

# CHEMICAL SYNTHESIS OF PORPHOBILINOGEN AND STUDIES OF ITS BIOSYNTHESIS

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Abstract . . . . .	36
I. Introduction . . . . .	36
A. Pyrroles as Natural Products . . . . .	36
B. The Importance of Porphobilinogen . . . . .	38
C. The Detection and Structure Determination of Porphobilinogen . . . . .	41
D. Reactivity of Porphobilinogen . . . . .	44
II. Synthesis of Porphobilinogen . . . . .	46
A. Introduction . . . . .	46
B. Synthesizing the Pyrrole Ring in a Knorr Reaction . . . . .	48
C. Synthesizing the Pyrrole Ring with Modified Knorr Reactions . . . . .	52
D. Starting from Pyrrole . . . . .	55
E. Starting from a Pyridine Ring . . . . .	57
III. Biosynthesis of Porphobilinogen . . . . .	59
A. Detection of 5-Aminolevulinic Acid Dehydratase . . . . .	59
B. Properties of Porphobilinogen Synthase . . . . .	60

C.	Mechanistic Studies of Porphobilinogen Synthase . . . . .	63
D.	Conclusions . . . . .	90
IV.	Efforts to Imitate the Biosynthetic Pathway . . . . .	90
A.	General Remarks . . . . .	90
B.	Known Pyrrole Synthesis Forming the Carbon–Carbon Bond First . . . . .	92
C.	Synthesis of Pyrroles Using Enamine Chemistry . . . . .	95
D.	First Synthesis of Pyrroles Via the Crossed-Aldol Reaction . . . . .	99
E.	Second Synthesis of Pyrroles Via the Crossed-Aldol Reaction . . . . .	103
F.	Discussion and Conclusions . . . . .	113
V.	Inhibition Studies of Porphobilinogen Synthase . . . . .	116
A.	General Remarks . . . . .	116
B.	Known Inhibitors of PBGS . . . . .	117
C.	Remarks Concerning Studies of the Inhibition Kinetics of PBGS . . . . .	123
D.	General Remarks on Our Inhibition Studies . . . . .	127
E.	Analogues of $\delta$ -Aminolevulinic Acid . . . . .	127
F.	Analogues of Porphobilinogen . . . . .	131
G.	Analogues of a Potential Intermediate . . . . .	132
H.	Conclusions . . . . .	133
I.	Postulated Mechanism for PBGS from <i>Rhodospseudobacter spheroides</i> . . . . .	136
	Acknowledgments . . . . .	138
	References . . . . .	138

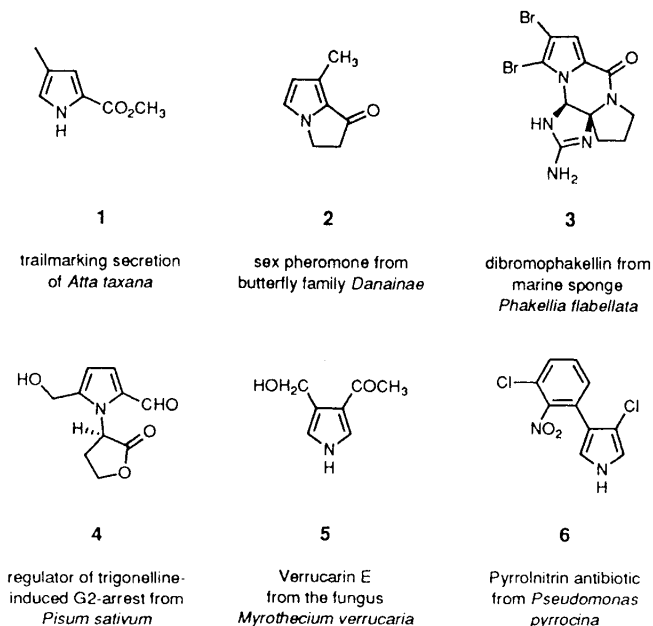
## ABSTRACT

Porphobilinogen is the second dedicated intermediate in the biosynthesis of tetrapyrroles. The substitution pattern attributes a high reactivity to porphobilinogen (= PBG 9). The published synthesis of this unusual pyrrole is reviewed and analyzed according to the synthetic strategies used. In order to compare the chemical synthesis with the biosynthetic process, the knowledge about the enzyme porphobilinogen synthase (= PBGS) is reviewed. Special importance is given to observations, which may be significant to understand the mechanism of the enzyme catalyzed process. Efforts, which have been undertaken to imitate the enzyme catalyzed reaction are described. Two novel pyrrole syntheses have been developed in this study. To elucidate the enzyme catalyzed reaction the known inhibitors of PBGS are comprehensively reviewed and our experiments with specially designed inhibitors are reported. As a consequence of our studies and following a proposal from *Shemin*, a chemically attractive mechanism is discussed.

## I. INTRODUCTION

### A. Pyrroles as Natural Products

Despite the fact that pyrrole belongs to the structurally simple five-membered heterocycles, relatively few natural products containing only one pyrrole ring are known (Figure 1).<sup>1-4</sup> Their biological functions are as varied as are their structures



**Figure 1.** Natural products containing a pyrrole ring.

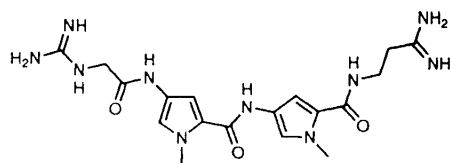
**1–6.**<sup>5–7</sup> Some natural pyrroles are pheromones **2**,<sup>8,9</sup> plant hormones **4**<sup>10</sup> or can be used as antibiotics **6**.<sup>11</sup>

An important class of pyrrole-derived natural products containing more than one pyrrole ring are neotropsin **7** and distamycin **8** which bind to the minor groove of DNA (Figure 2).<sup>12</sup> They contain a series of pyrrole rings which are linked by amide bonds with each other.

In spite of the differences in the structure of these natural products most of them have one common feature: they are stabilized by an electron-withdrawing substituent or by another aromatic ring. Without these stabilizing substituents the electron-rich pyrrole ring is easily attacked. Under the influence of small amounts of acids unstabilized pyrroles are polymerized and auto-oxidized to give so-called “pyrrole-red.”<sup>13–16</sup>

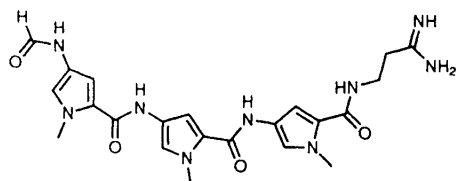
Porphobilinogen (PBG) is a remarkable exception to this rule (Figure 3). PBG **9** is a trisubstituted pyrrole which contains only alkyl substituents.

The lack of substituents which can stabilize the pyrrole ring via conjugation attributes a high reactivity to PBG. The reactivity of PBG is used in the biosynthesis of the tetrapyrrolic pigments. More than  $10^{10}$  tons of chlorophyll are synthesized each year.<sup>17–25</sup> As can be deduced from the  $K_M$  values of porphobilinogen synthase and of uroporphyrinogen I synthase, only small concentrations of the pyrrole **9** are



7

Netropsin



8

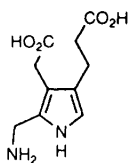
Distamycin A

**Figure 2.** Polypyrrolic natural products interacting with DNA.

present in living organisms.<sup>26</sup> This precaution avoids undesired side reactions due to the high reactivity of PBG. Only if the normal biosynthetic pathway is severely disturbed do large quantities of PBG accumulate. In humans this accumulation leads to acute intermittent porphyria, a rare but severe illness.<sup>27,28</sup>

### B. The Importance of Porphobilinogen

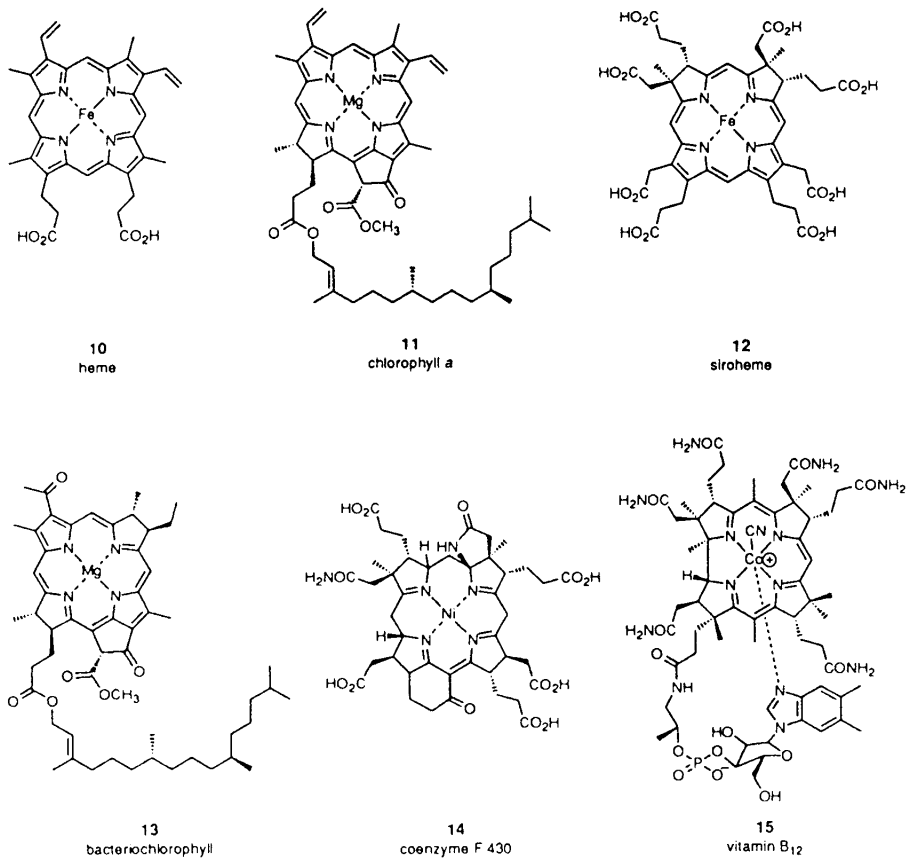
PBG is a dedicated intermediate in the biosynthesis of tetrapyrroles.<sup>29–35</sup> The tetrapyrrolic pigments **10–15** play an important role for the central processes of life (Figure 4). They are universally distributed and have therefore been named “pigments of life.”<sup>36,37</sup>



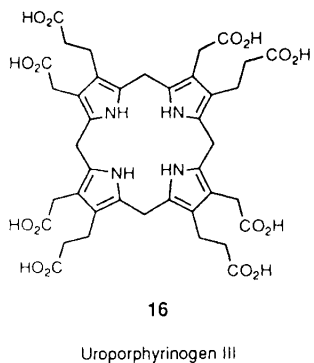
9

Porphobilinogen

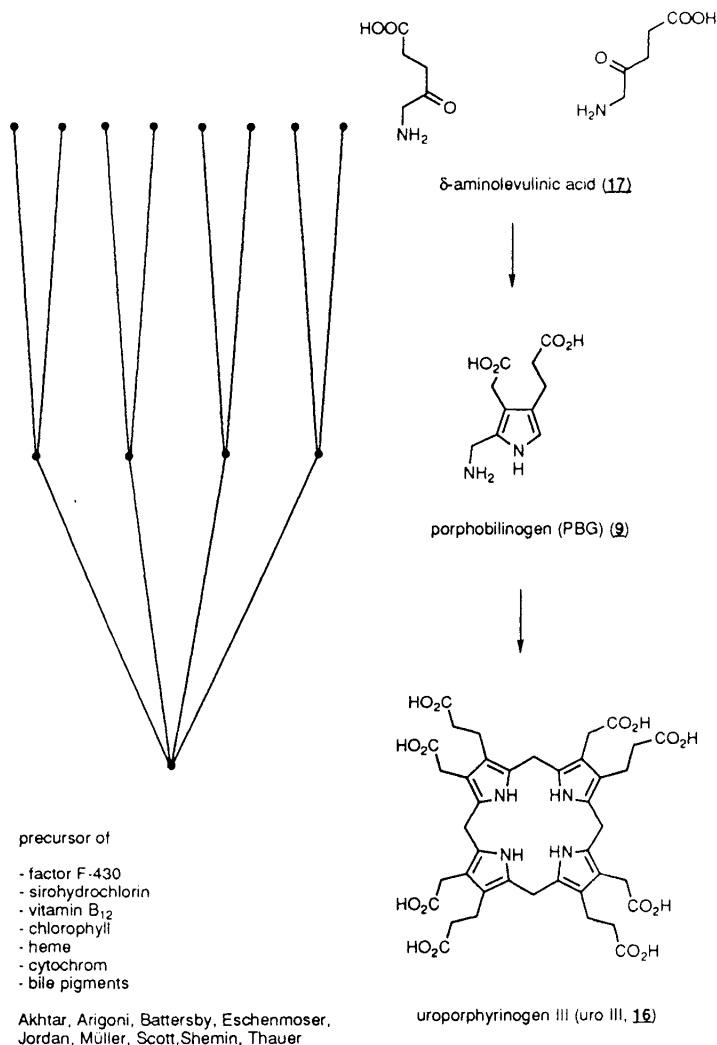
**Figure 3.**



**Figure 4.** Tetrapyrrolic pigments.



**Figure 5.**



**Figure 6.** Biosynthesis of the skeleton of the “pigments of life”: 8 molecules  $\delta$ -aminolevulinic acid are transformed into one molecule uroporphyrinogen III.

Tetrapyrrolic pigments function as indispensable cofactors for the transport of oxygen or of electrons (**10**); they harvest light and transform the energy of the photons into redox energy (**11,13**); they are catalysts for methane production (**14**), the reduction of nitrate and sulfite (**12**), and for many methylation steps; and they catalyze a series of unusual rearrangements (**15**).<sup>38,39</sup>

Tetrapyrroles are involved in the oldest metabolic pathways. This fact has been used to claim that the biosynthesis of these pigments was contemporary with the appearance of life.<sup>40–46</sup>

All tetrapyrroles are derived from a common tetrapyrrolic precursor uroporphyrinogen III (**16**) (Figure 5).<sup>29–31</sup> Uroporphyrinogen III (**16**) itself is biosynthesized in three steps from eight molecules of 5-aminolevulinic acid (**17**) (Figure 6).

There are two major biosynthetic pathways to synthesize **17**.<sup>32,47–49</sup> The final structural complexity of the tetrapyrrolic pigments is produced by modifying **16** in a series of chemical transformations (Figure 7). These transformations allow the tetrapyrrole structure to adapt to the specific task it has to fulfill. The size of the central coordination hole modulates the chelation properties of the ligand and most remarkably the redox potential of the metal complexes.<sup>50–52</sup> Modification of the functional groups attached to the side chains of the disk-like macrocycle attributes hydrophilic or hydrophobic properties to the metal complexes. Esterifying one of the carboxylic acids with phytol (**11,13**) allows the complexes to incorporate into membranes. The vinyl groups can also be used to covalently link the chelates to central proteins (**10**).

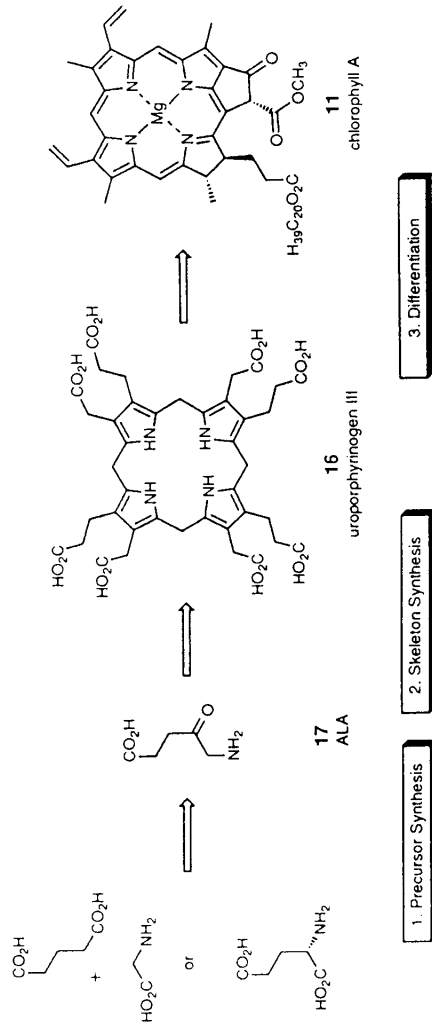
The first step in the biosynthesis differs between plants and animals, while *Euglena gracilis* can use both pathways (Figure 7).<sup>32,48</sup> The final steps of the biosynthesis starting from **16** vary according to the pigment which has to be synthesized. In contrast the central part of the biosynthesis is common to all organisms and for all biosynthetic pathways studied to whatever tetrapyrrole they lead.

The physiological importance of the tetrapyrroles and the fact that tetrapyrroles possess a dedicated biosynthetic pathway has stimulated the interest in PBG (**9**).

### C. The Detection and Structure Determination of Porphobilinogen

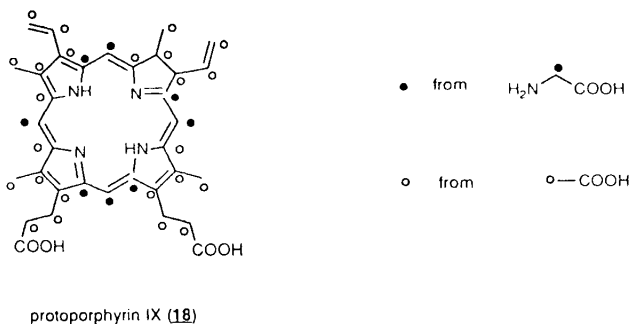
The spectacular development of our knowledge of the biosynthesis of tetrapyrroles has been intimately related to the availability of isotopes and the use of isotopically labeled precursors in the studies of biochemical problems in the United States.<sup>53</sup> In 1946, Shemin and Rittenberg published a paper entitled “The Life Span of Red Blood Cells”.<sup>54</sup> They reported the results of Shemin’s seminal self experiment, which showed that <sup>15</sup>N-labeled glycine was the precursor of some of the nitrogen atoms of protoporphyrin IX (**18**). Starting from this observation, Rittenberg, Wittenberg, Shemin, and Neuberger could deduce within 5 years the origin of all the atoms in **18**.<sup>55–62</sup> It could be shown that **18** is synthesized in nucleated blood cells of birds by the condensation of eight molecules of glycine with eight molecules of a four-carbon-atom compound derived from acetic acid via the Krebs cycle (Figure 8).

Before the advent of isotope-labeling studies, several widely different precursors of **18** had been postulated<sup>63</sup> without any experimental evidence. However the first experiments using isotopically labeled precursors showed that most of these speculations were wrong. In 1950 Neuberger<sup>64</sup> proposed a mechanism for the



**Figure 7.** The three parts of the biosynthetic pathways to the “pigments of life”: (1) synthesis of the first dedicated precursor; (2) synthesis of the tetrapyrrolic skeleton; (3) differentiation.

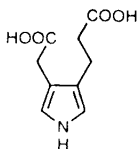




**Figure 8.** Labeling pattern of protoporphyrin IX starting from labeled glycine and labeled acetic acid.

biosynthesis based on a hypothesis of Turner,<sup>65</sup> who had suggested in 1940 one and only one general pyrrole precursor for the formation of protoporphyrin IX (**18**). In their paper, Neuberger and collaborators postulated that the first pyrrolic compound in the biosynthetic pathway was a dicarboxylic pyrrole **19** bearing an acetic acid residue in one  $\beta$ -carbon and a propionic acid side chain on the other  $\beta$ -position (Figure 9). They assumed that the last four carbon atoms derived from glycine were added later during the formation of the porphyrin ring.

In 1951, as a consequence of a series of highly elegant experiments, Shemin and Wittenberg suggested that only one common pyrrole was the precursor of all four rings in the biosynthesis of protoporphyrin IX (**18**).<sup>61</sup> They concluded that if the same compound is utilized for the synthesis of each side of the hypothetical pyrrole intermediate, then a four-carbon compound (e.g. an unsymmetrical succinate) is needed for the condensation with glycine. This hypothesis led Shemin and Russell to synthesize 5-aminolevulinic acid (**17**) and to study its incorporation into heme (**10**).<sup>66,67</sup> Parallel to the biosynthetic studies, in 1952 Westall isolated and crystallized PBG **9** from the urine of patients with acute porphyria.<sup>68</sup> PBG **9** had been first identified in 1931 by Sachs.<sup>69</sup> In 1953, Cookson, Rimington,<sup>70,71</sup> and Kennard<sup>72</sup> recognized the major structural features of PBG **9** and an X-ray crystallographic



**19**

**Figure 9.**

determination finally established the structure.<sup>72</sup> This same year in a series of publications by Falk and Rimington,<sup>73</sup> and by Shemin<sup>66</sup> and Neuberger,<sup>74</sup> PBG **9** was shown to be a highly specific precursor for tetrapyrroles and to be efficiently biosynthesized from 5-aminolevulinic acid (**17**). Thus within one year the structure and the biosynthetic role of PBG **9** was proven.

#### D. Reactivity of Porphobilinogen

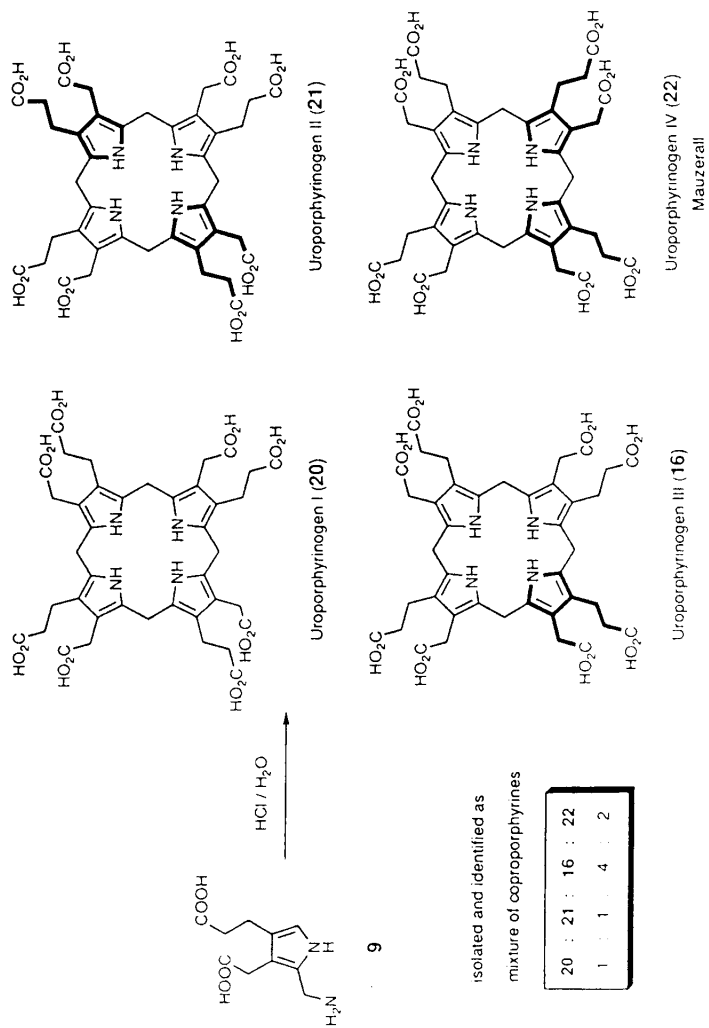
Early reactivity studies showed that tetrameroidization of PBG **9** could be achieved easily even without the help of an enzyme (Figure 10).<sup>75-78</sup>

Eschenmoser coined the expression tetrameroidization (or in general: oligomeroidization) for reactions where four (or several) molecules react with each other, but the product differs in its molecular formula from the sum of the molecular formula of the starting materials.<sup>79</sup> This expression was introduced to differentiate these reactions from processes where the molecular formula of the product is identical with the sum of the molecular formulae of the starting materials.

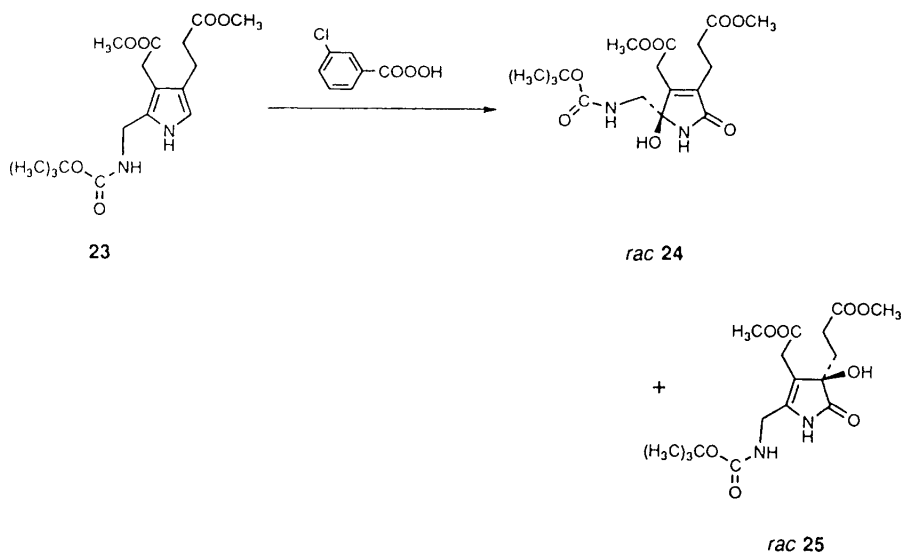
In the absence of oxygen, tetrameroidization of PBG **9** gave the statistical mixture of the uroporphyrinogens **20**, **21**, **16**, and **22**. The uroporphyrinogens were transformed into the uroporphyrins and then into the four corresponding coproporphyrins. These were identified via paper chromatography.<sup>77</sup> The most important aspect of these experimental observations is the fact that PBG **9** has a strong tendency to form the uroporphyrinogens. The mixture consists mainly of the uroporphyrinogen III (**16**), which represents 50% of the product. The next intermediate in the biosynthesis of tetrapyrroles is uroporphyrinogen III (**16**). In the biosynthesis the high selectivity for the formation of the type III product **16** is achieved in a highly elegant way by two distinct enzyme-catalyzed steps.<sup>80</sup> The fact that the chemical reactivity of PBG **9** leads to the formation of the next biosynthetic intermediate without the help of an enzyme is remarkable. In a chemist's view this enzymatic transformation might be called an example of a chemomimetic biosynthesis.<sup>81-84</sup>

With the exception of the first step, the condensation of glycine and succinic acid to 2-amino-3-ketoadipic acid, all the steps from glycine and succinic acid to uroporphyrin are thermodynamically favorable.<sup>83,84</sup> For the dimeroidization of 5-aminolevulinate to PBG **9**, a  $\Delta G = -16.9$  kcal/mol, and for the tetrameroidization of PBG **9** to uroporphyrinogen, a  $\Delta G = -34.6$  kcal/mol were calculated for the gas phase reactions.<sup>83</sup> Thus, tetrapyrrole biosynthesis belongs to the class of reactions where the formation of bigger molecules liberates free energy. This fact has attracted the interest of scientists and has led to the hypothesis on "the spontaneous formation of tetrapyrroles".<sup>85</sup>

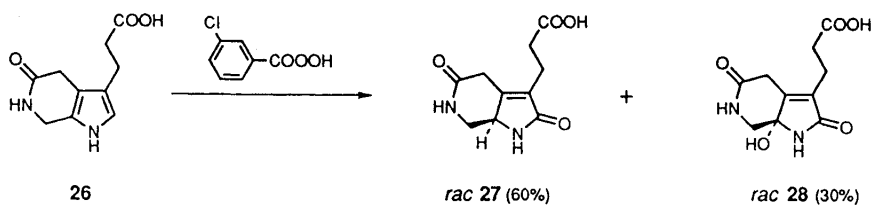
The second transformation of PBG **9** which has been studied to some extent, is the oxidation of the pyrrole ring. If the *N*-*tert*-butoxycarbonyldimethylester **23** is oxidized with *m*-chloroperbenzoic acid, two isomeric pyrrolinones *rac*-**24** (16% yield) and *rac*-**25** (15% yield) are isolated (Figure 11).<sup>86</sup>



**Figure 10.** Chemical ( $\approx$  statistical) synthesis of uroporphyrinogen I–IV.



**Figure 11.** Oxidation of a protected porphyrinogen derivative.



**Figure 12.** Oxidation of the lactam formed from porphyrinogen.

Using the lactam **26** as starting material, a 2:1 mixture of pyrrolinones *rac-27* (60% yield) and *rac-28* (30% yield) was obtained (Figure 12).

These oxidation studies were conducted to imitate the product formation of the enzymatic oxidation of PBG **9** by porphobilinogen oxygenases.

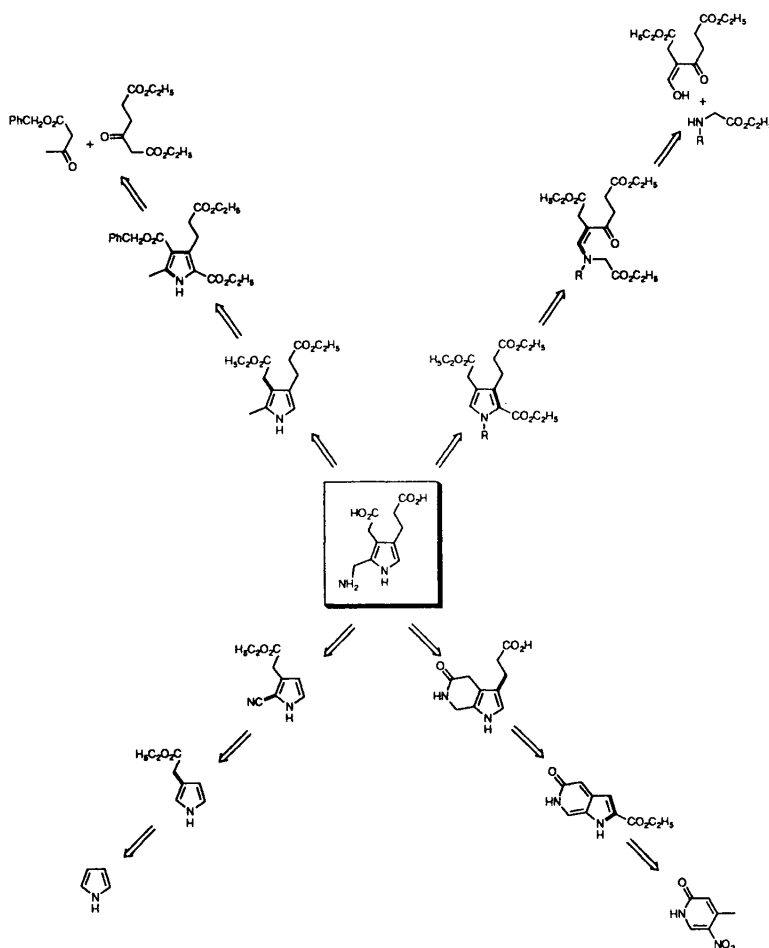
## II. SYNTHESIS OF PORPHOBILINOGEN

### A. Introduction

The synthesis of PBG **9** has attracted the attention of chemists for different reasons: Originally the synthetic efforts were undertaken to prove the structure<sup>87,88</sup>; later the interest was mainly in the synthesis of PBG **9** labeled at a specific position

in order to use it in the studies of the biosynthesis.<sup>89–91</sup> Labeled and unlabeled PBG **9** or precursors thereof were used in the synthesis of pyrromethanes, tripyrrines, bilanes, and porphyrinogens.<sup>92–99</sup> Despite an exorbitant price—almost 1000-fold greater than the price of gold<sup>100</sup>—there have only been a limited number of fundamentally different approaches to the synthesis of PBG **9** or its analogues reported in the literature. Especially surprising is the fact that since 1979, when the synthesis of PBG **9** was reviewed by Frydman,<sup>101</sup> only very few new results have been reported.

Four synthetic strategies have been used for the synthesis of PBG **9** (Figure 13). The first strategy uses a classic Knorr synthesis to obtain a suitable precursor. As



**Figure 13.** Retrosynthetic analysis of porphyrinogen.

the functional groups on the pyrrole ring which are compatible with the Knorr synthesis are not those present in PBG 9, a considerable amount of work has gone into the modifications of the functional groups to obtain the natural substituents. PBG 9 was first synthesized in a reasonable amount according to this strategy.<sup>87,88</sup> The development of this strategy is mainly due to MacDonald.<sup>102</sup> He developed the chemistry necessary to modify the suitable precursors. The major problems are the introduction of the acetic acid side chain, the introduction of the methylamino group, and finally the removal of the ester used as protecting group of the  $\alpha$ -position of the pyrrole.

The second strategy stems from Plieninger<sup>103</sup> and in a later version from Evans.<sup>104</sup> In both syntheses the pyrrole ring is formed by condensation of a C<sub>3</sub> unit with a C–N unit. In one case it is the Kleinspehn variant of the Knorr synthesis, 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. When the pyrrole ring is formed the methods developed by MacDonald are used to obtain the natural product.

The third strategy is due to Frydman and Rapoport.<sup>105</sup> 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 hydrolyzed to PBG 9. The major problems using this strategy is the introduction of the propionic acid side chain and the reduction to the right oxidation state.

The fourth strategy stems from Anderson and collaborators, who started with the unsubstituted pyrrole.<sup>106,107</sup> Introducing step-by-step (1) the acetic acid side chain in the  $\beta$ -position, (2) the nitrile group as precursor of the methylamino group in the  $\alpha$ -position, and (3) the propionic acid side chain in the  $\beta'$ -position finally gave PBG 9.

## B. Synthesizing the Pyrrole Ring in a Knorr Reaction

With a considerable effort MacDonald and his collaborators worked out different sequences which allowed the synthesis of PBG 9,<sup>108,109</sup> its regioisomers,<sup>102</sup> and also the necessary precursors<sup>92,93,110</sup> to obtain dipyrrenes and porphyrins in a regioselective manner (Figure 14).

They started with diethyl  $\beta$ -keto adipate (**29a**) which they obtained in three steps from the reaction of ethyl succinyl chloride and magnesium diethyl malonate in 51% overall yield.<sup>111</sup> This substance was treated with amyl nitrite and the resulting oxime was treated with zinc in acetic acid in the presence of benzyl acetoacetate (**30**) which gave in 68% yield the tetrasubstituted pyrrole **31**.<sup>108</sup> All the remaining nine steps are needed to adjust the substituents on the pyrrole ring. To obtain PBG 9 from pyrrole **31** requires introducing the acetic acid side chain, transforming the methyl group into the methylamino group, removing the 5-ethylcarboxylate, and hydrolyzing the esters. To achieve these goals the following sequence has been developed as the result of a long series of optimization. Removal of the benzyl protecting group by treating the pyrrole **31** with Raney nickel at 100 °C and under

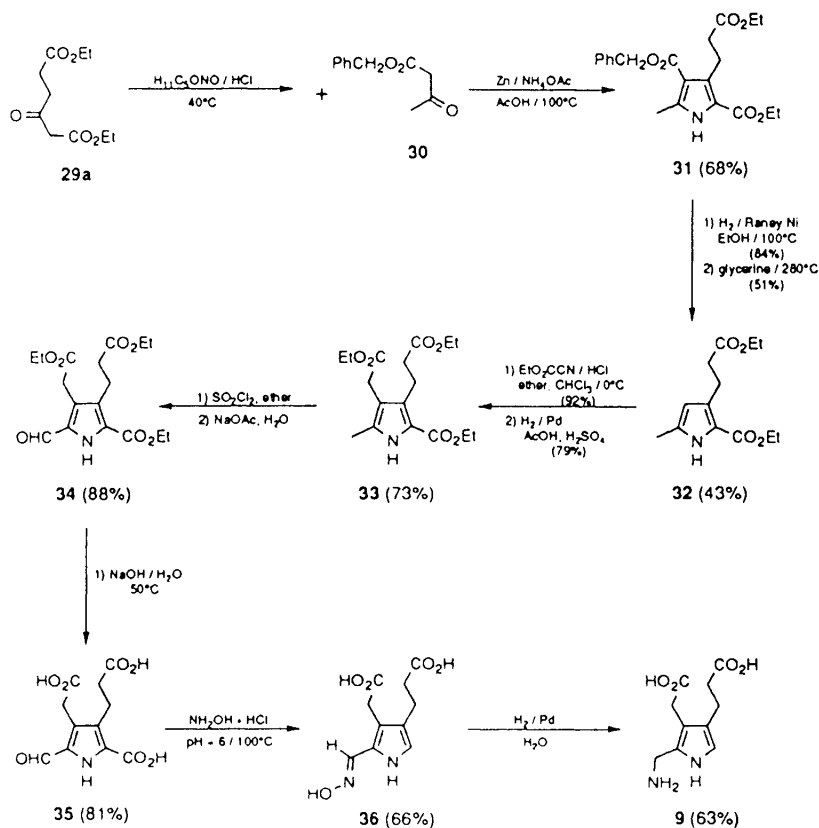


Figure 14.

100 atm in ethanol gave the acid in good yield which could be decarboxylated in glycerol at 260 to 280 °C to the  $\beta$ -unsubstituted diester 32. Pyrrole 32 could be acylated in a sort of Houben Hoesch reaction with ethyl cyanoformate which produced the glyoxalate triester in 92% yield.<sup>109,112</sup> The glyoxalate was reduced catalytically in 79% yield to give the triester 33, which contains now the complete carbon skeleton plus the 5-ethylcarboxylate which functions as protecting group for the  $\alpha$ -position of the pyrrole ring. Treatment of pyrrole 33 with sulfuryl chloride in ether followed by hydrolysis furnished the aldehyde 34 in excellent yield which was then hydrolyzed to the tricarboxylic acid 35. Treatment of this pyrrole 35 with hydroxylamine hydrochloride at almost neutral pH gave the oxime 36 and at the same time decarboxylation was effected.<sup>87,88</sup> The oxime was reduced in a dilute solution in the presence of palladium black as catalyst to give a good yield of PBG 9.

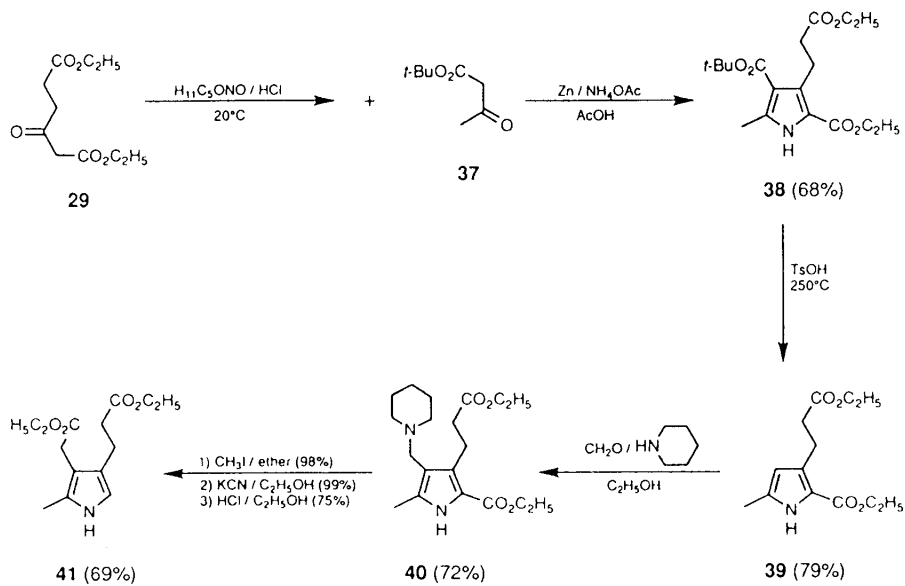


Figure 15.

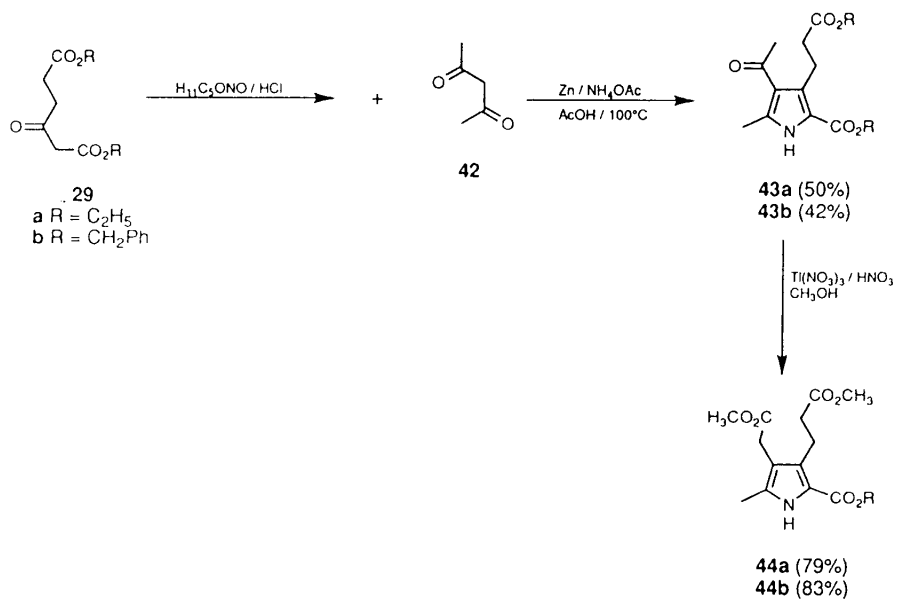


Figure 16.



Treibs and Ott developed in parallel a modification of the synthetic pathway (Figure 15).<sup>113</sup> They used the *tert*-butyl ester of acetoacetate (**37**)<sup>114</sup> as partner in the Knorr pyrrole synthesis.<sup>115</sup> Deprotection and decarboxylation of **37** was immediately followed by a Mannich-type alkylation leading to the pyrrole **40**. Quaternization with methyl iodide followed by substitution with potassium cyanide in ethanol produced the acetic acid side chain. The nitrile was then hydrolyzed to the ethylcarboxylate, which gave the central precursor **41** for the PBG-synthesis.

Another modification of this synthetic pathway is due to Kenner (Figure 16).<sup>91,116</sup> Also in this synthesis the  $\beta$ -oxoadipates **29a** and **29b** were used in the Knorr pyrrole synthesis, but this time with acetylacetone (**42**) as a partner. The advantage of this sequence is that **42** is a highly reactive precursor which usually gives good and reproducible yields of the corresponding pyrroles **43a** and **43b**.

Another advantage of Kenner's synthetic scheme is the fact that all carbon atoms necessary for the synthesis of porphobilinogen are put in place right from the beginning. The thallium-mediated oxidative rearrangement of the acetyl group furnishes the ester of the acetic acid side chain **44a** and **44b**. This gives in a few steps the central intermediate for the porphobilinogen synthesis according to MacDonald. Kenner also developed a modified sequence from this central pyrrole

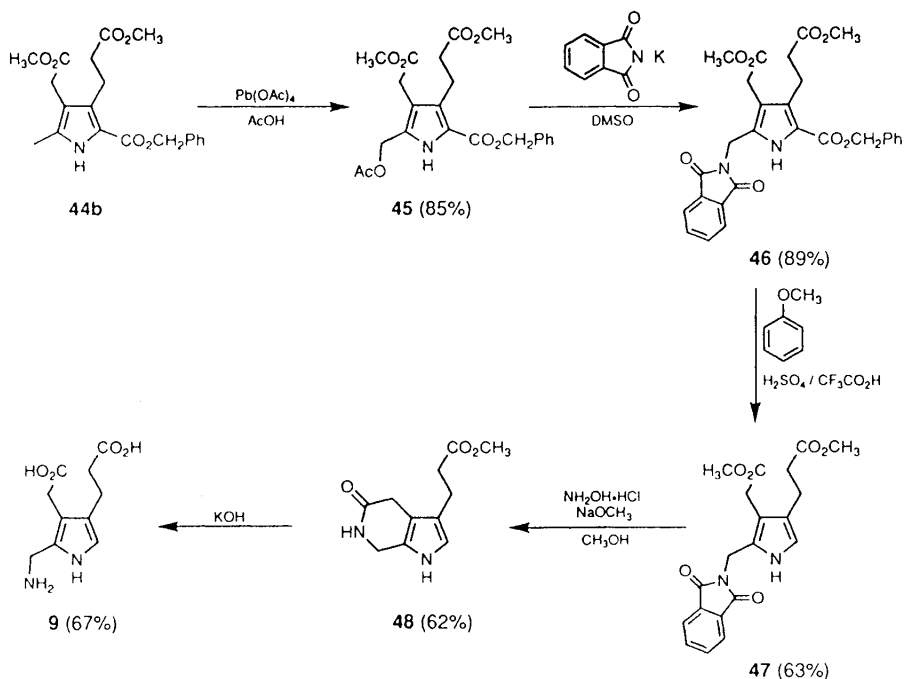


Figure 17.

to PBG **9**, due mainly to the fact that the initial sequence was sometimes not very reproducible (Figure 17).<sup>116</sup>

Kenner introduced the benzyl ester as protecting group for the  $\alpha$ -position of the pyrrole ring.<sup>91</sup> Oxidation with lead tetraacetate selectively modified the methyl group to give **45** in good yield. Substitution with potassium phthalimide in DMSO yielded the precursor **46**. The most delicate step was the deprotection of the benzyl ester. It was necessary to work in anisole as solvent in order to trap the benzyl group before it was going to react with the pyrrole. Under these conditions a 63% yield of the fully protected pyrrole **47** could be obtained. Deprotecting the phthalimide was possible with hydroxylamine or *N*-methylhydrazine which gave ester lactam of PBG **48**. Hydrolysis of this precursor allowed Kenner to obtain PBG **9** in good and reproducible yields.

There are a series of reports in the literature which used these general synthetic schemes and slight variants of them in order to obtain PBG **9** or pyrroles derived from porphobilinogen labeled at different positions.<sup>89-91,117,118</sup>

### C. Synthesizing the Pyrrole Ring with Modified Knorr Reactions

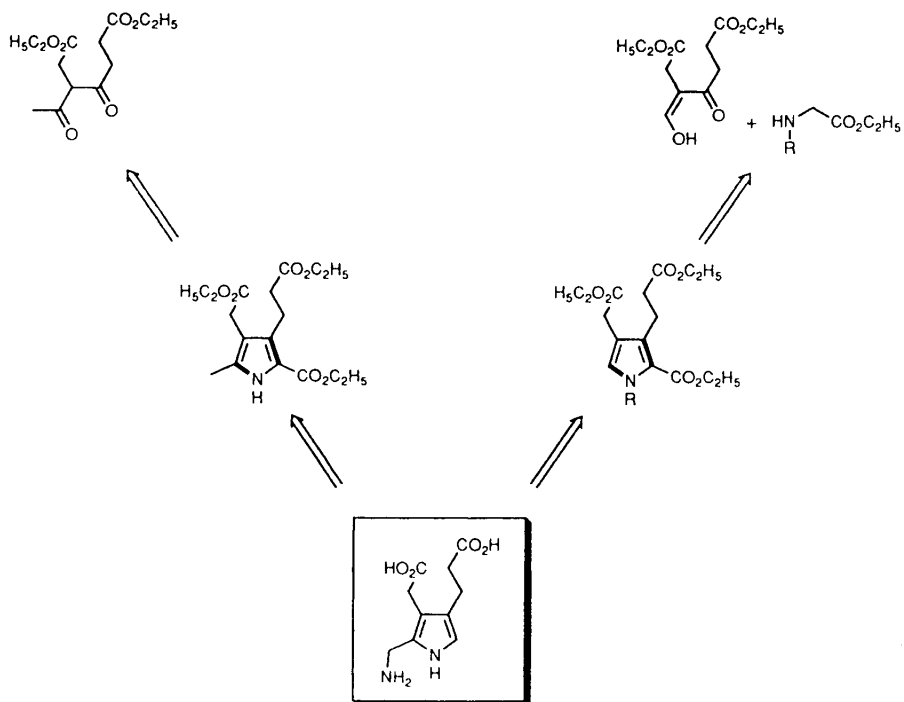
An alternative for the synthesis of the central intermediate of the MacDonald synthesis of porphobilinogen has been developed by Plieninger<sup>103</sup> and by Evans.<sup>104</sup> Both syntheses resemble each other. The major difference to the classical work of MacDonald is that the carbon skeleton is constructed first, and then the pyrrole ring is created by a condensation of a C–N unit with a  $\beta$ -diketone already possessing the rest of the carbon skeleton (Figure 18).

The difference between the two syntheses using this tactic is that in Plieninger's work all the carbons necessary for the creation of PBG **9** are assembled before the creation of the pyrrole ring, whereas in the second synthesis the carbon atom of the methylamino group is introduced after the creation of the pyrrole ring.

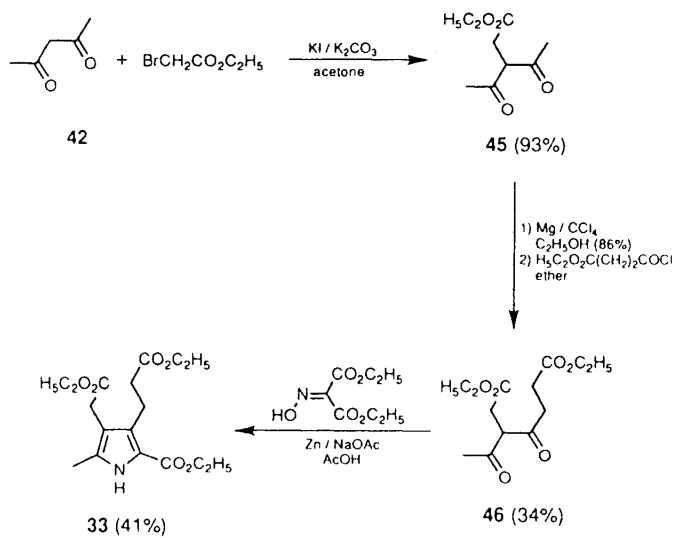
Plieninger<sup>103</sup> synthesized the diester **46** necessary for the Kleinspehn version of the Knorr synthesis starting from acetylacetone (**42**) in three steps: alkylation with ethyl bromoacetate and acylation of the magnesium salt of the  $\beta$ -diketone **45** with the ethyl ester of chlorosuccinate (Figure 19). An alternative synthesis of this compound starts from diethyl 4-oxopimelate (**47**), transformation of this compound into its silylenolether **48**, and acylation with acetyl chloride in the presence of zinc chloride to give the  $\beta$ -diketone **45** in excellent yield (Figure 20).<sup>119</sup> To form the pyrrole ring the diethyl ester of oximinomalonic acid is used.

Evans<sup>104</sup> starts from the commercially available diethyl ester of 4-oxopimelate (**47**) (Figure 21). Acylation of the enolate with ethyl formate yields the precursor **49** which is transformed in two steps into the *N*-substituted  $\alpha$ -free pyrrole ring **50**.

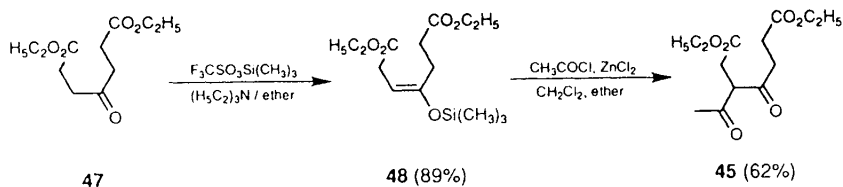
Condensation of the  $\beta$ -diketone with the *N*-dimethoxybenzyl ethyl glycinate gave the vinylogous amide which condensed to pyrrole **50** in the presence of acetic acid and sodium acetate. The yield of this two-step pyrrole synthesis is low (20 to 30%) and the procedure is delicate to reproduce. Deprotection of the dimethoxybenzyl group has to be done with a mixture of sulfuric acid and trifluoroacetic acid



**Figure 18.** Retrosynthetic analysis of porphobilinogen.

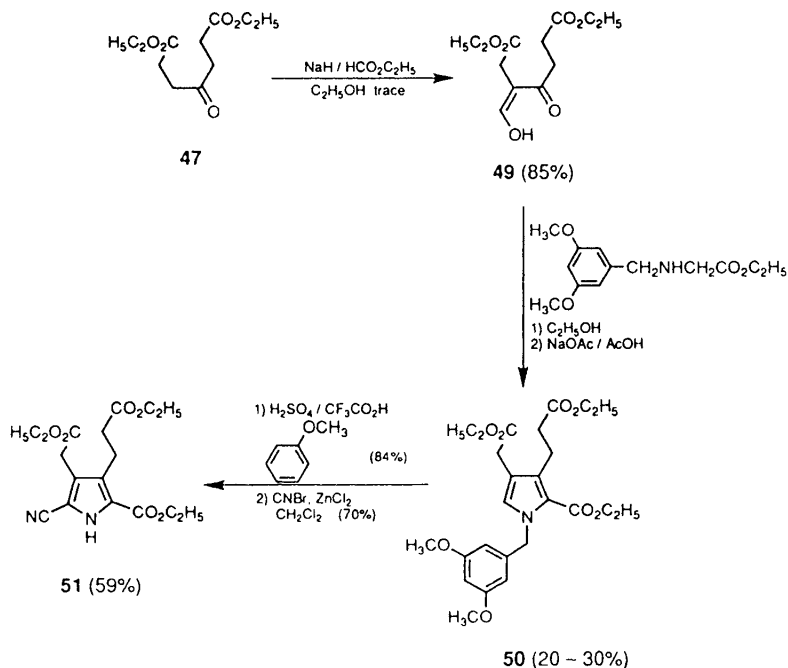


**Figure 19.**



**Figure 20.**

in anisole as a solvent to trap the dimethoxybenzyl cation. The  $\alpha$ -free pyrrole is then treated with cyanogen bromide in the presence of zinc chloride to obtain the cyano pyrrole **51**, which had already been obtained by another route in MacDonald's group. This precursor **51** can be transformed in six steps into PBG **9** using MacDonald's protocol<sup>109</sup> in a total yield of 6% for the six steps.



**Figure 21.**

### D. Starting from Pyrrole

Instead of trying to synthesize a suitably substituted pyrrole via a ring closure reaction, Anderson and his collaborators<sup>106</sup> decided to start from pyrrole itself and to introduce the side chains sequentially. They developed two synthetic pathways to PBG **9** starting from pyrrole (Figures 22 and 23). Vilsmeier–Haack acylation of pyrrole followed immediately by Friedel–Crafts type acylation with ethyl oxalyl chloride gave the 2,4-disubstituted pyrrole **52** in good yield (Figure 22). Palladium-catalyzed decarbonylation and then reduction with Raney nickel in a two-phase system—aqueous ethanol and toluene—gave the 3-acetic acid side chain as its ester in three steps in 40% yield.

Starting from this intermediate **53**, Anderson's group developed two synthetic pathways to PBG **9**. In the first reported synthesis<sup>106</sup> pyrrole **53** was treated with

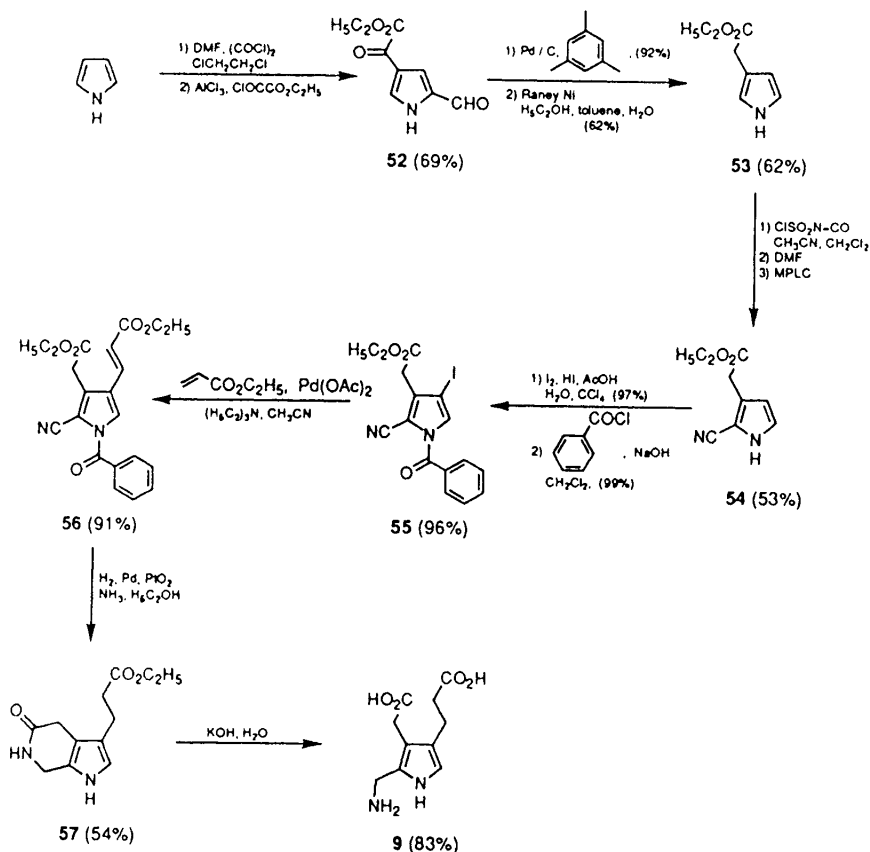
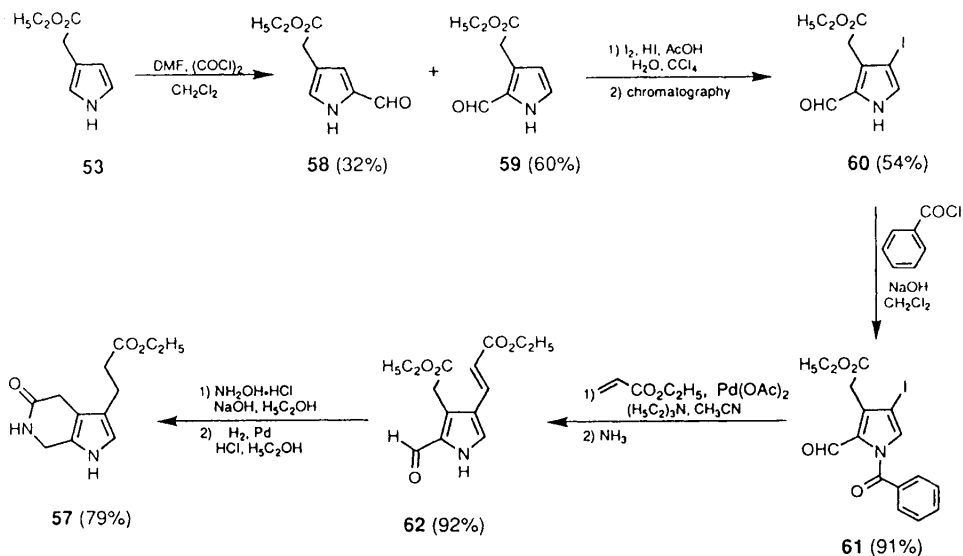


Figure 22.



**Figure 23.**

chlorosulfonyl isocyanate at  $-42\text{ }^\circ\text{C}$  and the intermediate obtained was directly decomposed with DMF to give a mixture of the regioisomeric cyano compounds. This mixture had to be separated with medium-pressure liquid chromatography, and the thus obtained 2-cyano pyrrole **54** was iodinated. The iodo compound was protected with the benzoyl group to give pyrrole **55**, which was reacted with ethyl acrylate in the presence of palladium acetate in a Heck reaction to give the cinnamate **56**. Cinnamate **56** was transformed into the porphobilinogen lactam ester **57**, which could be hydrolyzed according to the procedure of MacDonald.

A clear improvement of the synthetic sequence could be obtained using a Vilsmeier–Haack reaction to introduce the carbon at C2 as an aldehyde. (Figure 23).<sup>107</sup> The two regioisomers **58** and **59** could be separated by flash chromatography.

The identical sequence as before, iodination, introduction of the *N*-benzoyl group and Heck reaction yielded the corresponding cinnamate partially in the deprotected form **62**. Work-up with aqueous ammonia produced the *N*-deprotected pyrrole **62** as the only product. The aldehyde was transformed into its oxime, which was mainly present as its *syn*-isomer. The oxime was reduced in the presence of palladium black in a slightly acidic ethanol solution to give the ammonium salt by simultaneous reduction of the cinnamate and the oxime. The ammonium salt could be transformed into the desired lactam ester **57** by treatment with sodium ethoxide.

### E. Starting from a Pyridine Ring

A completely different approach has been developed by Frydman and Rapoport,<sup>105</sup> and modified versions of this synthetic sequence were reported later by Battersby's group.<sup>90,120</sup> PBG **9** is synthesized via a relatively rare 6-azaindole, starting with a pyridone ring **64** (Figure 24).

The missing propionic acid side chain is introduced into the azaindole **66** via a Mannich-type alkylation, giving compound **67**, followed by alkylation with malonate to give **68**. The remaining synthetic steps are needed to deprotect the precursor and to obtain the porphobilinogen lactam **70**.

The starting material in all three versions of this synthesis is 4-methyl-5-nitro-2-pyridone (**64**) which can be obtained in two steps from 2-amino-4-methylpyridine (**63**). The pyridone is the building block for the lactam ring of **70**.

In the Frydman–Rapoport synthesis,<sup>105</sup> pyridone **64** is transformed into its *O*-methyl ether which is then acylated at the methyl group with diethyl oxalate to obtain the  $\alpha$ -ketoester **65**, mainly present in its enol form. Reduction of this intermediate with palladium on carbon or better with zinc in acetic acid gives the

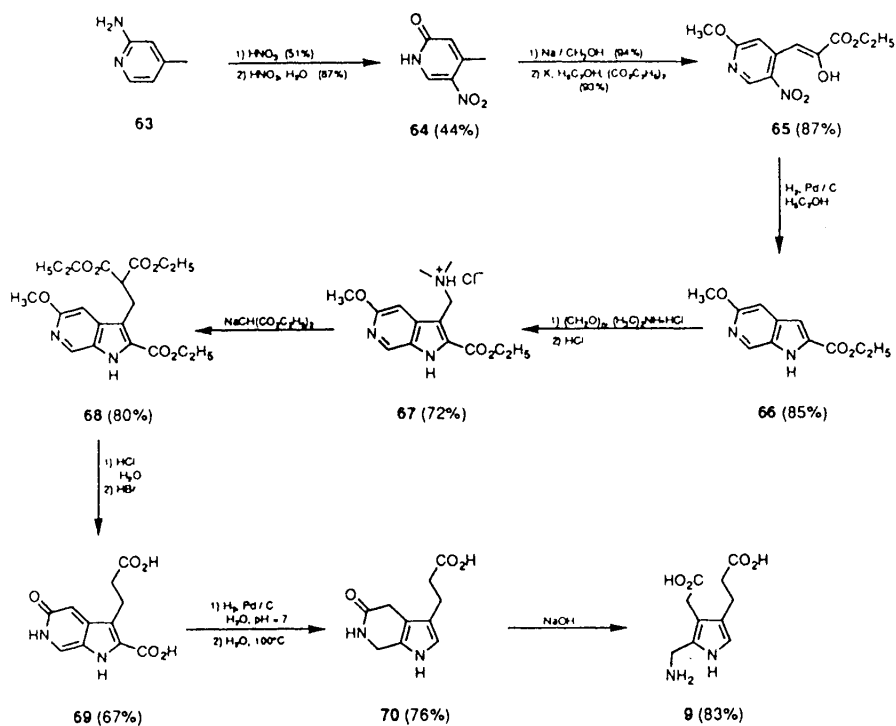


Figure 24.

central intermediate, azaindole **66**. This central intermediate **66** is alkylated in a Mannich reaction either with formaldehyde and dimethylamine or with formaldehyde and morpholine to give **67**. Alkylation with diethylmalonate yields **68**, which is hydrolyzed to give the diacid 2-pyridone **69**. The hydrolysis seems not to be straightforward. Reduction of the pyridone followed by heating the lactam diacid in water gives the porphobilinogen lactam **70**.

Starting from the same pyridone as Frydman, Battersby's group developed a modification of this synthesis (Figure 25).<sup>90,120</sup> Treating pyridone **64** under Vilsmeier–Haack conditions (phosphorylchloride and DMF) gives in one step

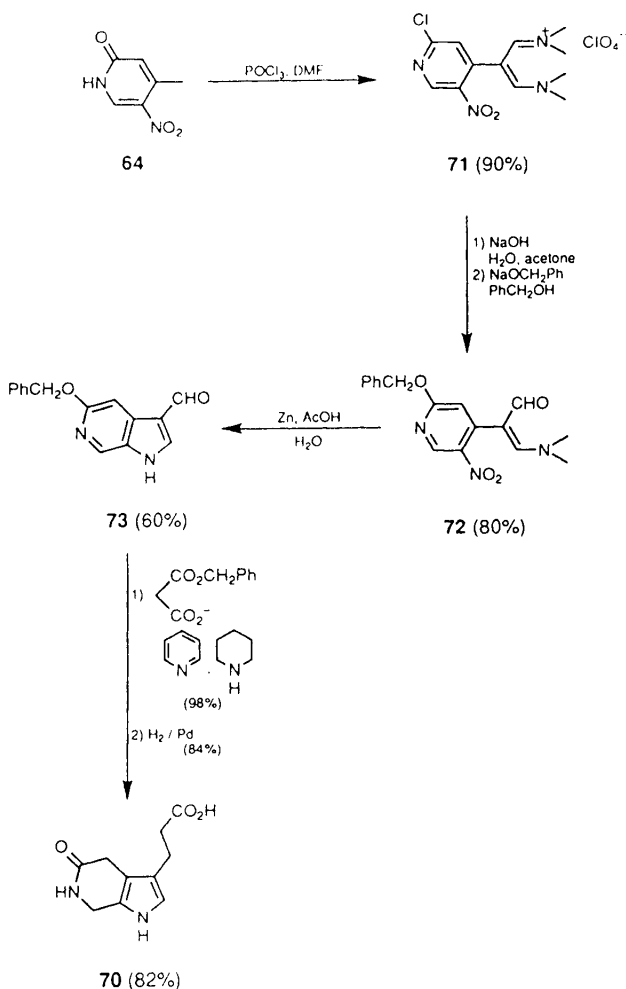


Figure 25.

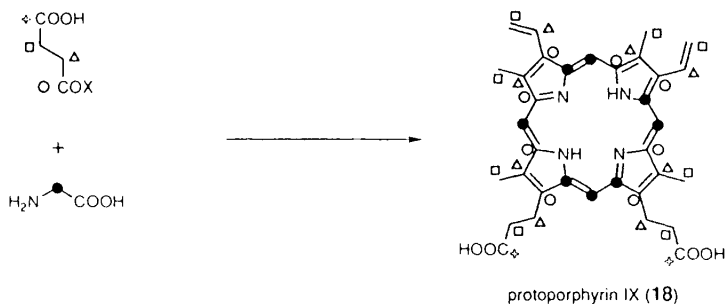


chlorination of the pyridone and the attachment of two carbon atoms on the methyl group, compound **71**. Stepwise hydrolysis with sodium hydroxide in water acetone and treatment with sodium benzyl oxide in benzyl alcohol gave directly the precursor **72** for the azaindole formation. The azaindole **73** was obtained via reduction with zinc in acetic acid. The Knoevenagel condensation gave the dibenzyl-protected cinnamate. Palladium-catalyzed reduction leads directly to the porphobilinogen lactam **70**.

### III. BIOSYNTHESIS OF PORPHOBILINOGEN

#### A. Detection of 5-Aminolevulinic Acid Dehydratase<sup>121-124</sup>

The intensive search in the early 1950s for the suspected biosynthetic precursor(s) of the tetrapyrroles motivated Shemin and Russell<sup>66</sup> and in parallel Neuberger and Scott<sup>74</sup> to synthesize 5-aminolevulinic acid (**17**) and to study its incorporation into heme. Based on the results of earlier labeling experiments, Shemin and Wittenberg had proposed in 1951 that the precursor for heme was formed from the reaction of an unsymmetrically substituted succinate and glycine.<sup>61</sup> In 1952, Westhall<sup>68</sup> succeeded to isolate and crystallize porphobilinogen, a compound which had been known since 1931.<sup>69</sup> He isolated PBG **9** from the urine of patients with acute porphyria. PBG was known to form uroporphyrin chemically by heating at any pH between 1 and 8.<sup>125</sup> The transformation of pure porphobilinogen into uroporphyrin was studied and described by Westhall in collaboration with Rimington in his first paper.<sup>68</sup> A year later Cookson and Rimington postulated the correct constitution of PBG **9**,<sup>70</sup> based on chemical transformations and on the determination of the molecular weight with the help of X-ray crystallographic data from Kennard.<sup>72</sup> As Shemin had shown that 5-aminolevulinic acid was a biosynthetic precursor of heme, it became immediately clear that PBG **9** was the much sought intermediate in the biosynthetic pathway.<sup>66</sup> In the same year Falk, Dresel, and Rimington showed that incubating PBG **9** with lysed bird erythrocytes indeed



**Figure 26.** Labeling pattern of protoporphyrin IX obtained from labeled glycine and labeled succinate.

led to its incorporation into porphyrins.<sup>73</sup> The combination of isolation work, structure determination, enzymatic transformations, and mechanistic reasoning allowed deduction of the major steps leading to the construction of the basic tetrapyrrole skeleton within a period of just over one year (Figures 6 and 26).

The basis to this astonishing development had been led by Shemin in collaboration with Rittenberg in 1945. Shemin studied in a seminal experiment the turnover of <sup>15</sup>N-labeled glycine in his own blood.<sup>126</sup> The label stayed in his blood much longer (127 days) than expected (5 days), assuming that the glycine would have been incorporated only into proteins. This observation was correctly interpreted as the consequence of the incorporation of glycine into heme. Starting from this result the groups of Shemin,<sup>54,59-61,127-130</sup> Bloch,<sup>131</sup> Rittenberg,<sup>132,133</sup> Neuberger,<sup>62,134-136</sup> Kamen,<sup>137</sup> Altman,<sup>138</sup> and Rimington<sup>139</sup> could establish in a fascinating series of experiments the metabolic origin of all the atoms in protoporphyrin IX (**18**) (Figure 26).

From the finding that comparable carbon atoms in each pyrrole had the same <sup>14</sup>C activity, Shemin concluded that the acetic acid had been converted into an unsymmetrical four-carbon compound, from which each side of a common pyrrole precursor was derived<sup>61</sup> (Figure 26).

In early work, blood of ducks,<sup>128,140</sup> blood of patients with sickle-cell anemia,<sup>141</sup> or immature reticulocytes from rabbits<sup>142</sup> were used to study the heme biosynthesis. These *in vitro* systems were highly efficient. Knowing the structures of the precursors in tetrapyrrole biosynthesis, in 1953 Dresel and Falk detected an enzyme which was capable of converting 5-aminolevulinic acid (**17**) into PBG **9**.<sup>143</sup> The enzyme was named aminolevulinic acid dehydratase<sup>144</sup> (ALAD), or porphobilinogen synthase (PBGS), or systematically 5-aminolevulinic acid hydro-lyase (adding 5-aminolevulinic acid and cyclizing) E.C.4.2.1.24. After its first description, the enzyme PBGS was quickly detected in animals, plants and bacteria.<sup>145-147</sup>

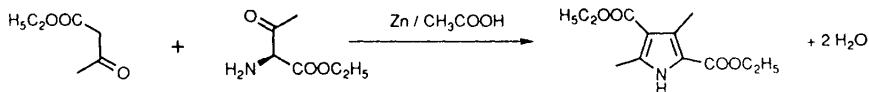
## B. Properties of Porphobilinogen Synthase

Porphobilinogen synthase (PBGS) catalyzes the formation of PBG **9**, the first pyrrolic compound in the biosynthetic pathway to the tetrapyrroles. The biosynthesis of PBG **9** essentially corresponds to a Knorr type reaction (Figure 27).

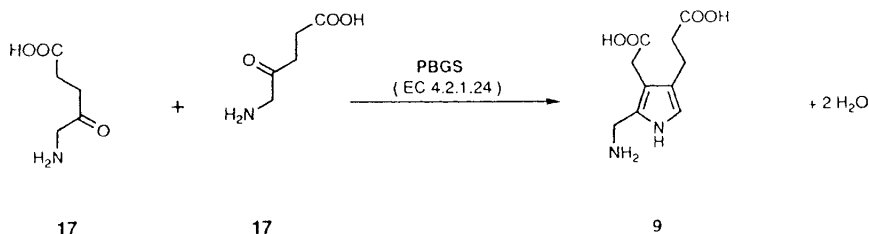
In the Knorr reaction, as well as in the biosynthetic transformation, a series of steps have to occur: (a) imine formation between the amino group of one molecule and the carbonyl group of the other molecule, (b) aldol reaction between C3 of one molecule and the carbonyl group of the other, (c) elimination of two water molecules and, if necessary, eventually (d) deprotonation to form the aromatic pyrrole ring. This complex series of reactions is under the control of one enzyme. It is therefore of interest to know the sequence of these steps during the enzymatic transformation. The enzyme was found to be widely distributed. It has been isolated and purified from different sources as described in the cited review articles.<sup>63,101,148-163</sup>

The properties of PBGS isolated from widely different organisms are similar. Already early on in the studies of PBGS the enzymes from different sources were classified according to the differences in their structure, the metal ion requirement

## Knorr Synthesis



## Porphobilinogen Biosynthesis



**Figure 27.** Comparison of the Knorr pyrrole synthesis with the biosynthesis of PBG from  $\delta$ -aminolevulinic acid.

for catalysis, and the presence or the absence of free thiols.<sup>148,153,159,162</sup> The first classification was essentially based on the apparent metal ion requirements and inhibition studies with EDTA.<sup>164,165</sup> The synthases were categorized into two classes, one which is inhibited by EDTA, suggesting a metal enzyme, and another which is not inhibited by EDTA. This classification was proposed because of the analogy to the classification of the aldolases,<sup>166</sup> which exist as metalloaldolases and non-metalloaldolases.

The synthases from animal cells, yeast, and *E. coli*, which require  $\text{Zn}^{2+}$  for maximal catalytic activity,<sup>153,167-171</sup> consist of eight identical subunits of about 37 kDa each.<sup>153,156,167,172-176</sup> PBGS from animal cells has a pH optimum between 6.3 and 7.0, whereas the yeast enzyme has a more alkaline pH optimum at 9.8. The plant synthases have an alkaline pH optimum at 8.0. They differ also in their structure and apparent metal ion requirement.<sup>177-179</sup> The plant synthases are reported to be hexameric proteins formed of six identical subunits of 50 to 40 kDa.<sup>177,179-181</sup> The plant enzyme needs the addition of  $\text{Mg}^{2+}$  or  $\text{Mn}^{2+}$  to be fully active.

Enzymes isolated from *Rhodospseudobacter spheroides* and *Rhodospseudomonas capsulatus* show quite a different behavior. The pH optimum lies between 8.0 and 8.5. The enzyme from *R. spheroides* needs monovalent cations like  $\text{K}^+$  or related ions like  $\text{Li}^+$ ,  $\text{Rb}^+$ , or  $\text{NH}_4^+$  for activation at low substrate concentrations.<sup>148,164,165,182-184</sup> The synthase from *R. capsulatus* does not appear to require metallic cations for activation.<sup>185</sup> It should be noted that only bovine, yeast, and *E. coli* PBGS's have been analyzed for their metal ion con-

Table 1. Properties of PBGS<sup>a</sup>

Source	Molecular Weight	Molecular Weight of the Subunit	Protein Sequence Known	Metal Requirement	SH Requirement	pH Optimum	K <sub>M</sub> (mM)	Reference
<i>Chlorella regularis</i>	316,000			Mg <sup>2+</sup> , Mn <sup>2+</sup>	-	8.5	0.5	181
<i>Rhodospseudobacter spheroides</i>	250,000	39,500		K <sup>+</sup>	+	8.5	0.7	182,189
<i>Rhodospseudomonas capsulatus</i>	260,000				-	8.3	0.7	185
<i>Propionibacterium shermanii</i>							0.0036	192
<i>Escherichia coli</i> K 12	270,000	36,000	+	Zn <sup>2+</sup> and Mg <sup>2+</sup>	+	8.5 (7.7)	0.8 (1.9)	193,194
<i>Erythrobacter</i> sp OCH 114	260,000	40,000				8.2	0.29	177
Yeast ( <i>Saccharomyces cerevisiae</i> )	275,000	37,800	+	Zn <sup>2+</sup>		9.6	1.5 (0.4)	168,169,195,196
Wheat leaves ( <i>Triticum</i> )				Mg <sup>2+</sup> , Mn <sup>2+</sup>	+	7.5	1.0	197
Tabacco leaves ( <i>Nicotiana</i> )				Mg <sup>2+</sup> , Mn <sup>2+</sup>	+	7.4	0.63	198
Radish cotyledon ( <i>Raphanus</i> )	282,000			Mg <sup>2+</sup> , Mn <sup>2+</sup>	+	8.0	0.39 (0.85)	180,199
Soyabean callus ( <i>Glycine</i> )	280,000			Mg <sup>2+</sup>	-(+)	8.6	0.35	200,201
Spinich leaves ( <i>Spinacia</i> )	324,000	50,000	+	Mg <sup>2+</sup> , Mn <sup>2+</sup>	+	8.2	0.24	178,202,203
Peas ( <i>Pisum sativum</i> )	50,000	50,000	+					204
<i>Phaseolus</i>						7.7	0.3	205
Chicken erythrocytes						6.7	0.5	206
Mouse erythroid								207
Mouse liver	35,000		+	Zn <sup>2+</sup>	+	6.5	0.48	185,208,209
Rat liver	270,000		+				0.15	210,211
Rat Harderian gland	250,000	35,000	+			6.8	0.36	212
Bovine liver	290,000	35,000	+	Zn <sup>2+</sup>	+	6.8	0.15	151,213-217
Rabbit erythrocytes						6.3	0.5	206
Human erythrocytes	283,000	35,000	+	Zn <sup>2+</sup>	+	7.2 (6.8)	0.08 (0.29)	167,218-222

Note: <sup>a</sup>From refs. 148, 153, 180, 191.

tent.<sup>170,171,186,187</sup> For these enzymes it could be shown that more than one metal ion is present per protein subunit. Mitchell and Jaffe have proposed that the PBGS's may have three different types of metal ion binding sites.<sup>170,233</sup> For PBGS from all other sources the role of metal ions was deduced from studies of the enzyme activity as a function of adding metal ions or chelators, a procedure which has its pitfalls as has been shown recently.<sup>188</sup>

The enzyme from *R. spheroides* is believed to be a hexameric protein.<sup>189</sup> In the presence of  $K^+$  the enzyme associates to dimers and trimers,<sup>183,190</sup> whereas 1 M urea dissociates the enzyme into two subunits of about 120 kDa compared to the enzyme of 240 kDa.<sup>190</sup> The observed properties of the synthases from various organisms are sufficiently different that for a long time it wasn't clear how relevant the data from one specific enzyme were compared to the whole class of porphobilinogen synthases (see Table 1).

In recent years molecular biology has contributed considerably to our knowledge about PBGS.<sup>160,193,194,196,203,204,207,211,221-227,227-233</sup> The primary structure derived from cDNAs suggest that the porphobilinogen synthases are relatively similar to each other. The sequences in the vicinity of the active site lysine are highly conserved independent of the organism from which the enzyme has been isolated. One postulated  $Zn^{2+}$ -binding domain, which corresponds to the consensus sequence for the so-called zinc fingers<sup>234,235</sup> is not present in the porphobilinogen synthases isolated from plants<sup>162,170,204,223</sup> where the cysteines have been replaced by aspartic acid.<sup>204,211,222</sup> In accordance with the Extended X-ray Absorption Fine Structure (EXFAS) analysis of Dent et al.,<sup>236</sup> Jaffe has proposed that the PBGS's should have at least two different metal-binding regions.<sup>231,237</sup> In view of the results obtained from PBGS from *Escherichia coli*, where a stimulation by  $Mg(II)$  could be shown, Mitchell and Jaffe proposed the existence of three different types of metal ion binding sites.<sup>170,233</sup> The sequences of the human and the animal synthases are highly homologous. Even the sequences of the plant enzymes show quite a substantial degree of conservation. The major difference is the fact that the coding sequence of pea PBGS corresponds to a protein which is at least 50 amino acids longer than the human enzyme. This does not necessarily prove that the plant protein is bigger than the proteins isolated from other sources. It is still conceivable that the N-terminal region of plant PBGS may be a chloroplast targeting sequence which is latter cleaved. On the whole these results seem to indicate that the similarities between the synthases are substantial and that the initial classification into two groups may be not very important.

### C. Mechanistic Studies of Porphobilinogen Synthase

The following analysis concentrates on the chemical transformations undergone by the substrates. Focusing the attention to the transformation of the substrates, some details of the enzyme catalyzed mechanism have to be neglected. Many details of the enzyme catalyzed reaction are either not yet known or may differ between the PBGS's from various sources. At this stage of our knowledge it is of some

advantage to analyze the enzymatic process mainly seen from the substrates point of view. If all the PBGS's use the same mechanistic pathway, but differ in the functional groups and metal ions they are using, this stripped version (ignoring the discussion of the interactions between the active site and the substrates) has the advantage to be sufficiently general to allow to fill in the neglected details the moment they are becoming available. If on the other hand there are fundamentally different pathways for different classes of PBGS's, then the analysis, which is mainly based on the chemical transformations, can be used as the measuring stick against which the "modifications" applied by different enzymes can be measured.

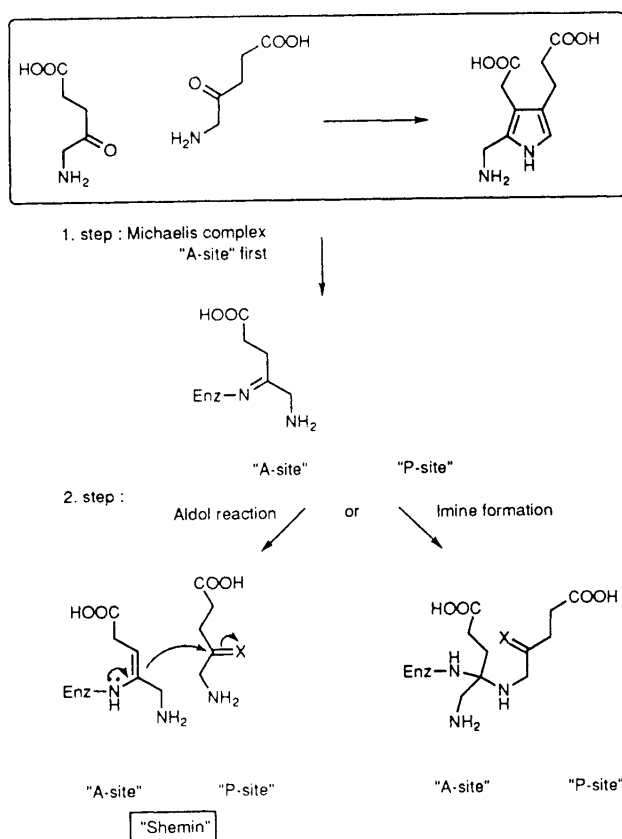
Porphobilinogen synthase is a special enzyme in so far as it uses the same substrate twice and incorporates them in different positions in the product. To our knowledge all of the PBGS's studied to date have been shown to form a binary complex between one substrate molecule and the amino group of the active site lysine (see page 73). We therefore assume that a substituted enzyme mechanism is working (in that a Schiff base is on the enzymatic pathway). In order to understand the catalytic process the answers to the following questions must be determined:

1. Which is the sequence of recognition of the two substrates? The two identical substrates end up in different positions in the product. The two sites are identified by the side chain they will form in the product ("A-site" for the recognition site for the 5-aminolevulinic acid which will form the acetic acid of the product and the "P-site" for the recognition site for the 5-aminolevulinic acid, which will end up as part of the propionic acid side chain in porphobilinogen. Another mnemonic has been brought to our attention by Jaffe stating that the "A-site" 5-aminolevulinic acid retains the free amino group while the nitrogen of the "P-site" 5-aminolevulinic acid is incorporated into the pyrrole ring).
2. What does the Michaelis complex look like for each of the two substrates? When and how is (are) the Schiff base(s) formed between the enzyme and the substrate(s)?
3. Which is the sequence of the different reaction steps? The number of possible pathways is large, so that some conditions limiting the possibilities have to be introduced.
4. How are the substrates activated and related to this question what is the function of the metal ions? Which basic sites and the Lewis acids at the active site are responsible for the catalytic action?

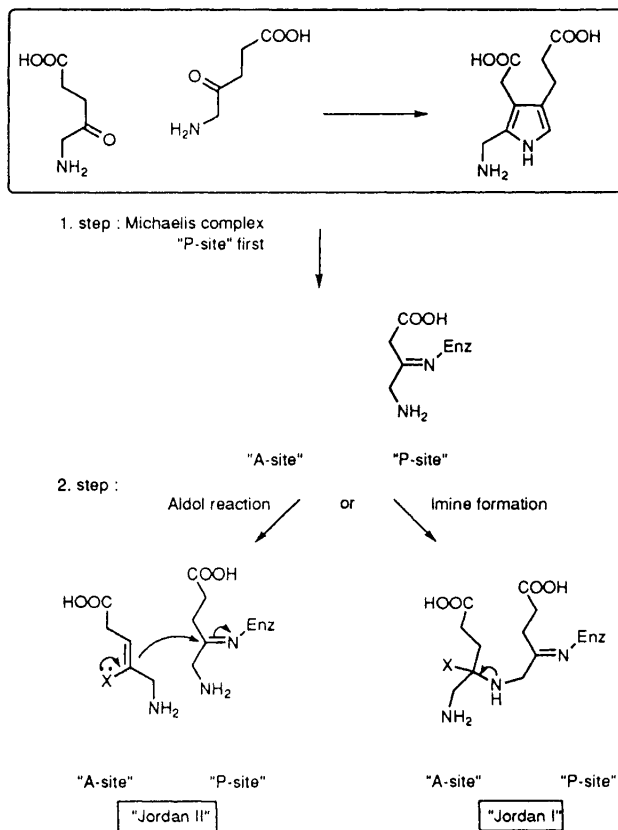
There are only very few restrictions which can be used *a priori* to reduce the number of possible mechanisms. The deprotonation on C2, aromatization, and product release have to be the last steps in the sequence. As mentioned before we assume that a substituted enzyme mechanism is working (see page 73) and implicitly that the sequence of binding (formation of the Michaelis complex) also determines which 5-aminolevulinic acid will form the Schiff base with the enzyme

(binary complex). Taking this limitation into account, at least four different mechanistic schemes have to be discussed. One arrives at these four mechanistic proposals assuming that the substrate can either bind to the “A-site” or to the “P-site” first and then form the binary complex, and secondly proposing that either the aldol reaction or the intermolecular imine formation between the two substrate molecules are the next step (see Figures 28 and 29):

1. “A-site” first; aldol reaction next (Shemin’s mechanism<sup>184</sup>).
2. “A-site” first; imine formation next.
3. “P-site” first; aldol reaction next (Jordan’s mechanism II<sup>162</sup>).
4. “P-site” first; imine formation next (Jordan’s mechanism I<sup>159,171,238–240</sup>).



**Figure 28.** Proposed mechanism for PBCS, assuming that the Michaelis complex forms at the “A-site” first immediately followed by the formation of the Schiff base.



**Figure 29.** Proposed mechanism for PBGS, assuming that the Michaelis complex forms at the "P-site" first immediately followed by the formation of the Schiff base.

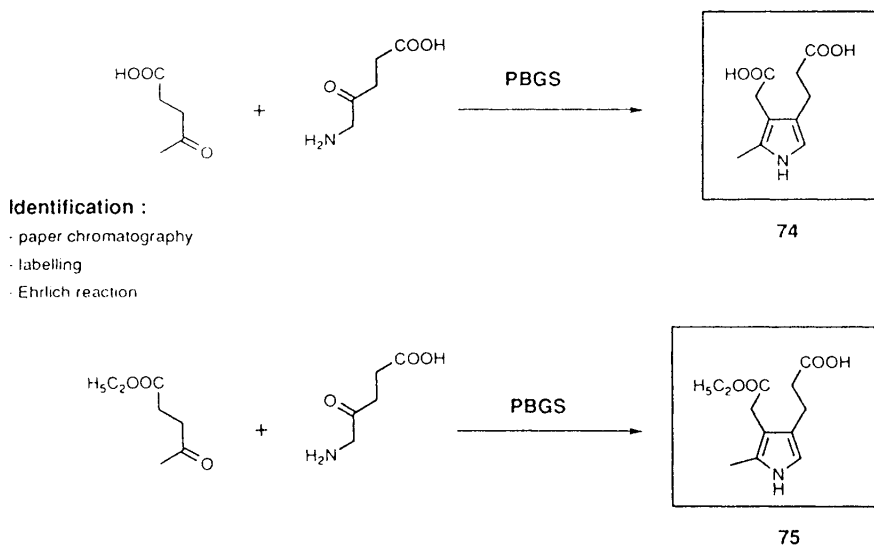
With the exception of the second hypothesis, the three other proposals have all been postulated in the literature. However, one further remark has to be made. Even if one would know the mechanistic outline of the PBG formation on the enzyme, meaning if one would know which of the four above mentioned alternatives corresponds to the catalytic process (answers to the questions 1 and 3 above), one would still not know the answers to questions 2 and 4. Conversely, knowing the structure of the Michaelis complex (answer to question 2 above), or knowing the catalytic groups at the active site or the locations of the metal ions (answer to question 4 above), does not allow one to deduce unequivocally the answer to question 3. Even knowing the sequence of recognition at the active site (answer to question 1) does not allow one to deduce the answer to question 3. So the answers to the four questions above are to a large extent independent of each other.



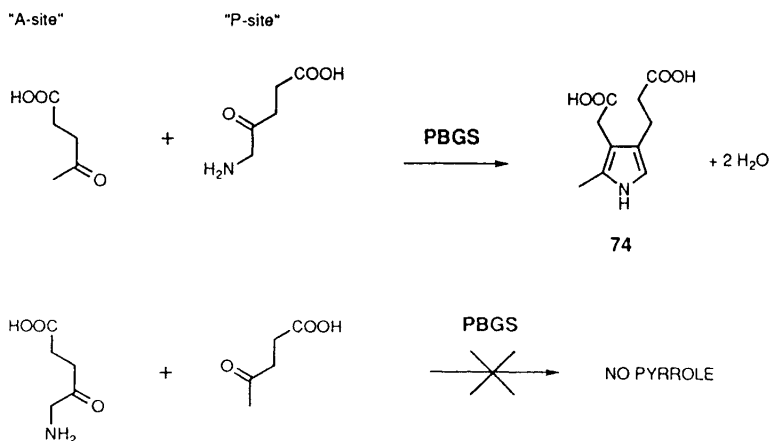
### Sequence of Binding the Two 5-Aminolevulinic Acids to the Enzyme (question 1)

The usual way to study the order of binding of two substrates to an enzyme is to determine the steady-state kinetics by varying the concentrations of the two substrates independently.<sup>241</sup> Since PBGS uses the same molecule twice, the kinetic approach to study the order of binding cannot be applied. Shemin deduced from his studies of the enzyme isolated from *R. spheroides* that the sequence of binding is “A-site” first and “P-site” second. He arrived at this conclusion in an indirect way: mixtures of levulinic acid or the ethyl ester of levulinic acid and 5-aminolevulinic acid formed “mixed” or heterologous pyrroles **74** and **75** under the influence of the bacterial synthase (Figure 30).<sup>148,184,190</sup> Shemin postulated the structure of the “mixed” pyrrole **74** based on the following observations:

1. Levulinic acid is a reasonably efficient competitive inhibitor for the synthase.
2. Incubation of radioactive 5-aminolevulinic acid and nonradioactive levulinic acid or of nonradioactive 5-aminolevulinic acid and radioactive levulinic acid with the enzyme led to a new product which was separated on paper chromatography.
3. The pyrroles were located with Ehrlich’s reagent and the radioactive products by radioautography.



**Figure 30.** Enzymatic formation of “mixed pyrroles” by PBGS from *R. spheroides*.



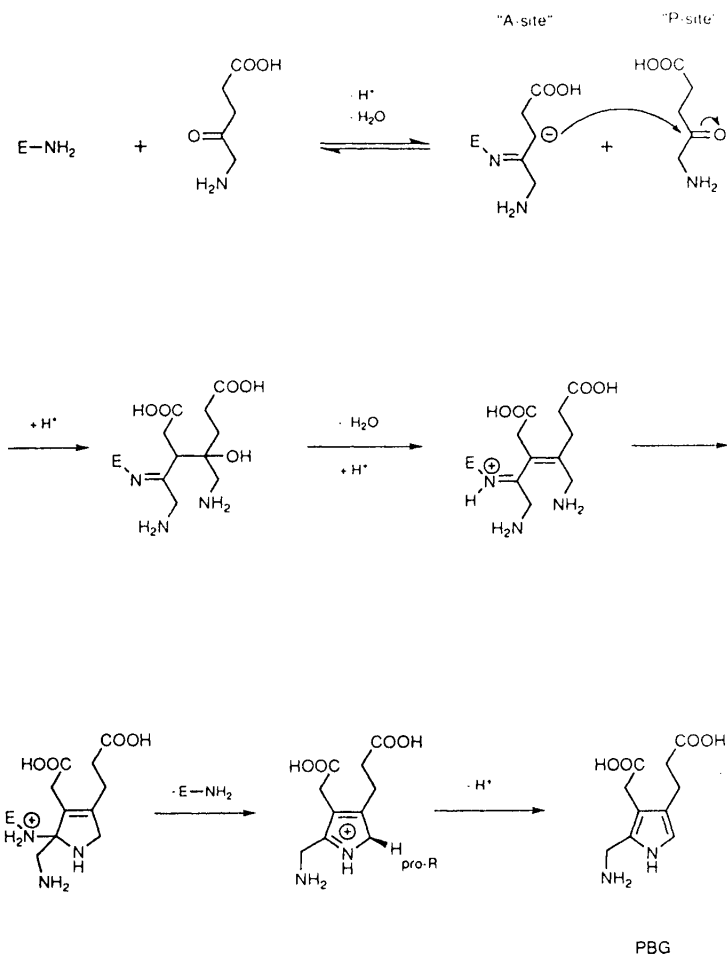
**Figure 31.** Deduction drawn from the formation of the "mixed pyrrole" according to Shemin.

4. In addition to the natural product, PBG, a new product was observed which was radioactive in both experiments and which reacted positive with Ehrlich's reagent.
5. Incubation of  $^{14}\text{C}$ -labeled 5-aminolevulinic acid with ethyl levulinate led to two new products. One product, formed in a minor amount, migrated in paper chromatography with the same  $R_f$  as the products formed in the first two experiments. The major product was less polar and had therefore a higher  $R_f$ -value. Both products were radioactive and reacted positive with Ehrlich's reagent.

Shemin interpreted his experimental results in the following way. In all experiments one or two pyrrole(s) other than PBG had been formed. In the case of the ester the major product is the monoester of the pyrrole formed in the first experiments and the minor product is the acid which has been formed via hydrolysis.

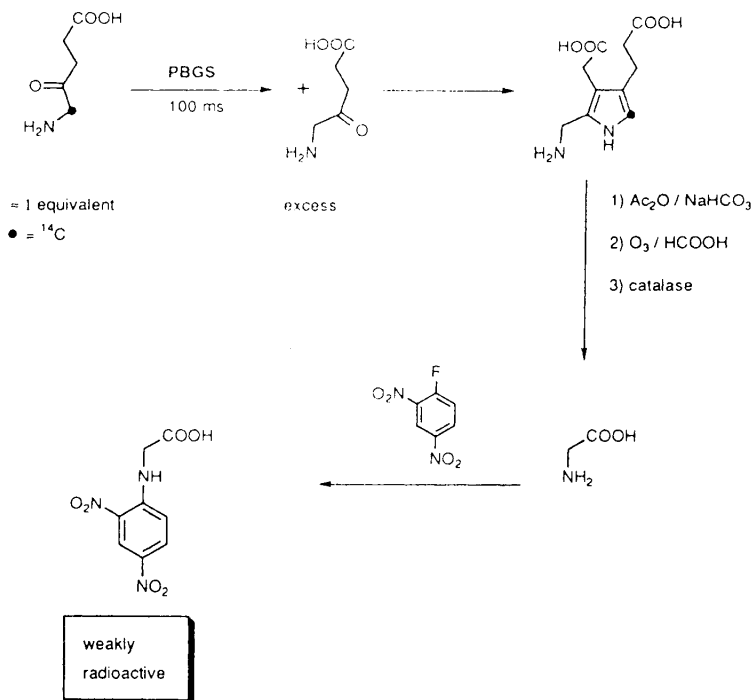
A pyrrole analogous to PBG can only be formed between levulinic acid and 5-aminolevulinic acid if the levulinic acid is using the "A-site." If the levulinic acid would be bound to the "P-site" the formation of a "mixed" pyrrole is impossible (Figure 31).<sup>190</sup>

As Shemin had shown that 5-aminolevulinic acid as well as levulinic acid formed an imine with the enzyme,<sup>184</sup> he proposed a mechanism for the enzyme catalyzed formation of porphobilinogen (Figure 32).<sup>148,184,190</sup> According to his mechanism the substrate on the "A-site" is forming the Schiff base as the first chemical step (formation of the binary complex). Implicitly he assumes that the substrate binding (formation of the Michaelis complex) to the "A-site" has to be first as well.



**Figure 32.** Mechanism for the biosynthesis of porphobilinogen postulated by Shemin.

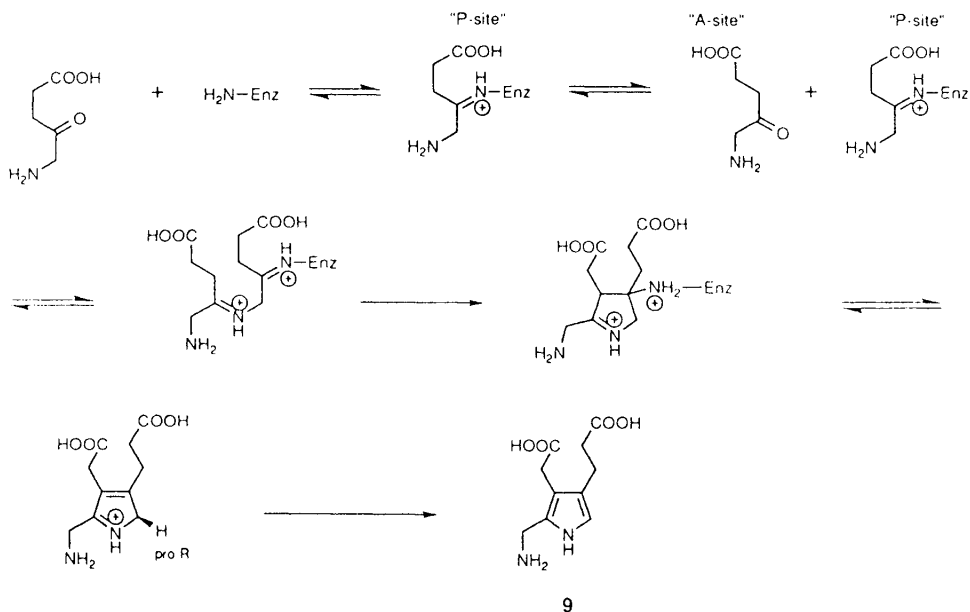
He used the following arguments to deduce his mechanism. The synthase reaction resembles that of one of the two classes of aldolases which use Schiff's bases as a central intermediate. In analogy to the mechanism postulated for this class of aldolases, Shemin postulated that the activation of the methylene group at C3 occurs through an imine–enamine tautomerization of the substrate bound to the "A-site." Since the imine formation between the two substrate molecules would destroy the covalent linkage to the enzyme, Shemin assumed that the aldol reaction was the



**Figure 33.** Pulse labeling of porphobilinogen using one equivalent of labeled 5-aminolevulinic acid according to Jordan.

next step, followed by the elimination of water. Replacing the enzyme substrate Schiff base by the Schiff base between the two substrate molecules follows, and finally the product is produced by deprotonation. The rationale behind this mechanistic scheme is twofold: first, this mechanism allows explanation of the formation of the “mixed” pyrrole, and second, the proposed sequence keeps the covalent bond between the substrate and the enzyme as long as possible up to the penultimate intermediate.

The first experiments designed to study the order of substrate binding were Jordan’s highly elegant single-turnover experiments.<sup>220,238,239</sup> Stoichiometric equivalents of labeled substrate and porphobilinogen synthase were rapidly mixed and after about 100 ms were added to a large excess of unlabeled substrate. Using [5- $^{14}\text{C}$ ]-5-aminolevulinic acid for the pulse, [2- $^{14}\text{C}$ ]-porphobilinogen could be isolated using 8 moles of labeled substrate per mol of octameric enzyme isolated from bovine liver or human erythrocytes (Figure 33).



**Figure 34.** Mechanism for the biosynthesis of porphobilinogen postulated by Jordan taking into account the results of the pulse labeling experiment.

The position of the radioactive label was determined by degradation. Treating PBG **9** with acetic anhydride to form the lactam, followed by ozonolysis. The aminomethyl side chain was degraded to glycine.<sup>238</sup> The glycine was derivatized with 2,4-dinitro-1-fluorobenzene. Subsequently, the radioactivity of this glycine derivative was determined. Using the human enzyme, 17% of the radioactivity was located at C11. In the experiment with the bovine enzyme only 14% of the radioactivity could be detected at C11.<sup>220</sup> In the control experiment 48% of the radioactivity was found at position C11. Using the enzyme isolated from *R. spheroides* the amount of radioactivity found at C11 was 40%. This value is considerably higher than the values observed for the two other enzymes. In spite of this high value of radioactivity at C11 or the low incorporation of the label at C2, Jordan interpreted his results as indicating that the substrate also binds first to the “P-site” for the enzyme from *R. spheroides*.

In order to prove that a Schiff base could be formed at all under the conditions of the single-turnover experiment, Jordan demonstrated that under the conditions of the rapid-mixing experiment the Schiff base could be trapped with  $\text{NaBH}_4$ .<sup>220</sup> Eight equivalents of  $[4\text{-}^{14}\text{C}]$  5-aminolevulinic acid were treated with one equivalent of the enzyme octamer for 100 ms and then quenched with  $\text{NaBH}_4$ ; roughly three equivalents of 5-aminolevulinic acid were covalently linked to the enzyme. These

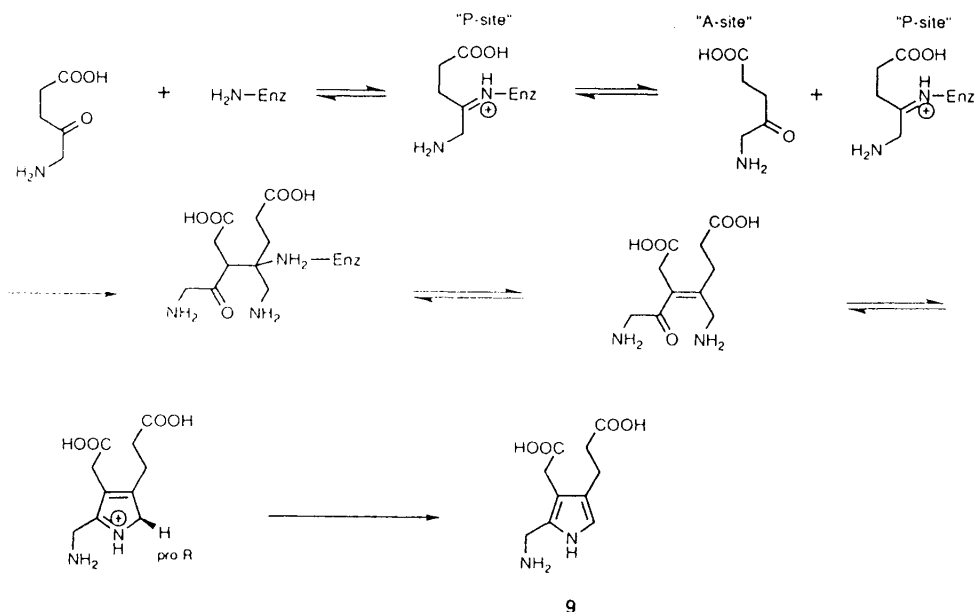
results were taken as support of the hypothesis that the enzyme substrate Schiff base is a catalytically viable intermediate.

The pulse labeling could also be done using [5- $^{13}\text{C}$ ] 5-aminolevulinic acid. The  $^{13}\text{C}$  NMR spectrum of the product allowed the position of the label to be identified without degradation.<sup>239</sup>

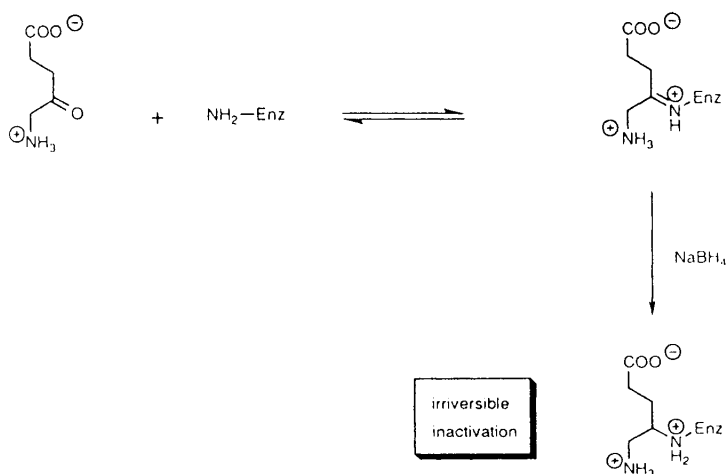
Starting from his observations, Jordan postulated an alternative mechanism for the formation of PBG<sup>220,238,239</sup> (Figure 34). In his first mechanism Jordan postulates that after the formation of the Schiff base between the enzyme and the first substrate molecule, the second substrate molecule forms a new Schiff base to the enzyme-bound 5-aminolevulininate. Only after this step follows the aldol reaction and the elimination which leads after deprotonation to the product.

In parallel, Jordan has discussed a second proposal<sup>159,162</sup> (see also ref. 220) (Figure 35).

After the formation of the substrate enzyme Schiff base, an aldol reaction between the second substrate and the iminium function follows. The elimination of the amino group of the active site lysine gives the  $\alpha,\beta$ -unsaturated ketone which has lost its covalent bond to the enzyme. The Schiff base formation between the amino group from the "P-site" substrate and the keto function of the "A-site" substrate produces the five-membered ring and deprotonation finally yields PBG.



**Figure 35.** Alternative mechanism for the biosynthesis of porphobilinogen postulated by Jordan taking into account the results of the pulse labeling experiment.



**Figure 36.** Inactivation of PBGS by treatment with NaBH<sub>4</sub>.

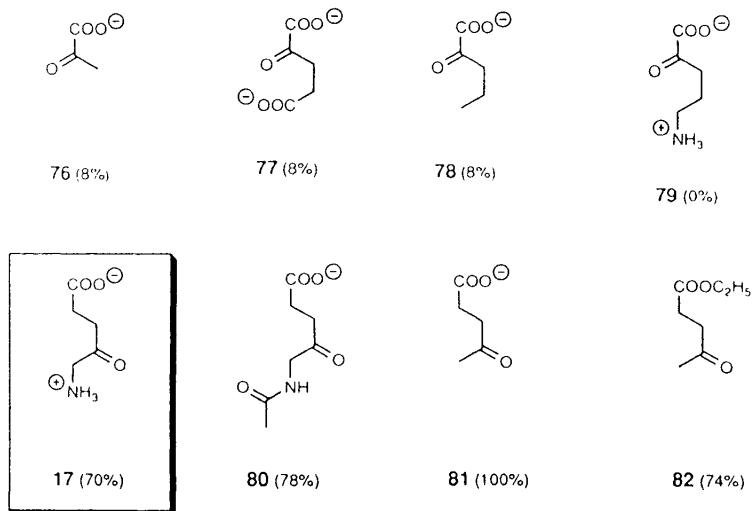
In his most recent review,<sup>162</sup> Jordan cites nonspecified stereoelectronic reasons as to why he prefers this second version (see also ref. 242).

### **Modification the Enzyme by Forming Covalent Bonds**

*Trapping of the Schiff Base by Reduction* (questions 2 and 4). Assuming that PBGS followed a similar reaction pathway as certain aldolases, Shemin reported in his first series of papers his experimental efforts to prove the presence of a Schiff base during the enzyme-catalyzed process. Treating the enzyme with the natural substrate, radioactively labeled [4-<sup>14</sup>C] 5-aminolevulinic acid in the presence of sodium borohydride, inactivated the enzyme and led to the incorporation of the radioactively labeled substrate into the protein (Figure 36).<sup>184,190</sup>

Under these conditions inactivation was between 60 and 93%. Studies using other keto acids indicated clearly that  $\alpha$ -keto acids do not form Schiff bases with the enzyme, whereas  $\gamma$ -keto acids are capable of forming Schiff bases as indicated by the high percentage of inactivation (Figure 37).<sup>184</sup>

Even the ethyl ester of levulinic acid, which was unable to act as an inhibitor, showed a high percentage of inactivation. It could be shown that lysine was the amino acid involved in the formation of the Schiff base between the natural substrate and the enzyme isolated from *R. spheroides*.<sup>243</sup> PBGS isolated from human erythrocytes was similarly incubated with NaBH<sub>4</sub> in the presence of 5-amino [4-<sup>14</sup>C] levulinic acid.<sup>244</sup> The modified active site amino acid residue was identified by comparison with authentic lysyl-aminolevulinic acid, prepared chemically. The CNBr-cleavage peptide, containing the <sup>14</sup>C-labeled modified lysine,



**Figure 37.** Inactivation of PBGS on reduction with  $\text{NaBH}_4$  in the presence of different ketoacids.

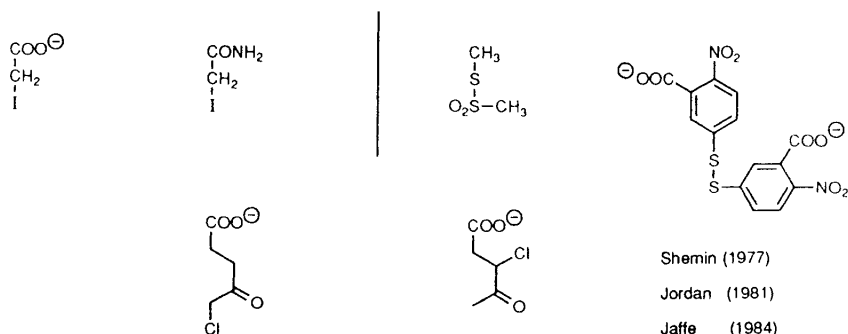
could be isolated and sequenced. On the basis of the sequence of the active site peptide<sup>196</sup> the position of the active site lysine in the protein could be identified.<sup>162,244</sup>

*Modification of the Thiol Groups.* To be fully active, all PGB synthases have to be incubated in the presence of reagents such as mercaptoethanol or dithiothreitol, indicating that free thiol groups are required.<sup>153</sup> The enzymes isolated from mammalian sources are especially sensitive to oxygen. In a careful study by Shemin et al., beef liver porphobilinogen synthase was treated with iodoacetate, iodoacetamide, and 5,5'-dithiobis(2-nitrobenzoic acid)<sup>245</sup> (Figure 38). The enzyme was inactivated with iodoacetate or with iodoacetamide. If PBGS was first treated with 5,5'-dithiobis(2-nitrobenzoic acid), then it was protected against inactivation by iodoacetate or iodoacetamide. The full catalytic power could be restored, treating the thio(2-nitrobenzoic acid)-modified enzyme with an excess of dithiothreitol.

It could be demonstrated that levulinic acid partially protects the enzyme from inactivation by iodoacetate, whereas the inactivation by iodoacetamide is amplified. In contrast to these observations PBG 9, the product of the enzymatic process, partially protects the enzyme against inactivation by both alkylating reagents. The incorporation of  $^{14}\text{C}$ -labeled alkylating reagents showed that the reaction of one fast reacting cysteinyl residue per monomer ( $M_r = 35,000$ ) is responsible for the loss of activity by both reagents. Analysis of the tryptic digest of the enzyme treated with iodo[ $^{14}\text{C}$ ]acetate or by iodo[ $^{14}\text{C}$ ]acetamide demonstrated that each reagent



**Reagents directed towards thiol groups  
on PBGS**



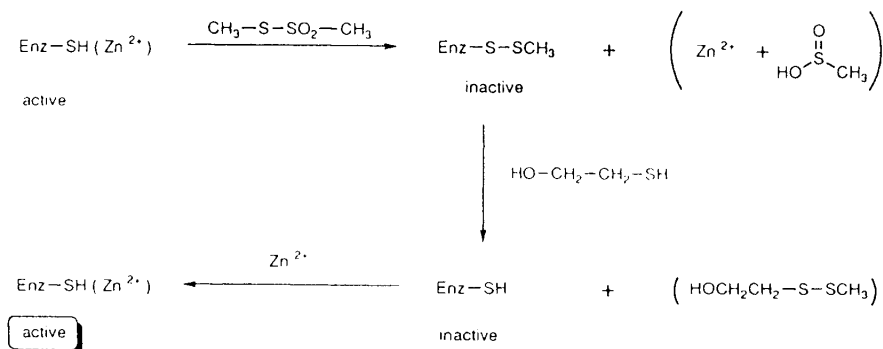
**Figure 38.** Reagents directed toward thiol groups used to inactivate PBGS.

gave rise to a different labeled tryptic peptide.<sup>245</sup> In a later paper, Shemin showed that Zn binding directly depends on the presence of free thiol groups.<sup>186</sup>

Using an enzyme from the same source, Jordan and Tsukamoto independently studied the inactivation by 5,5'-dithiobis(2-nitrobenzoic acid).<sup>176,246</sup> Four thiol groups per subunit were reacted with the reagent. Two thiol groups reacted very rapidly to form a disulfide as could be demonstrated titrating the enzyme with one equivalent of the reagent per subunit. Two molar equivalents of 2-nitrobenzoic acid 5-thiolate were liberated in this experiment. The two other thiol groups reacted considerably slower. Denaturing the enzyme with sodium dodecyl sulphate allows the reaction with three or four more thiol groups.<sup>176,246</sup>

Jaffe used methyl-methanethiosulfonate (MMTS) to modify PBGS from bovine liver in a highly controlled manner (Figure 39).<sup>187</sup> Three thiomethyl groups were introduced/mol of subunit of the octameric enzyme. It could also be shown that the intrinsic eight zinc per octamer are lost during the reaction of PBGS with MMTS.<sup>187</sup> The methylmercapto group blocks the thiol groups, but it is unlikely to interfere with the protein structure as shown by the following experiments. The modified enzyme is still able to bind four substrate molecules/octamer with a dissociation constant of 150  $\mu\text{M}$ .<sup>232</sup> The blocking of the thiol groups is readily reversible. Using mercaptoethanol allows recovery of the thiol groups and the enzyme obtains full activity enzyme if  $\text{Zn}^{2+}$  is added.<sup>187</sup>

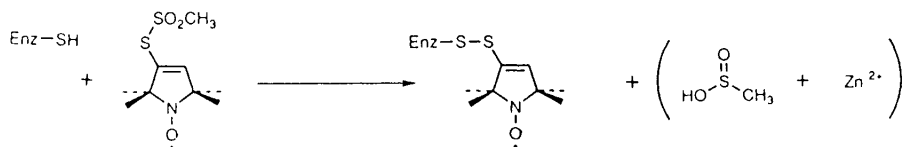
In order to study the spatial proximity and sequence localization of the thiol groups of bovine PBGS (1-oxy-2,2,5,5-tetramethyl- $\Delta^3$ -pyroline-3-methyl)methanethiosulfonate was used in order to introduce a nitroxide spin label into the enzyme (Figure 40).<sup>232</sup>



**Figure 39.** Reversible inhibition of PBGS with MMTS according to Jaffe.

EPR spectroscopy allowed observation of the modified enzyme and detection of the peptides obtained after digesting the modified enzyme with an Asp-N protease. From the gene derived protein sequences of other mammalian PBGS (human, mouse, and rat<sup>207,211,221</sup>) it is known that the 35-kDa subunit of the octameric enzyme contains eight uniformly conserved cysteines/mol of subunit. As with methyl methaniosulfonate,<sup>187</sup> only three of the eight cysteines could be modified.<sup>232</sup> The observation of a forbidden EPR transition indicates that at least two of the spin labels are within 7.6 Å of each other at most. The spin label allowed easy identification of the peptides containing modified cysteine residues, which were produced by an Asp-N protease digest of labeled PBGS.<sup>232</sup> Sequencing of these peptides allowed identification of two of the three modified cysteines as Cys-119 and Cys-223. The modified enzyme is able to bind four aminolevulinic acid molecules/octamer with a dissociation constant of 90 μM.<sup>232</sup>

The protein sequence of PBGS isolated from *E. coli* is known<sup>193,194</sup> where the latter sequence has been recognized to be more correct.<sup>171</sup> The gene-derived protein sequence of Li et al.<sup>194</sup> predicts the presence of six cysteine residues/mol of subunit. Using PBGS isolated from *E. coli* allowed Jordan to interpret the results of the modification of the enzyme in view of the known sequence. Titration of denatured *E. coli* PBGS with 5,5'-dithiobis-(2'-nitrobenzoic acid) under reducing conditions indicated the presence of six cysteine residues. Similar results have been obtained



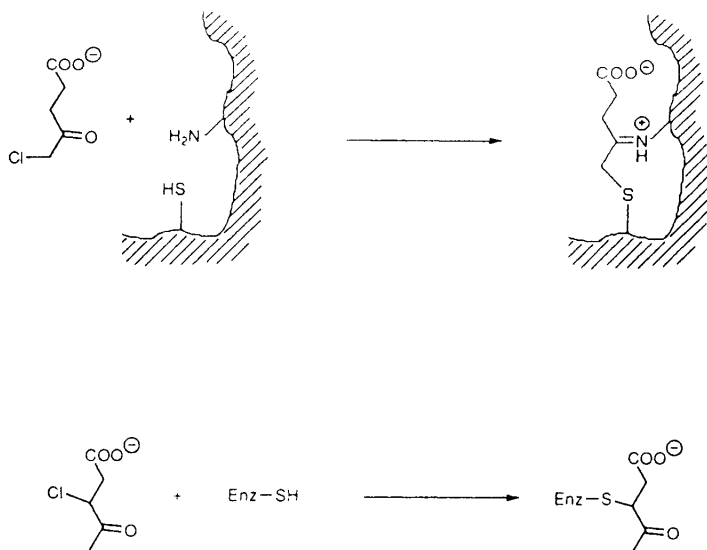
**Figure 40.** Inactivation of PBGS using a spin label derivative of MMTS according to Jaffe.

with PBGS from other sources.<sup>167,168,171,172,186</sup> Only four residues were accessible using the native holo enzyme.

Titration of apo-PBGS (enzyme where the  $Zn^{2+}$  has been removed) with two equivalents of 5,5'-dithiobis-(2'-nitrobenzoic acid) indicated the formation of two disulfide bridges. No evidence of dimer formation between the subunits could be found, indicating that the disulfide bridges are intrasubunit. Most of the activity was lost after the formation of the first disulfide bridge. The cysteines involved in the formation of the disulfide bridges were identified using a combination of chemospecific cleavage with cyanothiolnitrobenzoate, and determination of the numbers and the molecular weights of the produced peptides with SDS/PAGE. In a second study, either the cysteine not involved in the formation of the disulfide bridge or only the cysteines involved in the formation of the first disulfide bridge were labeled with 2-iodo[ $^{14}C$ ]acetic acid.<sup>171</sup> Treatment of the radioactively labeled enzymes with proteases and isolation of the peptides allowed determination of the sequence of the peptides and comparison with the gene derived sequence.<sup>171,194</sup> It could be shown that cysteines 105 and 242 are not involved in disulfide bond formation, whereas the cysteines 119, 121, 129, and 133 form, in a random way, the first disulfide bond.<sup>171</sup> This part of the gene-derived protein sequence had been presumed to be the metal-binding domain by comparison with the zinc-finger motif<sup>193,194,221</sup> (for one consensus sequence of some  $Zn^{2+}$ -binding proteins see refs. 234,247). The first sequence of PBGS from plants (pea *Pisum sativum* L.), an enzyme which appears to use  $Mg^{2+}$  instead of  $Zn^{2+}$ , consistently lacks the cysteines of the putative  $Zn^{2+}$ -binding domain<sup>204</sup> (compare with the recent three metal ion hypothesis proposed by Jaffe<sup>233</sup>).

*Modification Using Chlorolevulinic Acids* (question 4). The use of  $\alpha$ -halo-ketones to alkylate active-site nucleophiles is well documented.<sup>39,241</sup> Jordan used 5- and 3-chlorolevulinic acids to inactivate bovine PBGS (Figure 41).<sup>248</sup> Incorporation of four equivalents of either of the two chloroketones/octamer almost completely inactivated the enzyme.

3-Chlorolevulinic acid is a considerably less effective inactivating agent than 5-chlorolevulinic acid. The 5-chloro acid does not only alkylate the enzyme but is also a highly efficient competitive inhibitor with  $K_1 = 11 \mu M$ , whereas 3-chlorolevulinic acid did not inhibit PBGS even at high concentrations. The inhibition took place at much lower concentrations (11  $\mu M$ ) than those which were necessary for the irreversible inhibition of the enzyme (5 mM). PBGS could be protected against alkylation with 3-chlorolevulinic using levulinic acid, a competitive inhibitor, PBG 9, the product, or 5-aminolevulinic acid (17), the natural substrate. Only the natural substrate, 5-aminolevulinic acid (17), was able to protect the enzyme against inactivation using 5-chlorolevulinic acid. Reduction of the mixture between the enzyme and 5-chloro[3,5- $^3H_4$ ]levulinic acid with sodium borohydride resulted in loss of activity. Four equivalents/octamer of the labeled compound had been incorporated. Using the corresponding 3-chloro-derivative, inhibition and incorpo-



**Figure 41.** Covalent binding of 5-chlorolevulinic acid and of 3-chlorolevulinic acid as proposed by Jaffe.

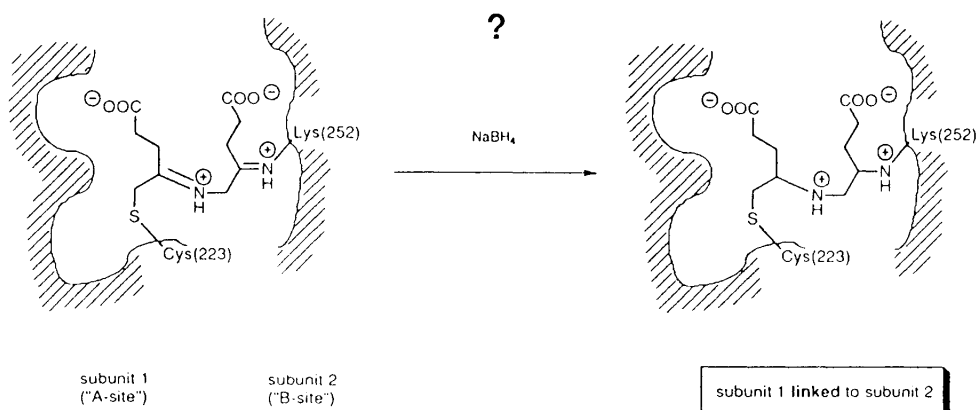
ration of the label into the enzyme were negligible. Electrophoretic analysis of the tryptic peptides obtained from the enzyme modified with both chloroderivatives separately gave two different radioactive peptides. The peptide, obtained using 3-chloro[3,5-<sup>3</sup>H<sub>4</sub>]levulinic acid, had similar properties to the peptide isolated after modification with iodo[2-<sup>14</sup>C]acetate. Based on these results, Jordan postulates that 3-chlorolevulinic acid behaves similar to iodoacetate, alkylating the most reactive (most accessible) thiol group in the primary sequence.<sup>248</sup> The 5-chlorolevulinic can be compared in its behavior to iodoacetamide, a competitive inhibitor of the enzyme. Jordan supposes that 5-chlorolevulinic modifies a thiol group present at the active site binding the future propionic acid side chain of PBG (“P-site”). The function of this active site thiol is not clear.

Almost ten years later Jaffe and collaborators characterized PBGS modified by 5-chlorolevulinic acid.<sup>231</sup> Their interpretation of the experimental results is different from the one given by Jordan. Jaffe used highly elegant experiments to get a deeper insight into the deactivation of PBGS by 5-chlorolevulinic acid. After modification the PBGS octamer was still able to bind three to four molecules of 5-aminolevulinic acid (17), with a dissociation constant  $K_d = 60 \mu\text{M}$ . This value lies between the dissociation constants determined for the natural substrate, 5-aminolevulinic acid ( $K_1 = 3.8 \mu\text{M}$  constant for the “P-site,”  $K_2 = 242 \mu\text{M}$ , constant for the “A-site”). The comparison of the dissociation constants was taken as a hint that 5-chlorolevulinic acid binds to and irreversibly inactivates at the “A-site” and leads to a reduced

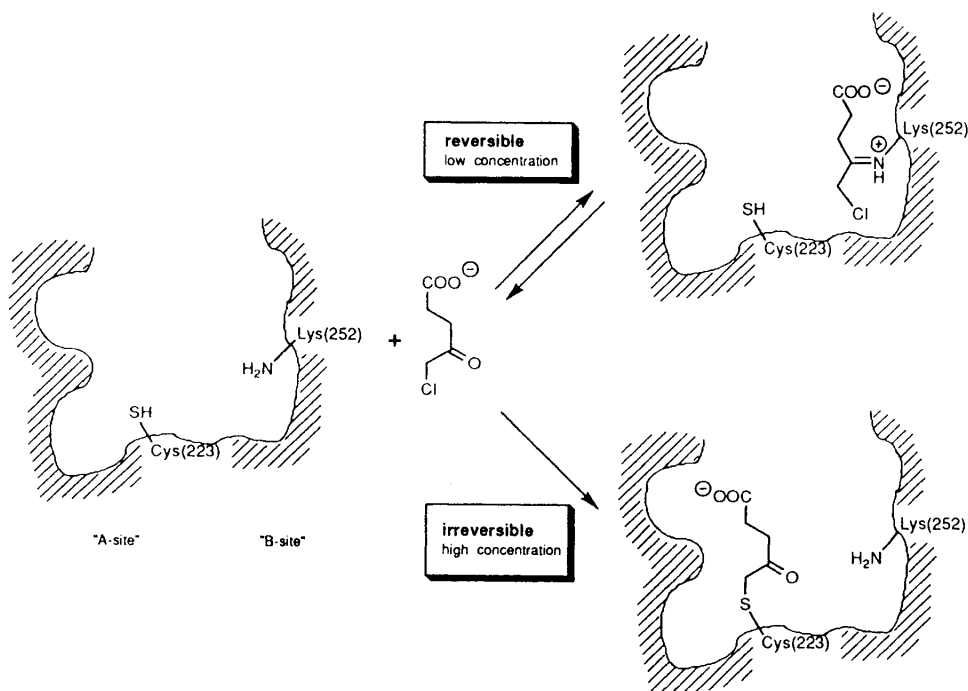
binding of the natural substrate at the “P-site.” In contrast to the substrate, the 5-chloro-levulinic acid modified enzyme could no longer bind the product PBG **9**. This observation indicates that 5-chloro-levulinic acid indeed binds to the active site, thereby blocking product recognition. Treating the modified enzyme with 5-aminolevulinic acid in the presence of  $\text{NaBH}_4$  allowed covalent linkage of  $3.4 \pm 0.7$  molecules of substrate/octamer. As the imine formation between the enzyme and the substrate has been shown to occur at the “P-site,”<sup>220,238,239</sup> this observation indirectly hints that the reaction of 5-chloro-levulinic acid is at the “A-site.”

The binding of  $\text{Zn}^{2+}$  to PBGS and to the 5-chloro-levulinic acid modified enzymes was studied. For the unmodified enzyme the  $\text{Zn}^{2+}$  binding can be described by two dissociation constants,  $K_1 \leq 0.1 \mu\text{M}$  and  $K_2 = 5.1 \mu\text{M}$ , where each binding site is capable of binding 4  $\text{Zn}^{2+}$ /octamer. The binding of the tightly bound  $\text{Zn}^{2+}$  is sufficient to largely restore the catalytic activity of the enzyme.<sup>168,187</sup> The modified enzyme can only bind 4  $\text{Zn}^{2+}$  with a dissociation constant of  $K = 5.0 \mu\text{M}$ . The modification of PBGS with 5-chloro-levulinic acid blocked the complexation of the octameric protein to the tightly bound (catalytic)  $\text{Zn}^{2+}$  and destroyed the catalytic activity. Methyl methanethiosulfonate, a reagent which interferes with binding at the “A-site” of the enzyme protected the enzyme against the irreversible inactivation by 5-chloro-levulinic acid. This result suggests that methyl methanethiosulfonate and 5-chloro-levulinic acid compete for the same cysteine residue which is at the “A-site”. The modified PBGS was subjected to digestion by Asp-N protease and the modified peptides were sequenced. It could be shown that 5-chloro-levulinic acid binds to cysteine 223 on one-half of the subunits.

Finally NMR studies using  $[4\text{-}^{13}\text{C}]\text{-ALA}$  and  $[^{15}\text{N}]\text{-ALA}$  showed similar spectra to that observed for the enzyme modified with methyl methanethiosulfonate (see section on NMR studies below). The NMR studies did not allow detection of a



**Figure 42.** Rationale for the unsuccessful experiment where PBGS was incubated with 5-chloro-levulinic acid, 5-aminolevulinic acid and  $\text{NaBH}_4$  in the hope of trapping an intermediate linking two subunits (according to Jaffe).



**Figure 43.** Interpretation of the different behavior as inhibitor of PBGS of 5-chlorolevulinic acid as a function of the used concentration as proposed by Jaffe. Note that at high concentration (20 mM) and very long reaction times (3–5 hours) 8 moles/octamer of 5-chlorolevulinic acid are incorporated.

covalent bond between the irreversibly bound inhibitor and added natural substrate (Figure 42). In the hope to detect cross-linking between two subunits, 5-aminolevulinic acid was added to PBGS modified with 5-chlorolevulinate. This complex was treated with NaBH<sub>4</sub> in the hope of detecting a di-Schiff base linking the two subunits. The electrophoretic analysis did not provide any sign of cross-linking.

All of the above observations were taken to support the idea that 5-chlorolevulinate at low concentrations (11 μM) is a competitive inhibitor of PBGS binding at the "P-site", whereas at high concentration (20 mM) 5-chlorolevulinate is irreversibly binding to the "A-site" and modifying cysteine 223 (Figure 43).<sup>231</sup>

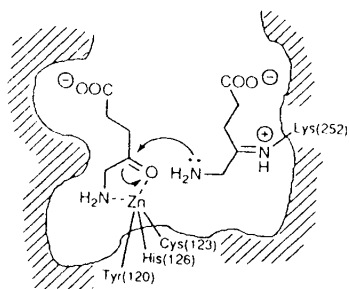
#### **Requirement of Zn<sup>2+</sup> for Enzyme Activity** (question 4)

Treating the enzyme from bovine liver with chelating agents like EDTA or 1,10-phenanthroline markedly reduced the enzymatic activity.<sup>186</sup> The enzyme

activity could be restored if at least four equivalents of  $Zn^{2+}$  per mole of enzyme ( $M_r = 280,000$ ) were added. A maximum of eight  $Zn^{2+}$  ions/octamer are bound with high affinity to the enzyme.<sup>176,186,246,249,250</sup> Besides  $Zn^{2+}$ ,  $Cd^{2+}$  was the only metal studied which was able to give back activity to the apo enzyme.<sup>186</sup> Modifying PBGS with methanethiosulfonate led to an immediate and complete loss of  $Zn^{2+}$  and of the enzymatic activity.<sup>187</sup> The catalytic activity could be fully reconstituted using 2-mercaptoethanol to remove the methyl disulfide group and incorporate at least four  $Zn^{2+}$ /octameric apo enzymes. Studying PBGS using EXAFS (extended X-ray absorption fine structure spectroscopy), Beyersmann and co-workers originally postulated that the zinc is coordinated to three sulfur atoms.<sup>251</sup> A second investigation by the same authors revealed that there exist two different zinc-binding sites per octamer,<sup>236</sup> in agreement with  $Zn^{2+}$ -binding studies.<sup>231</sup> The binding of four  $Zn^{2+}$  to the apo enzyme is crucial to restore the catalytic activity. Ligands for tighter bound zinc, denoted  $Zn_A$ , were proposed to be two or three histidines; one or two oxygens, probably stemming from tyrosine or aspartate or eventually from water, and one cysteine sulfur in a pentacoordinate geometry. The coordination sphere of the second  $Zn^{2+}$ , denoted  $Zn_B$ , is suggested to consist of four cysteine sulfurs. This result can be interpreted as a hint to two respective functions of  $Zn_A$  and  $Zn_B$ : a catalytic and a structural.

The studies of 5-chlorolevulinate modified bovine liver PBGS allowed the characterization of a protein which was capable of only binding the weakly bound  $Zn^{2+}$ , assumed to be the structural Zn (see above and ref. 231). From the sequence analysis of the peptides containing 5-chlorolevulinate modified cysteine, and especially from the EXAFS data,<sup>236</sup> the ligands for the catalytic Zn were proposed to be cysteine 223, histidine 129, and tyrosine 126. The role of the catalytic Zn is assumed to be to bind ALA to the "A-site" to activate the carbonyl group of the substrate for formation of the second Schiff base (Figure 44).

The synthase isolated from *Escherichia coli* which are overexpressing PBGS, allowed the study of the metal dependence of the catalytic activity.<sup>170,171,252</sup> *E. coli* PBGS contains 2  $Zn^{2+}$ /mol of subunit. To obtain the original activity 2  $Zn^{2+}$ /mol of



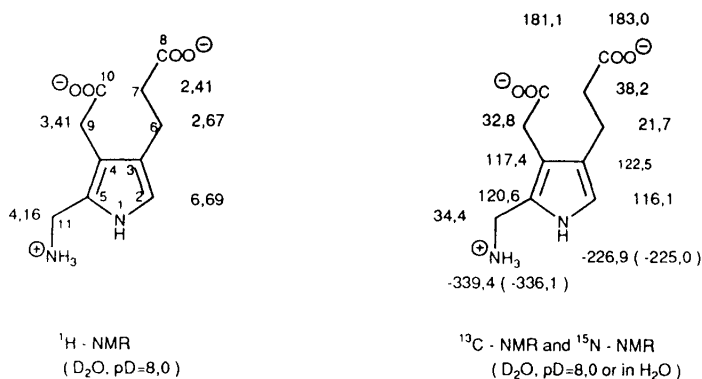
**Figure 44.** Putative coordination and function of the catalytic Zn (II) at the "A-site" of PBGS postulated by Jaffe.

subunit had to be incorporated.<sup>171</sup> The second mole of  $Zn^{2+}$  could be substituted by  $Mg^{2+}$  giving an enzyme which had a 4 times higher activity than the Zn-only species. In contrast to  $Zn^{2+}$ ,  $Mg^{2+}$  alone could not restore the activity of PBGS. Recent results show that  $Mg(II)$  can be replaced by  $Mn(II)$  giving a comparable increase in catalytic activity.<sup>252</sup> The EPR spectra of the enzyme modified with  $Mn(II)$  showed a single type of binding site, which was not affected by substrate binding.<sup>252</sup> 2  $Co^{2+}$  ions/mol of subunit could be incorporated into the apo enzyme. The incorporation of  $Co^{2+}$  into the enzyme could be detected by the UV/Vis absorption and with atomic absorption. The  $Co^{2+}$ -containing enzyme was completely inactive. One equivalent of  $Zn^{2+}$  was released when one disulfide bridge was formed by titration with 5,5'-dithiobis-(2'-nitrobenzoic acid). Forming a second disulfide bridge did not significantly change the zinc content (almost one equivalent of  $Zn^{2+}$ /mol of subunit).<sup>171</sup>

### NMR-Studies (question 1)

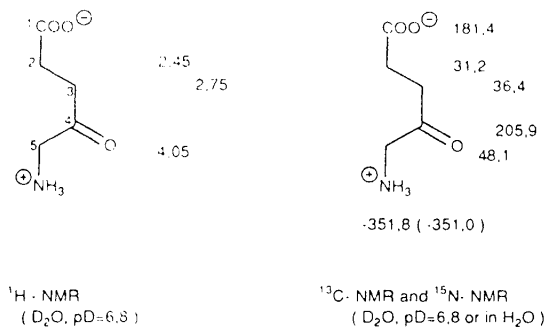
Despite the fact that the molecular weight of PBGS ( $M_w = 280,000$ ) seemed to be prohibitively high for NMR measurements, the group of Jaffe reported a series of elegant  $^{13}C$  and  $^{15}N$  NMR experiments where they observed the enzyme substrate and enzyme product complexes.<sup>170,231,253-255</sup> In order to compare and interpret the data obtained from the substrate enzyme and the product enzyme complexes, NMR data of 5-aminolevulinic acid (**17**) and of PBG **9** were studied under different pH and solvent conditions<sup>256-258</sup> (Figures 45 and 46).

The assignment of the signals was made using specifically labeled molecules, and, in the case of the  $^{13}C$  NMR spectra of porphobilinogen, analyzing the  $^1H$  coupled  $^{13}C$  NMR spectrum.<sup>256</sup> This latter method allowed the workers to differentiate between C8 and C10. The signals due to C3 and C5 were discriminated by



**Figure 45.**  $^1H$ -,  $^{13}C$ -,  $^{15}N$ -NMR of porphobilinogen in  $D_2O$  or in  $H_2O$ .

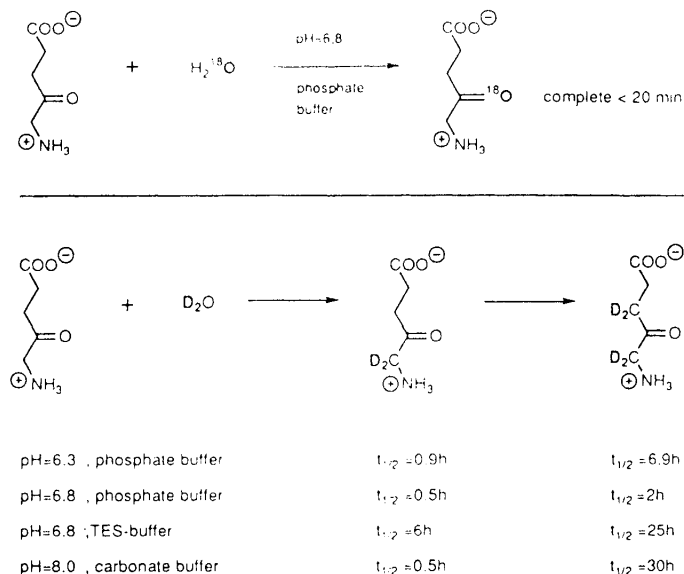




**Figure 46.**  $^1\text{H}$ -,  $^{13}\text{C}$ -,  $^{15}\text{N}$ -NMR of 5-aminolevulinic acid in  $\text{D}_2\text{O}$  or in  $\text{H}_2\text{O}$ .

selective  $^1\text{H}$  decoupling at  $\delta = 4.16$  ( $\text{H}_2\text{C}11$ ) and at  $\delta = 2.67$  ( $\text{H}_2\text{C}6$ ). The  $^{13}\text{C}$  NMR signal for C6 of PBG **9** appears at high field ( $\delta = 21.7$ ) compared to the signal for C7 ( $\delta = 38.2$ ), whereas the  $^1\text{H}$  NMR signal for  $\text{H}_2\text{C}6$  has to be attributed to the low field signal ( $\delta = 2.67$ ) compared to the signal for  $\text{H}_2\text{C}6$  ( $\delta = 2.41$ ). Comparison with model compounds where the signals have been independently attributed show a similar behavior.<sup>259,260</sup>

The exchange of the carbonyl oxygen at C4 and the deuterium exchange at C3 and C5 were studied (Figure 47).<sup>257,261</sup> The exchange of the carbonyl oxygen at C4



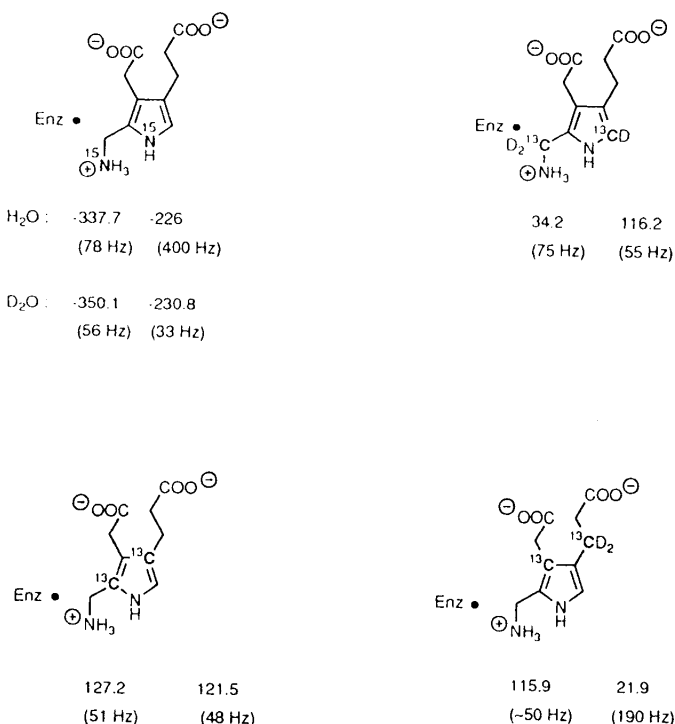
**Figure 47.** Rate of exchange for  $\text{H}_2\text{C}(5)$  and  $\text{H}_2\text{C}(3)$  as a function of the pH and the buffer.

using  $\text{H}_2^{18}\text{O}$  was complete within 20 minutes at  $\text{pH} = 6.8$ . The incorporation of  $^{18}\text{O}$  into the carbonyl group at C4 could be observed in the  $^{13}\text{C}$  NMR with the help of the isotope induced shift.

The deuterium exchange is faster at C5 compared to the exchange at C3 by a factor ranging from 4 to 60. The exchange rate depends on the pH but the influence of the buffer is even more important.

Incubation of fully active PBGS with a series of specifically labeled 5-aminolevulinate allowed observation of the  $^{13}\text{C}$  NMR and the  $^{15}\text{N}$  NMR spectra of the enzyme product complex. The following labeled substrates were used:  $^{15}\text{N}$ -5-aminolevulinate,<sup>253,255</sup>  $[5,5\text{-}^2\text{H},5\text{-}^{13}\text{C}]$ -5-aminolevulinate,<sup>254</sup> and  $[3,3\text{-}^2\text{H},3\text{-}^{13}\text{C}]$ -5-aminolevulinate<sup>255</sup> (Figure 48). In all cases the NMR spectra were obtained at 37 °C in order to obtain reasonably small line widths.

Deuteration of the  $^{13}\text{C}$  labeled methylene carbons led to a considerably smaller dipolar interaction and therefore to a reduced line width. In macromolecular complexes the rapid relaxation of deuterium effectively decouples  $^{13}\text{C}$  from  $^2\text{H}$ .<sup>254,255,262,263</sup> Only the concomitant deuteration of the  $^{13}\text{C}$ -labeled carbon atoms

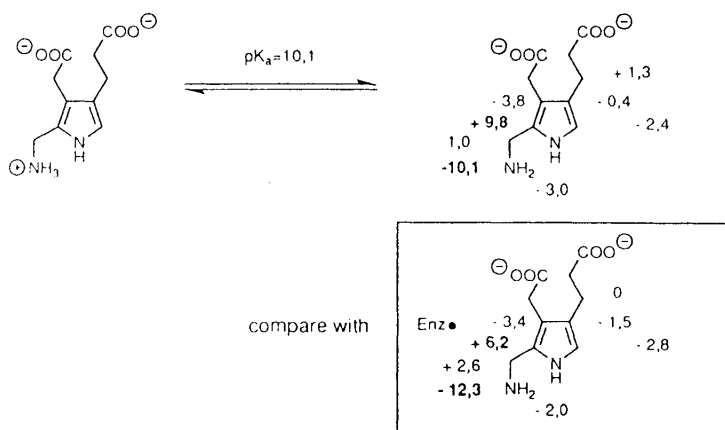


**Figure 48.** Chemical shift and line width of the complex between PBGS and porphobilinogen.

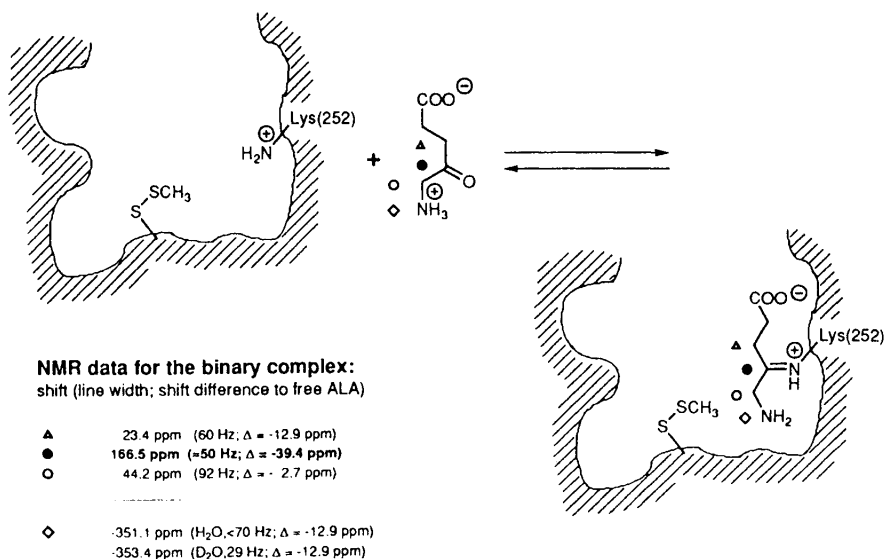
allows observation of the signals of the labeled product, despite the size of the enzyme product complex. The line width of the  $^{13}\text{C}$ -signals of the bound PBG **9** is roughly 50 Hz. This allows calculation of an estimated rotational correlation time of 245 ns,<sup>254</sup> which is consistent with rotational correlation time calculated for a 280-kDa protein using Stokes' law. The observed line width is therefore in accordance with the line width predicted for a totally immobilized PBG. If an undeuterated  $^{13}\text{C}$ -labeled substrate was used the expected line width would be in the range of several hundred Hertz and therefore unobservable! The narrow line width (78 Hz) of the amino group of porphobilinogen measured in  $\text{H}_2\text{O}$  suggests a shorter correlation time and thereby a higher mobility of this amino group compared to the rest of the molecule, which seems to be immobilized on the enzyme surface.<sup>255</sup>

Careful comparison of all chemical shifts of the enzyme-bound PBG with free PBG in water at pH = 7.0 (amino group in protonated form) and with PBG in water at pH = 12.0 (free amino group) led to the conclusion that the product is bound in the deprotonated form to the active site of the enzyme (Figure 49).<sup>253,254,256</sup> It was suggested that the product amino group was a ligand to the active site  $\text{Zn}^{2+}$ , called  $\text{Zn}_A$ . An analogous experiment using  $[4\text{-}^{13}\text{C}]\text{-5-aminolevulinate}$  and PBGS isolated from *E. coli* led to an NMR spectrum which was virtually identical to those observed with bovine PBGS.<sup>170</sup> The conclusion was that the environment of the product bound to the enzyme is very similar.

The same series of labeled compounds were incubated with PBGS modified by treatment with methyl methanethiosulfonate. The reversible modification of three cysteine thiol groups of the enzyme with this reagent leads to immediate loss of all  $\text{Zn}^{2+}$  and thereby also to complete loss of the catalytic activity.<sup>187,237</sup> The modified enzyme is still capable to bind ALA and to form a Schiff base with four equivalents



**Figure 49.** Comparison of the shift differences between porphobilinogen at neutral pH, under basic conditions, and in the enzyme product complex according to Jaffe.



**Figure 50.** Putative structure for the Schiff base covalently bound intermediate of the MMTS form of PBGS according to Jaffe and interpretation of the observed NMR data for the binary complex between PBGS and 5-aminolevulinic acid. Note the MMTS form of the enzyme  $-SCH_3$  at three cysteines/subunit!

of substrate/octamer. The predominant species observed in the NMR spectrum under those conditions is a Schiff base adduct (Figure 50).<sup>253–255</sup>

From the observed chemical shift differences compared with the free 5-aminolevulinic acid, it is postulated that the observed intermediate is a protonated imine in its *E*-form.<sup>254,255</sup> The major arguments for the attribution of this structure to the observed enzyme-bound intermediate are the chemical shifts of C3 and C5 indicating  $sp^3$  carbons and the shift of C4 ( $\delta = 166.5$  ppm) (these observations are a strong indication for the imine rather than an enamine form) and the bigger shift difference of C3 ( $\Delta = -12.9$  ppm) compared to C5 ( $\Delta = -1.2$  ppm) which indicates the configuration at the nitrogen atom. To assign the site of protonation (imine or iminium vs. amine or ammonium) the hydrazone and the oxime of 5-aminolevulinic acid were studied by  $^{13}C$  and  $^{15}N$  NMR.<sup>255</sup> Using these data a preliminary attribution of the protonation state was made.

Treating the MMTS modified PBGS Schiff base complex with mercaptoethanol,  $Cd^{2+}$  or  $Zn^{2+}$  and more substrate allowed the restoration of the catalytic activity and observation of the formation of PBG bound to the enzyme.<sup>253,254</sup>

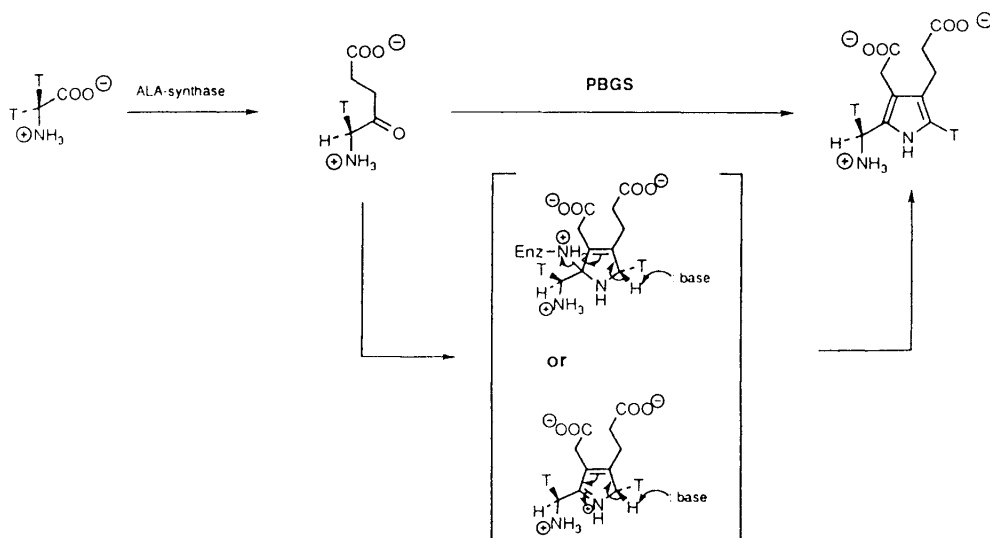
The enzyme modified with 5-chlorolevulinic acid was incubated with  $[4-^{13}C]$ -5-aminolevulinic acid and with  $[^{15}N]$ -5-aminolevulinic acid. The NMR spectra of the enzyme-bound intermediate showed a signal at  $\delta = 165.4$  ppm (line width 100 Hz) for C4 and at  $\delta = -353.0$  ppm (line width 21 Hz).<sup>231</sup> These data are almost identical

to those obtained with the methyl methanethiosulfonate-modified enzyme. The authors conclude that in both cases a Schiff base with the lysine of the active site is formed. Modification of the enzyme with 5-chlorolevulinate might have allowed the formation of a second Schiff base between the amino group of the bound substrate at the "P-site" and the keto function of 5-chlorolevulinate at the "A-site." The  $^{15}\text{N}$ -NMR clearly excludes this possibility.<sup>231</sup>

Finally it is worthwhile to note that experiments to observe  $^{113}\text{Cd}$  NMR spectra of an enzyme, where Cd should have replaced Zn, were unsuccessful. One report in the literature of a  $^{113}\text{Cd}$  NMR spectrum of the enzyme was later reported to be an artifact.<sup>251</sup>

### ***Incorporation of Enantiospecifically Labeled 5-Amino-5-Monodeutero-Levulinic Acid into Porphobilinogen***

Incubation of [ $5\text{RS-}^3\text{H}_2$ ]-5-aminolevulinic acid with PBGS leads to the stereospecific loss of half of the label at C2 of PBG.<sup>264</sup> These results suggest that the loss of the proton at C2 occurs enzymatically. Using tritium labeled glycine and the two enzymes, 5-aminolevulinate synthase and porphobilinogen synthase, allowed Jordan to determine the stereochemistry of the final elimination which leads to the pyrrole. Under these conditions [ $5\text{S-}^3\text{H}$ ]-5-aminolevulinic acid is formed and the label is incorporated intact into C11 of porphobilinogen ([ $11\text{S-}^3\text{H}$ ]-porphobilinogen is formed).<sup>265,266</sup> Careful work-up and separation methods had to be developed to



**Figure 51.** Incorporation of stereospecifically labeled tritiated 5-aminolevulinic acid according to Akthar.

determine the amount of incorporation into 5-aminolevulinic acid and into the product.<sup>267</sup> Using  $^3\text{H}$ :  $^{14}\text{C}$  ratios it could be shown that nearly half of the tritium is lost in the biosynthetic samples of 5-aminolevulinic acid and of PBG. These results indicate that the *pro-R* proton is removed from the last intermediate before the formation of the aromatic pyrrole ring. This indicates that this deprotonation must be enzyme catalyzed (Figure 51).<sup>267</sup>

This result and the hypothesis that the last intermediate in the biosynthetic sequence is still enzyme-bound has obtained strong support from the observation of the enzyme product complex using NMR techniques.<sup>253--255</sup>

### **Quaternary Structure**

PBGS from most sources has been shown to be an octamer as could be demonstrated determining the molecular weight of the intact enzyme ( $M_r \approx 275,000$ ) and of the subunits ( $M_r \approx 37,000$ ).<sup>167,171,172,186</sup> The mammalian enzymes were known for quite some time to be octameric. Recently it could be shown that also the bacterial enzyme from *Escherichia coli* is also an octamer<sup>171</sup> (for recent studies which indicate that the equilibrium between octamer, hexamer, tetramer, and dimer can be influenced by the presence of Mg (II) ions see ref. 252). Similar results were obtained for the PBGS isolated from genetically engineered yeast.<sup>168</sup> The yeast PBGS is also a homo-octamer. By studying crystals obtained of the bovine liver enzyme with electron microscopy it could be shown that the eight subunits were arranged at the corner of a cube.<sup>174,175</sup> The subunits were indistinguishable which is in contrast to the report indicating that half of the  $\text{NH}_2$ -terminal residues of bovine PBGS are acylated, whereas the rest of the subunits possess a free terminal amino group.<sup>230</sup>

For the enzyme isolated from *Rhodobacter spheroides* it is not clear if the active enzyme is a hexamer or an octamer.<sup>175</sup> PBGS from this source associates to higher aggregates depending on the  $\text{K}^+$  concentration.<sup>183,189</sup> In the absence of  $\text{K}^+$  ions PBGS from *R. spheroides* dissociates into functional tetramers which is an indication that this enzyme in its native state is also an octamer.<sup>268</sup> On the other hand studies of the spinach enzyme were interpreted as to indicate a hexameric structure.<sup>178</sup>

### **Half-Site Reactivity**

Incubation of PBGS with low concentrations of  $^{14}\text{C}$ -5-aminolevulinate and  $\text{NaBH}_4$ , followed by titrating the residual enzyme activity, allowed Choh and Neilands to make half-site reactivity of PBGS plausible.<sup>153</sup> The result of the titrations suggest that only four sites/octamer need to be labeled to eliminate the enzyme activity completely.

Using the technique developed to immobilize PBGS,<sup>269,270</sup> a very elegant experiment was executed by Shemin which strongly indicate half-site reactivity.<sup>268,271</sup>

PBGS was immobilized on sepharose. This immobilized enzyme showed roughly half of the specific activity of the native soluble enzyme. Under denaturing conditions (treatment with 0.1% sodium dodecyl sulfate or with 8 M guanidine-HCl) only two subunits/octamer remained fixed on the sepharose, indicating that on average two subunits/octamer are covalently bound. Upon treating the column containing the immobilized octameric enzyme with 4 M urea, half the protein was released, whereas the other half of the protein stayed on the column, maybe as a tetramer. The protein remaining on the column was still functional with a reduced activity (roughly 1/6 of the specific activity of the native enzyme). It seems that the binding between the subunits forming the dimer is considerably stronger than the binding between the tetramers. The full activity of the bound enzyme could be restored by reassociation under suitable conditions using either pure native enzyme or crude bovine liver extract. Almost the same activity as prior to the dissociation could be obtained. This experiment seems to indicate a hierarchy of the association process. The monomeric subunits strongly associate to form dimers, which are only weak catalysts. The dimers dimerize themselves to form tetramers, and the tetramers finally dimerize to form the fully active octamer. The association constants become weaker as one goes from dimer to tetramer, and finally to the octamer.

To gain a hint at the preferences of the formation of the different oligomeric species of PBGS, hybridization experiments were carried out. Phthalylated protein was hybridized with native enzyme. If a statistical mixture of all possible octamers were formed, there should be nine different variants observable. Only five variants could be detected by electrophoresis, indicating that the dimers are formed either from two native or two acylated enzymes, and the hybridization occurs preferentially between these dimeric units rather than between single subunits.<sup>268</sup>

Finally treating PBGS with [4-<sup>14</sup>C]-5-aminolevulinic acid and NaBH<sub>4</sub> allows determination of the stoichiometry of Schiff base trapping. Subsequent determination of the remaining reactivity allows one to titrate the number of deactivated active sites against the reactivity. Jaffe could show that using fully activated PBGS (including dithioerythritol and Zn<sup>2+</sup> in the incubation) 2.3 equivalents/octamer of the Schiff base could be irreversibly trapped.<sup>237</sup> The enzyme still had a relative activity of 38 to 50%. Using the apo enzyme, where Zn<sup>2+</sup> has been removed with EDTA, four equivalents/octamer of the Schiff base were irreversibly trapped and the remaining relative activity was as low as 4 to 6%. For the enzyme, which was modified with methyl methanethiosulfonate, the stoichiometry of Schiff base trapping was 3.4 equivalents/octamer, and the remaining reactivity after addition of reducing agent was only 3 to 7%. These results were interpreted as hinting at half-site reactivity.<sup>237</sup> The conclusion of all these experiments is that blocking the active site of one subunit blocks the catalytic potential of two subunits.

Based on this evidence, it has been speculated that the active sites sit at the interfaces of the two subunits forming a dimer which might be the minimal structure necessary for catalytic activity.<sup>175,272,273</sup> Each of the subunits would execute a different role ("A-site" and "P-site") in the catalytic cycle.<sup>272,273</sup> Jaffe has suggested

another hypothesis where the differentiation of the two binding sites is induced by the two different types of zinc bound to the enzyme.<sup>231</sup>

#### D. Conclusions

The mechanism of the enzymatic synthesis of PBG is not yet established. It is clear that the sequence of recognition of the two substrate molecules is "P-site" first and "A-site" second, at least for the bovine liver and the human erythrocyte enzyme. The substrate at the "P-site" forms a Schiff's base to a lysine of the active site and  $Zn^{2+}$  is not required for this reaction. The second substrate, which binds not as tightly, may be bound noncovalently to the enzyme. The second substrate also may be coordinated to  $Zn_A$  as has been proposed. One  $Zn^{2+}$  probably complexed to one cysteine helps in the catalytic step. There has been no speculation about the role of the second  $Zn^{2+}$ , complexed to four cysteines. There is good circumstantial evidence that the enzyme shows half-site reactivity. An active site formed at the interface of a dimer is an attractive interpretation of these observations. Finally, the product forms a relatively stable complex to the enzyme ( $K_d = 0.15$  mM), which is in agreement with the observation that the last chemical step, the deprotonation, is under the influence of the enzyme.

The above knowledge provides answers to the question of the sequence of recognition (question 1, pages 67, 82), to the question of the structure or part of the structure of the Michaelis complex (question 2, page 73), and partly to the question of the activation of the substrate (question 4, pages 73, 77, 80). The actual understanding does not provide an answer to the question concerning the sequence of chemical steps which led to the formation of PBG (question 3, pages 65, 69, 71). The postulated mechanisms are all reasonable working hypotheses, without having any definitive experimental proof.

### IV. EFFORTS TO IMITATE THE BIOSYNTHETIC PATHWAY

#### A. General Remarks

As discussed in the section on the biosynthesis of PBG (9), there have been a series of different mechanisms proposed for this deceptively simple transformation. Concentrating only on the step connecting the two substrate molecules via a covalent bond for the first time, there have been three mechanisms postulated to date for the biosynthesis of PBG. 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  $\delta$ -aminolevulinic acid (17) reacting as a nucleophile and the keto function of the other  $\delta$ -aminolevulinic acid reacting as an electrophile. In the postulated mechanisms it was assumed that the enzyme bound  $\delta$ -aminolevulinic acid (17) forms the nucleophilic partner during the reaction, and that the Schiff base complex between the enzyme amino group and ALA is in its enamine form and reacts as the electrophilic partner.



The steps which follow after this C–C bond-forming reaction are the elimination of water from the  $\beta$ -hydroxyketone, intramolecular aminal formation, imine formation, and deprotonation to yield the product. General mechanistic knowledge assumes, that all of these steps should be fast and reversible reactions. Therefore, there should exist an equilibrium between the “aldol product” resulting from the carbon–carbon bond forming step and the final product the pyrrole. If the reaction steps from the “aldol product” to the final product PBG are reversible, the equilibrium constant should be clearly in favor of the aromatic pyrrole ring. The aromaticity should provide the necessary driving force for the formation of the pyrrole ring.

In contrast to these steps, the formation of the carbon–carbon bond is probably an irreversible step. As a consequence of this mechanistic analysis one would expect that the formation of the central carbon–carbon bond should be slowest and therefore the rate-determining step of the chemical transformation in the biosynthetic sequence. The central intermediate would be the imine or the iminium ion derived from the “aldol reaction.” The inherent chemical reactivity of this intermediate is predicted to be such that it will directly led to the pyrrole with or without the help of the enzyme as a catalyst. Assuming all this to be true, the essential function of the enzyme would therefore be to act as an aldolase. The rest of the steps leading to the product would not need the help of the enzyme, at least not to the same extent as the “aldol reaction.” This sequence is in remarkable contrast to the mechanism of the Knorr pyrrole synthesis (Figure 52).<sup>274,275</sup>

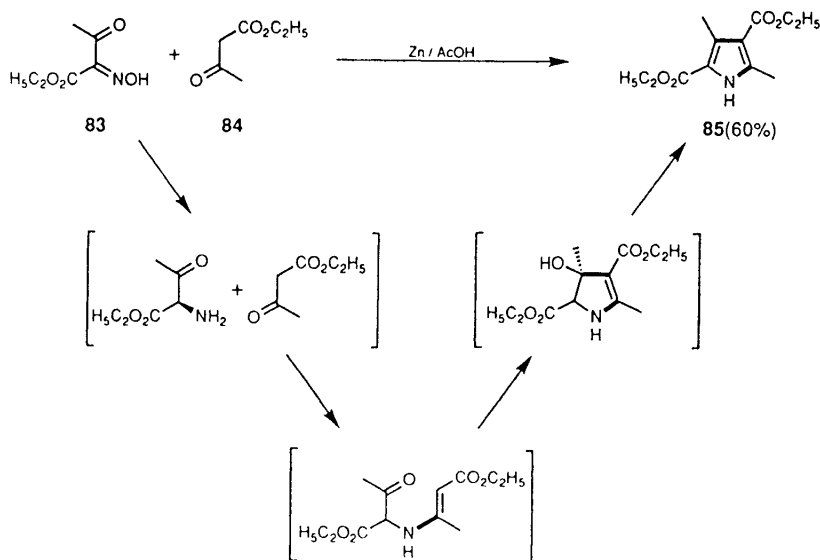


Figure 52.

For the Knorr pyrrole synthesis, the steps leading to the pyrrole nucleus are the same, but the sequence is clearly different from the postulated mechanism for the biosynthesis as described above. 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.

### B. Known Pyrrole Synthesis Forming the Carbon–Carbon Bond First

Two classical pyrrole synthesis, both known for a long time, have been reported, involve the formation of the carbon–carbon bond as probably the first step of the reaction sequence: the pyrrole synthesis, according to Hantzsch,<sup>276</sup> and its variant due to Feist (Figure 53).<sup>277,278</sup>

In recent years there have been several reports on pyrrole synthesis where the carbon–carbon bond is formed first. The following reactions were used to form the central carbon–carbon bond: treatment of the anion of imines with  $\alpha$ -haloketones according to Wittig<sup>279</sup>; the alkylation of  $\alpha$ -halooximes or  $\alpha$ -haloketones essentially with  $\beta$ -dicarbonyl compounds, e.g. the newest version according to Monforts;<sup>280</sup> the Michael additions to nitroolefins according to Yoshikoshi,<sup>281</sup> and the aldol reaction between ketones and glyoxalhydrazones.

In the Hantzsch synthesis<sup>276</sup> a vinylogous carbaminic acid ester formed from a  $\beta$ -ketoester with ammonia is treated with a bromoketone or a bromoaldehyde to produce a pyrrole substituted with an ester in the C3 position. In the variant, according to Feist,<sup>277,278</sup> an acyloin is used instead of the bromoketone, and the reaction is induced by the presence of a catalytic amount of zinc chloride.

Wittig and collaborators<sup>279</sup> developed a very interesting pyrrole synthesis starting from the deprotonated form of the *N*-cyclohexylacetaldimine which is treated with  $\alpha$ -halogenated ketones (Figure 54). The regioisomer **88** formed in this reaction is

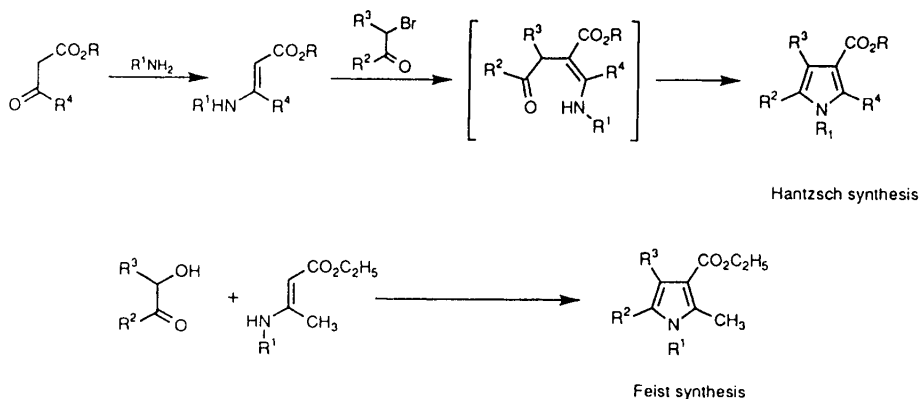
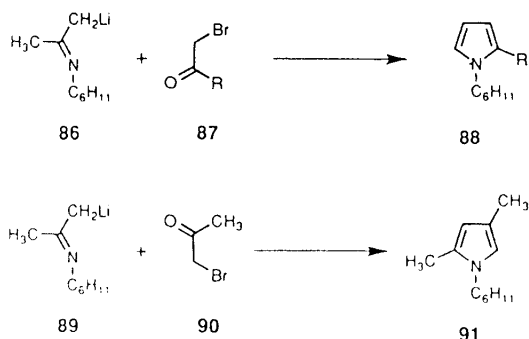


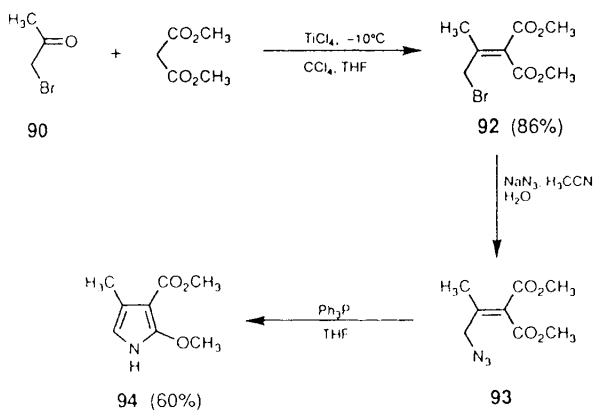
Figure 53.



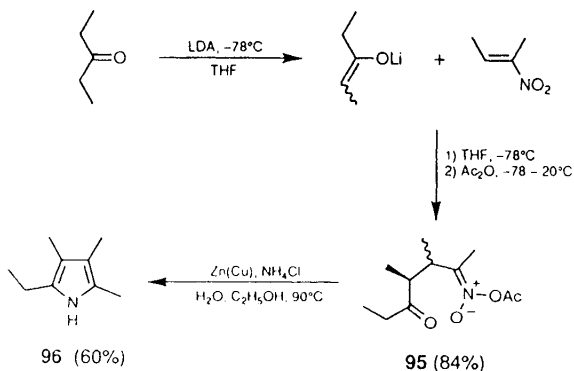
**Figure 54.** Pyrrole synthesis according to Wittig.

in accordance with the assumption that the carbon–carbon bond is formed first. However, there is no formal proof that this mechanistic assumption is correct. In contrast to the above example, the other regioisomer **91** is formed if the anion of the *N*-cyclohexylacetoneketenimine is used as nucleophile. This pyrrole synthesis is specially suited for the synthesis of alkylypyrroles.

A very interesting synthesis of 2-alkoxypyrrroles has been recently developed by Monforts and his group (Figure 55).<sup>280</sup> Treating  $\alpha$ -bromoacetone **90** with dimethyl malonate in the presence of an excess of titanium tetrachloride yields the  $\gamma$ -bromo- $\alpha,\beta$ -unsaturated diester **92**, which is transformed into the azido compound **93** by treatment with sodium azide in acetonitrile/water. The Staudinger reaction directly leads to the methoxypyrrrole **94** in good yield. In the synthesis of Monforts the substituent at position C3 has to be an ester.



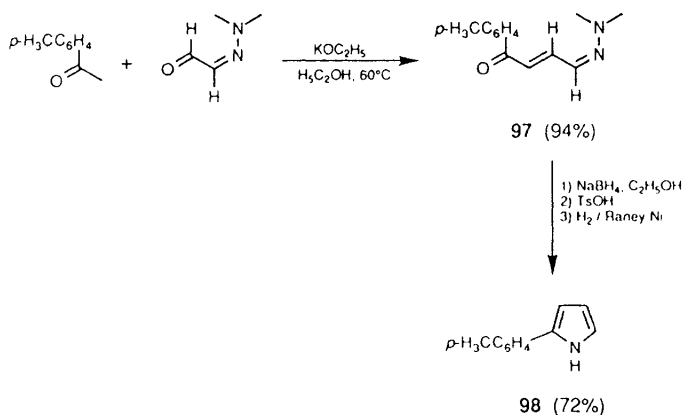
**Figure 55.** Pyrrole synthesis according to Montforts.



**Figure 56.** Pyrrole synthesis according to Yoshikoshi.

The Michael addition of lithium enolates of ketones to nitroolefins and trapping of the adduct with acetic acid anhydride was reported by Yoshikoshi (Figure 56).<sup>281</sup> Reduction of the resulting aceto nitronate **95** with zinc in slightly acidic water ethanol mixtures at 90 °C gave good yields of alkyl-substituted pyrroles **96**.

Severin and collaborators<sup>282</sup> synthesized a series of pyrroles by treating the mixture of an aromatic ketone and the hydrazone of glyoxal with sodium ethoxide in ethanol (Figure 57). The resulting product **97** was then reduced to give pyrroles substituted by aromatic rings **98**. Without the aromatic substituent, the formation of the pyrrole ring was unsuccessful. The double bond was reduced preferentially. In this synthesis one substituent has to be an aromatic ring.



**Figure 57.** Pyrrole synthesis according to Severin.

In summary, the number of pyrrole syntheses where the carbon–carbon bond formation is the central step is relatively small. If one wants to synthesize pyrroles which have only alkyl substituents, there are only very few methods available. Most classical pyrrole syntheses work only if an ester or an acyl substituent is present, or in the best cases the yields for the alkylypyrroles are very low.

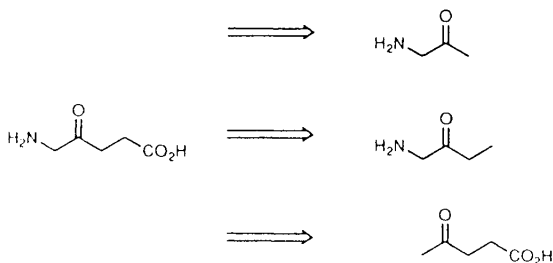
### C. Synthesis of Pyrroles Using Enamine Chemistry

The only proven intermediate in the synthesis of PBG **9**, which has been trapped, is the Schiff base between lysine(252) of the enzyme and the keto function of the substrate. The Schiff base at the active site may be present as imine, iminium salt, or even as enamine. Following the mechanistic reasoning first proposed by Shemin,<sup>184</sup> one can ask the question if pyrroles can be synthesized using enamines as starting material, and if it will finally be possible to synthesize even PBG **9** in a biomimetic way using such a methodology?

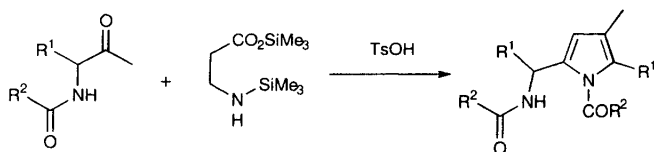
In order to study the feasibility of such a process a series of model studies were undertaken by us. It was decided to study the reaction conditions using the following compounds as models: aminoacetone, aminobutanone, and levulinic acid (Figure 58). To be able to use the amino ketones for these studies we had to suitably protect the amino group. We chose the acetamido group, mainly because this group is easy to synthesize and sufficiently stable even under relatively harsh reaction conditions.

The Steglich group<sup>283,284</sup> had already condensed  $\alpha$ -acylaminoalkyl methyl ketones to obtain *N*-acylpyrroles (Figure 59). Using *N*-(trimethylsilyl)  $\beta$ -alanine as the condensing agent and *p*-toluenesulfonic acid as the catalyst, Steglich's group was able to isolate the corresponding pyrroles in 40 to 70% yield.

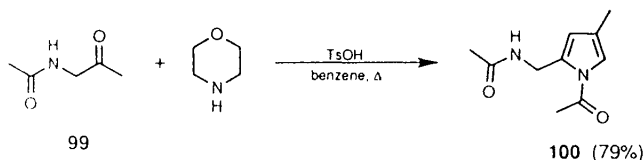
Zav'yalov and co-workers<sup>285–288</sup> had already shown that acetamido acetone (**99**) could be transformed into the pyrrole **100**, and had published their results under the attractive title, "Simplest Chemical Model of PBG Synthesis." Unfortunately they have published no further studies. Our first results using optimized conditions showed that the yield of the *N*-acetylpyrrole **100** could be improved to 80% (Figure 60).



**Figure 58.** Model compounds used in place of 5-aminolevulinic acid.



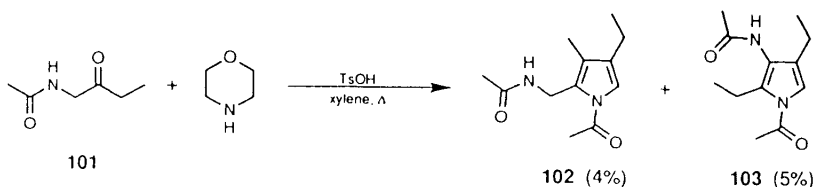
**Figure 59.** Pyrrole synthesis according to Steglich.



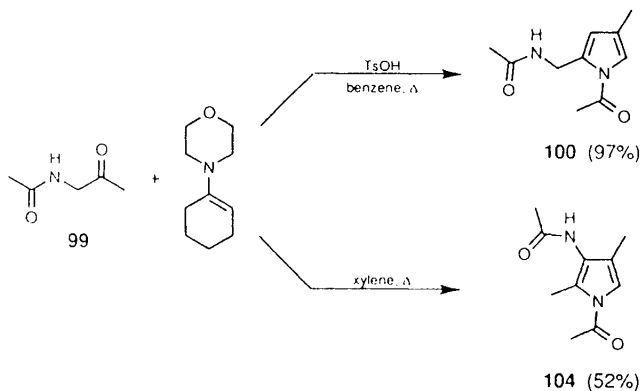
**Figure 60.**

Treating the acetamido acetone (**99**) with morpholine and a catalytic amount of *p*-toluenesulfonic acid in benzene using molecular sieves to remove the water with a Kutscher Steudel apparatus proved to us to be optimal for the synthesis of the pyrrole **100**. We assume that the less-substituted enamine formed from acetamido acetone (**99**) and morpholine is an intermediate which reacts with the keto function of another acetamido acetone (**99**). A possible alternative could be the reaction between the enamine of one and the iminium salt of the other acetamido acetone (**99**). This reaction would form the central carbon–carbon bond. A series of straightforward steps would then lead to the required pyrrole **100**. The most astonishing reaction in this sequence is the ring closure between the amide nitrogen from one acetamido acetone (**99**) reacting with the carbonyl function of the other acetamido acetone (**99**).

Using the same reaction conditions but changing the starting material to 1-acetamido-2-butanone (**101**) gave less encouraging results (Figure 61). To initiate the reaction we had to use much higher temperatures with xylene as a solvent. The yields of the two regioisomeric pyrroles **102** and **103** were low (4 and 5%,



**Figure 61.**



**Figure 62.**

respectively). We obtained a mixture of the two possible regioisomers, **102** and **103**, in a ratio of almost 1 : 1.

The lack of reactivity and of selectivity in the reaction starting with the butanone **101** might be due to the well-known difference in reactivity between unsubstituted and substituted enamines. It is also well known that in the presence of acids at higher temperatures regioisomeric enamines are readily equilibrated.

In order to verify the influence of Brønsted acid on the reaction we modified the reaction conditions (Figure 62). Treating acetamido acetone (**99**) with the enamine formed from cyclohexanone and morpholine in the presence of *p*-toluenesulfonic acid in benzene gave the pyrrole **100** in an excellent yield of 97%. Using the same starting material with xylene as solvent and in the absence of the acid catalyst, a 52% yield of the isomeric pyrrole **104** was obtained. Experiments to use the methyl ester of 5-acetamido levulinic acid under the aforementioned reaction conditions were unsuccessful.

In order to have a clearer picture of the properties of the enamines formed, we synthesized the mixture of regioisomeric enamines **105** and **106** in 55% yield starting from the ethyl ester of levulinic acid (**82**) with titanium tetrachloride as catalyst (Figure 63). Trying to synthesize the enamines using *p*-toluenesulfonic acid as catalyst, higher temperatures were needed, and we directly obtained the condensation product **107** between two levulinic acid esters in 85% yield.

The major isomer **105** formed in the titanium tetrachloride catalyzed reaction was the more highly substituted regioisomer. Under acidic conditions we were unable to isolate any product derived from this major more stable isomer **105**. At the same time the condensation product **107** starting from the minor isomer **106** could be isolated in 85% yield. This showed that under the conditions necessary

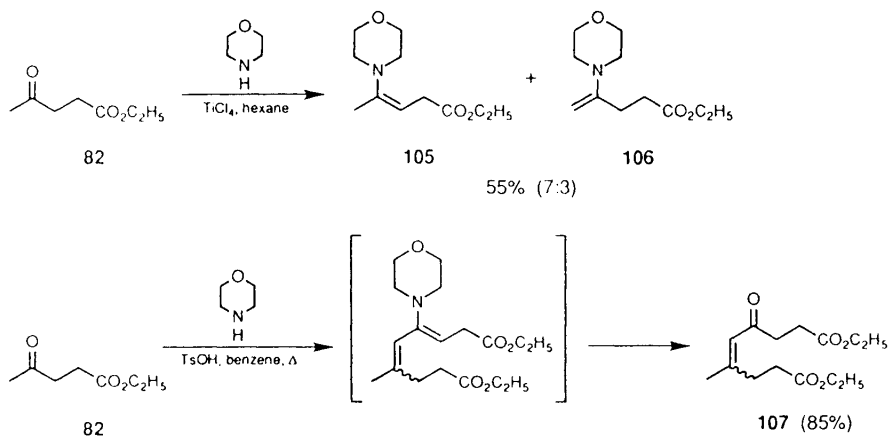


Figure 63.

for the carbon-carbon bond formation equilibration must be fast, and the higher reactivity of the unsubstituted enamine **106** controls the outcome of this reaction.

Using the 7:3 mixture of regioisomeric enamines we could show that the product distribution of the reaction with bromine at  $-78^\circ\text{C}$  corresponds nicely to the ratio of the regioisomeric starting material (Figure 64). For the acylation of the mixture

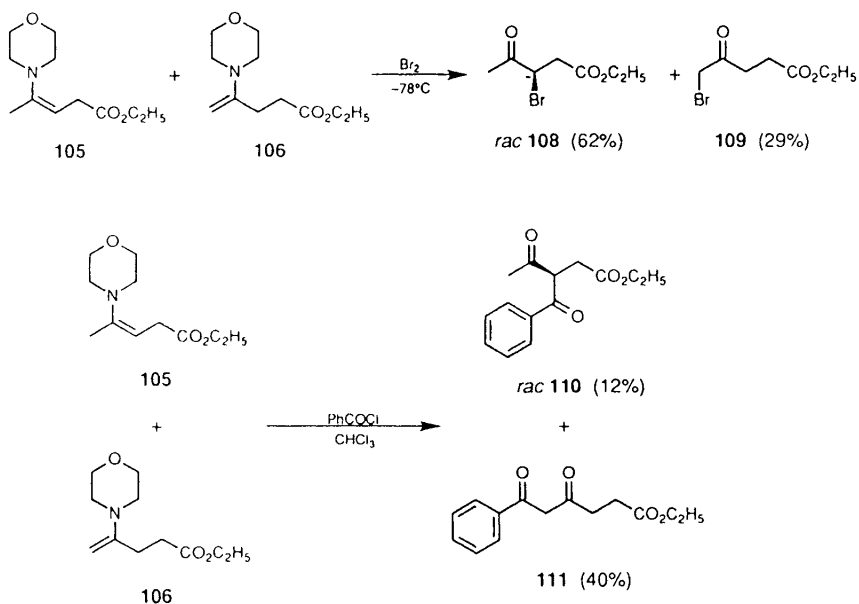
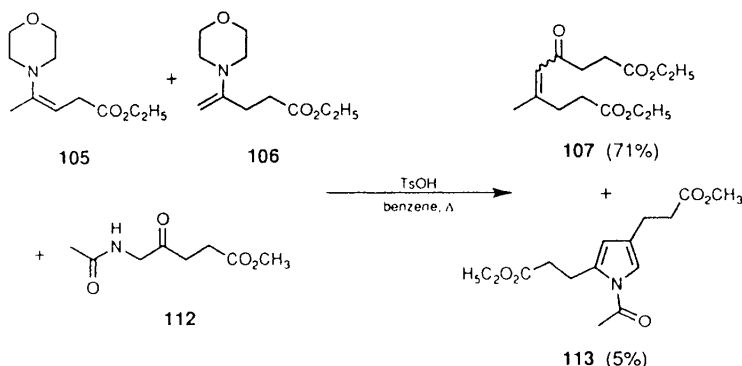


Figure 64.





**Figure 65.**

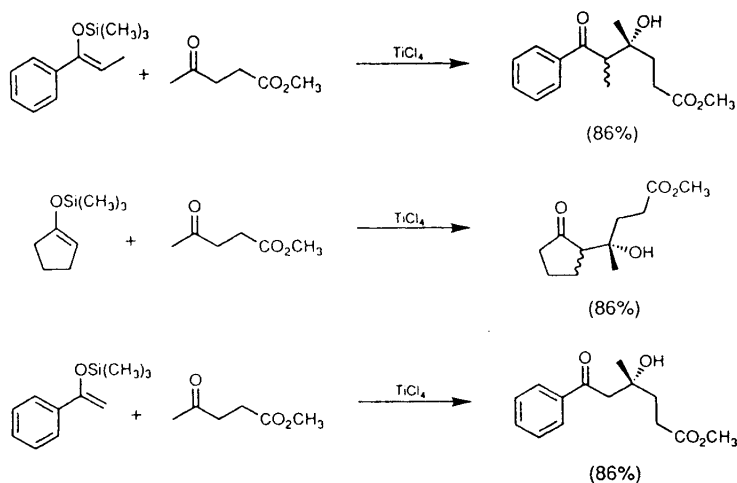
of enamines with benzoyl chloride, we had to go to room temperature. This reaction leads to a product mixture **110** and **111**, where both regioisomers can be isolated, but the minor less-substituted enamine has reacted preferentially (Figure 64). Reaction of the same mixture of enamines **105** and **106** with the methyl ester of 5-acetamido levulinic acid (**112**) leads to the condensation product between two levulinic acids **107** in 71% yield, and only 5% yield of the *N*-acetylpyrrole **113** was obtained (Figure 65). Both reactions have to pass via the less-substituted enamine.

We concluded that the reactivity of the enamines formed either from 5-acetamido levulinic acid or from levulinic acid are not high enough. In order to form the crucial carbon–carbon bond, relatively harsh reaction conditions have to be applied (higher temperatures and presence of Brønsted acids). Under these conditions the equilibration between the two possible regioisomers is fast compared to the carbon–carbon bond formation. We decided to study synthetic equivalents of the enamine which hopefully should be stable under the reaction conditions.

#### D. First Synthesis of Pyrroles Via the Crossed-Aldol Reaction

The Mukaiyama crossed-aldol reaction seemed to be ideally suited for our purpose.<sup>289,290</sup> Mukaiyama had reported the successful use of levulinic acid methyl ester as ketone component in one of his papers (Figure 66).<sup>291</sup> Using the method described by Miller,<sup>292</sup> we transformed levulinic acid methyl ester (**114**) into the mixture of the two silyl enolethers **115** and **116** using trimethylsilyl iodide as the silylating reagent and hexamethyldisilazene as the base (Figure 67).

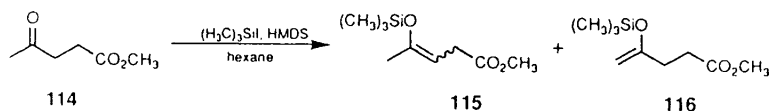
The Mukaiyama reaction of the resulting silyl enolethers **115** and **116** with levulinic acid methyl ester (**114**) gave a mixture of products. We isolated the two isomeric olefins **117** and **118** in 3% yield each. The isolation and identification of the olefin **117** was the first sign of the carbon–carbon bond formation at the higher substituted position (Figure 68).



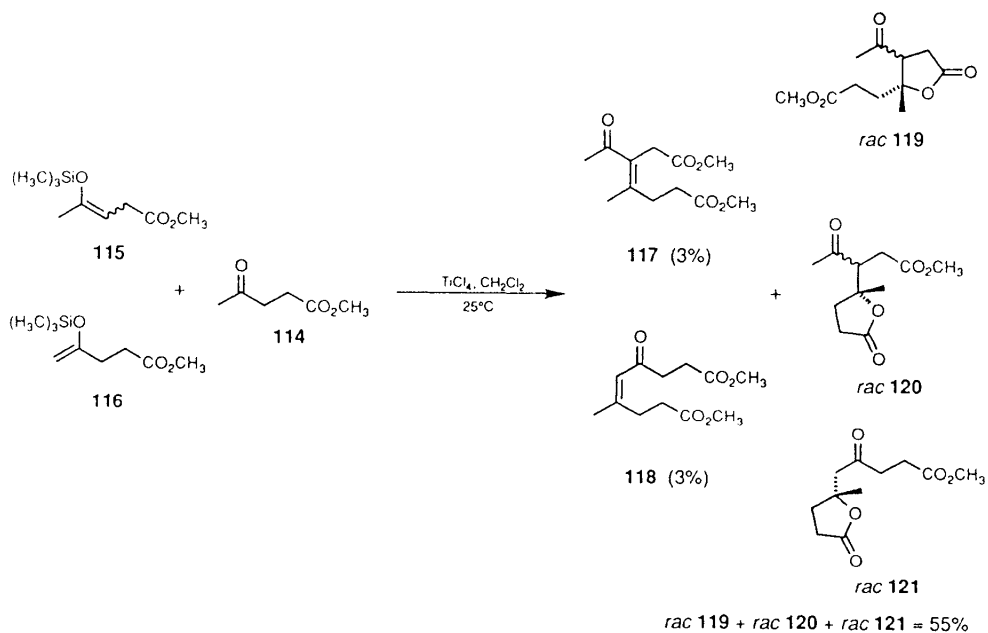
**Figure 66.** Crossed aldol reaction according to Mukaiyama.

Even more interesting was the fact that we isolated a mixture of three  $\gamma$ -lactones *rac*-**119**, *rac*-**120**, and *rac*-**121** in 55% yield. Two of these  $\gamma$ -lactones, *rac*-**119** and *rac*-**120** were formed via the quintessential aldol reaction at C3 of the enoether, whereas the third isomer, *rac*-**121**, was formed by the aldol reaction starting from C5. In contrast to the reports of Mukaiyama<sup>289,290</sup> who had been able to isolate the  $\beta$ -hydroxyketones, our aldol products (probably in their deprotonated form) reacted with one of the ester groups present to form the lactones *rac*-**119**, *rac*-**120** and *rac*-**121**.

Much better results were obtained using the acetal **122** in the place of the ketone **114** (Figure 69).<sup>293–297</sup> The  $\beta$ -methoxyketones **123** and **124** thus obtained were easier to isolate, and the absence of lactone formation made identification much easier. The two isomeric  $\beta$ -methoxyketones **123** and **124** were obtained in a total yield of 62% by this procedure. The ratio between the two products—**123** : **124**  $\approx$  4 : 1—nicely reflected the distribution between the two regioisomeric silyl enoethers of levulinic acid methyl ester **115** and **116**.

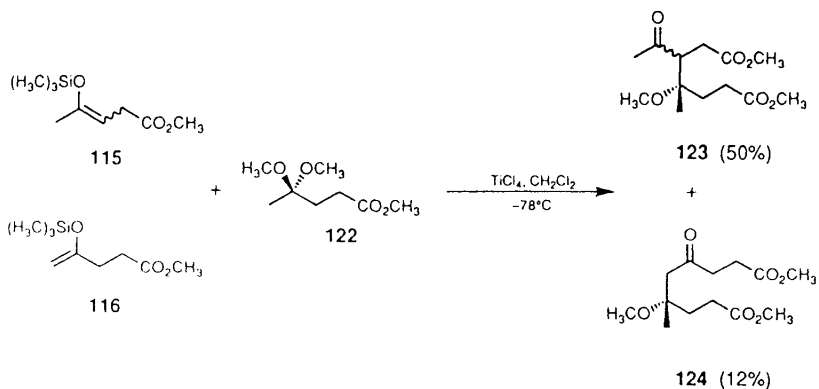


**Figure 67.** Thermodynamically controlled synthesis of the silyl enoethers from methyl levulinate according to Miller.



**Figure 68.**

The next step was to apply these reaction conditions to the carbon–carbon bond formation between **115** and **116** and the acetal of acetamido acetone (**126**) (Figure 70).<sup>298</sup> We were able to isolate small quantities of pyrrole **128** in our first experiments. Optimizing the reaction conditions, we could isolate up to 48% of the pyrrole



**Figure 69.**

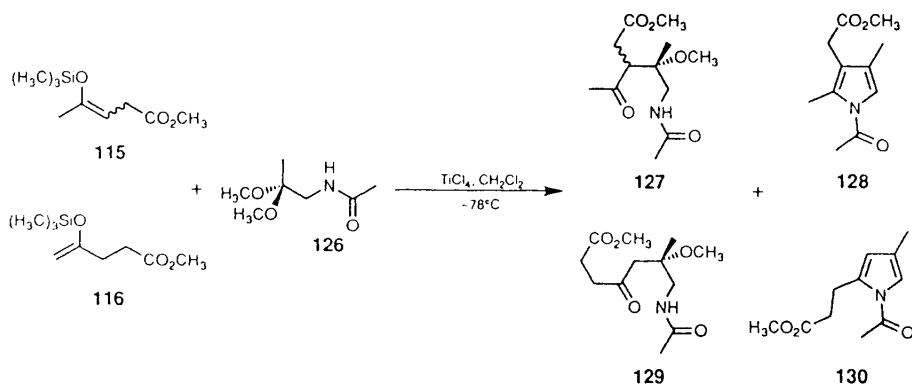


Figure 70.

**128**. A large excess of titanium tetrachloride was necessary to obtain good yields of the product. Besides the pyrrolic product **128**, we were also able to isolate the aldol products **127** and **129**, which could be cyclized in excellent yield in a separate step using benzene as a solvent and *p*-toluenesulfonic acid as catalyst.

Attempts to use this new pyrrole synthesis for the formation of other pyrroles met with mixed success (Figure 71).<sup>299</sup> Treating the silyl enoether of cyclopent-

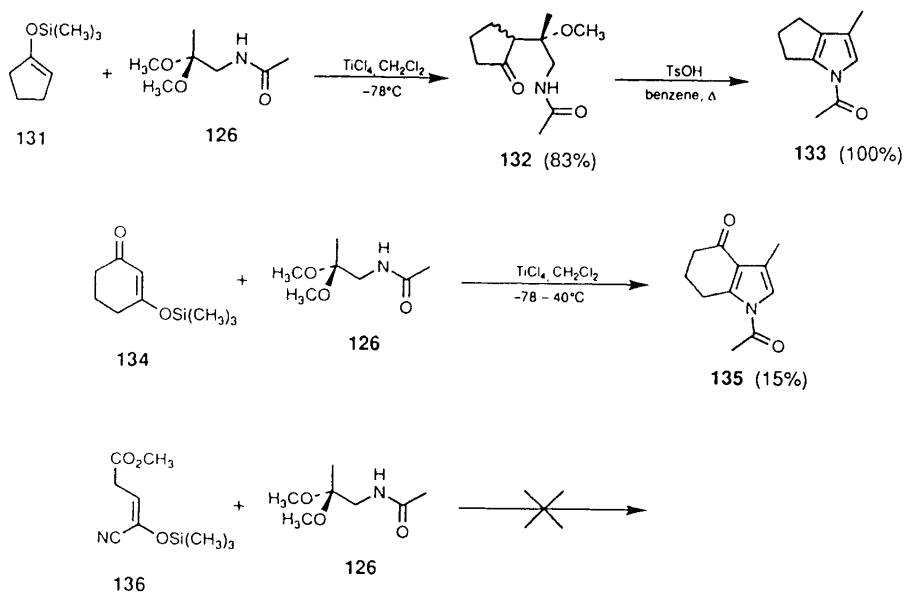


Figure 71.

tanone (**131**) with the acetal of acetamido acetone (**126**) gave a mixture of the diastereoisomeric aldol products **132** in 83% yield. Products **132** could be quantitatively transformed into the annelated pyrrole **133** using benzene as solvent and *p*-toluenesulfonic acid as catalyst. Using deactivated silyl enolethers, such as the silylenolether from 1,3-cyclohexanedione (**134**), the reaction conditions had to be much harsher, and the yield of the pyrrole **135** was disappointingly low. Using the silyl enolether **136** deactivated by the cyano group, we were unable to isolate any pyrrolic product.

The difficulties could derive from the fact that acetamide was used as protecting group. As already stated by Mukaiyama, the crossed-aldol reaction does not work if one of the components contains a labile hydrogen atom. Under the influence of titanium chloride, hydrogen chloride is formed and thereby the silyl enolether is hydrolyzed.<sup>300</sup>

In order to avoid these problems we changed to a more inert protecting group, the phthalimido protecting group (Figure 72). As we had hoped, the yield of the crossed-aldol reaction using the dimethyl acetal of phthalimido acetone (**137**) gave a considerably better yield for the aldol reaction. A definite reason for the better yield is that the aldol products **138** and **139** are more stable. However, the hydrolysis of the protecting group proved to be difficult. By treating **138** and **139** with hydrogen chloride in methanol, we finally succeeded transforming them into two isomeric tricyclic pyrroles **140** and **141** in relatively low yield. The removal of the protecting group had not been successful because the partially hydrolyzed intermediate first formed the *N*-acylated pyrrole first which undergoes an intramolecular Friedel–Crafts reaction. These results induced us to search for still another protecting group.

## E. Second Synthesis of Pyrroles Via the Crossed-Aldol Reaction

We studied two possible ways to improve the crossed-aldol reaction. One possible improvement would be to synthesize the silyl enolether in a regioselective manner. This would give only one regioisomeric aldol product. We hoped that the isolation of the aldol products should be easier. We decided to look also for another protecting group to fulfill two conditions at the same time: (1) no interference with the aldol reaction, and (2) easy to deprotect.

We used for the preliminary experiments the mixture of the silyl enolethers **115** and **116** starting from levulinic acid. For the synthesis of the isomerically pure silyl enolether we decided to follow the procedure for the reductive silylation of the corresponding bromoketone of Rubottom.<sup>301</sup> Treating the 3-bromoketone *rac*-**142** with activated zinc in diethyl ether in the presence of TMEDA and TMS chloride gave only the elimination product (Figure 73). To increase the rate of the heterogeneous reaction we intensified the stirring specially activating the zinc, but without success. Using the thermodynamic mixture of silyl enolethers obtained according to Miller<sup>292</sup> we could show that the silylenol ether in pure form is stable against

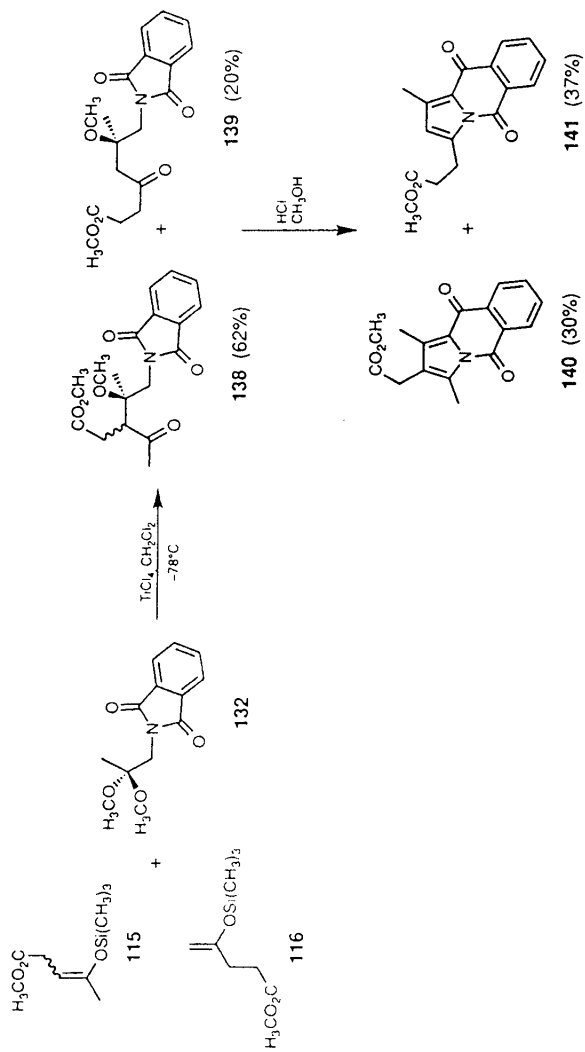
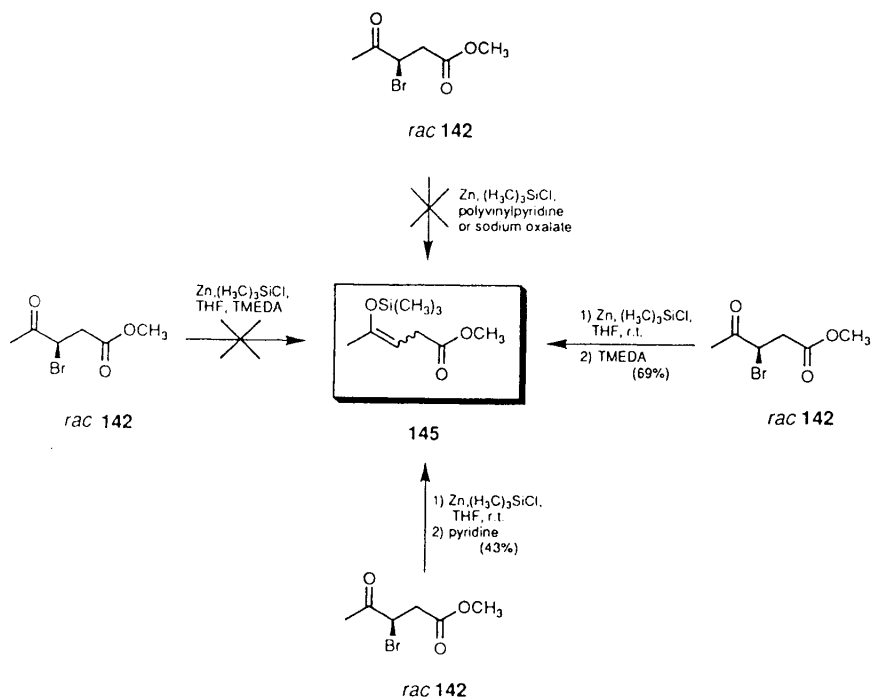


Figure 72.



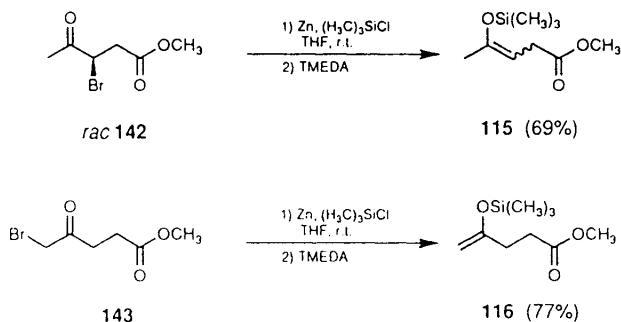
**Figure 73.**

extraction with aqueous bases. Therefore the elimination induced by TMEDA must be faster than the heterogeneous reduction with zinc.

Using the procedure of Itoh,<sup>302,303</sup>—silylation with zinc in the absence of a base, quenching the reaction with pyridine, followed by an aqueous work-up—allowed us to isolate the silylenol ether in 49% yield by distillation (Figure 73). Trying to isolate the silylenol ether avoiding the aqueous work-up met with no success. After the addition of pyridine, filtration of the precipitate and distillation of products allowed only the isolation of levulinic acid and polymerized material. Attempts to use other bases, such as polyvinyl pyridine or sodium oxalate, did not permit the removal of zinc salts completely.

The following procedure allowed us to isolate **115** in 69% yield (Figures 73 and 74): reductive silylation without a base in THF at rt, treating the reaction mixture with TMEDA, precipitating the zinc salts by the addition of pentane, and finally distilling the product. As long as most of the zinc salts could be removed by filtration then **115** could be distilled, avoiding the problems of hydrolysis.

To synthesize the regioisomeric silyl enolether **116**, the 5-bromolevulinic acid methyl ester (**143**) was treated according to the same procedure, and a 77% yield of **116** could be isolated (Figure 74).



**Figure 74.**

Instead of using the amide protecting group, we opted for the azido group.<sup>304</sup> In our preliminary experiments with the mixture of the silyl enolethers we were able to isolate the aldol products (Figure 75).

The separation of the isomeric aldol products *rac*-**145** and *rac*-**146** was delicate and therefore the isolated yields were not satisfactory. The aldol products were treated with triphenylphosphine in benzene to induce a Staudinger reaction.<sup>298,305–309</sup> Formation of nitrogen gas could be observed and we were able to isolate the alkyl pyrroles **147** and **148**. The isolation of the pyrroles was difficult as well; separation of the triphenylphosphine oxide from **147** and **148** was delicate, and in addition, the pyrroles were partly destroyed on the silica gel column.

The substitution of the amido group by the azido group offered a solution to our problems using the crossed-aldol reaction, but the overall yield of reaction sequence was still not satisfactory. Using regioisomerically pure silyl enolethers **115** and **116** considerably improved the yield of the aldol process (Figure 76). In most cases the best yields of the Mukaiyama reaction could be obtained if the starting materials were added to titanium tetrachloride.

Adding the mixture of the silyl enolether together with the acetal to a strongly stirred suspension of titanium tetrachloride in dichloromethane avoided problems associated with the formation of precipitates. At least one equivalent of titanium tetrachloride per complexing functional group was used. In all cases studied this procedure gave the best yields.

The yields of the crossed-aldol reaction varied between 10% for *rac*-**151** and 94% for *rac*-**157** (see Table 2). The major side products isolated were the (*Z*)- and (*E*)-diastereoisomers of the corresponding olefin (yields of the olefins are indicated in parenthesis). Especially in the case of *rac*-**151** obtained from the silyl enolether of cyclopentanone, only 16% of the aldol product could be detected, whereas 84% was present as elimination product. Assuming that the formation of the elimination product is due to hydrogen chloride formed during the work-up, the conditions for aqueous extraction were changed. In some cases the yield of the aldol products



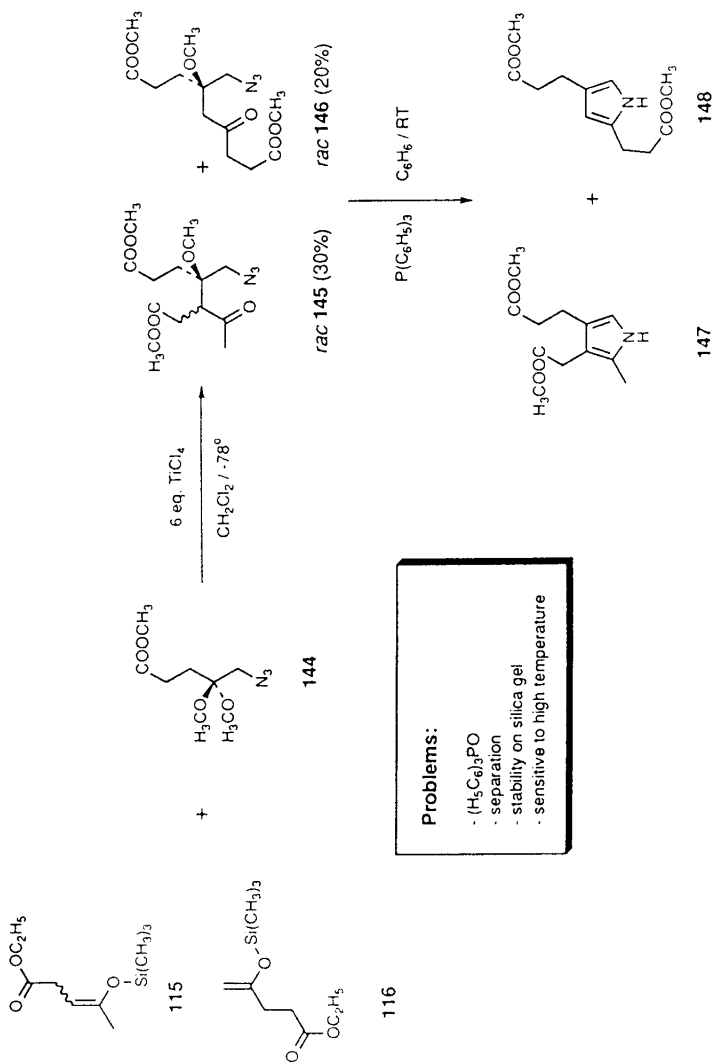


Figure 75.

## 1. modification: use of one regioisomer of the silylenolether

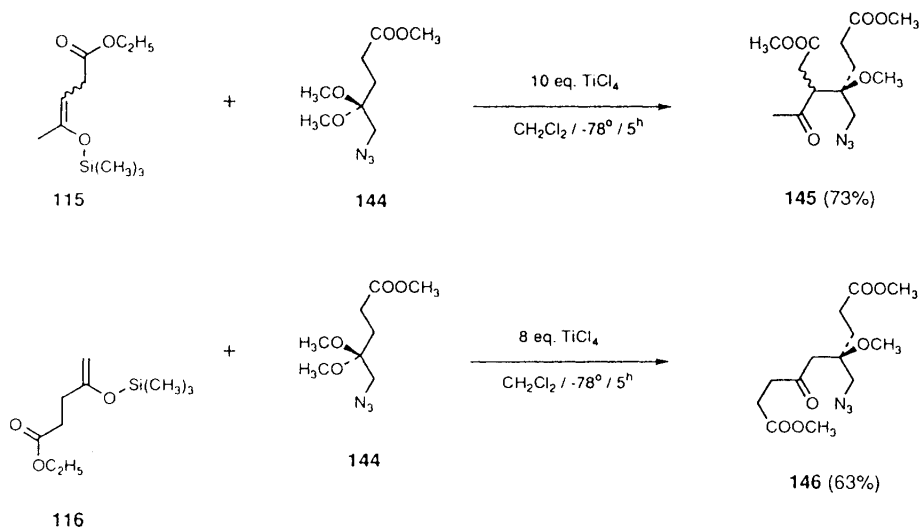


Figure 76.

could be slightly increased by substituting saturated sodium chloride with saturated bicarbonate in the extraction process. The disadvantage of this procedure is the formation of a titanium oxide precipitate. For large quantities, the initial conditions using saturated sodium chloride proved to be easier to apply.

Another problem was encountered when we used the silyl enolether derived from phenylacetaldehyde (**165**) (Figure 77). In this case the main products isolated were the carboxylic acids **167** and **168** derived by oxidation of the corresponding aldehyde. In our first attempts we used the Staudinger reaction<sup>298,305-309</sup> followed by an intramolecular aza-Wittig reaction. This type of reaction had been used to

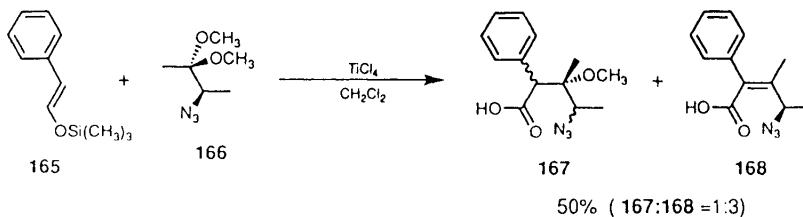
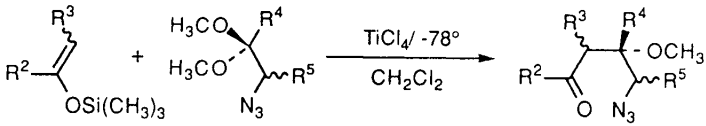
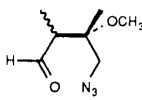
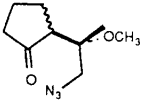
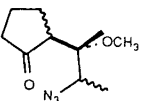
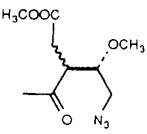
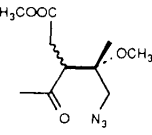
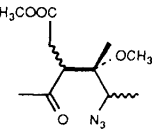
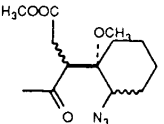


Figure 77.

Table 2.

			
Structure		Yield aldol product (ratio of isomers)	Yield olefin
	<i>rac</i> 149	44 % (1:2:1)	
	<i>rac</i> 150	36 % (2:1)	30 %
	<i>rac</i> 151	16 % (4:1)	84 %
	<i>rac</i> 152	72 % (2:1)	
	<i>rac</i> 153	93 % (1:1:2)	
	<i>rac</i> 154	49 % (2:1:1)	32 %
<b>154</b>			
	<i>rac</i> 155	38 %	

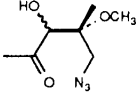
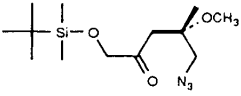
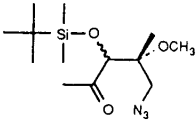
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**Table 2.** (Continued)

Structure		Yield aldol product (ratio of isomers)	Yield olefin
	<i>rac</i> <b>145</b>	71 % (1:3:1)	4 %
	<i>rac</i> <b>156</b>	88 %	
	<i>rac</i> <b>157</b>	94 %	
	<i>rac</i> <b>158</b>	64 % (1:1:1)	21 %
	<i>rac</i> <b>146</b>	64 %	
	<i>rac</i> <b>159</b>	32 %	57 %
	<i>rac</i> <b>160</b>	31 %	45 %
	<i>rac</i> <b>161</b>	39 %	

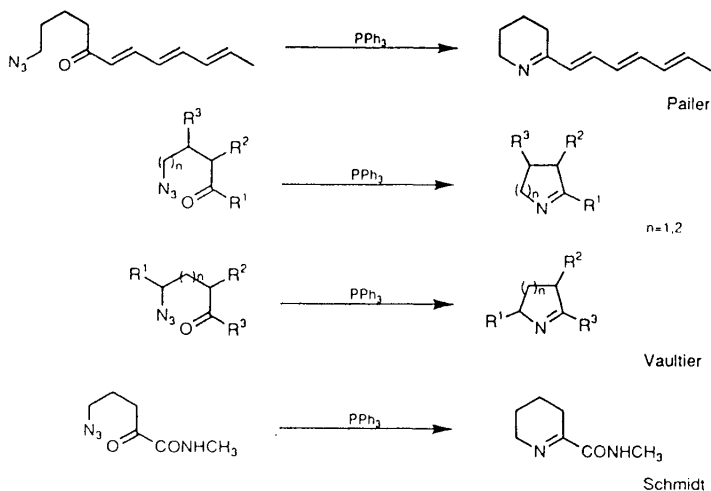
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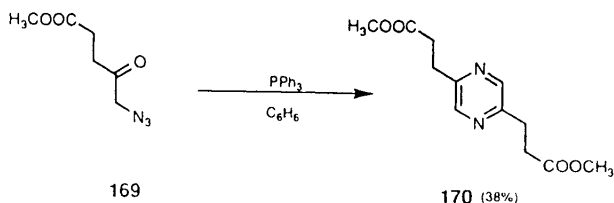
**Table 2.** (Continued)

Structure		Yield aldol product (ratio of isomers)	Yield olefin
	<i>rac</i> <b>162</b>	30 % (1 : 1.3)	
	<i>rac</i> <b>163</b>	23 %	
	<i>rac</i> <b>164</b>	28 %	

form imines from  $\omega$ -azido ketones via the intramolecular version of the aforementioned sequence (Figure 78).<sup>310–314</sup>

In the case of the  $\alpha$ -azido ketone, Zbiral had shown that pyrazines can be formed via an intermolecular condensation followed by oxidation.<sup>315</sup> This method can be applied to obtain the diester of the pyrazine which spontaneously forms by dimerization of 5-aminolevulinic acid at pH  $\geq 8$ <sup>257,261,316</sup> (Figure 79).

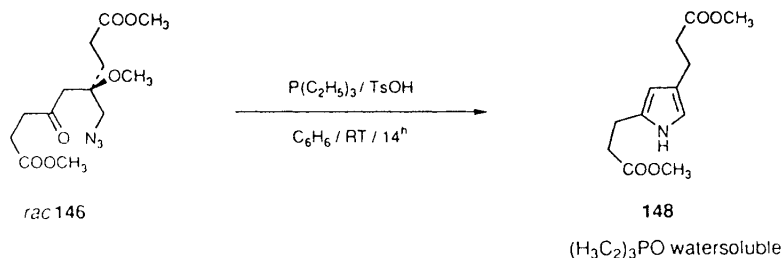
**Figure 78.** Synthesis of cyclic imines via intramolecular aza-Wittig reaction.



**Figure 79.**

Treating the aldol product with triphenylphosphine in benzene yielded the desired pyrrole. Two major problems arose using these reaction conditions (Figure 75). The isolation of the pyrroles in pure form proved to be difficult. The major obstacle was the presence of triphenylphosphine oxide. Distillation often could not be applied because of the heat sensitivity of the pyrroles. Chromatography is notoriously difficult in the presence of triphenylphosphine oxide, and some of the pyrroles were not stable to silica gel. The acidity of the surface probably led to the formation of pyrrole black. In order to avoid these problems we replaced the triphenylphosphine with triethylphosphine (Figure 80). This reagent had two major advantages: (1) the triethylphosphine is more reactive than the triphenylphosphine, and (2) the work-up was much easier because the triethylphosphine oxide is water-soluble. Especially in those cases where the separation of the phosphine oxide from the pyrrole was difficult, it proved to be a great advantage to be able to separate the phosphine oxide by simple extraction. Finally, in some cases the Staudinger reaction occurred as indicated by the development of nitrogen, but cyclization and aromatization were by far not complete. In these cases it proved to be helpful to add a catalytic amount of *p*-toluenesulfonic acid to induce aromatization.

Another way to transform the azido group into the amino group was by catalytic reduction.<sup>304,317-320</sup> Using palladium on charcoal as catalyst and methanol as solvent the aldol products could be reduced (Figure 81). The work-up using these



**Figure 80.**

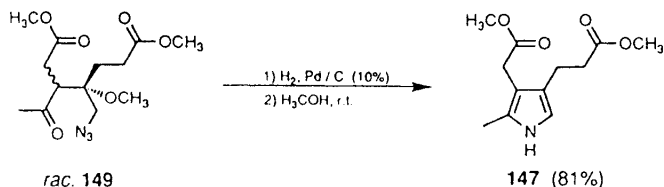


Figure 81.

conditions was extremely easy. After removal of the catalyst and solvent, the products were recovered quantitatively showing an <sup>1</sup>H-NMR which was identical with the spectrum obtained for the analytically pure samples. The purity of the raw material obtained in this way was in most cases greater than 90%. Isolation either by flash chromatography or by Kugelrohr distillation gave, in most cases, analytically pure samples. In a few cases the stability of the products was not sufficient so that the pyrroles were derivatized with methanesulfonyl chloride or di-*tert*-butyldi-carbonate (see Table 3).<sup>321</sup>

As with the Staudinger reaction, the yield of pyrrole obtained by reduction was extremely low for the annelated pyrroles. In the case of the cyclopentenyl derivative, no pyrrole could be isolated. The aldol reaction had not given the aldol product but mainly the olefins derived from the aldol reaction. Probably in the catalytic reduction the  $\alpha,\beta$ -unsaturated olefin is reduced first which could explain why no pyrrole was isolated.

## F. Discussion and Conclusions

The new two-step pyrrole synthesis allows the synthesis of mono-, di-, tri-, and tetraalkylpyrroles in good yield (see Table 3).

The synthesis is complementary to the classical pyrrole synthesis, like the Knorr synthesis, where at least one ester or an acyl group is necessary. It allows introduction of side chains at the correct positions and with the needed functionalities already present in the pyrrole-forming step. The advantage of this synthesis compared to the classical procedures is that the skeleton and the functionalities do not have to be adapted after the formation of the pyrrole ring. In addition, the reaction conditions for pyrrole formation are sufficiently mild to allow for the isolation of highly sensitive pyrroles.

The motivation for the development of this synthesis has been the proposed pathway of the biosynthesis of PBG 9. The results of our experimental studies prove clearly that the intermediate **186** postulated by Shemin<sup>184</sup> would be transformed into PBG 9 even without the help of an enzyme (Figure 82).

The natural reactivity of the intermediate formed by the carbon-carbon building process is such that the other steps—carbon-nitrogen bond formation, elimination

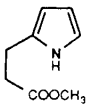
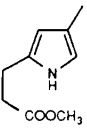
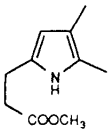
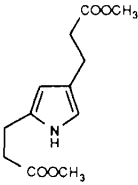
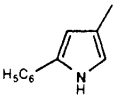
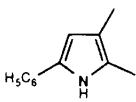
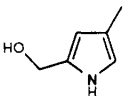
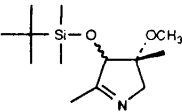
**Table 3.**

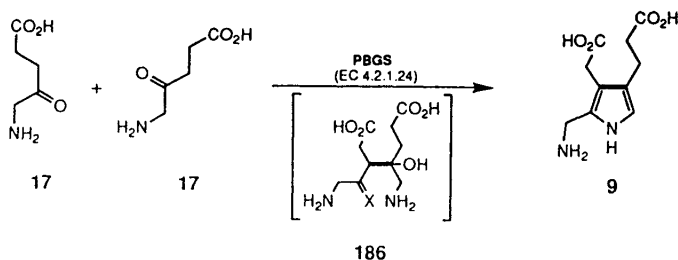
<i>Structure</i>	<i>Yield of reduction</i>	<i>Yield of the Staudinger reaction</i>	
	171	65 % / P(C <sub>6</sub> H <sub>5</sub> ) <sub>3</sub>	
	172	15 % <sup>a</sup> /P(C <sub>6</sub> H <sub>5</sub> ) <sub>3</sub>	
	173	63 %	89 % <sup>b</sup> /P(C <sub>2</sub> H <sub>5</sub> ) <sub>3</sub>
	174	72 %	93 % <sup>b</sup> /P(C <sub>2</sub> H <sub>5</sub> ) <sub>3</sub>
	175	52 %	99 % <sup>b</sup> /P(C <sub>2</sub> H <sub>5</sub> ) <sub>3</sub>
	176	14 % <sup>c</sup>	
	177	81 %	73 % <sup>b</sup> /P(C <sub>2</sub> H <sub>5</sub> ) <sub>3</sub>

(continued)



**Table 3.** (Continued)

<i>Structure</i>		<i>Yield of reduction</i>	<i>Yield of the Staudinger reaction</i>
	<b>178</b>	26 %	50 % /P(C <sub>2</sub> H <sub>5</sub> ) <sub>3</sub>
	<b>179</b>	46 %	84 % /P(C <sub>2</sub> H <sub>5</sub> ) <sub>3</sub>
	<b>180</b>	72 %	87 % /P(C <sub>2</sub> H <sub>5</sub> ) <sub>3</sub>
	<b>181</b>	64 %	76 % /P(C <sub>2</sub> H <sub>5</sub> ) <sub>3</sub>
	<b>182</b>		86 % /P(C <sub>6</sub> H <sub>5</sub> ) <sub>3</sub>
	<b>183</b>		87 % /P(C <sub>4</sub> H <sub>9</sub> ) <sub>3</sub>
	<b>184</b>		28 % /P(C <sub>6</sub> H <sub>5</sub> ) <sub>3</sub>
	<b>185</b>		43 %



**Figure 82.** Putative central intermediate for the biosynthesis of porphobilinogen according to Shemin.

of water or methanol, and aromatization—follow naturally at room temperature. In view of the results of our model studies we can interpret the postulated mechanisms for the enzymatic information. The function of the enzyme would be to bring the two identical partners together. The foremost task of the enzyme would be to induce the crucial carbon–carbon bond formation. The moment this central bond has been formed (intermediate **186**) the rest of the sequence follows the path of the natural reactivity. As shown in the experiments performed by Akthar,<sup>267</sup> all the intermediates stay at the active site of the enzyme until the formation of the product **9**. The major task for the enzyme is to catalyze the chemical transformation with the slowest rate. The chemical logic of the postulated mechanism increases the attractiveness of Shemin's mechanistic proposal.

## V. INHIBITION STUDIES OF PORPHOBILINOGEN SYNTHASE

### A. General Remarks

The results of model studies for the formation of pyrroles from appropriately substituted aldol products can be used for the interpretation of the postulated mechanism of PBGS. Neither the model studies nor the chemical logic of the interpretation proves the correctness of the postulated mechanism. To obtain more insight into the mechanism of the enzyme-catalyzed process, biosynthetic transformations have to be studied and the results have to be interpreted in view of the known chemical properties of the substrate molecules and the postulated intermediates.

The usual way to analyze enzyme-catalyzed reactions in which two substrates are used is through study of the kinetics of the process. In most cases, the order of complexation of two substrates at the active site can be determined this way. The unusual feature of the PBGS-catalyzed reaction is that the two substrates, which are incorporated differently into the product, are chemically the same. This complication has seriously hindered kinetic studies. In view of this difficulty, alterna-

tives had to be developed to obtain more information on the mechanism of the enzymatic transformation. Not surprisingly, progress has been slow.

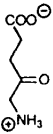
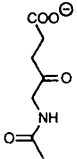
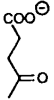

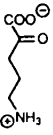
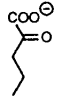

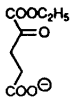

To contribute to the knowledge of enzymatic transformation, we decided to start a systematic inhibition study with substrate analogues, analogues of the product, and analogues of possible reaction intermediates. We decided to use the enzyme isolated from the photosynthetic bacterium *R. spheroides* for which no sequence data are known. Despite the lack of information on PBGS from *R. spheroides*, the properties of this enzyme seemed to be of sufficient interest (high specific activity, formation of mixed pyrrole) that we decided to initiate our studies with the enzyme isolated from this source. Those enzymes, where the sequence has been determined, possess a high degree of homology. The amino acid sequences around the active site of lysine (lys 252) and the cysteines proposed as ligands for one of the zinc ions (cys 223) seem to be well preserved.<sup>162,233,322</sup> Despite this fact, the properties of enzymes from different sources vary sufficiently that results obtained with different enzymes have to be compared with caution.

## B. Known Inhibitors of PBGS

The first studies of inhibitors of PBGS were reported by Granick in 1958.<sup>206,323</sup> The inhibition studies were done using whole cells or homogenates of chicken erythrocytes<sup>206,323</sup> or preparations purified from rabbit reticulocytes.<sup>206</sup> Ethyl levulinate (**82**) and  $\delta$ -aminovalerate (**187**) showed no inhibition at concentrations below  $10^{-1}$  M (see Table 4). Oxaloacetate (**197**) and  $\alpha$ -ketoglutarate (**77**) were weak inhibitors. Moderate inhibition of the enzymatic activity could be measured for levulinic acid (**81**) and succinic acid semi-aldehyde (**195**). Iodoacetamide (**202**) inhibited the enzyme completely at  $10^{-3}$  M. EDTA (**202**) and the other chelating agents **204** and **201** were strong inhibitors. Granick had already shown that the inhibition probably stems from chelation of metal atoms which are necessary for the functioning of the enzyme. Dialysis of EDTA-treated enzyme in the presence of 0.01 M  $Mg^{2+}$  resulted in the recovery of most of the activity.<sup>206</sup> The recovery could be due to the introduction of the 1–10  $\mu$ M  $Zn^{2+}$  together with the chemical grade 0.01 M  $Mg^{2+}$ . These low concentrations of  $Zn^{2+}$  are known to be essential, at least for the animal PBGS.

A major contribution to the knowledge of PBGS stems from Shemin's group who used purified enzyme isolated from *R. spheroides* and beef during the 1960s and 1970s.<sup>148</sup> To obtain further insight into the enzyme-catalyzed reaction mechanism, inhibition experiments were undertaken with the bacterial enzyme<sup>184</sup> (Figure 83; see Table 4).

Shemin demonstrated that  $\gamma$ -ketoacids were good inhibitors, whereas  $\alpha$ -ketoacids such as **77**, **78**, and **76** revealed no inhibition in his kinetic studies. Modifying the amino end of 5-aminolevulinic acid to, for example, *N*-acetyl-5-aminolevulinate (**80**) reduced the inhibition. Surprisingly enough, levulinic acid (**81**) was the most effective of all inhibitors studied. The same series of compounds were examined in kinetic studies and incubated with PBGS in the presence of  $NaBH_4$ .<sup>184</sup> The

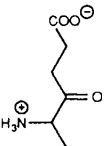
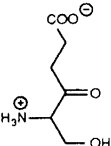
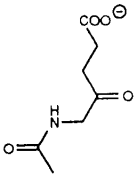
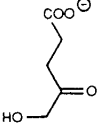
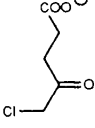
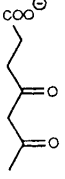
					
<b>17</b>	<b>80</b>	<b>81</b>	<b>82</b>		
Inhibition :	natural substrate	10% (3,3 mM)	50% (3,3 mM)	0% (10 mM)	
Inactivation with NaBH <sub>4</sub> :	60% (2.5 μM)	62% (2.5 μM)	95% (2.5 μM)	67% (2.5 μM)	
					
<b>79</b>	<b>78</b>	<b>76</b>	<b>77</b>	<b>187</b>	
Inhibition :	10% (3.3 mM)	0% (10 mM)	5% (10 mM)	3% (10 mM)	
Inactivation with NaBH <sub>4</sub> :	0% (5 μM)	8% (6 μM)	10% (5 μM)	8% (5 μM)	0% (5 μM)

**Figure 83.** Comparison of inhibition and inactivation studies of PBGS with a series of ketoacids and their derivatives.

conclusions drawn from these results were that the enzyme needs a  $\gamma$ -ketoacid for good recognition (Figure 83 and Table 4). Even relatively poor inhibitors can inactivate the enzyme in the presence of NaBH<sub>4</sub>. In most cases the inactivation reaches 50 to 60%, an interesting result in view of the previous discussion on the “half-site” reactivity. The only exception is levulinic acid (**80**) which inactivated the enzyme totally.

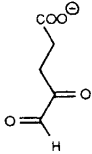

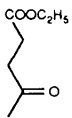

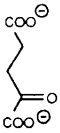
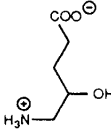
With the exception of the chlorolevulinic acid, most of the known inhibitors isolated from natural sources have not been studied in an effort to understand PBGS. The most potent inhibitor of PBGS known, succinylacetone (**196**), was identified during a study of hereditary tyrosinemia, a severe inborn metabolic disorder, where the degradation of tyrosine is severely hindered.<sup>332</sup> The realization that patients with this disease inevitably show an increased excretion of 5-aminolevulinic acid helped to identify the tyrosine degradation product responsible for this effect.<sup>333,334</sup> Using intact cells or broken cell preparations, **196** was found to be a very effective and rather specific inhibitor of PBGS.<sup>210,325–327,329</sup> It could be shown that **196** reacts with 5-aminolevulinic acid to form succinylpyrrole (**201**), an even more efficient inhibitor of PBGS.<sup>325</sup> The structure of the pyrrole has not been secured. Succinylacetone (**196**) was also used to induce higher excretion of 5-aminolevulinic acid from two bacteria, *Methanosarcina bakeri* and *Methanobacterium thermoautotrophicum*.<sup>335</sup> Studying the effect of **196** and levulinic acid on the metabolism of

Table 4.

Inhibitor	Inhibition Constant	Enzyme Source	Michaelis Constant	Ref.
1  191	$K_I = 12 \mu\text{M}$	<i>P. shermanii</i>	$K_M = 0.36 \mu\text{M}$	192
2  192	$K_I = 0.73 \mu\text{M}$	<i>P. shermanii</i>	$K_M = 0.36 \mu\text{M}$	192
3  80	10% (3.3 mM)	<i>R. spheroides</i>	$K_M = 0.7 \text{mM}$	184
4  193	83% (33 mM)	<i>Mycobacterium phlei</i>		324
5  194	$K_I = 11 \mu\text{M}$	bovine liver		248
6  196	$K_I = 11 \mu\text{M}$	<i>Erythrobacter sp</i> OC 114	$K_M = 0.29 \text{mM}$	177
	$K_I = 0.4 \text{mM}$	<i>Clostridium tetanomorphum</i>		325
	24 % (0.01 $\mu\text{M}$ )	Murine erythroleukemia cells		326
	38% (0.08 $\mu\text{M}$ )	rat liver		210
	72% (0.01 $\mu\text{M}$ )	L-1210 leukemia cells		327
	56% (1 $\mu\text{M}$ )	<i>Hardeum vulgare</i> L. (barley)		328
	50% (10 $\mu\text{M}$ )	rabbit reticulocytes		329

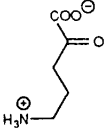
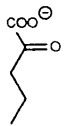

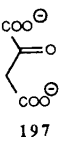
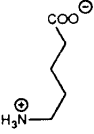
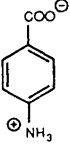
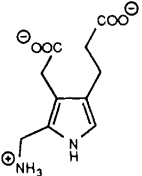
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Table 4. (Continued)

Inhibitor	Inhibition Constant	Enzyme Source	Michaelis Constant	Ref.
7  198	$K_I = 1.4 \text{ mM}$	<i>Chlorella regularis</i>		330
	$K_I = 0.65 \text{ nM}$	<i>Erythrobacter sp</i> OCH 114	$K_M = 0.29 \text{ mM}$	177
8  81	$K_I = 60 \text{ }\mu\text{M}$	<i>Scendesmus obliquus</i>		23
	$K_I = 0.8 \text{ mM}$	<i>Erythrobacter sp</i> OCH 114	$K_M = 0.29 \text{ mM}$	177
	$K_I = 5 \text{ mM}$	<i>Clostridium tetanomorphum</i>		325
	50% (3 mM)	rabbit reticulocytes	$K_M = 0.5 \text{ mM}$	323
	50% (3.3 mM)	<i>R. spheroides</i>	$K_M = 0.7 \text{ mM}$	184
36% (1 mM)	<i>Hardeum vulgare L.</i> (barley)		328	
9  82	30% (33 mM)	<i>Mycobacterium phlei</i>		192
	0% (<100 mM)	rabbit reticulocytes	$K_M = 0.5 \text{ mM}$	323
	0% (10 mM)	<i>R. spheroides</i>	$K_M = 0.7 \text{ mM}$	184
10  195	50% (1 mM)	rabbit reticulocytes	$K_M = 0.5 \text{ mM}$	323
11  77	50% (10 mM)	rabbit reticulocytes	$K_M = 0.5 \text{ mM}$	323
	$K_I = 4 \text{ mM}$	?	$K_M = 0.85 \text{ mM}$	199
12  199	$K_I = 17 \text{ mM}$	<i>Erythrobacter sp.</i> OCH 114	$K_M = 0.29 \text{ mM}$	177
	$K_I = 17 \text{ mM}$	<i>Erythrobacter sp.</i> OCH 114		331

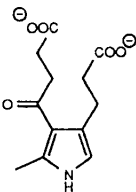
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**Table 4.** (Continued)

Inhibitor	Inhibition Constant	Enzyme Source	Michaelis Constant	Ref.
13  79	10% (3.3 mM)	<i>R. spheroides</i>	$K_M = 0.7$ mM	184
14  78	0% (10 mM)	<i>R. spheroides</i>	$K_M = 0.7$ mM	184
15  76	5% (10 mM)	<i>R. spheroides</i>	$K_M = 0.7$ mM	184
16  197	50% (20 mM)	rabbit reticulocytes	$K_M = 0.5$ mM	323
17  187	0% (<100 mM) 3% (10 mM)	rabbit reticulocytes <i>R. spheroides</i>	$K_M = 0.5$ mM $K_M = 0.7$ mM	323 184
18  200	50% (3 mM) 7% (3.3 mM)	rabbit reticulocytes <i>R. spheroides</i>	$K_M = 0.5$ mM $K_M = 0.7$ mM	323 184
19  9	$K_I = 2$ mM	bovine liver		26, 162

(continued)

Table 4. (Continued)

Inhibitor	Inhibition Constant	Enzyme Source	Michaelis Constant	Ref.
20  201	$K_I = 5 \mu\text{M}$	<i>Clostridium tetanomorphum</i>		325
21 heme 10	50% (0.33 $\mu\text{M}$ )	<i>R. spheroides</i>	$K_M = 0.7 \text{ mM}$	182
22 protoporphyrin IX 18	27% (0.1 mM)	<i>Erythrobacter sp.</i> OCH 114	$K_M = 0.29 \text{ mM}$	177
23 ICH <sub>2</sub> CONH <sub>2</sub> 202	100% (1 mM)	rabbit reticulocytes	$K_M = 0.5 \text{ mM}$	323
24 EDTA 203	$K_I = 40 \mu\text{M}$	rabbit reticulocytes	$K_M = 0.5 \text{ mM}$	323
25 N(CH <sub>2</sub> COOH) <sub>3</sub> 204	$K_I = 0.8 \text{ mM}$	rabbit reticulocytes	$K_M = 0.5 \text{ mM}$	323
26 H <sub>3</sub> CH(CH <sub>2</sub> COOH) <sub>2</sub> 205	$K_I = 8 \text{ mM}$	rabbit reticulocytes	$K_M = 0.5 \text{ mM}$	323

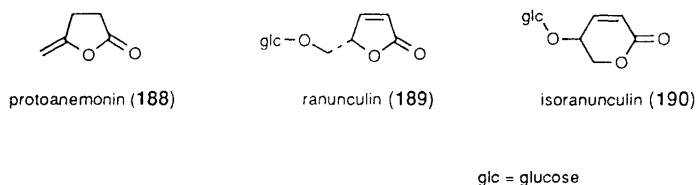
etiolated and greening barley leaves indicated not only a strong inhibition of PBGS but also a strong inhibition of the uptake of amino acids and a reduction of their incorporation into proteins.<sup>328,336</sup>

Another interesting inhibitor of PBGS is the 4,5-dioxovalerate (**198**),<sup>23,177,330</sup> which is a supposed intermediate in the biosynthesis of 5-aminolevulinic acid from glutamate in certain organisms.<sup>23,337</sup> 4,5-Dioxovalerate (DOVA, **198**) is only a moderate inhibitor roughly of the same strength as levulinic acid (**81**). If its function as biosynthetic precursor of 5-aminolevulinic acid can be confirmed, then DOVA would have a surprising double function as precursor for the biosynthesis and as regulator of the transformation of 5-aminolevulinic acid (**17**).<sup>23</sup>

Studying the metabolism of glyoxylic acid in *Mycobacterium takeo*, the formation of 5-hydroxylevulinic acid (**193**) was detected,<sup>338</sup> which is formed in a thiamine-catalyzed reaction from  $\alpha$ -ketoglutarate (**77**) followed by decarboxylation.<sup>339</sup> 5-Hydroxylevulinic acid (**193**) could be isolated from *Hellegorus foetidus* plants and derivatives of **193** as the glycosides **189**, **190**, and protoanemin (**188**) could be identified (Figure 84).<sup>340,341</sup>

When cell-free extracts of *Mycobacterium phlei* were incubated with **193** or with the substrates, the cofactors and the carboligase necessary for the synthesis of **193**, a remarkable inhibition of the porphyrin biosynthesis was observed.<sup>324</sup>





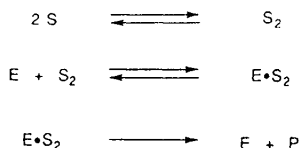
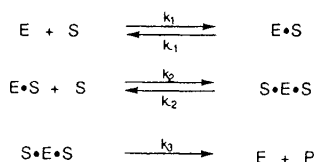
**Figure 84.** Natural products derived from 5-hydroxylevulinate.

Most of the other inhibitors isolated from different natural sources contain the succinyl moiety. It could be shown that 4-keto-5-amino-hexanoic acid (**191**), a homologue of the natural substrate, and 4-keto-5-amino-6-hydroxy hexanoic acid (**192**) were reasonably good inhibitors of PBGS.<sup>192</sup> 5-Chlorolevulinic acid (**194**) could not only act as a suicide inhibitor at high concentration but it was also shown to be a competitive inhibitor at low concentrations.<sup>248</sup> These results are in good agreement with the widespread use of levulinic acid (**17**), the prototype compound containing the minimal necessary recognition structure.<sup>47,148,184,342</sup> Ketoglutarate (**77**) also formally belongs to this class of compounds. A recent report indicates that **77** is also a reasonable inhibitor.<sup>199</sup> This report is not in agreement with earlier findings, which consistently reported that **77** did not inhibit PBGS.<sup>148,184,206,323</sup> A compound derived from the natural substrate by reduction the 4-hydroxy-5-amino-valeric acid (**199**) is also reported to be a good inhibitor even though this compound lacks the succinyl moiety recognized to be essential for good inhibition.<sup>331</sup> Finally it has been reported that a protein acts as an inhibitor for PBGS and it was speculated that this protein has some regulatory functions.<sup>343</sup>

### C. Remarks Concerning Studies of the Inhibition Kinetics of PBGS

The examination of many of the possible mechanisms of PBGS is complex and leads to kinetics which cannot be analyzed according to a simple Michaelis–Menten approach. This difficulty has been clearly recognized by Granick and Mauzerall in a lucid discussion in a very early paper.<sup>206</sup> They discuss two kinetic schemes, for PBGS A and B. In proposal A, the ketimine-dimer of 5-aminolevulinate is the immediate substrate for the enzyme-catalyzed step. In proposal B, the two substrate molecules are sequentially bound to the enzyme (Figure 85).

Proposal A is dismissed for the following reasons.<sup>206</sup> The chemical condensation cannot be rate-determining, otherwise the enzyme concentration would have no influence on product formation. A fast spontaneous formation of the dimer in small quantities would lead to second-order dependence of the rate as a function of the substrate concentration. This second-order dependence is not observed so the only interpretation compatible with the kinetic data would be that the dimer  $S_2$  is rapidly formed, and is at the same time the predominant species present during the

Proposal A:Proposal B:

$$\Rightarrow v = \frac{k_3 \times E_0}{1 + \left[ \frac{k_{-2} + k_3}{k_2} + \frac{k_3}{k_1} \right] \frac{1}{S_0} + \frac{k_{-1}}{k_1} \left[ \frac{k_{-2} + k_3}{k_2} \right] \frac{1}{S_0^2}}$$

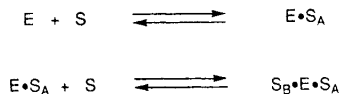
$$\text{to be able to neglect } \frac{1}{S_0^2} \quad S_0 > \frac{k_1}{k_2 \times k_3} \frac{k_{-1}}{k_{-2} + k_3}$$

$$\text{if } k_3 \text{ small} \quad \frac{k_{-1}}{k_1} < 10^{-4}$$

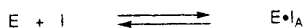
**Figure 85.** Mechanistic proposals and kinetic analysis of the PBGS catalyzed synthesis of porphobilinogen as discussed by Garnick and Mauzerall.

incubations. This can be rejected because only small quantities of 5-aminolevulinic acid are present as dimers.

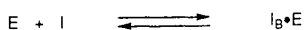
Proposal B only leads to the observed kinetic behavior, if the product formation is the slow step, and at the same time the dissociation constant of the first complex E·S must be smaller than the dissociation constant of the second complex S·E·S. The general equation obtained for the initial rate contains an expression depending on  $S^2$ . In order to be able to neglect the term  $S^2$ , the product formation must be rate-determining ( $k_3$  is small compared to  $k_{-1}$  and  $k_{-2}$ ) and the dissociation constant of the first complex must be small as well (Figure 85). Granick and Mauzerall suggested in 1958 that both molecules of 5-aminolevulinic acid form complexes with the enzyme, the first substrate being held more tightly than the second. They cite the possibility that the second substrate molecule "spontaneously" forms a ketimine with the first substrate. They invoke implicitly that the aldol condensation is the rate-determining step, which might be catalyzed by the presence of a metal ion.<sup>206</sup>

**General Mechanism:****Possible Sites for Inhibition:**

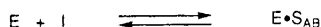
I) Inhibitor binds at site "A"



II) Inhibitor binds at site "B"



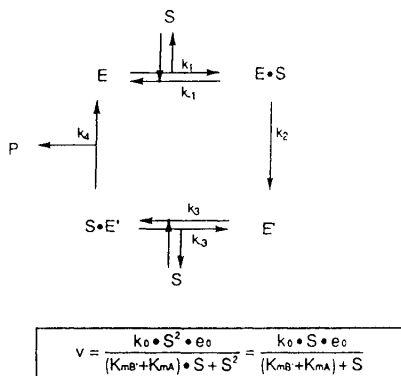
III) Inhibitor blocks site "A" and "B"



IV) 2 Molecules of inhibitor can bind sequentially

**Figure 86.** Possible sites for inhibition as analyzed by Tschudy.

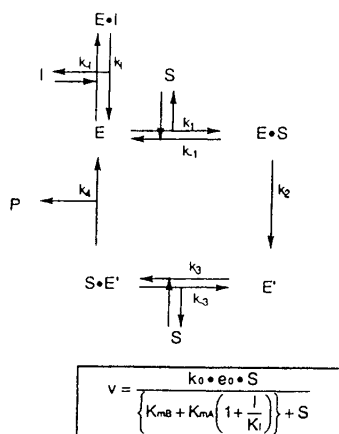
In the context of the inhibition of PBGS with succinylacetone, Tschudy et al.<sup>210</sup> discuss the different possibilities for an inhibitor to interfere with the catalytic process of an enzyme with two sites. The authors propose four possible sites for an inhibitor to bind to the enzyme (Figure 86).

**Figure 87.** Kinetics of the substituted enzyme mechanism.

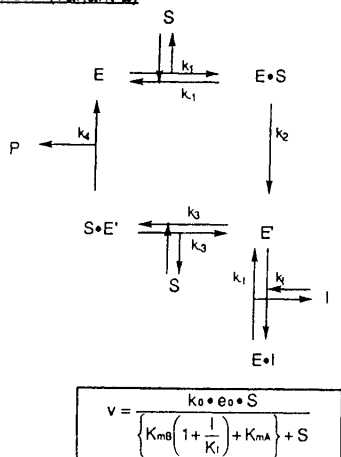
For the inhibition with succinylacetone they discard the possibility of blocking the enzyme with two molecules of inhibitor (site IV). Using the mechanistic proposals of Batlle,<sup>272</sup> they give preference to the suggestion that the inhibitor interacts at the "A-site." Since the inhibition of PBGS with succinylacetone is time-dependent, they assume an irreversible inhibition process.<sup>210</sup>

The assumption that the first substrate is undergoing a reversible association with the enzyme, immediately followed by a chemical bond formation (ketimine formation with the lysine amino group) so that this step becomes virtually irreversible, leads to a rate equation for a substituted-enzyme mechanism (Figure 87).

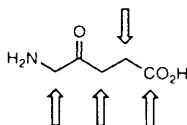
#### Inhibition (variant 1)



#### Inhibition (variant 2)



**Figure 88.** Kinetics for inhibition at the "A-site" or at the "B-site" of PBGS.



**Figure 89.** Different sites for structural variation of the substrate 5-aminolevulinic acid.

Kinetic analysis of this mechanistic scheme automatically leads to an expression which is not dependent on  $S^2$ . This explains the observed Michaelis–Menten kinetics for the normal transformation. This proposal easily accounts for the different inhibition behaviors as discussed by Tschudy<sup>210</sup> (Figure 88).

#### D. General Remarks on Our Inhibition Studies

It is clear that the inhibition constants  $K_I$  are no longer the simple dissociation constants of the inhibitor enzyme complex, but instead are more complicated expressions. As long as structurally similar inhibitors are used, the experimentally determined inhibition constants should still reflect the recognition between the enzyme and the inhibitor.

Keeping this *caveat* in mind, we engaged in a systematic study of specifically designed inhibitors for PBGS. Three categories of inhibitors were studied: analogues of the substrate, analogues of the product, and analogues of postulated intermediates.

#### E. Analogues of $\delta$ -Aminolevulinic Acid

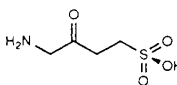
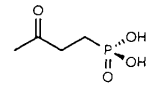
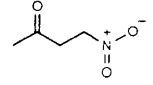
We synthesized and screened substrate analogues where all positions of the  $\delta$ -aminolevulinic acid had been modified with the exception of the ketofunction at the C4 position (Figure 89).

The inhibition of the compounds were evaluated by a UV/Vis assay monitoring product formation.  $K_I$  values were determined using Lineweaver–Burk analysis. The Lineweaver–Burk plot of all inhibitors was typical for a reversible competitive inhibition.<sup>344–346</sup>

##### Variations at Position C1

Three analogues were tested: the sulfonic acid analogue of  $\delta$ -aminolevulinic acid **206**, the phosphonic acid analogue **207** and the nitro analogue of levulinic acid **208** (Figure 90).

The sulfonic acid analogue **206** showed no inhibition up to concentrations 100 times higher than the value of the Michaelis constant. The phosphonic acid analogue of levulinic acid **207** was an extremely weak inhibitor as well. The inhibition constant for the phosphonic acid analogue was almost a factor 100 higher

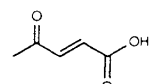
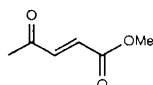
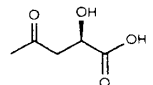
Structure	$K_m$	$K_i$	Inhibitor type
	<b>206</b> 0.26 mM $\pm 0.02$	–	–
	<b>207</b> 0.37 mM $\pm 0.01$	25 mM $\pm 3$	competitive
	<b>208</b> 0.26 mM $\pm 0.01$	18 $\mu$ M $\pm 3$	competitive

**Figure 90.**  $K_i$  for the inhibitors and  $K_M$  for the substrate determined from inhibition experiments for substrate analogues where the position C1 has been varied.

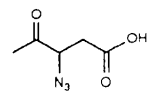
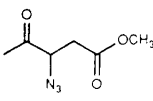
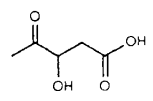
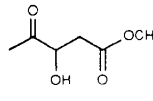
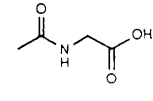
than the Michaelis constant of the substrate, indicating a negligible interaction between the enzyme and the inhibitor. The most powerful inhibitor was obtained when the carboxylic acid was replaced by the isosteric nitro group **208**.

### Variations at Positions C2 and C3

A series of eight compounds were tested where functional groups had been introduced at C2 and or C3 of levulinic acid (Figures 91 and 92).

Structure	$K_m$	$K_i$	Inhibitor type
	<b>209</b> 0.35 mM $\pm 0.03$	–	–
	<b>210</b> 0.25 mM $\pm 0.02$	–	–
	<b>211</b> 0.40 mM $\pm 0.1$	0.43 mM $\pm 0.13$	competitive

**Figure 91.**  $K_i$  for the inhibitors and  $K_M$  for the substrate determined from inhibition experiments for substrate analogues where the position C2 has been varied.

Structure		$K_M$	$K_I$	Inhibitor type
	<i>rac</i> - <b>212</b>	0.30 mM ± 0.01	2.2 mM ± 0.4	competitive
	<i>rac</i> - <b>213</b>	0.38 mM ± 0.02	5.5 mM ± 0.9	competitive
	<i>rac</i> - <b>214</b>	0.31 mM ± 0.03	1.2 mM ± 0.4	competitive
	<i>rac</i> - <b>215</b>	0.38 mM ± 0.09	7.3 mM ± 0.6	competitive
	<b>216</b>	0.38 mM ± 0.03	28 mM ± 2	competitive

**Figure 92.**  $K_I$  for the inhibitors and  $K_M$  for the substrate determined from inhibition experiments for substrate analogues where the position C3 has been varied.

The racemic 2-hydroxy levulinic acid **211** proved to be a good inhibitor with a  $K_I$  in the same order of magnitude as the  $K_M$  of the natural substrate. Surprisingly, the  $\beta$ -acetyl acrylic acid (**209**) did not show any inhibition up to a concentration of 13 mM. In contrast, at concentrations higher than 13 mM a complete inhibition of the product formation could be observed. The 3-azido (*rac*-**212**) and the 3-hydroxy levulinic acids (*rac*-**214**) were good inhibitors as well. The corresponding methyl esters *rac*-**213** and *rac*-**215** showed inhibition constants 2 to 6 times higher than the acids. *N*-Acetyl glycine (*rac*-**216**), the only inhibitor where the keto function at C4 was replaced by an amide function, is a poor substitute for the natural substrate.

### Variations at Position C5

Nine analogues where C5 of  $\delta$ -aminolevulinic acid had been varied were studied (Figure 93). As has been known from the studies of Shemin,<sup>184</sup> levulinic acid (**81**) is a quite good inhibitor. Replacing the ammonium group of the natural substrate by a hydroxy group **193** yields an inhibitor with an  $K_I$ -value, which is about equivalent to the Michaelis constant. Under the conditions of the inhibition studies, the 5-azido acid **218** was transformed by the thiols present into the 5-aminolevulinic

Structure		$K_m$	$K_i$	Inhibitor type
	<b>81</b>	0.36 mM ± 0.04	1.0 mM ± 0.1	competitive
	<b>114</b>	0.35 mM ± 0.02	–	–
	<b>217</b>	0.20 mM	–	–
	<b>218</b>	0.35 mM	3.1 mM	competitive
	<b>193</b>	0.40 mM ± 0.04	0.25 mM ± 0.05	competitive
	<b>214</b>	0.36 mM ± 0.04	2.7 mM ± 0.4	competitive
	<b>220</b>	0.30 mM ± 0.02	60 μM ± 15	competitive
	<b>77</b>	0.37 mM ± 0.01	19 mM ± 2	competitive
	<b>221</b>	0.28 mM ± 0.03	27 mM ± 4	competitive

**Figure 93.**  $K_i$  for the inhibitors and  $K_M$  for the substrate determined from inhibition experiments for substrate analogues where the position C5 has been varied.

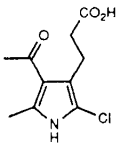
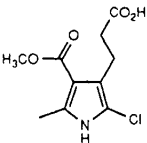
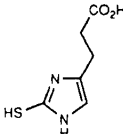
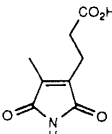
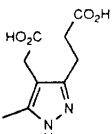
acid (**17**), the natural substrate. Therefore the inhibition constant could not be determined for the 5-azido acid **218**. In contrast to the other substrate analogues with substituents at C5, the 2-oxoglutarate **77** and the phthalimido derivative **221** were only very weak inhibitors.



## F. Analogues of Porphobilinogen

To be able to use the Ehrlich reaction to determine the amount of product formed, the product analogues had to be chosen so as not to interfere with the test reaction. Therefore the  $\alpha$ -position of the pyrroles had to be blocked (Figure 94).

The imidazole **224** and the maleimide **225** imitate only the propionic acid side chain and the aromatic NH-group of PBG **9**. The pyrazole **226** in contrast imitates the product of the enzyme catalyzed reaction nicely with the exception of the lacking amino group. Astonishingly enough, the pyrazole which structurally is nearest to the natural product is by far the weakest inhibitor. The maleimide **225**

Structure	$K_m$	$K_i$	Inhibitor type
	<b>222</b> 0.30 mM $\pm 0.05$	13 mM $\pm 2$	competitive
	<b>223</b> 0.34 mM $\pm 0.04$	15 mM $\pm 0.5$	competitive
	<b>224</b> 0.31 mM $\pm 0.01$	20 mM $\pm 5$	competitive
	<b>225</b> 0.30 mM $\pm 0.02$	12 mM $\pm 2$	competitive
	<b>226</b> 0.25 mM $\pm 0.03$	32 mM $\pm 4$	competitive

**Figure 94.**  $K_i$  for the inhibitors and  $K_M$  for the substrate determined from inhibition experiments for product analogues.

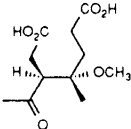
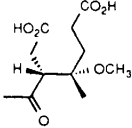
and the two pyrroles **222** and **223**, which both lack the acetic acid side chain and the amino function, are almost 3 times better inhibitors than the pyrazole **226**.

### G. Analogues of a Potential Intermediate

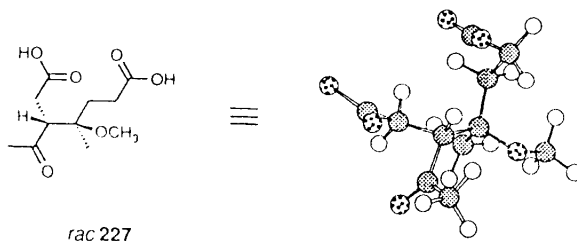
The two major alternatives for the mechanism of PBGS can be differentiated according to the first covalent bond formed between the two 5-aminolevulinic acid units. The first bond can be the formation of the intermolecular Schiff base or the product of the aldol reaction.

The chemical reactivity of the aldol product is such that, even without the presence of the enzyme, pyrrole formation could occur.<sup>321</sup> Therefore we had to use analogues of the postulated intermediate which lack the amino group involved in the pyrrole formation. The other amino group in the  $\alpha$ -position from the ketone also had to be left out because  $\alpha$ -amino ketones dimerize forming dihydropyrazines.<sup>315,316</sup> Thus analogues were synthesized which lack both amino groups (Figure 95).

The intermediate postulated by Shemin<sup>184</sup> can exist as four stereoisomers. In order to check if the aldol product is a potential intermediate we needed analogues which are chemically and configurationally stable. To assure that both conditions are fulfilled we decided to study the products *rac*-**227** and *rac*-**228** of the Mukaiyama crossed-aldol reaction between the silyl enolether of levulinic acid **115** with the acetal of levulinic acid **122**. The two diastereoisomeric pairs of enantiomers, *rac*-**227** and *rac*-**228**, were studied separately. The inhibition studies showed that both pairs of enantiomers *rac*-**227** and *rac*-**228** were weak inhibitors. One of the two pairs of enantiomers, *rac*-**228**, possessed an inhibition constant, which was more than 2 times smaller than the constant for the other *rac*-**227**. The determination

Structure		$K_m$	$K_i$	Inhibitor type
	<i>rac</i> - <b>227</b>	0.28 mM $\pm 0.04$	25 mM $\pm 2$	competitive
	<i>rac</i> - <b>228</b>	0.23 mM $\pm 0.01$	11 mM $\pm 1$	competitive

**Figure 95.**  $K_i$  for the inhibitors and  $K_M$  for the substrate determined from inhibition experiments for analogues of one of the putative intermediates postulated by Shemin.



**Figure 96.** X-ray-structure of the compound *rac* 227 determined by the group of Kratky.

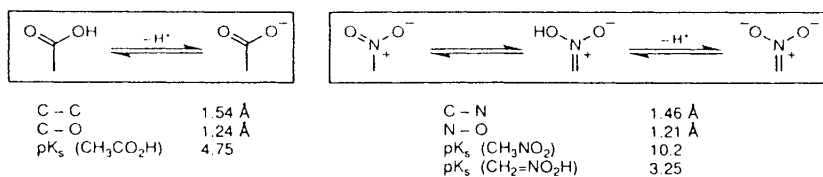
of the relative configuration was difficult. Happily enough, the less efficient inhibitor *rac*-227 crystallized and the structure could therefore be determined via X-ray (Figure 96).<sup>347</sup>

## H. Conclusions

The inhibition studies with substrate analogues allow us to draw the following conclusions. The carboxylic acid function at C1 is very important for the recognition process. Sulfonic acid and phosphonic acid analogues show a drastically decreased inhibition potency. In these two analogues the planar trigonal carboxylate has been replaced by tetragonal substituents. Sulfonic acid will be present as sulfonate, whereas phosphonic acid should mainly be present as a dianion. In contrast to these results the  $sp^2$ -hybridized nitro group showed the smallest inhibition constant of all inhibitors studied. Nitro groups are isosteric with carboxylate groups.<sup>348,349</sup> The inhibition constant of the nitro analogue is almost 15 times smaller than the Michaelis constant. This cannot be explained by sheer isosteric replacement (Figure 97).

In the case of the nitro analogues of citrate, the bonding of the analogues to aconitase is considerably better than the bonding of the substrates. This has been explained assuming that the nitronate form was an analogue of the transition state.<sup>350</sup> This leads to a much higher affinity to the active site of the enzyme, and thereby explains the highly successful competitive inhibition. Neither the nitro analogue **208** of levulinic acid nor the nitronate form of this inhibitor are analogues of any possible PBGS transition state. It is therefore *a priori* not obvious why the nitro compound possesses this high affinity to the enzyme.

Additional substituents at the positions C2 and C3 are tolerated as long as the inhibitors keep their flexibility around the C2–C3 bond. The acrylate **209** shows a very weak inhibition, whereas the hydroxy derivatives, *rac*-**212** and *rac*-**211**, and the azido derivative, *rac*-**214**, are good inhibitors. The interpretation of the inhibition results is difficult because it is not obvious if the “A-site,” the “P-site,” or both sites are blocked by the inhibitors. If the inhibitors preferentially block the “P-site”, which would be in accordance with the results of Jordan,<sup>238,239</sup> the interpretation is

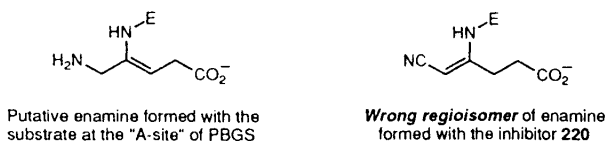


**Figure 97.** Comparison of the structure and the pK<sub>s</sub> of acetic acid, nitro methane, its aci-form, and the corresponding deprotonated forms.

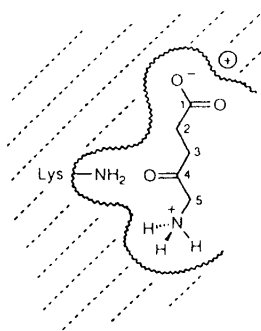
delicate as long as no X-ray structure or extensive NMR study is known. Assuming that the “A” site” or both sites can be blocked renders the interpretation easier. If the substrate analogue is fixed to the “A” site” of the active cleft, there should be space available for an additional substituent at the C3 position. Substrate analogues with additional substituents at C3 like *rac*-**212** and *rac*-**214** are indeed good inhibitors. The methylene group at C2 is not involved in any of the proposed mechanistic schemes. A rational interpretation of the inhibition that results with inhibitors modified at C2 is not yet possible. Variations at C5 are tolerated as shown by the inhibition results of **81**, **217**, **193**, and **220**.

The amino or ammonium group at C5 is not an essential element of recognition. This is in surprising contrast to other enzyme-catalyzed reactions where the presence of the charged ammonium group is crucial for the recognition process. Neutral and polar substituents can be used to replace the amino group. Switching to a negatively charged carboxylate **77**, or to a sterically demanding group such as the monophthalimide **221**, resulted in inhibitors with a low affinity. The nitrile derivative **220** is an excellent inhibitor. The protons at the methylene group at C5 are very acidic. In D<sub>2</sub>O as well as in CD<sub>3</sub>OH the <sup>1</sup>H NMR spectra showed complete deuterium hydrogen exchange. The excellent inhibition by the nitrile might arise from the formation of the enamine into the “wrong” position at the active site of the enzyme (Figure 98).

The picture of the recognition site of the enzyme porphobilinogen synthase from *Rhodospseudobacter spheroides* has to be adapted to our results (Figure 99). Besides the carbonyl group at position C4, which is essential for the recognition, there is a high specificity for the planar carboxylate group at C1. Some space is available for



**Figure 98.** Putative explanation for the strong inhibition observed for the inhibitor **220**.



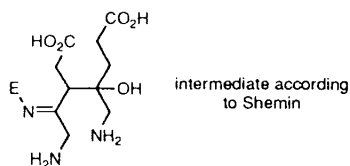
**Figure 99.** Putative structure at the active site as deduced from the inhibition studies.

additional substituents at positions C2 and C3. As long as the second substrate does not interact with the active site, thereby blocking the additional space available, this interpretation of the results is reasonable. The recognition of the 5-amino group is not very strong.

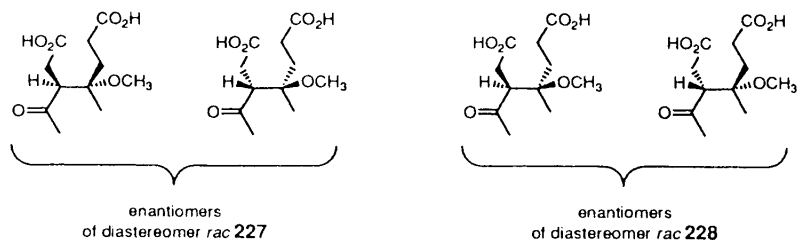
The product analogues studied were all only moderate inhibitors. The inhibition constants determined are larger by a factor of at least 5 than the preliminary values reported by either Shemin<sup>26</sup> or Jordan<sup>162</sup> from studies with radioactively marked PBG. Also the NMR studies of Jaffe<sup>253,255</sup> indicate a good recognition of the product by the active site of the enzyme. The steric and electronic differences of the product analogues used in this study compared with PBG are substantial. This may explain the difference in inhibition potency. Surprising in this respect is the result of the studies with the pyrazole derivative **226** where two of the three side chains correspond exactly to those of the natural product. Using product analogues, our inhibitors intervene at a different stage of the reaction sequence than the analogues of the substrate. Therefore the values of the inhibition constant cannot be directly compared between the two classes of inhibitors. The interpretation of the results has therefore to be made with caution.

The most interesting result is the inhibition studies with the analogues *rac*-**227** and *rac*-**228** of the potential intermediate. Inhibition studies with the racemate of one diastereoisomer, *rac*-**227**, showed no or only a very weak inhibition (Figures 99 and 100).

The inhibition studies with the racemate of the other diastereoisomer, *rac*-**228**, gave an inhibition constant of 11 mM, which corresponds to the value we determined for our best product analogues **222**, **223**, and **225**. As we used the racemic mixture we have to assume that the inhibition stems from only one of the enantiomers. For the pure enantiomer this would lead to an inhibition constant of approximately 5–6 mM, which is comparable to  $K_M$  of the substrate and to the  $K_I$ 's of other substrate analogues. In order to get a chemically and configurational stable inhibitor



Analogues of the postulated intermediate :

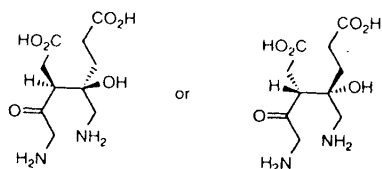


**Figure 100.** Structure of the two diastereoisomeric analogues of the postulated intermediate.

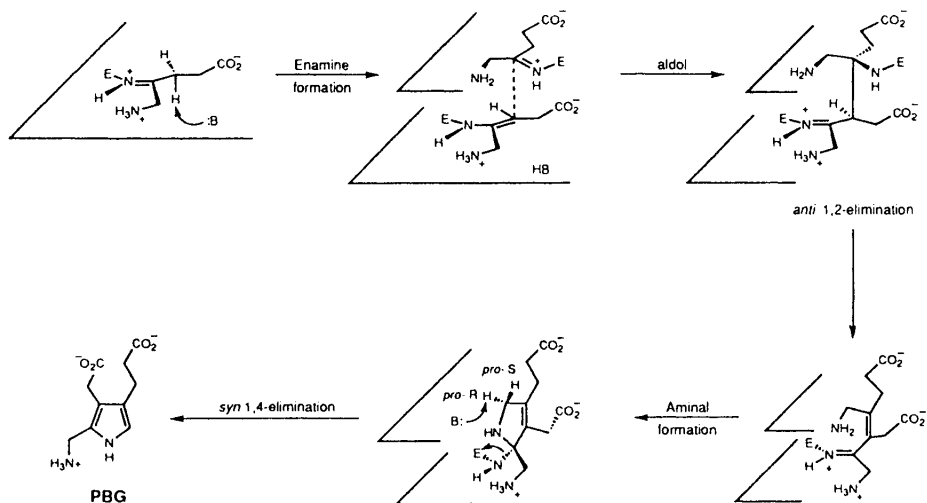
we have suppressed both amino groups. From the comparison between levulinic acid (**81**) with 5-aminolevulinic acid (**17**) we know that we lose roughly a factor of 3 in suppressing the amino group. Assuming that further studies will prove that our hypotheses are correct, we postulate that the aldol intermediate possesses either of the two drawn relative and absolute configurations (Figure 101).

### I. Postulated Mechanism for PBGS from *Rhodopseudobacter spheroides*

Using the information known from the literature and assuming that the relative configuration of the aldol intermediate corresponds to that of our better inhibitor, *rac*-228, the mechanism first postulated by Shemin<sup>184</sup> and later adjusted by Jordan<sup>220,238,239</sup> can now be modified so as to include the newly accumulated knowledge (Figure 102).



**Figure 101.** The two enantiomeric structures of the intermediate postulated by Shemin as deduced from the results of the inhibition studies.



**Figure 102.** Tentative mechanism for the biosynthesis of porphobilinogen including the postulates deduced from the inhibition studies. The role of the Zn-ion(s) has been consciously omitted in order to focus the attention on the bond-breaking and bond-making steps of the two substrate molecules (see also the discussion at the beginning of Section III.C).

The postulated mechanism is based on the following assumptions: Imine or enamine formation between the lysine of the enzyme and the carbonyl group occurs with both substrates, and the relative configuration at the two chiral centers corresponds to the relative configuration of the aldol intermediate. To illustrate the postulated mechanism the (3*R*,4*S*)-stereoisomer has been arbitrarily chosen, which automatically leads to a stereoelectronically preferred *trans* elimination and to the fact that the *pro-R* hydrogen is eliminated from the position C5 of the substrate bound to the “P-site.” It is assumed that the intermediate is still bound to the enzyme and that consequently the deprotonation and the elimination of the lysine amino group is the last step in the sequence, which forms the pyrrole ring via a *syn* 1,4-elimination and liberates the product at the same time.

This mechanism concentrates almost exclusively on the chemical transformations of the two substrate molecules. It consciously neglects the role of the different type Zn<sup>2+</sup>-ions which has been shown for the mammalian and the *E. coli* enzyme, but not as yet for other enzymes. Since it is not known if PBGS's from all sources work according to the same mechanism, the concentration on the chemical reactivity of the postulated intermediates allows a general description. As soon as more details about the enzymatic mechanism(s) are known, one will be able to compare these observations with the conclusions based on the chemistry. This comparison

will allow one to evaluate the influence of the enzyme on the dimerization of 5-aminolevulinic acid to PBG.

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