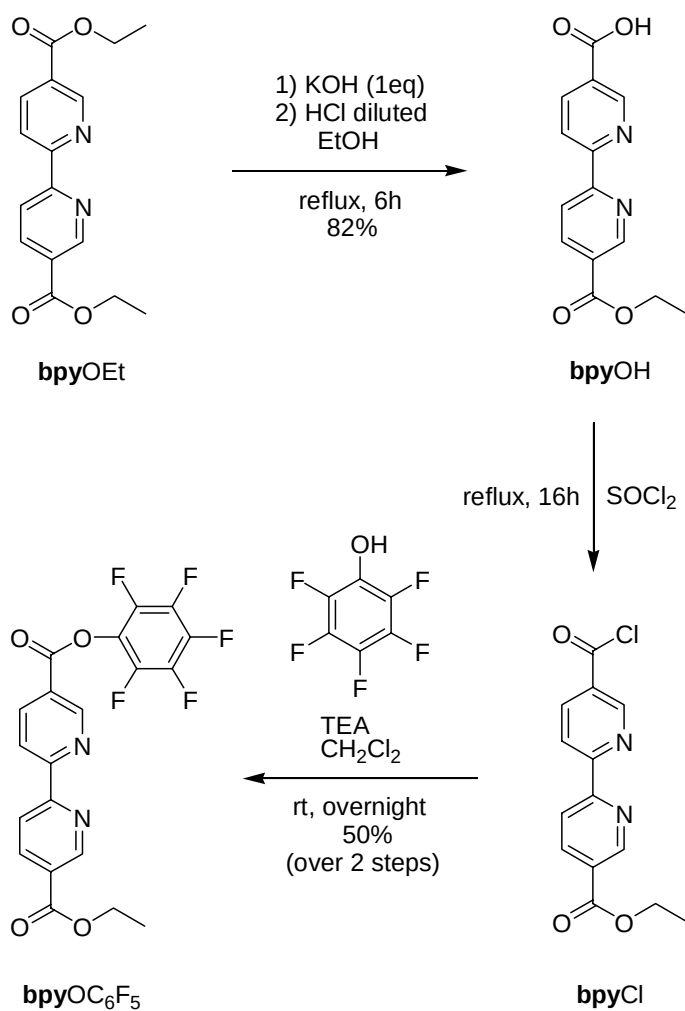


Figure 3-3. Synthesis of the activated pentafluorophenylester **bpyOC₆F₅**.

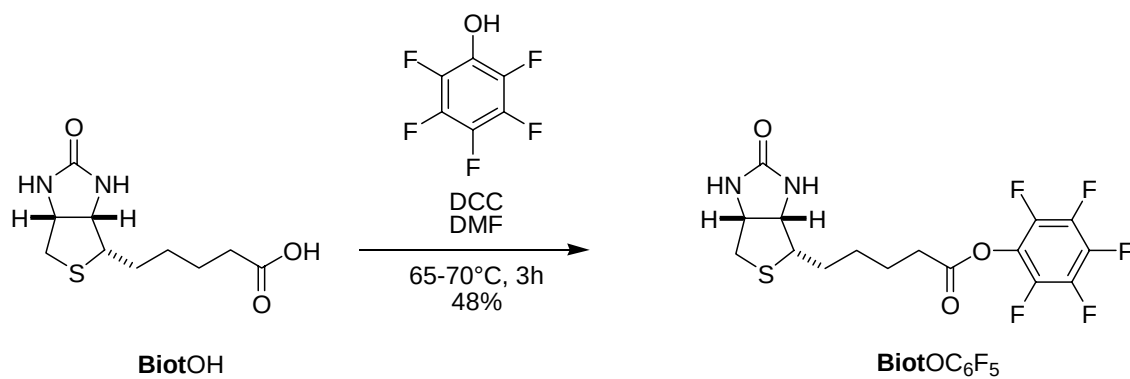


Figure 3-4. Synthesis of the activated pentafluorophenylester **BiotOC₆F₅** of biotin.

The next step was the synthesis of the biotinylated amines **Biot-1ⁿ** and **Biot-2^q** by coupling one side of the diamine **1ⁿ** or **2^q** (different **spacers**) through an amide bond to biotin (**Figure 3-5**).

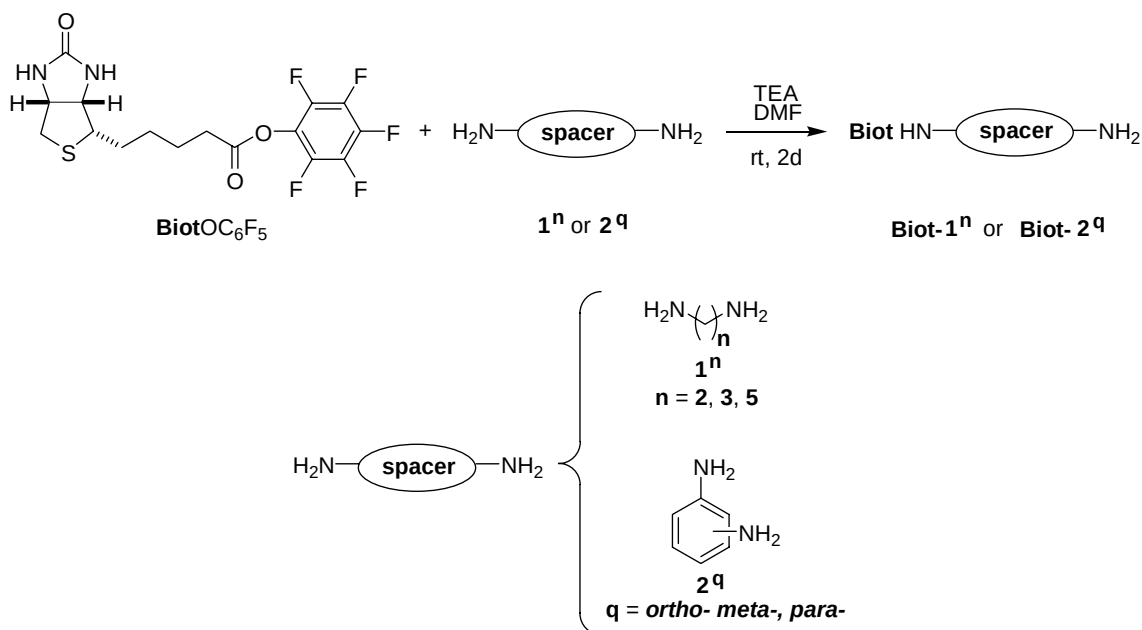


Figure 3-5. Synthesis of the biotinylated precursors by incorporating either an aliphatic **1ⁿ** or an aromatic **2^q** diamine.

Using the activated species **BiotNHS**, as in the case of $[\text{Rh}(\text{COD})(\text{Biot-NP}_2)]^+$, is another possibility for biotinylated amide coupling, but the yields were smaller than starting from the biotinylated pentafluorophenylester **BiotOC₆F₅**.

The isolated yields for the biotinylated amines are listed in **Table 3-1**.

Biotinylated Amine	Yield (%)
Biot-1²	72
Biot-1³	54
Biot-1⁵	68
Biot-2^{ortho}	82
Biot-2^{meta}	76
Biot-2^{para}	92

Table 3-1. Isolated yields for **Biot-1ⁿ** ($n = 2, 3, 5$) and **Biot-2^q** ($q = \text{ortho-}, \text{meta-}, \text{para-}$).

In order to obtain the biotinylated ligands **Biot-1ⁿ-bpy** and **Biot-2^q-bpy**, the remaining amino group of the diamine was coupled via an amide bond to the activated bipyridine moiety **bpyOC₆F₅**. The reaction scheme is depicted in **Figure 3-6**.

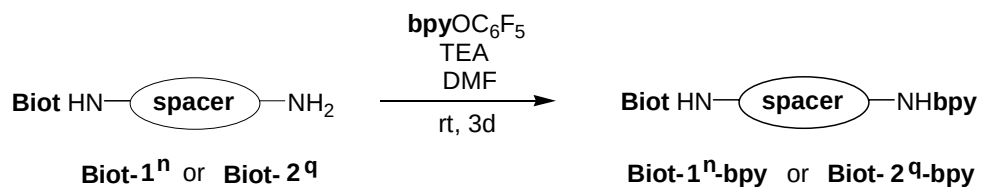


Figure 3-6. Synthesis of **Biot-1ⁿ-bpy** and **Biot-2^q-bpy**.

The isolated yields for the biotinylated ligands are listed in **Table 3-2**.

Biotinylated Ligand	Yield (%)
Biot-1²-bpy	70
Biot-1³-bpy	57
Biot-1⁵-bpy	65
Biot-2^{ortho}-bpy	63
Biot-2^{meta}-bpy	42
Biot-2^{para}-bpy	96

Table 3-2. Isolated yields for **Biot-1ⁿ-bpy** ($n = 2, 3, 5$) and **Biot-2^q-bpy** ($q = \textit{ortho-}, \textit{meta-}, \textit{para-}$).

3.2.2. Complex Synthesis

3.2.2.1. Synthesis of $\textit{rac}$ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺

The starting material $\textit{cis}$ -Ru(bpy)₂Cl₂ was synthesized by refluxing RuCl₃ and 2,2'-bipyridine in DMF (**Figure 3-7**).¹³⁸

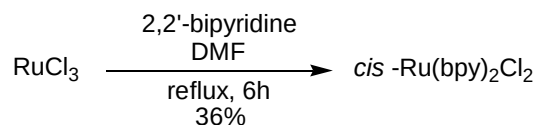


Figure 3-7. Synthesis of $\textit{cis}$ -Ru(bpy)₂Cl₂.

The synthesis of $\textit{rac}$ -[Ru(bpy)₂(**Biot-spacer-bpy**)]²⁺ was performed with the biotinylated ligands using $\textit{cis}$ -Ru(bpy)₂Cl₂. Of all ligands used to prepare $\textit{rac}$ -[Ru(bpy)₂(**Biot-spacer-bpy**)]²⁺, only one corresponding complex displayed an induced CD signal upon incorporation in (strept)avidin. We thus focus on $\textit{rac}$ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ for the remainder of the

chapter. The synthesis of the rac -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ complex is shown in **Figure 3-8**.

The reaction was performed under reflux in EtOH during three days. The isolated yield was 80%.¹³⁹

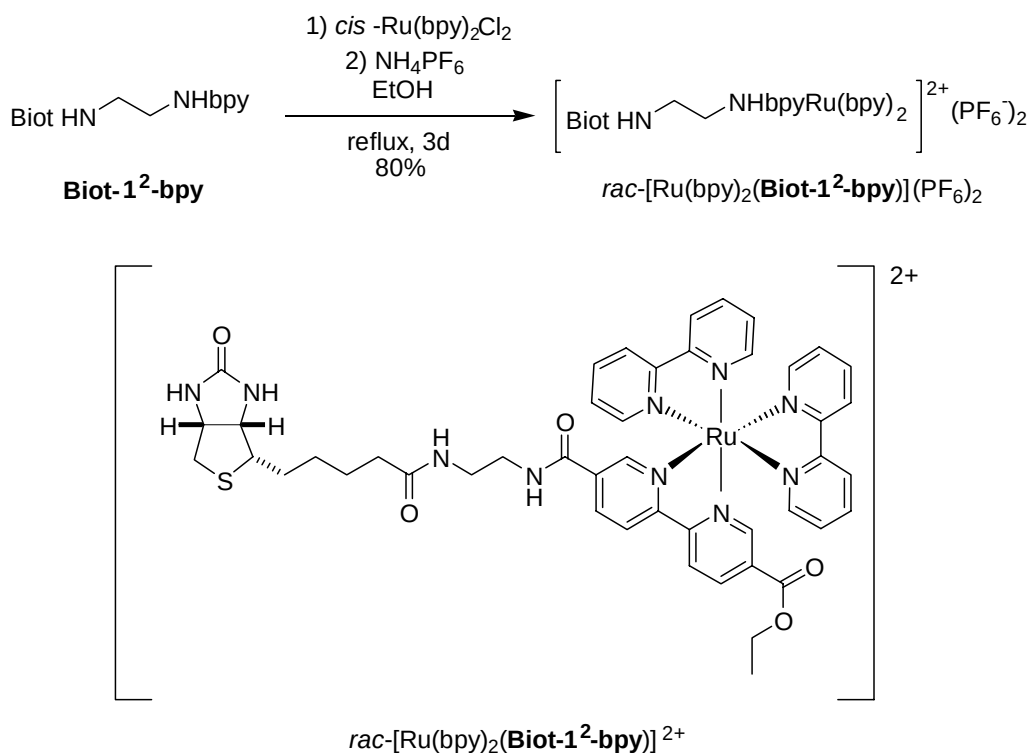


Figure 3-8. Synthesis of the rac -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ complex.

During the determination of the stability constant of the racemic ruthenium complex, we experienced difficulties in fitting the data (section 3.2.3.1.). We suspected that this arose from the fact that both pseudo-enantiomers may have different affinities for the host proteins, thus leading to a highly complex tetrameric system. We thus attempted an pseudo-enantiomer separation by ion exchange chromatography (*SP Sephadex C-25*) using sodium tartrate. Using this method, the pseudo-enantiomer separation was only partial.

The relative evolution of the optical purity of $[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$ during elution chromatography is depicted in **Figure 3-9**. $\Delta\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$ is eluted before $\Lambda\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$. The assignment of the absolute configuration was done by comparison with CD spectra of related species.¹⁴⁰⁻¹⁴⁵ For the Λ -form, the CD signal appears in the region of the long-axis-polarized transition of the bpy ligands strongly positive at lower energies and strongly negative at higher energies (by applying exciton theory).

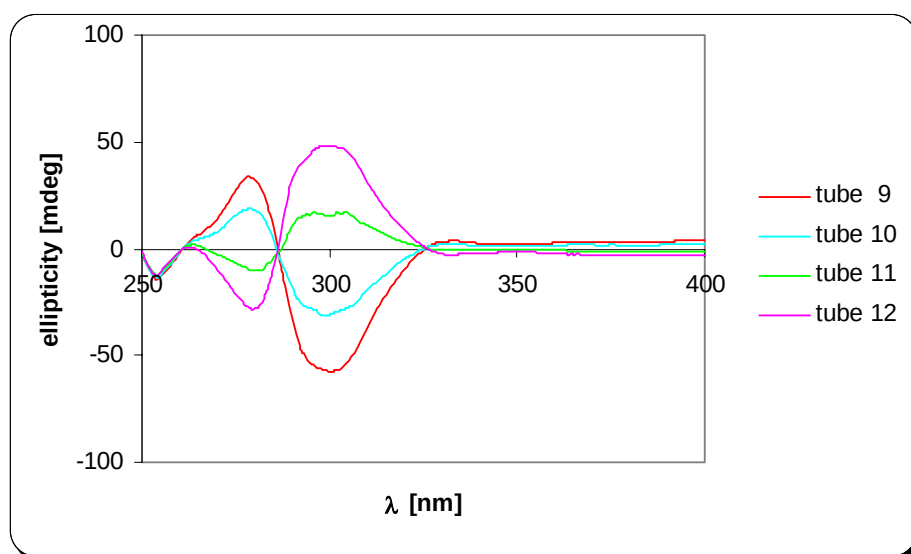


Figure 3-9. Elution profile of enantiomerically enriched $\Delta\text{-}$ or $\Lambda\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$ depending on arbitrary tube numbers (tubes 9 and 10: enantiomerically enriched $\Delta\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$; tubes 11 and 12: enantiomerically enriched $\Lambda\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$). Type of ion exchanger: *SP* Sephadex C-25; column length: 120cm; column width: 4cm; eluent: aqueous sodium tartrate (0.05M) at pH $\sim$ 5.

By plotting the ratio (signal CD / signal UV) of the elution profile as a function of tube number, it is possible to estimate whether a sample is enantiopure or merely enantioenriched. (**Figure 3-10**). If the product is enantiopure, the plot should be a straight horizontal line. If the ratio (signal CD / signal UV) in- or decreases, this suggests that the solution is merely enantiomerically enriched.

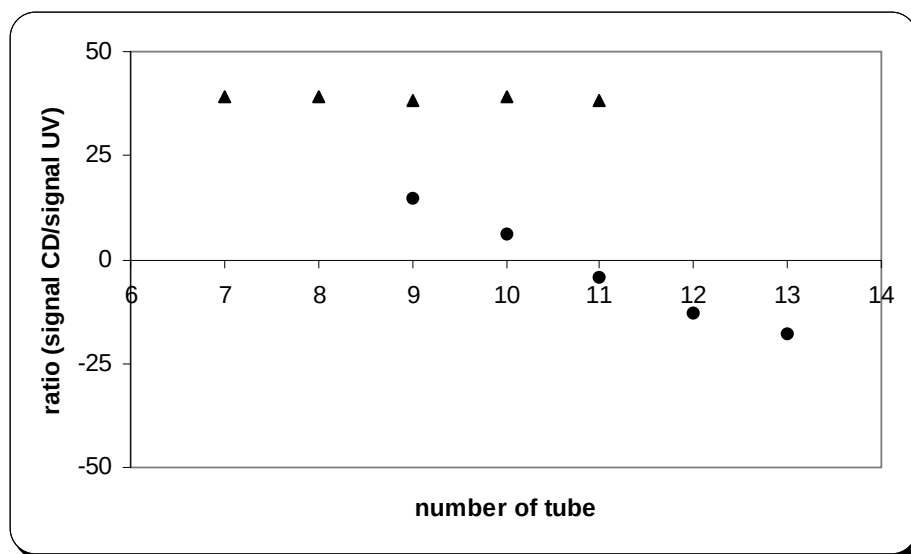


Figure 3-10. By plotting the ratio (signal CD / signal UV) of the elution profile as a function of arbitrary tube number (at 430nm), it is possible to estimate whether a sample is enantiopure or merely enantioenriched.

● : enantioenriched (measured data points from **Figure 3-9**)

▲ : enantiopure (measured data points of Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ from section 3.2.2.2.)

Because only partial separation of the racemate into both enantiomers could be achieved by ion exchange chromatography, the direct synthesis of Δ - and Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺, respectively, was attempted.

3.2.2.2. Synthesis of Enantiopure Δ - and Λ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺

von Zelewsky and co-workers have developed a method for the resolution of racemic *cis*-[Ru(bpy)₂(py)₂]²⁺ (bpy = 2,2'-bipyridine, py = pyridine) with dibenzoyltartrate.¹⁴⁰⁻¹⁴² It has been shown that the two pyridine ligands in *cis*- Δ/Λ -[Ru(bpy)₂(py)₂]²⁺ can be substituted by a bidentate ligand (such as bpy) with retention of the absolute configuration in the absence of light. The synthesis of racemic *cis*-[Ru(bpy)₂(py)₂]Cl₂ was performed following the method of Dwyer and co-workers (**Figure 3-11**).¹⁴⁶

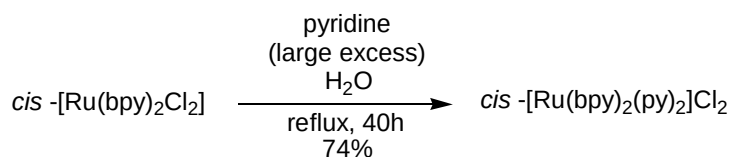


Figure 3-11. Synthesis of racemic *cis*-[Ru(bpy)₂(py)₂]Cl₂.

The racemic *cis*-[Ru(bpy)₂(py)₂]Cl₂ complex shows in its optical spectrum two absorption bands in the visible region as well as two in the ultraviolet region. The two signals in the visible region could be assigned to strong metal-to-ligand charge transfer bands (MLCT: d_{Ru} → π^*_{bpy}), whereas the two strong charge transfer bands (at 245nm and at 290nm) in the ultraviolet region are coming from intraligand transitions ($\pi_{\text{bpy}} \rightarrow \pi^*_{\text{bpy}}$) (**Figure 3-12**).¹⁴⁷⁻¹⁴⁹

The UV/Vis spectra of Δ - and Λ -[Ru(bpy)₂(py)₂]²⁺ are superimposable within the margin of error (below 250nm the absorption was too strong for comparison), whereas the CD spectra (**Figure 3-13**) of both enantiomers are the mirror images from each other (in form and in intensity), which suggest that both compounds have been synthesized in enantiopure quality.

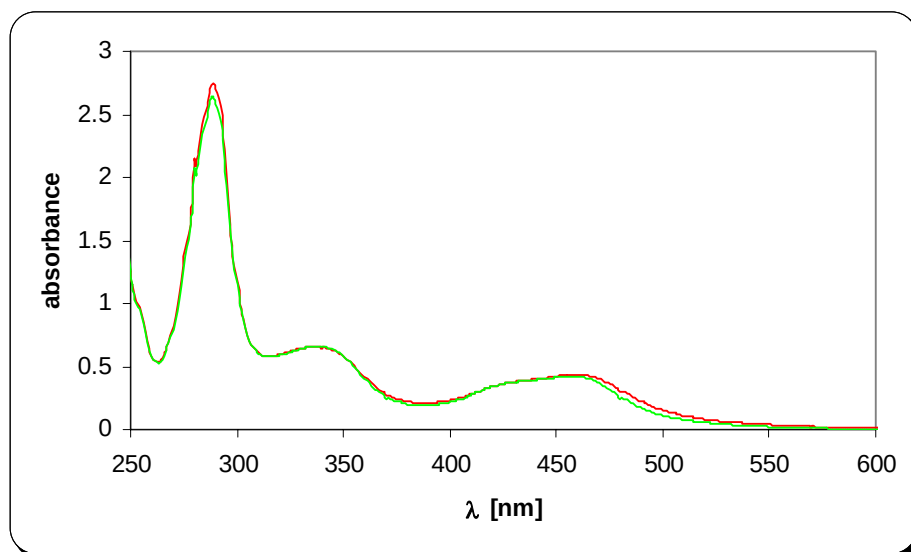


Figure 3-12. UV/Vis spectra of both $cis-\Delta$ -[Ru(bpy)₂(py)₂]²⁺ (in green) and $cis-\Lambda$ -[Ru(bpy)₂(py)₂]²⁺ (in red).

Concentration: 50 μ M; solvent: phosphate buffer (pH = 7.0, I = 0.3 M).

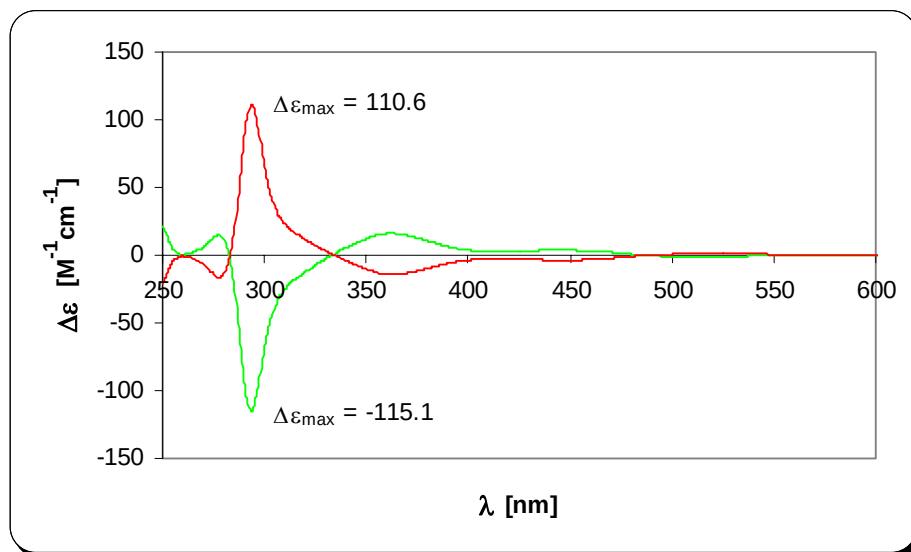


Figure 3-13. CD spectra of both $cis-\Delta$ -[Ru(bpy)₂(py)₂]²⁺ (in green) and $cis-\Lambda$ -[Ru(bpy)₂(py)₂]²⁺ (in red). Solvent:

phosphate buffer (pH = 7.0, I = 0.3 M).

The synthesis of Δ - and Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺, starting from *cis*-[Ru(bpy)₂(py)₂]Cl₂, is sketched in **Figure 3-14**. The crystallization of *cis*- Δ -[Ru(bpy)₂(py)₂][(+)-O,O'-dibenzoyl-D-tartrate] · 12H₂O (*cis*- Λ -[Ru(bpy)₂(py)₂][(-)-O,O'-dibenzoyl-L-tartrate] · 12H₂O) was performed with an aqueous solution of 0.5M disodium (+)-O,O'-dibenzoyl-D-tartrate (disodium (-)-O,O'-dibenzoyl-L-tartrate), which was prepared by addition of a NaOH solution to the corresponding acid, until a pH of 8-9 was obtained, during 8-10 days at room temperature in the dark.^{140-142,150} The yields were 65% (Δ -enantiomer) and 64% (Λ -enantiomer), respectively.

The final step in the synthesis of Δ - and Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ was achieved by heating a solution of *cis*- Δ -[Ru(bpy)₂(py)₂][(+)-O,O'-dibenzoyl-D-tartrate] · 12H₂O (*cis*- Λ -[Ru(bpy)₂(py)₂][(-)-O,O'-dibenzoyl-L-tartrate] · 12H₂O) in the presence of **Biot-1²-bpy** in ethylene glycol (10% H₂O) at 120°C for 6h (**Figure 3-14**).¹⁴¹ The resulting mixture was purified by ion exchange chromatography (*SP Sephadex C-25*) using sodium perchlorate. The elution yielded two main bands. The first band (singly charged product Δ -[Ru(bpy)₂(**Biot-1²-bpy'**)]⁺ (Λ -[Ru(bpy)₂(**Biot-1²-bpy'**)]⁺) where **Biot-1²-bpy'** is the hydrolyzed ligand carrying one negative charge) was eluted with a 0.05M NaClO₄ solution while the second band (desired doubly charged product Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ (Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺) was eluted with a 0.2M (0.5M) NaClO₄ solution.

Both UV/Vis and CD spectra of Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ and Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺, respectively, are shown in **Figure 3-15** as well as in **Figure 3-16**.

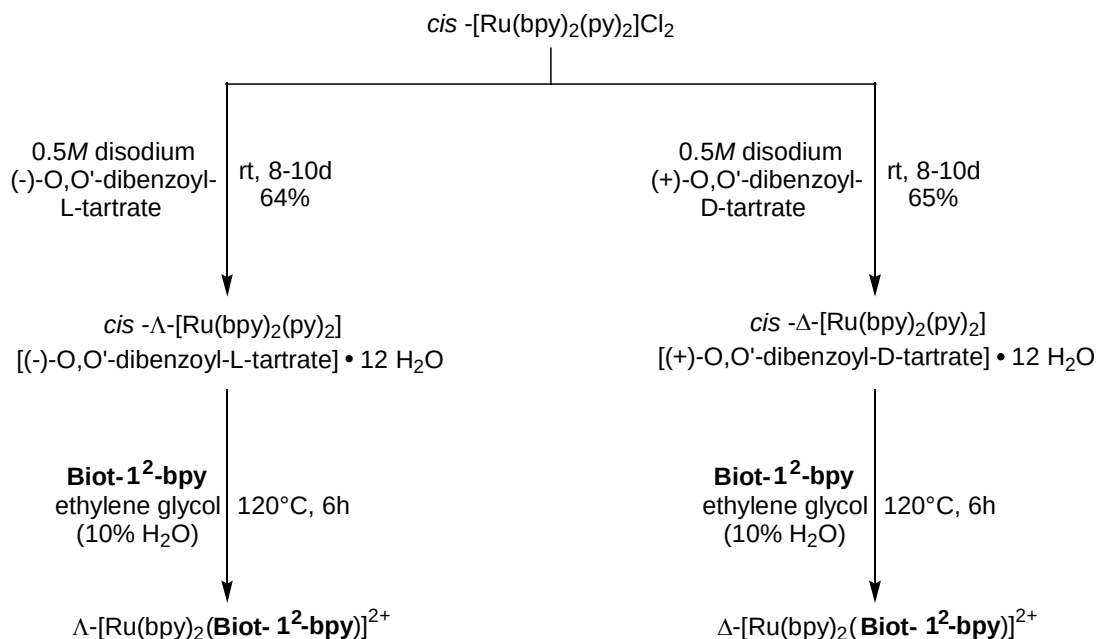


Figure 3-14. Synthesis of $\Delta\text{-}$ and $\Lambda\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$ starting from racemic $cis\text{-}[\text{Ru}(\text{bpy})_2(\text{py})_2]\text{Cl}_2$. All reactions involving chiral ruthenium complexes were carried out in the dark.

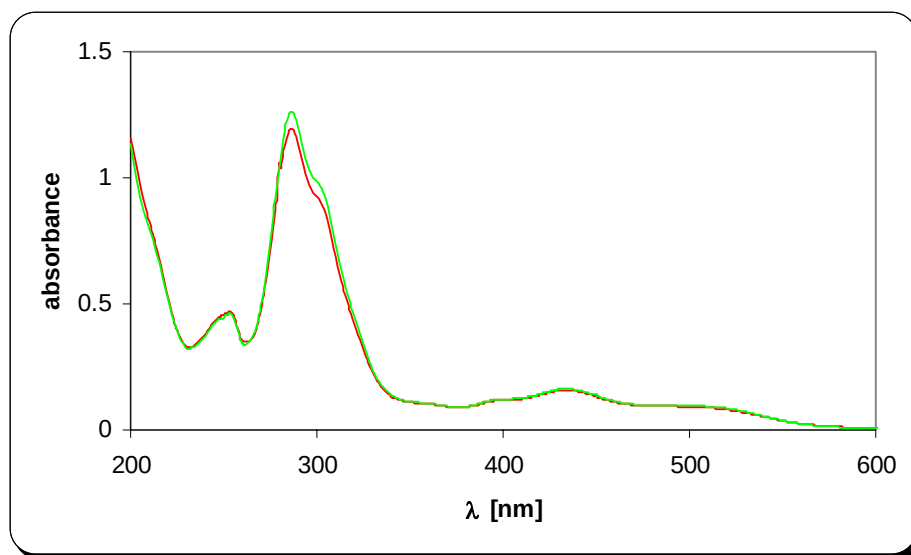


Figure 3-15. UV/Vis spectra of $\Delta\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$ (in green) and $\Lambda\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$ (in red), respectively. Concentration: 20 μM ; solvent: phosphate buffer (pH = 7.0, I = 0.3M).

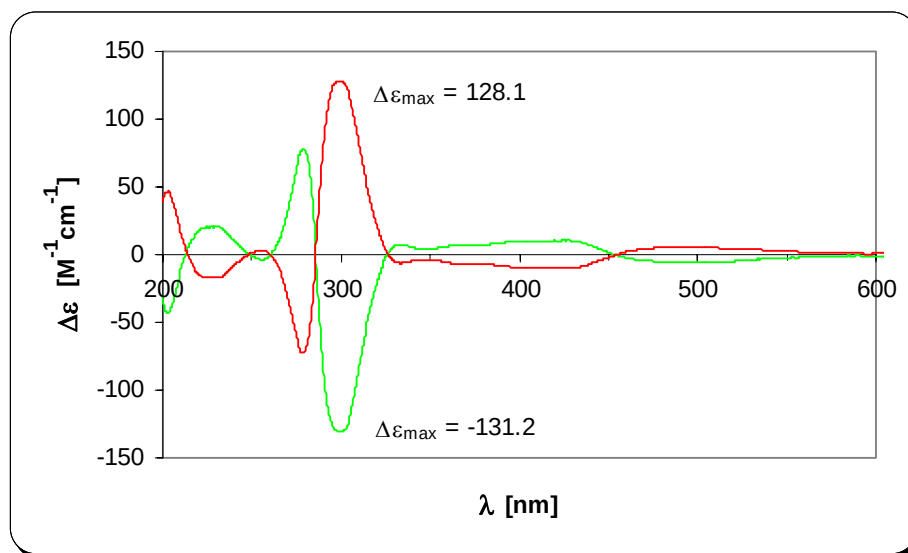


Figure 3-16. CD spectra of Δ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺ (in green) and Λ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺ (in red), respectively. Solvent: phosphate buffer (pH = 7.0, I = 0.3M).

As expected for pseudo-enantiomers, the UV/Vis spectra are superimposable (**Figure 3-15**), whereas the CD spectra of both Δ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺ and Λ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺ are mirror images from each other (**Figure 3-16**).

3.2.3. Thermodynamics: Titration Measurement and Determination of

$$K_{[\text{Ru}(\text{bpy})_2(\text{Biot}-1^2-\text{bpy})]^{2+} \subset (\text{strept})\text{avidin}}$$

3.2.3.1. The *rac*-[Ru(bpy)₂(Biot-1²-bpy)]²⁺ $\subset$ (Strept)Avidin System

Adding aliquots of *rac*-[Ru(bpy)₂(Biot-1²-bpy)]²⁺ to (strept)avidin leads to the appearance of a CD signal ($\lambda_{\text{max}} = 313\text{nm}$) caused by the chiral second coordination sphere provided by the host protein. This signal steadily increases up to 3.5-4.0eq vs tetrameric protein concentration. This signal is an **induced CD signal** having its origin probably in a charge-transfer from the

protein to the rac -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ complex as it is the case for the HABA⇌(strept)avidin system. A typical series of CD titration spectra obtained by adding the racemic ruthenium complex in 0.5eq steps to avidin (hence forming the rac -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⇌avidin complex) is depicted in **Figure 3-17**. The increase of the CD signal ($\lambda_{\text{max}} = 313\text{nm}$) is clearly seen. The CD band on the left, between 250nm and 300nm, is protein centered, coming mostly from the tryptophan residues. Similar results are obtained for streptavidin (**Figure 3-18**). For both series of CD titration spectra, one could notice a displacement of the peak maximum by about ~15nm in comparison with the enantiopure Δ - and Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ complexes (**Figure 3-16**), suggesting different interactions between these ones and (strept)avidin.

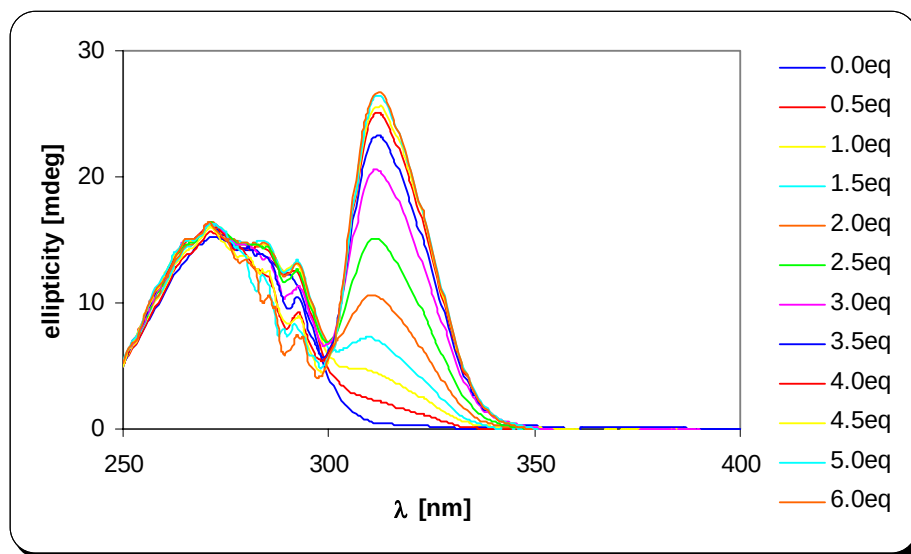


Figure 3-17. Appearance of a CD signal ($\lambda_{\text{max}} = 313\text{nm}$) upon addition of rac -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ (0.96mM) to avidin. Initial protein concentration: 8.00μM; solvent: phosphate buffer (pH = 7.0, I = 0.3M).

The increase in ellipticity (in millidegrees = [mdeg]) at $\lambda_{\text{max}} = 313\text{nm}$ as a function of added equivalents of rac -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ is depicted in **Figure 3-19**. The most striking feature of this titration curve is its sigmoidal behaviour, suggesting cooperativity.

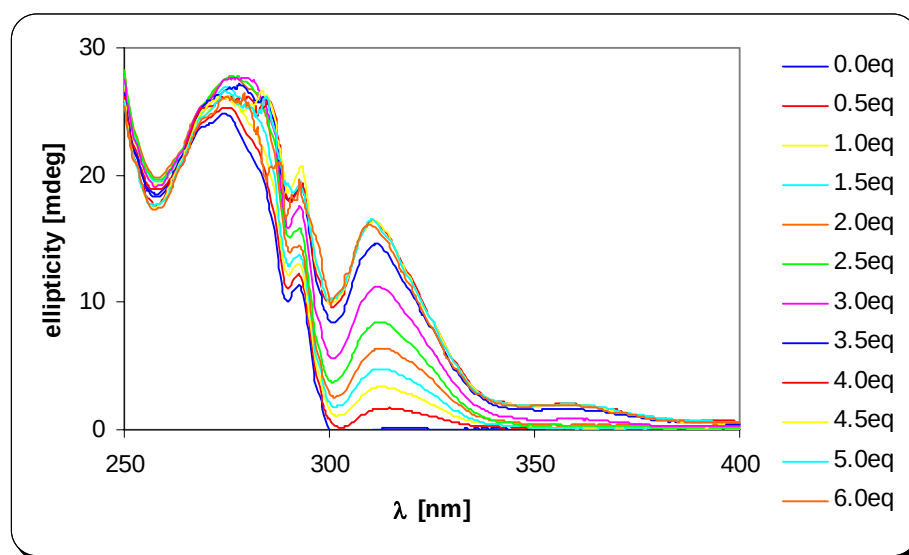


Figure 3-18. Appearance of a CD signal ($\lambda_{\text{max}} = 313\text{nm}$) upon addition of $\text{rac-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$ (0.96mM) to streptavidin. Initial protein concentration: 8.00 μM ; solvent: phosphate buffer (pH = 7.0, I = 0.3M).

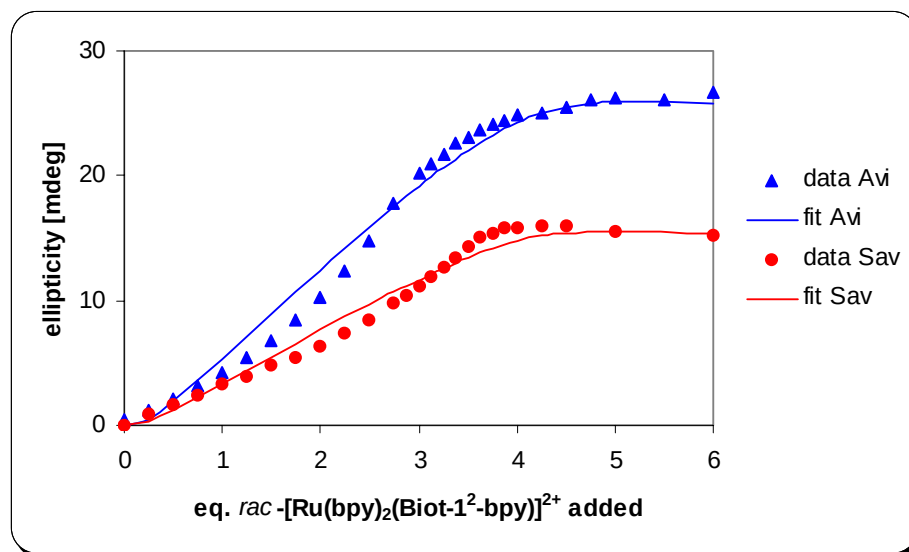


Figure 3-19. Appearance of a CD signal (at $\lambda_{\text{max}} = 313\text{nm}$) upon addition of $\text{rac-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$ (0.96mM) to (strept)avidin (data Avi (Sav) = measured points for WT (strept)avidin; fit Avi (Sav) = calculated points for WT (strept)avidin) using the “two-step” model). Initial protein concentration: 8.00 μM ; solvent: phosphate buffer (pH = 7.0, I = 0.3M).

All attempts to fit this sigmoidal curve in order to obtain the (cumulative) stability constants $K_a(\beta)$ using PSEQUAD¹²⁸ (section 5.4.2.) as well as using the method of Srivastava and co-workers failed.¹³⁰ The latter is a spectroscopic titration method for determining dissociation constants and stoichiometries of ligand-protein complexes. However, looking at the hyperbolic titration curve of HABA-avidin used in that work, it is assumed that all four subunits of avidin behave totally independently and that the obtained dissociation constant is an average value for each of the four binding sites. In the case of *rac*-[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ we can assume from the sigmoidal curve that cooperativity occurs in the *rac*-[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺-(strept)avidin system. Therefore four different models, which were implemented by J. Sauser (section 5.4.1.),¹²⁹ were considered as working hypothesis for the determination of the (cumulative) stability constant(s) $K_a(\beta)$ to get information on the cooperativity of the system. In the following, P refers to the protein (avidin or streptavidin) and L to the ligand (*rac*-, Δ - or Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺).

a) “Monomer-like” model. The four subunits of the tetrameric protein are assumed to behave totally independently.



b) “One-step” model. All the successive binding events are assumed to occur with the same K_a , leading to a single global constant β_4 .



c) “Two-step” model. The first two binding events are assumed to have a different β value than the last two.



d) “Four-step” model. All four binding events are assumed to take place with their own K_a .



Model a) is non-cooperative, whereas models b), c) and d) represent three different degrees of cooperativity. Determination of the different (cumulative) stability constant(s) K_a (β) was performed by minimizing the sum of the squares of the differences between observed and calculated absorbance values, *i.e.* changing K_a (β) values until

$$\sum (\text{signal}_{\text{calc}} - \text{signal}_{\text{meas}})^2 = \text{minimum} \quad (3.9)$$

Assuming cooperativity from the sigmoidal curve, model a) is excluded. Attempts to fit the (cumulative) stability constant(s) K_a (β) for *rac*-[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⋯(strept)avidin were performed with the help of the models c) and d). **Figure 3-19** represents the best fit for both avidin and streptavidin by applying the “two-step” model. From these fits, it was concluded that model c) (“two-step” model) and model d) (“four-step” model, not graphically shown) do not correspond to the actual binding mechanism. One assumption why the models

were not yielding acceptable fits was, by using the racemate of $[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$ (composed of Δ - and Λ -enantiomers), the presence of several combinations of Δ - and Λ - $[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$ within the same (strept)avidin tetramer. Thus, the observed titration curve would not only correspond to several species of formed $[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+} \subset (\text{strept})\text{avidin}$ (species PL_2 and PL_4 in the “two-step” model (**Equations 3.3** and **3.4**) and species PL , PL_2 , PL_3 and PL_4 in the “four-step” model (**Equations 3.5** to **3.8**)), but also to different diastereoisomers of Δ/Λ - $[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-Bpy})]^{2+} \subset (\text{strept})\text{avidin}$. Stability constants of such complex systems can not be determined using simplified models like the “two-step” or the “four-step” model.

Therefore, titrations with both enantiopure Δ - and Λ - $[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$ complexes were carried out to simplify the fitting of the titration curves.

3.2.3.2. The Δ - and Λ - $[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+} \subset (\text{Strept})\text{Avidin}$ System

A typical series of CD titration spectra by adding the enantiopure Δ - or Λ -form of the ruthenium complex in 0.5eq steps to avidin is shown in **Figure 3-20** and **Figure 3-21**, respectively. At most wavelengths, the CD signal increases or decreases linearly, suggesting no interaction between the host and the guest. Nevertheless, there is an important difference comparing the spectra of the two titration series. One can recognize that the form of the peak at 300nm of Λ - $[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+} \subset (\text{strept})\text{avidin}$ differs from that of Δ - $[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+} \subset (\text{strept})\text{avidin}$. It was concluded that there is a correlation between the difference of these two titration series and the appearing induced CD signal ($\lambda_{\text{max}} = 313\text{nm}$) of the *rac*- $[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+} \subset (\text{strept})\text{avidin}$.

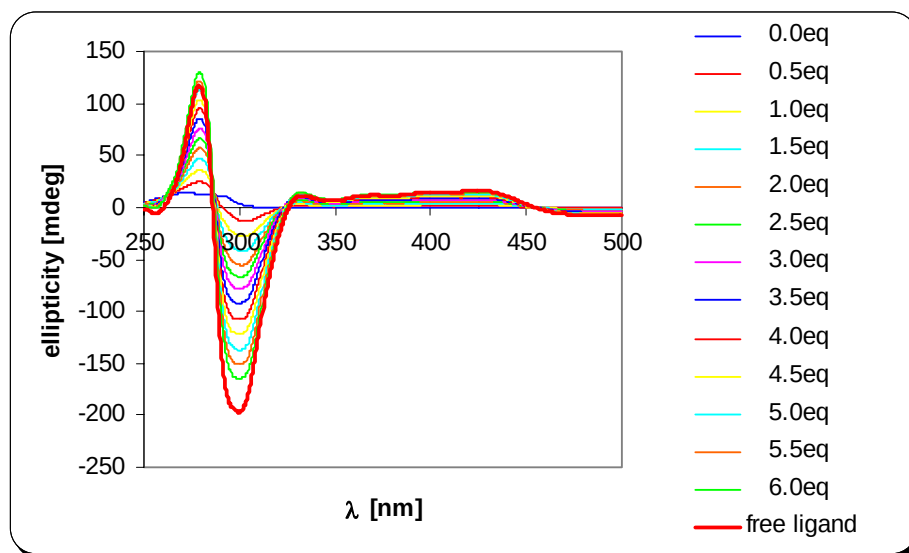


Figure 3-20. Increase of a CD signal upon addition of Δ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺ (0.64mM) to WT Avi. The red bold line represents the enantiopure ruthenium complex devoid of protein at 6.0eq (free ligand). Initial protein concentration: 8.00μM; solvent: phosphate buffer (pH = 7.0, I = 0.3M).

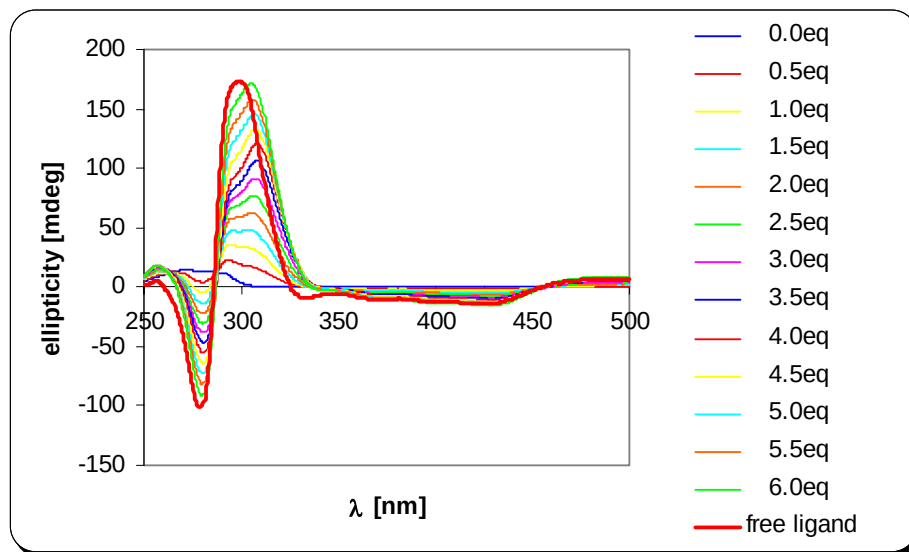


Figure 3-21. Increase of a CD signal upon addition of Δ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺ (0.96mM) to WT Avi. The red bold line represents the enantiopure ruthenium complex devoid of protein at 6.0eq (free ligand). Initial protein concentration: 8.00μM; solvent: phosphate buffer (pH = 7.0, I = 0.3M).

By the addition of the spectra of both Δ - and Λ -[Ru(bpy)₂(Biot-1²-Bpy)]²⁺ (Figure 3-20 and Figure 3-21) in the range of 300-340nm (no contribution to the CD signal of the protein) the spectra obtained with the racemic compound (Figure 3-18) with exactly twice its intensity is reproduced. This means that the CD activity obtained by the racemic compound is due to the difference between the spectra obtained with the two enantiomeric Ru(bpy)₃-units and that the latter exhibit identical enantiomeric purities.

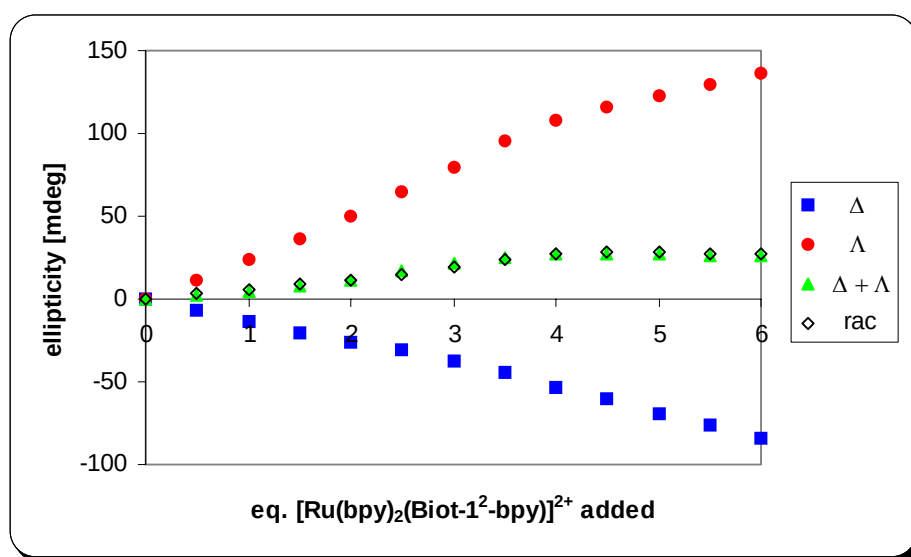


Figure 3-22. CD titration series at 313nm upon addition of Δ - and Λ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺ (0.64mM for the Δ -complex and 0.96mM for the Λ -complex), respectively, to WT Avi and their difference spectra, obtained by adding the spectra of both titration series together, giving the same plot as for the CD titration series at 313nm upon addition of *rac*-[Ru(bpy)₂(Biot-1²-bpy)]²⁺ to avidin (Figure 3-19). Initial protein concentration: 8.00 μ M; solvent: phosphate buffer (pH = 7.0, I = 0.3M).

Moreover, looking more exactly at the curves of Δ - and Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin in **Figure 3-22** and especially at the titration series in both **Figure 3-20** and **Figure 3-21**, it can be noticed that the interaction of Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin with the protein gives a much more positive induced CD signal than Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin (contrasting changes of slope between Δ - and Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin), where the plot between 1.0 to 4.0eq equivalents is almost linearly, suggesting only a weak induced CD signal.

As for *rac*-[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂(strept)avidin, the determination of the stability constant of Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂(strept)avidin as well as for Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂(strept)avidin was not possible by using the “two-step” or the “four-step” model.

3.2.3.2.1. “Two-Step” and “Four-Step” Model (Direct Method)

For the “two-step” model, the measured and fitted plots of both Δ - and Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂(strept)avidin are depicted in **Figure 3-23** as well as in **Figure 3-24**, respectively. All plots with the exception of Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin (only slightly sigmoidal), show sigmoidal behaviour. The only plot that could be more or less fitted had no sigmoidal behaviour (Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin). An overview of all determined cumulative stability constants β_4 (for PL₄), using the “two-step” and the “four-step” model, is represented in **Table 3-3**. All overall $\log(\beta_{rac-, \Delta- \text{ and } \Lambda-[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+} \subset (\text{strept})\text{avidin}})$ values (cumulative stability constants) for WT Avi and WT Sav, respectively (hence six different systems), are around 23.

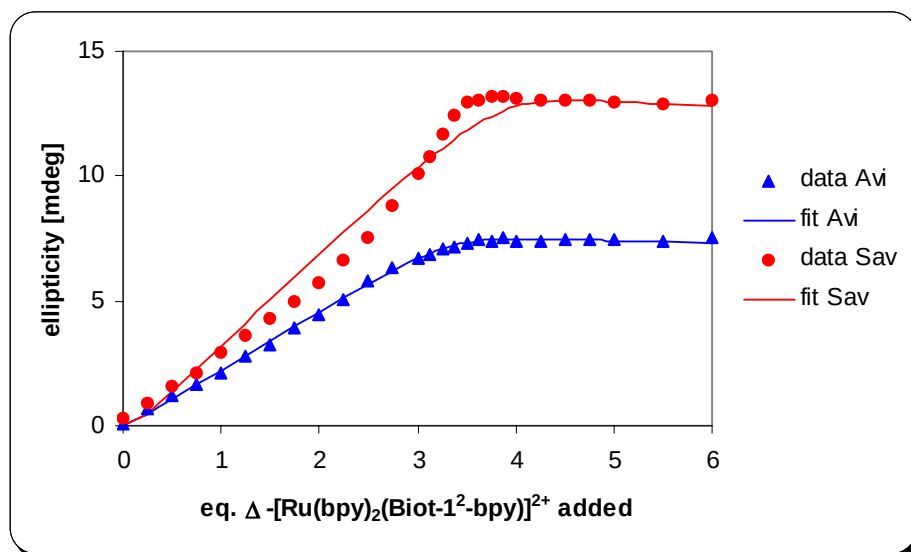


Figure 3-23. Appearance of a CD signal (at $\lambda = 327\text{nm}$, where the CD signal of the free ruthenium complex is absent) upon addition of $\Delta\text{-[Ru(bpy)}_2\text{(Biot-1}^2\text{-bpy)}]^{2+}$ (0.64mM) to (strept)avidin (data Avi (Sav) = measured points for WT (strept)avidin; fit Avi (Sav) = calculated points for WT (strept)avidin using the “two-step” model). Initial protein concentration: $8.00\mu\text{M}$; solvent: phosphate buffer (pH = 7.0, I = 0.3M).

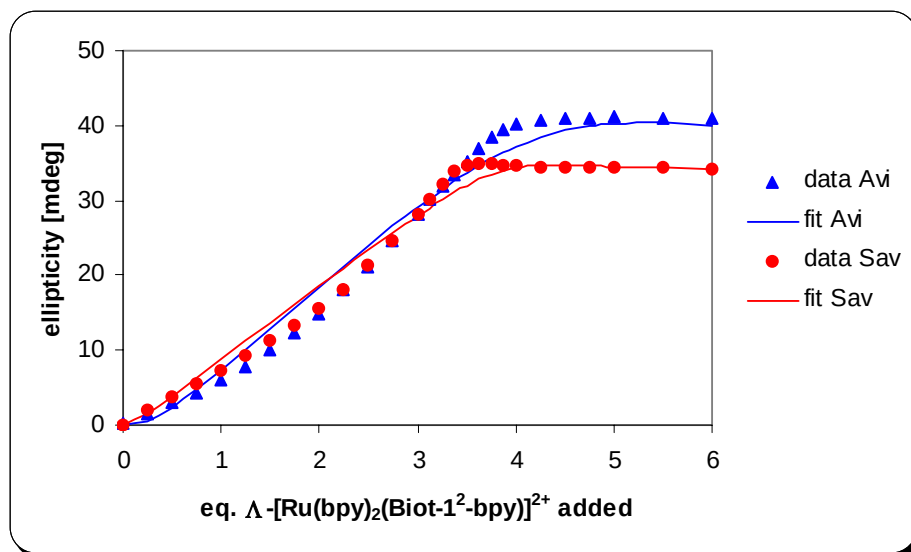


Figure 3-24. Appearance of a CD signal (at $\lambda = 325\text{nm}$, where the CD signal of the free ruthenium complex is absent) upon addition of $\Delta\text{-[Ru(bpy)}_2\text{(Biot-1}^2\text{-bpy)}]^{2+}$ (0.96mM) to (strept)avidin (data Avi (Sav) = measured points for WT (strept)avidin; fit Avi (Sav) = calculated points for WT (strept)avidin using the “two-step” model). Initial protein concentration: $8.00\mu\text{M}$; solvent: phosphate buffer (pH = 7.0, I = 0.3M).

	WT Avi	WT Sav	Model
$\log(\beta_{rac-[Ru(bpy)_2(Biot-1^2-bpy)]^{2+}})$	23.3	23.3	“two-step” model
$\log(\beta_{rac-[Ru(bpy)_2(Biot-1^2-bpy)]^{2+}})$	23.3	23.3	“four-step” model
$\log(\beta_{\Delta-[Ru(bpy)_2(Biot-1^2-bpy)]^{2+}})$	23.5	22.4	“two-step” model
$\log(\beta_{\Delta-[Ru(bpy)_2(Biot-1^2-bpy)]^{2+}})$	23.3	22.4	“four-step” model
$\log(\beta_{\Lambda-[Ru(bpy)_2(Biot-1^2-bpy)]^{2+}})$	21.0	22.9	“two-step” model
$\log(\beta_{\Lambda-[Ru(bpy)_2(Biot-1^2-bpy)]^{2+}})$	21.0	22.9	“four-step” model

Table 3-3. Overview of all determined cumulative stability constants β_4 (for PL₄) using the “two-step” and the “four-step” model for *rac*-, Δ -, Λ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺⊂(strept)avidin.

3.2.3.2.2. Exchange Procedure with 2-(4'-Hydroxyazobenzene)benzoic acid (HABA) (Indirect Method)

Another approach to determine the stability constant K_a is to apply the same exchange procedure as for the determination of $K_{[Rh(COD)(Biot-NP_2)]^+ \subset (strept)avidin}$ (section 2.2.) using 2-(4'-hydroxyazobenzene)benzoic acid (HABA). Knowing the stability constant K_{HABA} (**Equation 2.3**) for WT Avi ($\log(K_{HABA}) = 5.5$; $R^2 = 0.998$) and WT Sav ($\log(K_{HABA}) = 3.5$; $R^2 = 0.999$), the stability constant $K_{rac-, \Delta- \text{ and } \Lambda-[Ru(bpy)_2(Biot-1^2-bpy)]^{2+} \subset (strept)avidin}$ (average value for each of the four binding sites) could be estimated (**Equations 2.4 to 2.6** and **Figures 3-25 to 3-27**) using the “monomer-like” model (section 5.4.1.). The sigmoidal behaviour of the fits for streptavidin in **Figure 3-26** and **Figure 3-27** is probably due to another batch used of different streptavidin in comparison with the exchange procedure with the racemic ruthenium complex (**Figure 3-25**), where no similar behaviour was observed. Moreover, one could not obtain such fits by applying the simple “monomer-like” model.

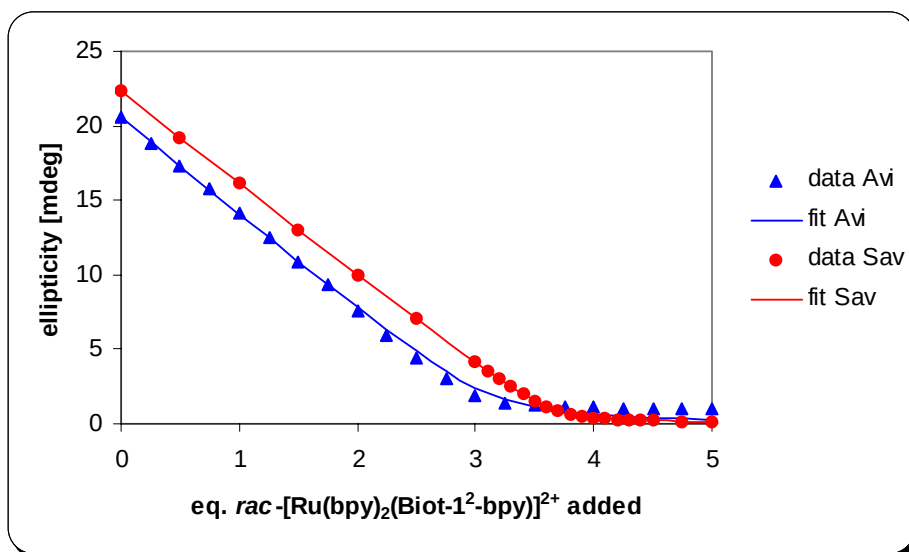


Figure 3-25. Disappearance of a CD band ($\lambda_{\text{max}} = 506\text{nm}$) upon addition of $\text{rac-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$ aliquots (0.96mM) to $\text{HABA-}(\text{strept})\text{avidin}$ (data Avi (Sav) = meas. points for WT (strept)avidin; fit Avi (Sav) = calculated points for WT (strept)avidin). Initial protein conc.: $7.87\mu\text{M}$ for Avi and $7.11\mu\text{M}$ for Sav; initial HABA conc.: $157.38\mu\text{M}$ (for Avi) and 1.07mM (for Sav); solvent: phosphate buffer ($\text{pH} = 7.0$, $I = 0.3\text{M}$).

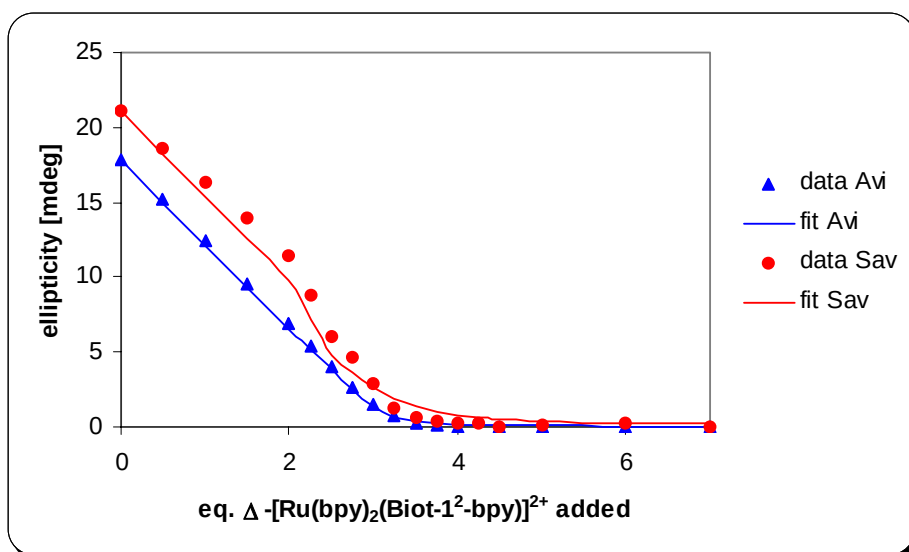


Figure 3-26. Disappearance of a CD band ($\lambda_{\text{max}} = 506\text{nm}$) upon addition of $\Delta\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$ (0.64mM) aliquots to $\text{HABA-}(\text{strept})\text{avidin}$ (data Avi (Sav) = meas. points for WT (strept)avidin; fit Avi (Sav) = calculated points for WT (strept)avidin). Initial protein conc.: $7.87\mu\text{M}$ for Avi and $7.11\mu\text{M}$ for Sav; initial HABA conc.: $157.38\mu\text{M}$ (for Avi) and 1.07mM (for Sav); solvent: phosphate buffer ($\text{pH} = 7.0$, $I = 0.3\text{M}$).

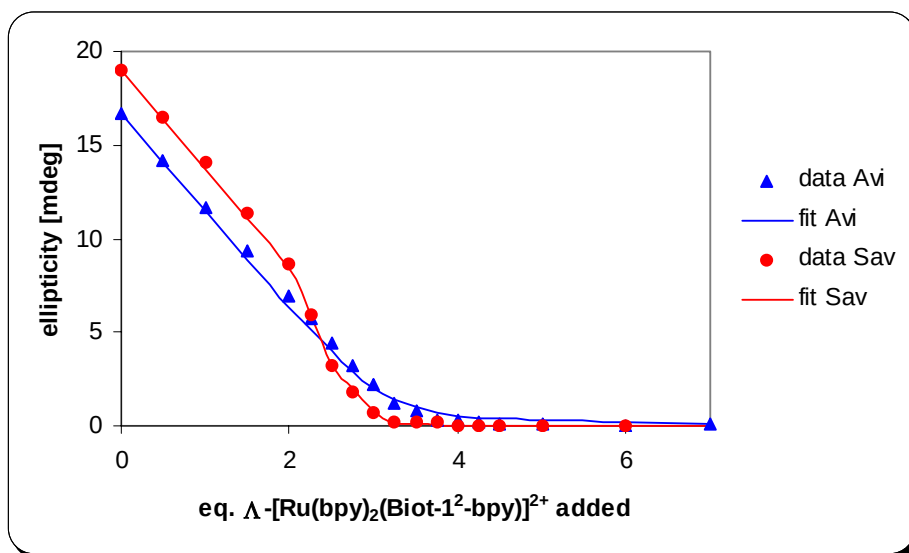


Figure 3-27. Disappearance of a CD band ($\lambda_{\text{max}} = 506\text{nm}$) upon addition of Δ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺ (0.96mM) aliquots to HABA $\subset$ (strept)avidin (data Avi (Sav) = meas. points for WT (strept)avidin; fit Avi (Sav) = calculated points for WT (strept)avidin). Initial protein conc.: 7.87 μM for Avi and 7.11 μM for Sav; initial HABA conc.: 157.38 μM (for Avi) and 1.07mM (for Sav); solvent: phosphate buffer (pH = 7.0, I = 0.3M).

An overview of all determined stability constants ($\log(K_a)$ values) is presented in **Table 3-4**. Hereby, it is worthy to have a look at the value of $\log(K_{\text{rac-[Ru(bpy)}_2\text{(Biot-1²-bpy)]}^{2+}\subset\text{(strept)avidin}})$. For both host proteins, the $\log(K_{\text{rac-[Ru(bpy)}_2\text{(Biot-1²-bpy)]}^{2+}\subset\text{(strept)avidin}})$ value lies between the $\log(K_{\Delta\text{-[Ru(bpy)}_2\text{(Biot-1²-bpy)]}^{2+}\subset\text{(strept)avidin}})$ and $\log(K_{\Lambda\text{-[Ru(bpy)}_2\text{(Biot-1²-bpy)]}^{2+}\subset\text{(strept)avidin}})$ values, suggesting that the $\log(K_a)$ value of the racemate is an average value of the $\log(K_a)$ values of both Δ - and Λ -enantiomer. Looking at the **Table 3-4**, another interesting point is evident. The $\log(K_a)$ values of Δ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺ $\subset$ avidin and Λ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺ $\subset$ streptavidin are in the same order of magnitude, whereas the $\log(K_a)$ values of Δ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺ $\subset$ avidin and Δ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺ $\subset$ streptavidin are quite different from each other. It seems that the Δ -enantiomer interacts in a markedly different way

with avidin than with streptavidin. In contrast, the affinity of the Λ -enantiomer for avidin is in the same order of magnitude as for streptavidin.

	WT Avi	WT Sav	Model
$\log(K_{\text{HABA}})$	5.5 ($R^2=0.998$)	3.5 ($R^2=0.999$)	“monomer-like” model
$\log(K_{\text{exch}\{\text{rac-}[\text{Ru}(\text{bpy})_2(\text{Biot-I}^2\text{-bpy})]^{2+}\}})$	3.0 ($R^2=0.996$)	4.3 ($R^2=0.997$)	“monomer-like” model
$\log(K_{\text{exch}\{\Delta\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-I}^2\text{-bpy})]^{2+}\}})$	3.4 ($R^2=0.999$)	3.6 ($R^2=0.984$)	“monomer-like” model
$\log(K_{\text{exch}\{\Lambda\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-I}^2\text{-bpy})]^{2+}\}})$	2.8 ($R^2=0.996$)	5.1 ($R^2=0.999$)	“monomer-like” model
$\log(K_{\text{rac-}[\text{Ru}(\text{bpy})_2(\text{Biot-I}^2\text{-bpy})]^{2+}})$	8.5	7.8	“monomer-like” model
$\log(K_{\Delta\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-I}^2\text{-bpy})]^{2+}})$	8.9	7.1	“monomer-like” model
$\log(K_{\Lambda\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-I}^2\text{-bpy})]^{2+}})$	8.3	8.6	“monomer-like” model

Table 3-4. Overview of all determined stability constants (average $\log(K_a)$ values for one binding site) for WT Avi and WT Sav: $\log(K_{\text{HABA}})$ and $\log(K_{\text{exch}})$ are obtained from each single step of the two-step exchange procedure and $K_{\text{rac-}, \Delta\text{-} \text{ and } \Lambda\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-I}^2\text{-bpy})]^{2+} \subset (\text{strept})\text{avidin}}$ by taking the sum of the $\log(K_{\text{HABA}})$ and $\log(K_{\text{exch}})$ values. The applied model was the “monomer-like” model.

A disadvantage of using the exchange procedure is that only an average stability constant K_a for each of the four binding sites could be determined instead of calculating separately the stepwise formation constants of PL, PL₂, PL₃ and PL₄. Moreover, all calculations were performed only at the maximum of the CD band, hence only at one single wavelength. The application of SPECFIT/32¹⁵¹ could circumvent this obstacle.

3.2.3.2.3. SPECFIT/32¹⁵¹ (Direct Method)

SPECFIT/32 is a program for fitting complex Metal/Ligand/H⁺ equilibria from spectrophotometric data.¹⁵¹⁻¹⁵⁶ Using SPECFIT/32, the titration series of all four systems (Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin, Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin, Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂streptavidin, and Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂streptavidin) could be fitted in a very good way. Reliable results (hence stability constants) are obtained by using the model consisting of the three species PL₂ (two-liganded protein), PL₃ (three-liganded protein) and PL₄ (four-liganded protein) where P refers to the protein (avidin or streptavidin) and L to the ligand (Δ - or Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺). All other tested models diverged, especially that one with the species PL (either one or more stability constants became negative or the molar circular dichroic absorption coefficient ($\Delta\epsilon$) values for all species including the protein were unreasonable). The measured and the fitted spectra are depicted in **Figure 3-28** (for the Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin system), **Figure 3-29** (for the Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin system), **Figure 3-30** (for the Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂streptavidin system) and **Figure 3-31** (for the Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂streptavidin system). The wavelengths for the fits were chosen in regions, where the ellipticity changed in a nonlinear fashion upon addition of Δ - or Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ to the protein. Typical wavelengths displaying curvature are found at 285nm, 316nm, 325nm and 334nm for Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂(strept)avidin, and at 272nm, 289nm, 322nm and 331nm for Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂(strept)avidin. In all figures, the fitted data correspond very well to the measured data.

The advantage of SPECFIT/32 is that it does not only calculate the CD spectra of the titration series, but also the concentration profiles and $\Delta\epsilon$ of all CD active species. The concentration

profiles are shown in **Figure 3-32** (for the Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin system), **Figure 3-33** (for the Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin system), **Figure 3-34** (for the Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂streptavidin system) and **Figure 3-35** (for the Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂streptavidin system), whereas $\Delta\epsilon$ of all CD active species are represented for Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin in **Figure 3-36**, for Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin in **Figure 3-37**, for Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂streptavidin in **Figure 3-38** and for Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂streptavidin in **Figure 3-39**.

Looking at the concentration profiles, all of them are similar with each other except that of Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin. The free protein concentration (protein; P) decreases at the beginning, while the concentration of the species PL₂, PL₃ and PL₄ start to increase (PL₂ and PL₃ disappears afterwards) in this order until only the species PL₄ is left at the end. After almost all binding sites are occupied (formation of PL₄) the concentration of the free ruthenium complex (ligand; L) increases, first slightly and then proportional to the addition of the ruthenium complex. For Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin, one can recognize that at the end, all of the species except the free protein are present, which means that the final equilibrium is not reached. Thus, all calculations based on this measurement are doubtful if not wrong. But the concentration profile of Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin suggests that the affinity of Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ for WT Avi is smaller than that of Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺.

Comparing the measured and calculated $\Delta\epsilon$ spectra of the proteins and of the free complexes, it was found that the spectra for avidin are coincident in form and intensity (**Figure 3-40** and **Figure 3-41**), whereas for streptavidin the agreement is, slightly worse, but still good in form, but a bit different in intensity (**Figure 3-42** and **Figure 3-43**). In contrary, the agreement for the measured and the calculated $\Delta\epsilon$ spectra for the free ruthenium complexes is variable for all

four systems. Very good agreement in form and intensity has been found for the free ruthenium complex in Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin (**Figure 3-41**). In Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂streptavidin (**Figure 3-42**), the coincidence was still good (in both form and intensity), whereas in Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin (**Figure 3-40**) and Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂streptavidin (**Figure 3-43**) there are differences in form (slightly) as well as in intensity (largely) between the measured and calculated $\Delta\epsilon$.

Therefore one should keep a skeptical eye on the obtained cumulative stability constants β by SPECFIT/32. It seems that for the Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ enantiomer, the determination of the cumulative stability constants β is more accurate for avidin, whereas for the Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ enantiomer, it is the case for streptavidin. In general, one should keep in mind that there are only slight interactions between the protein and the Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ enantiomer, in contrary to the Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂(strept)avidin where one can observe strong stereospecific interactions. Moreover, the interactions between PL₂ (form of spectra of the free ligand as well as of PL₂ are similar) and PL₃ (form of spectra of PL₃ as well as of PL₄ are similar) are in this system different from each other, suggesting a possible change in conformation within Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂(strept)avidin between PL₂ and PL₃ (**Figure 3-37** and **Figure 3-39**). This change in CD signal within Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂(strept)avidin leads also to the induced CD signal (in the range of 300-340nm) observed in the series of CD titration spectra by adding the racemic ruthenium complex to the (strept)avidin (**Figure 3-17** and **Figure 3-18**).

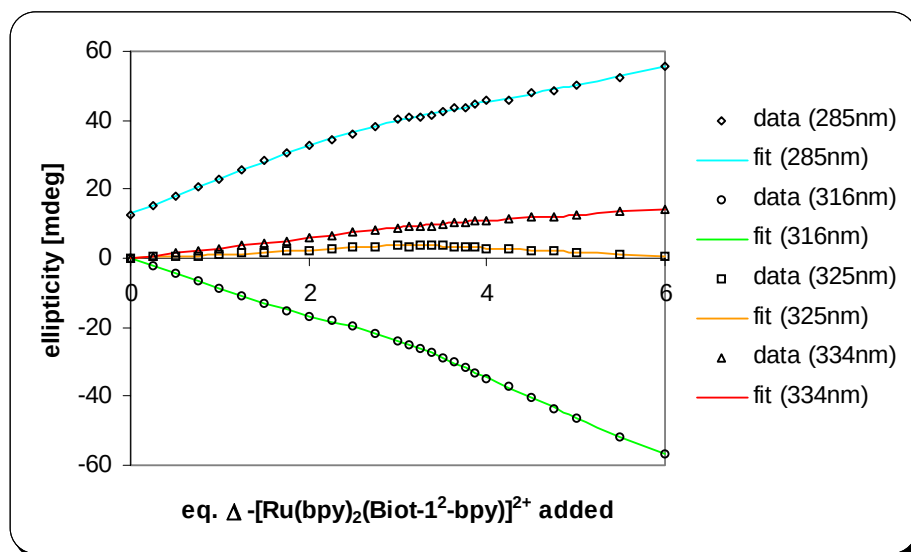


Figure 3-28. Measured and fitted (by SPECFIT/32) data points at different wavelengths for the formation of Δ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺⊂avidin upon addition of Δ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺ (0.64mM) to avidin. Initial avidin concentration: 8μM; solvent: phosphate buffer (pH = 7.0, I = 0.3M).

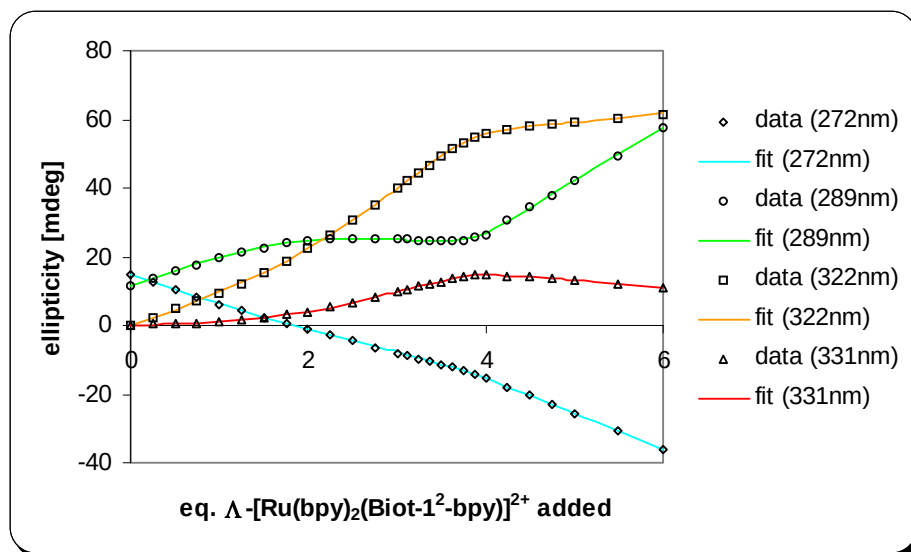


Figure 3-29. Measured and fitted (by SPECFIT/32) data points at different wavelength for the formation of Δ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺⊂avidin upon addition of Δ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺ (0.96mM) to avidin. Initial avidin concentration: 8.00μM; solvent: phosphate buffer (pH = 7.0, I = 0.3M).

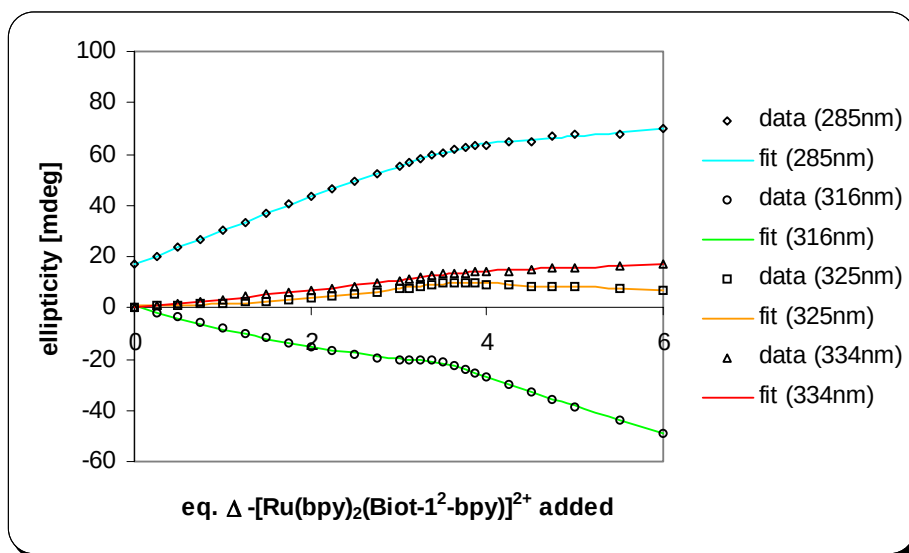


Figure 3-30. Measured and fitted (by SPECFIT/32) data points at different wavelengths for the formation of Δ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺ streptavidin upon addition of Δ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺ (0.64mM) to streptavidin. Initial streptavidin concentration: 8.00 μ M; solvent: phosphate buffer (pH = 7.0, I = 0.3M).

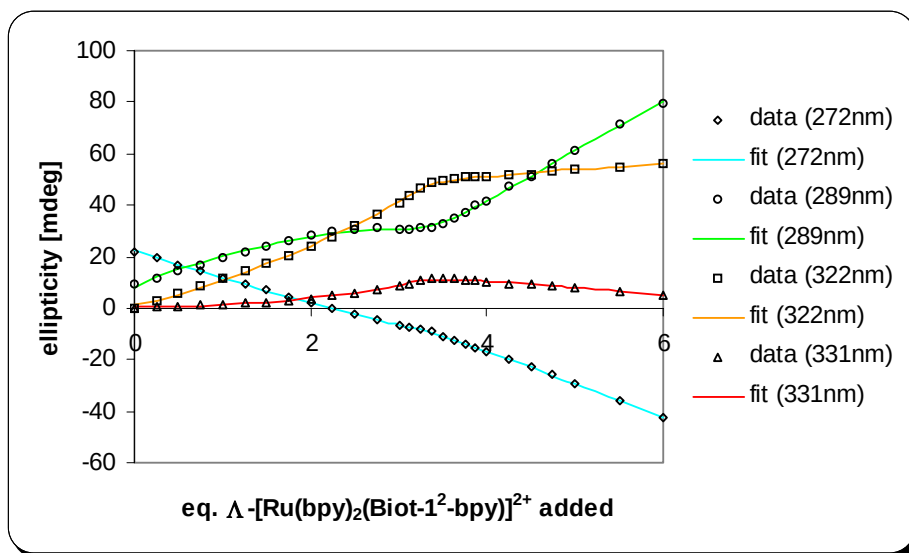


Figure 3-31. Measured and fitted (by SPECFIT/32) data points at different wavelengths for the formation of Λ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺ streptavidin upon addition of Λ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺ (0.96mM) to streptavidin. Initial streptavidin concentration: 8.00 μ M; solvent: phosphate buffer (pH = 7.0, I = 0.3M).

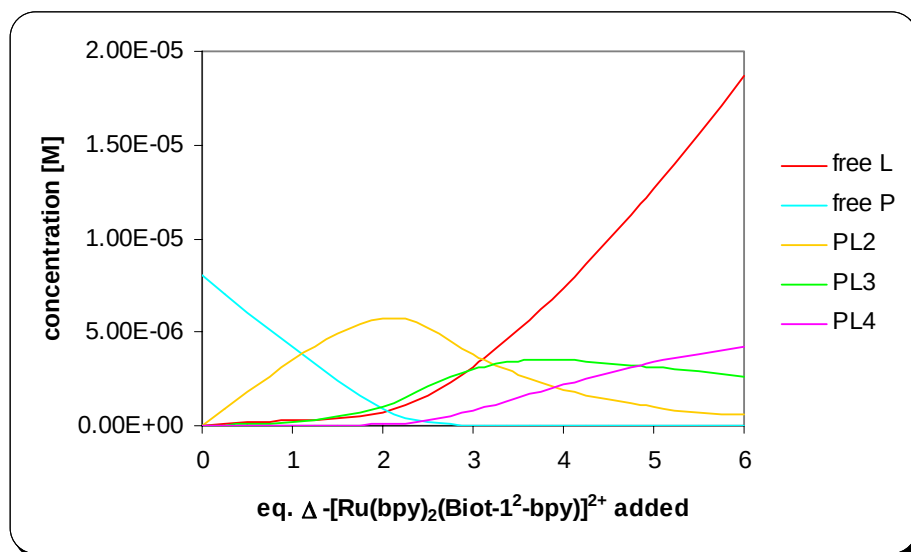


Figure 3-32. Concentration profiles (by SPECFIT/32) of the CD active species free L, free P, PL₂, PL₃ and PL₄, where L refers to the ligand (Δ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺) and P to the protein (avidin). PL₂, PL₃ and PL₄ correspond to the di-, tri- and tetra-liganded protein. Solvent: phosphate buffer (pH = 7.0, I = 0.3M).

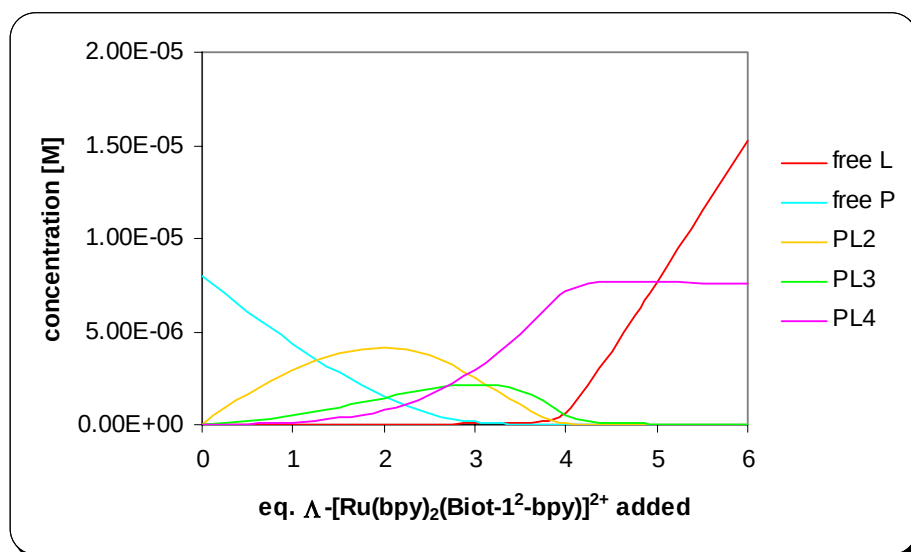


Figure 3-33. Concentration profiles (by SPECFIT/32) of the CD active species free L, free P, PL₂, PL₃ and PL₄, where L refers to the ligand (Δ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺) and P to the protein (avidin). PL₂, PL₃ and PL₄ correspond to the di-, tri- and tetra-liganded protein. Solvent: phosphate buffer (pH = 7.0, I = 0.3M).

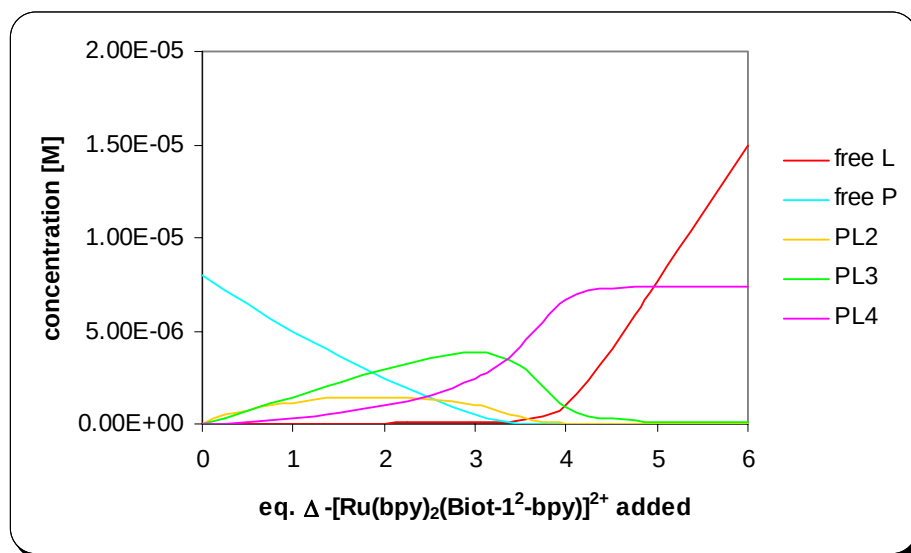


Figure 3-34. Concentration profiles (by SPECFIT/32) of the CD active species free L, free P, PL₂, PL₃ and PL₄, where L refers to the ligand (Δ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺) and P to the protein (streptavidin). PL₂, PL₃ and PL₄ correspond to the di-, tri- and tetra-liganded protein. Solvent: phosphate buffer (pH = 7.0, I = 0.3M).

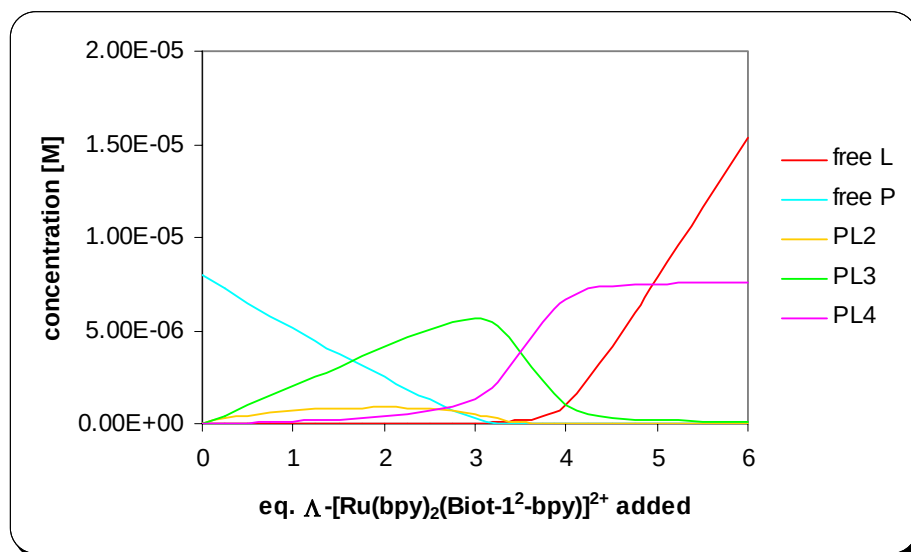


Figure 3-35. Concentration profiles (by SPECFIT/32) of the CD active species free L, free P, PL₂, PL₃ and PL₄, where L refers to the ligand (Δ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺) and P to the protein (streptavidin). PL₂, PL₃ and PL₄ correspond to the di-, tri- and tetra-liganded protein. Solvent: phosphate buffer (pH = 7.0, I = 0.3M).

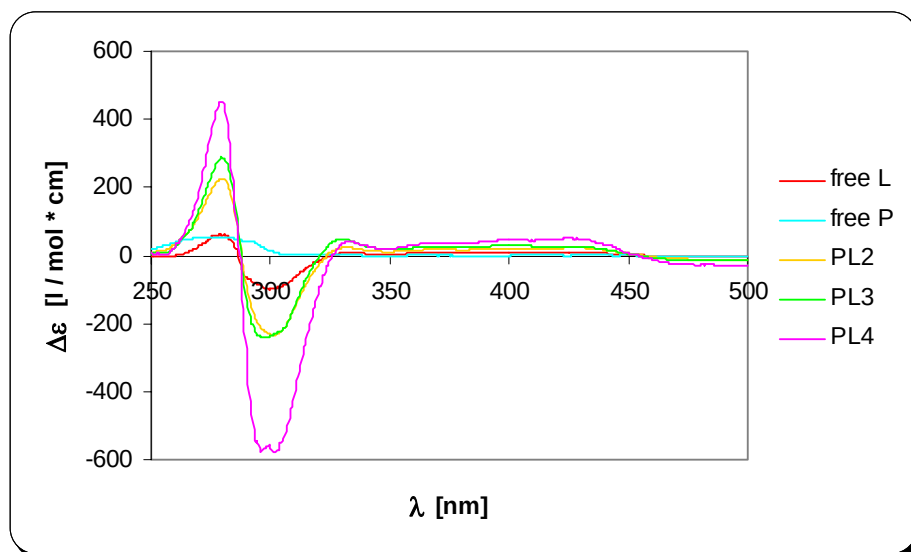


Figure 3-36. Calculated CD ($\Delta\epsilon$) spectra (by SPECFIT/32) of the CD active species free L, free P, PL₂, PL₃ and PL₄, where L refers to the ligand (Δ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺) and P to the protein (avidin). PL₂, PL₃ and PL₄ correspond to the di-, tri- and tetra-liganded protein. Solvent: phosphate buffer (pH = 7.0, I = 0.3M).

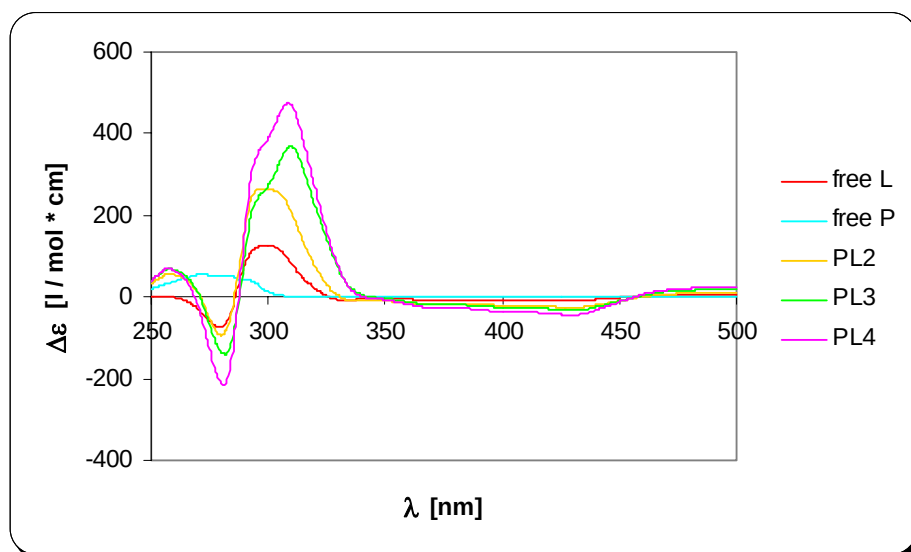


Figure 3-37. Calculated CD ($\Delta\epsilon$) spectra (by SPECFIT/32) of the CD active species free L, free P, PL₂, PL₃ and PL₄, where L refers to the ligand (Δ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺) and P to the protein (avidin). PL₂, PL₃ and PL₄ correspond to the di-, tri- and tetra-liganded protein. Solvent: phosphate buffer (pH = 7.0, I = 0.3M).

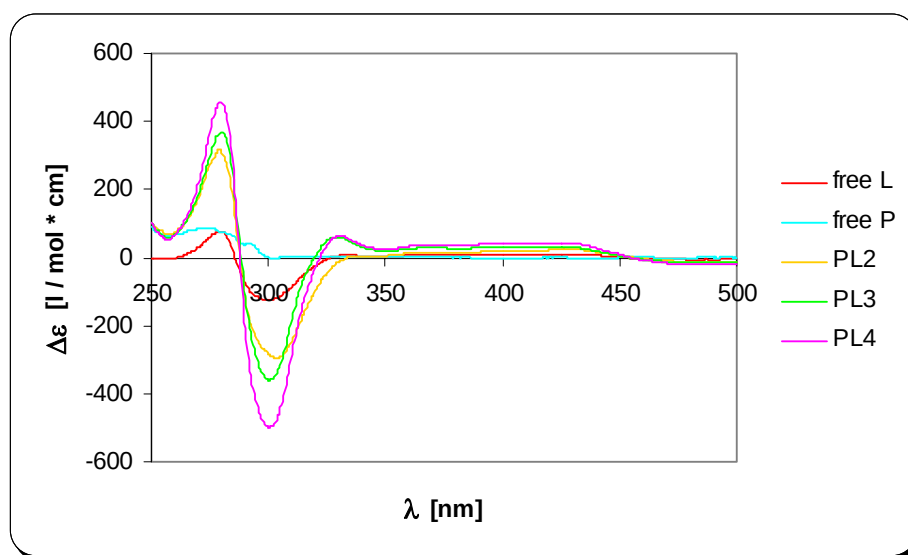


Figure 3-38. Calculated CD ($\Delta\epsilon$) spectra (by SPECFIT/32) of the CD active species free L, free P, PL₂, PL₃ and PL₄, where L refers to the ligand (Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺) and P to the protein (streptavidin). PL₂, PL₃ and PL₄ correspond to the di-, tri- and tetra-liganded protein. Solvent: phosphate buffer (pH = 7.0, I = 0.3M).

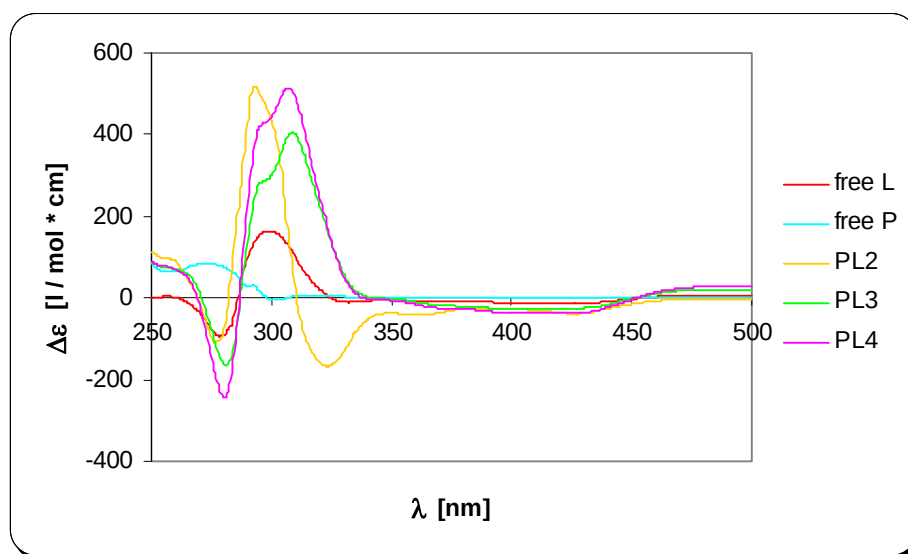


Figure 3-39. Calculated CD ($\Delta\epsilon$) spectra (by SPECFIT/32) of the CD active species free L, free P, PL₂, PL₃ and PL₄, where L refers to the ligand (Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺) and P to the protein (streptavidin). PL₂, PL₃ and PL₄ correspond to the di-, tri- and tetra-liganded protein. Solvent: phosphate buffer (pH = 7.0, I = 0.3M).

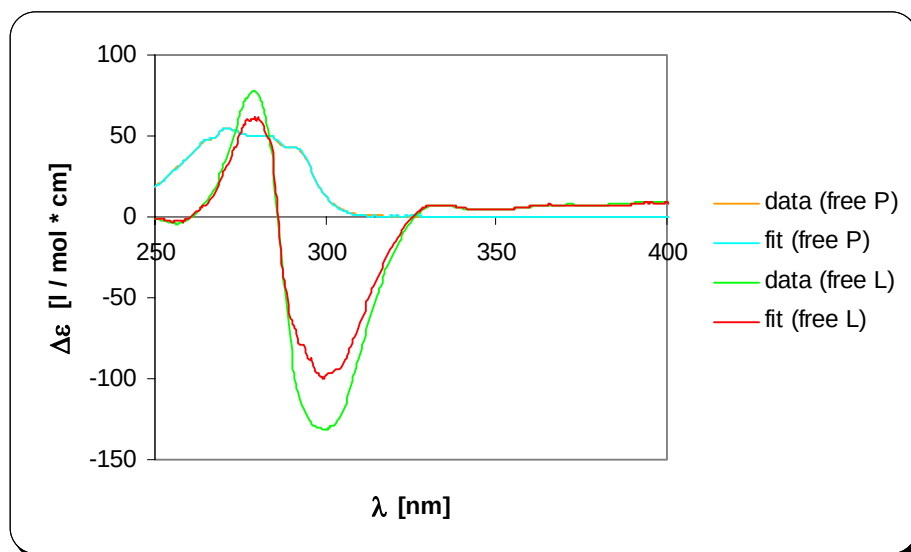


Figure 3-40. Measured and calculated (by SPECFIT/32) CD ($\Delta\epsilon$) spectra for the free protein (free P: avidin) and the free ligand (free L: $\Lambda\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$). Solvent: phosphate buffer (pH = 7.0, I = 0.3M).

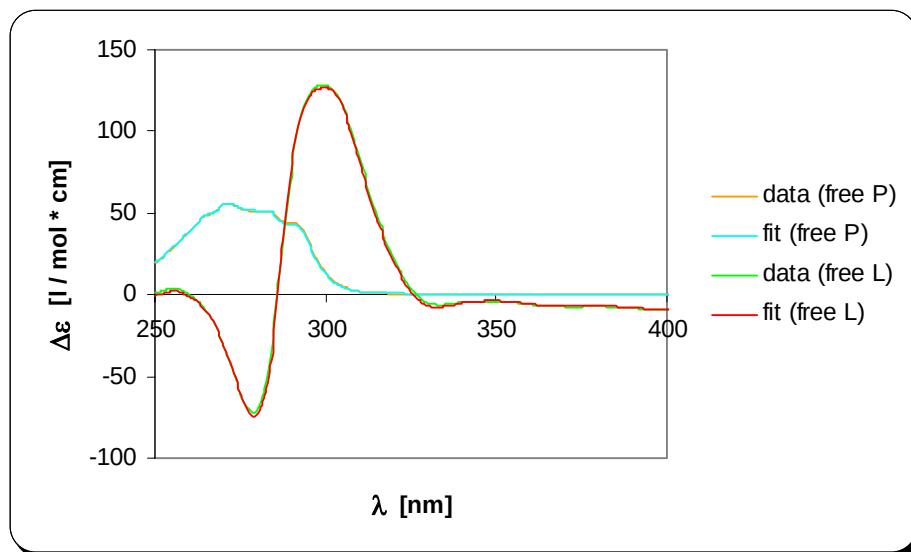


Figure 3-41. Measured and calculated (by SPECFIT/32) CD ($\Delta\epsilon$) spectra for the free protein (free P: avidin) and the free ligand (free L: $\Lambda\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$). Solvent: phosphate buffer (pH = 7.0, I = 0.3M).

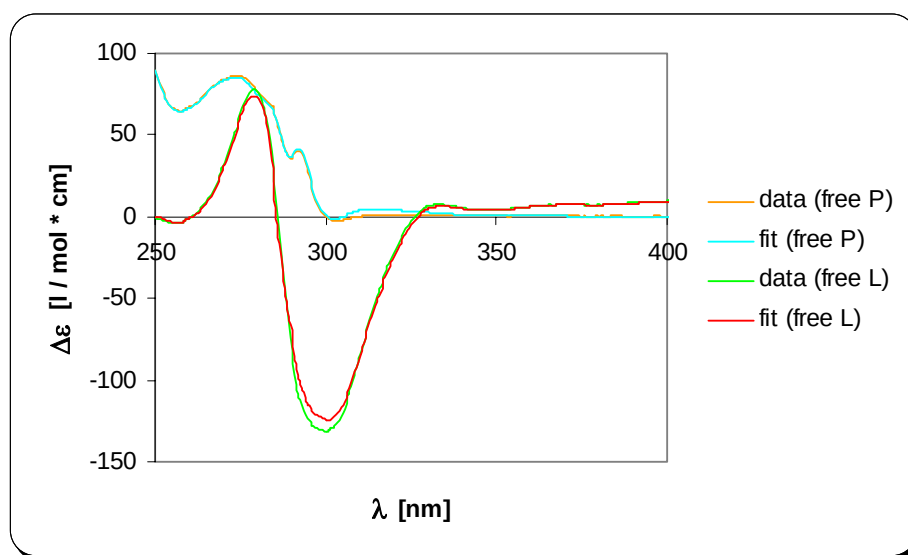


Figure 3-42. Measured and calculated (by SPECFIT/32) CD ($\Delta\epsilon$) spectra for the free protein (free P: streptavidin) and the free ligand (free L: Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺). Solvent: phosphate buffer (pH = 7.0, I = 0.3M).

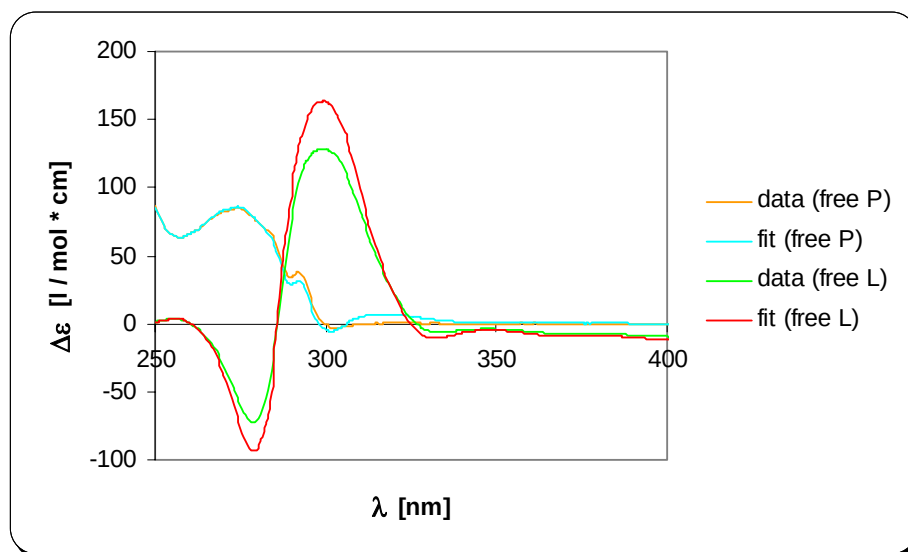


Figure 3-43. Measured and calculated (by SPECFIT/32) CD ($\Delta\epsilon$) spectra for the free protein (free P: streptavidin) and the free ligand (free L: Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺). Solvent: phosphate buffer (pH = 7.0, I = 0.3M).

An overview of all by SPECFIT/32 determined cumulative stability constants β for the formed species PL_2 , PL_3 and PL_4 are listed in **Table 3-5**. The cumulative stability constants β are larger for both host proteins with the Δ -enantiomer than with the Λ -enantiomer, whereby the difference in avidin is much more pronounced, in contrary to the exchange procedure with HABA, where it is the case for streptavidin.

	WT Avi	WT Sav	Species
$\log(\beta_{2\Delta-[Ru(bpy)_2(Biot-1^2-bpy)]^{2+}})$	13.1 ± 0.4	14.4 ± 0.6	PL_2
$\log(\beta_{3\Delta-[Ru(bpy)_2(Biot-1^2-bpy)]^{2+}})$	18.5 ± 0.6	22.1 ± 0.7	PL_3
$\log(\beta_{4\Delta-[Ru(bpy)_2(Biot-1^2-bpy)]^{2+}})$	23.4 ± 0.7	28.9 ± 0.8	PL_4
$\log(\beta_{2\Lambda-[Ru(bpy)_2(Biot-1^2-bpy)]^{2+}})$	15.7 ± 0.3	15.1 ± 0.7	PL_2
$\log(\beta_{3\Lambda-[Ru(bpy)_2(Biot-1^2-bpy)]^{2+}})$	22.9 ± 0.4	23.6 ± 0.9	PL_3
$\log(\beta_{4\Lambda-[Ru(bpy)_2(Biot-1^2-bpy)]^{2+}})$	30.3 ± 0.4	30.3 ± 1.0	PL_4

Table 3-5. Overview of all by SPECFIT/32 determined cumulative stability constants β ($\log(\beta)$ values) for the formed species PL_2 , PL_3 and PL_4 upon addition of both Δ - or Λ - $[Ru(bpy)_2(Biot-1^2-bpy)]^{2+}$ to both WT Avi and WT Sav.

As mentioned above, the stability constant K_1 ($=\beta_1$) for the formation of PL could not be determined. Possibilities to achieve this aim are either to gather more data points at the beginning of the titration series, where PL is mostly present (between 0 and 1 equivalent of added Δ - or Λ - $[Ru(bpy)_2(Biot-1^2-bpy)]^{2+}$ complex) or to perform a reverse titration series by adding the protein to the Δ - or Λ - $[Ru(bpy)_2(Biot-1^2-bpy)]^{2+}$ complex. A reason for not having been able to determine the stability constant K_1 of PL could be the presence of a strong

cooperativity between PL and PL₂. Looking at the affinity constants in **Table 3-5**, it seems that all listed logβ values are more or less statistical, suggesting non-cooperativity between PL₂ and PL₃ as well as between PL₃ and PL₄.

3.2.4. Cooperativity: Scatchard and Hill Plot

The Scatchard plots for the four investigated systems are depicted for Δ-[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin in **Figure 3-44**, for Δ-[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin in **Figure 3-45**, for Δ-[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂streptavidin in **Figure 3-46** and for Δ-[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂streptavidin in **Figure 3-47**. All of these Scatchard plots (using concentrations obtained by SPECFIT/32), showing positive cooperativity, are markedly different from those discussed in the introduction (**Figure 1-8** and **Figure 1-9**) for independent, non-interacting sites.

At low values of n, the curve increases and reaches a maximum at

$$v_{\max} = \frac{n \cdot (\alpha_H - 1)}{\alpha_H} \quad (3.10)$$

and decreases again to the intercept $v = n$ (except for Δ-[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin).¹⁰⁶

The Hill coefficient (Hill constant) α_H can be determined by rearranging **Equation 3.10**. The obtained values for α_H following **Equation 3.11** are listed in **Table 3-6**.

$$\alpha_H = \frac{n}{n - v_{\max}} \quad (3.11)$$

Though the Hill coefficient α_H can be determined from the Scatchard plot, Hill plots are usually used to do this. By plotting $\ln[\gamma/(1-\gamma)]$ vs $\ln[L]_{\text{free}}$, where $[L]_{\text{free}}$ is the free ligand concentration and γ the fractional saturation, the α_H parameter can be directly obtained from the slope of the plot. In general, this plot does not hold for the entire range of γ values.

Looking at the Hill plot, the Hill coefficient α_H is normally evaluated at $\gamma = 1/2$, thus at $\ln = 1$ ($y = 0$). Using this type of Hill plot, the average value of the stability constant $-\ln(K_a)$ can be estimated (crossing point with the x-axis).

ligand $\subset$ protein system	α_H (by Hill plot)	α_H (by Scatchard plot)
Δ -[Ru(bpy) ₂ (Biot-1²-bpy)] ²⁺ $\subset$ WT Avi	—	$\approx 1.4^a$
Λ -[Ru(bpy) ₂ (Biot-1²-bpy)] ²⁺ $\subset$ WT Avi	1.35	$\approx 1.6^a$
Δ -[Ru(bpy) ₂ (Biot-1²-bpy)] ²⁺ $\subset$ WT Sav	2.06	≈ 2.0
Λ -[Ru(bpy) ₂ (Biot-1²-bpy)] ²⁺ $\subset$ WT Sav	1.94	≈ 2.0
O ₂ $\subset$ hemoglobin	2.80 ^b	—

Table 3-6. Hill coefficients for the four systems Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ $\subset$ avidin, Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ $\subset$ avidin, Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ $\subset$ streptavidin and Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ $\subset$ streptavidin (using the free ligand concentration obtained by SPECFIT/32).

^a value is not reliable ^b S. J. Edelstein¹⁵⁷

The Hill plots are shown for Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ $\subset$ avidin in **Figure 3-48**, for Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ $\subset$ avidin in **Figure 3-49**, for Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ $\subset$ streptavidin in **Figure 3-50** and for Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ $\subset$ streptavidin in **Figure 3-51**. The obtained α_H coefficient value for the Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ $\subset$ avidin system are not reliable (Scatchard plot) or undetermined (Hill plot). This comes from the fact that it seems that the final equilibrium is not reached (see the concentration profile in **Figure 3-29**).

By applying the Hill plot, where $\ln[L]_{\text{free}}$ is a function of $\ln[(n/v)-1]$, the slope corresponds to $-1/\alpha_H$ and the intercept corresponds to $-\ln(K_a)$. The stability constant K_a (average value for one binding site) should be the same as that one determined by SPECFIT/32 because the free ligand concentration used in the Scatchard and the Hill plots, respectively, are derived from the values predicted by SPECFIT/32.

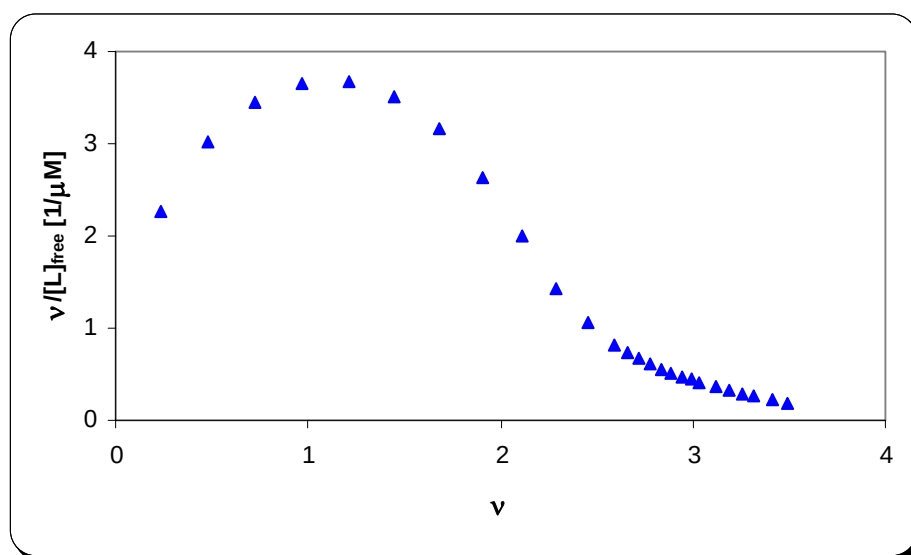


Figure 3-44. Scatchard plot for Δ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺-avidin.

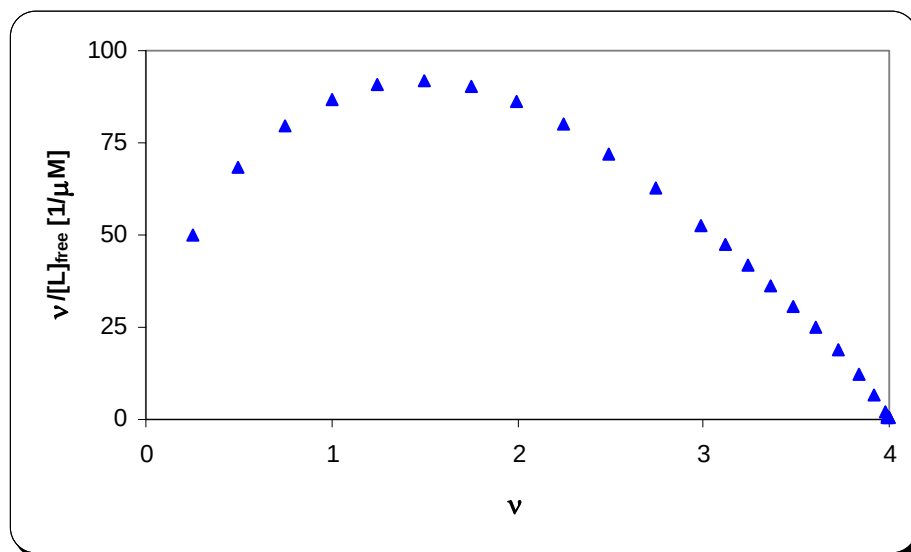


Figure 3-45. Scatchard plot for Δ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺-avidin.

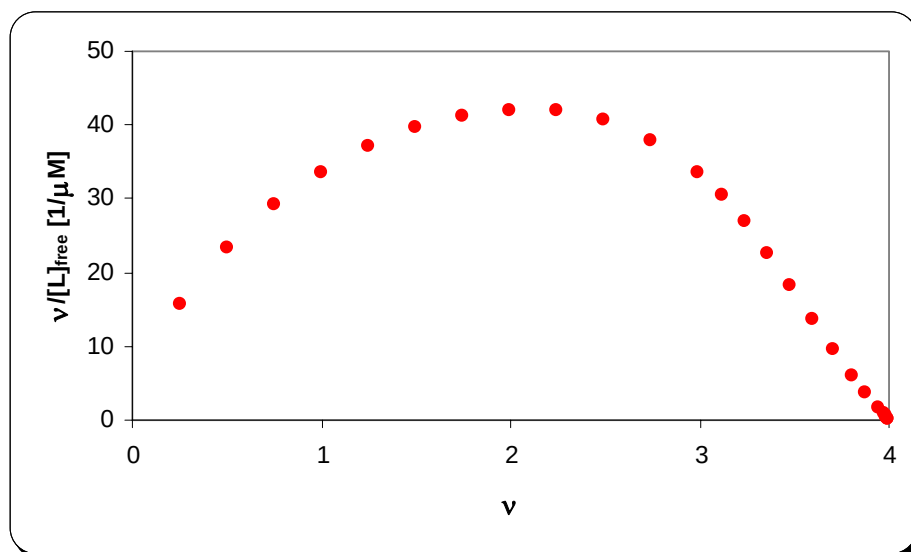


Figure 3-46. Scatchard plot for $\Delta\text{-[Ru(bpy)}_2\text{(Biot-1}^2\text{-bpy)]}^{2+}\text{-streptavidin}$.

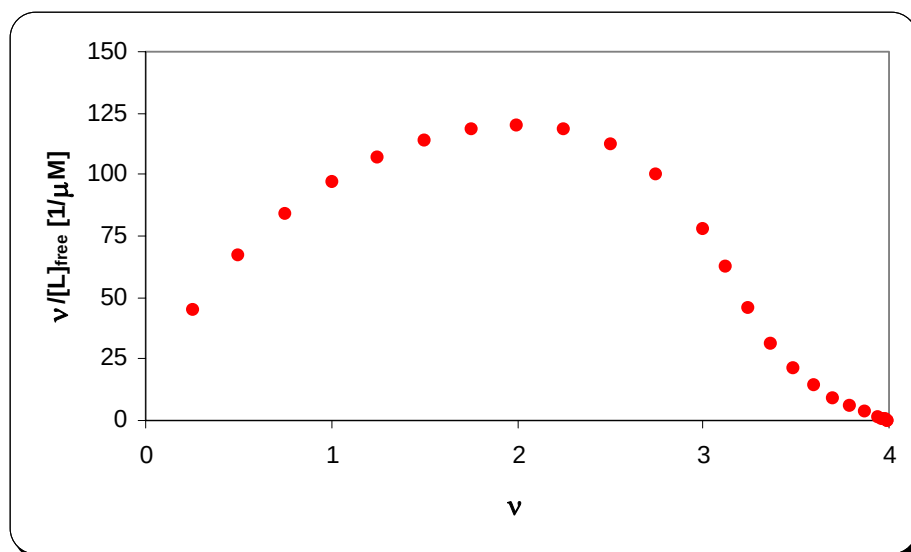


Figure 3-47. Scatchard plot for $\Lambda\text{-[Ru(bpy)}_2\text{(Biot-1}^2\text{-bpy)]}^{2+}\text{-streptavidin}$.

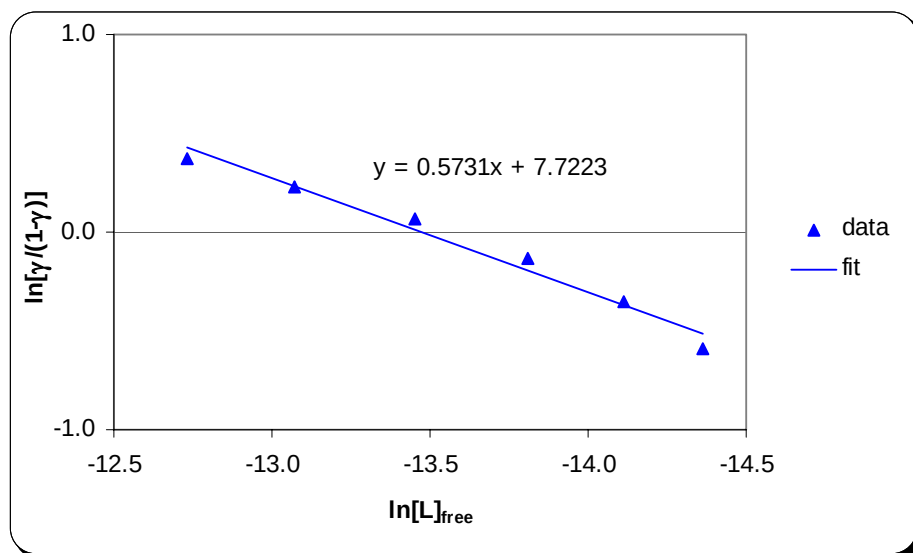


Figure 3-48. Hill plot for Δ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺-avidin.

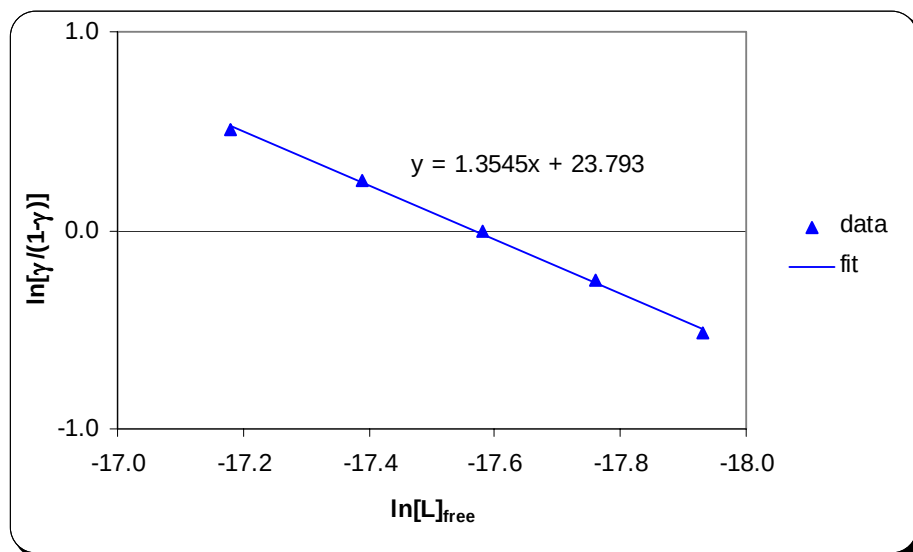


Figure 3-49. Hill plot for Δ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺-avidin.

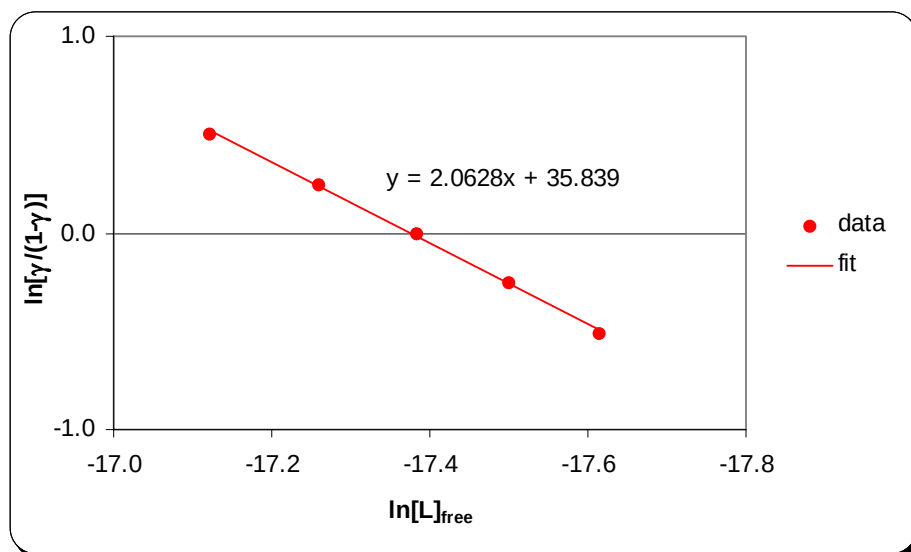


Figure 3-50. Hill plot for Δ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺-streptavidin.

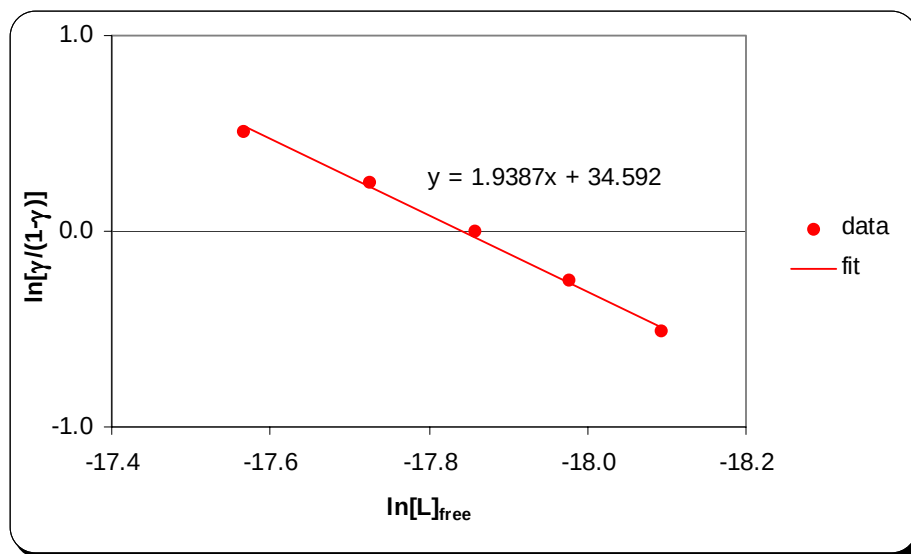


Figure 3-51. Hill plot for Λ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺-streptavidin.

Comparing the Hill coefficients obtained from both Scatchard and Hill plot, it seems that the positive cooperativity in streptavidin is larger than in avidin for both pseudo-enantiomers. For streptavidin, the value of the Hill parameter is around 2, whereas for avidin it is only around 1.5. The cooperative binding of Δ/Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂(strept)avidin does not act in an “all-or-none” fashion, analogously to oxygen binding to hemoglobin, where the Hill coefficient is around 2.8. A significant difference between the investigated systems and the O₂⊂hemoglobin system is the kind and the order of magnitude of cooperativity. In the O₂⊂hemoglobin system, the second and the third O₂ molecule are by a factor 3 and the fourth O₂ molecule by a factor 300 stronger bound to hemoglobin than the first O₂ molecule, showing a strong positive cooperativity at the end (between PL₃ and PL₄), whereas in our investigated systems it seems that the positive cooperativity occurs at the beginning (between PL and PL₂) because all in **Table 3-5** listed logβ values are more or less statistical, suggesting non-cooperativity between PL₂ and PL₃ as well as between PL₃ and PL₄.

3.2.5. Kinetics: Measurement and Determination of

$$k_{\text{off}} [\Delta\text{- and } \Lambda\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+} \subset (\text{strept})\text{avidin}]^{158}$$

All kinetic measurements were performed at 298K. Thereby, the host proteins were first loaded with an excess of $\Delta\text{-}$ or $\Lambda\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$ (1.2eq relative to the tetrameric protein) to ensure full saturation of the active sites. Then a large excess of biotin was added to $\Delta\text{-}$ or $\Lambda\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+} \subset (\text{strept})\text{avidin}$ to favour the exchange process. The kinetic parameters (k_{off} values) were determined by following the disappearing signal centered either at 325nm ($\Lambda\text{-}$ enantiomer, no CD signal at this wavelength for the free complex) or 327nm ($\Delta\text{-}$ enantiomer, no CD signal at this wavelength for the free complex) caused by the $\Delta\text{-}$ or $\Lambda\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+} \subset (\text{strept})\text{avidin}$ interaction. The measured and fitted (by ORIGIN)¹⁵⁸ curves of the kinetic measurement in avidin is depicted in **Figure 3-52** (for both $\Delta\text{-}$ and $\Lambda\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$). The same is shown in **Figure 3-53** for streptavidin, also for both $\Delta\text{-}$ and $\Lambda\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$.

The dissociation rate constants of all four systems (both $\Delta\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$ and $\Lambda\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$ in WT Avi and WT Sav, respectively) are listed in **Table 3-7**.

ligand $\subset$ protein system	k_1 [min^{-1}]	k_2 [min^{-1}]
$\Delta\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+} \subset \text{WT Avi}$	1.8×10^{-2} ($R^2 = 0.982$)	6.2×10^{-3} ($R^2 = 0.982$)
$\Lambda\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+} \subset \text{WT Avi}$	7.3×10^{-3} ($R^2 = 0.999$)	8.2×10^{-4} ($R^2 = 0.999$)
$\Delta\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+} \subset \text{WT Sav}$	7.6×10^{-3} ($R^2 = 0.996$)	1.3×10^{-3} ($R^2 = 0.996$)
$\Lambda\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+} \subset \text{WT Sav}$	3.5×10^{-3} ($R^2 = 0.999$)	5.6×10^{-4} ($R^2 = 0.999$)

Table 3-7. Dissociation rate constants k_1 and k_2 (by ORIGIN) for both $\Delta\text{-}$ and $\Lambda\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$ upon addition of biotin to $\Delta\text{-}$ or $\Lambda\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+} \subset (\text{strept})\text{avidin}$ at 298K.

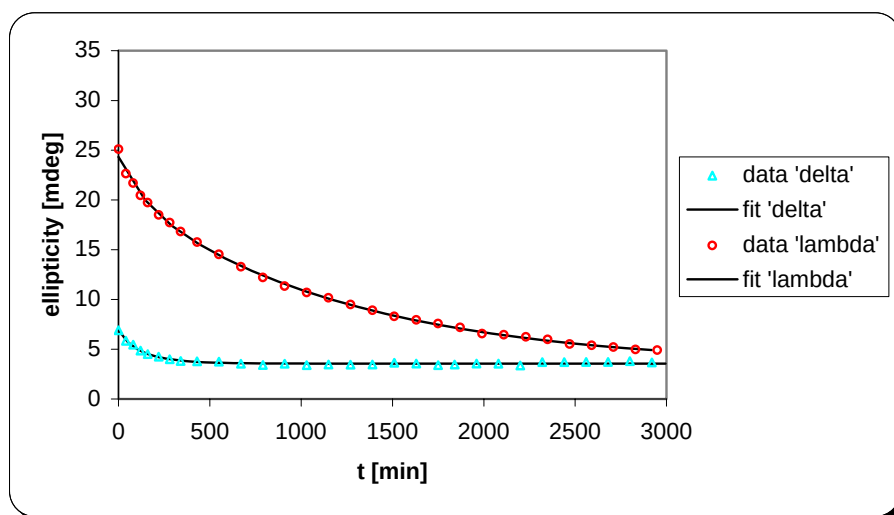


Figure 3-52. Measured and fitted (by ORIGIN) data points of the CD signal at either 325nm (for the Λ -enantiomer, 'lambda') or 327nm (for the Δ -enantiomer, 'delta') upon addition of biotin to Δ - or Λ - $[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$ -cavidin at 298K. Solvent: phosphate buffer (pH = 7.0, I = 0.3M).

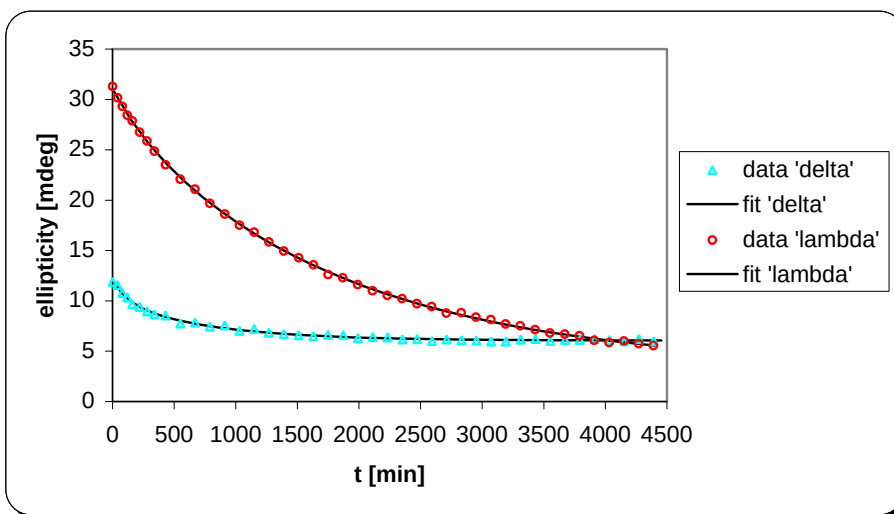


Figure 3-53. Measured and fitted (by ORIGIN) data points of the CD signal at either 325nm (for the Λ -enantiomer, 'lambda') or 327nm (for the Δ -enantiomer, 'delta') upon addition of biotin to Δ - or Λ - $[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$ -streptavidin at 298K. Solvent: phosphate buffer (pH = 7.0, I = 0.3M).

The difference between Δ - and Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ is, as in the determination of the stability constants, larger in avidin than in streptavidin. In avidin, the dissociation rate constant k_1 (release of third and fourth bound “ligands”) and k_2 (release of first and second bound “ligands”) differ by a factor 2.5 and 7.6, respectively, whereas in streptavidin the difference between both pseudo-enantiomers is for k_1 and for k_2 a factor 2.2 and 2.3, respectively. Comparing k_1 and k_2 within the same system, the difference for Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin is a factor 9, whereas for Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin it is only a factor 3. The difference for Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂streptavidin as well as for Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂streptavidin is a factor of about 6. The best fit was achieved by fitting the curve with a two-step reaction. The two-step reaction is coincident with conformational changes (after the occupation of the first two binding sites of (strept)avidin) found in thermodynamics. From the kinetic data, it seems that first, the third and forth bound Δ - or Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ “ligands” are displaced by biotin, and then, upon structural changes within the host protein, also the first and second bound Δ - or Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ “ligands”.

Thermodynamics and kinetics are thus coincident with the assumption that the binding in all four systems (Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin, Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂avidin, Δ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂streptavidin and Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺⊂streptavidin) occurs with structural changes after the occupation of the first two binding sites. Moreover, the difference between Δ - and Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ is in thermodynamics as well as for kinetics larger for avidin than for streptavidin.

Alea iacta est!

Gaius Iulius Caesar, 100 BC – 44 BC

CHAPTER 4:

SUMMARY AND OUTLOOK

In the field of enantioselective catalysis, the second coordination sphere (solvent, counterion, added salt etc.) often dramatically influences the properties of a catalyst. Incorporation of an achiral catalyst precursor in a protein ensures that any level of enantioselection is induced by the second coordination sphere provided by the protein. However, in order to ensure the localization of the catalyst precursor within the protein, a very strong non-covalent host-guest (*i.e.* protein-inhibitor) system should be selected. This one is found in the biotin-(strept)avidin system. The principle of the biotin-(strept)avidin technology (often referred to as molecular velcro) relies on the extraordinary affinity of biotin for either avidin ($K_a = 10^{15} \text{ M}^{-1}$) or streptavidin ($K_a = 10^{13} \text{ to } 10^{14} \text{ M}^{-1}$).

In our research group, the biotin-(strept)avidin technology is applied by using modified biotin-(strept)avidin systems for:

- a) enantioselective hydrogenation catalysis
- b) photosystem II analogues

In the past four years, we have demonstrated that in a) enantioselection by the achiral catalyst $[\text{Rh}(\text{COD})(\text{Biot-NP}_2)]^+$ is induced by the second coordination sphere provided by (strept)avidin, whereas in b) the microenvironment of the protein can influence excited state lifetime properties of the prototypical biotinylated coordination complex $[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$. For both these areas of research, a fundamental concern is the affinity of the biotinylated organometallic moieties towards (strept)avidin.

In case a), it was shown that the affinity of the achiral catalyst $[\text{Rh}(\text{COD})(\text{Biot-NP}_2)]^+$ for (strept)avidin is markedly reduced ($K_a = \text{ca. } 10^7\text{-}10^8 \text{ M}^{-1}$), but almost independent of the isoelectric point of the protein and essentially constant. Thus, at a given pH, the affinity of $[\text{Rh}(\text{COD})(\text{Biot-NP}_2)]^+$ for a protein does not depend on the isoelectric point of the host protein. Therefore, all investigated proteins (avidin, streptavidin) are suitable for enantio-selective hydrogenation catalysis. The difference in performance is not caused by a difference in affinity but, most probably, by a difference in the host protein's binding pocket topography.

In case b), during the determination of the stability constant, we experienced difficulties in fitting the data to get information on the affinity of racemic $[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$ towards (strept)avidin. It was suspected that this arose from the fact that both pseudo-enantiomers of $[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$ may have different affinities for the host proteins. By using von Zelewsky's procedure for the enantiomer separation of *cis*- Δ -/ Λ - $[\text{Ru}(\text{bpy})_2(\text{py})_2]^{2+}$, both pseudo-enantiomers of $[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$ could be synthesized in enantiomerically pure form. Cumulative stability constant determinations using SPECFIT/32 yielded for avidin dramatically different $\log(\beta)$ values between Δ - and Λ - $[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$, whereas for streptavidin the $\log(\beta)$ values for both Δ - and Λ - $[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$ were much more similar, but still different. Avidin showed therefore much stronger chiral discrimination properties between both biotinylated enantiopure probes than streptavidin. Moreover, the spectra of the di-liganded protein and the tri-liganded protein (tetra-liganded protein), are different from each other (for Λ - $[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+} \subset (\text{strept})\text{avidin}$), suggesting stronger stereospecific interactions and a possible change in conformation within the Λ - $[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+} \subset (\text{strept})\text{avidin}$ system.

Scatchard and Hill plot have shown that there is positive cooperativity between Δ - or Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ and (strept)avidin. The determined $\log(\beta_2)$, $\log(\beta_3)$ and $\log(\beta_4)$ values seem to be more or less statistical, suggesting that positive cooperativity takes place at the beginning, hence between the mono- liganded protein and the di-liganded protein.

From the kinetic data, it seems that first the third and forth bound Δ - or Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ “ligands” are displaced by biotin, and then, upon structural changes within the host protein, also the first two bound Δ - or Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ “ligands”.

Thermodynamics and kinetics are thus coincident with the assumption that the binding in all investigated systems occurs with structural changes within the protein.

There are several possibilities to proceed:

By performing titration measurements at several temperatures, one could express the rate constant as a function of temperature. Two forms are commonly used to show the temperature dependence of the rate constant.

The first is the familiar Arrhenius equation,

$$k = A \cdot \exp\left(-\frac{E_a}{RT}\right) \quad (4.1)$$

If a data set containing {k,T}-pairs is fitted to this equation, the values of A, the pre-exponential factor, and E_a, the Arrhenius activation energy (sometimes simply the activation energy), are obtained.

The second equation used to express the temperature dependence of a rate constant results from transition state theory (TST). Its form is

$$k = \kappa \frac{k_B T}{h} \cdot \exp\left(\frac{\Delta S^\ddagger}{R}\right) \cdot \exp\left(-\frac{\Delta H^\ddagger}{RT}\right) \quad (4.2)$$

where k_B is Boltzman's constant, h Planck's constant and R the gas constant. The data fit allows the evaluation of the parameters $\Delta S^\ddagger$, the standard entropy of activation, and $\Delta H^\ddagger$, the standard enthalpy of activation.

The traditional method of fitting data to these equations uses logarithmic forms. Thus, applying the Arrhenius equation (**Equation 4.1**), $\ln(k)$ will vary linearly with $1/T$. This plot yields A from the intercept and E_a from the slope. Taking the logarithmic form for the TST equation (**Equation 4.2**), $\ln(k/T)$ will be a linear function of $1/T$ if the data set follows this equation, as most do. The slope yields $\Delta H^\ddagger$, a quantity akin to E_a . The intercept, after allowance for $\ln(k_B/h) = 23.760$, provides the value of $\Delta S^\ddagger$. Moreover, knowing K_a (or K_d) from thermodynamics and k_{off} from kinetics, one could determine k_{on} from the relation according to **Equation 4.3**:

$$K_a = \frac{k_{\text{on}}}{k_{\text{off}}} \quad \left(K_d = \frac{k_{\text{off}}}{k_{\text{on}}} \right) \quad (4.3)$$

- By varying the spacer of $[\text{Ru}(\text{bpy})_2(\text{Biot-spacer-bpy})]^{2+}$, the protein's "active site" could be probed systematically to determine, which spacer leads to the largest chiral discrimination, as reflected in the difference in thermodynamic and kinetic data for both biotinylated enantiomers of $[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})]^{2+}$. Having identified the spacer, which affords the largest difference in binding affinity for both enantiomers, substitution of the $[\text{Ru}(\text{bpy})_2]$ -

moiety by a catalytically active species could be done for testing whether this spacer indeed gives the best enantiomeric excess in transfer hydrogenation.

- By optimizing the spacer and eventually the bipyridine moieties to produce $[\text{Ru}(\text{bpy})_2(\text{Biot-spacer-bpy})]^{2+} \subset (\text{strept})\text{avidin}$ systems with long lived excited states, one could envisage introduction of a biotinylated energy or electron acceptor into the host protein. Several readily accessible biotinylated energy or electron acceptors could be incorporated to produce two component supramolecular systems.¹⁵⁹

Nulla sine exitu via est.

Lucius Annaeus Seneca, 3 BC – AD 65

CHAPTER 5:

EXPERIMENTAL PART

5.1. Materials, Instrumentation and Techniques

5.1.1. Abbreviations Used

DMF	<i>N,N'</i> -dimethylformamide
CD	circular dichroism
CH ₂ Cl ₂	dichloromethane
CH ₃ CN	acetonitrile
d	day
DCC	1,3-dicyclohexylcarbodiimide
DMSO	dimethylsulfoxide
EA	ethyl acetate
eq	equivalent
ESI	electron spray ionisation
EtOH	ethanol
g, mg	gram, milligram
h	hour
HABA	2-(4'-hydroxyazobenzene)benzoic acid
H ₂ O	water
HCl	hydrochloride acid
KOH	potassium hydroxide
L, mL, µL	liter, milliliter, microliter
LiCl	lithium chloride
MeOH	methanol
min	minute
MLCT	metal-to-ligand charge transfer

mol, mmol, μ mol, nmol	mole, millimole, micromole, nanomole
MS	mass spectrometry
NaOH	sodium hydroxide
NH ₄ PF ₆	ammonium hexafluorophosphate
NHS	<i>N</i> -hydroxysuccinimide
NMR	nuclear magnetic resonance
rt	room temperature
s	second
TEA	triethylamine
TMS	tetramethylsilane
UV/Vis	ultraviolet/visible

5.1.2. Reagents and Solvents

Commercial reagents were purchased from Acros, Geel (B), Aldrich, Steinheim (D), Belovo, Bastogne (B), Chanzhou Huaren, Jiangsu (RC), Fluka, Buchs SG (CH), Merck, Darmstadt (D), Prochimie, Avenches (CH), Riedel-de-Haën, Seelze (D), SDS, Peypin (F) and Sigma, St Louis MO (USA) according to **Table 5-1**.

Table 5-1. Origin and purity of the reagents used.

Substance	Origin	Quality Grade and Purity
ammonium hexafluorophosphate	Fluka	$\geq 98\%$ (gravimetric)
avidin	Belovo	
(+)-biotin (<i>d</i> -biotin)	Chanzhou Huaren	
2,2'-bipyridine	Fluka	purum $\geq 98\%$ (NT)
1,3-diaminopropane	Aldrich	$\geq 99\%$
1,5-diaminopentane	Aldrich	$\geq 95\%$
(+)-O,O'-dibenzoyl-D-tartaric acid	Fluka	$\geq 99\%$ (T)

(-)-O,O'-dibenzoyl-L-tartaric acid	Fluka	≥ 99% (T)
1,3-dicyclohexylcarbodiimide	Aldrich	≥ 99%
N,N'-dimethylformamide	Fluka	≥ 99.8% (GC)
ethanol	Merck	p.a. ≥ 99.8% (GC)
ethyl nicotinate	Fluka	≥ 98% (GC)
ethylene glycol	Fluka	≥ 99.5% (GC)
ethylenediamine	Fluka	≥ 99% (GC)
hydrochloric acid	Prochimie	solution 32%
2-(4'-hydroxyazobenzene)benzoic acid	Sigma	≥ 98% (TLC)
lithium chloride hydrate anhydrous	Fluka	puriss. p.a.
2,3,4,5,6-pentafluorophenol	Fluka	≥ 99% (GC)
<i>m</i> -phenylenediamine	Aldrich	≥ 99%
<i>o</i> -phenylenediamine	Fluka	≥ 98%
<i>p</i> -phenylenediamine	Aldrich	≥ 97%
phosphate buffer solution pH 7.0	Merck	Titrisol®
potassium hydroxide, pellets	Fluka	≥ 85% (T)
pyridine	SDS	≥ 99.5%
Raney nickel	Fluka	puriss. p.a.
ruthenium(III) chloride hydrate	Acros	35-40% Ru
sodium hydroxide, pellets	Fluka	puriss. p.a. ACS ≥ 98%
sodium perchlorate	Fluka	puriss. p.a. ACS ≥ 99%
streptavidin	own production	
thionyl chloride	Fluka	purum ≥ 99%
triethylamine	Riedel-de-Haën	≥ 99% (GC)
xylene (mixtures of isomers)	Fluka	puriss. p.a.

For column chromatography, extractions and crystallizations, solvents of technical grade were distilled and for reactions, solvents of technical grade were absolved and under nitrogen distilled before use. Thionyl chloride was distilled under nitrogen.

For spectroscopic measurements (UV/Vis, CD, NMR), solvents of analytical grade were employed. In the case of phosphate buffer, milliQ-water was used.

All reactions and manipulations involving rhodium were performed under a nitrogen atmosphere using glove box and standard Schlenk techniques.

Nitrogen (99.995%; commercial gas cylinders) for air-sensitive syntheses and CD measurements was purchased from Carbagas, Bern (CH) and was used without further purification.

5.1.3. Preparative Methods

5.1.3.1. Column Chromatography

- Flash chromatography was carried out on silica gel 60, 32-63 μ m from Brunschwig, Basel (CH).
- *SP Sephadex C-25* from Pharmacia LKB, Uppsala (S), were used for ion exchange chromatography. *SP Sephadex* is composed of a cross-linked dextran matrix, functionalized with strongly acidic propanesulfonate groups. The cations to be separated are adsorbed onto the anionic stationary phase, and separation is achieved as a result of differential ion-exchange equilibria involving the components of the mixture and the cations of the eluting electrolytic solution (typically a sodium salt).

5.1.4. Analyses

5.1.4.1. Absorption Spectrophotometry (UV/Vis)

The measurements were performed on a UVIKON 930 spectrophotometer from Kontron/BioTech (manufacturer exists no more). The sample solutions were introduced in 1cm quartz cells from Hellma, Müllheim (D). The spectrophotometric data were treated with Excel.

Indications in the spectroscopical data collection: $\lambda_{\max}$ [nm] ($\epsilon_{\max}$ [$\text{M}^{-1}\text{cm}^{-1}$], attribution)

5.1.4.2. Circular Dichroism (CD)

CD spectra were recorded on a J-710 spectropolarimeter from JASCO, Tokyo (J). The sample solutions were introduced in 1cm quartz cells from Hellma, Müllheim (D). The spectrophotometric data were treated with Excel.

Indications in the spectroscopical data collection: $\lambda_{\max}$ [nm] ($\Delta\epsilon_{\max}$ [$\text{M}^{-1}\text{cm}^{-1}$])

5.1.4.3. Mass Spectrometry (MS)

Mass spectra were recorded on a mass spectrometer LCQ from ThermoFinnigan, San José (USA), and on a mass spectrometer FTMS 4.7T BioAPEX II from Bruker, Billerica (USA) (at the University of Fribourg). The ionisation mode was for both ESI (electron spray ionisation).

Indications in the spectroscopic data collection: M_r of species (relative intensity, attribution).

The relative intensity is given in % with respect to the strongest peak of the spectrum.

5.1.4.4. Nuclear Magnetic Resonance (NMR)¹⁶⁰⁻¹⁶²

Proton and carbon NMR spectra were recorded on spectrometers Gemini (200.00MHz for ^1H , 50.29MHz for ^{13}C) from Varian, Palo Alto (USA), AC-300 (300.00MHz for ^1H , 75.43MHz for ^{13}C ; at the University of Berne) and AMX-400 (400.00MHz for ^1H , 100.58MHz for ^{13}C , 376.31MHz for ^{19}F , 161.93MHz for ^{31}P), both from Bruker, Billerica (USA). The samples were measured either in deuterated chloroform (99.8% D) or in deuterated dimethylsulfoxide (99.5% D) from Cambridge Isotope Laboratories, Andover MA (USA).

Indications in the spectroscopical data collection : δ [ppm] relative to external standards TMS (^1H , ^{13}C), CFCl_3 (^{19}F) and H_3PO_4 (^{31}P) (multiplicity (^1H only), J [Hz] (^1H only), normalized integration values (^1H only), attribution (^1H and ^{13}C only)). The following abbreviations were used: s for singlet, d for doublet, dd for doublet of doublet, ddd for doublet of doublet of doublet, dt for doublet of triplet, t for triplet, q for quadruplet and m for multiplet.

5.1.4.5. Thin Layer Chromatography (TLC)

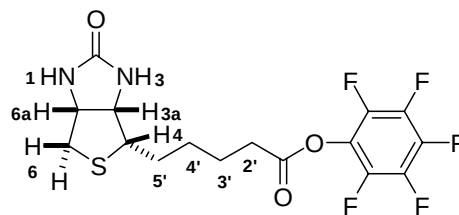
The substances were applied on 0.2mm silicagel 60 F₂₅₄ aluminium sheets from Merck, Darmstadt (D).

5.2. Synthesis Protocols

5.2.1. Synthesis of the Ligands

5-(2-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoic acid pentafluorophenyl ester (**BiotOC₆F₅**)¹³⁷

Biotin (**BiotOH**) (2.00g, 8.2mmol) was dissolved in DMF (25mL) under heating (70°C). To this transparent solution, DCC (2.40g, 11.60mmol, 1.4eq) and pentafluorophenol (2.00g, 10.80mmol, 1.1eq) were added.



Upon cooling to room temperature, the mixture was stirred (3h). The white precipitate (1,3-dicyclohexyl urea) was filtered off. The solvent was evaporated to dryness. The residue was dissolved in MeOH (50mL) and allowed to stand overnight (5°C). The resulting white precipitate (**BiotOC₆F₅**) was filtered, washed with cold MeOH and dried *in vacuo* (yield: 1.61g, 3.92mmol, 48%).

¹H NMR (400.00MHz, DMSO-*d*₆), δ [ppm]: 1.41-1.74 (m, 6H, H-3' & H-4' & H-5'), 2.59 (d, $J = 12.4\text{Hz}$, 1H, H-6), 2.78-2.87 (m, 3H, H-2' & H-6), 3.11-3.15 (m, 1H, H-4), 4.14-4.18 (m, 1H, H-3a), 4.30-4.34 (m, 1H, H-6a), 6.38 (s, 1H, biotin NH), 6.46 (s, 1H, biotin NH)

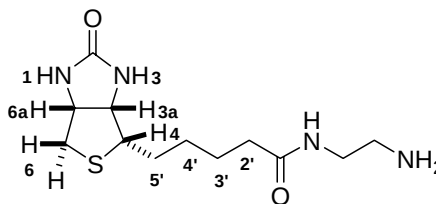
¹³C NMR (100.58MHz, DMSO-*d*₆), δ [ppm]: 25.2 (C-5'), 28.5 & 28.8 (C-3' & C-4'), 33.2 (C-2'), 40.8 (C-6), 56.1 (C-4), 66.0 (C-3a), 61.9 (C-6a), 163.6 & 170.4 (CO)

¹⁹F NMR (376.31MHz, DMSO-*d*₆), δ [ppm]: -153.4, -158.4, -162.9

MS (ESI (positive mode), for C₁₆H₁₅F₅N₂O₃S, $M_w = 410.3\text{g/mol}$), m/z : 821.2 (13, [2M+H]⁺), 411.0 (100, [M+H]⁺)

5-(2-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoic acid (2-amino-ethyl)-amide
(Biot-1²)¹⁶³

A solution of **BiotOC₆F₅** (300mg, 0.73mmol) in DMF (20mL) was added slowly (30min) at 0°C to a solution of ethylenediamine **1²** (490μL, 7.31mmol, 10.0eq) and TEA (230μL,



1.64mmol, 2.2eq) in DMF (10mL). Upon stirring the reaction mixture (rt, 2d), the solvent was removed under vacuum. The pale brown residue was dissolved under heating in MeOH (20mL). The almost colourless product (**Biot-1²**) was precipitated overnight upon cooling (5°C), filtered, washed with cold MeOH and cold CH₃CN and dried *in vacuo* (yield: 150mg, 0.52mmol, 72%).

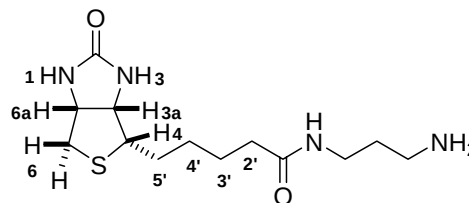
¹H NMR (400.00MHz, DMSO-*d*₆), δ [ppm]: 1.25-1.67 (m, 6H, H-3' & H-4' & H-5'), 2.10 (t, *J* = 7.4Hz, 2H, H-2'), 2.59 (d, *J* = 12.3Hz, 1H, H-6), 2.79-2.86 (m, 3H, H-6 & spacer H), 3.08-3.19 (m, 1H, H-4), 3.23-3.28 (m, 2H, spacer H), 4.13-4.16 (m, 1H, H-3a), 4.32 (m, 1H, H-6a), 6.40 (s, 1H, biotin NH), 6.45 (s, 1H, biotin NH), 8.03 (s, 1H, amide NH)

¹³C NMR (100.58MHz, DMSO-*d*₆), δ [ppm]: 25.9 (C-5'), 28.9 & 29.1 (C-3' & C-4'), 36.0 (C-2'), 39.8 (spacer C), 40.0 (spacer C), 40.7 (C-6), 56.3 (C-4), 60.1 (C-3a), 61.9 (C-6a), 163.6 & 173.6 (CO)

MS (ESI (positive mode), for C₁₂H₂₂N₄O₂S, M_w = 286.3g/mol), *m/z*: 287.2 (100, [M+H]⁺), 270.3 (51, [daughter product of 287.2]⁺)

5-(2-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoic acid (3-amino-propyl)-amide (Biot-1³**)¹⁶³**

A solution of **BiotOC₆F₅** (300mg, 0.73mmol) in DMF (20mL) was added slowly (30min) at 0°C to a solution of 1,3-diaminopropane **1³** (610μL, 0.73mmol, 10.0eq) and TEA (245μL,



1.75mmol, 2.4eq) in DMF (10min). The reaction mixture was stirred (rt, 2d), and the solvent was evaporated to dryness. The slightly yellowish residue (**Biot-1³**) was dissolved under heating in MeOH (20mL), precipitated overnight upon cooling (5°C), filtered, washed with ether, cold MeOH and cold CH₃CN and dried *in vacuo* (yield: 118mg, 0.39mmol, 54%).

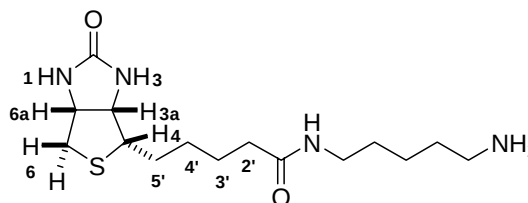
¹H NMR (400.00MHz, DMSO-*d*₆), δ [ppm]: 1.24-1.66 (m, 8H, H-3' & H-4' & H-5' & spacer H), 2.05 (t, *J* = 1.9Hz, 2H, H-2'), 2.56-2.65 (m, 1H, H-6), 2.80-2.85 (m, 1H, H-6), 3.00-3.12 (m, 5H, H-4 & spacer H), 4.11-4.15 (m, 1H, H-3a), 4.29-4.32 (m, 1H, H-6a), 6.36 (s, 1H, biotin NH), 6.43 (s, 1H, biotin H), 7.73-7.76 (m, 1H, amide NH)

¹³C NMR (100.58MHz, DMSO-*d*₆), δ [ppm]: 26.2 (C-5'), 28.9 & 29.1 (C-3' & C-4'), 30.2 (spacer C), 36.1 (C-2'), 37.1 (spacer C), 40.7 (C-6), 56.3 (C-4), 60.1 (C-3a), 61.9 (C-6a), 163.6 & 172.8 (CO)

MS (ESI (positive mode), for C₁₃H₂₄N₄O₂S, *M_w* = 300.4g/mol), *m/z*: 300.2 (100, [M+H]⁺)

5-(2-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoic acid (5-amino-pentyl)-amide (Biot-1⁵)¹⁶³

A solution of **BiotOC₆F₅** (600mg, 1.46mmol) in DMF (40mL) was added dropwise (1h) at 0°C to a solution of 1,5-diaminopentane **1⁵** (525μL, 4.39mmol,



3.0eq) and TEA (450μL, 3.27mmol, 2.2eq) in DMF (15mL). Upon stirring the reaction mixture (rt, 2d), the solvent was evaporated under vacuum. The residue was dissolved under heating in MeOH (40mL) and precipitated overnight (5°C) upon addition of ether. The resulting colourless precipitate (**Biot-1⁵**) was filtered, washed with ether, cold MeOH and cold CH₃CN and dried *in vacuo* (yield: 599mg, 1.83mmol, 68%).

¹H NMR (400.00MHz, DMSO-*d*₆), δ [ppm]: 1.19-1.75 (m, 12H, H-3' & H-4' & H-5' & spacer H), 2.05 (t, *J* = 7.4Hz, 2H, H-2'), 2.63-2.73 (m, 2H, H-6 & spacer H), 2.80-2.90 (m, 2H, spacer H), 3.01 (d, *J* = 2.5Hz, 1H, spacer H), 4.09-4.18 (m, 1H, H-3a), 4.25-4.36 (m, 1H, H-6a), 6.38 (s, 1H, biotin NH), 6.44 (s, 1H, biotin NH), 7.77 (s, 1H, amide NH)

¹³C NMR (100.58MHz, DMSO-*d*₆), δ [ppm]: 24.2 (spacer C), 26.2 (C-5'), 28.9 & 29.1 (C-3' & C-4'), 29.6 (spacer C), 36.1 (C-2'), 39.0 (spacer C), 39.2 (spacer C), 40.2 (spacer C), 40.7 (C-6), 56.3 (C-4), 60.0 (C-3a), 61.9 (C-6a), 163.6 & 172.7 (CO)

MS (ESI (positive mode), for C₁₅H₂₈N₄O₂S, M_w = 328.4g/mol), *m/z*: 329.3 (100, [M+H]⁺), 351.3 (30, [M+Na]⁺), 369.4 (26, [M+Na+H₂O]⁺)

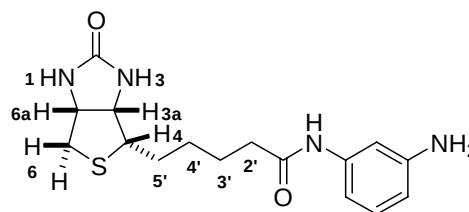
¹H NMR (400.00MHz, DMSO-d₆), δ [ppm]: 1.37-1.72 (m, 6H, H-3' & H-4' & H-5'), 2.32 (t, *J* = 7.4Hz, 2H, H-2'), 2.58 (d, *J* = 12.4Hz, 1H, H-6), 2.84 (dd, *J* = 5.1Hz & 12.4Hz, 1H, H-6), 3.12-3.16 (m, 1H, H-4), 4.14-4.17 (m, 1H, H-3a), 4.30-4.34 (m, 1H, H-6a), 4.79 (s, 2H, amine NH₂), 6.33 (s, 1H, biotin NH), 6.40 (s, 1H, biotin NH), 6.54 (dt, *J* = 1.3Hz & 7.6Hz, 1H, spacer H), 6.72 (dd, *J* = 1.3Hz & 7.9Hz, 1H, spacer H), 6.89 (dt, *J* = 1.3Hz & 7.6Hz, 1H, spacer H), 7.16 (dd, *J* = 1.0Hz & 7.9Hz, 1H, spacer H), 9.05 (s, 1H, amide NH)

¹³C NMR (100.58MHz, DMSO-d₆), δ [ppm]: 26.2 (C-5'), 29.0 & 29.1 (C-3' & C-4'), 36.5 (C-2'), 40.7 (C-6), 56.3 (C-4), 60.1 (C-3a), 61.9 (C-6a), 116.7, 117.0, 124.4, 126.1, 126.6 & 142.8 (spacer C), 163.6 & 171.9 (CO)

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5-(2-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoic acid (3-amino-phenyl)-amide (Biot-2^{meta}**)**¹⁶³

A solution of **BiotOC₆F₅** (300mg, 0.73mmol) in DMF (20mL) was added drop by drop (30min) at 0°C to a solution of *m*-phenylenediamine **2^{meta}** (158mg, 1.46mmol, 2.0eq) and TEA (245μL, 1.75mmol, 2.4eq) in



DMF (10mL). Upon stirring the reaction mixture (rt, 2d), the solvent was evaporated to dryness. The residue was dissolved under heating in MeOH (20mL) and, after addition of ether, precipitated overnight (5°C). The resulting precipitate (**Biot-2^{meta}**) was filtered, washed with ether, cold MeOH and cold CH₃CN and dried under vacuum (yield: 185mg, 0.55mmol, 76%).

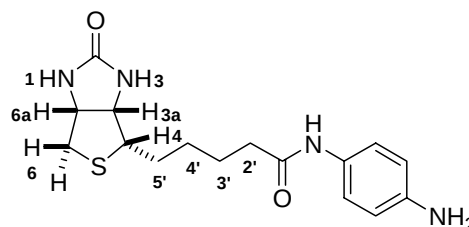
¹H NMR (400.00MHz, DMSO-*d*₆), δ [ppm]: 1.33-1.69 (m, 6H, H-3' & H-4' & H-5'), 2.26 (t, *J* = 7.4Hz, 2H, H-2'), 2.58 (d, *J* = 12.4Hz, 1H, H-6), 2.82 (dd, *J* = 5.1Hz & 12.4Hz, 1H, H-6), 3.09-3.14 (m, 1H, H-4), 4.12-4.16 (m, 1H, H-3a), 4.29-4.32 (m, 1H, H-6a), 5.01 (s, 2H, amine NH₂), 6.21-6.24 (dd, *J* = 1.3Hz & 8.0Hz, 1H, spacer H), 6.36 (s, 1H, biotin NH), 6.43 (s, 1H, biotin NH), 6.66 (d, *J* = 8.0Hz, 1H, spacer H), 6.88 (t, *J* = 8.0Hz, 1H, spacer H), 6.93 (s, 1H, spacer H), 9.53 (s, 1H, amide NH)

¹³C NMR (100.58MHz, DMSO-*d*₆), δ [ppm]: 26.1 (C-5'), 29.0 & 29.1 (C-3' & C-4'), 37.1 (C-2'), 40.7 (C-6), 56.3 (C-4), 60.0 (C-3a), 61.9 (C-6a), 105.7, 107.9, 109.9, 129.7, 140.8 & 149.8 (spacer C), 163.6 & 171.7 (CO)

MS (ESI (negative mode), for C₁₆H₂₂N₄O₂S, M_w = 334.4g/mol), *m/z*: 333.2 (100, [M-H]⁻)

5-(2-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoic acid (3-amino-phenyl)-amide (Biot-2^{para}**)¹⁶³**

A solution of **BiotOC₆F₅** (600mg, 1.46mmol) in DMF (40mL) was added slowly (30min) at 0°C to a solution of *p*-phenylenediamine **2^{para}** (316mg, 2.92mmol, 2.0eq) and TEA (490μL, 3.50mmol, 2.4eq) in DMF (20mL). The reaction mixture was stirred (rt, 2d), and the



solvent was evaporated to dryness. The slightly brown residue was dissolved under heating in MeOH (20mL). After addition of ether, a pale violet solid precipitated overnight (5°C). The resulting product (**Biot-2^{para}**) was filtered, washed with ether, cold MeOH and cold CH₃CN and dried under vacuum (yield: 465mg, 1.39mmol, 92%).

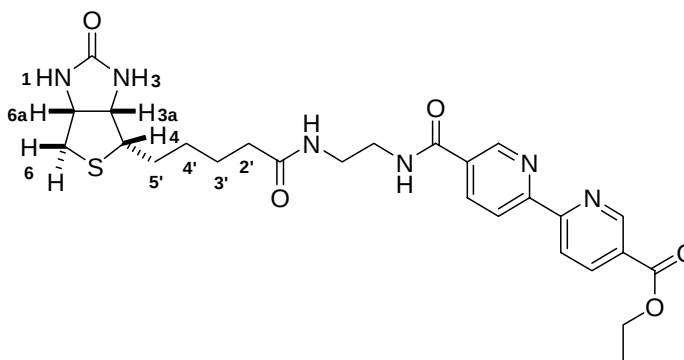
¹H NMR (400.00MHz, DMSO-*d*₆), δ [ppm]: 1.23-1.69 (m, 6H, H-3' & H-4' & H-5'), 2.21 (t, *J* = 7.4Hz, 2H, H-2'), 2.58 (d, *J* = 12.4Hz, 1H, H-6), 2.82 (dd, *J* = 5.1Hz & 12.4Hz, 1H, H-6), 3.10-3.18 (m, 1H, H-4), 4.10-4.32 (m, 1H, H-3a), 4.29-4.32 (m, 1H, H-6a), 4.81 (s, 2H, amine NH₂), 6.37 (s, 1H, biotin NH), 6.45 (s, 1H, biotin NH), 6.46-6.50 (m, 2H, spacer H), 7.19-7.22 (m, 2H, spacer H), 9.41 (s, 1H, amide NH)

¹³C NMR (100.58MHz, DMSO-*d*₆), δ [ppm]: 26.2 (C-5'), 29.0 & 29.1 (C-3' & C-4'), 36.9 (C-2'), 40.7 (C-6), 56.3 (C-4), 60.0 (C-3a), 61.9 (C-6a), 114.6, 121.7, 129.4 & 145.4 (spacer C), 163.6 & 171.0 (CO)

MS (ESI (positive mode), for C₁₆H₂₂N₄O₂S, M_w = 334.4g/mol), *m/z*: 357.2 (100, [M+Na]⁺)

5'-{2-[5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)pentanoylamino]-ethylcarbamoyl}-2,2'-bipyridinyl-5-carboxylic acid ethyl ester (Biot-1²-bpy)

A solution of **Biot-1²** (100mg, 0.31mmol) and TEA (130μL, 0.93mmol, 3.0eq) in DMF (15mL) was added dropwise to a solution of **bpyOC₆F₅** (272mg, 0.62mmol, 2.0eq)



in DMF (15mL), which led to a change of yellow to orange-yellowish. Upon stirring the reaction mixture (rt, 3d), the solvent was evaporated. The residue was purified by washing with ether, cold CH₂Cl₂ and cold CH₃CN. The resulting solid (**Biot-1²-bpy**) was dried *in vacuo* (yield: 118mg, 0.22mmol, 70%).

¹H NMR (400.00MHz, DMSO-d₆), δ [ppm]: 1.23-1.63 (m, 6H, H-3' & H-4' & H-5'), 1.37 (t, *J* = 7.1Hz, 3H, CH₂CH₃), 2.07-2.10 (t, *J* = 7.4Hz, 2H, H-2'), 2.56 (d, *J* = 12.4Hz, 1H, H-6), 2.78 (dd, *J* = 5.1Hz & *J* = 12.4Hz, 1H, H-6), 3.22-3.28 (m, 4H, spacer H), 3.02-3.11 (m, 1H, H-4), 4.07-4.11 (m, 1H, H-3a), 4.26-4.29 (m, 1H, H-6a), 4.39 (q, *J* = 7.1Hz, CH₂CH₃), 6.34 (s, 1H, biotin NH), 6.40 (s, 1H, biotin NH), 7.95 (t, *J* = 5.6Hz, 1H, amide NH), 8.36-8.39 (m, 1H, bpy H), 8.44-8.48 (m, 1H, bpy H), 8.52-8.60 (m, 2H, bpy H), 8.80 (t, *J* = 5.6Hz, 1H, amide NH), 9.13 (s, 1H, bpy H), 9.20 (s, 1H, bpy H)

¹³C NMR (100.58MHz, DMSO-d₆), δ [ppm]: 15.0 (CH₂CH₃), 26.1 (C-5'), 28.9 & 29.0 (C-3' & C-4'), 36.1 (C-2'), 38.9 (spacer C), 40.2 (spacer C), 40.7 (C-6), 56.2 (C-4), 60.0 (C-3a), 61.8 (C-6a), 62.2 (CH₂CH₃), 121.7, 121.8, 127.0, 131.5, 137.3, 139.1, 149.4, 150.9, 157.1 & 158.7 (bpy C), 163.5, 165.4, 166.8 & 173.3 (CO)

MS (ESI (positive mode), for $C_{26}H_{32}N_6O_5S$, $M_w = 540.6\text{g/mol}$), m/z : 563.3 (100, $[M+Na]^+$)

5'-{3-[5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)pentanoylamino]-propylcarbamoyl}-2,2'-bipyridinyl-5-carboxylic acid ethyl ester (Biot-1³-bpy)

A solution of **Biot-1³** (100mg, 0.33mmol)

and TEA (139 μ L, 1.00mmol, 3.0eq) in

DMF (10mL) was added to a solution of

bpyOC₆F₅ (219mg, 0.50mmol, 1.5eq) in

DMF (15mL), which gave a dark orange

solution. The reaction mixture was stirred

(rt, 3d), leading to a formation of a

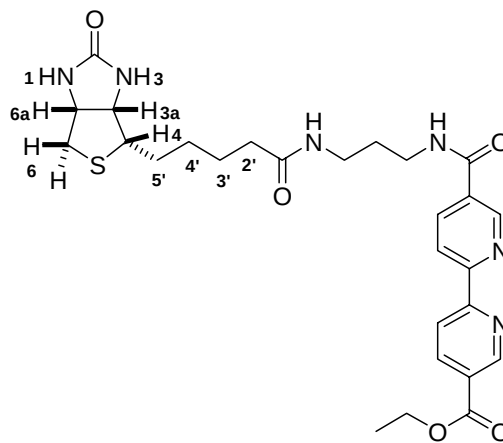
colourless precipitate. The solvent was

removed *in vacuo*. The residue was

dissolved under heating in MeOH (20mL). Upon addition of ether, a colourless solid was

obtained. The resulting precipitate (**Biot-1³-bpy**) was filtered, washed with ether and

ether/CH₃CN and dried under vacuum (yield: 105mg, 0.19mmol, 57%).



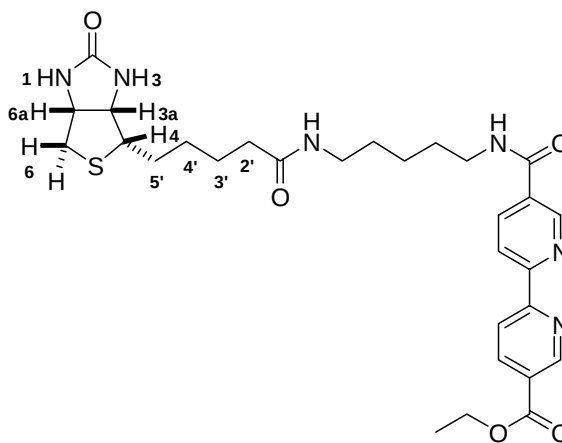
¹H NMR (400.00MHz, DMSO-d₆), δ [ppm]: 1.24-1.66 (m, 8H, H-3' & H-4' & H-5' & spacer H), 1.37 (t, $J = 7.1\text{Hz}$, 3H, CH₂CH₃), 2.03-2.09 (t, $J = 7.4\text{Hz}$, 2H, H-2'), 2.57 (d, $J = 12.4\text{Hz}$, 1H, H-6), 2.82 (dd, $J = 5.1\text{Hz}$ & 12.4Hz , 1H, H-6), 3.00-3.13 (m, 5H, H-4 & spacer H), 4.11-4.14 (m, 1H, H-3a), 4.29-4.32 (m, 1H, H-6a), 4.39 (q, $J = 7.1\text{Hz}$, 2H, CH₂CH₃), 6.35 (s, 1H, biotin NH), 6.41 (s, 1H, biotin NH), 7.74 (s, 1H, amide NH), 8.42-8.48 (m, 2H, bpy H), 8.57 (dd, $J = 8.2\text{Hz}$ & 19.2Hz , 2H, bpy H), 9.20 (dd, $J = 1.3\text{Hz}$ & 10.7Hz , 2H, bpy H)

^{13}C NMR (100.58MHz, DMSO- d_6), δ [ppm]: 15.0 (CH_2CH_3), 26.2 (C-5'), 28.9 & 29.1 (C-3' & C-4'), 30.2 (spacer C), 36.1 (C-2'), 37.1 (spacer C), 40.7 (C-6), 56.3 (C-4), 60.0 (C-3a), 61.9 (C-6a), 62.2 (CH_2CH_3), 121.8, 122.0, 127.1, 132.0, 137.1, 139.3, 150.9, 151.2, 156.6 & 158.5 (bpy C), 163.6, 165.3, 166.8 & 172.8 (CO)

MS (ESI (positive mode), for $\text{C}_{27}\text{H}_{34}\text{N}_6\text{O}_5\text{S}$, $M_w = 554.6\text{g/mol}$), m/z : 549.4 (100, $[\text{M-ethyl}+\text{Na}+\text{H}]^+$)

5'-{5-[5-(2-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)pentanoylamino]-pentylcarbamoyl}-2,2'-bipyridinyl-5-carboxylic acid ethyl ester (Biot-1⁵-bpy)

A solution of **Biot-1⁵** (164mg, 0.50mmol) and TEA (230 μL , 1.65mmol, 3.0eq) in DMF (10mL) was added to a solution of **bpyOC₆F₅** (330mg, 0.75mmol, 1.5eq) in DMF (20mL). The reaction mixture was stirred (rt, 3d), giving a colourless precipitate. The solvent was evaporated to dryness. The residue



was dissolved under heating in MeOH (20mL). Upon addition of ether to the solution, a colourless solid was precipitated. The resulting precipitate (**Biot-1⁵-bpy**) was filtered, washed with ether and ether/ CH_3CN and dried *in vacuo* (yield: 191mg, 0.33mmol, 65%).

^1H NMR (400.00MHz, DMSO- d_6), δ [ppm]: 1.16-1.60 (m, 12H, H-3' & H-4' & H-5' & spacer H), 1.97 (t, $J = 7.1\text{Hz}$, 3H, CH_2CH_3), 2.03-2.09 (m, 2H, H-2'), 2.57 (d, $J = 12.6\text{Hz}$, 1H,

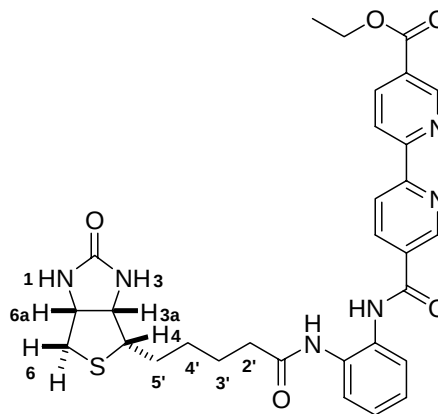
H-6), 2.81 (dd, $J = 5.1\text{Hz} \& 12.4\text{Hz}$, 1H, H-6), 3.02-3.10 (m, 3H, H-4 & spacer H), 3.27-3.33 (m, 2H, spacer H), 4.10-4.13 (m, 1H, H-3a), 4.28-4.31 (m, 1H, H-6a), 4.39 (q, $J = 7.1\text{Hz}$, 2H, CH_2CH_3), 6.37 (s, 1H, biotin NH), 6.43 (s, 1H, biotin NH), 7.77 (t, $J = 5.4\text{Hz}$, 1H, amide NH), 8.37-8.61 (m, 4H, bpy H), 8.77 (t, $J = 5.4\text{Hz}$, 1H, amide NH), 9.13 (d, $J = 1.5\text{Hz}$, 1H, bpy H), 9.20 (d, $J = 1.5\text{Hz}$, 1H, bpy H)

^{13}C NMR (100.58MHz, DMSO- d_6), δ [ppm]: 15.0 (CH_2CH_3), 24.8 (spacer C), 26.2 (C-5'), 28.9 & 29.1 (C-3' & C-4'), 29.6 (spacer C), 29.8 (spacer C), 36.1 (C-2'), 39.1 (spacer C), 40.1 (spacer C), 40.8 (C-6), 56.3 (C-4), 60.0 (C-3a), 61.9 (C-6a), 62.2 (CH_2CH_3), 121.8, 122.0, 127.0, 131.6, 137.3, 139.1, 149.4, 150.9, 156.7, 158.7 (bpy C), 163.6, 165.1, 165.4 & 172.7 (CO)

MS (ESI (negative mode), for $\text{C}_{29}\text{H}_{38}\text{N}_6\text{O}_5\text{S}$, $M_w = 582.7\text{g/mol}$), m/z : 581.9 (100, $[\text{M-H}]^-$)

5'-{2-[5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)pentanoylamino]-phenylcarbamoyl}-2,2'-bipyridinyl-5-carboxylic acid ethyl ester (Biot-2^{ortho}-bpy)

A solution of **Biot-2^{ortho}** (300mg, 0.90mmol) and TEA (375μL, 2.70mmol, 3.0eq) in DMF (15mL) was added to a solution of **bpyOC₆F₅** (590mg, 1.35mmol, 1.5eq) in DMF (30mL), leading to an orange solution. Upon stirring the reaction mixture (rt, 4d), the solvent was removed under vacuum. The residue was



purified by washing with ether/MeOH and ether/CH₃CN. The resulting solid (**Biot-2^{ortho}-bpy**) was dried under vacuum (yield: 332mg, 0.56mmol, 63%).

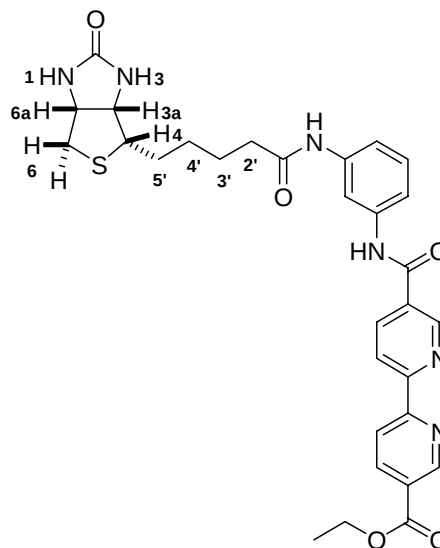
¹H NMR (400.00MHz, DMSO-*d*₆), δ [ppm]: 1.24-1.61 (m, 6H, H-3' & H-4' & H-5'), 1.38 (t, *J* = 7.1Hz, 3H, CH₂CH₃), 2.36-2.40 (m, 2H, H-2'), 2.68-2.75 (m, 1H, H-6), 2.94-2.99 (m, 1H, H-4), 4.02-4.05 (m, 1H, H-3a), 4.20-4.23 (m, 1H, H-6a), 4.40 (q, *J* = 7.1Hz, 2H, CH₂CH₃), 6.32 (s, 1H, biotin NH), 6.40 (s, 1H, biotin NH), 7.23-7.26 (m, 2H, spacer H), 7.58 (d, *J* = 8.5Hz, 1H, spacer H), 7.64 (d, *J* = 8.5Hz, 1H, spacer H), 8.44-8.63 (m, 4H, bpy H), 9.20-9.27 (m, 2H, bpy H), 9.64 (s, 1H, amide NH)

¹³C NMR (100.58MHz, DMSO-*d*₆), δ [ppm]: 15.0 (CH₂CH₃), 26.1 (C-5'), 28.9 & 29.1 (C-3' & C-4'), 36.7 (C-2'), 40.7 (C-6), 56.2 (C-4), 60.0 (C-3a), 61.8 (C-6a), 62.2 (CH₂CH₃), 121.8, 123.4, 127.1, 131.5, 137.8, 139.2, 149.6, 150.9, 157.1 & 158.6 (bpy C), 163.5, 165.4, 166.8 & 173.3 (CO)

MS (ESI (negative mode), for C₃₀H₃₂N₆O₅S, M_w = 588.6g/mol), *m/z*: 587.2 (100, [M-H]⁻)

5'-{3-[5-(2-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)pentanoylamino]-phenylcarbamoyl}-2,2'-bipyridinyl-5-carboxylic acid ethyl ester (Biot-2^{meta}-bpy)

A solution of **Biot-2^{meta}** (75mg, 0.22mmol) and TEA (94μL, 0.67mmol, 3.0eq) in DMF (10mL) was added to a solution of **bpyOC₆F₅** (148mg, 0.34mmol, 1.5eq) in DMF (15mL). The reaction mixture was stirred (rt, 5d), and the solvent was evaporated to dryness. The residue was purified by washing with ether/MeOH and ether/CH₃CN. The resulting solid (**Biot-2^{meta}-bpy**) was dried *in vacuo* (yield: 55mg, 0.09mmol, 42%).



¹H NMR (400.00MHz, DMSO-*d*₆), δ [ppm]: 1.36-1.67 (m, 6H, H-3' & H-4' & H-5'), 1.37 (t, *J* = 7.1Hz, 3H, CH₂CH₃), 2.31-2.35 (m, 2H, H-2'), 2.58 (d, *J* = 12.4Hz, 1H, H-6), 2.83 (dd, *J* = 5.1Hz & 12.4Hz, 1H, H-6), 3.11-3.16 (m, 1H, H-4), 4.14-4.17 (m, 1H, H-3a), 4.30-4.33 (m, 1H, H-6a), 4.40 (q, *J* = 7.1Hz, 2H, CH₂CH₃), 6.35 (s, 1H, biotin NH), 6.44 (s, 1H, biotin NH), 7.28 (t, *J* = 8.0Hz, 1H, spacer H), 7.36 (d, *J* = 8.5Hz, 1H, spacer H), 7.46 (d, *J* = 8.0Hz, 1H, spacer H), 8.15 (s, 1H, spacer H), 8.48-8.52 (m, 2H, bpy H), 8.58-8.62 (m, 2H, bpy H), 9.22-9.25 (m, 2H, bpy H), 10.56 (s, 1H, amide NH), 10.71 (s, 1H, amide NH)

¹³C NMR (100.58MHz, DMSO-*d*₆), δ [ppm]: 15.0 (CH₂CH₃), 26.0 (C-5'), 29.0 & 29.1 (C-3' & C-4'), 37.1 (C-2'), 40.7 (C-6), 56.3 (C-4), 60.1 (C-3a), 61.9 (C-6a), 62.2 (CH₂CH₃), 112.3, 116.1, 116.6, 129.2, 139.9 & 140.4 (spacer C), 121.7, 121.9, 127.1, 132.1, 137.9, 139.2, 149.8, 150.9, 157.0 & 158.6 (bpy C), 163.6, 164.5, 165.4 & 172.1 (CO)

MS (ESI (positive mode), for C₃₀H₃₂N₆O₅S, M_w = 588.6g/mol), *m/z*: 611.3 (100, [M+Na]⁺)

5'-{4-[5-(2-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)pentanoylamino]-phenylcarbamoyl}-2,2'-bipyridinyl-5-carboxylic acid ethyl ester (Biot-2^{para}-bpy)

A solution of **Biot-2^{para}**

(300mg, 0.90mmol) and

TEA (375μL,

2.70mmol, 3.0eq) in

DMF (15mL) was

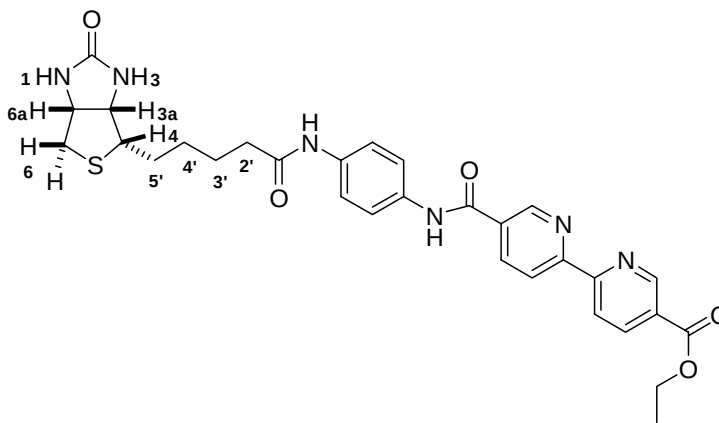
added to a solution of

bpyOC₆F₅ (590mg,

1.35mmol, 1.5eq) in DMF (30mL). Upon stirring the reaction mixture (rt, 4d), the solvent was

evaporated to dryness. The residue was purified by washing with ether/MeOH and

ether/CH₃CN. The resulting solid (**Biot-2^{para}-bpy**) was dried under vacuum (yield: 510mg, 0.86mmol, 96%).



¹H NMR (400.00MHz, DMSO-*d*₆), δ [ppm]: 1.35-1.72 (m, 6H, H-3' & H-4' & H-5'), 1.38 (t, *J* = 7.1Hz, 3H, CH₂CH₃), 2.32 (t, *J* = 7.3Hz, 2H, H-2'), 2.59 (d, *J* = 12.4Hz, 1H, H-6), 2.83 (dd, *J* = 5.1Hz & 12.4Hz, 1H, H-6), 3.11-3.16 (m, 1H, H-4), 4.14-4.17 (m, 1H, H-3a), 4.30-4.37 (m, 1H, H-6a), 4.39 (q, *J* = 7.1Hz, 2H, CH₂CH₃), 6.36 (s, 1H, biotin NH), 6.44 (s, 1H, biotin NH), 7.59 (d, *J* = 9.0Hz, 2H, spacer H), 7.71 (d, *J* = 9.0Hz, 2H, spacer H), 8.44-8.51 (m, 2H, bpy H), 8.56-8.62 (m, 2H, bpy H), 9.20-9.25 (m, 2H, bpy H), 9.88 (s, 1H, amide NH), 10.49 (s, 1H, amide NH)

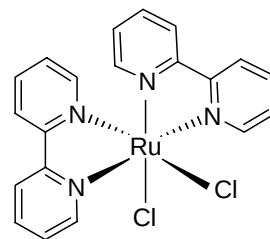
¹³C NMR (100.58MHz, DMSO-*d*₆), δ [ppm]: 15.0 (CH₂CH₃), 26.0 (C-5'), 29.0 & 29.1 (C-3' & C-4'), 37.0 (C-2'), 40.7 (C-6), 56.3 (C-4), 60.1 (C-3a), 61.9 (C-6a), 62.2 (CH₂CH₃), 120.2, 121.7, 134.7, 136.5, 137.7 & 139.3 (spacer C), 121.7, 121.8, 122.0, 127.0, 132.0, 139.2, 149.7, 150.9, 156.9 & 158.7 (bpy C), 163.6, 164.1, 165.4 & 171.8 (CO)

MS (ESI (negative mode), for $C_{30}H_{32}N_6O_5S$, $M_w = 588.6\text{g/mol}$), m/z : 609.1 (18, $[M+Na-2H]^+$), 587.3 (100, $[M-H]^+$)

5.2.2. Synthesis of the Complexes

***cis*-[Ru(bpy)₂Cl₂]¹³⁸**

Commercial ruthenium trichloride (5.00g, 24.10mmol) and 2,2'-bipyridine (6.00g, 38.41mmol, 1.6eq) were heated at reflux temperature (6h) in DMF (200mL). Most of the solvent was distilled off. The remaining solution was cooled to room temperature, treated with acetone (170mL) and kept overnight (0°C). The crystals formed were collected by suction filtration and washed with cool water. The crude product was suspended in H₂O/EtOH 1:1 (800mL) and heated at reflux temperature (1h), filtered from insoluble solid and treated carefully with LiCl (100g). EtOH was evaporated and the resulting water solution was cooled in an ice bath giving dark crystals. These crystals were dried *in vacuo* (1d) (yield: 4.13g, 8.53mmol, 36%).



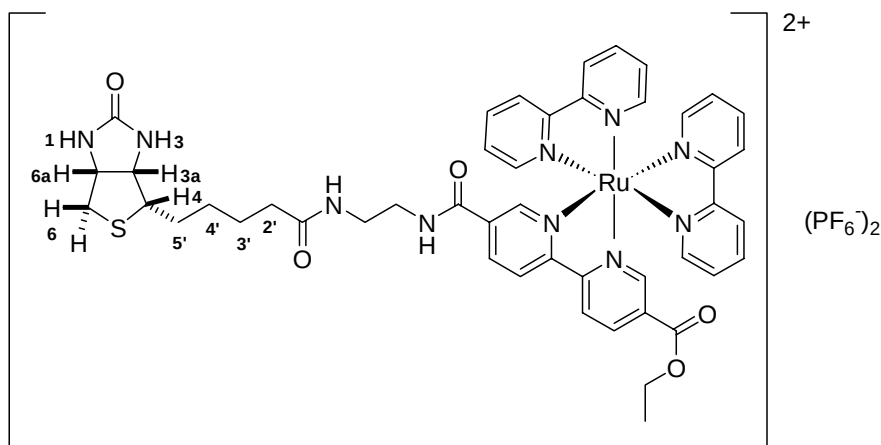
¹H NMR (400.00MHz, DMSO-d₆), δ [ppm]: 7.09-7.12 (t, $J = 6.0\text{Hz}$, 2H, bpy H), 7.50 (d, $J = 5.4\text{Hz}$, 2H, bpy H), 7.68 (t, $J = 7.4\text{Hz}$, 2H, bpy H), 7.77 (t, $J = 6.0\text{Hz}$, 2H, bpy H), 8.07 (dt, $J = 1.3\text{Hz}$ & 7.3Hz , 2H, bpy H), 8.50 (d, $J = 7.8\text{Hz}$, 2H, bpy H), 8.66 (d, $J = 7.8\text{Hz}$, 2H, bpy H), 9.97 (d, $J = 5.0\text{Hz}$, 2H, bpy H)

¹³C NMR (100.58MHz, DMSO-d₆), δ [ppm]: 123.4, 123.7, 126.1, 126.2, 134.2, 135.4, 152.9, 154.0, 159.0 & 161.1 (bpy C)

MS (ESI (positive mode), for $C_{20}H_{16}Cl_2N_4Ru$, $M_w = 484.4\text{g/mol}$), m/z : 486.2 (100, $[M+H]^+$), 449.1 (30, $[M-Cl]^+$)

UV/Vis (EtOH), $\lambda[\text{nm}](\epsilon[\text{M}^{-1}\text{cm}^{-1}])$: 519 (5400, [MLCT: $d_{Ru} \rightarrow \pi^*_{bpy}$]), 359 (5300, [MLCT: $d_{Ru} \rightarrow \pi^*_{bpy}$], 296 (34500, [intraligand: $\pi_{bpy} \rightarrow \pi^*_{bpy}$]), 246 (12800, [intraligand: $\pi_{bpy} \rightarrow \pi^*_{bpy}$])

***rac*-[Ru(bpy)₂(Biot-1²-bpy)](PF₆)₂¹³⁹**



cis-[Ru(bpy)₂Cl₂] (89.6mg, 0.19mmol) and the biotinylated ligand **Biot-1²-bpy** (100.0mg, 0.19mmol, 1.0eq) were added to EtOH (30mL). The solution was heated at reflux temperature (3d) and the solvent was evaporated. The residue was washed three times with ether and redissolved in EtOH (4mL). To this solution, NH₄PF₆ (127.0mg, 0.78mmol, 8.0eq relative to the formed intermediate product) in H₂O (0.5mL) was added. The resulting precipitate (*rac*-[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺) (the structure above is shown for the Λ -enantiomer) was filtered, washed with ether and dried *in vacuo* (yield: 0.18g, 0.15mmol, 80%).

¹H NMR (400.00MHz, DMSO-*d*₆), δ [ppm]: 1.26-1.84 (m, 6H, H-3' & H-4' & H-5'), 1.28 (t, $J = 7.1\text{Hz}$, 3H, CH₂CH₃), 2.14-2.19 (m, 2H, H-2'), 2.68 (dd, $J = 3.6\text{Hz}$ & 12.9Hz, 1H, H-6),

2.89-2.94 (m, 1H, H-6), 3.14-3.21 (m, 1H, H-4), 4.27-4.32 (m, 1H, H-3a), 4.30 (q, $J = 7.1\text{Hz}$, 2H, CH_2CH_3), 4.48-4.52 (m, 1H, H-6a), 7.48-7.59 (m, 4H, bpy H), 7.85-7.92 (m, 4H, bpy H), 8.14-8.27 (m, 6H, bpy H (4H) & biotbpy H (2H)), 8.47 (dd, $J = 1.7\text{Hz}$ & 8.5Hz , 1H, biotbpy H), 8.60 (dd, $J = 1.8\text{Hz}$ & 8.5Hz , 1H, biotbpy H), 8.72-8.78 (m, 4H, bpy H), 8.88 (d, $J = 8.2\text{Hz}$, 2H, biotbpy H)

^{13}C NMR (100.58MHz, DMSO- d_6), δ [ppm]: 13.8 (CH_2CH_3), 25.2 (C-5'), 28.0 & 28.1 (C-3' & C-4'), 35.1 (C-2'), 37.8 (spacer C), 39.3 (spacer C), 39.8 (C-6), 55.4 (C-4), 59.2 (C-3a), 61.0 (C-6a), 61.9 (CH_2CH_3), 124.5, 124.6, 125.2, 125.3, 127.9, 128.1, 135.9, 137.6, 138.2, 138.3, 150.7, 151.0, 151.4, 151.6, 151.8, 152.0, 156.1, 156.3, 156.5, 156.7, 157.3 & 159.3 (bpy C), 162.2, 162.4, 162.7 & 172.2 (CO)

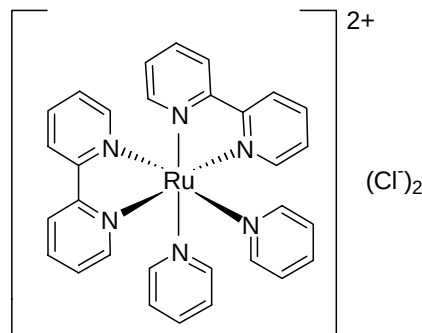
MS (ESI (positive mode), for $\text{C}_{46}\text{H}_{48}\text{N}_{10}\text{O}_5\text{RuS}$, $M_w = 954.0\text{g/mol}$), m/z : 1099.1 (18, $[\text{M}+\text{PF}_6]^+$), 477.2 (100, $[\text{M}/2]^{2+}$)

MS (ESI (negative mode), for PF_6 , $M_w = 145.0\text{g/mol}$), m/z : 145.1 (100, $[\text{M}]^-$)

UV/Vis (EtOH), $\lambda[\text{nm}](\epsilon[\text{M}^{-1}\text{cm}^{-1}])$: 434 (8120), 287 (63000), 253 (23200)

***cis*-[Ru(bpy)₂(py)₂]Cl₂¹⁴⁶**

cis-[Ru(bpy)₂Cl₂] (1.00g, 2.06mmol) was heated at reflux temperature (40h) in a solution of H₂O (20mL) and pyridine (10mL, large excess). The colour of the solution changed from brown-red to red-orange. After filtration of the solution, the solvent was evaporated to dryness. The brown-orange residue was taken



up in MeOH (10mL). Upon addition of ether (20mL), a red-orange solid was precipitated overnight (5°C). The resulting product was filtered, washed with ether and dried *in vacuo* (1d) (yield: 0.98g, 1.53mmol, 74%).

¹H NMR (400.00MHz, DMSO-d₆), δ [ppm]: 7.41 (t, *J* = 7.0 Hz, 4H, pyridine H), 7.50 (t, *J* = 6.6Hz, 2H, bpy H), 7.88-7.96 (m, 6H, pyridine H), 8.03 (t, *J* = 7.8Hz, 2H, bpy H), 8.25 (t, *J* = 7.8Hz, 2H, bpy H), 8.40 (d, *J* = 5.3Hz, 4H, bpy H), 8.70 (d, *J* = 8.0Hz, 2H, bpy H), 8.77 (d, *J* = 8.0Hz, 2H, bpy H), 9.03 (d, *J* = 5.3Hz, 2H, bpy H)

¹³C NMR (100.58MHz, DMSO-d₆), δ [ppm]: 127.0 (2x), 138.9 & 154.3 (2x) (pyridine C), 124.7, 125.0, 128.7, 129.0, 138.5, 138.8, 153.2, 153.4, 157.7 & 157.9 (bpy C)

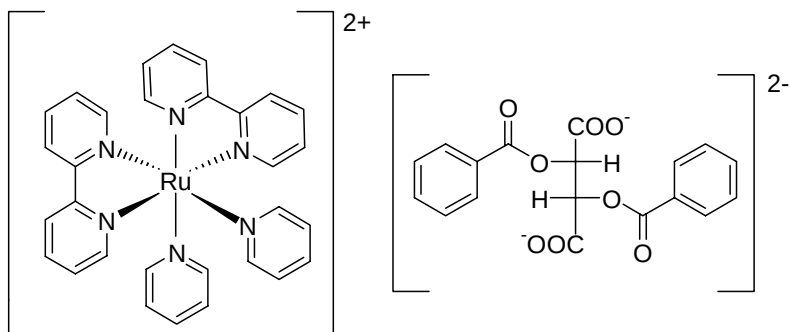
MS (ESI (positive mode), for C₃₀H₂₆N₆Ru, M_w = 571.6g/mol), *m/z*: 285.9 (100, [M/2]²⁺)

MS (ESI (negative mode), for Cl, M_w = 35.5g/mol), *m/z*: 35.4 (100, [M]⁻)

UV/Vis (EtOH), λ[nm](ε[M⁻¹cm⁻¹]): 458 (8100, [MLCT: d_{Ru} → π*_{bpy}]), 339 (12700, [MLCT: d_{Ru} → π*_{bpy}]), 290 (50000, [intraligand: π_{bpy} → π*_{bpy}]), 245 (24000, [intraligand: π_{bpy} → π*_{bpy}])

Δ -[Ru(bpy)₂(py)₂][(+)-O,O'-dibenzoyl-D-tartrate] · 12H₂O^{140-142,150}

An aqueous solution of 0.5M disodium (+)-O,O'-dibenzoyl-D-tartrate (7mL) was added to a solution of *cis*-



[Ru(bpy)₂(py)₂]Cl₂ (0.70g, 1.09mmol) in H₂O (14mL). The disodium (+)-O,O'-dibenzoyl-D-tartrate solution was prepared by addition of a NaOH solution to the corresponding acid, until a pH of 8-9 was obtained. The reaction mixture was stirred for a short time (10min). In a dark room, the solvent was allowed to evaporate naturally. Red crystals of Δ -[Ru(bpy)₂(py)₂][(+)-O,O'-dibenzoyl-D-tartrate] · 12H₂O were formed (8-10d) and collected by filtration. The Δ -diastereoisomer was washed with cold H₂O and air-dried (yield: 0.41g, 0.35mmol, 65%).

¹H NMR (400.00MHz, DMSO-d₆), δ [ppm]: 5.47 (s, 2H, C*H counterion), 7.38-7.49 (m, 10H, phenyl (6H) & pyridine (4H)), 7.55-7.59 (m, 2H, bpy H), 7.86-8.03 (m, 12H, bpy (6H) & phenyl (4H) & pyridine (2H)), 8.20-8.24 (m, 2H, bpy H), 8.38 (d, J = 5.3Hz, 4H, pyridine H), 8.65 (d, J = 8.1Hz, 2H, bpy H), 8.72 (d, J = 7.9Hz, 2H, bpy H), 9.00 (d, J = 5.0Hz, 2H, bpy H)

¹³C NMR (100.58MHz, DMSO-d₆), δ [ppm]: 78.2 (C*H counterion), 124.7, 125.0, 138.8, 138.9, 153.2, 153.3, 157.6 & 157.9 (bpy C), 128.6, 133.1 & 138.6 (pyridine C), 127.0, 129.0, 130.2, 132.9 & 154.2 (phenyl C), 166.2 & 169.3 (CO)

MS (ESI (positive mode), for C₃₀H₂₆N₆Ru, M_w = 571.6g/mol), m/z: 286.1 (100, [M/2]²⁺), 246.5 (8, [(M-pyridine)/2]²⁺)

MS (ESI (negative mode), for $C_{18}H_{14}O_8$, $M_w = 358.3\text{g/mol}$), m/z : 357.1 (100, $[M-H]^-$)

UV/Vis (H_2O), $\lambda[nm](\epsilon[M^{-1}cm^{-1}])$: 459 (8500), 337 (13200), 289 (52900), 239 (43500)

CD (H_2O), $\lambda[nm](\Delta\epsilon[M^{-1}cm^{-1}])$: 519 (-1.1), 444 (3.7), 362 (16.1), 294 (-115.1), 278 (15.0)

Λ -[Ru(bpy)₂(py)₂][(-)-O,O'-dibenzoyl-L-tartrate] · 12 H₂O^{140-142,150}

An aqueous

solution of 0.5M

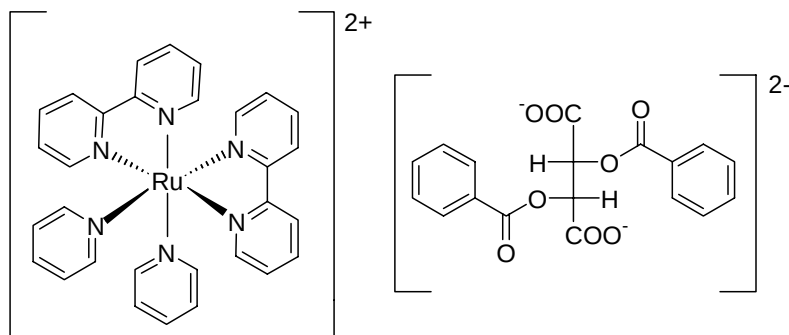
disodium (-)-

O,O'-dibenzoyl-

L-tartrate (10mL)

was added to a

solution of *cis*-



[Ru(bpy)₂(py)₂]Cl₂ (0.96g, 1.49mmol) in H₂O (20mL). The disodium (-)-O,O'-dibenzoyl-L-tartrate solution was prepared by addition of a NaOH solution to the corresponding acid, until a pH of 8-9 was obtained. The reaction mixture was stirred for a short time (10min). In a dark room, the solvent was allowed to evaporate naturally. Red crystals of Λ -[Ru(bpy)₂(py)₂][(-)-O,O'-dibenzoyl-L-tartrate] · 12H₂O were formed (8-10d) and collected by filtration. The Λ -diastereoisomer was washed with cold H₂O and air-dried (yield: 0.55g, 0.48mmol, 64%).

¹H NMR (400.00MHz, DMSO-d₆), δ [ppm]: 5.48 (s, 2H, C*H counterion), 7.38-7.49 (m, 10H, phenyl (6H) & pyridine (4H)), 7.55-7.59 (m, 2H, bpy H), 7.86-8.03 (m, 12H, bpy (6H) & phenyl (4H) & pyridine (2H)), 8.20-8.24 (m, 2H, bpy H), 8.38 (d, $J = 5.3\text{Hz}$, 4H, pyridine H), 8.65 (d, $J = 8.1\text{Hz}$, 2H, bpy H), 8.72 (d, $J = 7.9\text{Hz}$, 2H, bpy H), 9.00 (d, $J = 5.0\text{Hz}$, 2H, bpy H)

^{13}C NMR (100.58MHz, DMSO- d_6), δ [ppm]: 78.1 (C*H counterion), 124.7, 125.0, 138.8, 138.9, 153.2, 153.3, 157.6 & 157.9 (bpy C), 128.6, 133.1 & 138.6 (pyridine C), 127.0, 129.0, 130.2, 132.9 & 154.2 (phenyl C), 166.2 & 169.3 (CO)

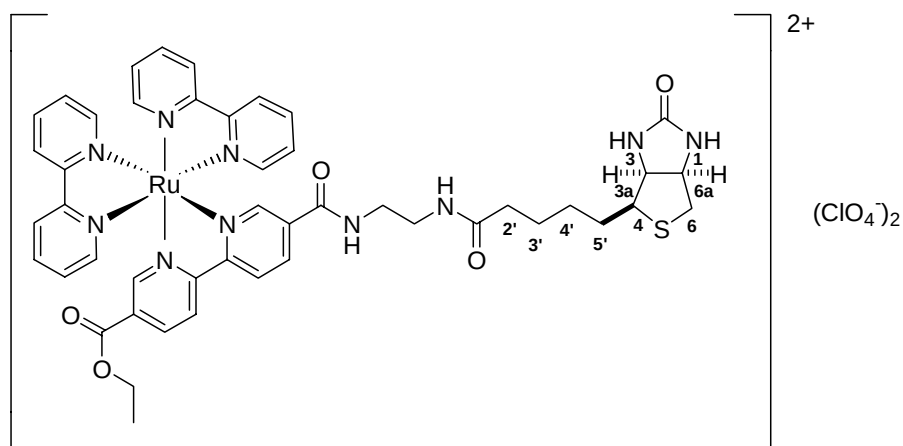
MS (ESI (positive mode), for $\text{C}_{30}\text{H}_{26}\text{N}_6\text{Ru}$, $M_w = 571.6\text{g/mol}$), m/z : 286.1 (100, $[\text{M}/2]^{2+}$), 246.5 (18, $[(\text{M-pyridine})/2]^{2+}$)

MS (ESI (negative mode), for $\text{C}_{18}\text{H}_{14}\text{O}_8$, $M_w = 358.3\text{g/mol}$), m/z : 357.1 (100, $[\text{M-H}]^-$)

UV/Vis (H_2O), $\lambda[\text{nm}](\epsilon[\text{M}^{-1}\text{cm}^{-1}])$: 459 (8700), 337 (13100), 289 (55000), 239 (41000)

CD (H_2O), $\lambda[\text{nm}](\Delta\epsilon[\text{M}^{-1}\text{cm}^{-1}])$: 521 (0.9), 448 (-3.6), 363 (-14.4), 294 (110.6), 278 (-16.5)

$\Delta\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot-1}^2\text{-bpy})](\text{ClO}_4)_2$ ¹⁴¹



$\Delta\text{-}[\text{Ru}(\text{bpy})_2(\text{py})_2][(+)\text{-O,O'}\text{-dibenzoyl-D-tartrate}] \cdot 12\text{H}_2\text{O}$ (56.3mg, 0.049mmol) and ligand **Biot-1²-bpy** (40.0mg, 0.074mmol, 1.5eq) were added to ethylene glycol (10% H_2O) (4mL) in a 25mL flask. The solution was heated (120°C, 6h), cooled to room temperature and diluted with H_2O (16mL). The resulting mixture was filtered and purified by ion exchange chromatography (*SP Sephadex C-25*) using sodium perchlorate. The elution yielded two main

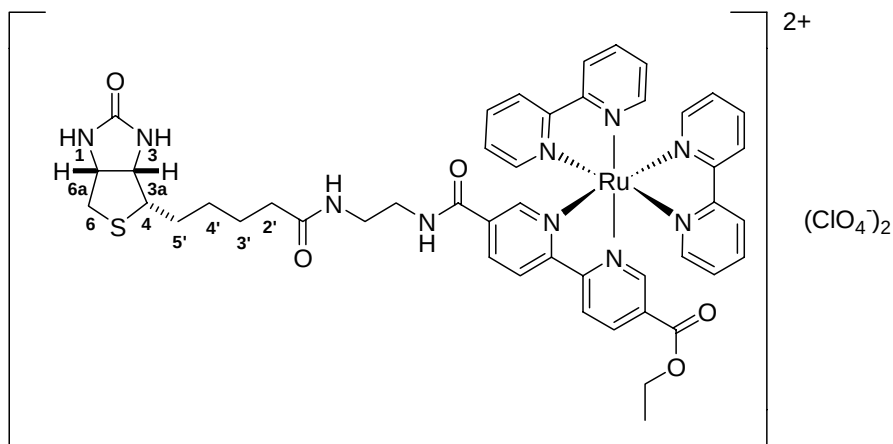
bands. The first band (singly charged product $\Delta\text{-[Ru(bpy)}_2(\text{Biot-1}^2\text{-bpy}')\text{]}^+$ where **Biot-1²-bpy'** is the hydrolyzed ligand carrying one negative charge) was eluted with a 0.05M NaClO₄ solution while the second band (desired doubly charged product $\Delta\text{-[Ru(bpy)}_2(\text{Biot-1}^2\text{-bpy})\text{]}^{2+}$) was eluted with a 0.2M NaClO₄ solution.

MS (ESI (positive mode), for C₄₆H₄₈N₁₀O₅RuS, M_w = 954.0g/mol), m/z: 1314.0 (12, [M+((ClO₄)₃Na₂ · H₂O)]⁺), 1193.1 (31, [M+((ClO₄)₂Na · H₂O)]⁺), 1069.2 (100, [M+(ClO₄ · H₂O)]⁺), 485.1 (88, [M/2]²⁺)

UV/Vis (H₂O), λ[nm](ε[M⁻¹cm⁻¹]): 434 (8200), 287 (63800), 254 (23200)

CD (H₂O), λ[nm](Δε[M⁻¹cm⁻¹]): 488 (-5.7), 427 (10.5), 300 (-131.2), 279 (78.2), 256 (-4.0), 230 (20.5)

$\Delta\text{-[Ru(bpy)}_2(\text{Biot-1}^2\text{-bpy})\text{]}(\text{ClO}_4)_2$ ¹⁴¹



$\Delta\text{-[Ru(bpy)}_2(\text{py})_2\text{]}[(-)\text{-O,O'-dibenzoyl-L-tartrate}] \cdot 12 \text{ H}_2\text{O}$ (42.3mg, 0.037mmol) and ligand **Biot-1²-bpy** (40.0mg, 0.074mmol, 2.0eq) were added to ethylene glycol (10% H₂O) (4mL) in a 25mL flask. The solution was heated (120°C, 6h), cooled to room temperature and diluted

with H₂O (16mL). The resulting mixture was filtered and purified by ion exchange chromatography (*SP Sephadex C-25*) using sodium perchlorate. The elution yielded two main bands. The first band (singly charged product Λ -[Ru(bpy)₂(**Biot-1²-bpy'**)]⁺ where **Biot-1²-bpy'** is the hydrolyzed ligand carrying one negative charge) was eluted with a 0.05M NaClO₄ solution while the second band (desired doubly charged product Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺) was eluted with a 0.5M NaClO₄ solution.

MS (ESI (positive mode), for C₄₆H₄₈N₁₀O₅RuS, M_w = 954.0g/mol), m/z: 1069.2 (100, [M+(ClO₄ · H₂O)]⁺), 1169.2 (63, [M+((ClO₄)₂ · H₂O)]⁺), 1271.2 (24, [M+((ClO₄)₃ · H₂O)]⁺)

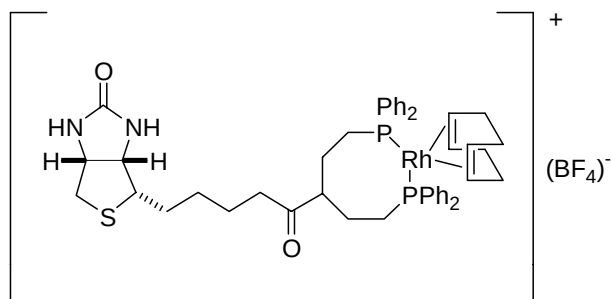
UV/Vis (H₂O), λ[nm](ε[M⁻¹cm⁻¹]): 434 (8200), 287 (62200), 254 (24400)

CD (H₂O), λ[nm](Δε[M⁻¹cm⁻¹]): 487 (5.3), 427 (-10.3), 300 (128.1), 279 (-72.6), 256 (3.4), 230 (-17.3)

[Rh(COD)(Biot-NP₂)]BF₄ (COD = cycloocta-1,5-dien)

The metal source [Rh(COD)₂]BF₄

(4.10mg, 10.10μmol) (complex was synthesized within the research group) was dissolved in MeOH (5mL). An aliquot (2.02mM, 1mL, 2.02μmol, 0.2eq)



of this solution was added to the ligand **Biot-NP₂** (1.35mg, 2.02μmol, 1.0eq) in MeOH (1mL) to obtain [Rh(COD)(**Biot-NP₂**)]BF₄. The resulting solution was diluted with MeOH (0.96mM, 2.1mL final volume, 2.02μmol, 0.2eq) and used for the CD measurements.

5.3. Titration Protocols

5.3.1. Stability Constant Determination of *rac*-, Δ - and Λ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺⊂(Strept)Avidin

5.3.1.1. General Considerations

All solutions were prepared and measured at room temperature in a phosphate buffer (pH = 7.0, I = 0.3M). All aliquots were added using micropipettes. The activity of the host proteins was determined using Gruber's and Kirsch's protocols¹⁶⁴⁻¹⁶⁷ (section 5.3.4.). The data were analyzed and fitted using the programs SPECFIT/32¹⁵¹ and PSEQUAD¹²⁸ (section 5.4.2.) as well as Excel's solver functionality¹²⁹ (sections 5.4.1. and 5.4.3.).

5.3.1.2. Determination of $K_{rac-, \Delta- \text{ and } \Lambda-[Ru(bpy)_2(Biot-1^2-bpy)]^{2+} \subset (strept)avidin}$

a) Direct Method (One-Step Process):

Aliquots of a *rac*-, Δ - or Λ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺ solution (0.96mM for the *rac*- and the Λ -complex, 0.64mM for the Δ -complex) were added to (strept)avidin (8.00μM initial concentration, 2400μL, 19.20nmol) in 0.25eq steps (5μL per step, up to 6.00eq) relative to the tetrameric protein. The CD measurements (250-500nm, band width 2nm, step resolution 0.5nm, response time 4s, 3 accumulations) were recorded at 50nm/min after thorough vortex mixing (1min at rt).

b) Indirect Method (Two-Step Process)**- Determination of K_{HABA} for Avidin (First Step)**

Aliquots of a HABA solution (0.32mM) were added in 0.25eq steps (5 μ L per step, up to 6.00eq) relative to the tetrameric avidin solution (2.67 μ M initial concentration, 2400 μ L, 6.40nmol). The UV/Vis (200-700nm) measurements were recorded at 50nm/min after thorough vortex mixing (1min at rt).

HABA $\subset$ avidin: $\lambda_{\text{max}} = 495\text{nm}$

- Determination of K_{HABA} for Streptavidin (First Step)

Aliquots of a HABA solution (0.96mM) were added first in 0.50eq and then in 1.00eq steps, respectively (10 μ L and 20 μ L, respectively, per step, up to 6.00eq), relative to the tetrameric streptavidin solution (8.00 μ M initial concentration, 2400 μ L, 19.20nmol). The UV/Vis (200-700nm) measurements were recorded at 50nm/min after thorough vortex mixing (1min at rt).

HABA $\subset$ streptavidin: $\lambda_{\text{max}} = 490\text{nm}$

- Determination of K_{exch} (Second Step)

The host proteins avidin and streptavidin (8.00 μ M initial concentration, 2400 μ L, 19.20nmol) were loaded with a large excess of a HABA solution (9.60mM stock solution, 20.00eq vs avidin and 150.00eq vs streptavidin, respectively, relative to the tetrameric protein) to ensure full saturation of the active sites (7.87 μ M, 2440 μ L, 19.20nmol for avidin and 7.11 μ M, 2700 μ L, 19.20nmol for streptavidin). Aliquots of a *rac*-, Δ - or Λ -[Ru(bpy)₂(**Biot-1²-bpy**)]²⁺ solution (0.96mM for the *rac*- and the Λ -complex (in MeOH), 0.64mM for the Δ -complex (in MeOH)) were added to HABA $\subset$ (strept)avidin in 0.25eq steps (5 μ L per step, up to 7.00eq)

relative to the tetrameric protein. The CD measurements (400-600nm, band width 2nm, step resolution 0.5nm, response time 4s, 3 accumulations) were recorded at 50nm/min after thorough vortex mixing (1min at rt).

5.3.2. Rate Constant Determination of Δ - and Λ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺ $\subset$ (Strept)Avidin (at 25°C)

5.3.2.1. General Considerations

All solutions were prepared at room temperature in a phosphate buffer (pH = 7.0, I = 0.3M). All aliquots were added using micropipettes. The activity of the host proteins was determined using Gruber's and Kirsch's protocols¹⁶⁴⁻¹⁶⁷ (section 5.3.4.). The data were analyzed and fitted using the program ORIGIN.¹⁵⁸

5.3.2.2. Determination of k_{off} [Δ - and Λ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺ $\subset$ (strept)avidin]

The host proteins avidin and streptavidin (8.00μM initial concentration, 2400μL, 19.20nmol) were loaded with an excess of Δ - or Λ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺ solution (0.96mM stock solution for the Λ -complex and 0.64mM stock solution for the Δ -complex, respectively, 1.20eq relative to the tetrameric protein) to ensure full saturation of the active sites. Upon addition of a large excess of a biotin solution (4.80mM stock solution, 600μL, 150.00eq relative to the tetrameric protein) to Δ - or Λ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺ $\subset$ (strept)avidin, the measurement was immediately started after thorough vortex mixing (10s). The CD measurements (250-400nm, band width 2nm, step resolution 0.5nm, response time 2s) were recorded at 200nm/min every 10min (the first 2-3h), 30min (the second 2-3h) and 60min (all measurements after the first 5-6h) at a temperature of 298K.

5.3.3. Stability Constant Determination of $[\text{Rh}(\text{COD})(\text{Biot-NP}_2)]^+ \subset (\text{Strept})\text{Avidin}$

5.3.3.1. General Considerations

All solvents were flushed with nitrogen for 2h prior to use. All solutions were prepared and measured at room temperature in a phosphate buffer (pH = 7.0, I = 0.3M). All aliquots were added using micropipettes in the glove box. The activity of the host proteins was determined using Gruber's and Kirsch's protocols¹⁶⁴⁻¹⁶⁷ (section 5.3.4.). The data were analyzed and fitted using PSEQUAD¹²⁸ (section 5.4.2.) and Excel's solver functionality¹²⁹ (section 5.4.3.).

5.3.3.2. Determination of $K_{[\text{Rh}(\text{COD})(\text{Biot-NP}_2)]^+ \subset (\text{strept})\text{avidin}}$ (Two-Step Process)

- Determination of K_{HABA} for Avidin (First Step)

Aliquots of a HABA solution (0.32mM) were added in 0.25eq steps (5 μ L per step, up to 6.00eq) relative to the tetrameric avidin solution (2.67 μ M initial concentration, 2400 μ L, 6.40nmol). The UV/Vis (200-700nm) measurements were recorded at 50nm/min after thorough vortex mixing (1min at rt).

HABA $\subset$ avidin: $\lambda_{\text{max}} = 495\text{nm}$

- Determination of K_{HABA} for Streptavidin (First Step)

Aliquots of a HABA solution (0.96mM) were added first in 0.50eq and then in 1.00eq steps, respectively (10 μ L and 20 μ L, respectively, per step, up to 6.00eq), relative to the tetrameric streptavidin solution (8.00 μ M initial concentration, 2400 μ L, 19.20nmol). The UV/Vis (200-700nm) measurements were recorded at 50nm/min after thorough vortex mixing (1min at rt).

HABA $\subset$ streptavidin: $\lambda_{\text{max}} = 490\text{nm}$

- Determination of K_{exch} (Second Step)

The host proteins avidin and streptavidin (8.00 μ M initial concentration, 2400 μ L, 19.20nmol) were loaded with a large excess of a HABA solution (9.60mM stock solution, 20.00eq vs avidin and 150.00eq vs streptavidin, respectively, relative to the tetrameric protein) to ensure full saturation of the active sites (7.87 μ M, 2440 μ L, 19.20nmol for avidin and 7.11 μ M, 2700 μ L, 19.20 μ mol for streptavidin). Aliquots of a $[\text{Rh}(\text{COD})(\text{Biot-}\mathbf{NP}_2)]^+$ solution (0.96mM in MeOH) were added to HABA $\subset$ (strept)avidin in 0.25eq steps (5 μ L per step, up to 7.00eq) relative to the tetrameric protein. The CD measurements (300-700nm, band width 2nm, step resolution 0.5nm, response time 4s, 3 accumulations) were recorded at 100nm/min after thorough vortex mixing (1min at rt).

5.3.4. Determination of the Active Site Concentration of (Strept)Avidin of a Given Solution**5.3.4.1. General Considerations**

During the calculation proceedings we had usually to correct the concentration of the (strept)avidin solution. The reason for this was an amount of salt impurities or endogenous biotin in the (strept)avidin powder after the purification steps. The effective concentration was therefore lower than the calculated one. The determination of the effective concentration of (strept)avidin was performed by a displacement titration (HABA was displaced by biotin) following the decreasing signal of HABA at 506nm.

5.3.4.2. Determination of the Active Site Concentration of (Strept)Avidin by Biotin Titration

The host proteins avidin and streptavidin (8.00 μ M initial concentration, 2400 μ L, 19.20nmol) were loaded with a large excess of a HABA solution (9.60mM stock solution, 20.00eq vs avidin and 150.00eq vs streptavidin, respectively, relative to the tetrameric protein) to ensure full saturation of the active sites (7.87 μ M, 2440 μ L, 19.20nmol for avidin and 7.11 μ M, 2700 μ L, 19.20 μ mol for streptavidin). Aliquots of a biotin solution (9.60mM) were added to HABA $\subset$ (strept)avidin in 0.25eq steps (5 μ L per step, up to 6.00eq) relative to the tetrameric protein. The CD measurements (480-520nm, band width 2nm, step resolution 0.5nm, response time 4s, 3 accumulations) were recorded at 100nm/min after thorough vortex mixing (1min at rt). An example of such a determination of the active site concentration of avidin is shown in **Figure 5-1**.

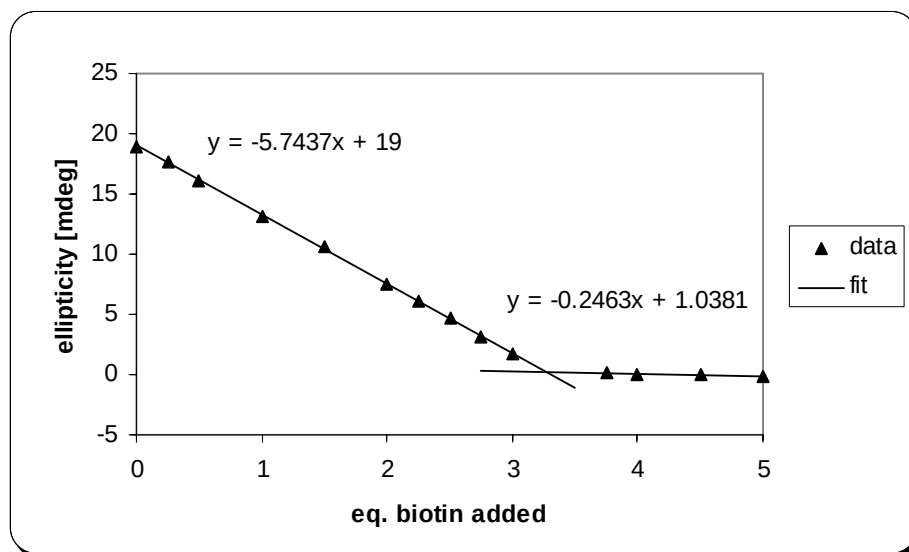


Figure 5-1. Disappearance of a CD band ($\lambda_{\text{max}} = 506\text{nm}$) upon addition of biotin aliquots to HABA $\subset$ protein allows the determination of the active site concentration of the protein. In the represented example (for avidin), the active site concentration is equal to $x = 3.27$ (3.27 of 4.00 binding sites).

5.4. Calculations

5.4.1. Determination of $K/\beta_{rac-, \Delta-}$ and Λ -[Ru(bpy)₂(Biot-1²-bpy)]²⁺ $\subset$ (strept)avidin (with Models a-d)

The complete systems of equations and polynomial equations for the four models a-d, as mentioned in **Chapter 3**, are the following:

a) “Monomer-like” model:

$$K_1 = \frac{[PL]}{[P] \cdot [L]} \quad (5.1)$$

$$[P]_{\text{tot}} = [P] + [PL] \quad (5.2)$$

$$[L]_{\text{tot}} = [L] + [PL] \quad (5.3)$$

$$K_1 \cdot [L]^2 + (1 + K_1 \cdot [P]_{\text{tot}} - K_1 \cdot [L]_{\text{tot}}) \cdot [L] - [L]_{\text{tot}} = 0 \quad (5.4)$$

b) “One-step” model:

$$\beta_4 = \frac{[PL_4]}{[P] \cdot [L]^4} \quad (5.5)$$

$$[P]_{\text{tot}} = [P] + [PL_4] \quad (5.6)$$

$$[L]_{\text{tot}} = [L] + 4 \cdot [PL_4] \quad (5.7)$$

$$\beta_4 \cdot [L]^5 + (4 \cdot \beta_4 \cdot [P]_{\text{tot}} - \beta_4 \cdot [L]_{\text{tot}}) \cdot [L]^4 + [L] - [L]_{\text{tot}} = 0 \quad (5.8)$$

c) “Two-step” model:

$$\beta_2 = \frac{[PL_2]}{[P] \cdot [L]^2} \quad (5.9)$$

$$\beta_4 = \frac{[PL_4]}{[PL_2] \cdot [L]^2} \quad (5.10)$$

$$[P]_{\text{tot}} = [P] + [PL_2] + [PL_4] \quad (5.11)$$

$$[L]_{\text{tot}} = [L] + 2 \cdot [PL_2] + 4 \cdot [PL_4] \quad (5.12)$$

$$\begin{aligned} & \beta_2 \cdot \beta_4 \cdot [L]^5 + (4 \cdot \beta_2 \cdot \beta_4 \cdot [P]_{\text{tot}} - \beta_2 \cdot \beta_4 \cdot [L]_{\text{tot}}) \cdot [L]^4 + \beta_2 \cdot [L]^3 + \dots \\ & \dots (2 \cdot \beta_2 \cdot [P]_{\text{tot}} - \beta_2 \cdot [L]_{\text{tot}}) \cdot [L]^2 + [L] - [L]_{\text{tot}} = 0 \end{aligned} \quad (5.13)$$

d) “Four-step” model:

$$K_1 = \frac{[PL]}{[P] \cdot [L]} \quad (5.14)$$

$$K_2 = \frac{[PL_2]}{[PL] \cdot [L]} \quad (5.15)$$

$$K_3 = \frac{[PL_3]}{[PL_2] \cdot [L]} \quad (5.16)$$

$$K_4 = \frac{[PL_4]}{[PL_3] \cdot [L]} \quad (5.17)$$

$$[P]_{\text{tot}} = [P] + [PL] + [PL_2] + [PL_3] + [PL_4] \quad (5.18)$$

$$[L]_{\text{tot}} = [L] + [PL] + 2 \cdot [PL_2] + 3 \cdot [PL_3] + 4 \cdot [PL_4] \quad (5.19)$$

$$\begin{aligned} & K_1 \cdot K_2 \cdot K_3 \cdot K_4 \cdot [L]^5 + (K_1 \cdot K_2 \cdot K_3 + 4 \cdot K_1 \cdot K_2 \cdot K_3 \cdot K_4 \cdot [P]_{\text{tot}} - K_1 \cdot K_2 \cdot K_3 \cdot K_4 \cdot [L]_{\text{tot}}) \cdot [L]^4 + \dots \\ & \dots (K_1 \cdot K_2 + 3 \cdot K_1 \cdot K_2 \cdot K_3 \cdot [P]_{\text{tot}} - K_1 \cdot K_2 \cdot K_3 \cdot [L]_{\text{tot}}) \cdot [L]^3 + \dots \\ & \dots (K_1 + 2 \cdot K_1 \cdot K_2 \cdot [P]_{\text{tot}} - K_1 \cdot K_2 \cdot [L]_{\text{tot}}) \cdot [L]^2 + (1 + K_1 \cdot [P]_{\text{tot}} - K_1 \cdot [L]_{\text{tot}}) \cdot [L] - [L]_{\text{tot}} = 0 \end{aligned} \quad (5.20)$$

In order to solve the system of equations described above, an initial set of K_i values was needed. The optimization of the fit was done by varying systematically one or several fitting parameters, solving the polynomial in each data point with a Newton-Raphson algorithm, computing the sum of the squares of the distance to the measured curve, comparing this sum with the best obtained in previous steps and starting the cycle again by varying the parameters until a minimum of this sum was found.

5.4.2. Determination of K_{HABA} (with PSEQUAD) (First Step)

The spectrophotometric data were fitted with the PSEQUAD program.¹²⁸ The program uses the mass balance equations of the components and calculates the stability constants of the absorbing species. In this case $\text{HABA} \subset (\text{strept})\text{avidin}$ was the only absorbing species. Moreover, it was assumed that the four binding events were non-cooperative, giving following equilibrium:



Therefore, the K_{HABA} was

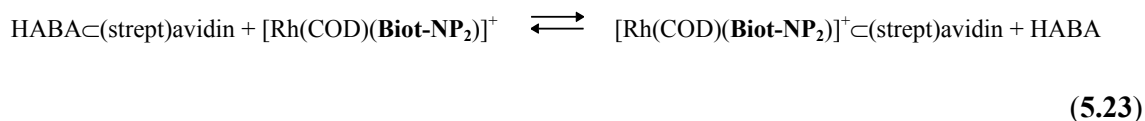
$$K_{\text{HABA}} = \frac{[\text{HABA} \subset (\text{strept})\text{avidin}]}{[\text{HABA}] \cdot [(\text{strept})\text{avidin}]} \quad (5.22)$$

PSEQUAD varies iteratively the stability constant to minimize the sum of the squares of the differences between observed and calculated absorbance values. The calculations were performed with the absorbance data in the 420-580nm range at 33 different wavelengths for avidin and in the 440-600nm range at 33 different wavelengths for streptavidin. For avidin (streptavidin), sets from 0.25eq to 6.00eq (from 0.50eq to 13.00eq) were used for the calculations with PSEQUAD.

Another way to determine the value of K_{HABA} was by applying the “monomer-like” model from the direct method assuming that the binding sites were independent from each other in the case of HABA (see section 5.4.1.).

5.4.3. Determination of K_{exch} (with Derived Mathematical Procedure) (Second Step)

In order to determine the overall stability constant $K_{[\text{Rh}(\text{COD})(\text{Biot}-\text{NP}_2)]^+ \subset (\text{strept})\text{avidin}}$ (also available for $K_{\text{rac-}, \Delta-}$ and $\Lambda\text{-}[\text{Ru}(\text{bpy})_2(\text{Biot}-\text{I}^2\text{-bpy})]^{2+} \subset (\text{strept})\text{avidin}$), (K_{HABA} (first step) and K_{exch} (second step) had to be calculated. K_{HABA} was already determined in a previous chapter (section 5.4.2.). For the determination of K_{exch} , a mathematical procedure for fitting the data was derived. The following equilibrium was assumed as a working hypothesis:



The fitting procedure required to calculate a “theoretical” signal and compare it to the measured value. Variation of the parameter K_{exch} in the signal derivation following a least-square distance procedure allowed to optimize the fitting curve and led to an estimated value of K_{exch} . The calculated signal was obtained by multiplying $[\text{HABA} \subset (\text{strept})\text{avidin}]$ by the extinction coefficient of the HABA complex (computed from the solution saturated in HABA). $[\text{HABA} \subset (\text{strept})\text{avidin}]$ was obtained from the following system of **Equations 5.24** to **5.27**.

$$K_{\text{exch}} = \frac{[[\text{Rh}(\text{COD})(\text{Biot}-\text{NP}_2)]^+ \subset (\text{strept})\text{avidin}] \cdot [\text{HABA}]}{[\text{HABA} \subset (\text{strept})\text{avidin}] \cdot [[\text{Rh}(\text{COD})(\text{Biot}-\text{NP}_2)]^+]} \quad (5.24)$$

$$[(\text{strept})\text{avidin}]_{\text{tot}} = [[\text{Rh}(\text{COD})(\text{Biot}-\text{NP}_2)]^+ \subset (\text{strept})\text{avidin}] + [\text{HABA} \subset (\text{strept})\text{avidin}] \quad (5.25)$$

$$[\text{HABA}]_{\text{tot}} = [\text{HABA}] + [\text{HABA} \subset (\text{strept})\text{avidin}] \quad (5.26)$$

$$[[\text{Rh}(\text{COD})(\text{Biot}-\text{NP}_2)]^+]_{\text{tot}} = [[\text{Rh}(\text{COD})(\text{Biot}-\text{NP}_2)]^+] + [[\text{Rh}(\text{COD})(\text{Biot}-\text{NP}_2)]^+ \subset (\text{strept})\text{avidin}] \quad (5.27)$$

Rearranging the system, a single quadratic equation depending only on $[HABA \subset (strept)avidin]$ was obtained:

$$(1 - K_{exch}) \cdot [HABA \subset (strept)avidin]^2 + (K_{exch} \cdot [(strept)avidin]_{tot} - [Rh(COD)(Biot - NP_2)]^+_{tot}) \cdot [HABA \subset (strept)avidin] - [HABA]_{tot} \cdot [(strept)avidin]_{tot} = 0 \quad (5.28)$$

For given values of the fitting parameter K_{exch} , $[HABA \subset (strept)avidin]$ (and hence the signal) was calculated and the least-square distance procedure was performed until the sum on all data points of $(signal_{calc} - signal_{measured})^2$ reached a minimum.

Est rerum omnium magister usus.

Gaius Iulius Caesar, 100 BC – 44 BC

CHAPTER 6:

APPENDIX

Introduction to Chiroptical Methods^{122,168-172}

To understand the basis of chiroptical measurements, one has to recall that light is an electromagnetic radiation, which can be considered to behave as two wave motions: an electric and a magnetic one. Electric and magnetic waves (or fields $\vec{E}$ and $\vec{M}$), propagated in time, are generated by oscillating electric and magnetic vectors (dipoles) that lie perpendicular to one another. Although the energies associated with the electric and magnetic waves are equal, most optical measurements (UV/Vis, CD, etc.) are concerned only with the electric field $\vec{E}$.

Ordinary electromagnetic radiation is unpolarized (or isotropic), meaning that the electric field $\vec{E}$ (vectors) associated with the light oscillates in all directions perpendicular to the direction of propagation. Thus, ordinary light consists of different wavelengths vibrating in many different planes. However, if the light is filtered so as to remove all oscillations or waves other than in one direction, the light is anisotropic and linearly or plane polarized (oscillating axis and propagation axis give the polarization plane). Linearly polarized light can be resolved into two circularly polarized components $\vec{E}_L$ and $\vec{E}_R$ (called left circularly and right circularly polarized components). As long as the phases and the intensities of the two circularly polarized components remain the same (the components are counterrotating at equal angular rates ω_L and ω_R), their resultant will lie in a plane and oscillate in magnitude. This is the case, when linearly polarized light passes through an achiral and non-absorbing medium,

where both angular rates vary in the same way that after a certain time t , the angle $\varphi_L(t)$ ($=\omega_L t$) is exactly the opposite of $\varphi_R(t)$ ($=\omega_R t$), that means that the initial phase relationship is maintained (see **Figure 6-1a**).¹⁷³

When linear polarized light passes through an optically active (chiral: enantiopure or enantioenriched) but non-absorbing medium, $\varphi_L(t)$ and $\varphi_R(t)$ vary differently ($\omega_L \neq \omega_R$) (**Figure 6-1b**).¹⁷³ The anisotropic medium does not transmit left and right circularly polarized light equally, where the initial phase relationship is not maintained upon emergence from the sample. An optically active substance thus exhibits circular birefringence, where the indices of refraction for left (n_L) and right (n_R) circularly polarized light are not equal, $n_L \neq n_R$. The difference of time Δt [s] between the two circularly polarized rays, after having passed through an optical active medium of thickness d [cm], is therefore, by taking into account that the indice of refraction is in inverse proportion to the propagation of rate v :

$$\Delta t = \frac{d}{v_L} - \frac{d}{v_R} = \frac{d \cdot (n_L - n_R)}{c} \quad (6.1)$$

where v_L [cm/s] and v_R [cm/s] are the propagation rates of the left and right circularly polarized light that are displaced afterwards by c/n_L and c/n_R (c = propagation rate of light [cm/s]).

The phase difference Φ between the two rays is:

$$\Phi = 2 \cdot \pi \cdot v \cdot \Delta t = \frac{2 \cdot \pi \cdot v \cdot d}{c} \cdot (n_L - n_R) \quad (6.2)$$

And with $v/c = 1/\lambda$ (λ in [cm]):

$$\Phi = 2 \cdot \pi \cdot v \cdot \Delta t = \frac{2 \cdot \pi \cdot d}{\lambda} \cdot (n_L - n_R) \quad (6.3)$$

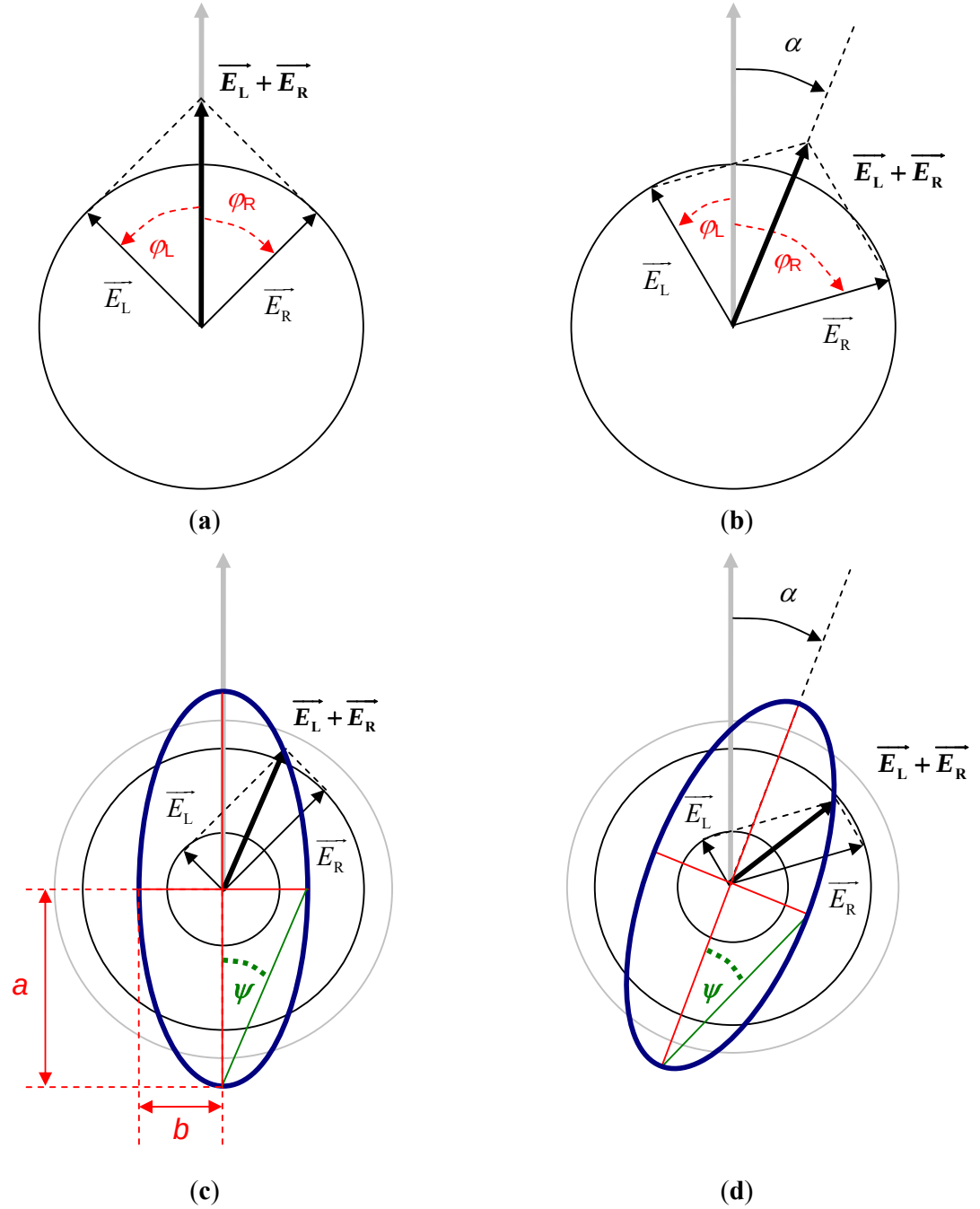


Figure 6-1. Modification of the electric field vectors of (initially) linear polarized monochromatic light with time after having passed through a medium. The initial resultants are traced in grey. Four cases are considered:

- a) The medium is not optically active and does not absorb the light of a given wavelength ($\omega_L = \omega_R$; $\varepsilon_L = \varepsilon_R$).
- b) The medium is optically active and does not absorb the light of a given wavelength ($\omega_L \neq \omega_R$; $\varepsilon_L = \varepsilon_R$).
- c) The medium is optically active and light-absorbing at a given wavelength ($\omega_L = \omega_R$ ($\alpha = 0$); $\varepsilon_L \neq \varepsilon_R$).
- d) The medium is optically active and light-absorbing at a given wavelength ($\omega_L \neq \omega_R$ ($\alpha \neq 0$); $\varepsilon_L \neq \varepsilon_R$).¹⁷³

After the two circularly polarized rays having passed through an optically active medium, the resultant plane of polarization is rotated because of the phase difference from its original orientation by the angle $\alpha = \phi/2$ [°]. The rotation of the polarization plane is therefore:

$$\alpha = \frac{\pi \cdot d}{\lambda} \cdot (n_L - n_R) = \frac{180 \cdot d}{\lambda} (n_L - n_R) \quad (6.4)$$

When reporting rotations of optically active solutions, one typically normalizes α against the sample concentration c' [g/cm³] or c'' [g/100cm³] and path length d (by convention in [dm]) and cites the specific rotation $[\alpha]$ as a function of temperature T [°C] and wavelength λ [nm]:

$$[\alpha]_{\lambda}^T = \frac{\alpha}{c' \cdot d} = \frac{100 \cdot \alpha}{c'' \cdot d} \quad (6.5)$$

Imagine two homologous compounds whose molecules contain the same “chiral” part, but are attached to two different alkyl chains, a short and a long one. The specific rotation of the latter compound will then be smaller than that of the first, since the long (supposedly achiral) chain acts like a solvent molecule. One corrects, therefore, by multiplication with the relative molar mass M [g/mol]. Since these values often would become very large, it was proposed that they be divided by 100, so the molecular rotation $[\alpha_M]$ is defined by following formula:

$$[\alpha_M]_{\lambda}^T = \frac{[\alpha]_{\lambda}^T \cdot M}{100} \quad (6.6)$$

As for the specific rotation $[\alpha]$, the molar rotation $[\alpha_M]$ is cited as a function of temperature T [°C] and wavelength λ [nm]. Note that division by 100 is introduced here for convenience, whereas in **Equation 6.5** the factor is necessitated by the units' convention!

A plot of rotation as a function of wavelength is called optical rotatory dispersion (ORD). For a non-absorbing substance the absolute values of α decrease asymptotically with increasing λ (normal ORD) according to **Equation 6.4**.

When light passes through an absorbing optically active medium, the right circularly polarized component can be absorbed differently from the left circularly polarized component ($\vec{E}_L \neq \vec{E}_R$ and $\varepsilon_L \neq \varepsilon_R$) (**Figures 6-1c and 6-1d**).¹⁷³ The difference is called circular dichroism (CD), and in all commercially available “dichrographs”, the difference in absorption for the two helical rays is recorded. The difference of the absorbance ΔA ($A_L - A_R$) is directly related to the differences of the molar circular dichroic absorption coefficient $\Delta \varepsilon$ [$M^{-1}cm^{-1}$] by the law of Bouguer-Lambert-Beer (c is the concentration in $[M]$ and d is the path length in $[cm]$):

$$\Delta \varepsilon = \varepsilon_L - \varepsilon_R = \frac{A_L - A_R}{c \cdot d} \quad (6.7)$$

If $\vec{E}_L$ is less intense (absorbs more, $\varepsilon_L > \varepsilon_R$) than $\vec{E}_R$, the resultant of the electric field vector follows an elliptical path, corresponding to elliptically polarized light (**Figures 6-1c and 6-1d**).¹⁷³ The ellipse is characterized by a major axis of length $2a$, where a is:

$$a = \left| \vec{E}_L + \vec{E}_R \right| \quad (6.8)$$

and a minor axis of length $2b$, where b is:

$$b = \left| \vec{E}_L - \vec{E}_R \right| \quad (6.9)$$

The angle whose tangent is equal to the ratio between the minor axis b and the major axis a of the elliptically polarized light is called ellipticity ψ :

$$\psi = \arctan \frac{b}{a} \quad (6.10)$$

The numerical values of ψ are in general very small (the ellipses in **Figures 6-1c and 6-1d** would appear as line when using real numerical values).¹⁷³ For this reason, ψ is mainly given in m° (millidegrees). In principle, the ellipticity can be measured directly (and has

occasionally been in the past), but none of the commercially available instruments does this, although they are sometimes calibrated in ψ . As already mentioned, in all commercially available “dichrographs” the difference of the absorbance ΔA is recorded. It can easily be shown that ellipticity ψ is directly proportional to the circular dichroic absorption ΔA :

$$\psi = \frac{360 \cdot 1000 \cdot m^\circ}{2 \cdot \pi} \cdot \frac{\ln 10}{4} \cdot (A_L - A_R) = 32980 \cdot (A_L - A_R) \quad (6.11)$$

Since ellipticity ψ is an angle like α , analogous definitions were introduced for ellipticity ψ as for rotation α , the so-called specific ellipticity $[\psi]$:

$$[\psi]_\lambda^T = \frac{\psi}{c' \cdot d} \quad (6.12)$$

Molecular ellipticity $[\Theta]$ is expressed as follows:

$$[\Theta]_\lambda^T = \frac{[\psi]_\lambda^T \cdot M}{100} \quad (6.13)$$

In other terms, $\Delta \varepsilon$ is called circular dichroism and ellipticity is an effect caused by circular dichroism.

A plot of α , $[\alpha]$ or $[\Theta]$ vs λ of an absorbing substance gives an anomalous dispersion curve (anomalous ORD effect). With the peak appearing at longer wavelength than the trough, the ORD curve is said to have a positive Cotton effect. The mirror image curve, with the trough at the longer wavelength, would correspond to a negative Cotton effect. A zero angle point in the anomalous ORD spectrum corresponds to an extremum in the CD spectrum and to a maximum in the normal absorption spectrum. The vertical distance from peak to trough, measured in $[\Theta]$ units is called the amplitude of the ORD Cotton effect and is used as a quantitative measure of the ORD transition. The signed order of an ORD curve (e.g. peak of long wavelength and trough of short wavelength) can often be correlated with the

stereochemistry of a chiral molecule. However, CD curves have definite advantages over ORD curves for quantitative measurements. For optically active samples, although ΔA and $[\theta]$ (and hence CD) can be detected only in the region of an absorption band, $n_L - n_R$, or the change in optical rotation α , is everywhere finite and thus can be detected throughout the electromagnetic spectrum. Hence, CD spectroscopy offers the distinct advantages of few overlapping signals, whereas in ORD all of the transitions overlap at every wavelength.

Note:

- **Optical Rotatory Dispersion (ORD)** measures circular birefringence, the unequal transmission (velocity) of left and right circularly polarized light as it travels through a sample. An ORD spectrum plots the variation of rotation of the plane of polarization (of plane polarized light) as a function of wavelength.
- **Circular Dichroism (CD)** is the unequal absorption of left and right circularly polarized light as it travels through a sample. A CD spectrum plots the absorption difference ($\Delta A = A_L - A_R$), the molar circular dichroic absorption coefficient difference ($\Delta \epsilon = \epsilon_L - \epsilon_R$) or the change in angle of **ellipticity** ψ as a function of wavelength.
- Linearly and circularly (pure left or pure right) polarized light are two special cases of elliptically polarized light: in the first case, the ellipticity ψ is equal to 0° , meaning that the ellipse is indefinitely thin and degenerates to the major axis, whereas in the second case, the ellipticity ψ is 45° , meaning that the ellipse degenerates to a circle.

- The three effects (anomalous ORD, CD and ellipticity) are called “Cotton effect” in honour of the French physicist Aimé Cotton (1869-1951).

Non omnia possumus omnes.

Gaius Lucilius, 180 BC – 101 BC

CHAPTER 7:

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Finis coronat opus.

Publius Ovidius Naso, 43 BC – AD 17

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Errare humanum est !

Lucius Annaeus Seneca, 3 BC – AD 65