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[C0003]

A NOVEL SYNTHESIS OF PORPHOBILINOGEN: SYNTHETIC AND BIOSYNTHETIC STUDIES

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References

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Introduction

Importance of the "pigments of life" The studies of the tetrapyrrolic dyes, which have been called the "pigments of life"[1] have attracted the attention of chemists and biologists since their discovery. The importance of the "pigments of life" as crucial cofactors for processes like photosynthesis, oxygen transport, oxidation processes, methane synthesis and for a series of unusual rearrangements illustrates well the central role played by this class of natural products 1 - 3 (Figure 1).[2]

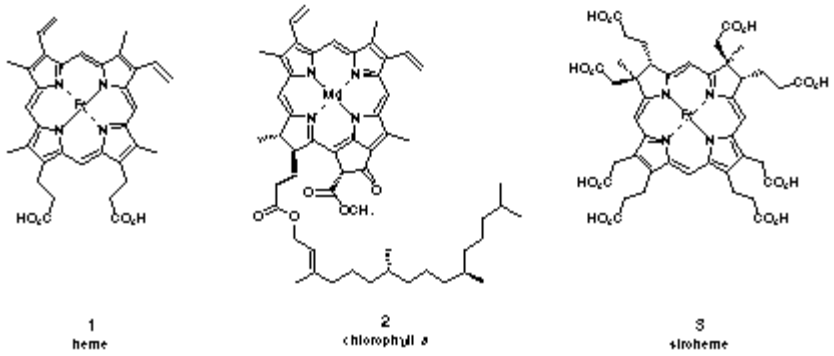


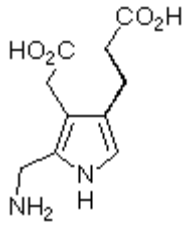
Figure 1: Some important "pigments of life"

Over a period of about 70 years at least six Nobel prices were awarded in Chemistry alone for research obtained working on problems related to the tetrapyrrolic natural products.(R. Willst&#65533;tter, H. Fischer, D. Crowfoot-Hodgkin, M.F. Perutz , J.C. Kendrew, R.B. Woodward, J. Deissenhofer, R. Huber, H. Michl).

Monopyrroles as natural products

Relatively few mono-pyrrolic natural products have been reported in the literature. Most of these natural mono-pyrroles are stabilised by an electron-withdrawing substituent or by an aromatic ring. Without these substituents the electron rich pyrrole ring is easily polymerised or auto oxidised.

Porphobilinogen (4) a trialkylsubstitued pyrrole is a remarkable exception to this rule (Figure 2). The lack of stabilising substituents confers a high reactivity to porphobilinogen (4). The biosynthesis of the tetrapyrrolic "pigments of life" makes use of this high reactivity. About 1010 tons of chlorophyll and more than 4*105 tons of hem are synthesised each year [3,4].



4

- Porphobilinogen

Figure 2: Porphobilinogen (4).

Porphobilinogen (4) is the second dedicated intermediate in the biosynthesis of tetrapyrroles.[5]

Biosynthesis of porphobilinogen (4)

The tetrapyrrolic skeleton of all "pigments of life" is synthesised in a highly convergent way, starting with 8 molecules of d-aminolevulinic acid (5). 5-aminolevulinic acid (5) is then condensed to porphobilinogen (4), which itself tetramerises to form uroporphyrinogen III (6) (Figure 3).

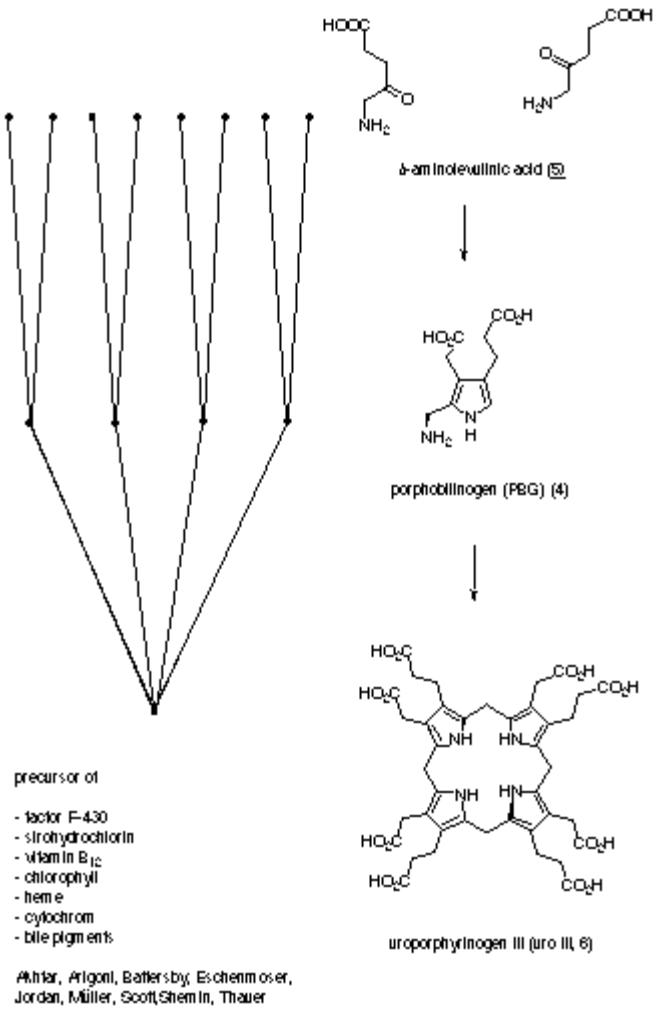


Figure 3: Biosynthesis of Uroporphyrinogen III (6)

The tetramerisation of porphobilinogen (4) could be achieved without the help of an enzyme (Figure 4).[6] Porphobilinogen (4) forms uroporphyrinogens 6 - 9 induced by heat and in the presence of mineral acid. The chemical reactivity of porphobilinogen (4) leads to the formation of the next biosynthetic intermediate without the help of an enzyme. This enzymatic transformation might be called an example of a chemomimetic biosynthesis.[7]

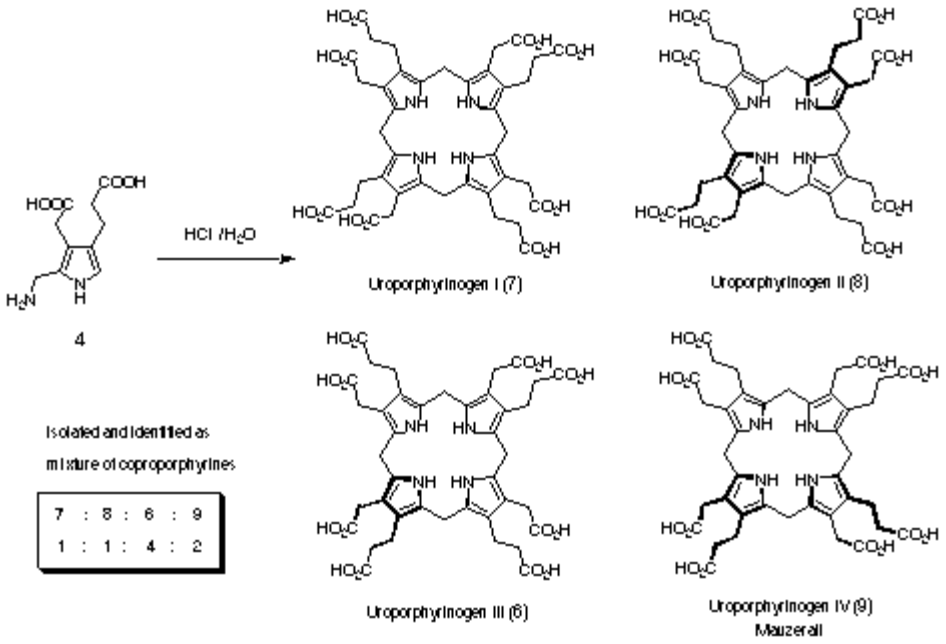


Figure 4: Tetramerisation of porphobilinogen (4).

These observations immediately raise the question of the mechanism for the transformation catalysed by porphobilinogen synthase (=PBGS) and of the comparison between the enzyme catalysed mechanism and its chemical analogue the Knorr pyrrole synthesis (see Figure 5).

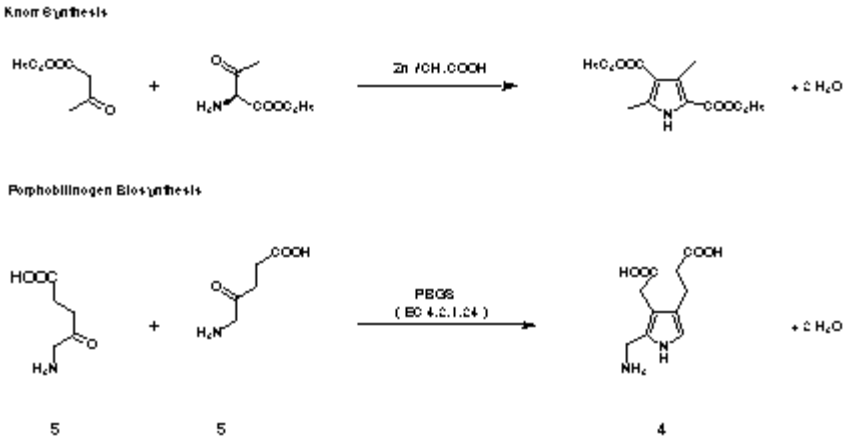


Figure 5: Comparison between Knorr pyrrole synthesis and porphobilinogen (4) biosynthesis.

For the dimerisation of 5-aminolevulinate(5) to porphobilinogen (4) a *G = -16.9 kcal/mol and for the tetramerisation of porphobilinogen (4) to uroporphyrinogen a *G = -34.6 kcal/mol were calculated for the gas phase reactions.[8] The biosynthesis of tetrapyrroles liberates free energy. This observations were taken as arguments in favour of a spontaneous formation of tetrapyrroles [9].

The importance of the "pigments of life" and the elegance of the biosynthetic pathway was a strong motivation to develop chemical synthesis of porphobilinogen (4). In recent years the need for good analytical methods to determine low levels of lead poisoning has renewed the interest in the synthesis of porphobilinogen (4).[10]

Previous synthesis of porphobilinogen (4)

The synthesis of porphobilinogen (4) has attracted the attention of the chemists for different reasons: In the beginning the synthetic efforts were undertaken to prove the structure;[11] afterwards the interest was mainly focused on the synthesis of porphobilinogen (4) labelled at a specific positions in order to use it in the studies of the biosynthesis.[12,13] Finally the interest to develop synthesis of porphobilinogen (4) was renewed, when it became clear that the enzyme synthesising porphobilinogen (4), porphobilinogen synthase, is a very sensitive indicator for lead poisoning. Despite an exorbitant price which is almost three factors of 10 higher than the price for gold there have been only a limited number of fundamentally different approaches to porphobilinogen (4) or to analogues of porphobilinogen (4) been reported in the literature. Especially surprising is the fact, that since 1979 when the synthesis of porphobilinogen (4) has been reviewed by Frydman the last time,[14] only very few new results have been reported.[15]

Six synthetic strategies have been reported for the synthesis of porphobilinogen (4) (see Figure 6).

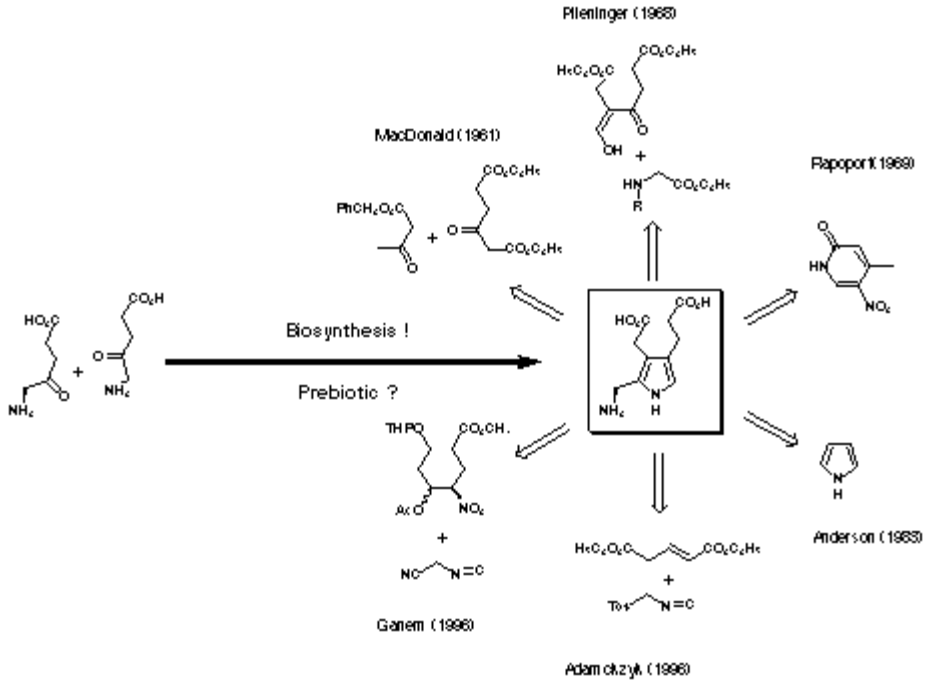


Figure 6: Synthetic strategies for the synthesis of porphobilinogen (4) compared with the biosynthesis.

The first and historically the oldest strategy uses a classic Knorr synthesis to obtain a suitable precursor.[16] To obtain the correct substitution pattern the group of MacDonald has invested a considerable amount of work into the modification of the side chains obtained directly from the Knorr synthesis.

In the second strategy developed by the groups of Plieninger[17] and of Evans[18] the pyrrole ring is formed by condensation of a C3-unit with a C-N-unit. In one case the variant of Kleinspehn of the Knorr synthesis is used, whereas in the second case the ring closure is achieved in a stepwise fashion. In this strategy both the acetic acid and the propionic acid side chains are in place right from the beginning.

The third strategy is due to Frydman and Rapoport.[12] They started with a pyridine derivative, which they successfully transformed into a suitably substituted azaindole. Hydrogenation of the azaindole led to the porphobilinogen lactam, which could be hydrolysed to porphobilinogen (4).

The forth strategy stems from Anderson and collaborators, who started with the unsubstituted pyrrole.[19,20] Introducing step by step the acetic acid side chain in the b-position, the nitrile group as precursor of the methylamino group in the a-position and the propionic acid side chain in the b'-position finally gave porphobilinogen (4).

The fifth and the sixth strategies were developed by the groups of Adamckzyk[21] and Ganem[22] and were published in the same year. Both strategies use a formal [2 + 3]-cycloaddition of the anion generated from a substituted methyl isocyanide to construct the pyrrole ring. In one approach the propionic acid side chain has to be built up, after the cycloaddition, whereas in the other approach all carbon atoms are present already at this stage.

Comparing the strategy used in the biosynthesis with the strategies developed during the last 40 years by chemists allows to draw two important conclusions:
1) The efficiency and beauty of the one step, 100% yield biosynthesis has not been reached by any of the reported synthesis so far;[23]
2) The sensitivity of porphobilinogen (4) has forced the chemists to introduce stabilising (protecting) groups onto the pyrrole ring in order to be able to manipulate and to isolate the advanced intermediates. The second restriction by necessity leads to longer synthetic pathways and the yield of the necessary deprotection step is often rather low.

Therefore the synthesis of porphobilinogen (4) is a double challenge:
1) Will chemists be able to use the beautiful strategy developed by nature and
2) an even more fundamental question: How did nature find this highly efficient and convergent synthetic strategy?

References

Development of a potentially biomimetic methodology

"A Novel Synthesis of Porphobilinogen: Synthetic And Biosynthetic Studies"

Christiane Bobillier Neier / August 1999

Development of a potentially biomimetic methodology

General remarks and comparison of the Shemin mechanism with the Knorr pyrrole synthesis

Novel pyrrole synthesis based on Shemin's proposal for the biosynthesis

General remarks and comparison of the Shemin mechanism with the Knorr pyrrole synthesis Concentrating only on the step connecting the two substrate molecules via a covalent bond for the first time, there have been three mechanisms postulated so far for the biosynthesis of porphobilinogen (4).[24-26] In two of these mechanisms the central decisive step for the building of the pyrrole ring is the formation of the carbon-carbon bond between C3 of one 5-aminolevulinic acid (5) reacting as a nucleophile and the keto function of the other 5-aminolevulinic acid. It was assumed that the nucleophilic 5-aminolevulinic acid partner is bound to the enzyme as an enamine. One would expect that the formation of the central carbon-carbon bond should be slowest and therefore the rate determining step of the biosynthetic sequence. This sequence is in remarkable contrast to the mechanism of the Knorr pyrrole synthesis (see Figure 7).[27]

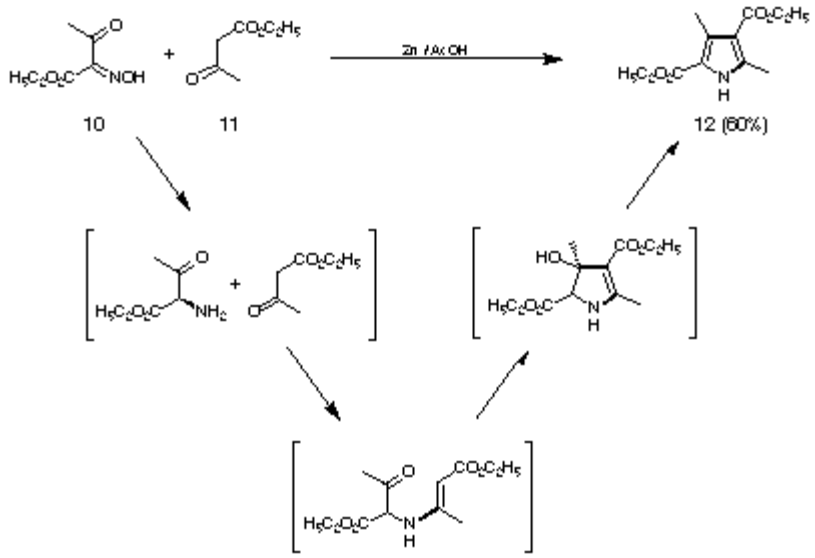


Figure 7: Mechanism for the Knorr pyrrole synthesis.

For the Knorr pyrrole synthesis the steps leading to the pyrrole nucleus are the same, but the sequence is clearly different from the Shemin mechanism for the biosynthesis (see Figure 8). In the Knorr synthesis the carbon-nitrogen bond is formed first and the aldol-like carbon-carbon bond forming reaction is therefore an intramolecular process.

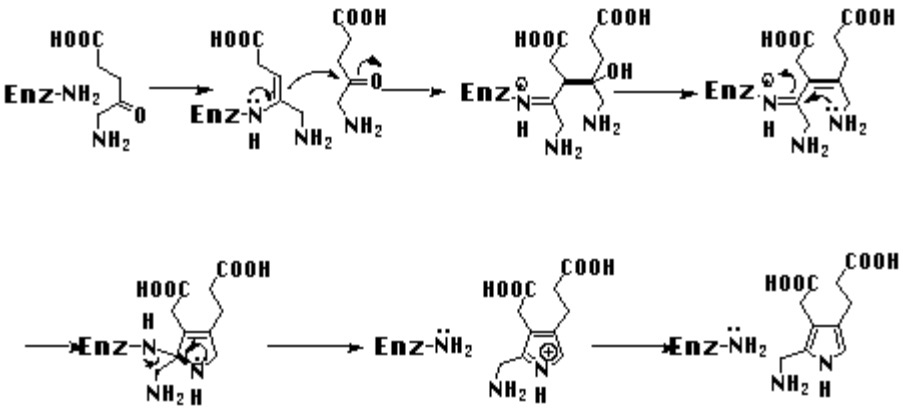


Figure 8: Mechanism proposed by Shemin for the biosynthesis of porphoblinogen (4).

Novel pyrrole synthesis based on Shemin's proposal for the biosynthesis

Following the mechanistic reasoning first proposed by Shemin, one can ask the question if pyrroles can be synthesised using the same sequence of transformations and if it will be possible to synthesise even porphobilinogen (4) in a biomimetic way using such a methodology?

Based on this mechanistic question a novel synthesis of pyrroles could be developed. The key step is the Mukaiyama crossed aldol reaction between regioselectively generated silyl enol ethers 13 and 16 and azido ketals 14 (see Figure 9).[28] The Mukaiyama crossed aldol reaction forms the crucial carbon carbon bond.[29]

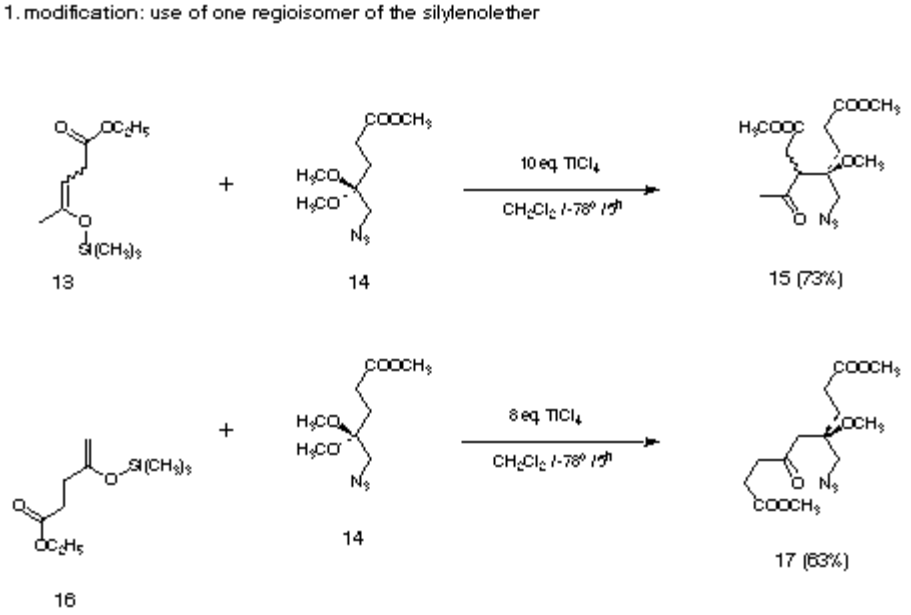


Figure 9: The crossed-aldol reaction using the pure silyl enol ethers 13 and 16.

In the second step the azido group is reduced either using triethylphosphine to induce the Staudinger reaction followed by an aza-Wittig reaction. The triethylphosphine gives the water soluble triethylphosphine oxide, which can be easily removed by extraction (see Figure 10).

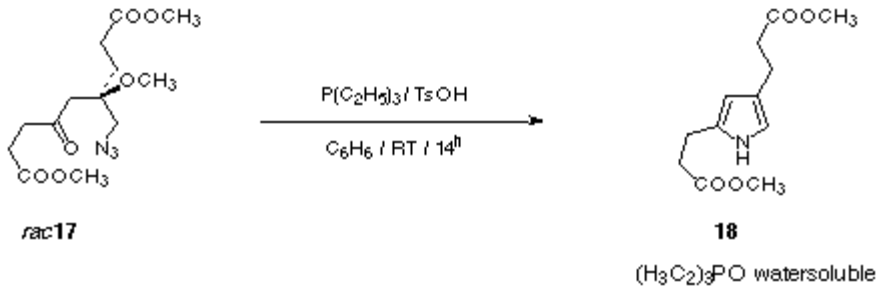


Figure 10: Modified Staudinger reaction of ***rac***-17 forming the pyrrole 18.

Catalytic reduction is another mild method to transform the azido group into the corresponding amine. Using palladium on charcoal as catalyst and methanol as solvent the aldol product ***rac***-15 could be reduced to 19 (see Figure 11). The amino ketone formed spontaneously the corresponding pyrrole. The work-up using these conditions was very convenient.

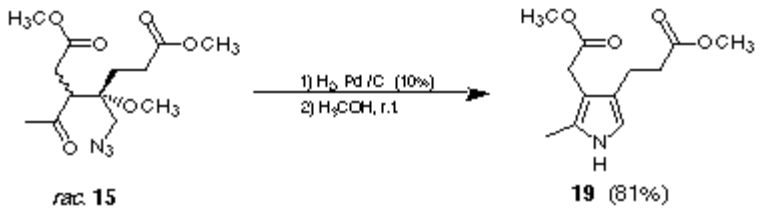


Figure 11: Catalytic reduction of 15 forming the pyrrole 19.

The new two-step pyrrol synthesis is especially effective for the synthesis of mono-, di-, tri- and tetraalkylpyrroles in good yield (see Figure 12).[28]

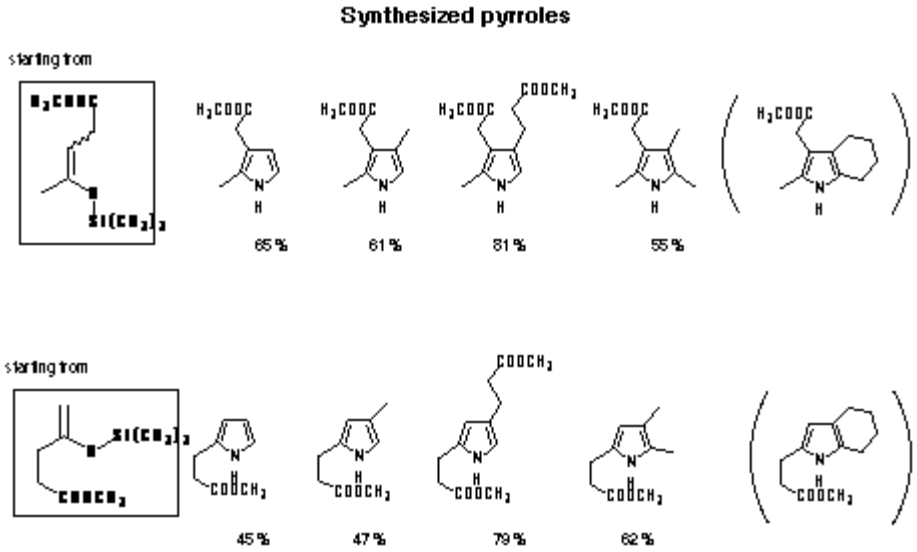


Figure 12: Pyrroles synthesised using the Mukaiyama crossed aldol condensation.

The synthesis is complementary to the classical Knorr pyrrole synthesis. It allows to introduce the side chains at the correct positions and with the needed functionalities already in the pyrrole forming step. The reaction conditions for the pyrrole formation are sufficiently mild to allow also the isolation of highly sensitive pyrroles. At this stage of the project we hoped to be able to apply our reaction conditions to a synthesis of porphobilinogen (4) avoiding many of the pitfalls of the former synthesis.

References

Chemical Synthesis of Porphobilinogen

- "A Novel Synthesis of Porphobilinogen: Synthetic And Biosynthetic Studies"

Christiane Bobillier Neier / August 1999

Chemical Synthesis of Porphobilinogen

Regioselective synthesis of the silyl enol ether component

For the planned synthesis of porphobilinogen (4) and of structural analogues of porphobilinogen we needed the corresponding silyl enol ether of a protected derivative of 5-aminolevulinic acid (see Figure 13).

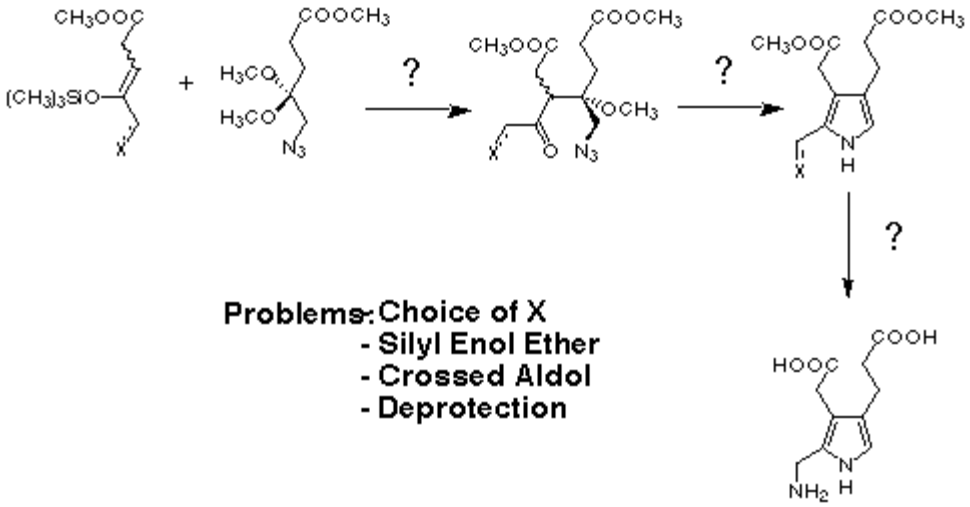


Figure 13: Retrosynthesis for the planned biomimetic approach to porphobilinogen (4) or to structural analogues thereof.

Trials to submit the 5-azido levulinic acid methyl ester, or the 5-***tert***.-butyldimethylsilyloxy-levulinic acid methyl ester to the conditions worked out by Miller[30] inevitably led to the unwanted regioisomers of the silyl enol ether. The synthetic problem could be finally solved using the 5-phthalimido-levulinic acid methyl ester 20 submitting it to Miller's conditions but changing the solvent to chloroform (see Figure 14).[31] Adding after 17 hours dry hexane allowed to precipitate the salts which had been formed. The wanted silyl enol ether 22 was obtained in 93 % yield as 1 : 1 mixture of the diastereoisomers containing only 4 % of the unwanted silyl enol ether 21 as side product.

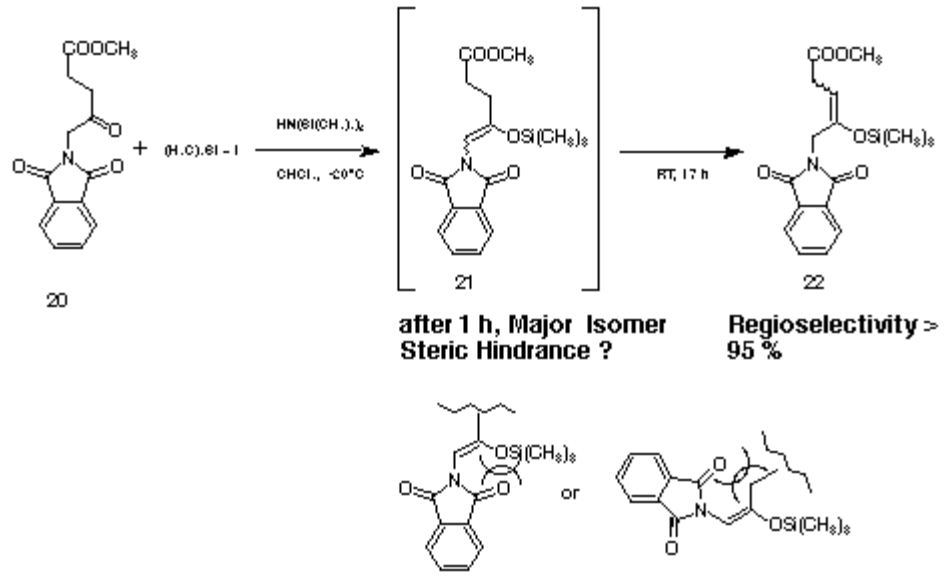


Figure 14: Synthesis of the silyl enol ether 22.

The silyl enol ether 22 could be stored for months at - 20 °C in the refrigerator. The regioselectivity of the formation of the silyl enol ether is surprising, because one has to assume the methylene group in the a-position to the phthalimido group should be clearly more acidic than the protons at the C3 methylene group. The regioselectivity can probably be attributed to the steric hindrance which is created if the silyl enol ether towards the position C3 is formed.

Trials to couple the silyl enol ether 22

Having the correct regioisomer 22 in our hands experiments were undertaken to couple the silyl enol ether with the acetal of the 5-azido levulinic acid methyl ester under standard conditions.[28,32] Unfortunately all our trials to couple these two precursors of 5-amino levulinic acid according to Mukaiyama were totally unsuccessful. Despite our considerable efforts to achieve also the Mukaiyama Aldol coupling using a protected form of d-aminolevulinate, we were unable to isolate products which could be traced back to the central C-C-bond formation. Using TiCl4 as a catalyst for the aldol coupling starting from the silyl enol ether 22 at temperatures below -40 °C no reaction could be observed. Increasing the temperature above -40°C rapid destruction of the reaction partner was observed. Using Lewis acids like TMSOTf[33,34] or the "super-Lewis acid" B(OTf)4TMS according to Davis[35] the aldol reaction between 22 and the dimethyl acetal of levulinic acid methyl ester could be achieved. Using Noyori's conditions [33] whereby 0.11 equivalents of TMSOTf are utilised 30% of one pure diastereoisomer could be isolated. Even when these stronger Lewis acids were used we were unable to achieve the crucial C-C-bond forming process starting from an adequate precursor of 5-aminolevulinate. The use of the more reactive, but also more aggressive catalyst TMSI[36] at -80 °C lead to the destruction of both starting materials: the silyl enol ether 2e and the acetal.

Synthesis of porphobilinogen (4)

The only way out seemed to be to increase the inherent reactivity of the carbonyl component.

In order to obtain porphobilinogen (4) we tried to use the monocyanide of succinic acid monomethyl ester (23) as activated carbonyl component. Using this strategy it should be possible to combine two partners which contain all the carbon, oxygen and nitrogen atoms necessary for the construction of porphobilinogen. Deprotection of the aldol product should then induce the ring closing and aromatisation process. In view of this analysis we reacted the silyl enol ether 22 with the monocyanide of succinic acid monomethyl ester (23). The cyano hydrine 27 could be detected in the raw product of the reaction. Extraction against water and purification with column chromatography yielded 35 % of the b-diketon ***rac***-24 as hydrolysis product.

The diketon could be easily transformed in two steps into the pyrazole 26 which is a close structural analogue of porphobilinogen (see Figure 15).[15,37]

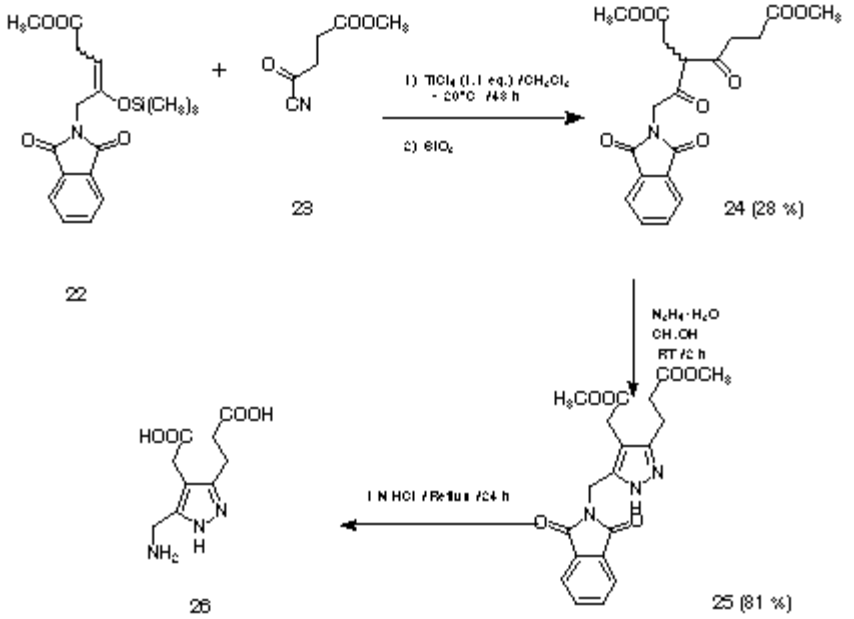


Figure 15: Synthesis of the pyrazole 26, a structural analogue of porphobilinogen.[37]

Under optimised conditions at -20° C using TiCl4, which had been freed from HCl by distillation over polyvinyl pyridine, the aldol product ***rac***-27 could be obtained in 60 to 87 % (see Figure 16). One diastereoisomer of the aldol product ***rac***-27 could be obtained analytically pure by crystallisation in 47 % yield. Trials to reduce the cyano hydrine directly met with limited success. For the synthesis we protected the unpurified aldol product using acetone enol acetate. The acetylated aldol product ***rac***-28 could be obtained in 56 % yield. Even the reduction of the acetylated cyano hydrine ***rac***-28 proved to be difficult. Finally the cyano hydrine ***rac***-28 could be reduced smoothly at 65° C under 120 atm H2 in the presence of Raney nickel. After column chromatography we obtained the fully protected porphobilinogen 29[31] in 54 % yield analytically pure. Removal of the protecting groups over two steps has already been described in the literature.[18]

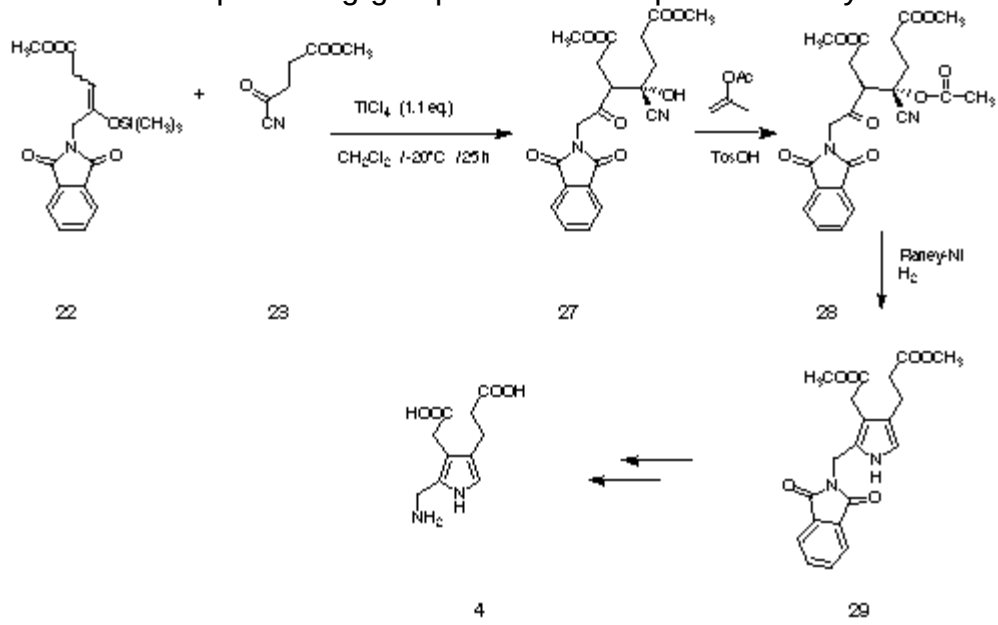


Figure 16: Synthesis of a protected form of porphobilinogen 29.

In conclusion we were able to obtain the protected porphobilinogen 29 in a convergent way starting from two easily obtainable starting materials. The central step of the synthesis is the Mukaiyama aldol reaction between the regioselectively formed silyl enol ether 22 as the nucleophile with the monocyanide of succinic

acid monomethyl ester (23) as electrophile. Reducing the acetylated cyano hydrine ***rac***-28 yields directly the protected porphobilinogen 29. This synthesis follows the proposal for the biosynthesis made by Shemin almost 30 years ago. The correctly functionalised side chains are introduced on the level of the two starting materials used for the synthesis of the pyrrole ring. Subsequent functionalisation is therefore not necessary. In this synthetic scheme the same bonds are formed as in the biosynthesis catalysed by porphobilinogen synthase. The overall yield starting from 5-phthalimido methyl levulinate is 25 %. The synthesis can be used to obtain selectively labelled porphobilinogen (4).

References

Biosynthesis of Porphobilinogen

"A Novel Synthesis of Porphobilinogen: Synthetic And Biosynthetic Studies"

Christiane Bobillier Neier / August 1999

Biosynthesis of Porphobilinogen

Mechanistic studies of porphobilinogen synthase The step leading to porphobilinogen (4) in the biosynthesis of the "pigments of life" has been intensively studied during the last thirty years.[38] Before the report on the first high resolution X-ray structures of the enzyme porphobilinogen synthase appeared in the literature end of 1997[39] a series of important experimental findings relevant for the understanding of the enzyme mechanism have been reported (see Figure 17).[40] Almost sixty gene derived protein sequences for porphobilinogen synthase from different sources are known.[41] The gene derived protein sequences show a relatively high degree of homology. The amino acids at the active site and at the postulated sites for the binding of the metal ions which are necessary for the activity are highly conserved.

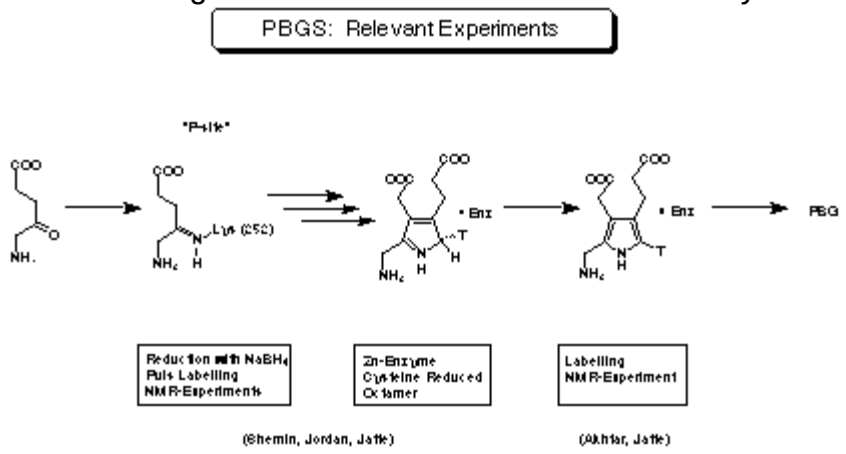


Figure 17: Experiments relevant for the mechanism of porphobilinogen synthase.

The results reported by the groups of Shemin, Jordan and Jaffe can be summarised as follows: The enzyme is usually a homo-octamer. The minimal active species is a dimer. Most of the enzymes isolated so far need Zn^{2+} or Mg^{2+} as essential cofactors. The enzymes can be categorised as a function of the metal ion needed for their activity. For those enzymes binding Zn^{2+} the cysteines have to be reduced to keep the enzyme in its active form. The substrate forming the propionic acid side chain is interacting first. At least one of the substrates is forming a covalent bond with the enzyme via a Schiff base. To study the order in which the two substrate molecules bind to the enzyme, Jordan performed highly elegant single-turnover experiments.[42] Stoechiometric equivalents of labelled substrate and porphobilinogen synthase were rapidly mixed and after about 100 ms added to a large excess of unlabelled substrate. The position of the radioactive label was determined by degradation. The pulse labelling could also be done using [5-^{13}C] 5-aminolevulinic acid. The ^{13}C-NMR spectrum of the product allowed to identify the position of the label directly.[26] The deprotonation leading from the pyrrolenine tautomer to the aromatic pyrrole is enantioselective and occurs therefore on the surface of the enzyme. This observation has been confirmed by NMR results showing that porphobilinogen (4) bound to the active site can be observed.[43] Despite the intensive efforts of several groups the exact mechanism of the biosynthesis of porphobilinogen (4) could not be deduced based on the biochemical knowledge accumulated so far.

Proposals for the mechanism of porphobilinogen synthase

For the detailed analysis of an enzyme mechanism the following methods are usually applied:

1) X-ray structure determination;
2) site-directed mutagenesis;
3) spectroscopic studies;
4) kinetic and inhibition studies.[44]
Until recently most of these methods could not be applied to study porphobilinogen synthase because no secured structural information was available. Therefore it was often difficult to deduce clear mechanistic conclusions from the experimental results. Despite these difficulties it was possible to analyse the sequence of the possible mechanisms concentrating on the transformations of the substrate molecules alone.[38] This approach provides a frame-work for the chemical analysis of the sequence of events.

Shemin has been the first to propose a mechanism for porphobilinogen synthase drawing a close analogy between this enzyme and class I aldolases (see Figure 18).[24] Shemin was the first to proof that porphobilinogen synthase forms a Schiff base between the e-amino group a lysine of the active site and the carbonyl group of one of the two substrate molecules. His postulated mechanism is mainly based on two arguments: the transformation has been formulated in strict analogy of porphobilinogen synthase with aldolases and the sequence of recognition is based on the observed formation of a compound which Shemin called a mixed pyrrole.[24] Later experiments however showed that the proposed structure was wrong.[45]

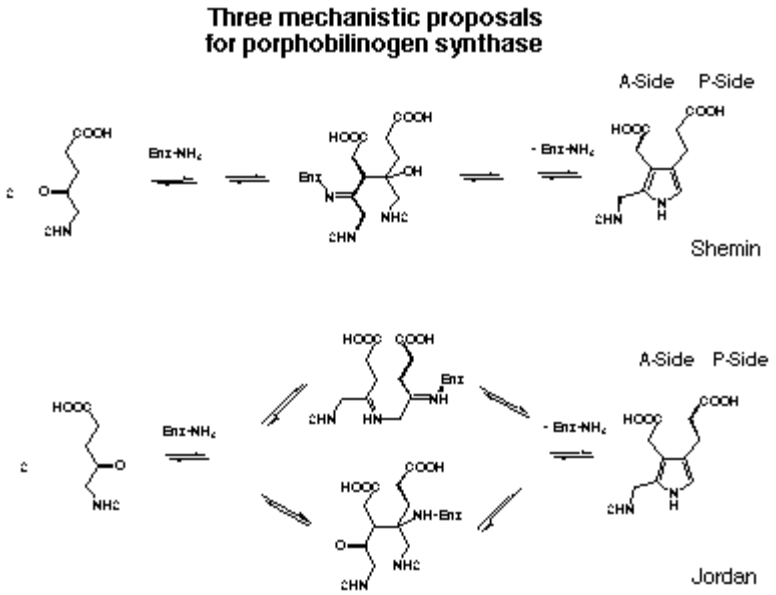


Figure 18: The three mechanistic proposals for porphobilinogen synthase.

Starting from the results of his highly elegant single turn-over experiments Jordan postulated two alternative mechanisms for the formation of porphobilinogen (4) (see Figure 18).[25,26,40] Both mechanisms include the finding that the first substrate bound to the enzyme will be incorporated into the P-side of the product porphobilinogen (4). The first alternative mechanism proposed by Jordan postulates that the first bond between the two substrate molecules is a Schiff base between the two substrate molecules. Only after this step follows the aldol reaction and the elimination which leads after deprotonation to the product. This mechanism follows closely the known mechanism for the Knorr pyrrole synthesis. In the second alternative mechanism proposed by Jordan the aldol reaction forming the carbon carbon bond is the step joining the two substrates for the first time.

Despite the efforts of several research groups, the mechanism of the enzymatic synthesis of porphobilinogen (4) is not established yet. The sequence of recognition of the two substrate molecules is: "P-site" first, "A-site" second, at least for the bovine liver and the human erythrocyte enzyme. The substrate at the "P-site" is forming a Schiff's base to a lysine of the active site. The second substrate may

be bound non-covalently to the enzyme. One Zn probably complexed to cysteines helps in the catalytic step. There is same circumstantial evidence, that the enzyme shows half-the-site reactivity. Assuming that the active site is formed at the interface of a dimer had been considered to be an attractive interpretation of these observations. Finally the product forms a relatively stable complex with the enzyme. This observation is in agreement with the fact that the last chemical step, the deprotonation, is still occurring at the enzyme.[46]

X-ray structures of porphobilinogen synthase

Since the first report on the X-ray structure determination of porphobilinogen synthase from yeast,[39] the structures of two more enzymes isolated from ***Escherichia coli*** and ***Pseudomonas aeruginosa*** have been published.[47,48] More importantly the structures of porphobilinogen synthase co-crystrallized with levulinic acid were solved as well.[47-49] The crystal structures increased our knowledge about porphobilinogen synthase considerably. Many aspects which had been inferred so far can now be clearly interpreted and correlated with the structural data. Some of the tentative conclusions, like the proposal that the active sites might be located at the interface between the monomeric units, were proven to be wrong

All structures determined are similar despite very interesting differences between the individual shapes. All three enzymes form octamers composed of dimers. The tertiary structure of all porphobilinogen synthase is dominated by a TIM-barrel.[50] The N-terminal end is not part of the TIM-barrel, but wraps around the neighbouring molecule. The N-terminal ends make major contributions to the dimer interface contacts. The eight active sites of the octamer are all exposed to the solvent. As expected for the TIM-barrel structure, the active site was found at the C-terminal end of a b-sheet. The active site lysine could be identified as well in the structure of the "empty" enzyme as well as in the structure of the co-crystals with levulinic acid. A second lysine was found to be present nearby in the active centre. This second lysine had not been identified before, but its presence seems to be crucial for the functioning of porphobilinogen synthase.[39] The yeast and ***Eschierichia coli*** enzyme contain a metal binding site near to the active site (= active site Zn) and a secondary metal binding site further removed from the active site (= structural Zn). In the structure determined for ***Pseudomonas aeruginosa*** the Mg^{2+}-binding site is located at the surface of the subunit. The Mg^{2+}-binding is connected to the active site via a series of hydrogen bonds, which influence the structure of the active site by a series of subtle changes. The function of Mg^{2+} bound to this site has been interpreted as being allosteric. Finally the active sites having bound one molecule of levulinic acid are shielded by a "lid" whereas the structures of the "empty" active sites are open towards the outside and the "lid" region is much less well defined.

The nature and the role of the basic groups at the active site can now be assessed with reasonable confidence based on the structural data.[49] The authors attribute to the second lysine at the active site a role in the formation of the Schiff base. The hydroxide ion bound to the active-site Zn is considered to be a likely candidate for the deprotonation necessary for the C-C bond formation. Finally the same Zn-ion could also co-ordinate the oxygen of the carbonyl group of A-site substrate.

One conclusion drawn from this wealth of structural information is that it is still not possible to discriminate with certitude between the two possible mechanisms. This uncertainty is nicely demonstrated by the title of the first publication on the X-ray structure of porphobilinogen synthase, where the enzyme is called "a hybrid aldolase".[39] In this and the following publication more importance is given to the carbon-carbon bond forming process.[51,52] In the most recent publication of the same authors, reporting on the structure of porphobilinogen synthase from ***Escherichia coli*** complexed with levulinic acid, the conclusion drawn by the authors is the following: "The scheme in which the C-N bond formation occurs first may be more attractive from the mechanistic point of view since formation of the inter substrate Schiff base increases the acidity of the A-site C-3 protons".[49]

References

- Inhibition Studies of Porphobilinogen Synthase from Escherichia coli
- "A Novel Synthesis of Porphobilinogen: Synthetic And Biosynthetic Studies"

Christiane Bobillier Neier / August 1999

Inhibition Studies of Porphobilinogen Synthase from ***Escherichia coli***

Inhibition tests and inhibition type for porphobilinogen synthase In order to obtain kinetic information on the biosynthesis of porphobilinogen synthase we have undertaken a systematic search of the inhibition behaviour of this enzyme isolated from Rhodopseudomonas spheroides and from Escherichia coli.[38,53,54] The intention of these studies was to accumulate sufficient knowledge about the recognition site of this enzyme, so that this information will help to deduce conclusions about the mechanism. To be able to draw valid conclusions we had to analyse systematically the behaviour of analogues of the substrate and of analogues of the product. The result of these studies should allow us to interpret the findings obtained from studies of analogues of postulated intermediates. With a firm knowledge about the factors important for the recognition at the active site, it should be possible to interpret the whole body of information in a coherent way. Finally we hoped that the best of our inhibitors will contribute to the structural analysis of porphobilinogen synthase, as soon as the crystal structures become available and as soon as co-crystallization becomes feasible.

The majority of our studies were done with the enzyme isolated from an overproducing strain from ***Escherichia coli*** gratefully put at our disposal by Dr. Charles Roessner and Professor Ian A. Scott from Texas A&M (see Figure 19).[55]

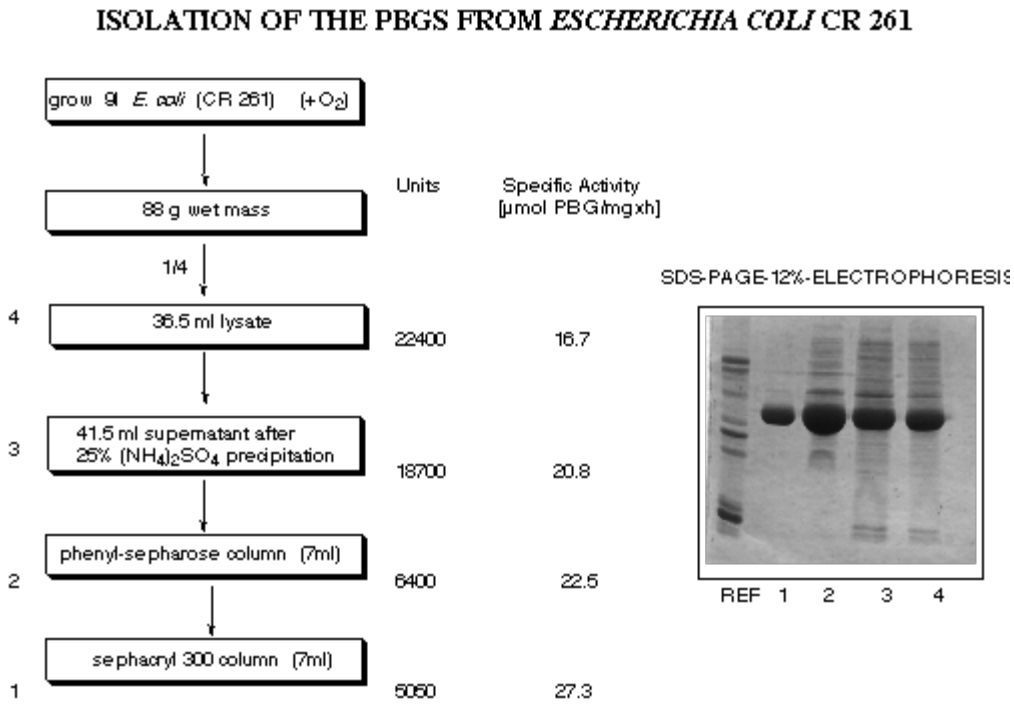


Figure 19: Isolation and purification of porphobilinogen synthase obtained from E. coli CR 261.

The test of the enzyme kinetics and activity is based on a modified Ehrlich test, which allows to determine the amount of porphobilinogen (4) formed by measuring the absorption at 554 nm of the chromophor obtained by treating the test solution with ***p***-dimethylamino benzaldehyde (see Figure 20).

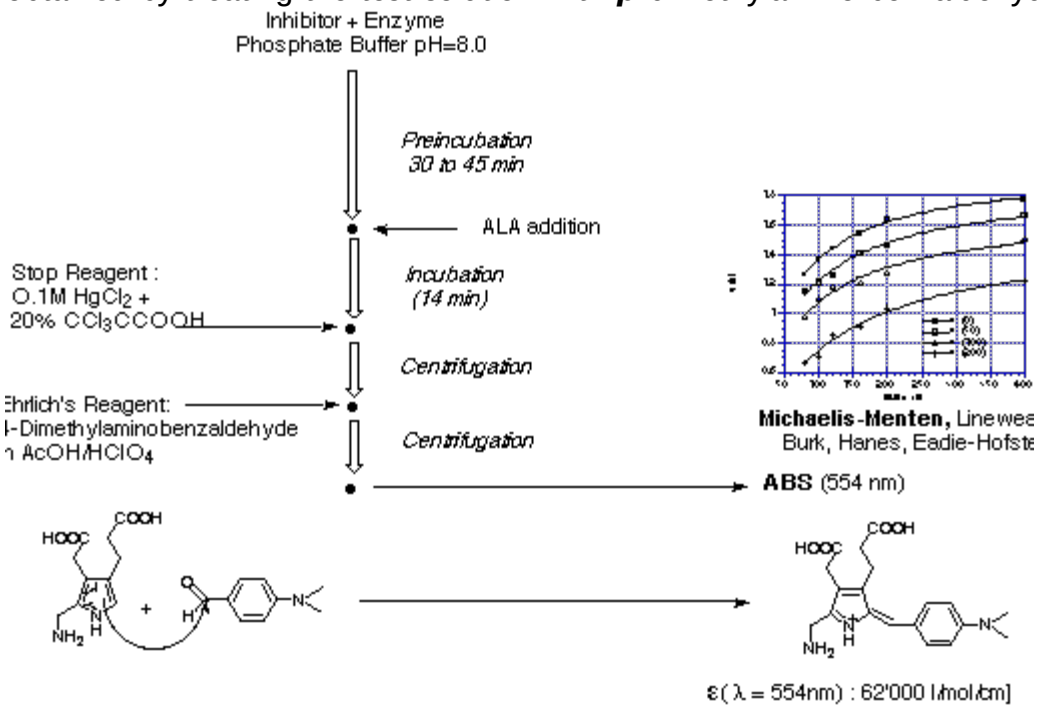


Figure 20: Ehrlich test for the determination of the kinetics of porphobilinogen synthase.

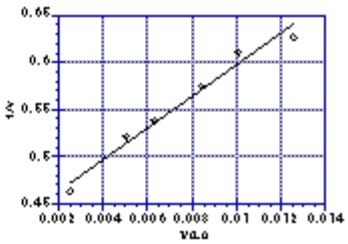
The kinetic data obtained are highly reproducible and followed nicely Michaelis-Menten kinetics. This result came as a surprise to us, because an enzyme using two equivalents of the very same molecule as substrate should follow Michaelis-Menten kinetics only under very special conditions. Testing the kinetics of porphobilinogen synthase under a variety of concentration conditions we could show that the simple Michaelis-Menten kinetics is nicely followed if the concentration of the natural substrate 5-aminolevulinate (5) is 80 mM or bigger. If however the concentration of 5-aminolevulinate (5) is varied between 4mM and 80 mM significant deviation from the linear dependence predicted by the Michaelis-Menten kinetics can be observed (see Figure 21).

Calculation of the two Michaelis-Menten Constants

1) $E + S \underset{}{\overset{Km}{\rightleftharpoons}} ES \longrightarrow E + P$

Linweaver-Burk: linear relation

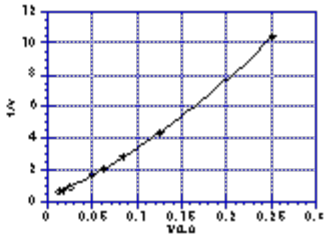
[S] = 80 - 400 µM



2) $E + S \underset{}{\overset{Km_1}{\rightleftharpoons}} ES + S \underset{}{\overset{Km_2}{\rightleftharpoons}} ES' \xrightarrow{k_{cat}} E + P$

Linweaver-Burk: parabolic relation

[S] = 4 - 80 µM



K_{m1} = 4.6 µM
K_{m2} = 66 µM

Figure 21: Dependence of the kinetic behaviour on the concentration range used for the substrate: a) linear Michaelis-Menten behaviour for concentrations of 5-aminolevulinate (5) of 80 mM or bigger;
b) parabolic behaviour for concentrations of 5-aminolevulinate of 4 - 80 mM.

The linear part of the correlation allows to determine the Michaelis constant K_M following the classic analysis of enzyme kinetics. The parabolic curve could be analysed using non-linear regression. The kinetic model used was a steady-state model based on the sequential formation of two Michaelis complexes with two different Michaelis constants. The two Michaelis constants K_{M1} and K_{M2} could be determined. The Michaelis constant K_{M1} for the tight binding substrate was 4.6 mM and the Michaelis constant K_{M2} for the loosely bound substrate was 66 mM which corresponds nicely to the value determined from the linear Michaelis-Menten correlation. This two values correlate perfectly well with the observation made for the single turn-over experiments which indicated that the recognition of the first substrate was sufficiently more tight, so that the sequence of recognition could be determined experimentally. The difference between the two K_M values explains also the fact that a standard Michaelis-Menten kinetics can be observed using substrate concentrations which are in the range of the second Michaelis-Menten constant or higher.

For the inhibition experiments experimental conditions are used, which should give normal Michaelis-Menten kinetics (see Figure 22). Under these conditions the recognition of the second substrate at the A-site of the enzyme is determined. Such a behaviour is characterised by a clear unequivocal competitive inhibition. When the inhibition behaviour 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 framework for the interpretation of the experimental results.

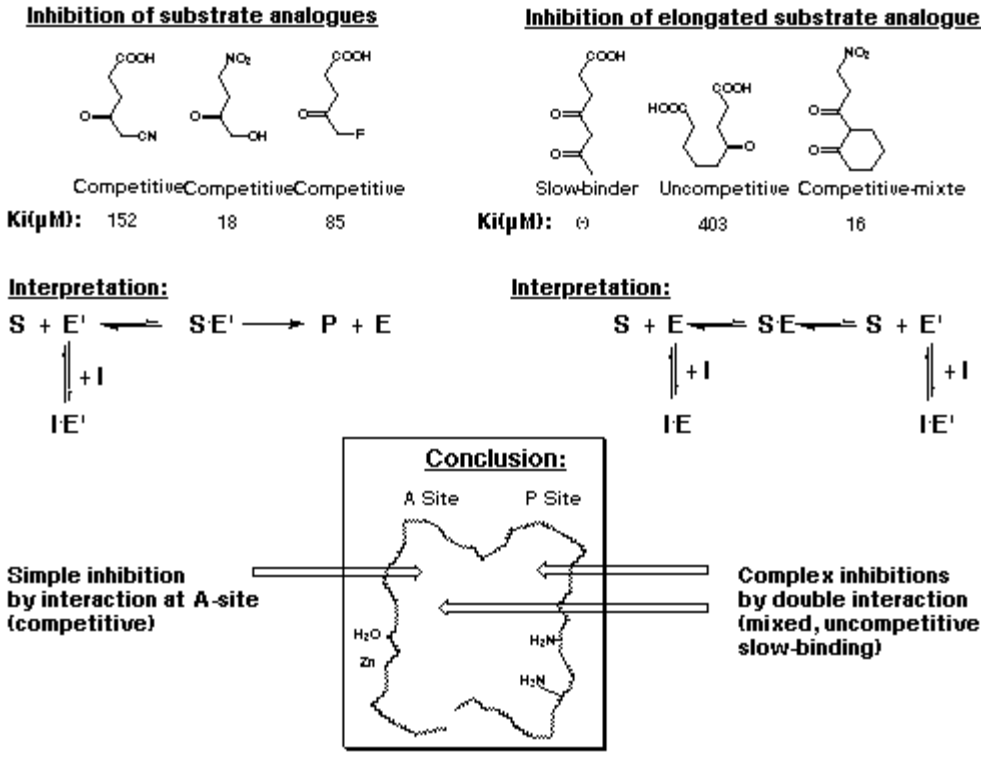


Figure 22: Inhibition type as a function of the sites of interaction

Inhibition tests with keto diacids

The most important application of this interpretation was the study of a series of diacids which were considered to be analogues of the postulated intermediates (see Figure 23).

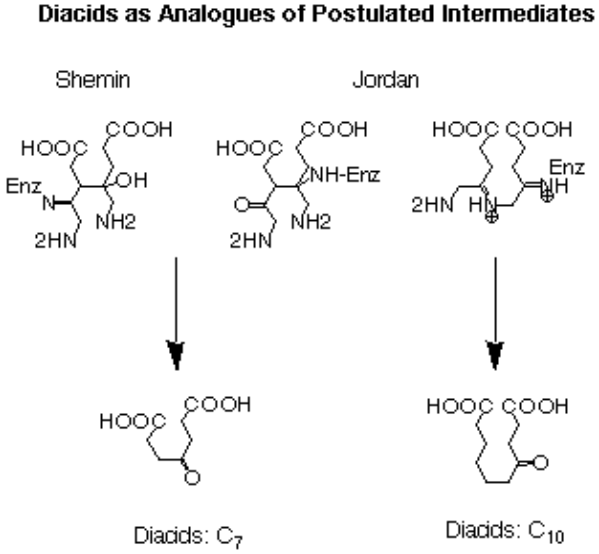


Figure 23: Comparison of the structures postulated as intermediates by Shemin and Jordan with diacids used as inhibitors.

After the formation of the first bond uniting the two substrates a diacid is created for both postulated mechanisms. The difference between the two proposals is the fact that in the Shemin mechanism the diacid is a derivative of pimelic acid, whereas in the Jordan mechanism the diacid intermediate is a derivative of sebacic acid. In order to obtain good recognition a g-keto function is added, which allows the inhibitor to interact at three points with the active site.

The systematic study of the g-keto dicarboxylic acids from C5 to C12 gave a clear picture (see Figure 24).

The first three dicarboxylic acids C5 to C7 are weak competitive inhibitors with inhibition constants K_I between 8'500 and 10'400 mM. Going to C8 and C9 the type of inhibition changes and the value for the inhibition constant diminishes dramatically. The inhibition constants K_I are almost a factor of 100 smaller and uncompetitive behaviour is observed. C10 is under our conditions an irreversible inhibitor, but it should probably be better classified as slow tight binding inhibitor. Finally the dicarboxylic acids C11 and C12 are slow binders.

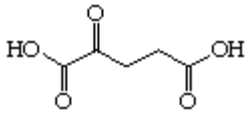
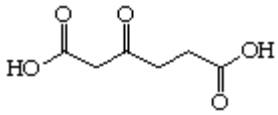
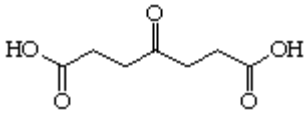
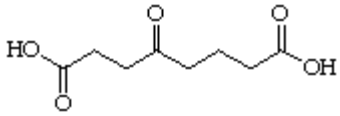
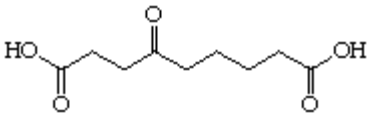
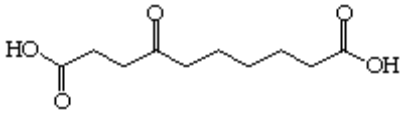
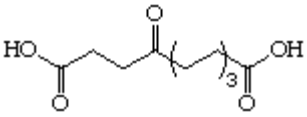
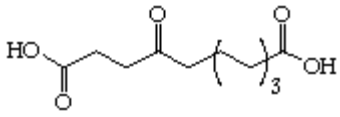
Cx	Compound	*Ki (µM)*	Inhibition type
C5		8'500	Competitive
C6		10'400	Competitive
C7		8'600	Competitive
C8		82	Uncompetitive
C9		450	Uncompetitive
C10		(-)	Irreversible
C11		(-)	Slow-binder
C12		(-)	Slow-binder

Figure 24: Results of the inhibition studies of the g-keto dicarboxylic acids C5 to C12.

The important conclusions from this series of inhibition studies is clear (see Figure 25). The g-keto dicarboxylic acids, which resembles the intermediates postulated by Jordan are tightly bound, so much so that they show an irreversible behaviour. The intermediate which imitates the intermediate postulated by Shemin however seems to be only recognised as analogue of the substrate without any additional site of interaction with the enzyme. As a consequence, only a weak interaction between the C7 dicarboxylic acid and the enzyme is observed, which is reflected by the high unspectacular value for K_I. An additional argument is the observation that the two diastereroisomers ***rac***-30 and ***rac***-31 which imitate more closely the intermediate postulated by Shemin are even weaker inhibitors than the simple 4-oxo-pimelic acid. The

values of K_I are 11'900 and 17'000 mM respectively. The obvious interpretation of these results is that the 4-oxo sebacic acid is recognised 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 additional point of recognition. This three point recognition leads to a quasi irreversible behaviour of this inhibitor. Inhibitors which are slightly too short or slightly too long still are strongly bound to the active site, but they show either slow-binding behaviour or good recognition, which means a small K_I value and uncompetitive behaviour. Inhibitors, where the distance between the two carboxylic acid ends is too small, are "only" recognised as substrate analogues and show therefore competitive and not very efficient inhibition behaviour. Based on these observations clearly the Jordan mechanism is preferred.

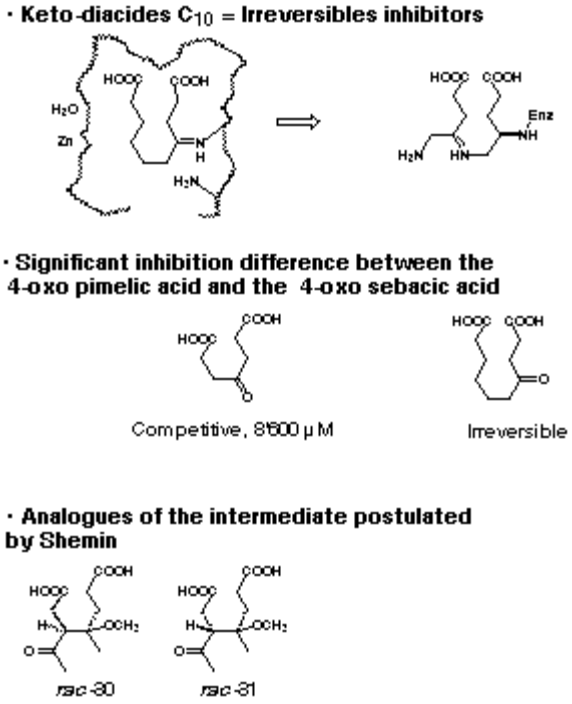


Figure 25: Interpretation of the inhibition results using the g-keto dicarboxylic acids.

References

Conclusion

"A Novel Synthesis of Porphobilinogen: Synthetic And Biosynthetic Studies"

Christiane Bobillier Neier / August 1999

Conclusions

Acknowledgement

Conclusions Based on our inhibition results and incorporating the information from the X-ray structures the following mechanism can be proposed for porphobilinogen synthase (see Figure 26).[49]

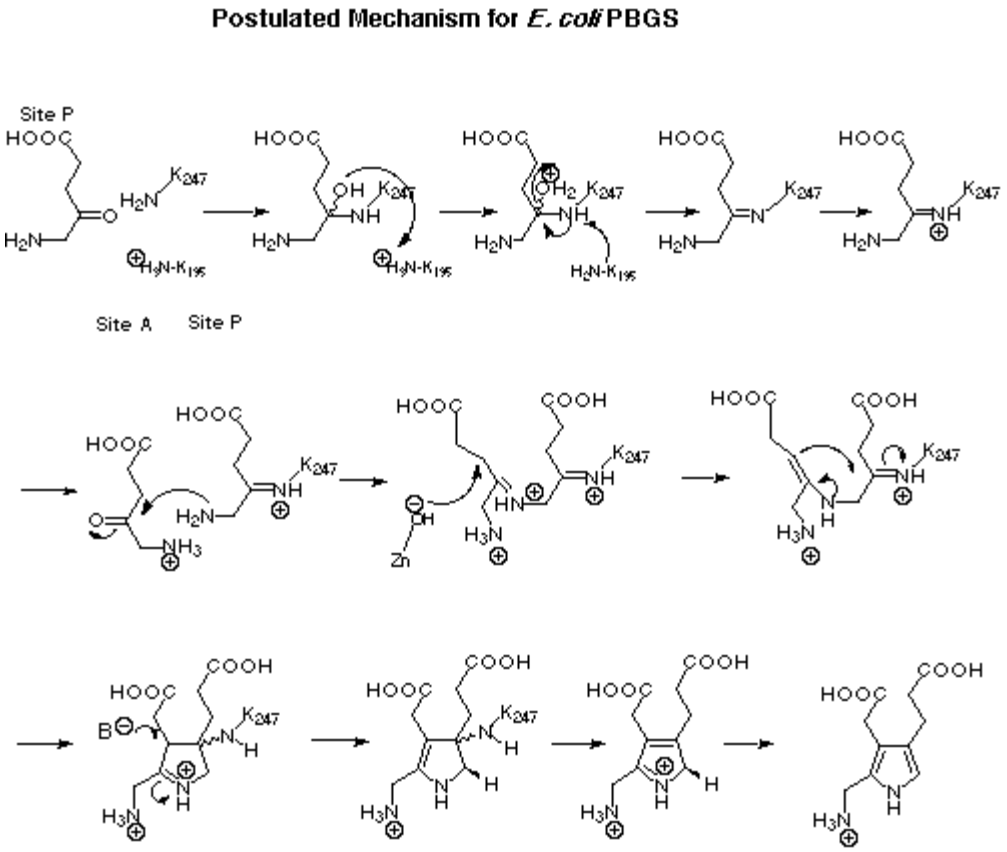


Figure 26: Proposed mechanism for the biosynthesis of porphobilinogen (4).[49]

The reaction sequence starts with the formation of the first Michaelis-Menten complex at the P-site of the enzyme. The first substrate forms then the Schiff base with the active-site lysine. As proposed by Jordan and Cooper the role of the second lysine could be to play the role of a proton donor and a proton acceptor during the formation of the Schiff base.[49] At this stage the second Michaelis-Menten complex is formed at the A-site. Once the second substrate has been recognised by the active site the crucial bond forming between the two substrate molecules has to occur. The Schiff base connecting the two 5-aminolevulinate molecules (5) should then be transformed into the corresponding enamine. The enamine contains all the reactive centres in the arrangement necessary to create the crucial carbon carbon bond. Once the carbon carbon bond is formed a series of reversible but chemically straight forward steps will lead to the product porphobilinogen (4). A plausible sequence could be: deprotonation a to the iminium ion forming the enamine, followed by elimination of the amino group of the active site lysine, finally the protonated form of porphobilinogen is deprotonated by a base at the active site of the enzyme, thereby forming porphobilinogen (4) bound to the enzyme.

This proposal incorporates a series of observations from the X-ray structures and is in accordance with most of our inhibition results. At the same time a series of new questions have arisen and a few chemical problems have still not found an answer so far.

The following structural and kinetic information has been incorporated:

The second lysine group found at the active site has a role as proton shuttle and as group influencing the pKa of the active site lysine. The attribution of this role is largely inferred indirectly. It is at the moment not possible to exclude that this lysine plays a different role e.g. forming a Schiff base with the second substrate. There is a major role attributed to the active site Zn. The active site Zn should be essentially the porter of a judicially placed a base, the hydroxide ion, which plays a crucial role in creating regioselectively the enamine needed for the ring closure. A sort of secondary role can be attributed to the Zn ion, which would be that of complexing and thereby fixing the carbonyl group of the A-site substrate. Up to this point the arguments in favour of this mechanism are based on the X-ray structures of the porphobilinogen synthase isolated from yeast and Escherichia coli and from the structures obtained by co-crystallisation with levulinic acid. The precise role of each unit mentioned in the mechanism above, is reasonable, for some of these roles even good analogies from other enzymes exist. The distances within the active cleft allow to propose these roles.

The contribution of the inhibition studies is mostly indirect, but rather compelling. In our trials to study analogues of the proposed intermediates we were confronted with the fact, that all the analogues to the Shemin sequence of events were weak inhibitors, whereas the inhibitors based on the Jordan sequence showed good inhibition potency or became even irreversible inhibitors. This analysis is the consequence of the study of more than 100 compounds and is therefore based on a reasonable large data set. Our results are compatible with the sequence of events first proposed by Jordan. The inhibition kinetics alone will however not be able to prove the sequence of events.

Problems arise from three lines of arguments: 1) What will be the mechanism of Pseudomonas aeruginosa? 2) What is the "chemical" logic of the proposed sequence? 3) What is the relation of the actual biochemical mechanism to the hypothesis of a prebiotic formation of tetrapyrroles?

The problem posed by the structure of Pseudomonas aeruginosa is quite evident. The position of the Mg^{2+} observed and thereby the role attributed to Mg-ion in porphobilinogen synthase from Pseudomonas aeruginosa is completely different from that of Zn^{2+} in the enzymes isolated from yeast and Escherichia coli. A probable consequence of this difference could be that the mechanisms are different for the Zn-enzymes and for the Mg-enzymes which would give an enhanced importance to the classification of porphobilinogen synthase according to their metal content made earlier by Jaffe.[41,56]

A question indirectly related to the first problem but already touching the problem of the "chemical" logic is the good recognition of the carbonyl group by the enzyme. All substrate analogues with good inhibition behaviour have to have the g-keto function. In the mechanistic proposal the recognition of the keto function is relatively vaguely defined. The Zn^{2+} is co-ordinated in a tetrahedral geometry to three cysteines and to a water molecule. Already Shoolingin-Jordan and Cooper observe that co-ordination only to cysteines is unusual for an active site Zn cation.[49] It is also not evident how the Zn^{2+} may play the double role of the porter of the hydroxide base, as well as the role of the co-ordination site. At a first glance it seems reasonable that the Zn cation plays either one of these two roles, but it is not at all obvious how the active site Zn^{2+} can play both roles at the same time?

The major problem in connection with the proposed sequence is the question of the ring closing process. This process does not follow Baldwins rules. It has been shown that this ring closure is possible but it is not a favourable process.[57] The obvious questions in this context are to find out how the enzyme has learned to overcome the unfavourable stereoelectronics of the ring closure and how the enzyme manages to accommodate the changes in hybridisation and therefore in the conformation of a relatively tightly bound intermediate. Or in other terms how can the enzyme keep the strong interaction with the two carboxylic acids during this process if the spatial arrangement of the central part of the molecule changes completely. A related question concerns the deprotonation which leads to the enamine. From the solution studies it is clear that deprotonation is thermodynamically favoured towards the ammonium group of 5-aminolevulinate.[58] The obvious answer to this question is that the enzyme has only a basic site juxtaposed for the deprotonation at carbon 3. Our actual knowledge does not allow us confirm this hypothesis, especially because we do not know how the keto function is interacting with the enzyme. So the conclusion from the mechanistic analysis of the proposed mechanism is that once the carbon carbon bond is formed the mechanism is chemically reasonable and easy to understand. The problems posed are why is

the crucial step coming relatively late in the mechanism and how does the enzyme circumvent a series of stereoelectronic obstacles connected with this mechanism.

The final question arising with this mechanism connects today's enzymatic mechanisms with the postulated prebiotic formation of tetrapyrroles.[23,59] Assuming that the hypothesis of the prebiotic formation of tetrapyrroles is correct, then there are immediately two questions which are connected with the enzymatic formation of porphobilinogen (4): as the chemical reactivity of 5-aminolevulinate (5) does not give any hint towards a pre biotic formation of porphobilinogen (4).[60] the question is: does the biochemical mechanism give us any hint towards the prebiotic formation of porphobilinogen (4) or an adequate prebiotic analogue? [59] At this stage the answer to this question is probably no. Then the second question is obvious: how did nature make the switch from prebiotic conditions to an enzyme controlled synthesis? The studies of the structure of the enzyme have not created a clear cut answer. At the moment we can only speculate and try to undertake experiments supporting our speculations. But the gap between our knowledge about prebiotic processes and the knowledge accumulated about the biochemistry seems to increase. This gap is all the more embarrassing as the synthesis of porphobilinogen (4) is central to all tetrapyrrole synthesis. We have good evidence that starting from porphobilinogen (4) tetrapyrrole synthesis is in principle easy to explain be it prebiotic, be it chemical, be it biochemical. It would therefor be important to be able to show that also the synthesis of porphobilinogen (4) itself is easy.

In conclusion the sum of chemical, biochemical and in the last two years X-ray studies has increased our knowledge about porphobilinogen synthase considerably. We are not yet in the position to have firm proof of one mechanism, but at least for porphobilinogen synthase from Escherichia coli the combination of structural data with the results of inhibition studies allows to propose the Jordan sequence as the most probable. The tools are probably now available to solve the question of the sequence of the enzyme catalysed reaction, by trying to co-crystallize the enzyme with some of the analogues of the postulated intermediates. We will hopefully be able to obtain definitive proof soon. We will then be able to infer reasonable answers to some of the questions posed above.

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