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Porphobilinogen Synthase: A Challenge for the Chemist?

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Abstract: The initial steps in the biosynthesis of the tetrapyrrolic dyes, called the 'pigments of life', are highly convergent. The formation of porphobilinogen, the pyrrolic precursor of the tetrapyrrolic skeleton, uses δ-aminolevulinate as the starting material. This amino acid is dedicated to the biosynthesis of tetrapyrroles. However, the chemical condensation of δ-aminolevulinate leads to a symmetric pyrazine. Attempts to imitate the biosynthesis using one of the proposed pathways for the biosynthesis of porphobilinogen as a guideline has allowed us to synthesize a protected precursor of porphobilinogen in an efficient way. Based on the two major proposals for the biosynthesis, a series of specifically synthesized inhibitors was also tested. The inhibition behavior and the potency of the inhibitors expressed as their K_i value has unraveled an interesting relationship between the structure of the inhibitor and the strength of its interaction with the active site. The concerted use of mechanistic analysis, synthesis and kinetic studies of inhibitors has increased our knowledge about the enzyme porphobilinogen synthase. Structural studies of enzyme-inhibitor complexes will hopefully complement the kinetic results accumulated so far.

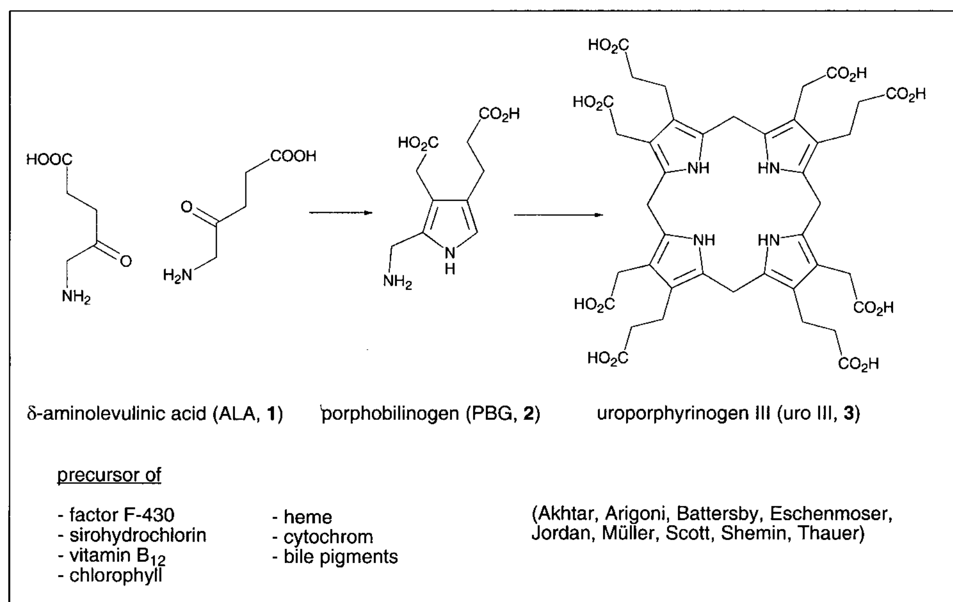
Keywords: δ-Aminolevulinate · Biosynthesis · Inhibition · Porphobilinogen · Tetrapyrroles

Introduction: Importance, Biosynthesis and Prebiotic Synthesis of the Tetrapyrrolic 'Pigments of Life'

The tetrapyrrolic dyes, which have been called the 'pigments of life' [1], have attracted the attention of chemists and biologists alike. The 'pigments of life' are crucial cofactors for processes like photosynthesis, oxygen transport, and many oxidation processes just to mention a few of the most important processes that require tetrapyrrolic cofactors [2].

The obvious structural similarity of these cofactors used for widely different processes was interpreted as an indication of a common biosynthetic pathway, maybe even of a common prebiotic history of this class of compounds [3–8]. And, indeed, the tetrapyrrolic skeleton of all 'pigments of life' is synthesized in a highly convergent way, starting with eight molecules of δ-aminolevulinic acid (**1**). δ-Aminolevulinic acid (**1**) is then condensed to porphobilinogen (**2**), which itself tetramerises to form uroporphyrinogen III (**3**) (Scheme 1).

The dimerization of δ-aminolevulinate (**1**) to porphobilinogen (**2**) and the tetramerization of **2** are both exergonic [9][10]. This observation was considered as an argument in favor of a spontaneous



Scheme 1. Biosynthesis of uroporphyrinogen III (**3**)

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formation of tetrapyrroles [4][5]. A series of remarkable experiments in the group of Eschenmoser at the ETH investigated whether tetrapyrroles can be formed under so called prebiotic conditions [11]. The astonishing outcome of these experiments was that it is easier to reproduce the more complex, latter stages of the biosynthesis under prebiotic conditions than to simulate the dimerization of **1**.

The Challenge of the Biosynthesis of Porphobilinogen

The tetramerization of **2** could be achieved without the help of an enzyme [12]. Porphobilinogen (**2**) forms uroporphyrinogens I to IV upon heating in the presence of mineral acid. Based on this observation the enzymatic transformation has been called an example of a chemomimetic biosynthesis [6].

Chemists have not had the same success imitating the seemingly simpler dimerization of δ-aminolevulinic acid (**1**) to porphobilinogen (**2**) [13–17]. Chemical dimerization of **1** can be easily achieved. Under conditions which allow oxidation either in air or by the addition of iodine, a symmetric dimer, pyrazine **4**, was obtained in good yields [18]. These observations raise questions about the mechanism of the transformation of **1** catalyzed by porphobilinogen synthase (= PBGS) and the reaction occurring in test-tube (Scheme 2).

The lack of success imitating the biosynthesis of porphobilinogen [19–22] is all the more surprising because the Knorr pyrrole synthesis is a synthetic method well-known in heterocyclic chemistry [23–25]. The Knorr pyrrole synthesis yields pyrroles starting from an α-aminoketone and an appropriate carbonyl component. The importance of the 'pigments of life' and the dichotomy between the chemical reactivity of δ-aminolevulinic acid (**1**) and the biochemical transformation were strong motivations to study the chemical synthesis and the biochemical formation of porphobilinogen (**2**) [15][20][21][26][27]. Since the beginning of organic chemistry, the desire to understand biological processes has been amalgamated with the wish to imitate nature [28–31].

Proposals for the Mechanism of Porphobilinogen Synthase

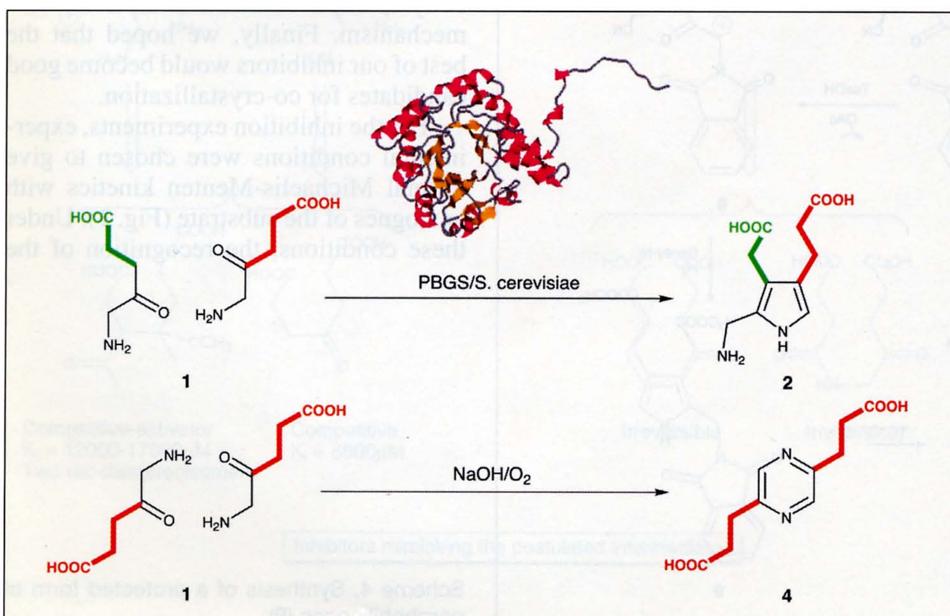
Kinetic and biochemical studies alone failed to reveal the sequence of mechanistic steps exploited by porphobilinogen synthase. As we now know, careful biochemical studies over a period of more than 30 years failed to identify all the important groups at the active site. X-ray structures of porphobilinogen synthase became available only very recently [32–34]. More importantly, the structures of porphobilinogen synthase co-crystallized with levulinic acid, a well-known competitive inhibitor, were solved as well [34–36]. These crystal structures increased our knowledge about porphobilinogen synthase dramatically.

Shemin was the first to propose a mechanism for porphobilinogen synthase drawing a close analogy between this enzyme and class I aldolases [37–39]. Starting from the results of his elegant single turn-over experiments, Jordan postulated two alternative mechanisms for the formation of porphobilinogen (**2**), which are the basis of our research today (Scheme 3) [40–43]. Jordan initially suggested that the first bond connecting the two substrate molecules is the carbon–nitrogen bond of the Schiff base. Only after this step, can the aldol reaction and subsequent elimination occur, leading to the five-membered nitrogen heterocycle. This mechanism closely follows the known mechanism of the Knorr pyrrole synthesis. In the alternative mechanism, the aldol reaction is postulated to occur first, forming the carbon-carbon bond joining the two substrates.

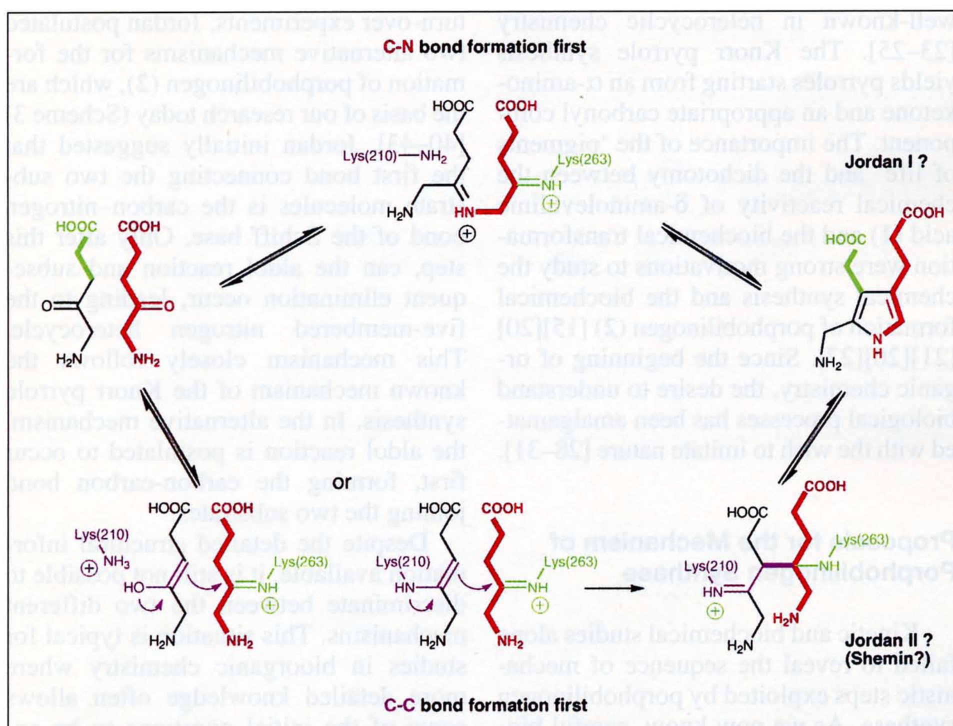
Despite the detailed structural information available, it is still not possible to discriminate between the two different mechanisms. This situation is typical for studies in bioorganic chemistry where more detailed knowledge often allows some of the initial questions to be answered, while raising new, even more intriguing problems. Under these circumstances, additional knowledge from model studies is necessary to guide future research on porphobilinogen synthase.

Novel Pyrrole Synthesis Based on the Shemin-Jordan Mechanism for the Biosynthesis

Following the mechanistic proposals of Shemin and Jordan, one can ask whether pyrroles can be synthesized by imitating the sequence of transformations proposed for the biosynthesis of porphobilinogen (**2**).



Scheme 2. Comparison of the chemical dimerization of δ-aminolevulinic acid (**1**) with the porphobilinogen (**2**) biosynthesis catalyzed by the enzyme PBGS



Scheme 3. The two main mechanistic proposals for porphobilinogen synthase

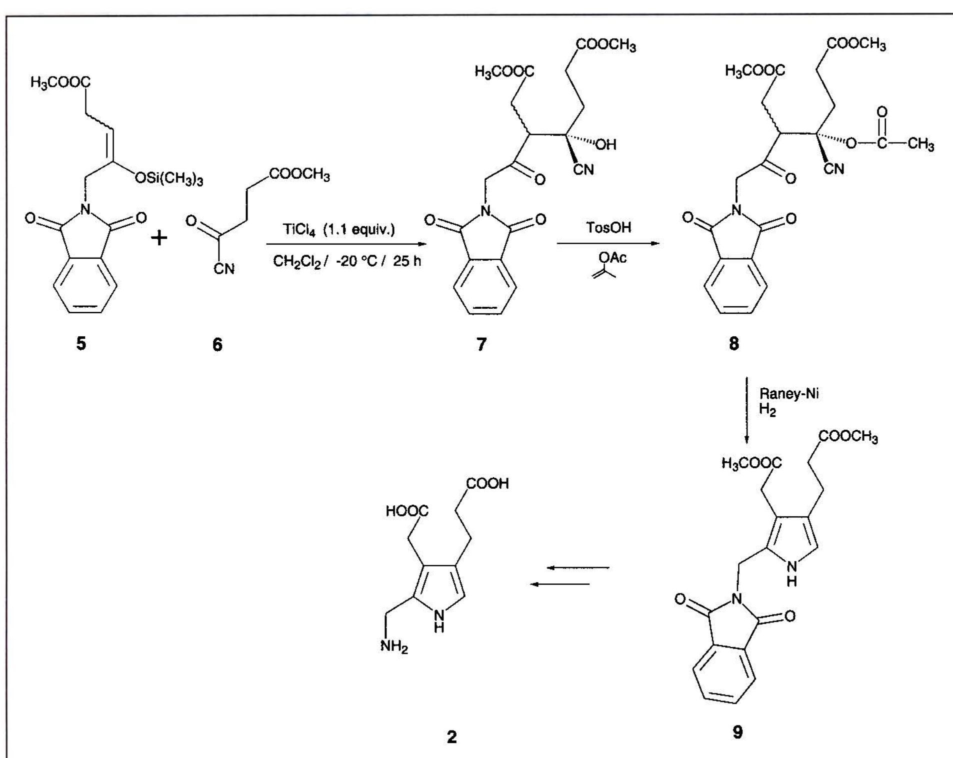
Based on this mechanistically motivated question, a novel synthesis of pyrroles has been developed. The key step is the Mukaiyama crossed aldol reaction between regioselectively generated silyl enol ethers and acylcyanide (Scheme 4) [20][21]. The Mukaiyama crossed aldol reaction forms the crucial carbon-carbon bond [44][45]. This special type of Mukaiyama crossed aldol reaction imitates the postulated carbon-carbon bond formation but the oxidation state of the substrate incorporated at the P-side of porphobilinogen (**2**) has to be modified. Adjustment of the oxidation state proved to be more difficult than initially predicted. Removal of the protecting groups from the porphobilinogen derivative **9** over two steps has already been described in the literature [46][47].

Our synthetic efforts have clearly shown that once the carbon-carbon bond has coupled the two substrates together, the rest of the process leading to the pyrrole is chemically easy and thermodynamically favored. However, the conditions necessary to achieve this central bond formation are such that one hesitates to push the analogy between the chemistry in the flask and the chemistry on the enzyme surface too far.

Inhibition Studies of Porphobilinogen Synthase from *Escherichia coli*

In recent years it has become possible to elicit catalytic antibodies using antigens imitating transition states of certain transformations [48–50]. This approach has its theoretical basis in Pauling's postulate that the active site of an enzyme should be complementary to the transition state of the catalyzed reaction [51][52]. A corollary of these findings is the fact that the potency of many excellent inhibitors can be interpreted if a strong resemblance of these compounds to the structure of the transition state of the rate determining step is assumed [52]. In order to obtain further information on the biosynthesis of porphobilinogen synthase, we have undertaken a systematic search of the inhibition behavior of this enzyme [19][53–56]. We intended to accumulate sufficient knowledge about the recognition site of porphobilinogen synthase so that conclusions about the mechanism could be deduced. Based on firm knowledge about the factors responsible for the recognition at the active site, it should be possible to interpret the whole body of experimental data in terms of a mechanism. Finally, we hoped that the best of our inhibitors would become good candidates for co-crystallization.

For the inhibition experiments, experimental conditions were chosen to give normal Michaelis-Menten kinetics with analogues of the substrate (Fig. 1). Under these conditions, the recognition of the



Scheme 4. Synthesis of a protected form of porphobilinogen (**9**)

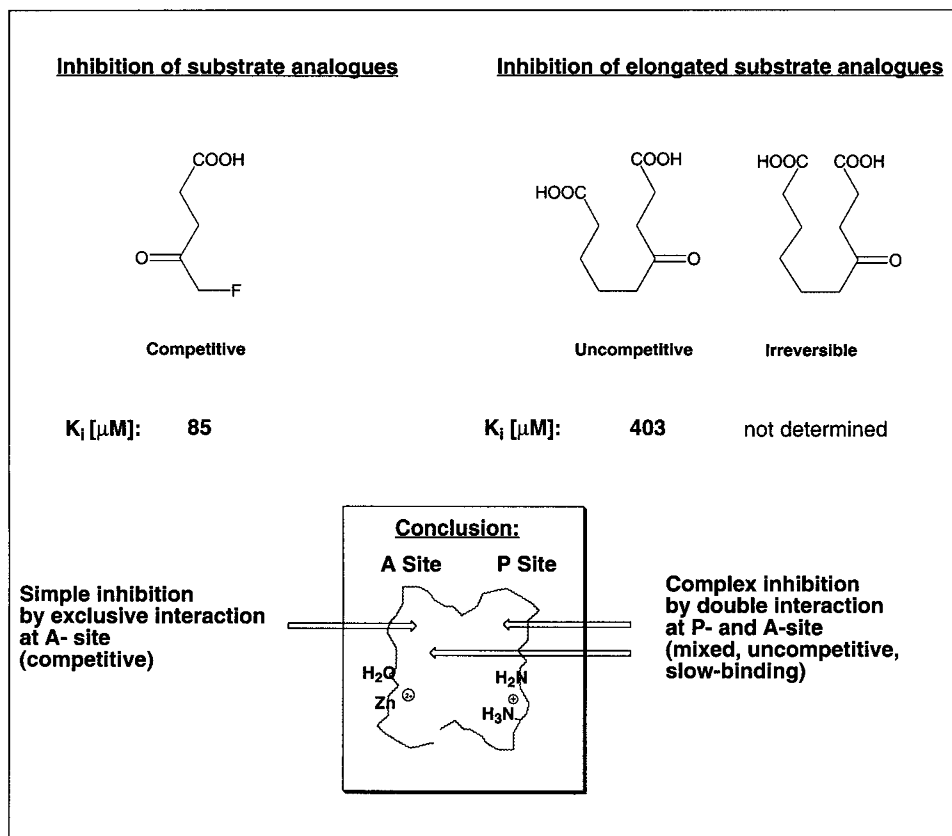
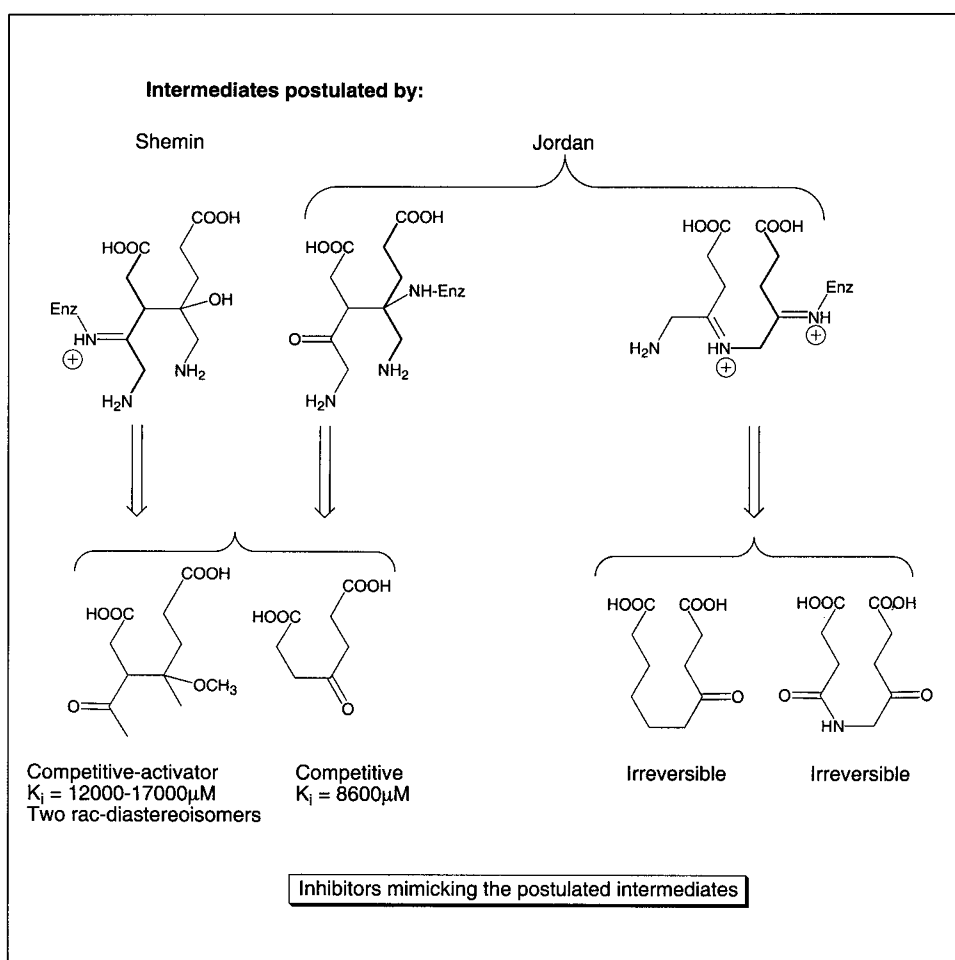


Fig. 1. Inhibition type as a function of the sites of interaction



Scheme 5. Comparison of the structures postulated as intermediates by Shemin and Jordan respectively with diacids used as inhibitors

second substrate at the A-site of the enzyme is determined. When the inhibition behavior of a specific inhibitor becomes more complex, this is a clear sign that not only competition for the binding of the second substrate at the A-site plays a role, but that a double interaction can occur. If an inhibitor interacts with the A- and the P-site, this double interaction leads to mixed or uncompetitive inhibition. If the interaction becomes thermodynamically stronger than the interaction with the natural substrate, then slow tight binding can be observed. Under these experimental conditions the type and the site(s) of interaction of a specific inhibitor can be deduced from the inhibition type determined with the help of the kinetic analysis. Studying more than 100 inhibitors, this correlation could be verified and has become an important foundation for the interpretation of the experimental results.

The most important application of this approach was the study of a series of diacids that were considered to be analogues of the postulated intermediates (Scheme 5).

After the first bond between the two substrates is formed, a diacid is created in both postulated mechanisms. The difference between the two proposals is the fact that one diacid is a derivative of pimelic acid, whereas the alternative diacid intermediate is a derivative of sebacic acid. In order to optimize recognition of our inhibitors, we added a γ-keto function, which allows the inhibitor to interact at three points with the active site.

Systematic study of the γ-keto dicarboxylic acids from C_5 to C_{12} was very informative (Fig. 2). The first three dicarboxylic acids C_5 to C_7 are weak competitive inhibitors with large inhibition constants corresponding to weak recognition. Going to C_8 and C_9, the type of inhibition changes and the magnitude of the inhibition constant diminishes dramatically. Under our conditions, C_{10} is an irreversible inhibitor, but it is probably better classified as a slow tight-binding inhibitor. Finally, the dicarboxylic acids C_{11} and C_{12} are slow binders.

The important conclusion from this series of inhibition studies is clear (Scheme 6). The γ-keto dicarboxylic acids, which resemble the intermediates initially postulated by Jordan, are tightly bound, and show essentially irreversible behavior. The intermediate which imitates the intermediate first postulated by Shemin and then incorporated in Jordan's second proposal seems to be recognized 'only' as an analogue of the substrate without any additional site of interaction with the enzyme. As a consequence, only a weak interaction between the C_7 dicarboxylic acid and the enzyme is observed. The obvious interpretation of these results is that the 4-oxo sebacic acid is recognized at the two carboxylic acid ends of the molecule and the keto function forms a Schiff base with the active site lysine of the enzyme as an additional point of recognition. This three point recognition leads to quasi irreversible inhibition. Inhibitors which are too short or too long still bind strongly to the active site, but they show either slow-binding behavior or good recognition, which means a small K_i value and uncompetitive behavior. Inhibitors, where the distance between the two carboxylic acid ends is too small, are only recognized as substrate analogues and therefore show competitive and not very efficient inhibition behavior.

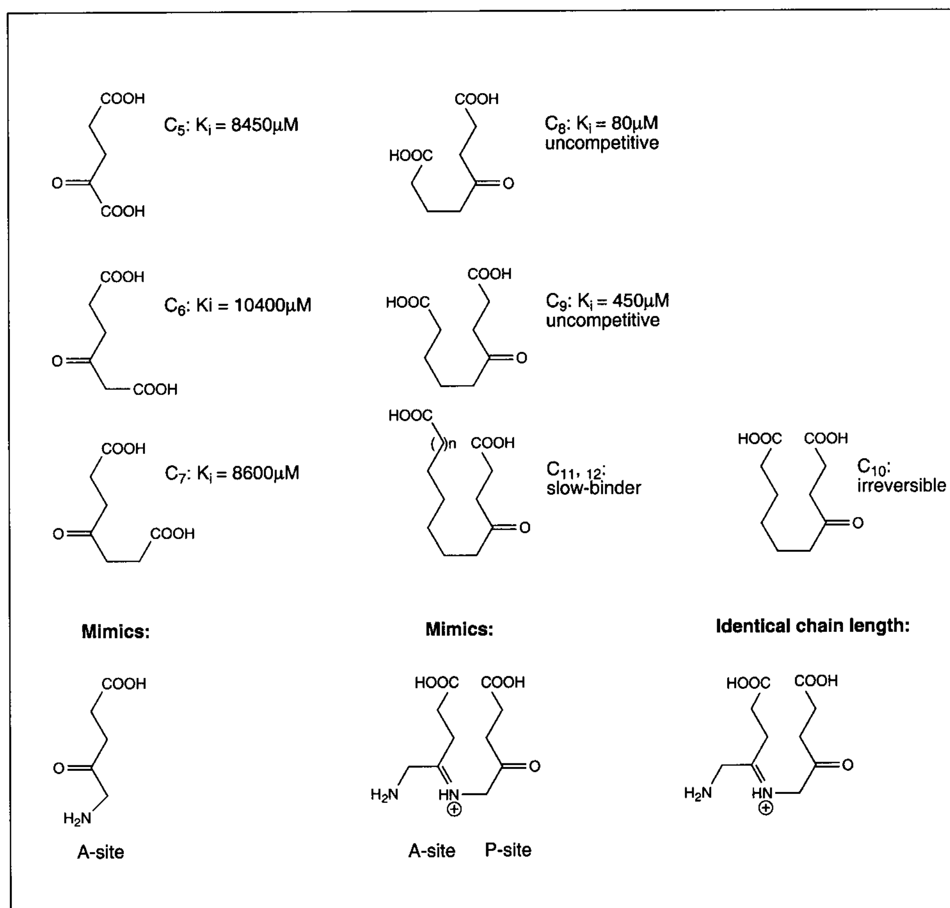
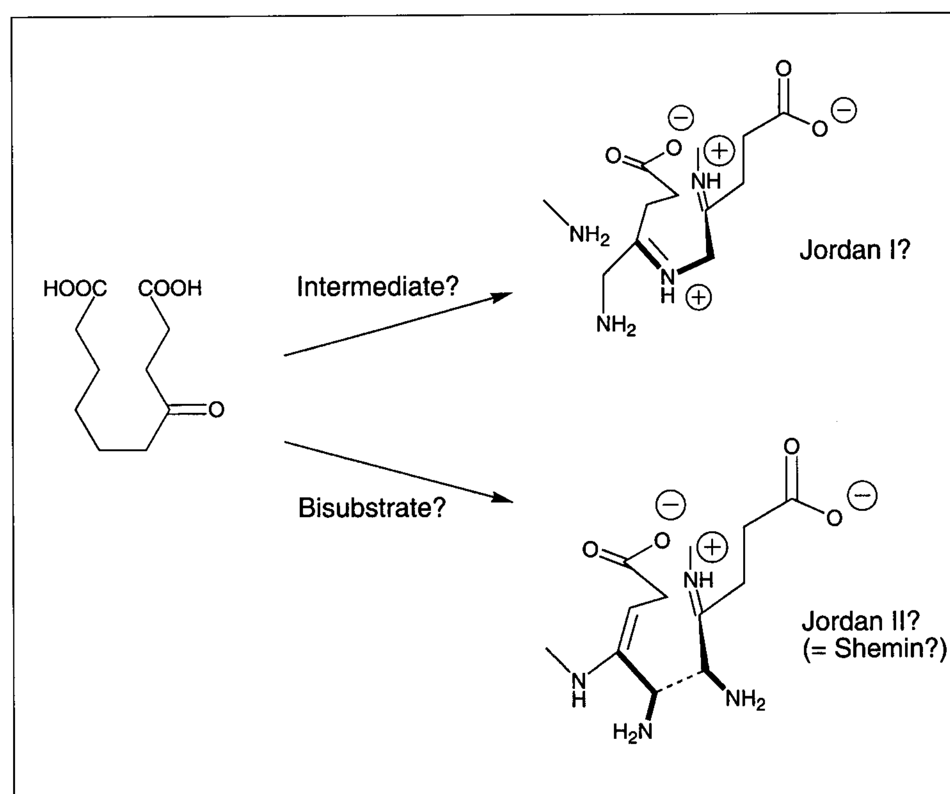


Fig. 2. Results of the inhibition studies of the γ-keto dicarboxylic acids C_5 to C_{12}

The insights provided by the inhibition studies are mostly indirect but rather compelling. All the analogues that mimic an initial aldol reaction are weak inhibitors, whereas the inhibitors based on initial formation of a Schiff base show good inhibition potency or are even irreversible inactivators. Interpreted only from a structural point of view, the results seem to be clear. Mechanistic interpretation of our findings is more difficult. At this stage we cannot be sure if our inhibitors are really analogues of the postulated intermediate or if they only happen to mimic the bisubstrate. We hope that co-crystallization of one or several of our inhibitors with the enzyme will be possible. These structures will contribute significantly to our understanding of the interaction of our inhibitors with the active site. The tools are probably now in place to solve the question of the sequence of the enzyme catalyzed reaction, but other problems are still not solved and we even do not know how to tackle them: 1) What is the chemical logic of this sequence? 2) What is the relation of the actual biochemical mechanism to the hypothesis of a prebiotic formation of tetrapyrroles?



Scheme 6. Are the dicarboxylic acids analogues of the intermediate or analogues of the bisubstrate?

In conclusion the sum of chemical, biochemical and X-ray studies has increased our knowledge about porphobilinogen synthase considerably. Despite this progress we are still quite far from the situation proposed by Feynman as a criterion for real understanding: What we cannot create we do not understand [49].

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