Normal and Abnormal Heme Biosynthesis. 1. Synthesis and Metabolism of Di- and Monocarboxylic Porphyrinogens Related to Coproporphyrinogen-III and Harderoporphyrinogen: A Model for the Active Site of Coproporphyrinogen Oxidase

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Coproporphyrinogen oxidase (copro'gen oxidase), which catalyses the conversion of coproporphyrinogen-III via a monovinylic intermediate to protoporphyrinogen-IX, is one of the least well understood enzymes in the heme biosynthetic pathway. To develop a model for the substrate recognition and binding recognition for this enzyme, a series of substrate analogues were prepared with two alkyl substituents on positions 13 and 17 in place of the usual propionate residues. Although the required substrate probes are porphyrinogens (hexahydroporphyrins), the corresponding porphyrin methyl esters were initialy synthesized via *a*,*c*-biladiene intermediates. These were hydrolyzed and reduced with 3% sodium amalgam to give the unstable porphyrinogens needed for the biochemical investigations. These modified structures were metabolized by avian preparations of copro'gen oxidase to give monovinylic products, but the second propionate residue was not further metabolized. In three cases, the metabolites were isolated and further characterized by proton NMR spectroscopy and mass spectrometry. When methyl or ethyl groups were placed at the 13 and 17 positions, the resulting porphyrinogens were very good substrates (although the ethyl version, mesoporphyrinogen-VI, gave slightly better results), but when propyl units were introduced metabolism was significantly inhibited and the butyl-substituted structure was only slightly transformed after long incubation periods. These results suggest the presence of an active-site lipophobic region near the catalytic site for copro'gen oxidase. The observation that the related 3-vinyl- and 3-ethylporphyrinogens with 13,17-diethyl substituents were not substrates for this enzyme confirmed the need for a second propionate residue to hold the substrate in place at the catalytic site.

Introduction

Metalloporphyrins, chlorins, and related tetrapyrroles play many essential roles in biology[1] (hemes, chlorophylls, vitamin B_{12}, etc.) and have been aptly designated as "the pigments of life".[2] The biosyntheses of protoheme and the chlorophylls share many of the same intermediary steps and only branch at the penultimate stage in heme formation (Scheme 1). In vertebrates, δ-aminolevulinic acid (δ-ALA), the first unique biosynthetic intermediate in the tetrapyrrole pathway, is formed from glycine and acetyl coenzyme A,[3] whereas plants and many bacteria generate δ-ALA by the 5-carbon glutamate pathway.[4] For vertebrates, the formation of δ-ALA occurs in the mitochondria, while the next four enzyme catalyzed steps in the tetrapyrrole pathway take place in the cytoplasm. Two δ-ALA units are self-condensed by PBG synthetase in a manner reminiscent of the Knorr pyrrole condensation to give the pyrrolic intermediate porphobilinogen (PBG).[5] In a well-studied two-enzyme controlled process, four units of PBG are combined to form the first macrocyclic intermediate uroporphyrinogen-III (uro'gen-III) via a hydroxymethylbilane intermediate.[6] It is of particular significance that this process occurs with inversion of the D ring to generate the so-called "type III" isomer pattern which provides the basic skeleton for nearly all of the biochemically significant tetrapyrroles. Uro'gen-III is a nonconjugated porphyrinogen or 5,10,15,20,22,24-hexahydroporphyrin, and it is noteworthy that the next two enzymic steps occur at this oxidation level. Uro'gen-III is also the precursor to the corrins, including vitamin B_{12},[7] but the metabolic route branches away at this stage. Sequential oxidative decarboxylation of the four acetate moieties by uro'gen decarboxylase affords coproporphyrinogen-III (copro'gen-III).[8]

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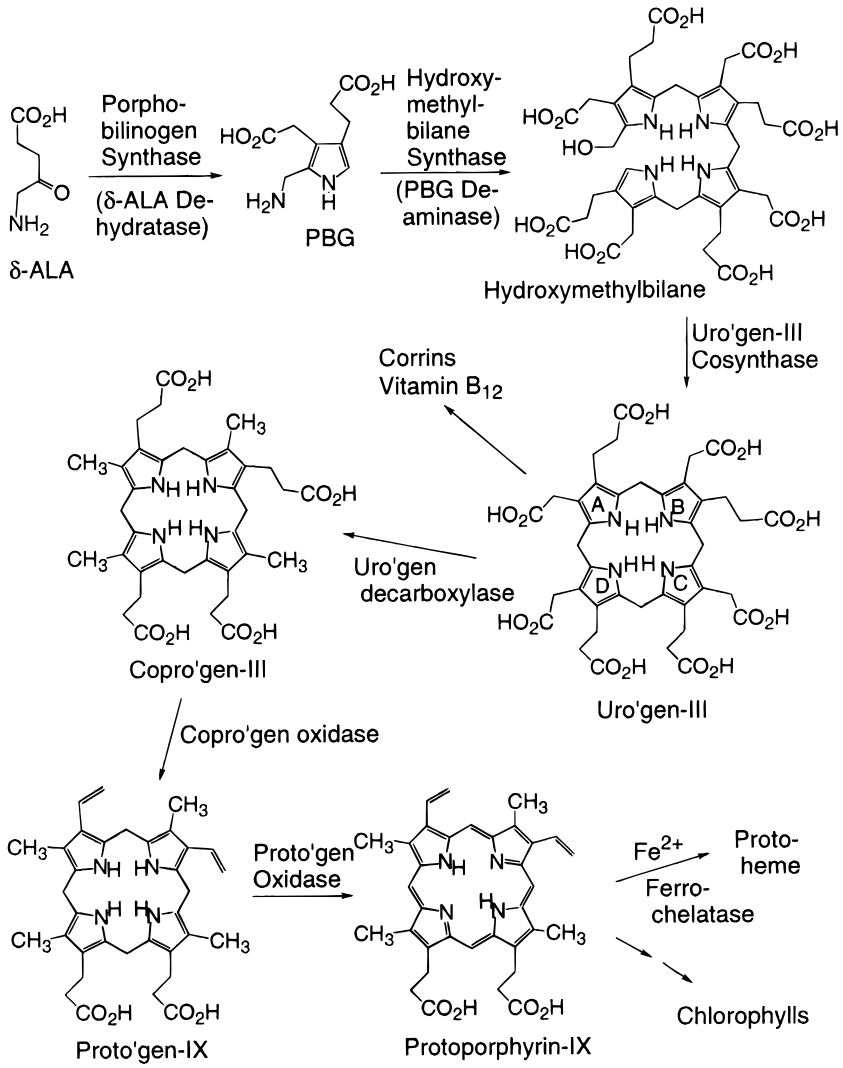
Scheme 1. Heme Biosynthetic Pathway



The final three steps in the heme biosynthetic pathway take place in the mitochondria. Oxidative decarboxylation of the upper two propionate side chains generates protoporphyrinogen-IX (proto'gen-IX).[9] Subsequent dehydrogenation by proto'gen oxidase produces the corresponding fully aromatic porphyrin, and insertion of Fe^{2+} by ferrochelatase then affords protoheme. Although most of the major steps in the heme biosynthetic pathway have been known for over 50 years, the latter stages remain relatively poorly understood. In addition to the compelling need to fully understand all of the enzymology for nature's major metabolic pathways, further studies of heme biosynthesis have clinical implications.[10] Defects in any one of the eight enzymes in the heme pathway leads to diseases known as porphyrias, which may manifest numerous pathological symptoms, including skin photosensitivity and lesions, liver damage, and neurological problems.[10] Hence, a better understanding of the enzymes involved in the heme pathway may lead to improved treatments for porphyric patients.

Perhaps the least well understood of the heme enzymes is coproporphyrinogen oxidase (EC 1.3.3.3). Copro'gen oxidase selectively transforms the A and B ring propionate units of copro'gen-III to vinyl moieties while leaving the structurally very similar side chains on the C and D rings untouched.[9] The conversion of a propionic acid unit to a vinyl group involves an oxidation as well as a decarboxylation, and while the stereochemistry for this process has been fully elucidated, the mechanism by which this transformation occurs is currently unknown.[9] In aerobic organisms oxygen is required for enzyme activity, but no other cofactors have been identified. The presence of copper(II) in a cloned enzyme has been claimed,[11] but numerous other reports strongly indicate the absence of any transition-metal ions in this oxidative

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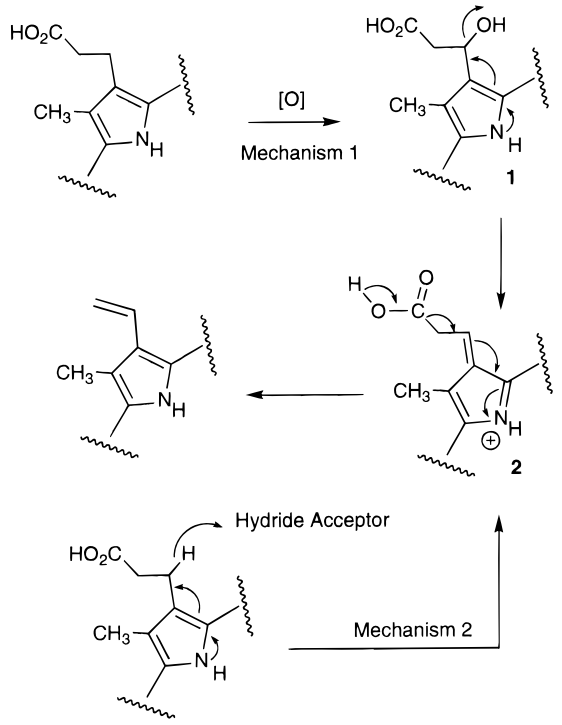
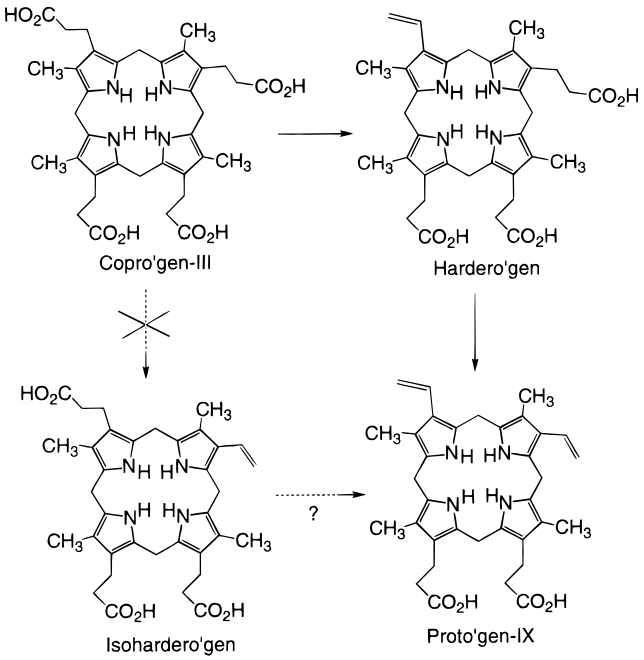
Scheme 2. Proposed Mechanisms for the Oxidative Decarboxylation of the Propionate Side Chains of Coproporphyrinogen-III



enzyme.[12−14] Some evidence for the formation of a hydroxypropionate intermediate (**1**; Scheme 2) has been presented,[13,15,16] although some authors contend that this type of species is not a true intermediate in this conversion.[9,17] Porphyrins with hydroxypropionate[18] and acrylate substituents[19] have been isolated from natural sources, including meconium,[19] but these structures may be chemical artifacts or biodegraded materials. Two principal mechanisms have been proposed to explain this chemistry (Scheme 2). In mechanism 1, Sano suggested that an initial hydroxylation takes place.[13] Elimination may lead to a cationic azafulvene intermediate **2** whereby the pyrrole ring can act as an "electron sink" and facilitate a subsequent decarboxylation to generate the vinyl unit. Alternatively, a concerted elimination of H_2O and CO_2 from structure 1 might be envisaged.[13] In

Scheme 3. Metabolism of Coproporphyrinogen-III



mechanism 2, first proposed in a slightly different form by Sano and Granick[20] and strongly supported by Akhtar et al.,[9] an unspecified hydride acceptor allows the direct formation of intermediate **2**. This latter possibility is perhaps the most likely in anaerobic organisms,[21] although no definitive data are presently available to differentiate between these mechanisms.

Substrate Recognition: A Model for the Active Site of Coproporphyrinogen Oxidase from Avian Erythrocytes

The two propionate groups of copro'gen-III undergo sequential oxidative decarboxylation, and this could lead to two different intermediary porphyrinogens depending on whether the A- or B-ring substituent is processed first. A tricarboxylate porphyrin has been isolated from various natural sources,[22−24] initially from the porphyrin-rich harderian gland of the rat,[22] and shown to be an A-ring-modified vinyl porphyrin named "harderoporphyrin".[22] Numerous studies using total synthesis,[25a] HPLC,[25] and MS[16] have demonstrated that harderoporphyrinogen (Scheme 3) is the only intermediate in the formation of proto'gen-IX, and the isomer "isoharderoporphyrinogen" has been shown to be at best a poor substrate for coproporphyrinogen oxidase.[13,26]

Despite the selectivity of this pathway, a number of mostly synthetic porphyrinogens have been shown to be

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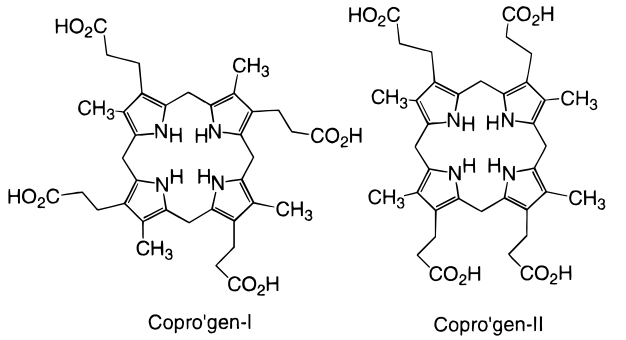
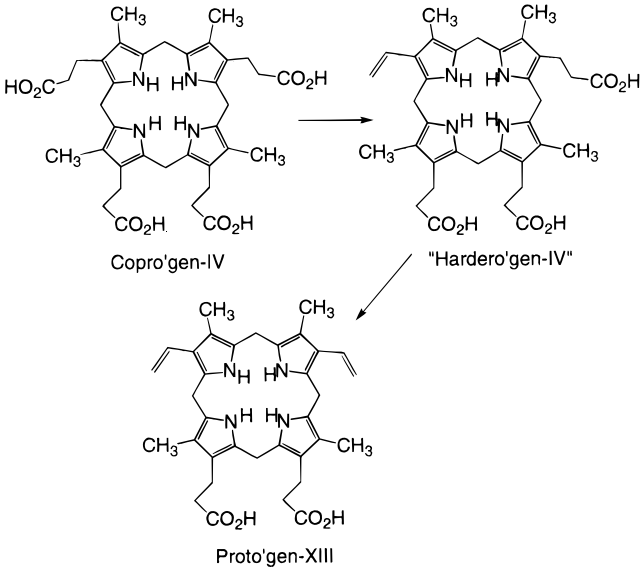
Chart 1. Type Isomers of Coproporphyrinogen That Are Not Metabolized by Copro'gen Oxidases



Scheme 4. Metabolism of Coproporphyrinogen-IV



substrates for copro'gen oxidase. If a porphyrinogen has four methyl and four propionate side chains, these can be arranged in four different ways to produce the type-I, -II, -III, and -IV isomers. The hemes and chlorophylls all derive from the type-III skeleton, but type-I isomers may arise particularly under abnormal conditions (e.g., in the porphyrias)[10] and type-II and -IV coproporphyrins have been detected in porphyric human urine and feces.[27] Copro'gens I and II (Chart 1) are not substrates for copro'gen oxidase,[28] but copro'gen-IV has been shown to be a good substrate for this enzyme (Scheme 4).[28−32] In kinetic studies using chicken red cell hemolysates as an enzyme source, copro'gen-III is converted into proto'gen-IX via hardero'gen (Scheme 2), although very little of the tricarboxylate intermediate accumulates at intermediary times.[29] In contrast, while copro'gen-IV appears to be an equally good substrate for this enzyme and is eventually converted into proto'gen-XIII, the type IV "hardero'gen" accumulates to approximately 40% of total porphyrinogen content part way through the metabolism.[29] Other substrates have been reported (Table 1),[28−35] and these suggest that a specific sequence of peripheral substituents are necessary for enzyme−substrate recognition. Initially, the sequence R Me-P Me (where R can be H, Me, vinyl (V) or Et but not a bulky propionate (P) side chain) was proposed as a minimal requirement for metabolism,[33] but the necessity of a second propionate residue was subsequently recognized.[13b,34,36] For the known substrates listed in Table 1, the necessary sequence R Me-P Me-P has been highlighted in bold; the selected nonsubstrates all interupt this sequence in one way or another.

These data provide clues about the nature of copro'gen oxidase, and this has allowed us to develop a model for the active site of this poorly understood enzyme (Scheme 5).[37] Three regions are defined for substrate binding. Region X is a binding site for the second propionate side chain and is envisaged as involving an ionic interaction perhaps to a lysine residue.[38] Region Y represents the catalytic site where the oxidative decarboxylation takes place, while Z has a steric requirement for a small alkyl residue (H, Me, V, Et) but cannot accommodate a propionate moiety. Following the formation of the enzyme−substrate complex, oxidative decarboxylation would occur at Y to give hardero'gen. This is set up to undergo a 90° rotation while remaining at the active site of copro'gen oxidase so that the C ring propionate can associate with X, and the B ring propionate slots into the catalytic site Y while Z can accommodate the vinyl moiety. Following metabolism of the B ring propionate side chain, proto'gen-IX could dissociate as the final enzymic product. The situation for copro'gen-IV is somewhat altered, and the proposed model provides a good explanation for the differences observed in its metabolism. Copro'gen-IV has a plane of symmetry and has two equivalent orientations where it can be compatible with the active site. Following association and oxidative decarboxylation, "hardero'gen-IV" is generated, but this structure cannot undergo a simple rotation so that a second propionate group can be held at Y while fulfilling the requirements at X and Z. Instead, this species would have to dissociate from the enzyme and flip over before the enzyme−substrate complex could be reformed (Scheme 6). Hence, proto'gen-XIII can be generated as the final

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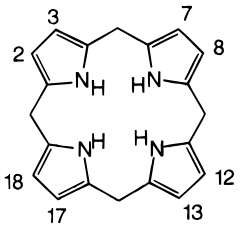
Table 1. Substrates and Selected Nonsubstrates for Coproporphyrinogen Oxidase



P = $CH_2CH_2CO_2H$

A = CH_2CO_2H

V = $CH{=}CH_2$

	Substituents							
porphyrinogen	2	3	7	8	12	13	17	18
known substrates								
coproporphyrinogen-III[13,28,29]	**Me**	**P**	**Me**	**P**	Me	P	P	**Me**
harderoporphyrinogen[13,25]	Me	**V**	**Me**	**P**	**Me**	**P**	P	Me
coproporphyrinogen-IV[28−32]	P	**Me**	**Me**	**P**	**Me**	**P**	P	Me
"harderoporphyrinogen-IV"[29−32]	**P**	**Me**	**Me**	V	Me	P	**P**	**Me**
dihydrohardero'gen[35]	Me	**Et**	**Me**	**P**	**Me**	**P**	P	Me
desvinylhardero'gen[28]	Me	**H**	**Me**	**P**	**Me**	**P**	P	Me
penta'gen dab[35]	**Me**	**P**	**Me**	**P**	A	P	P	**Me**
mesoporphyrinogen-VI[33] (**3a**)	**Me**	**P**	**Me**	**P**	Me	Et	Et	**Me**
nonsubstrates								
coproporphyrinogen-I[20]	Me	P	Me	P	Me	P	Me	P
coproporphyrinogen-II[29]	Me	P	P	Me	Me	P	P	Me
isocopro'gen[35]	Me	Et	Me	P	A	P	P	Me
proto'gen-IX[13]	Me	V	Me	V	Me	P	P	Me
meso'gen-IX[13]	Me	Et	Me	Et	Me	P	P	Me
meso'gen-XII[39]	Et	Me	Me	Et	Me	P	P	Me

product but the monovinylic intermediate will inevitably accumulate at earlier times in kinetic studies.

Although this model provides an excellent rationalization for nearly all of the reported substrate recognition studies, one report was seemingly at odds with this conjecture.[33] The C and D ring propionate residues are not metabolized by copro'gen oxidase, and in early work it was not clear whether they fulfilled any role in enzyme−substrate recognition. To investigate the possible role of these units, an analogue with ethyl substituents in place of the acidic side chains (mesoporphyrinogen-VI; **3a**) was synthesized and incubated with copro'gen oxidase (Scheme 7). Enzyme preparations from rat liver gave the monocarboxylate species **4a** as the major product, but chicken red cell hemolysates were reported to give further metabolism to generate a divinyl species **5**.[33] Although meso'gen-VI has the correct sequence of substituents to be recognized by copro'gen oxidase, monovinyl porphyrinogen **4a** does not have the structural requirements for further metabolism. Specifically, the second propionate residue that binds to region X is absent. It is notable that other meso'gen isomers such as meso'gens IX and XII (Chart 2) are not recognized by copro'gen oxidase.[39] In particular, meso'gen-XII represents a modified form of copro'gen-IV in much the same way as meso'gen-VI is conceptually derived from copro'gen-III, but this is still insufficient for this species to fulfill the requirements needed in our model for enzyme−substrate recognition. While we recognized that the model was a starting point for these investigations and could not be expected to be infallible, the inconsistencies required investigation. To carry out such as investigation, it was first necessary to synthesize the necessary porphyrinogen substrates.

Synthesis of Mesoporphyrin-VI and Related Di- and Monocarboxylate Porphyrins

Porphyrinogens are unstable molecules that are prone to both oxidation and isomerization reactions, and the required materials were synthesized in the form of the corresponding porphyrin methyl esters. Several routes for porphyrin synthesis are available for these studies, but we have favored the use of *a*,*c*-biladiene intermediates for this work.[40] The original study[33] made use of mesoporphyrin-VI (**6a**; Scheme 8), which has ethyl groups on the C and D rings in place of the propionate side chains of the natural substrate, but it seemed appropriate to extend the series to include structures with two additional methyl, propyl, or butyl substituents (**6b**−**d**). The lower half of these structures has an inherent symmetry, and this allows both the C and D rings to be constructed from a single pyrrole aldehyde in each case. The required aldehydes **7a**−**d** were prepared from the corresponding pyrrole ethyl esters **8**[41] by cleaving the ester moiety with sodium hydroxide in refluxing ethylene glycol, followed by formylation with benzoyl chloride in DMF[42] (Scheme 9).

The upper portion of the targeted porphyrins can be derived from a dipyrrylmethane **9a**, and this pivotal intermediate was easily prepared by condensing acetoxymethylpyrrole **10a**[43] with α-unsubstituted pyrrole *tert*-butyl ester **11**[44] in the presence of Montmorillonite clay[45] in dichloromethane (Scheme 8). Many dipyrrylmethanes with mixed benzyl and *tert*-butyl esters have been isolated as oils or gums that do not crystallize,[45] and this appeared to be the case for **9a** in our initial

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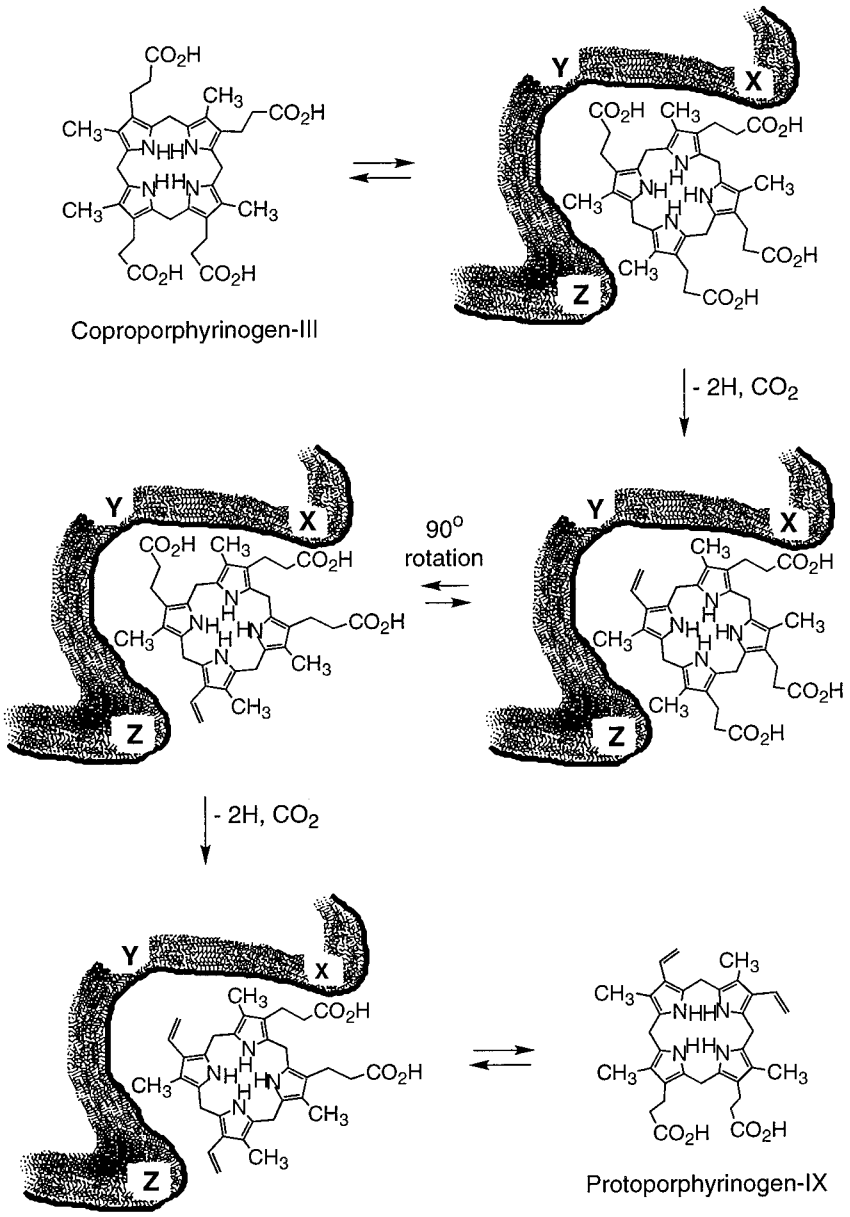
Scheme 5



investigations. However, after many attempts to crystallize a sample of **9a**, the material was to have been subjected to purification by flash chromatography using acetone−petroleum ether as the eluent. Surprisingly, this mixture induced immediate crystallization, and this proved to be a fortuitous and convenient method for purifying the dipyrrole. The benzyl ester was cleaved by hydrogenolysis over 10% palladium−charcoal to give the corresponding carboxylic acid **12a**. Treatment with TFA cleaved the *tert*-butyl ester and decarboxylated the pyrrole carboxylic acid, and subsequent condensation with formylpyrroles **7a**−**d** in the presence of HBr−acetic acid and precipitation with ether gave a series of *a*,*c*-biladiene dihydrobromide salts **13** as orange or red powders in 66−82% yield. Cyclization with copper(II) chloride in DMF at room temperature,[46] followed by demetalation with 15% H_2SO_4−TFA and reesterification with 5% H_2SO_4−methanol, gave the required porphyrin dimethyl esters **6a**−**d** in 54−64% yield.

To fully understand the biochemical transformations, a monovinyl porphyrin **14** corresponding to the oxidized form of the reported meso'gen-VI metabolite **4a** was needed, and a related triethylporphyrin **6f** was also targeted for synthesis. Pyrrolylmethyl acetates **10b**[47] and **10c**[48] were condensed with **11** using Montmorillonite clay as a catalyst to give the dipyrrylmethanes **9b** and **9c**, and subsequent hydrogenolysis over palladium-charcoal afforded the corresponding carboxylic acids **12**. Treatment with TFA, followed by condensation with 2 equiv of pyrrole aldehyde **7a** in the presence of HBr gave the *a*,*c*-biladienes **13e** and **13f**, respectively, in good yields. Cyclization with copper(II) chloride in DMF, followed by demetalation and reesterification, gave the 3-(chloroethyl)- and 3-ethylporphyrins **6e** and **6f** in 30−32% yield. Chloroethylporphyrin **6e** was dehydrohalogenated with

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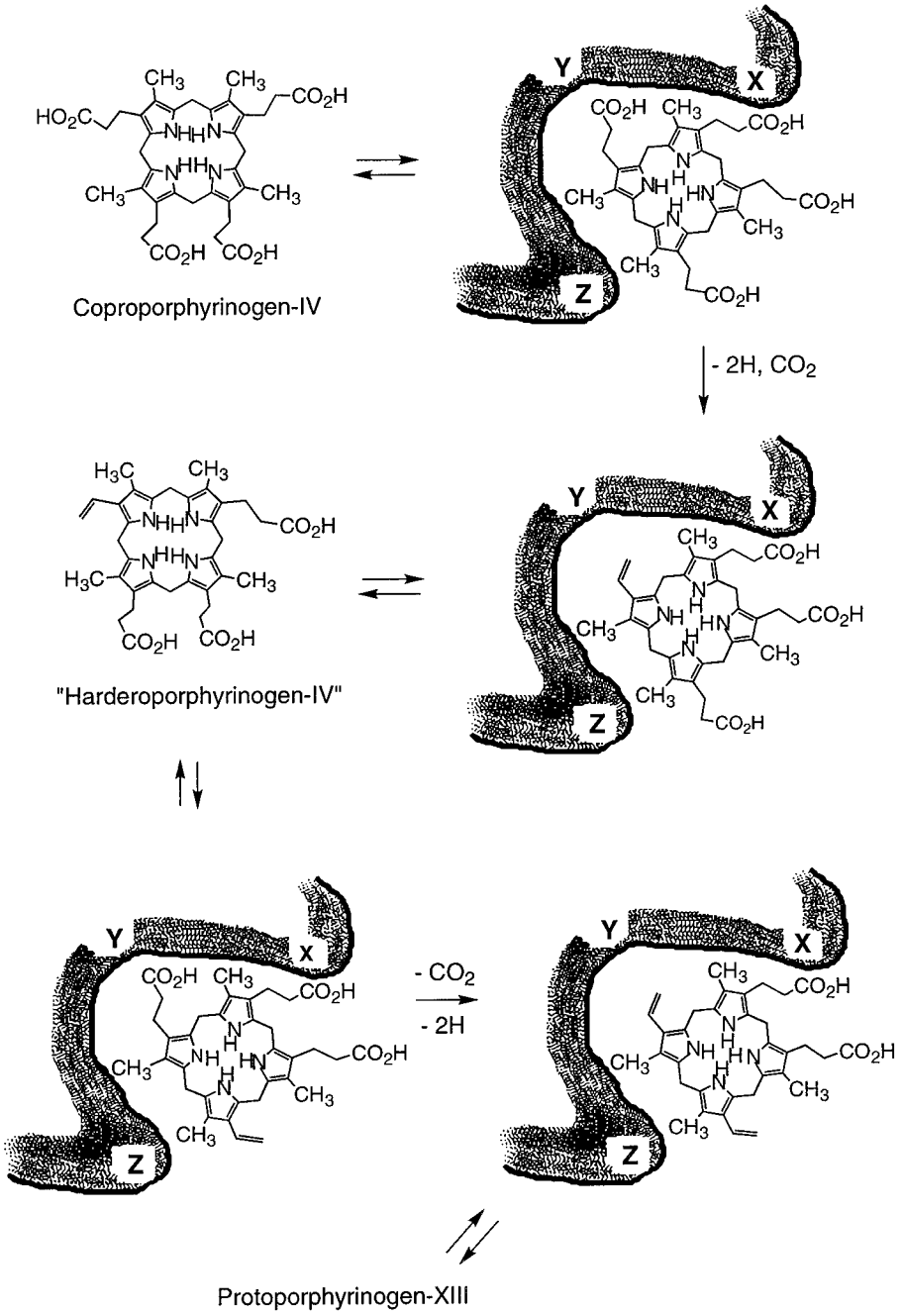
Scheme 6



potassium hydroxide in refluxing pyridine[49] to afford the monocarboxylate harderoporphyrin analogue **14a** in 82% yield.

Mesoporphyrin-XII dimethyl ester (**15**) was also required as a standard, as the corresponding porphyrinogen (Chart 2) has previously been shown to be unaffected by copro'gen oxidase. Condensation of dipyrrylmethanedicarboxylic acid **16**[50] with 2 equiv of pyrrole aldehyde **17** in the presence of HBr gave the *a*,*c*-biladiene **18**, and subsequent cyclization with copper(II) chloride in DMF afforded the required porphyrin in excellent yield (Scheme 10).

Biochemical Studies with Chicken Red Cell Hemolysates (CRH)

The natural substrates for coproporphyrinogen oxidase are porphyrinogen carboxylic acids, and these unstable compounds were generated as needed from the corresponding synthetic porphyrin methyl esters. Saponification of the esters is carried out in 25% hydrochloric acid (16 h at room temperature), and the resulting carboxylic acids are reduced with 3% sodium−amalgam under buffered conditions and incubated at 37 °C with crude enzyme preparations from chicken red blood cells.[15,51,52] The crude enzymic products reoxidize during isolation and for convenience are converted back into their methyl esters for analysis and characterization. Copro'gen-III was used as a standard in these studies and consistently

(50) Lash, T. D.; Armiger, Y. L. S.-T. *J. Heterocycl. Chem.* **1991**, *28*, 965.

(51) Dresel, E. I. B.; Falk, J. E. *Biochem. J.* **1956**, *63*, 388.

(52) Chicken blood provides a convenient source for copro'gen oxidase. While the substrate specificity is likely to vary for different sources, the avian enzyme appears to give results similar to those from mammalian sources.[13,29] Under the experimental conditions utilized for these studies, the mitochondria are disrupted.

Scheme 7. Metabolism of Mesoporphyrinogen-VI

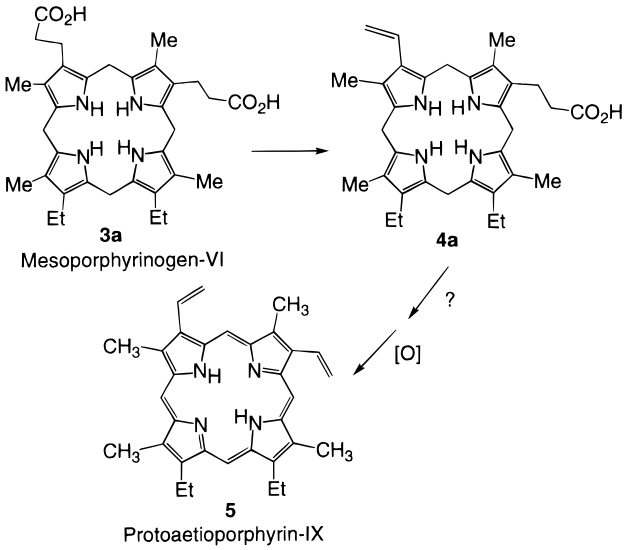
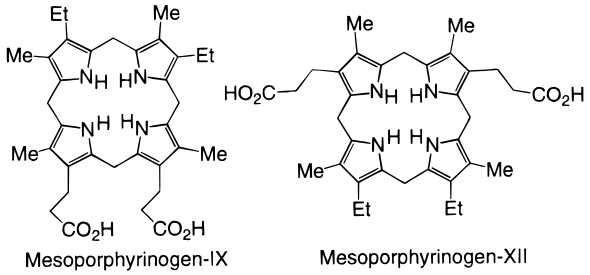


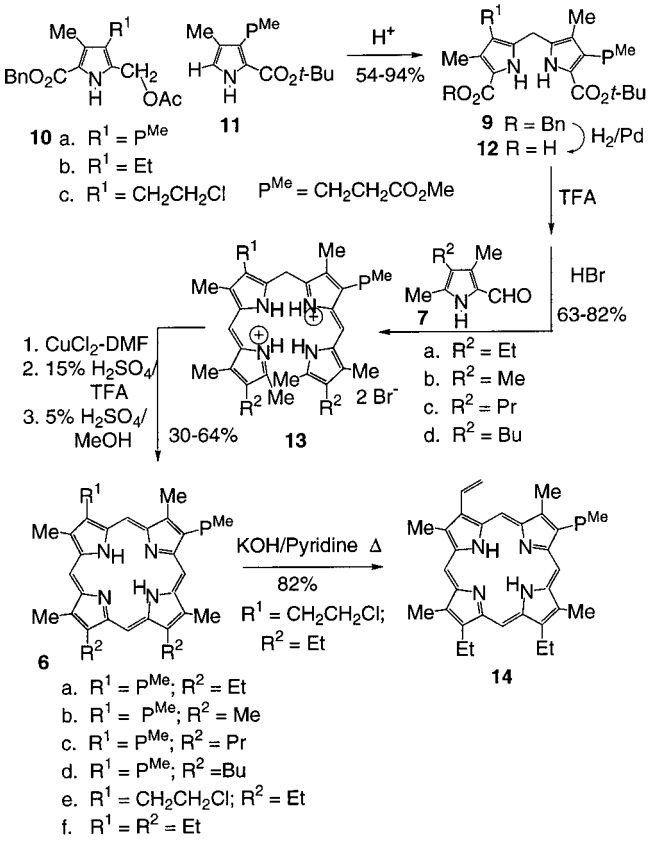
Chart 2. Two Type Isomers of Mesoporphyrinogen That Are Not Metabolized by Copro'gen Oxidase



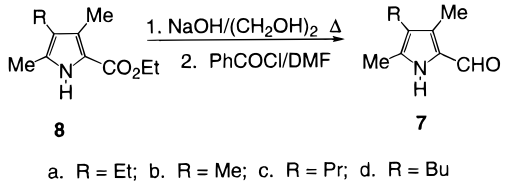
gave good conversions to protoporphyrin-IX. Endogenous protoporphyrin was also present in these isolates, and it was necessary to take this into account when assessing the extent of substrate conversion. For each experiment, a zero incubation time sample was also evaluated to quantify the endogenous protoporphyrin-IX content, and this value was used to adjust the data from the incubation studies.

Mesoporphyrinogen-VI (**3a**) was incubated with chicken red cell hemolysates (CRH) for varying times, and the metabolites were analyzed by TLC and HPLC. The initial data confirmed that **3a** was a good substrate for copro'gen oxidase, but the R_f values and retention times by HPLC indicated that the only product was a monocarboxylate species (Scheme 10) that was chromatographically indistinguishable from **14a**. Time course studies indicate that **3a** is converted to this metabolite at much the same rate as copro'gen-III is converted into proto'gen-IX (Figure 1). To further identify the metabolic product, larger scale incubations were performed, and the product (as the porphyrin methyl ester) was characterized by proton NMR spectroscopy and mass spectrometry. NMR confirmed the presence of one vinyl group and the retention of one propionate side chain; the data also were indistinguishable from those obtained for the synthetic material. FAB MS gave the expected $[M + H]^+$ ion at m/z 563. Similar kinetic results were obtained using duck and turkey blood, and none of our experiments showed even a trace of divinylporphyrin. Synthetic monovinylporphyrinogen **4a** (derived from **14a**) and the related porphyrinogen from porphyrin **6f** were similarly incubated with

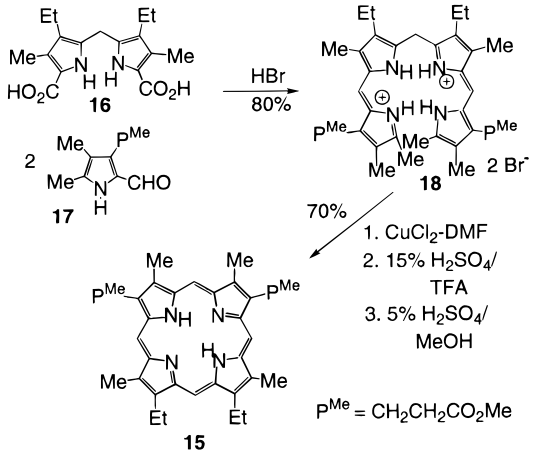
Scheme 8



Scheme 9



Scheme 10



CRH, but no metabolism to a less polar "zero-carboxylate" product could be detected by HPLC or TLC in any of the multiple experiments that we have conducted. In a control study, the "zero-carboxylate" standard etioporphyrin-IV was added to CRH incubations and taken through the standard isolation procedures. The relatively lipophilic etioporphyrin-IV was recovered unchanged from these studies, indicating that the divinylporphyrin

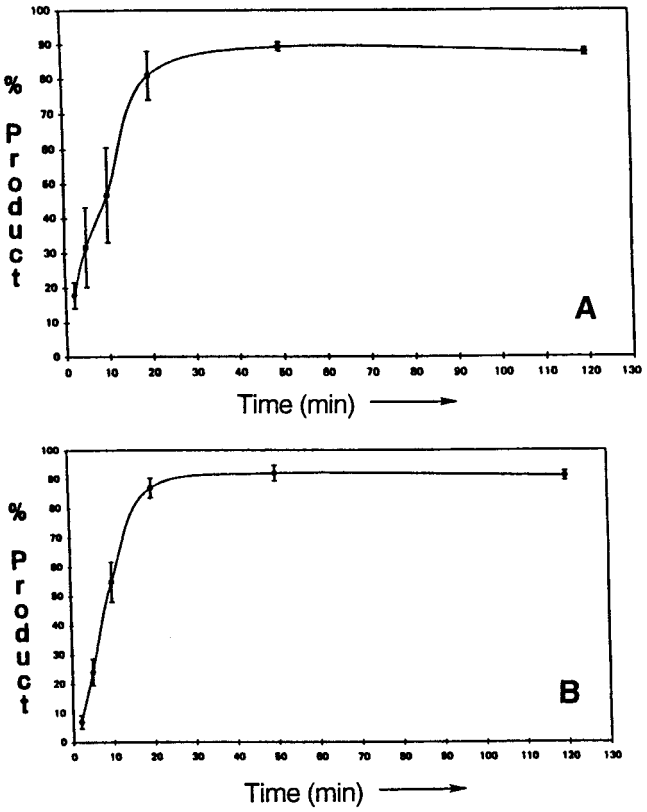


Figure 1. Time course study for incubations of copro'gen-III (A) and meso'gen-VI (B) showing the percentage of final product formation vs time.

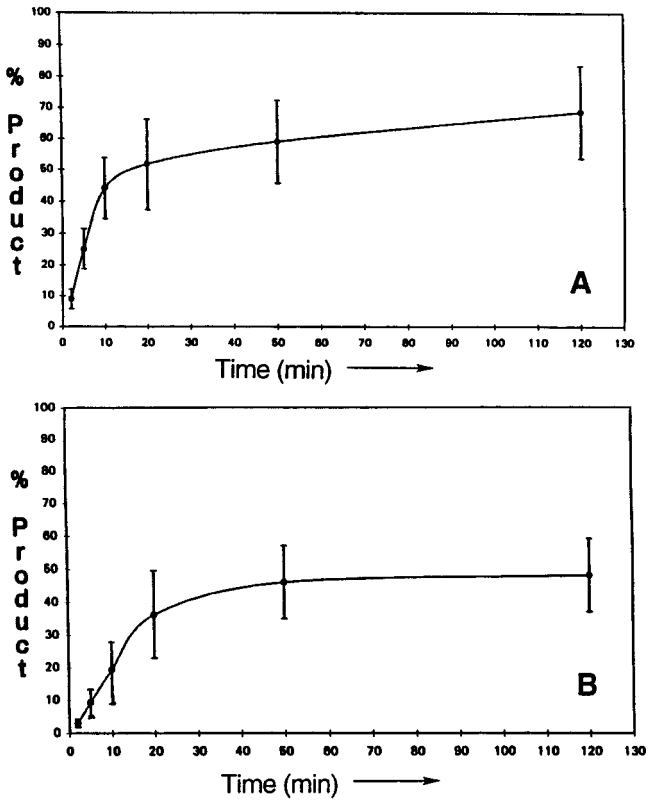
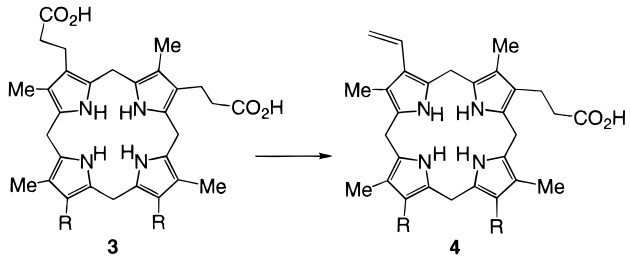


Figure 2. Time course study for incubations of **3b** (A) and **3c** (B) showing the percentage of vinylporphyrinogen product formation vs time.

5 would extract if it were generated in these investigations. Although the etioporphyrin-IV standard ran very close to the dichloromethane solvent peak in many of the HPLC chromatograms, raising the possibility that trace amounts of **5** might be obscured in these analyses, TLC indicated that no porphyrins (easily identified by their bright red fluorescence under long-wavelength UV light) were present in the isolates from these incubation studies with higher R_f values than **14a**.

While our results are self-consistent, it seemed appropriate to extend these investigations to a series of related porphyrinogens. Synthetic porphyrins with 13,17-dimethyl (**6b**), 13,17-dipropyl (**6c**) and 13,17-dibutyl substituents (**6d**) were readily available using the same synthetic methods for **6a** (vide infra). The selection of ethyl moieties on rings C and D in the meso'gen-VI studies was to a certain extent an arbitrary choice, and it was of some interest to see whether the presence alternative alkyl substituents would have any bearing on these results. Porphyrins **6b**−**d** were converted into the correponding porphyrinogens **3b**−**d** (Scheme 10) as before and incubated with CRH, and the products were analyzed by TLC and HPLC, and for **3b** and **3c** also by NMR spectroscopy and mass spectrometry. The hexamethylporphyrinogen **3b** was a good substrate for copro'gen oxidase, although the maximum conversion to monovinylic product was somewhat lower than for **6a** (Figure 2a), but again only one propionate side chain was processed to give **4b** as the final product (Scheme 11). In the case of the dipropyl species **3c**, the monovinyl compound was again the only product but the initial rate and overall conversion were greatly reduced (approximately 50%) for this porphyrinogen relative to meso'gen-VI (Figure 2b). Dibutylporphyrinogen **3d** was a particularly poor substrate, and only 6% was converted to a monovinylic product after 2 h at 37 °C. These results confirm our initial observations for meso'gen-VI and provide further insights into substrate selectivity. As the number of carbon atoms at positions 13 and 17 are increased, there is a slight increase (approximately 25%) in activity from methyl to ethyl, followed by a sharp decline in metabolism from ethyl to propyl to butyl (Figure 3). This suggests that further interactions besides those at regions X, Y, and Z (Scheme 5) have an influence on substrate binding at the active site of copro'gen oxidase. The factor cannot be primarily steric because the propionate groups on the natural substrate copro'gen-III are comparable in size to the butyl substituents in **3d**, and it seems likely that there is a region of lipophobicity where the carboxylate components of the normal propionate side chains usually reside. However, it should be noted that these data were obtained using a crude enzyme source that contains membrane fragments from the original red blood cells, and it is possible that this material can sequester the more lipophilic porphyrino-

Scheme 11. Metabolism of Dicarboxylate Porphyrinogens



a. R = Et; b. R = Me; c. R = Pr; d. R = Bu

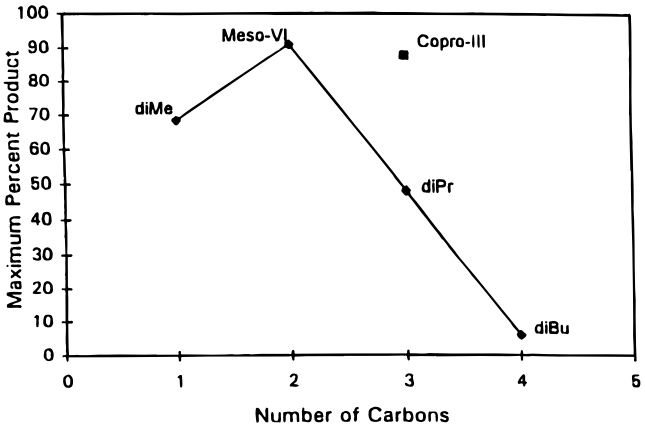


Figure 3. Maximum product formation for **3b** (diMe), **3a** (Meso-VI), **3c** (diPr), and **3d** (diBu) showing a sharp decline in metabolism as the number of carbons at positions 13 and 17 increase from 2 to 4.

Table 2. Comparison of Initial Velocities and Maximum Product Formation for 3a−d and Copro'gen-III[a]

substrates	no. of carbons at positions 13/17	initial velocity[e]	max % product
3b	1	4.4 ± 1.0[c]	69 ± 15
3a	2	4.4 ± 0.17[c]	92 ± 2.7[d]
3c	3	1.8 ± 0.71	48 ± 11
3d	4	ND[b]	6.0 ± 1.6
copro'gen-III	3	3.4 ± 0.15	88 ± 1.3[d]

[a] For each substrate, the means and standard deviations were determined for four separate experiments. [b] ND = not determined. [c,d] Values with the same letter are not significantly different at $p < 0.05$. [e] % product/min.

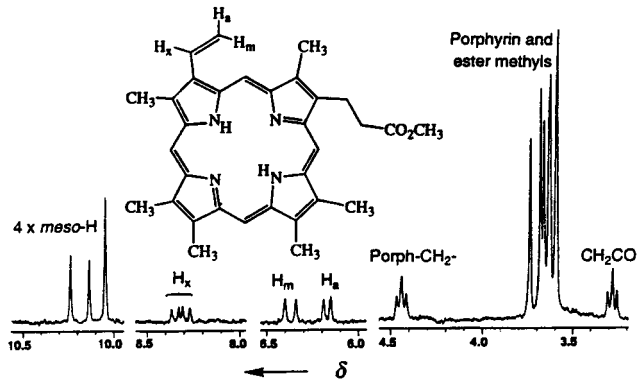


Figure 4. 300 MHz proton NMR spectrum of the esterified monovinylporphyrin product from incubations of **3b** with CRH.

gens, thereby reducing accessibility to the enzyme. Further studies will be required using highly purified copro'gen oxidase to assess this possibility. The lesser activity of the hexamethylporphyrinogen **6b** is puzzling, especially since the initial rate for this substrate is comparable to the value observed for meso'gen-VI. Table 2 compares the initial rates and maximum percentage of product formation for copro'gen-III and substrate analogues **3a**−**d**.

The structures of the new metabolites **14b** and **14c** were confirmed by high-resolution mass spectrometry and 300 MHz proton NMR spectroscopy (Figure 4). In both cases, the presence of one vinyl unit was evident from the proton NMR data by the presence of two 1H doublets at 6.2 and 6.4 ppm and a 1H doublet of doublets at 8.3 ppm. The remaining propionate side chain gave two 2H triplets at 3.3 and 4.5 ppm, in accord with the expected values for a porphyrin structure of this type, and the meso protons appeared as a series of downfield singlets near 10 ppm.

Conclusions

The new results show that meso'gen-VI and some related porphyrinogens are consistently metabolized by CRH preparations of copro'gen oxidase into monovinylic products, and this provides strong support for our proposed model for the active site of this important enzyme. The structures of the resulting monovinyl products have been confirmed by NMR and MS, and the relative activities of several dicarboxylate substrates have been contrasted by carrying out kinetic studies. Although the first oxidative decarboxylation of the A ring propionate unit takes place smoothly when the C and D ring propionates are replaced with methyl or ethyl groups, further extension of these alkyl substituents leads to a sharp decline in activity, suggesting the presence of an important lipophobic region. Further studies of this type are likely to lead to an increased understanding of this critical enzyme in the heme biosynthetic pathway.

Experimental Section

Hydrogenations were carried out using a Parr hydrogenator at 30−40 psi. Chromatography was performed using Grade 3 neutral alumina or 70−230 mesh silica gel, and 3% sodium−amalgam was prepared as described in the literature.[53] Unless otherwise indicated, reagents were purchased from Aldrich Chemical Co. and were not further purified. EI and FAB mass spectral determinations were made at the Mass Spectral Laboratory, School of Chemical Sciences, University of Illinois at Urbana−Champaign, supported in part by a grant from the National Institute of General Medical Sciences (GM 27029) or the Midwest Center for Mass Spectrometry at the University of Nebraska−Lincoln with partial support by the National Science Foundation, Biology Division (Grant No. DIR9017262). Elemental analyses were obtained from Micro-Analysis, Inc., Wilmington, DE, or the School of Chemical Sciences Microanalysis Laboratory at the University of Illinois.

Ethyl 3,5-Dimethyl-4-propylpyrrole-2-carboxylate (8c). Iodopropane (85.0 g) was added to a stirred mixture of 2,4-pentanedione (50.0 g), potassium carbonate (84.0 g), and acetone (125 mL) in a 500 mL three-necked round-bottom flask over a period of 5 min. The resulting mixture was stirred vigorously under reflux for 20 h. Upon cooling, the mixture was suction filtered, and inorganic solids were washed thoroughly with acetone. The solvent was evaporated under reduced pressure and the residue distilled to give 3-propyl-2,4-pentanedione (49.6 g; 70%) as a pale yellow oil, bp 185−190 °C.

A mixture of diethyl aminomalonate[41,54] (44.20 g) and 3-propyl-2,4-pentanedione (34.74 g) was added dropwise to refluxing acetic acid (91 mL), and the resulting light brown solution was stirred under reflux for 1 h. The mixture was cooled to 70 °C, poured into ice (800 mL), and allowed to stand for several hours. The precipitate was filtered off and washed with water. Recrystallization from 95% ethanol gave the title pyrrole as white crystals (34.64 g; 68%): mp 97−97.5 °C (lit.[55] mp 97.5−98.5 °C); ^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, J = 7.3 Hz), 1.35 (3H, t, J = 7.2 Hz), 1.45 (2H, sextet), 2.19 (3H, s), 2.27 (3H, s), 2.33 (2H, t, J = 7.5 Hz), 4.29 (2H, q, J = 7.2 Hz), 8.69 (1H, br s).

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(55) MacDonald, S. F.; Markovac, A. *Can. J. Chem.* **1965**, *43*, 3247.

3,5-Dimethyl-4-propylpyrrole-2-carboxaldehyde (7c). Ethyl 4-propyl-3,5-dimethylpyrrole-2-carboxylate (5.00 g) was refluxed for 45 min with sodium hydroxide (5.01 g) in ethylene glycol (50 mL). The cloudy yellow mixture was dispersed between hexane (100 mL) and water (50 mL), the aqueous layer was extracted with hexane (2 × 50 mL), and the combined organic layers were washed with water and dried over sodium sulfate. The solvent was evaporated under reduced pressure to give a yellow oil. *N*,*N*-Dimethylformamide (15 mL) was added to the residual oil and cooled to 0 °C in an ice−salt bath. Benzoyl chloride (1 mL) was added dropwise, maintaining the temperature below 5 °C. When the temperature dropped to −5 °C, the ice−salt bath was removed, and the mixture was stirred further for 15 min. Toluene (50 mL) was added to the mixture and cooled in the ice−salt bath for 1 h. The buff-colored precipitate was suction filtered and washed with cold toluene (20 mL). The solid was dissolved in ethanol (40 mL), a solution of aqueous sodium carbonate (5.0 g in 40 mL of H_2O) was added, and the mixture was stirred on a boiling water bath for 15 min. Water (100 mL) was added and the mixture stirred overnight at room temperature. The mixture was cooled in an ice-bath and filtered to give a yellow-brown solid. Recrystallization from 95% ethanol gave the title pyrrole aldehyde (3.62 g; 81%) as pale yellow needles: mp 101.5−102.5 °C (lit.[56] mp 105 °C); ^{1}H NMR ($CDCl_3$) δ 0.91 (3H, t, J = 7.3 Hz), 1.42−1.50 (2H, m), 2.24 (3H, s), 2.26 (3H, s), 2.36 (2H, t, J = 6 Hz), 9.44 (1H, s), 9.8 (1H, br s).

4-Butyl-3,5-dimethylpyrrole-2-carboxaldehyde (7d). The title compound was prepared from ethyl 4-butyl-3,5-dimethylpyrrole-2-carboxylate[57] (5.03 g) by the procedure detailed above. Recrystallization from 95% ethanol gave the aldehyde (3.68 g; 81%) as off-white needles: mp 84−85 °C (lit.[58] mp 85 °C); ^{1}H NMR ($CDCl_3$) δ 0.94 (3H, t, J = 7.0 Hz), 1.38 (4H, m), 2.25 (3H, s), 2.27 (3H, s), 2.37 (2H, t, J = 7.2 Hz), 9.20 (1H, br s), 9.47 (1H, s).

4-Ethyl-3,5-dimethylpyrrole-2-carboxaldehyde (7a). Compound **7a** was prepared from ethyl 4-ethyl-3,5-dimethylpyrrole-2-carboxylate[41] (5.00 g) by the procedure detailed above. Recrystallization from 95% ethanol gave the aldehyde (3.21 g; 82%) as pale yellow needles: mp 101−102 °C (lit.[59] mp 106 °C); IR (Nujol mull) ν 3363 (st, sh, NH), 1620 (st, sh, C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.05 (3H, t, J = 7 Hz), 2.25 (3H, s), 2.26 (3H, s), 2.42 (2H, q, J = 7 Hz), 9.45 (1H, s), 9.80 (1H, br s).

3,4,5-Trimethylpyrrole-2-carboxaldehyde (7b). Compound **7b** was prepared from ethyl 3,4,5-trimethylpyrrole-2-carboxylate[41] (5.00 g) by the procedure detailed above. Recrystallization from 95% ethanol gave the aldehyde (2.95 g; 78%) as off-white needles: mp 144−146 °C (lit.[60a] mp 145−146 °C; lit.[60b] 147 °C; mp lit.[60c] 144−145 °C); IR (Nujol mull) ν 3270 (st, sh, NH), 1620 (st, sh, C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.96 (3H, s), 2.27 (3H, s), 2.28 (3H, s), 9.4 (1H, br s), 9.49 (1H, s).

***tert*-Butyl 5′-(Benzyloxycarbonyl)-3′,4-bis(2-methoxycarbonylethyl)-3,4′-dimethyl-2,2′-dipyrrylmethane-5-carboxylate (9a).** Montmorillonite clay K-10 (37.00 g) was added to a stirred solution of benzyl 5-(acetoxymethyl)-4-(2-methoxycarbonylethyl)-3-methylpyrrole-2-carboxylate[43] (**10a**; 10.60 g) and *tert*-butyl 3-(2-methoxycarbonylethyl)-4-methylpyrrole-2-carboxylate[44] (**11**; 8.30 g; 1.1 equiv) in dichloromethane (400 mL). The resulting mixture was stirred vigorously for 24 h, although the reaction appeared to be complete after 5 h by TLC. The clay catalyst, which turned dark red in color, was filtered off and washed thoroughly with dichloromethane. The solvent was evaporated under reduced pressure to afford an orange gum. Recrystallization from 20% acetone−hexane gave the title dipyrrole (13.28 g; 77.5%) as pale pink crystals, mp 141−143 °C. Further crystallization using the same solvent system gave an analytical sample: mp 141−142 °C; ^{1}H NMR ($CDCl_3$) δ 1.52 (9H, s), 1.99 (3H, s), 2.26 (3H, s), 2.42 (2H, t, J = 7.5 Hz), 2.50 (2H, t, J = 7.5 Hz), 2.71 (2H, t, J = 7.5 Hz), 2.97 (2H, t, J = 7.5 Hz), 3.66 (3H, s), 3.67 (3H, s), 3.87 (2H, s), 5.25 (2H, s), 7.3−7.4 (5H, m), 8.63 (1H, br s), 8.91 (1H, br s); ^{13}C NMR ($CDCl_3$) δ 8.75, 10.99, 19.30, 21.01, 22.98, 28.45, 34.50, 35.12, 51.50, 51.98, 65.75, 80.89, 116.75, 117.87, 119.40, 120.45, 127.58, 128.01, 128.45, 128.94, 130.89, 136.51, 161.10, 161.60, 173.88. Anal. Calcd for $C_{32}H_{40}N_2O_8$: C, 66.19; H, 6.94; N, 4.82. Found: C, 66.09; H, 6.66; N, 4.55.

***tert*-Butyl 5′-(Benzyloxycarbonyl)-3′-(2-chloroethyl)-4-(2-methoxycarbonylethyl)-3,4′-dimethyl-2,2′-dipyrrylmethane-5-carboxylate (9b).** The title dipyrrylmethane was prepared by the previous procedure from benzyl 5-(acetoxymethyl)-4-(2-chloroethyl)-3-methylpyrrole-2-carboxylate[47] (**10b**; 3.01 g) and *tert*-butyl 3-(2-methoxycarbonylethyl)-4-methylpyrrole-2-carboxylate[44] (**11**; 2.52 g) in ethanol-free chloroform (165 mL). Recrystallization from dichloromethane−hexane (1:2 v/v) gave the title dipyrrole (3.03 g; 63%) as pale pink crystals: mp 137.5−138.5 °C; ^{1}H NMR ($CDCl_3$) δ 1.52 (9H, s), 1.97 (3H, s), 2.28 (3H, s), 2.51 (2H, t, J = 8 Hz), 2.83 (2H, t, J = 8 Hz), 2.97 (2H, t, J = 8 Hz), 3.44 (2H, t, J = 8 Hz), 3.66 (3H, s), 3.85 (2H, s), 5.27 (2H, s), 7.36 (5H, m), 8.68 (1H, br s), 8.91 (1H, br s); ^{13}C NMR ($CDCl_3$) δ 8.95, 10.94, 21.01, 23.51, 27.58, 28.51, 35.22, 45.02, 51.56, 66.00, 80.99, 117.25, 118.41, 118.64, 119.50, 127.62, 128.25, 128.87, 129.10, 131.01, 136.50, 161.01, 161.75, 173.90. Anal. Calcd for $C_{30}H_{37}N_2O_6Cl$: C, 64.68; H, 6.69; N, 5.03. Found: C, 64.40; H, 6.56; N, 4.91.

***tert*-Butyl 5′-(Benzyloxycarbonyl)-3′-ethyl-4-(2-methoxycarbonylethyl)-3,4′-dimethyl-2,2′-dipyrrylmethane-5-carboxylate (9c).** The dipyrrylmethane was prepared by the reaction of (acetoxymethyl)pyrrole **10c**[48] (0.91 g) and α-unsubstituted pyrrole **11**[44] (0.81 g) under the conditions described above for **9a**. The crude product was purified by chromatography on silica, eluting first with 1% and then 2% ethyl acetate−toluene. A small prefraction was collected that corresponded to a symmetrical dipyrrylmethane byproduct. The main fraction gave the title dipyrrylmethane (1.18 g; 78%) as a pale yellow-orange gum: ^{1}H NMR ($CDCl_3$) δ 1.01 (3H, t, J = 7 Hz), 1.51 (9H, s), 1.96 (3H, s), 2.28 (3H, s), 2.35 (2H, q, J = 7 Hz), 2.50 (2H, t, J = 8 Hz), 2.97 (2H, t, J = 8 Hz), 3.66 (3H, s), 3.80 (2H, s), 5.26 (2H, s), 7.34 (5H, m), 8.71 (1H, br s), 8.88 (1H, br s). Analytical data were obtained for the related carboxylic acid **12c**.

5′-(*tert*-Butoxycarbonyl)-3,4′-bis(2-methoxycarbonylethyl)-3′,4-dimethyl-2,2′-dipyrrylmethane-5-carboxylic Acid (12a). *tert*-Butyl 5′-(benzyloxycarbonyl)-3′,4-bis(2-methoxycarbonylethyl)-3,4′-dimethyl-2,2′-dipyrrylmethane-5-carboxylate (**9a**; 1.156 g) and triethylamine (20 drops) were dissolved in methanol (150 mL), and the solution was shaken with 10% palladium/charcoal (200 mg) under a hydrogen atmosphere at 44 psi and room temperature for 14 h. The catalyst was filtered off and the solvent evaporated under reduced pressure. The residue was taken up in aqueous ammonia (5%) and cooled to 0 °C using an ice−salt bath. The solution was neutralized with glacial acetic acid, maintaining the temperature at 0 °C, and the resulting precipitate was filtered off, washed thoroughly with water, and dried in vacuo overnight. The title dipyrrylmethanecarboxylic acid (0.930 g; 95%) was obtained as a pale pink powder: mp 143−144 °C; ^{1}H NMR ($CDCl_3$) δ 1.55 (9H, s), 2.10 (3H, s), 2.30 (3H, s), 2.32 (2H, t), 2.43 (2H, t), 2.77 (2H, t), 2.96 (2H, t), 3.67 (3H, s), 3.69 (3H, s), 3.89 (2H, s), 10.79 (1H, br s), 11.50 (1H, br s). The carboxylic acid proton did not show up in the NMR spectrum. Anal. Calcd for $C_{25}H_{34}N_2O_8$: C, 61.21; H, 6.99; N, 5.71. Found: C, 60.85; H, 6.70; N, 5.65.

5′-(*tert*-Butoxycarbonyl)-3-(2-chloroethyl)-4′-(2-methoxycarbonylethyl)-3′,4-dimethyl-2,2′-dipyrrylmethane-5-carboxylic Acid (12b). Chloroethyl dipyrrylmethane **9b** (501 mg) was hydrogenolyzed under the conditions described above. After being dried under vacuum overnight, the desired carboxylic acid (402 mg; 96%) was obtained as a pale pink powder: mp 145.5−147.5 °C; ^{1}H NMR ($CDCl_3$) δ 1.58 (9H, s), 2.15 (3H, s), 2.32 (3H, s), 2.50 (2H, t), 2.80−3.00 (4H,

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overlapping triplets), 3.38 (2H, t), 3.68 (3H, s), 3.90 (2H, s), 10.75 (1H, s), 11.15 (1H, s). Anal. Calcd for $C_{23}H_{31}N_2O_6Cl$: C, 59.16; H, 6.69; N, 6.00. Found: C, 58.79; H, 6.62; N, 5.88.

5′-(*tert*-Butoxycarbonyl)-3-ethyl-4′-(2-methoxycarbonylethyl)-3′,4-dimethyl-2,2′-dipyrrylmethane-5-carboxylic Acid (12c). Compound **12c** was prepared from **9c** by the procedure detailed above in 97% yield as a pale pink powder: mp 143 °C dec; ^{1}H NMR ($CDCl_3$) δ 1.23 (3H, t, J = 7 Hz), 1.53 (9H, s), 2.07 (3H, s), 2.30 (3H, s), 2.45 (2H, t, J = 7 Hz), 2.95 (4H, m), 3.66 (3H, s), 3.82 (2H, s), 10.80 (1H, br s), 11.58 (1H, br s). Anal. Calcd for $C_{23}H_{32}N_2O_6$: C, 63.87; H, 7.46; N, 6.48. Found: C, 63.41; H, 7.53; N, 6.08.

8,13-Bis(2-methoxycarbonylethyl)-1,2,3,7,12,17,18,19-octamethyl-10,23-dihydrobilin Dihydrobromide (13b). 5′-(*tert*-Butoxycarbonyl)-3,4′-bis(2-methoxycarbonylethyl)-3′,4-dimethyl-2,2′-dipyrrylmethane-5-carboxylic acid (**12a**; 502 mg) was treated with trifluoroacetic acid (2 mL) and stirred for 10 min at room temperature. A solution of 3,4,5-trimethylpyrrole-2-carboxaldehyde (**7b**; 280 mg; 2.0 equiv) in methanol (8.0 mL) was added, immediately followed by the addition of a mixture of aqueous 48% HBr and acetic acid (1:2.33 v/v; 1.6 mL), and the resulting dark red mixture was stirred at room temperature for 30 min. Anhydrous ether (40 mL) was added dropwise, and the resulting mixture was stirred for additional 2 h. The precipitate was filtered, washed thoroughly with ether, and dried in vacuo overnight to give the title *a*,*c*-biladiene dihydrobromide (628 mg; 80%) as a dark red powder: mp 220 °C, dec; UV−vis ($CHCl_3$) λ_{max} (log ϵ) 455 (4.45), 525 nm (5.23); ^{1}H NMR ($CDCl_3$) δ 1.90 (2H, t), 1.95 (3H, s), 1.99 (6H, s), 2.24 (3H, s), 2.27 (3H, s), 2.34 (3H, s), 2.48 (2H, t), 2.68 (6H, s), 2.78 (2H, t, J = 7 Hz), 2.94 (2H, t, J = 7 Hz), 3.42 (3H, s), 3.59 (3H, s), 5.19 (2H, s), 7.09 (1H, s), 7.30 (1H, s), 13.22 (2H, br s), 13.25 (1H, br s), 13.30 (1H, br s). Anal. Calcd for $C_{35}H_{46}N_4O_4Br_2 \cdot H_2O$: C, 54.98; H, 6.31; N, 7.32. Found: C, 54.59; H, 5.86; N, 6.98.

2,18-Diethyl-8,13-bis(2-methoxycarbonylethyl)-1,3,7,12,17,19-hexamethyl-10,23-dihydrobilin Dihydrobromide (13a). Compound **13a** was prepared from **12a** (501 mg) and 4-ethyl-3,5-dimethylpyrrole-2-carboxaldehyde (**7a**; 0.308 g; 2.0 equiv) by the procedure detailed above. The bright red precipitate was filtered, washed thoroughly with ether, and dried in vacuo overnight to give the title *a*,*c*-biladiene dihydrobromide (646 g; 82%) as red crystals: mp 194 °C dec; UV−vis ($CHCl_3$): λ_{max} (log ϵ) 455 (4.49), 525 nm (5.28); ^{1}H NMR ($CDCl_3$) δ 1.08 (6H, 2 overlapping triplets), 1.91 (2H, t), 1.94 (3H, s), 2.24 (3H, s), 2.29 (3H, s), 2.34 (3H, s), 2.4−2.5 (6H, m), 2.69 (6H, s), 2.78 (2H, t, J = 8 Hz), 2.93 (2H, t, J = 7 Hz), 3.42 (3H, s), 3.60 (3H, s), 5.20 (2H, s), 7.09 (1H, s), 7.31 (1H, s), 13.21 (2H, br s), 13.28 (1H, br s), 13.30 (1H, br s). Anal. Calcd for $C_{37}H_{50}N_4O_4Br_2 \cdot 0.5H_2O$: C, 56.71; H, 6.56; N, 7.15. Found: C, 56.35; H, 6.27; N, 6.89.

8,13-Bis(2-methoxycarbonylethyl)-1,3,7,12,17,19-hexamethyl-2,18-dipropyl-10,23-dihydrobilin Dihydrobromide (13c). Compound **13c** was prepared from **12a** (250 mg) and 4-propyl-3,5-dimethylpyrrole-2-carboxaldehyde (**7c**; 168 mg; 2.0 equiv) by the preceding method. The precipitate was filtered, washed well with ether, and dried in vacuo overnight to give the title *a*,*c*-biladiene dihydrobromide (283 mg; 69%) as a red-orange powder: mp 198 °C dec; UV−vis ($CHCl_3$) λ_{max} (log ϵ) 456 (4.48), 525 (5.27); ^{1}H NMR ($CDCl_3$) δ 0.93 (6H, 2 overlapping triplets), 1.50 (4H, m), 1.90 (2H, t), 1.95 (3H, s), 2.24 (3H, s), 2.28 (3H, s), 2.33 (3H, s), 2.40 (4H, t), 2.48 (2H, t), 2.70 (6H, s), 2.78 (2H, t, J = 8 Hz), 2.93 (2H, t, J = 7 Hz), 3.41 (3H, s), 3.59 (3H, s), 5.20 (2H, s), 7.09 (1H, s), 7.30 (1H, s), 13.23 (2H, s), 13.29 (1H, s), 13.32 (1H, s). Anal. Calcd for $C_{39}H_{54}N_4O_4Br_2$: C, 58.36; H, 6.78; N, 6.98. Found: C, 58.17; H, 6.66; N, 6.72.

2,18-Dibutyl-8,13-bis(2-methoxycarbonylethyl)-1,3,7,12,17,19-hexamethyl-10,23-dihydrobilin Dihydrobromide (13d). Compound **13d** was prepared from **12a** (254 mg) and 4-butyl-3,5-dimethylpyrrole-2-carboxaldehyde (**7d**; 187 mg; 2.0 equiv) by the procedure reported above. The bright-red precipitate was filtered, washed thoroughly with ether, and dried in vacuo overnight to give the title *a*,*c*-biladiene dihydrobromide (284 mg; 66%): mp 210 °C dec; UV−vis ($CHCl_3$) λ_{max} (log ϵ) 455 (4.45), 525 (5.25); ^{1}H NMR ($CDCl_3$) δ 0.93 (6H, 2 overlapping triplets), 1.40 (8H, m), 1.92 (2H, m), 1.96 (3H, s), 2.24 (3H, s), 2.28 (3H, s), 2.33 (3H, s), 2.42 (4H, t), 2.48 (2H, t), 2.70 (6H, s), 2.78 (2H, t, J = 8 Hz), 2.93 (2H, t, J = 7 Hz), 3.41 (3H, s), 3.60 (3H, s), 5.20 (2H, s), 7.09 (1H, s), 7.30 (1H, s), 13.22 (2H, s), 13.28 (1H, s), 13.33 (1H, s). Anal. Calcd for $C_{41}H_{58}N_4O_4Br_2 \cdot 0.5H_2O$: C, 59.28; H, 7.04; N, 6.74. Found: C, 58.91; H, 6.99; N, 6.62.

2,8,18-Triethyl-13-(2-methoxycarbonylethyl)-1,3,7,12,17,19-hexamethyl-10,23-dihydrobilin Dihydrobromide (13f). Dipyrrylmethane **12c** (602 mg) was dissolved in TFA (2.8 mL) and the resulting solution stirred at room temperature for 10 min. A solution of formylpyrrole **7a** (420 mg; 2.0 equiv) in methanol (11.1 mL) was added, followed immediately by 30% HBr in acetic acid (2.1 mL), and the mixture was stirred for a further 30 min. Anhydrous ether (40 mL) was added dropwise and the mixture stirred for an additional 2 h. The precipitate was filtered and washed with ether to give the *a*,*c*-biladiene (521 mg; 52%) as brick red crystals: mp 210.5−211.5 °C; UV−vis ($CHCl_3$) λ_{max} (log ϵ) 451 (5.05), 525 (4.98); ^{1}H NMR ($CDCl_3$): δ 0.61 (3H, t, J = 7 Hz), 1.09 (6H, 2 overlapping triplets), 1.94 (3H, s), 2.23 (3H, s), 2.29 (3H, s), 2.34 (3H, s), 2.2−2.3 (2H, m), 2.4−2.5 (6H, m), 2.70 (6H, s), 2.92 (2H, t), 3.58 (3H, s), 5.18 (2H, s), 7.09 (1H, s), 7.28 (1H, s), 13.18 (1H, br s), 13.26 (2H, br s), 13.35 (1H, br s). Anal. Calcd for $C_{35}H_{48}N_4O_2Br_2 \cdot H_2O$: C, 57.22; H, 6.86; N, 7.63. Found: C, 57.16; H, 6.26; N, 7.50.

8-(2-Chloroethyl)-2,18-diethyl-13-(2-methoxycarbonylethyl)-1,3,7,12,17,19-hexamethyl-10,23-dihydrobilin Dihydrobromide (13e). Compound **13c** was prepared by the previous procedure from dipyrrole **12b** (510 mg) and pyrrole aldehyde **7a** (350 mg). Precipitation with ether gave the *a*,*c*-biladiene (448 mg; 55%) as dark red crystals: mp 223.5−224.5 °C; UV−vis (CH_2Cl_2) λ_{max} (log ϵ) 450 (5.22), 521 (5.06); ^{1}H NMR ($CDCl_3$): δ 1.12 (6H, 2 overlapping triplets), 2.00 (3H, s), 2.31 (3H, s), 2.32 (3H, s), 2.36 (3H, s), 1.95−2.05 (2H, m), 2.45−2.55 (6H, m), 2.94 (2H, t), 3.03 (2H, t), 3.62 (3H, s), 5.22 (2H, s), 7.14 (1H, s), 7.33 (1H, s), 13.23 (1H, br s), 13.29 (1H, br s), 13.32 (1H, br s), 13.37 (1H, br s). Anal. Calcd for $C_{35}H_{47}N_4O_2Br_2Cl$: C, 55.97; H, 6.30; N, 7.46. Found: C, 56.09; H, 6.38; N, 6.96.

8,12-Diethyl-3,17-bis(2-methoxycarbonylethyl)-1,2,7,13,18,19-hexamethyl-20,23-dihydrobilin Dihydrobromide (18). Dipyrrylmethane dicarboxylic acid **16** (150 mg) was placed in a 50 mL pear-shaped flask and stirred with TFA (1 mL) at room temperature for 10 min. A solution of aldehyde **17** (197 mg; 2 equiv) in methanol (4 mL) was added, followed immediately by 0.8 mL of HBr in acetic acid (30%), and the resulting red solution was stirred for a further 40 min. Anhydrous diethyl ether (16 mL) was then added and the mixture stirred for 1 h at room temperature and a further 1 h while the flask was cooled with an ice bath. The precipitate was suction filtered and washed with ether to give the title *a*,*c*-biladiene (290 mg; 80%) as a red-brown powder: mp 222−223 °C; UV−vis ($CHCl_3$) λ_{max} (log ϵ) 457 (4.40), 529 nm (5.28); ^{1}H NMR ($CDCl_3$) δ 0.67 (6H, t), 2.03 (6H, s), 2.28 (6H, s), 2.56 (8H, m), 2.69 (6H, s), 3.00 (4H, t), 3. 64 (6H, s), 5.22 (2H, s), 7.26 (2H, s), 13.29 (2H, br s), 13.40 (2H, br s). Anal. Calcd for $C_{37}H_{50}N_4O_4Br_2 \cdot 0.5H_2O$: C, 56.71; H, 6.56; N, 7.15. Found: C, 56.04; H, 6.36; N, 7.05.

3,8-Bis(2-methoxycarbonylethyl)-2,7,12,13,17,18-hexamethylporphyrin (6b). 2,18-Dimethyl-8,13-bis(2-methoxycarbonylethyl)-1,3,7,12,17,19-hexamethyl-20,23-dihydrobilin dihydrobromide (**13b**; 620 mg) was added to a stirred solution of copper(II) chloride (1.89 g) in DMF (325 mL), and the resulting mixture was stirred in the dark for 2 h. The mixture was diluted with dichloromethane (350 mL) and washed with water (3 × 400 mL). The aqueous layers were back-extracted with dichloromethane, and the combined organic layers were dried over sodium sulfate and filtered. The solvent was evaporated on a rotary evaporator under aspirator pressure and then using a vacuum pump to remove residual DMF. The solid residue was taken up in 15% v/v sulfuric acid/trifluoroacetic acid (60 mL) and stirred in the dark at room temperature for 45 min. The reaction mixture was diluted with

dichloromethane (350 mL) and washed with water (2 × 400 mL) and 5% aqueous sodium bicarbonate solution. The aqueous layers were back-extracted with dichloromethane, the combined organic layers were dried over sodium sulfate, and the solvent was evaporated under reduced pressure. The crude product was reesterified by treatment with 5% sulfuric acid−methanol (60 mL) at room temperature in the dark overnight. The reaction mixture was diluted with dichloromethane and washed with water and then with 5% sodium bicarbonate solution. The aqueous layers were back-extracted with dichloromethane, the combined organic layers were dried over sodium sulfate, and the solvent was evaporated under reduced pressure. The residue was chromatographed on a grade-III alumina column (2.2 cm i.d. × 20 cm), eluting with dichloromethane. The dark violet fraction was evaporated under pressure and the residue recrystallized from chloroform−methanol to give the title porphyrin (295 mg; 64%) as purple crystals: mp 239−240 °C; UV−vis ($CHCl_3$) λ_{max} (log ϵ) 399 (5.20), 497 (4.11), 532 (3.97), 567 (3.78), 622 (3.68); ^{1}H NMR ($CDCl_3$) δ −3.91 (2H, s), 3.28 (4H, m), 3.55 (6H, s), 3.56 (3H, s), 3.58 (3H, s), 3.61 (3H, s), 3.64 (3H, s), 3.70 (6H, s), 4.39 (4H, t), 9.97 (1H, s), 10.00 (2H, s), 10.03 (1H, s); HRMS (FAB) calcd for $C_{34}H_{38}N_4O_4$ + H 567.2971, found 567.2968. Anal. Calcd for $C_{34}H_{38}N_4O_4 \cdot 0.5H_2O$: C, 70.93; H, 6.83; N, 9.73. Found: C, 70.84; H, 6.72; N, 9.44.

13,17-Diethyl-3,8-bis(2-methoxycarbonylethyl)-2,7,12,18-tetramethylporphyrin (Mesoporphyrin-VI dimethyl ester; 6a). Compound **6a** was prepared from *a*,*c*-biladiene **13a** (0.640 g) by the procedure reported above. Recrystallization of the residue from chloroform−methanol gave the title porphyrin (0.316 g; 64%) as violet crystals: mp 201.5−202.5 °C (lit.[61] mp 199 °C; lit.[33] mp 195−197 °C); UV−vis ($CHCl_3$) λ_{max} (log ϵ) 399 (5.22), 498 (4.10), 532 (3.96), 567 (3.80), 621 (3.65); ^{1}H NMR ($CDCl_3$) δ −3.81 (2H, br s), 1.88 (6H, t), 3.28 (4H, t), 3.62 (3H, s), 3.63 (3H, s), 3.64 (3H, s), 3.65 (3H, s), 3.69 (3H, s), 3.70 (3H, s), 4.10 (4H, m), 4.42 (4H, m), 10.05 (1H, s), 10.07 (2H, s), 10.08 (1H, s); EIMS *m*/*z* (relative intensity) 594 (100) (M^+), 521 (26); HRMS calcd for $C_{36}H_{42}N_4O_4$ 594.3206, found 594.3205.

3,8-Bis(2-methoxycarbonylethyl)-2,7,12,18-tetramethyl-13,17-dipropylporphyrin (6c). Compound **6c** was prepared from *a*,*c*-biladiene **13c** (405 mg) by the procedure reported above. Recrystallization from chloroform−methanol gave the title porphyrin (201 mg; 64%) as purple needles: mp 193−194 °C; UV−vis ($CHCl_3$) λ_{max} (log ϵ) 399 (5.27), 498 (4.15), 532 (4.01), 567 (3.85), 621 (3.71); ^{1}H NMR ($CDCl_3$): δ −3.78 (2H, br s), 1.29 (6H, t), 2.34 (4H, m), 3.27 (4H, m), 3.62 (3H, s), 3.64 (3H, s), 3.66 (3H, s), 3.68 (3H, s), 3.69 (3H, s), 3.70 (3H, s), 4.05 (4H, t), 4.41 (4H, m), 10.07 (3H, s), 10.09 (1H, s); EIMS *m*/*z* (relative intensity) 622 (100) (M^+), 593 (15), 549 (14); HRMS calcd for $C_{38}H_{46}N_4O_4$ 622.3519, found 622.3518. Anal. Calcd for $C_{38}H_{46}N_4O_4 \cdot H_2O$: C, 71.22; H, 7.55; N, 8.74. Found: C, 70.66; H, 7.44; N, 8.33.

13,17-Dibutyl-3,8-bis(2-methoxycarbonylethyl)-2,7,12,18-tetramethylporphyrin (6d). Compound **6d** was prepared from *a*,*c*-biladiene **13d** (269 mg) by the procedure reported above. Recrystallization from chloroform−methanol gave the title porphyrin (135 mg; 64%) as purple granules: mp 187−188 °C; UV−vis ($CHCl_3$) λ_{max} (log ϵ) 399 (5.21), 497 (4.11), 532 (3.96), 567 (3.81), 620 (3.66); ^{1}H NMR ($CDCl_3$): δ −3.79 (2H, br s), 1.13 (6H, t), 1.76 (4H, m), 2.25 (4H, m), 3.25 (4H, t), 3.59 (3H, s), 3.61 (3H, s), 3.63 (3H, s), 3.66 (3H, s), 3.69 (3H, s), 3.70 (3H, s), 4.05 (4H, m), 4.42 (4H, t), 10.06 (2H, s), 10.07 (2H, s); HRFABMS calcd for $C_{40}H_{50}N_4O_4$ + H 651.3910, found 651.3920. Anal. Calcd for $C_{40}H_{50}N_4O_4 \cdot 0.25H_2O$: C, 73.31; H, 7.77; N, 8.55. Found: C, 73.01; H, 7.68; N, 8.33.

3-(2-Chloroethyl)-13,17-diethyl-8-(2-methoxycarbonylethyl)-2,7,12,18-tetramethylporphyrin (6e). Cyclization of *a*,*c*-biladiene **13e** (414 mg) afforded porphyrin **6e** (172 mg; 32%) as purple crystals: mp > 300 °C; UV−vis (CH_2Cl_2) λ_{max} (log ϵ) 398 (5.22), 497 (4.13), 531 (3.97), 566 (3.80), 620 (3.63); ^{1}H NMR ($CDCl_3$) δ −3.79 (2H, br s), 1.88 (6H, t, J = 7.7 Hz), 3.29 (2H, t, J = 7.9 Hz), 3.64 (3H, s), 3.65 (3H, s), 3.68 (6H, s), 4.12 (4H, q, J = 7.8 Hz), 4.33 (2H, t, J = 7.7 Hz), 4.45 (2H, t, J = 8 Hz), 4.55 (2H, t, J = 7.6 Hz), 10.04 (1H, s), 10.10 (2H, s), 10.14 (1H, s); HRFABMS calcd for $C_{34}H_{39}N_4O_2Cl$ + H 571.2839, found 571.2832.

3,13,17-Triethyl-8-(2-methoxycarbonylethyl)-2,7,12,18-tetramethylporphyrin (6f). Compound **6f** was prepared by the previous procedure from *a*,*c*-biladiene **13f** (235 mg). Recrystallization from chloroform−methanol gave the triethylporphyrin (53 mg; 30%) as purple crystals: mp 218−219 °C; UV−vis ($CHCl_3$) λ_{max} (log ϵ) 397 (5.25), 497 (4.16), 531 (3.99), 566 (3.81), 620 (3.66); ^{1}H NMR ($CDCl_3$) δ −3.76 (2H, br s), 1.88 (9H, t, J = 7.4 Hz), 3.29 (2H, t), 3.63 (3H, s), 3.66 (6H, s), 3.67 (3H, s), 3.71 (3H, s), 4.11 (6H, m), 4.43 (2H, t, J = 7.9 Hz), 10.09 (1H, s), 10.10 (2H, s), 10.11 (1H, s); HRFABMS calcd for $C_{34}H_{40}N_4O_2$ + H 537.3229, found 537.3226. Anal. Calcd for $C_{34}H_{40}N_4O_2 \cdot 0.25H_2O$: C, 75.45; H, 7.54; N, 10.35. Found: C, 75.51; H, 7.54; N, 10.28.

13,17-Diethyl-2,8-bis(2-methoxycarbonylethyl)-2,7,12,18-tetramethylporphyrin (Mesoporphyrin-XII dimethyl ester; 15). Compound **15** was prepared from *a*,*c*-biladiene **18** (0.250 g) by the procedure reported above. Recrystallization of the residue from chloroform−methanol gave the title porphyrin (0.134 g; 70%) as fluffy maroon-colored crystals: mp 195−196 °C (lit.[62] mp 190−191 °C); ^{1}H NMR ($CDCl_3$) δ −3.75 (2H, br s), 1.88 (6H, t, J = 7.5 Hz), 3.28 (4H, t, J = 7.8 Hz), 3.64 (6H, s), 3.65 (6H, s), 3.68 (6H, s), 4.07 (4H, q, J = 7.6 Hz), 4.43 (4H, t, J = 7.9 Hz), 10.08 (2H, s), 10.09 (1H, s), 10.10 (1H, s).

13,17-Diethyl-8-(2-methoxycarbonylethyl)-2,7,12,18-tetramethyl-3-vinylporphyrin (14a). Chloroethylporphyrin **6e** (115 mg) was taken up in pyridine (25 mL) and stirred in the dark under reflux for 10 min. Aqueous sodium hydroxide (5%; 5 mL) was added and the resulting mixture refluxed for an additional 2 h. After cooling, 25% aqueous acetic acid (10 mL) was added, and the solvents were removed under reduced pressure. Toluene was added and then rotary evaporated to azeotrope away trace amounts of water. The residue was taken up in 5% sulfuric acid−methanol (25 mL) and stirred in the dark at room temperature overnight. The mixture was diluted with water (50 mL), neutralized with 5% sodium bicarbonate solution, and extracted with chloroform. The organic solution was dried over magnesium sulfate and evaporated and the residue chromatographed on an alumina column eluting with dichloromethane (Note: the column was covered with aluminum foil to limit the exposure of the photosensitive vinylporphyrin to ambient light). Crystallization from chloroform−methanol gave the vinylporphyrin (88 mg; 82%) as purple needles: mp 217−219 °C; UV−vis (CH_2Cl_2) λ_{max} (log ϵ) 400 (5.16), 501 (4.07), 536 (3.99), 570 (3.77), 624 (3.56); ^{1}H NMR ($CDCl_3$) δ −3.69 (2H, br s), 1.84 (6H, 2 overlapping triplets), 3.25 (2H, t, J = 7 Hz), 3.59 (3H, s), 3.63 (6H, s), 3.66 (3H, s), 3.70 (3H, s), 4.05 (4H, m), 4.41 (2H, t, J = 7 Hz), 6.19 (1H, d), 6.39 (1H, d), 8.31 (1H, dd), 10.06 (1H, s), 10.07 (1H, s), 10.14 (1H, s), 10.22 (1H, s); HRFABMS calcd for $C_{34}H_{38}N_4O_2$ + H 535.3073, found 535.3072. Anal. Calcd for $C_{34}H_{38}N_4O_2 \cdot 0.5H_2O$: C, 75.11; H, 7.23; N, 10.30. Found: C, 75.08; H, 7.14; N, 10.13.

Preparation of Chicken Blood Hemolysates. Fresh chicken blood was removed from the chicken neck after transection. Generally, the blood was obtained from three chickens and collected into a beaker that contained 10 mL of 0.9% NaCl with 10 mg of heparin. During collection, the mixture was swirled gently and continuously to prevent coagulation. Approximately 100 mL of the blood was collected before the blood was transferred into polypropylene tubes and cooled in ice. The blood was immediately centrifuged at 2000 rpm with a JA-17 rotor for 10 min at 4 °C. The plasma (upper phase) was discarded, and the pellet, which consisted of both red and white cells, was washed with an equal volume of 0.9% aqueous sodium chloride and recentrifuged for 10 min. The

(61) Fischer, H.; Rothhaas, A. *Liebigs Ann. Chem.* **1930**, *484*, 90.

(62) Fischer, H.; Friedrich, H.; Lamatsch, W.; Morgen-Roth, K. *Liebigs Ann. Chem.* **1928**, *466*, 147.

supernatant and white cell "buffy coat" were removed and the washing repeated (×2). The red cells were transferred into a beaker and cooled to 0 °C with an ice bath. Aliquots of water (21 mL for every 25 mL of original chicken blood) at 0 °C were added, and the red cells were hemolyzed by stirring with a glass rod for 5 min. To maintain isotonicity with the original blood, aliquots of aqueous 0.6 M KCl solution were then added (47 mL for every 225 mL of original chicken blood). At this stage, the crude hemolysate (CRH) was ready to be used as an enzyme source for coproporphyrinogen oxidase. The enzyme preparation was either used directly or stored for later use. For storage, the hemolysates were transferred to polypropylene tubes as 10 mL aliquots and immediately frozen at −80 °C.

Substrate Preparation. Porphyrin methyl esters (200−500 μg) were hydrolyzed to the corresponding carboxylic acids in 25% aqueous hydrochloric acid (1 mL) at room temperature in the dark for 16 h. The HCl solution was removed under high vacuum at <30 °C and the residue dissolved in aqueous KOH (0.05 M; 100 μL). The porphyrin solution was transferred to a microcentrifuge tube, sodium amalgam (3%, 400 mg) was added, and the mixture was vortexed in the dark for 2 min. The resulting colorless porphyrinogen solution was transferred to another microcentrifuge tube, and 0.9 mL of 0.25 M Tris−HCl buffer (pH 7) containing 0.1 M thioglycolate and 1 mM EDTA was added (note: thioglycolate, obtained as the sodium salt, is not stable in solution and must be added just prior to use). The contents of the tube were mixed well, and the pH of the final solution checked and adjusted to 7 if necessary. The unstable porphyrinogen solutions were used immediately.

Standard Enzyme Assay. In time course studies, 10 mL of the chicken blood hemolysate (CRH) was used per incubate. Aliquots of the substrate were added such that 1 μg of porphyrinogen was used for each 1 mL of hemolysate, mixed well, and incubated in the dark at 37 °C for selected time periods (5−120 min). The enzyme-catalyzed reaction was terminated by pouring the mixture into a polypropylene centrifuge tube and adding 100 mL of acetic acid/ethyl acetate (3:7 v/v). The "killed" hemolysates were centrifuged for 10 min at 614*g* and the supernatants collected into 500 mL separatory funnels. The pellets were reextracted with 40 mL of 3:7 v/v acetic acid/ethyl acetate, and the supernatants were combined and washed with 3% aqueous sodium acetate (70 mL) and water (2 × 70 mL). The ethyl acetate phase was extracted with aqueous hydrochloric acid (10%, 2 × 35 mL; then 5%, 35 mL) so that the porphyrin dication transferred to the aqueous phase. The combined HCl solutions were neutralized to Congo red with saturated sodium acetate and extracted with diethyl ether (2 × 50 mL). The combined ether extracts were washed with water (2 × 50 mL) and evaporated under a stream of nitrogen gas or by rotoevaporation. The residue was esterified with 5% sulfuric acid−methanol (10 mL) at room temperature overnight. The solution was diluted with water (10 mL) and 5% aqueous ammonia (5 mL) and extracted with chloroform. The chloroform extracts were washed with water (2 × 10 mL), and the solvent was evaporated. The porphyrin esters so obtained were analyzed by TLC (20% ethyl acetate−toluene on silica gel G plates) and HPLC. HPLC analyses were performed using normal-phase columns (5 μm partisil silica, Altech) eluting with appropriate ratios of ethyl acetate and cyclohexane (3:7 v/v for copro'gen-III and 2:8 v/v for **3a**−**d**). Kinetic data are reported as mean ± standard deviation for four replicate experiments (Table 2) and compared statistically using analysis of variance (ANOVA) following Fisher's LSD Post Test. Values are considered significantly different at $p < 0.05$.

Porphyrins Isolated from Preparative Enzymic Studies. These products were purified by flash chromatography, eluting with 2% ethyl acetate−toluene, and characterized by FAB MS and 300 MHz proton NMR spectroscopy.

8-(2-Methoxycarbonylethyl)-2,7,12,13,17,18-hexamethyl-3-vinylporphyrin (14b): ^{1}H NMR ($CDCl_3$) δ −3.65 (2H, s), 3.28 (2H, t, $J = J = 7.8$ Hz), 3.60 (6H, s), 3.63 (6H, s), 3.67 (3H, s), 3.69 (3H, s), 3.74 (3H, s), 4.44 (2H, t, $J = 7.7$ Hz), 6.17 (1H, d, $J = 11.5$ Hz), 6.37 (1H, $J = 18$ Hz), 8.32 (1H, dd, $J = 11.2$ Hz, 18 Hz), 10.05 (2H, s), 10.14 (1H, s), 10.24 (1H, s); HRFABMS calcd for $C_{32}H_{34}N_4O_2$ + H 507.2760, found 507.2761.

13,17-Diethyl-8-(2-methoxycarbonylethyl)-2,7,12,18-tetramethyl-3-vinylporphyrin (14a). This material was indistinguishable from synthetic samples of **14a** by HPLC and ^{1}H NMR spectroscopy: HRFABMS calcd for $C_{34}H_{38}N_4O_2$ + H 535.3073, found 535.3072.

8-(2-Methoxycarbonylethyl)-2,7,12,18-tetramethyl-13,17-dipropyl-3-vinylporphyrin (14c): ^{1}H NMR ($CDCl_3$) δ −3.64 (2H, s, 2 × NH), 1.3 (6H, obscured by impurity peak), 2.27−2.40 (4H, m), 3.28 (2H, t, $J = 7.8$ Hz), 3.62 (3H, s), 3.65 (3H, s), 3.66 (3H, s), 3.68 (3H, s), 3.75 (3H, s), 4.0−4.2 (4H, m), 4.45 (2H, t, $J = 7.8$ Hz), 6.18 (1H, d, $J = 11.8$ Hz), 6.37 (1H, $J = 18$ Hz), 8.32 (1H, dd, $J = 11.8$ Hz, 18 Hz), 9.95 (2H, s), 10.04 (1H, s), 10.12 (1H, s); HRFABMS calcd for $C_{36}H_{42}N_4O_2$ + H 563.3386, found 563.3385.

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Supporting Information Available: Copies of ^{1}H NMR spectra for compounds **7**, **8c**, **9**−**15**, and **18**, selected HPLC traces for incubations of **3a**−**d** and **14a**, and characterization for metabolites **14a**−**c** are provided (70 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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