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The Porphobilinogen Conundrum in Prebiotic Routes to Tetrapyrrole Macrocycles

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Abstract Attempts to develop a credible prebiotic route to tetrapyrroles have relied on enzyme-free recapitulation of the extant biosynthesis, but this process has foundered from the inability to form the pyrrole porphobilinogen (**PBG**) in good yield by self-condensation of the precursor δ-aminolevulinic acid (**ALA**). **PBG** undergoes robust oligomerization in aqueous solution to give uroporphyrinogen (4 isomers) in good yield. **ALA**, **PBG**, and uroporphyrinogen III are universal precursors to all known tetrapyrrole macrocycles. The enzymic formation of **PBG** entails carbon-carbon bond formation between the less stable enolate/enamine of one **ALA** molecule (3-position) and the carbonyl/imine (4-position) of the second **ALA** molecule; without enzymes, the first **ALA** reacts at the more stable enolate/enamine (5-position) and gives the pyrrole **pseudo-PBG. pseudo-PBG** cannot self-condense, yet has one open α-pyrrole position and is proposed to be a terminator of oligopyrromethane chain-growth from **PBG**. Here, 23 analogues of **ALA** have been subjected to density functional theoretical (DFT) calculations, but no motif has been identified that directs reaction at the 3-position. Deuteration experiments suggested 5-(phosphonooxy) levulinic acid would react preferentially at the 3- versus 5-position, but a hybrid condensation with **ALA** gave no observable uroporphyrin. The results suggest efforts toward a biomimetic, enzyme-free route to tetrapyrroles from **ALA** should turn away from structure-directed reactions and focus on catalysts that orient the two aminoketones to form **PBG** in a kinetically controlled process, thereby avoiding formation of **pseudo-PBG**.

Keywords δ-aminolevulinic acid · Porphyrinogen · Porphyrin · DFT · Biosynthesis · Biomimetic

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Introduction

The questions of whether tetrapyrrole macrocycles formed in the prebiotic era and may have contributed to the origin of life are not known and indeed may not be answerable with scientific certainty, yet the identification of routes that form porphyrins from simple precursors would augur in support of such chemistries. The inventory of tetrapyrroles produced by modern biosynthesis—the so-called pigments of life—includes cobalamin, F_{430}, heme, chlorophylls and bacteriochlorophylls (Battersby et al. 1980; Battersby 1985). A striking feature of this collection of macrocycles is that each member originates from a common precursor (Bali et al. 2014), uroporphyrinogen III (Fig. 1). Uroporphyrinogen III in turn derives from four
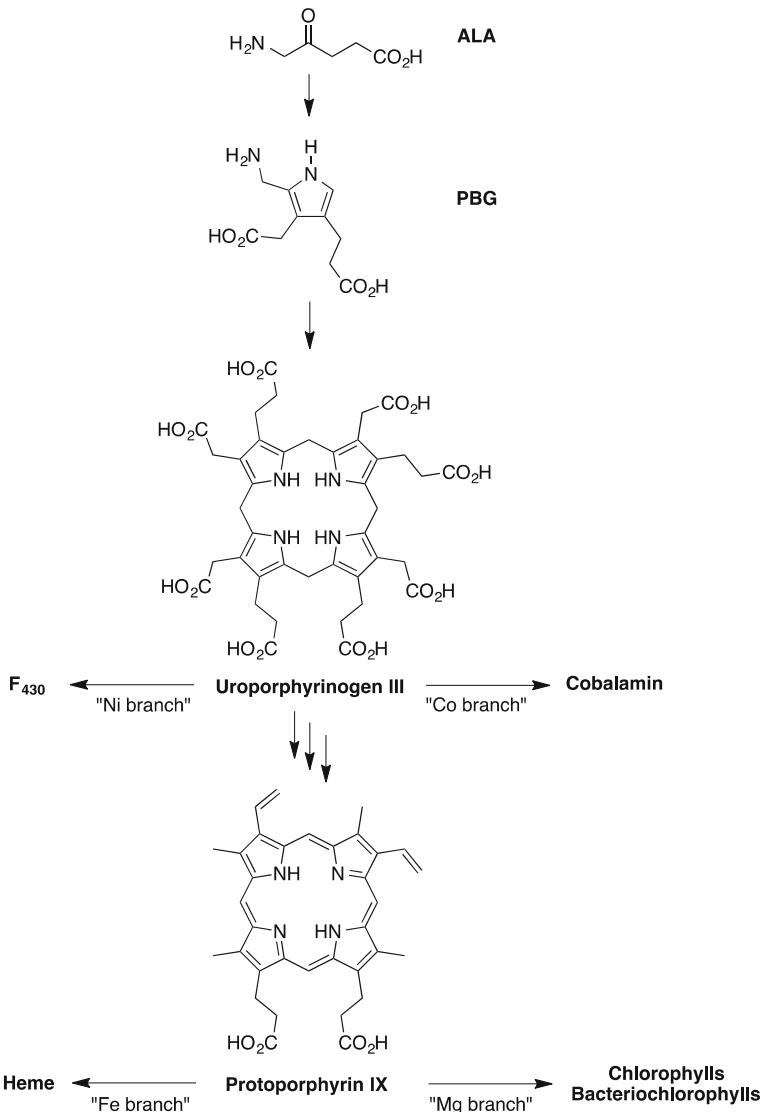

Fig. 1 Biosynthesis of tetrapyrrole macrocycles (all species shown in neutral, non-ionized form for clarity)

molecules of porphobilinogen (**PBG**), which itself forms from two molecules of δ-aminolevulinic acid (**ALA**). The elucidation of the early steps of tetrapyrrole biosynthesis leading to and from uroporphyrinogen III prompted parallel chemical studies to examine whether such a path to tetrapyrroles might be recapitulated in the absence of enzymes (Mauzerall 1960a). If so, such a "structure-directed" pathway (Eschenmoser 2007) would likely have compelling attributes for prebiotic prevalence.

PBG undergoes condensation in solution in the absence of enzymes to form a statistical mixture of uroporphyrinogen isomers (Fig. 2), of which uroporphyrinogen III is the statistically most likely member (Mauzerall 1960b; Taniguchi and Lindsey 2012). Chromatographic conditions have only been developed to separate 3 of the expected 4 isomers (Rideout et al. 1983). The reaction occurs under a variety of conditions (Cookson and Rimington 1954; Mauzerall 1960a; Frydman et al. 1971), including a typical concentration of ~1 mM, the pH range of at least pH 1 to pH 10, temperature range from 20 to 100 °C, aerobic or anaerobic environment, the presence of modest concentrations of zinc acetate, and duration from a few hours to several weeks. All known conditions for **PBG** reactions, include several reported here, are listed in the Appendix (see Table 7).
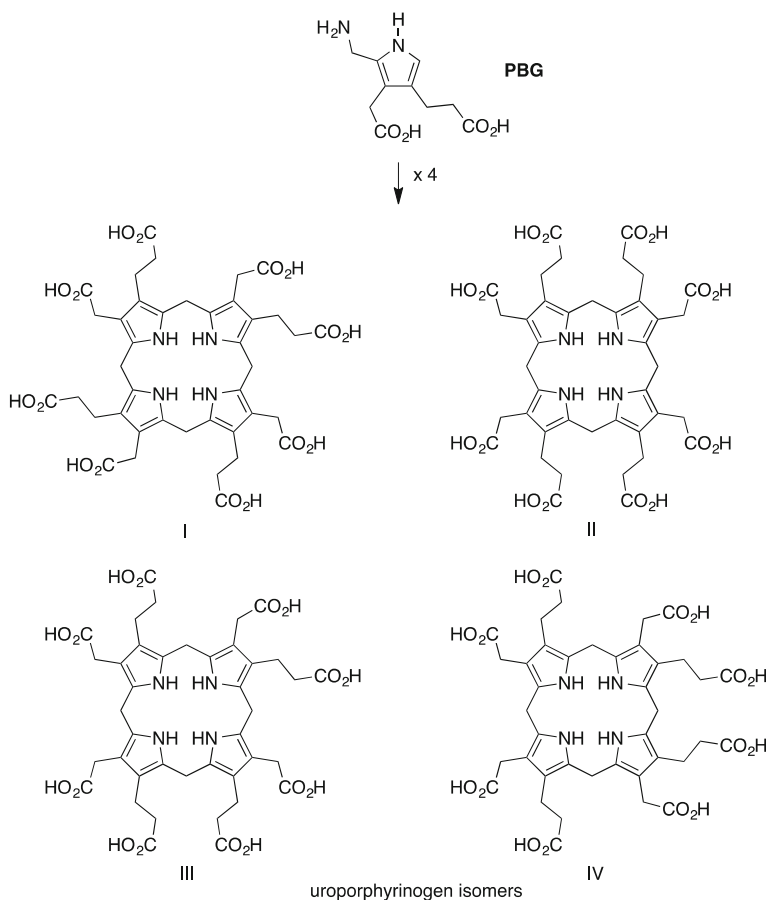

Fig. 2 Self-assembly of uroporphyrinogen isomers from **PBG** (all species shown in neutral, non-ionized form for clarity)

   The facile reactivity of **PBG** stems from a handful of factors (Fig. 3) (Lindsey et al. 2011). The features that underpin the reactivity include the following: (1) an aminomethyl group at one α-position, which is readily protonated (pK_a 10.1, Granick and Bogorad 1953) at neutral pH, leading to displacement of the resulting ammonium moiety to form a resonance-stabilized azafulvenium ion, a potent electrophile (see structures i and ii in Fig. 3); (2) no substituent at the other α-position, thereby enabling oligomerization at the two α-positions; (3) alkyl groups at both β-positions, which are electron-releasing and thereby render the pyrrole strongly reactive toward electrophiles; (4) two carboxylic acid groups at the termini of the β-alkyl groups (pK_a values 3.70 and 4.95, acetic and propionic acid respectively, Granick and Bogorad 1953), thereby affording a water-soluble compound given that at neutral pH, the dominant **PBG** species is the dicarboxylate–ammonium salt (**PBG** is typically isolated in solid form as the ·HCl salt as shown in the inset in Fig. 3); and (5) possibly, the intramolecular interaction of the β-acetate substituent with the adjacent α-methyl site, either stabilizing the incipient carbocation or cyclizing to form a six-membered lactone (see structure iii in Fig. 3). Together, these features provide ideal, if not almost magical, reactivity for a water-soluble precursor that underpins the self-assembly to afford uroporphyrinogens. Both types of macrocycles are photochemically active—uroporphyrinogens in the far ultraviolet region, and uroporphyrins in the ultraviolet and visible regions; and both have been proposed as valuable constituents in proto-photosynthesis (Granick 1967; Mauzerall 1978a, b, 1998; Mercer-Smith and Mauzerall 1981, 1984; Mercer-Smith et al. 1985).

   The successful condensation of **PBG** to form uroporphyrinogens led naturally to studies of the next earlier step, the solution condensation of **ALA** to form **PBG**. Such studies have led to conflicting reports over the years (vide infra), yet a general framework for understanding the course of reaction is shown in Fig. 4 (Franck and Stratmann 1981; Butler and George 1992). The reaction in solution leads chiefly to the dihydropyrazine (**I**) and **pseudo-PBG**, with no— or at most trace amounts of—**PBG**. Other minority species, such as hydrated **ALA** (Jaffe 2004) are not shown. The formation of **pseudo-PBG** rather than **PBG** stems from the enhanced reactivity of the 5-position versus 3-position in the initial condensate of two **ALA**
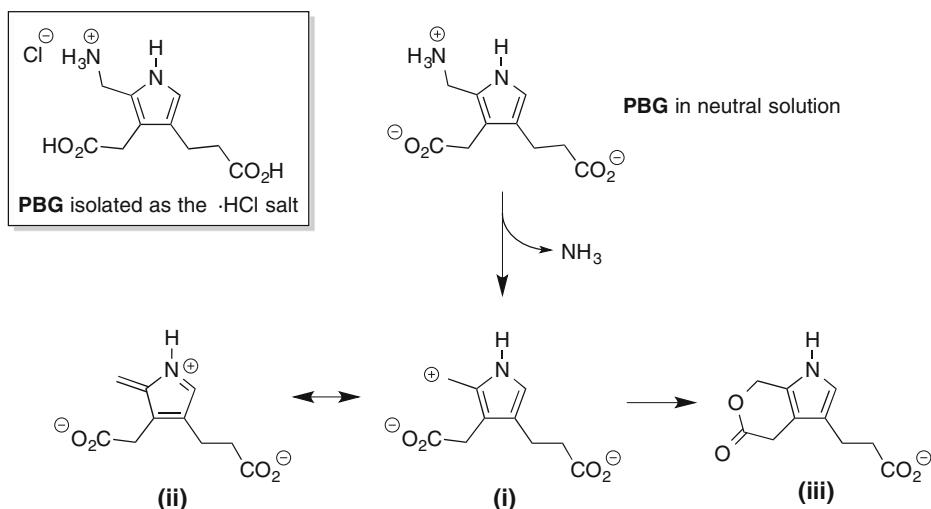

Fig. 3 Ionization and reactivity features of **PBG** (pH 7)

molecules. In the longstanding terminology of the heterocyclic chemistry field, **PBG** can be said to be a Knorr pyrrole (Knorr 1884) whereas **pseudo-PBG** is a Fischer-Fink pyrrole (Fischer and Fink 1944; Chandrashaker et al. 2012). The outcome of the self-condensation of **ALA** in solution is obviously at odds with the enzymic process mediated by PBG synthase, EC 4.2.1.24, which is also known as ALA dehydratase (Jaffe 2004; Jaffe and Lawrence 2014). While the outcome of the enzymic process is clear, the mechanism including the sequence of steps and whether both or only one **ALA** molecule is enzyme-bound via an imine, is not known with certainty (Frère et al. 2006; Erdtman et al. 2010; Tian et al. 2012).

The challenges associated with settling on a consensus enzyme mechanism include (1) enzyme diversity across eukarya, prokarya and archaea, including presence or absence of metals at the active site; (2) multimeric (e.g., octameric) assembly and quaternary allosterism to form the active enzyme, where each monomer is composed of ~300 amino acids; and (3) isolation of the enzyme active site from solvent. Representative mechanistic processes include the classic "Knorr mechanism" put forth nearly half a century ago (Shemin 1970), the "aldol mechanism" and what might be called the "dual imine mechanism" (lower half of Fig. 4) (Neier 1996; Jaffe 2004; Frère et al. 2006; Jaffe and Lawrence 2014). Regardless, the enzymic process fixes intermolecular carbon-carbon bond formation exclusively at the C3–C4 positions leading to **PBG**. In the structure-directed process, by contrast, intermolecular imine formation occurs first, whereupon intramolecular carbon-carbon bond formation occurs chiefly at the C5–C4 positions to close the ring, forming **pseudo-PBG**.

Loosening the strict constraint that **ALA** must also be the sole precursor to prebiotic tetrapyrroles opens the door to a number of possible alternative routes. Numerous routes are known for the synthesis of pyrroles (Bean 1990), although a one-flask, structure-directed synthesis to afford **PBG** from acyclic reactants is not known. Our approach toward a pathway for forming PBG-analogues has been to identify condensation processes of acyclic precursors, particularly with **ALA**, to ultimately form uroporphyrinogens or analogues thereof. A time-honored method for conversion of **ALA** to a pyrrole has entailed reaction with a β-dicarbonyl compound such as acetylacetone (Fig. 5) or ethyl acetoacetate (Mauzerall and Granick 1956; Mauzerall 1984). Such reaction succeeds because the β-dicarbonyl motif causes nucleophilic attack to occur at the carbon flanked by the two carbonyl groups thereby forming the Knorr pyrrole; only a small amount of the Fischer-Fink pyrrole forms (from the β-diketone), which is derived by condensation at the 5-position of **ALA** (Butler and George 1992; Chandrashaker et al. 2012). This phenomenon pointed the direction to the development of ALA-analogues that upon reaction with **ALA** would give rise to a PBG-analogue. At a minimum, such a route might constitute a chemical model for the prebiogenesis of tetrapyrrole macrocycles.

In a first route, we extended this robust chemistry by incorporation of a methoxy substituent at the 4-position of a β-ketoester (Fig. 6, left panel) (Lindsey et al. 2009). Upon reaction of methyl 4-methoxyacetoacetate (**MeO-AcEs**) with **ALA** in aqueous solution at 90 °C, condensation ensued to give the α-(methoxymethyl)-substituted pyrrole (**MeO-Pyr-Es**) upon elimination of two molecules of water. The pyrrole in turn underwent tetramerization to give the porphyrinogen **Pogen-Es**, which upon subsequent oxidation with I_2 gave the porphyrin in overall yield of ~5 % (not shown).

In a second route, a 1,5-dimethoxypentane-2,4-dione bearing a 3-carboxymethyl group (**1-AcOH**) underwent condensation with **ALA** in aqueous solution at 60 °C (Fig. 6, right panel) (Lindsey et al. 2011). The reaction process is more elaborate than that with methyl 4-methoxyacetoacetate: imine formation is accompanied by elimination of one molecule of water, then ring closure creates a quaternary center. The latter is relieved upon displacement

## Non-enzymic, structure-directed, solution synthesis
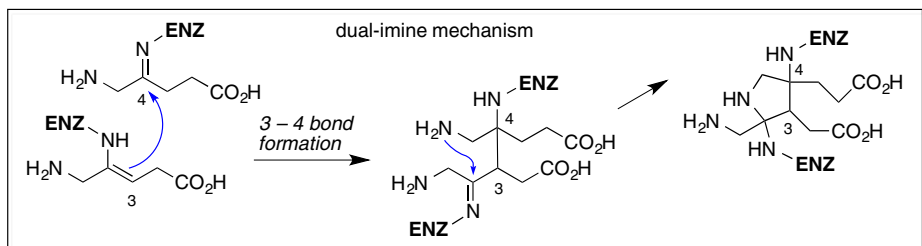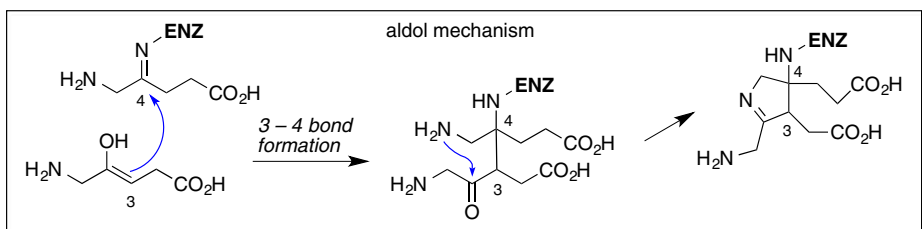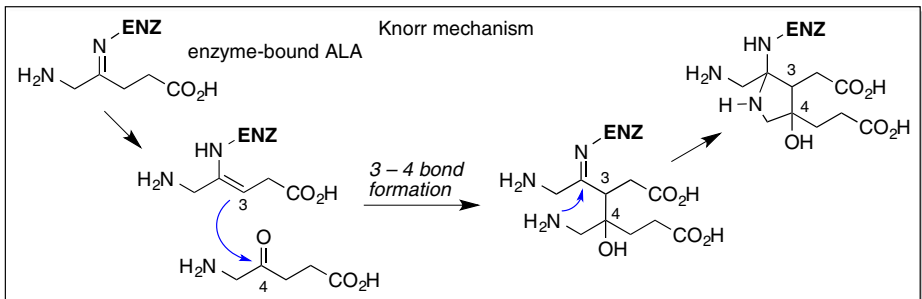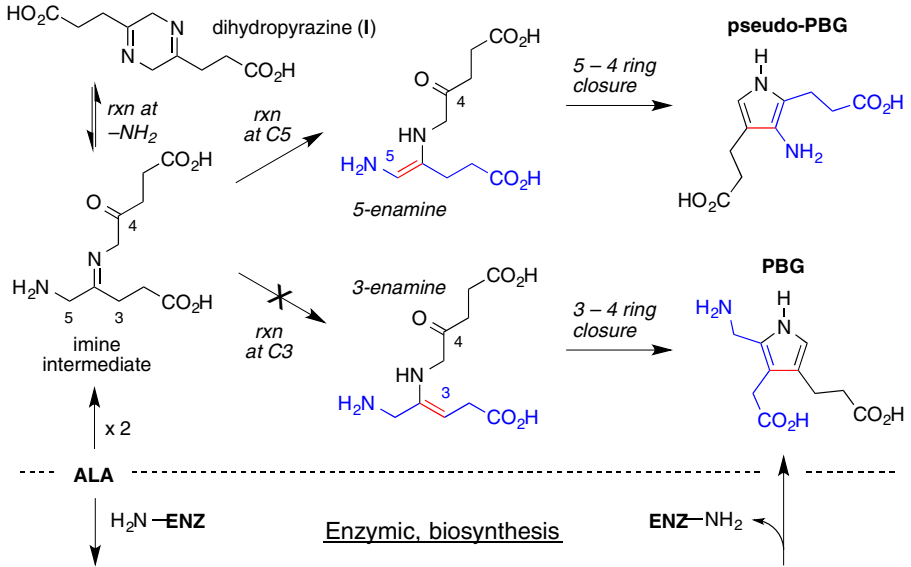
Knorr mechanism

aldol mechanism

dual-imine mechanism

of methoxyacetic acid, enabling aromatization to form the corresponding α-(methoxymethyl)-substituted pyrrole **MeO-PBG**. **MeO-PBG** differs from **PBG** solely in the presence of an α-methoxymethyl versus α-aminomethyl substituent. Subsequent tetramerization gave uroporphyrinogens, from which uroporphyrins have been obtained in yields as high as 15 %. The reaction also is robust, individually and combinatorially, toward a variety of 3-substituted analogues of **1-AcOH** (Taniguchi et al. 2012; Soares et al. 2012a, b, 2013a, b).

The two pyrroles formed via the two routes shown in Fig. 6 differ in the nature of one β-substituent, which profoundly affects the degree of activation toward electrophilic substitution—pyrrole **MeO-Pyr-Es** contains a deactivating carbomethoxy substituent, whereas pyrrole **MeO-PBG** contains an alkyl (acetic acid) substituent. In general, the presence of an alkyl pyrrole causes a substantial increase in reactivity toward electrophilic aromatic substitution (Jackson 1990a, b). Still, we wondered whether other ALA-analogues might afford condensation to give pyrroles equipped for self-condensation to form tetrapyrroles. The key for an α-aminoketone to form a pyrrole equipped for self-condensation is reaction at the position distal rather than adjacent to the amine (i.e., the 3-position rather than 5-position of **ALA**).

In this paper, we report density functional theoretical (DFT) calculations of 23 analogues of **ALA** to identify motifs that would enable self-condensation, or hybrid condensations with **ALA**, to form pyrroles and subsequently porphyrinogens. We also report reactivity studies of an ALA-analogue bearing a phosphonooxy (also termed dihydroxyphosphoryloxy) group at the 5-position of levulinic acid. No suitable reactants for a biomimetic, structure-directed, enzyme-free route to tetrapyrroles were identified, although the requirements for the non-enzymic conversion of **ALA** to **PBG** and then to uroporphyrinogens have been more sharply prescribed. In particular, the possible adverse effects of the Fischer-Fink pyrrole **pseudo-PBG** are described.

Fig. 5 Derivatization of **ALA** with a β-dicarbonyl compound (neutral, non-ionized species shown for clarity)
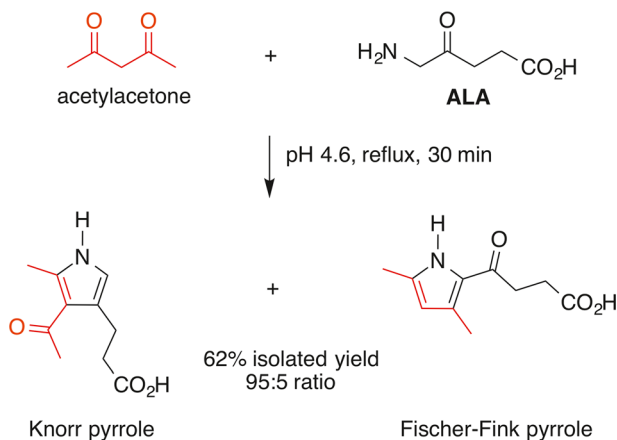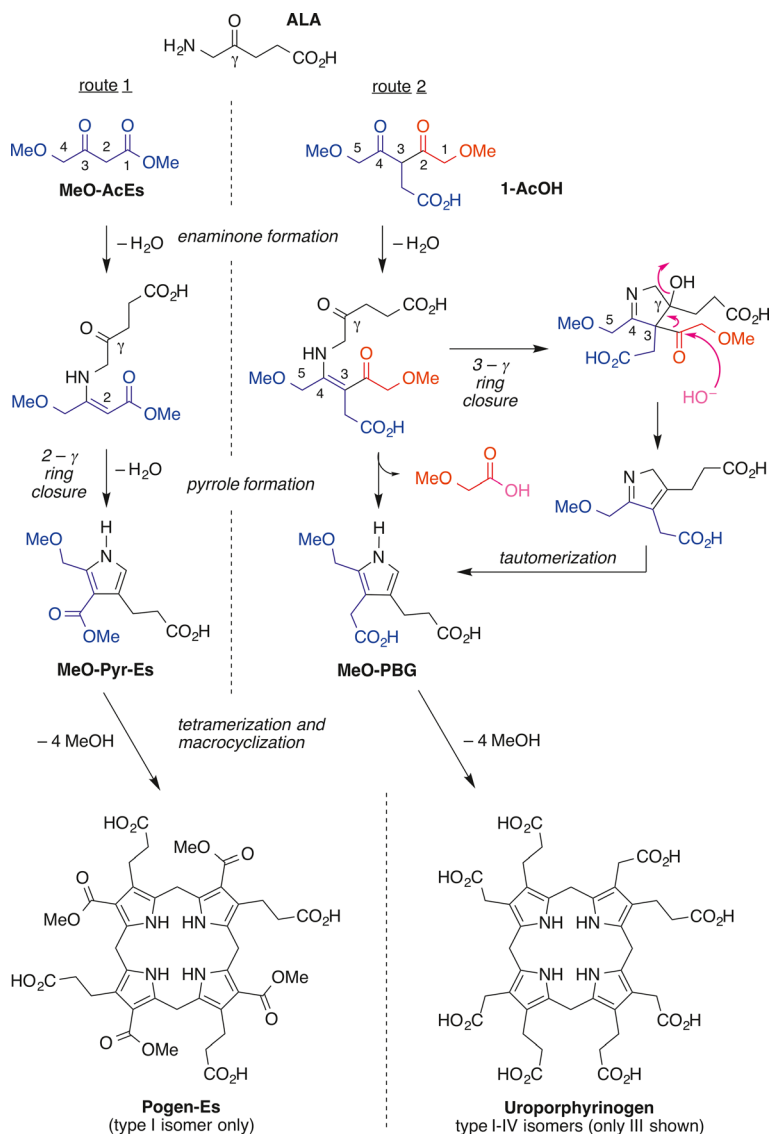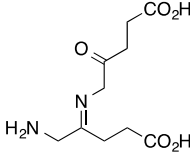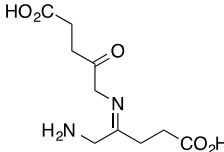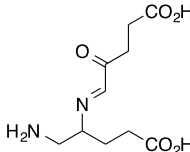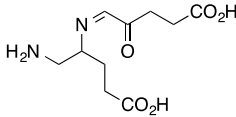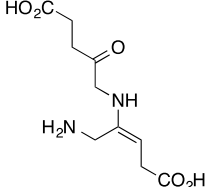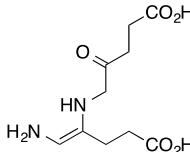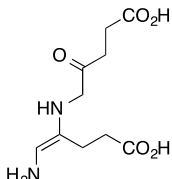
Fig. 6 ALA + β-dicarbonyl routes to porphyrinogens (neutral, non-ionized species shown for clarity)

Results

Assessment of Putative PBG Precursors by DFT Calculations

Calculations were carried out to identify the relative energies of the tautomers of the initial condensate derived from two molecules of **ALA**. The results for these structures are listed in Table 1. In this and subsequent tables and figures, all species are shown in the neutral, non-io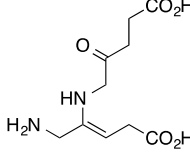nized form for clarity. In addition, the lowest energy value is displayed in red for clarity. The imine (entry 1) is set as the benchmark against which tautomers can be compared. Entry 2 also shows an imine,

Table 1 DFT calculations of (**ALA**)$_2$ initial condensates

Entry	Structure	Hartree	ΔE
1		−876.323721	0
2		−876.320040	+2.31
3		−876.329013	−3.32
4		−876.314424	+5.84
5		−876.313068	+6.68
6		−876.314698	+5.66
7		−876.320091	+2.28
8		−876.317960	+3.62

All ΔE values are in kcal/mol
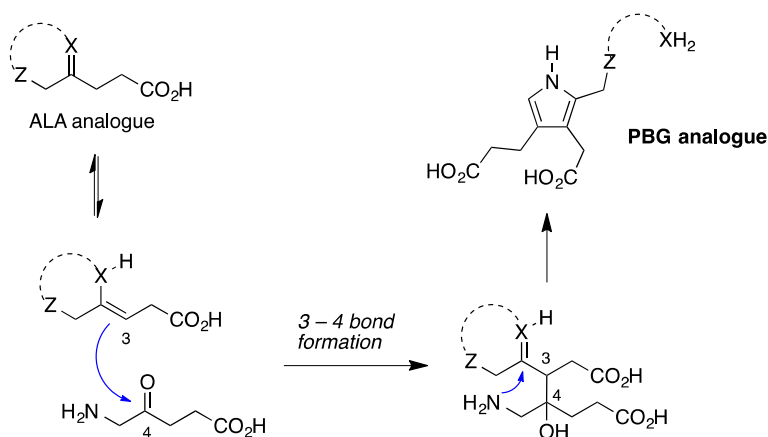
Fig. 7 Fictive hybrid route to a PBG analogue

in *cis* rather than *trans* configuration. The tautomer with the double bond conjugated with the carbonyl (entry 3) is the most stable entity in the table. The cis analogue of entry 3 is far less stable (entry 4). The 3-enamines (entries 5 and 6) are less stable than the 5-enamines (entries 7 and 8). Note that for coherence with expected sites of reactivity at the **ALA** 3- and 5-positions, we use the terms "3-enamine" and "5-enamine" to refer to the species wherein the carbon-carbon double bond encompasses the respective 3,4 and 4,5 positions in **ALA**. For ease of comparison, the same terms are used for ALA analogues regardless of the number of carbons therein.

A variety of structures were considered wherein the reactive terminus of the ALA-analogue is embedded in a cyclic motif. Here, the goal was to assess whether a given motif might cause reaction to prevail at the 3-position versus the 5-position. If so, a hybrid reaction with the cyclic ALA-analogue and **ALA** would give the pyrrole with a dangling substituent (attached to "Z") as shown in Fig. 7. The substituent Z (or ZH$^+$ upon protonation) must constitute a leaving group for the pyrrole to undergo self-condensation leading to pyrromethane oligomers. The dangling motif and leaving group (Z—XH$_2$) would then be displaced, having served the purpose of directing reaction in the initial stages of pyrrole formation. In this regard, a common view is that prebiotic chemistry may have broad parallels to extant biochemistry yet differ in specific aspects (de Duve 1991).

The ALA-analogues include 5-hydroxylevulinic acid (a known secondary metabolite, Tschesche et al. 1981) and 5-mercaptolevulinic acid. None of the 5-membered ring structures is a known compound, but could in principle be formed by the reactions shown in Fig. 8. The
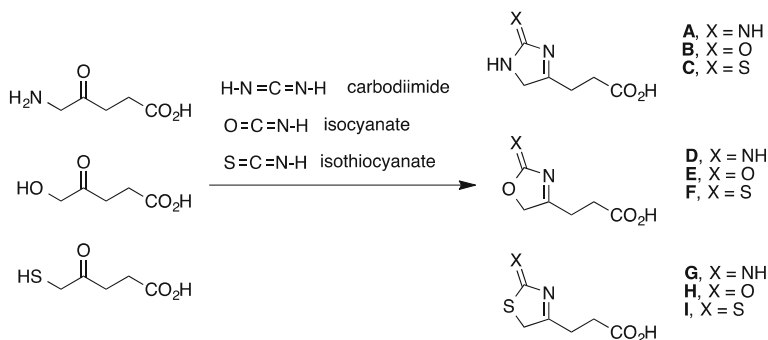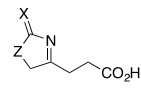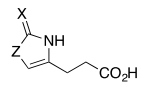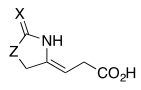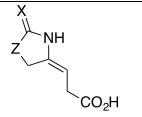

Fig. 8 Fictive reactions form 5-membered ring analogues of **ALA**

Table 2 DFT calculations of 5-membered ring derivatives of **ALA**

Cmpd	Imine	5-enamine	3c-enamine	3t-enamine
A: X = Z = NH				
Hartree	−548.754207	−548.752895	−548.748458	−548.749450
ΔE	0	+0.82	+3.61	+2.99
B: X = O, Z = NH				
Hartree	−568.648365	−568.654687	−568.646174	−568.650282
ΔE	0	−3.97	+1.37	−1.20
C: X = S, Z = NH				
Hartree	−891.602530	−891.618842	−891.604318	−891.607505
ΔE	0	−10.24	−1.12	−3.12
D: X = NH, Z = O				
Hartree	−568.623723	−568.615087	−568.615395	−568.615984
ΔE	0	+5.42	+5.23	+4.86
E: X = Z = O				
Hartree	−588.514491	−588.512244	−588.509802	−588.510081
ΔE	0	+1.41	+2.94	+2.77
F: X = S, Z = O				
Hartree	−911.457306	−911.467148	−911.460600	−911.464341
ΔE	0	−6.18	−2.07	−4.41
G: X =NH, Z = S				
Hartree	−891.595967	−891.591419	−891.586055	−891.586881
ΔE	0	+2.85	+6.22	+5.70
H: X = O, Z = S				
Hartree	−911.485088	−911.486479	−911.478885	−911.479027
ΔE	0	−0.87	+3.89	+3.80
I: X = Z = S				
Hartree	−1234.431799	−1234.445410	−1234.434791	−1234.438077
ΔE	0	−8.54	−1.88	−3.94

All ΔE values are in kcal/mol

results for these structures are listed in Table 2. No compound with a stable 3-enamine was identified.

A wider variety of species containing 5-membered rings was considered next. The possible formation of each species is shown in Fig. 9. In each case, only one tautomeric form is displayed. We emphasize that these are unproven reactions and hence are regarded as fictive. Regardless, some consideration of method of formation is warranted. The energies of the relevant tautomers of each compound are shown in Table 3. In no case was the desired enamine found to have the lowest energy.
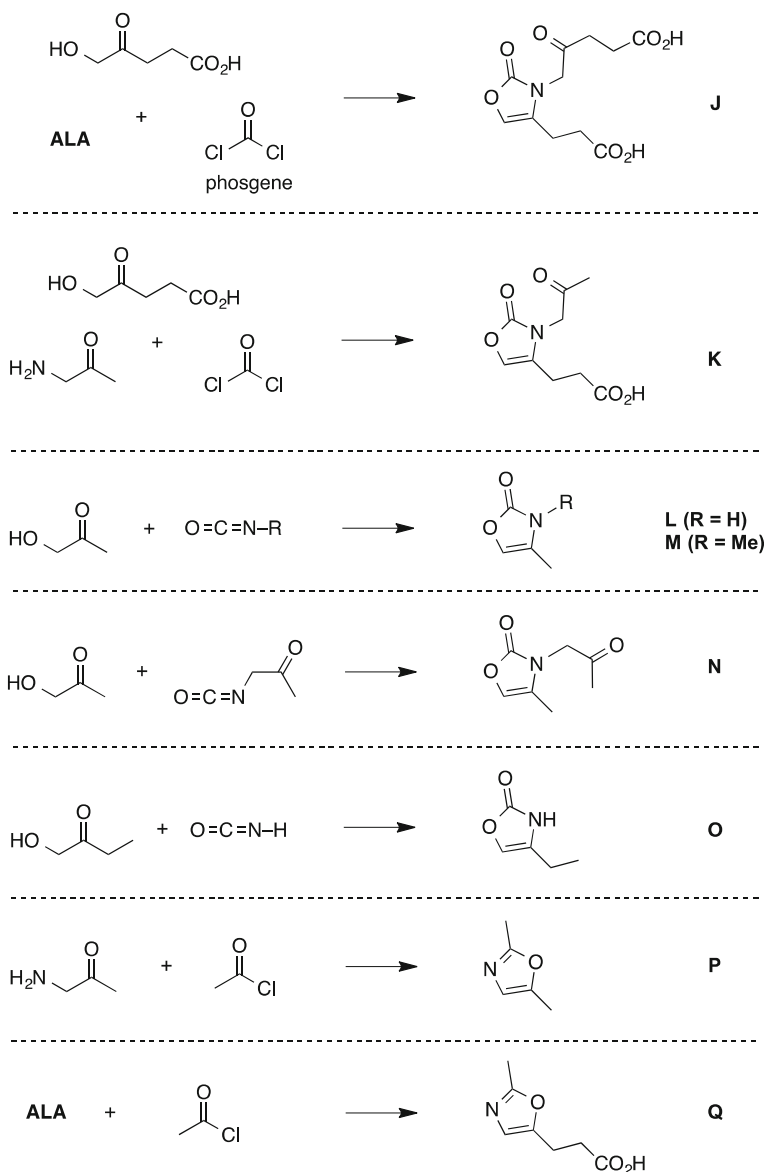

Fig. 9 Fictive routes to diverse 5-membered ring analogues of **ALA**
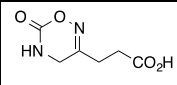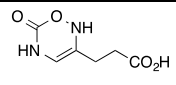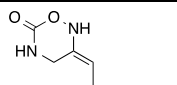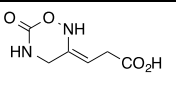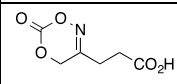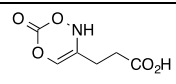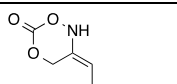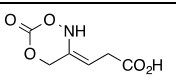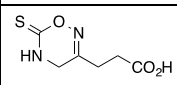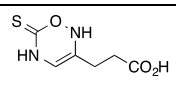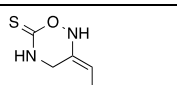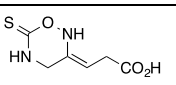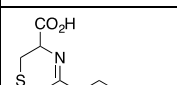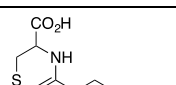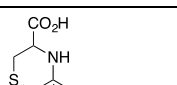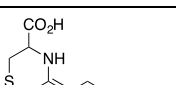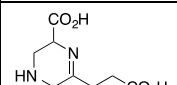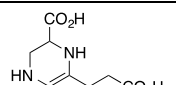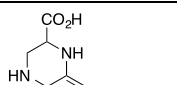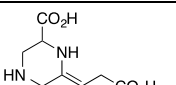
Table 3 DFT calculations of miscellaneous 5-membered ring derivatives of **ALA**

Cmpd	imine	5-enamine	3c-enamine	3t-enamine
J				
Hartree		−1008.349822	−1008.341553	−1008.343330
ΔE		0	+5.19	+4.07
K				
Hartree		−780.466303	−780.463444	−780.461529
ΔE		0	+1.79	+3.00
L				
Hartree	−360.633609	−360.635077	−360.628610	
ΔE	0	−0.92	+3.14	
M				
Hartree		−399.947754	−399.941440	
ΔE (kcal/mol)		0	+3.96	
N				
Hartree		−552.588537	−552.582614	
ΔE		0	+3.72	
O				
Hartree	−399.947691	−399.948415	−399.945673	−399.945805
ΔE	0	−0.45	+1.27	+1.19
P				
Hartree	−324.719072	−324.694361	−324.68699	−324.682763
ΔE	0	+15.51	+20.13	+22.78
Q				
Hartree	−552.589002	−552.571842	−552.5671	−552.554041
ΔE	0	+10.77	+13.74	+21.94

All ΔE values are in kcal/mol

Structures were next considered wherein the reactive terminus of the ALA-analogue is embedded in a 6-membered ring. The structures are considered without regard to how each might be formed, although it warrants mention that compound **U** could be derived from 5-bromolevulinic

Table 4 DFT calculations of 6-membered ring derivatives of **ALA**

Cmpd	imine	5-enamine	3c-enamine	3t-enamine
R				
Hartree	-643.819591	-643.802602	-643.797553	-643.795092
ΔE	0	+10.66	+13.83	+15.37
S				
Hartree	−663.676607	−663.655522	−663.652960	−663.653668
ΔE	0	+13.23	+14.84	+14.39
T				
Hartree	−966.770858	−966.754552	−966.749592	−966.746490
ΔE	0	+10.23	+13.34	+15.29
U				
Hartree	−1065.318202	−1065.315871	−1065.309832	−1065.311314
ΔE	0	+1.46	+5.25	+4.32
Hartree	−548.754207	−548.752895	−548.748458	−548.749450
V				
Hartree	−722.480287	−722.470417	−722.462792	−722.464783
ΔE	0	+6.19	+10.98	+9.73

All ΔE values are in kcal/mol

acid and cysteine. The results for these structures are listed in Table 4. In each case, the imine was the most stable species.

To avoid one site of tautomerization, the condensation of **ALA** with α-ketoglutaric acid was considered. α-Ketoglutaric acid is an intermediate in the Krebs cycle. The resulting imine and possible Knorr pyrrole (**Pyr-X**) are shown in Fig. 10. While such a pyrrole is blocked by the presence of the α-CO$_2$H unit from self-condensation to form a porphyrinogen, in principle a decarboxylation step could occur thereby opening the door to oligomerization.

The energies of various tautomers of the imine derived from the condensation of **ALA** with α-ketoglutaric acid are shown in Table 5. Note that α-ketoglutaric acid can form an imine and 3-enamine, but not a 5-enamine. Entry 6 shows the enamine that would lead to the α-carboxypyrrole **Pyr-X**; the enamine is more stable than the imine (entry 1), but an even more stable structure is shown in entry 4. Again, no compound with a stable 3-enamine (entries 5 and 6) was identified.

Studies of Kinetic Reactivity

The prior section examined the thermodynamic stability of various species including **ALA** condensation products and ALA-analogues. For a given compound, no "3-enamine" or equivalent was found to be more stable than other tautomers. An alternative approach is to consider whether a reactive site might be created at the 3-site preferentially by kinetic reaction even if such a reactive species is not the most stable among a set of possible reactants. As a proxy for such reactivity, we first examine literature data concerning deuteriation kinetics for relevant substrates.

The rate of enolization of **ALA** at 3- versus 5-positions, as measured by deuterium exchange, was studied by several groups. The results are as follows.

(a) A. I. Scott, Townsend, Okada, and Kajiwara (1973) reported studies in D$_2$O at pH 8.0 (Na$_2$CO$_3$ present, temperature not specified): **ALA** gave complete deuteriation at the 5-position in 30 to 45 min, while 30–35 h was required for complete deuteriation at the 3-position.

(b) Jaffe and Rajagopalan (1990) reported studies in D$_2$O at pH 6.8 (potassium phosphate buffer, 37 °C): levulinic acid underwent deuteriation 3-times faster at the 3-position versus the 5-position, although exchange at both positions was extremely slow. On the other hand, **ALA** underwent deuteriation ~4 times faster at the 5-position versus the 3-position. The rate constants for deuterium exchange of **ALA** were 1400 times (5-position) and ~130 times (3-position) faster than that of levulinic acid. 5-Chlorolevulinic acid underwent exchange at the 5-position at a rate almost identical to that of **ALA**, but the 3-position was three-times slower. In all of these cases, intramolecular catalysis by the terminal carboxylate could potentially play a role in the reaction at the 3-position, albeit via a 5-membered intermediate.
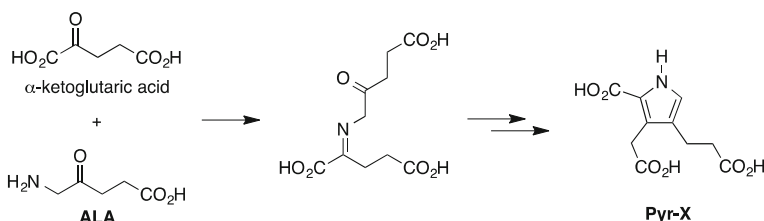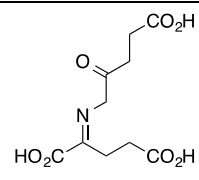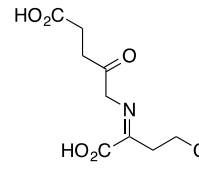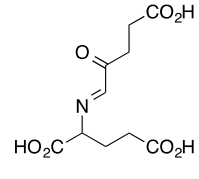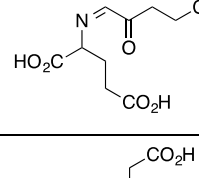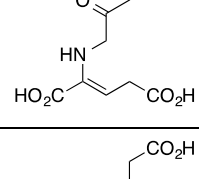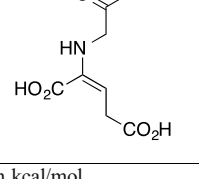
Fig. 10 Fictive reaction of **ALA** and α-ketoglutaric acid

Table 5 DFT calculations of α-ketoglutaric acid derivatives of **ALA**

Entry	Structure	Hartree	ΔE
1		−970.227497	0
2		−970.229090	+1.00
3		−970.235577	+5.07
4		−970.219838	−4.81
5		−970.227024	−0.30
6		−970.222647	−3.04

All ΔE values are in kcal/mol

(c) Under similar conditions to those of Jaffe and Rajagopalan (1990), Butler and George (1992) reported that deuteriation of **ALA** occurred about 6-times more readily at the 5-position than at the 3-position, but no rate constants or half-lives were reported.

(d) Other substrates of relevance here have also been subjected to deuteriation studies. For example, 1-aminoacetone upon complexation with cobalt-tren underwent

deuterium exchange 250-fold faster at the methylene versus methyl site (Gumm et al. 2007).

In summary, the deuteriation results indicate **ALA** reacts 4–6 times more rapidly at the 5-position versus the 3-position. These results cohere with the thermodynamic assessments, which together explain why **pseudo-PBG** is formed via solution self-condensation of **ALA**. We decided to consider analogues of **ALA** wherein the 5-substituent is negatively charged, which might suppress deuteriation at the 5-position. For these comparisons, we turned to phosphoesters of methyl 5-hydroxylevulinate. The hypothesis was that electrostatic repulsion near the 5-position due to the phosphate moiety would suppress attack of hydroxide (or other base) more so at the 5- versus 3-position. If so, the presence of a phosphate group at the 5-position of levulinic acid (**PiO-LA**) might cause reaction with **ALA** to proceed to the Knorr pyrrole **PiO-PBG** (Fig. 11).

Our first studies concerned deuterium exchange of **PiO-LA-Me**, the carboxylic acid methyl ester of **PiO-LA**. The experiments were carried out at 40 °C to achieve a meaningful degree of exchange and therefore make accurate determination of the rate constants and half-lives. The results for the kinetics of deuteriation are shown in Fig. 12. The deuterium exchange study was carried out for sufficient duration (>22, 45 h) to encompass ≥50 % exchange for the H^3 and H^5 sites of **PiO-LA-Me** and **ALA**. Under these reaction conditions, deuterium exchange was not detected at the 5-position of **PiO-LA-Me**, but was observed at the 3-position. Thus, the preferred position of enolization changed from H^5 to H^3 by the introduction of the phosphate group compared with that of **ALA**. The results for **ALA** and **PiO-LA-Me** are summarized in Table 6. The results have some commonality with those from analogous studies of aldolase mechanisms using dihydroxyacetone phosphate (Lowe and Pratt 1976; Pratt 1977; Motiu-DeGrood et al. 1979), where the hydroxymethyl undergoes more rapid exchange than the phosphonooxymethyl unit, although of course such reactions are enzyme-mediated.

The success of the deuteriation studies prompted examination of the condensation of **PiO-LA-Me** and **ALA**. The reaction was carried out under a variety of conditions, including 2.5–240 mM **ALA**, 40–500 mM **PiO-LA-Me**, room temperature (rt)–85 °C, 13–94 h, use of a 1° or 2° amine for imine catalysis, and various buffers (see the Appendix, Table 8, for a full listing). The reaction was assayed by absorption spectroscopy (following I_2 oxidation) for the presence of uroporphyrins. In each case, however, the characteristic absorption band of uroporphyrins was not detected. A control experiment was carried out with **ALA** and 2-butanone (150 mM each) in aqueous bicine (0.3 M) without or with DL-proline (50 mM). After reaction at room temperature for 19 h, ESI-MS gave no peak characteristic of the putative pyrrole product.

Fig. 11 Fictive reaction to form a reactive analogue of **PBG** (for clarity, the only ionized moiety displayed is the partially ionized phosphate moiety)
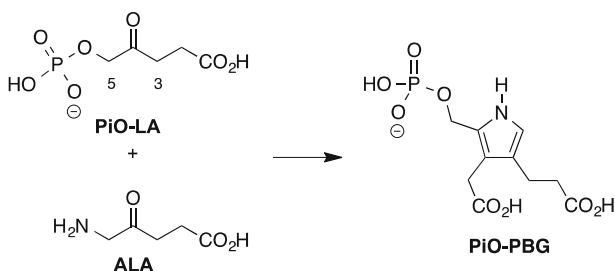
Panel (1):
$y = 0.0008159 - 2.607 \times 10^{-6}\ x$
$R = 0.9943$

Panel (2):
$y = -0.0574 - 1.206 \times 10^{-5}\ x$
$R = 0.9992$

Panel (3):
$y = -0.02575 - 4.725 \times 10^{-5}\ x$
$R = 0.9976$
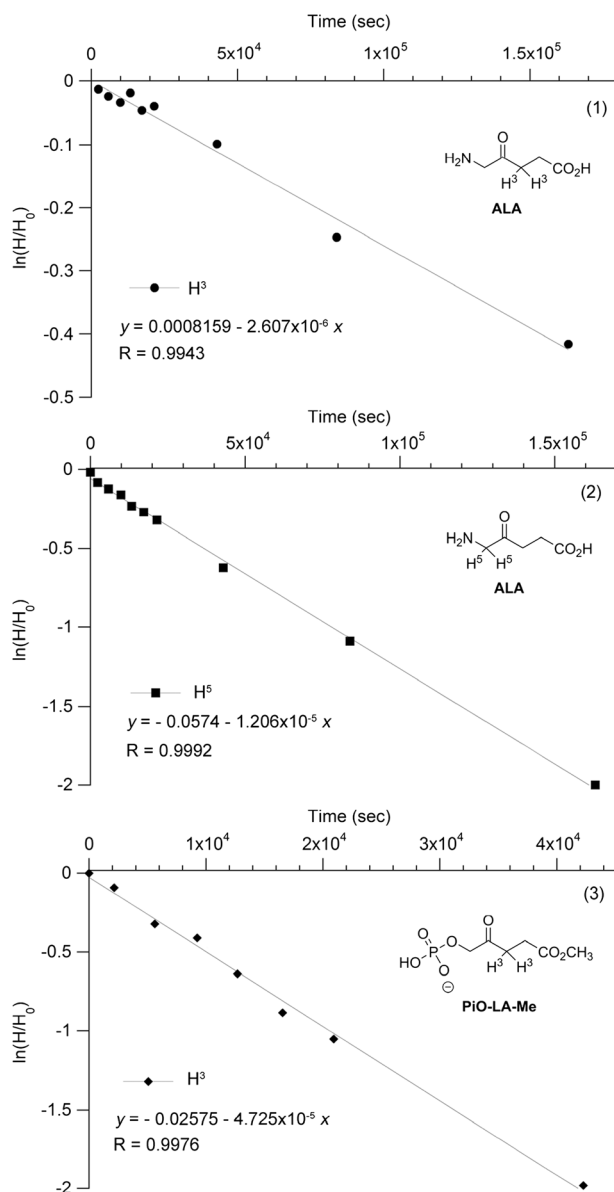
Discussion

The biosynthesis of tetrapyrroles has been regarded as a window on evolution, wherein the
first products served a role in early evolutionary stages, but were replaced with more suitable
molecules as further biosynthetic steps were added later in evolution (Granick 1950, 1957,
1965; Mauzerall 1976, 1978a, b, 1998). In this view, uroporphyrinogens and the dehydroge-
nation products, uroporphyrins, would have served as reactants in proto-photosynthesis
(Granick 1967; Mauzerall 1978a, b; Mercer-Smith and Mauzerall 1981, 1984; Mercer-Smith

Table 6 Pseudo-first-order rate constants for deuterium exchange of levulinic acid derivatives[a]
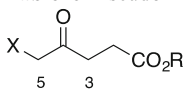
Compound	X	R	Temp	H[3]		H[5]	
				k (s^{-1} × 10^5)	$t_{1/2}$ (h)	k (s^{-1} × 10^5)	$t_{1/2}$ (h)
ALA	H_2N-	-H	40 °C	0.26	74	1.2	16
PiO-LA-Me	Na_2O_3PO-	-CH$_3$	40 °C	4.7	4	_[a]	_[a]

[a] 0.1 M sodium phosphate in D_2O

[b] No significant exchange was observed

et al. 1985; Mauzerall 1998). Such processes would occur in aqueous solution given the water solubility of uroporphyrinogens and uroporphyrins. Over the course of evolution, further steps cobbled on to the early steps would have led to more amphiphilic and lipophilic tetrapyrrole macrocycles that functioned in membraneous environments. Thus, the extant biosynthesis is a palimpsest with the function of early products no longer manifested.

This paradigm, as well as the logic and molecular concision of the extant biosynthesis, have prompted studies to recapitulate the early stages of the biosynthesis in the absence of enzymes. The conversion of **ALA** to **PBG** has been called the "Achille's heel" of the prebiotic process (Mauzerall 1990, 1998), given that both **ALA** and **PBG** are universal precursors to extant tetrapyrrole macrocycles. A complicating factor of such studies concerns what constitutes success in the reaction, and along with that, how the reaction is analyzed. Significant findings over the years concerning the conversion of **ALA** to **PBG** are as follows:

- Gibson (1955) reported that "at physiological pH values, temperatures and concentrations of **ALA**, no detectable amount of **PBG** is formed." The dihydropyrazine (**I**, Fig. 4) was proposed as an intermediate in the condensation of **ALA**.
- J. J. Scott (1956) reported that addition of alkali to **ALA** hydrochloride *in vacuo* for several days at 18 °C afforded **PBG** that was isolated by crystallization in 3 % yield. A. I. Scott, Townsend, Okada, and Kajiwara (1973) carried out several hundred attempts to repeat the experiment but "the only product isolated (eventually in over 70 % yield) was the 3-aminopyrrole isomer," dubbed Ψ-PBG or **pseudo-PBG**.
- Granick and Mauzerall (1958) reported that upon standing at pH 6.8, 40 °C for 2 h, **ALA** affords the dihydropyrazine **I** (<10 % yield), as observed by paper electrophoresis. Jaffe and Rajagopalan (1990), who confirmed this result by NMR spectroscopy, added that the amount of **I** depends on the concentration of **ALA**.
- Szutka (1966) reported that ultraviolet illumination of **ALA** afforded a polar "pyrrolic compound" on the basis of a positive (Ehrlich) color test with *p*-dimethylaminobenzaldehyde. Upon repeating this experiment one of us (J. S. L., unpublished) found that illumination does afford colored materials that may give a positive color test for pyrroles, but a subsequent self-condensation to form uroporphyrins does not occur.
- A. I. Scott, Townsend, Okada, and Kajiwara (1973) reported that incubation of **ALA** in the presence of Amberlite IR-45 ion-exchange resin and glutathione at pH 7.9 for 20 days gave **PBG** in 7–11 % yield. Amberlite IR-45 is a polystyrene bearing a substituent at the *p*-phenyl position

(7-amino-2,5-diazaheptyl) that contains primary and secondary alkylamines. The >200 experiments leading to this finding are described in the Ph.D. thesis by Townsend (1974), who noted that "even in the absence of IR-45 some **PBG** is produced, albeit in far lower yields than that produced under similar conditions in the presence of resin." The assay for **PBG** formation included an Ehrlich test, paper chromatographic mobility, and conversion (in strong acid with heating followed by I_2 oxidation) to uroporphyrins; attempts to isolate **PBG** by crystallization were not successful. The yield data were obtained entirely by the acid-mediated conversion to uroporphyrins. Townsend (1974) appropriately did not claim a prebiotic synthesis of uroporphyrinogens beginning with **ALA**, only a "biogenetically modified synthesis of porphobilinogen" upon the resin-mediated reaction of **ALA**.

- Franck and Stratmann (1981) reported that **ALA** under alkaline conditions (5 N NaOH, 60 °C, 8 h) afforded two products in 10:1 ratio; the major component was the dihydropyrazine **I** (isolated indirectly as the pyrazine), and the minor component was **PBG** (supported by paper chromatography and a positive Ehrlich test).
- Butler and George (1992) pointed out the unreliability of the Ehrlich test to distinguish **PBG** and **pseudo-PBG**.
- Butler and George (1992) reported that **ALA** at moderate pH (4.6, 9.7 or 11.6) afforded only the dihydropyrazine **I**, whereas under alkaline conditions **I** and **pseudo-PBG** were obtained in a 2:1 ratio as determined by ^{13}C and ^{1}H NMR spectroscopy.
- Neier and coworkers, who have developed efficient chemical syntheses of **PBG** (Soldermann et al. 2008) and have critically reviewed all existing chemical syntheses of **PBG** (Neier 1996; Bobal and Neier 1997), also have stated that the transformation of **ALA** to **PBG** essentially fails in solution (Stauffer et al. 2001; Chaperon et al. 2003).

The only significant positive result from the above studies is the finding that the aminoalkyl-containing resin facilitates conversion of **ALA** to **PBG** (hereafter referred to as the resin reaction). An interesting aspect of the chemistry is that the resin reactions to form **PBG** were carried out under near-neutral conditions whereas the yield assay was done in strong acid. Consider a reaction that produces both **PBG** and **pseudo-PBG**: the latter is expected to cap the chain-growth oligomerization of the former. The termination of pyrrole oligomerization on the path to porphyrinogens has been treated previously (Deans et al. 2015). Briefly, successful oligomerization to form uroporphyrinogens depends on the mole fraction of **PBG** to the fourth power [$(\chi_{PBG})^4$], where the mole fraction is diminished by any pyrrole that can terminate oligomerization, such as **pseudo-PBG**. As one example, if normal and defective monomers are present in 1:1 ratio and exhibit equal reactivity, their combinatorial reaction would cause the overall yield to be offset by a factor of 0.0625. Thus, if the yield of **PBG** and **pseudo-PBG** are each 10 %, the maximum yield of uroporphyrins would be a mere 0.625 %—due to chain termination by **pseudo-PBG**—and that value assumes quantitative yields for oligomerization (bilane formation), cyclization (porphyrinogen formation) and subsequent oxidation (porphyrin formation).

We speculate that in strong acid, **pseudo-PBG** would be deactivated toward electrophilic substitution and not interfere with oligomerization (Fig. 13). The diminished activity stems from protonation of the 3-amino group (pK_a values range from 2.5 to 6 depending on substituents) (Cirrincione et al. 1992; De Rosa et al. 2015), which strongly deactivates the pyrrole ring by induction, analogous to that upon protonation of aniline to form the unreactive anilinium unit. Protonation of 3-aminopyrroles also can occur at the adjacent carbon (2-position) to give the tautomer (De Rosa and Arnold 2013); regardless of the site, the protonated 3-aminopyrrole would be deactivated toward electrophilic aromatic substitution.
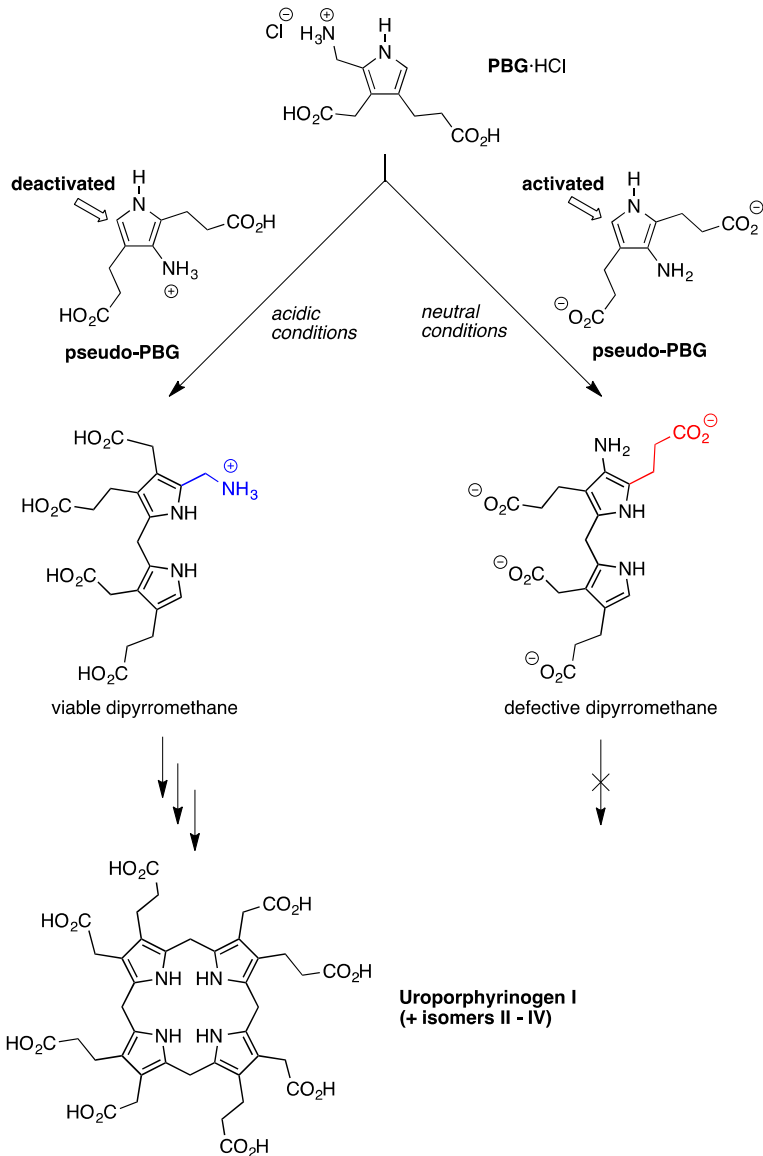
Fig. 13 Proposed reaction of **PBG** in the presence of **pseudo-PBG**. Uroporphyrinogen I is shown as one example of the four isomers. Under neutral conditions (*right*), **pseudo-PBG** reacts combinatorially with **PBG** and terminates **PBG** oligomerization; the defective dipyrromethane shown is one example of a defective oligopyrromethane. In acidic conditions (*left*), **pseudo-PBG** is deactivated whereupon **PBG** oligomerization proceeds without interference

This insight may be relevant to understanding the experimental data reported by A.I. Scott, Townsend, Okada, and Kajiwara: the resin reaction of **ALA** was carried out under near-neutral conditions whereas the yield assay relied on condensation in strong acid leading to uroporphyrins (Scott et al. 1973; Townsend 1974). Any **pseudo-PBG** present would not interfere in acid. If the reaction were done entirely under near-neutral conditions, the effects

of any **pseudo-PBG** would be manifested in diminished yield of uroporphyrins. We note that because **PBG** and **pseudo-PBG** are isomers, mass spectral identification of capped oligomers cannot be readily achieved. Investigation of this conjecture could be achieved with authentic samples (and sufficient quantity) of **PBG** and **pseudo-PBG**, one of which is isotopically substituted, or alternatively with an ester of one of the two species. Such a study is beyond the scope of the present paper.

If this conjecture is correct, any successful prebiotic synthesis of **PBG** (from **ALA**) that continues on to make uroporphyrinogens must do so with suppression of the yield of **pseudo-PBG** or removal of **pseudo-PBG** from the reaction mixture. In short, there is at present no demonstrated one-flask, non-enzymic conversion of **ALA** (via **PBG**) to uroporphyrinogens under a fixed set of conditions. The absence of a robust route from **ALA**, the universal precursor to **PBG**, or from any other credible prebiotic precursors, has presented an insurmountable obstacle to the notion of a spontaneous origin of a prebiotic parallel to the modern biosynthetic pathway.

The results reported herein suggest that none of the putative ALA-like reactants examined (Tables 2, 3, 4, and 5) would lead to **PBG**. The DFT calculations were employed as a first screen, where thermodynamic stability was assessed in the gas phase. We recognize that tautomeric forms may have differing degrees of polarity and hence may be differentially stabilized in a polar medium, which are not accommodated in these DFT calculations. The DFT calculations are not intended to constitute an exclusive screen; our intent was that any compound that exhibited a stable 3-enamine (or equivalent) would immediately rise to a premier target for reaction studies. No compounds were so identified. The greater stability of the imine versus enamine is well understood both as a concept in synthetic chemistry and has been supported by computation (Lammertsma and Prasad 1994); here, the issue was whether a particular structure would give lower energy for a 3-enamine versus 5-enamine. No such structural motif was identified.

Other studies concerning the energetics of porphyrin formation have been reported. George calculated the thermodynamics of each step from succinic acid and glycine to uroporphyrin. The reaction of 2 **ALA** → **PBG** + $2H_2O$ was assessed to give $\Delta H° = -12.6$ kcal/mol, $\Delta S° = +14.6$ e.u., and $\Delta G° = -16.9$ kcal/mol (George 1973). Erdtman et al. assessed the energetics of various possible enzymic pathways for formation of **PBG**, particularly the order of steps for imine and carbon-carbon bond formation (Erdtman et al. 2010, 2011; Tian et al. 2012). Aylward and Bofinger (2005) calculated the energetics for formation and reaction of an abiotic pyrrole. To our knowledge, no assessments have been done concerning the energetics of formation of **pseudo-PBG**.

One compound that we considered to have promise in a hybrid reaction with **ALA** was **PiO-LA** (or **PiO-LA-Me**) given chemical intuition and the results from deuteration studies. However, no uroporphyrins were detected upon reaction with **ALA** under a representative set of conditions. Butler and George (1992) examined the condensation of **ALA** with 5-(*N,N*-dimethylamino)levulinic acid, which is blocked from the normal path of self-condensation. The products included the two nonproductive self-condensation products from homo-dimerization of **ALA** (i.e., the dihydropyrazine and **pseudo-PBG**), as well as the Fischer-Fink product from **ALA** and 5-(*N,N*-dimethylamino)levulinic acid. Bulkier amino substituents,

which might suppress reaction at the 5-position, were not considered. Regardless, the minimum conclusion to date is that a *robust* route to **PBG** from **ALA** without enzymes under near-neutral conditions has not yet been identified.

Outlook

PBG is a gateway molecule to all known tetrapyrroles. The absence of a robust structure-directed route to **PBG** from **ALA** or analogues thereof suggests that the formation of tetrapyrroles via a prebiotic version of the extant biosynthesis would have required the presence of catalysts. Such catalysts could have been promiscuous and inefficient, but need to facilitate formation, and accelerate the reaction, of the 3-enamine or equivalent of an ALA-like reactant. The diminished formation of **pseudo-PBG** may be essential to avoid termination of oligopyrromethane formation. Indeed, suppressing **pseudo-PBG** formation may be of equal or greater importance than achieving a high yield of **PBG**. Catalysts that also enable formation of **ALA** likely would be required, as a prebiotic route to **ALA** has not yet been demonstrated. An alternative approach might entail fundamentally different routes to porphyrins altogether, but to date no robust route has emerged.

Experimental Section

Materials All reactants employed herein were obtained from commercial sources, except for **PiO-LA-Me**, the synthesis of which will be described elsewhere. Reactions of acyclic reactants in putative pathways to uroporphyrins were carried out as described previously in detail (Lindsey et al. 2011).

DFT Calculations DFT calculations were performed B3LYP with 6-31G* level in the gas phase using Spartan'08.

Deuterium Exchange Studies An exemplary procedure is as follows: A sample of a levulinic acid derivative (30 μmol) was placed in an NMR tube and 0.1 M sodium phosphate in D_2O (pD 7.2, 600 μL, final concentration of levulinic acid derivative was 5 mM) was added immediately before the measurement ($t = 0$). The ^1H NMR spectrum was recorded (40 °C) over time, and the resonances from the 3- and 5- positions were integrated. The deuterium exchange processes were followed relative to the non-exchanging protons (2-position). Pseudo-first order rate constants were obtained by nonweighted least-squares fitting of the log of the intensity of the resonance versus the elapsed time.

Appendix

Table 7 Condensations of porphobilinogen (**PBG**)

Entry[a]	[PBG], mM	pH	Medium	Atmosphere	Temp	Time, h	Analysis	Yield
1	2.0	>10	Na_2CO_3 + urine	Aerobic	20 °C	408	isolation	7.7 %
2	0.67	6.5	NaOAc + urine	Aerobic	20 °C	408	isolation	18.8 %
3	1.6	>10	Aq NH_3	Aerobic	20 °C	408	isolation	9.4 %
4	2.0	<1	Aq HCl	Aerobic	100 °C	0.33	isolation	77.5 %
5	10	<1	Aq HCl (1.0 M)	Anaerobic	98 °C	0.5	I_2, abs[b]	78 %
6	10	7.6	Aq NaPi (0.25 M)	Anaerobic	60 °C	21	I_2, abs[b]	55 %
7	10	10	Aq Na_2CO_3 (0.20 M)	Anaerobic	60 °C	20	I_2, abs[b]	70 %
8	10	>10	Aq NaOH (0.10 M)	Anaerobic	98 °C	2	I_2, abs[b]	40 %
9	0.26	7.4	phosphate (0.10 M)	Anaerobic	70 °C	2[c]	I_2, abs[b]	~55 %
10	0.26	3.5	acetate (0.10 M)	Anaerobic	70 °C	2[c]	I_2, abs[b]	~60 %
11	1.0	7.0	Aq NaPi (0.10 M)	Anaerobic[d]	60 °C	24	Abs[e]	27 %
12	1.0	7.0	MOPS[f] (0.10 M)	Aerobic	60 °C	3.75	Abs[e]	16 %
13	1.0	7.0	MOPS[f] (0.10 M) $Zn(OAc)_2 \cdot 2H_2O$ (0.25 or 1.25 mM)[g]	Aerobic	60 °C	3.75	Abs[h]	18 or 16 %

[a] Entries 1–4 (Cookson and Rimington 1954); entries 5–8 (Mauzerall 1960a); entries 9 and 10 (Frydman et al. 1971); entries 11–13 (reported here)

[b] Oxidation of reaction aliquots with I_2 followed by absorption spectroscopy of the uroporphyrins in conc. HCl (ε_{Soret} = 505,000 $M^{-1} cm^{-1}$ (Mauzerall 1960b)

[c] The yields (not shown) also were determined upon reaction for 1 h at temperatures from ~45–85 °C (Frydman et al. 1971)

[d] The reaction without I_2 oxidation gave 26 % yield, indicating the deaeration of the medium was incomplete. A timecourse for the reaction with I_2 oxidation gave yields of 19.3 % (3 h), 24.1 % (6 h), 26.0 % (12 h) and 27.2 % (24 h). The timecourse without I_2 treatment gave yields of 6.1 % (3 h), 16.6 % (6 h), 24.0 % (12 h) and 25.3 % (24 h). The corresponding $t_{1/2}$ points for formation of uroporphyrinogens are ~2.5 and 4.5 h, respectively. While oxidation in situ—presumably due to adventitious O_2—is unlikely to be relevant under prebiotic conditions, the results show the robustness to the presence of oxidants of the reaction leading to uroporphyrins

[e] The absorption spectrum of uroporphyrins can be affected to some extent by the medium. For uroporphyrin III in 1 M HCl, the Soret band is very sharp and has ε_{Soret} = 505,000 $M^{-1} cm^{-1}$ (Mauzerall 1960b). In neutral solvents, the band is broader; the ε_{Soret} values include 215,000 $M^{-1} cm^{-1}$ ($CHCl_3$) (Mauzerall 1960b); 225,000 $M^{-1} cm^{-1}$ (0.1 M Na-EDTA buffer, pH 6.05) (Mauzerall 1962); and 204,000 $M^{-1} cm^{-1}$ (0.1 M citrate buffer, pH 5.9) (Mauzerall 1965); 220,000 $M^{-1} cm^{-1}$ (phosphate buffer, pH 6.8) (Mercer-Smith et al. 1985). Uroporphyrin III octamethyl ester in $CHCl_3$ gives ε_{Soret} = 215,000 $M^{-1} cm^{-1}$ (Mauzerall 1960b). The spectra were recorded here in N,N-dimethylformamide using a consensus value for uroporphyrins in neutral solution of ε_{Soret} = 220,000 $M^{-1} cm^{-1}$

[f] MOPS is 3-(N-morpholino)propanesulfonic acid, a buffer that can be used in the presence of metals, unlike NaPi (phosphate buffer)

[g] The reaction with $Zn(OAc)_2 \cdot 2H_2O$ at higher concentration (6.25 mM) either in the presence of MOPS or with no buffer at all resulted in formation of an unknown precipitate with at most a trace of uroporphyrins formed

[h] The zinc chelate rather than the free base was formed exclusively. For the zinc chelate, ε_{Soret} values reported include 476,000 $M^{-1} cm^{-1}$ for Zn-Uroporphyrin I (three-fourths isomer I) in 0.10 M KCN (Carapellucci and Mauzerall 1975), and 340,000 $M^{-1} cm^{-1}$ for Zn-Uroporphyrin I in water (Shamim and Hambright 1983). The spectra were recorded here in N,N-dimethylformamide using ε_{Soret} = 340,000 $M^{-1} cm^{-1}$

Table 8 Evaluation of the reaction of phosphoester **PiO-LA-Me** with **ALA**

Entry	[**ALA**], mM	[**PiO-LA-Me**], mM	pH,[a] temp, time	Additive
1	5	40	7, 60 °C, 43 h	–
2	2.5	80	7, 60 °C, 43 h	–
3	10	40	7, 35 °C, 44 h	–
4	10	40	7, 35 °C, 44 h	GlyOEt, 50 mM
5	50	100	–,[b] 35 °C, 44 h	–
6	50	100	7,[c] 35 °C, 44 h	GlyOEt, 100 mM
7	50	100	7,[c] 35 °C, 44 h	–
8	150	150	8,[d] rt, 94 h	Proline, 50 mM
9	150	150	8,[d] rt, 94 h	–
10	300	–	8,[d] rt, 94 h	Proline, 50 mM
11	150	150	–,[b] rt, 94 h	Proline, 50 mM
12	5	500	–,[b] 85 °C, 13 h	–
13	240	240	7,[e] 65 °C, 43 h	CTAB, 10 mM
14	120	120	7,[e] 65 °C, 43 h	–

[a] pH 7 (6.88) was with 100 mM NaPi buffer unless noted otherwise

[b] No buffer added

[c] 250 mM NaPi

[d] 300 mM bicine buffer (pH 8.2)

[e] MOPS buffer (0.5 M, pH 7.00)

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