Normal and Abnormal Heme Biosynthesis. 3.[1] Synthesis and Metabolism of Tripropionate Analogues of Coproporphyrinogen-III: Novel Probes for the Active Site of Coproporphyrinogen Oxidase

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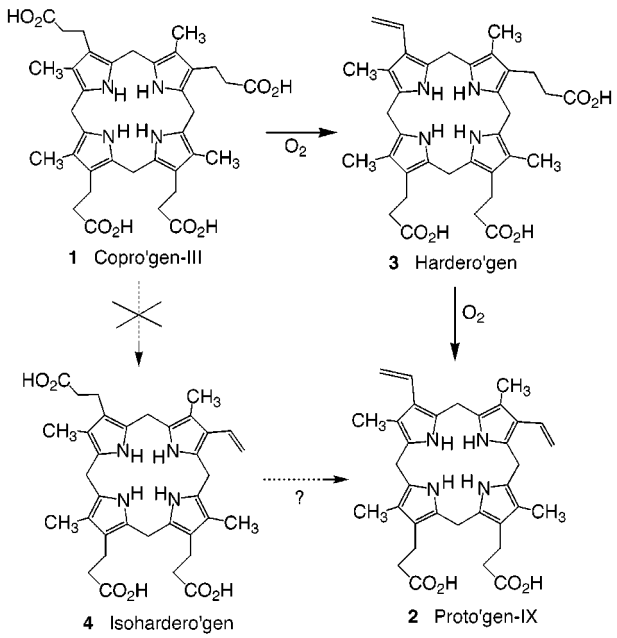
Coproporphyrinogen oxidase (copro'gen oxidase) catalyses the oxidative decarboxylation of two propionate side chains on coproporphyrinogen-III to produce protoporphyrinogen-IX. This process is very poorly understood at a molecular level, and copro'gen oxidase remains one of the least well-characterized enzymes in the heme biosynthetic pathway. To provide a rigorous test for a proposed model for substrate recognition and binding by this enzyme, two tripropionate analogues of copro'gen-III were prepared where an ethyl group replaced one of the usual propionate residues on positions 13 or 17. Although the required substrate probes are porphyrinogens (hexahydroporphyrins), the corresponding porphyrin methyl esters were initially synthesized via tripyrrene and a,c-biladiene intermediates. These were hydrolyzed and reduced with 3% sodium-amalgam to give the unstable porphyrinogens needed for the biochemical investigations. The modified structure with a 13-ethyl moiety was metabolized by avian preparations of copro'gen oxidase to give a monovinylic product, but the isomeric 17-ethylporphyrinogen afforded a divinylic product, albeit with poorer overall conversion. These results strongly support the proposed model for substrate binding at the active site of copro'gen oxidase.

Introduction

Coproporphyrinogen oxidase, a key enzyme in the heme biosynthetic pathway, is responsible for the conversion of coproporphyrinogen-III (copro'gen-III; **1**) to protoporphyrinogen-IX (proto'gen-IX; **2**) (Scheme 1).[2−4] This metabolic process involves the sequential oxidative decarboxylation of two propionate side chains to generate vinyl groups on the A and B pyrrole rings.[2−4] In aerobic organisms, molecular oxygen is an obligatory requirement for enzymatic activity, but there are no known cofactors and the mechanism for this reaction is presently unknown. However, it has long been known that the A ring propionate residue undergoes the first oxidative decarboxylation to produce harderoporphyrinogen (**3**), rather than the B ring modified regioisomer isoharderoporphyrinogen (**4**) (Scheme 1), and subsequent metabolism produces the divinylporphyrinogen **2**.[5−7] In addition, isohardero'gen (**4**) appears to be a very poor substrate for coproporphyrinogen oxidase, while synthetic samples of **3** are excellent substrates, providing further evidence for the involvement of **3** as a true intermediate.[5−7] Although copro'gen oxidase has been isolated from several sources[8] and the enzyme sequenced and cloned for a number of different organisms,[9−11] it remains poorly characterized[8,12] and while some evidence for the presence of copper(II) in the native enzyme has been presented,[10] this has been strongly refuted for the human-

Scheme 1. Metabolism of Coproporphyrinogen-III



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(1) Part 2: Lash, T. D.; Mani, U. N.; Lyons, E. A.; Thientanavanich, P.; Jones, M. A. *J. Org. Chem.* **1999**, *64*, 478.

(2) Akhtar, M. In *The Biosynthesis of Tetrapyrrole Pigments*; Ciba Foundation Symposium 180; Wiley: Chichester, 1994; pp 130−155.

(3) *Biosynthesis of Tetrapyrroles*; Jordan, P. M., Ed.; Elsevier: London, 1991.

(4) Lash, T. D.; Mani, U. N.; Drinan, M. A.; Hall, T.; Zhen, C.; Jones, M. A. *J. Org. Chem.* **1999**, *64*, 464.

(5) Kennedy, G. Y.; Jackson, A. H.; Kenner, G. W.; Suckling, C. J. *FEBS Lett.* **1970**, *6*, 9. Smith, S. G.; Belcher, R. V.; Mahler, R. F.; Yudkin, J. *Clin. Chim. Acta* **1969**, *23*, 241. Belcher, R. V.; Smith, S. G.; Mahler, R. F. *Enzyme* **1974**, *17*, 53. Jackson, A. H.; Rao, K. R. N.; Smith, S. G.; Lash, T. D. *Biochem. J.* **1985**, *227*, 327.

(6) Cavaleiro, J. A. S.; Kenner, G. W.; Smith, K. M. *J. Chem. Soc., Perkin Trans. 1* **1974**, 1188. Evans, N.; Jackson, A. H.; Matlin, S. A.; Towill, R. *J. Chromatog.* **1976**, *125*, 345. Jackson, A. H.; Jones, D. M.; Philip, G.; Lash, T. D.; Battle, A. M. del C.; Smith, S. G. *Int. J. Biochem.* **1980**, *12*, 681.

(7) Games, D. E.; Jackson, A. H.; Jackson, J. R.; Belcher, R. V.; Smith, S. G. *J. Chem. Soc., Chem. Commun.* **1976**, 187.

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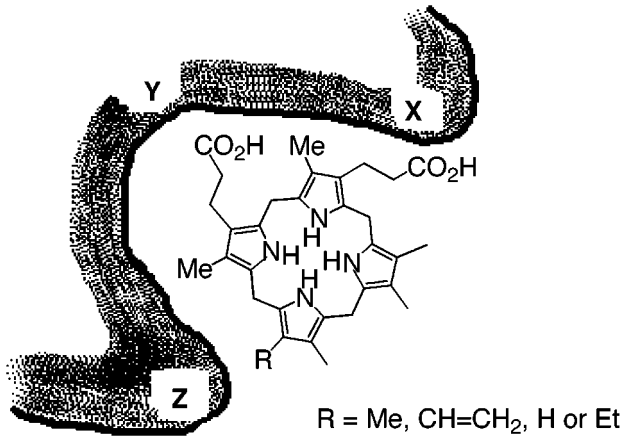
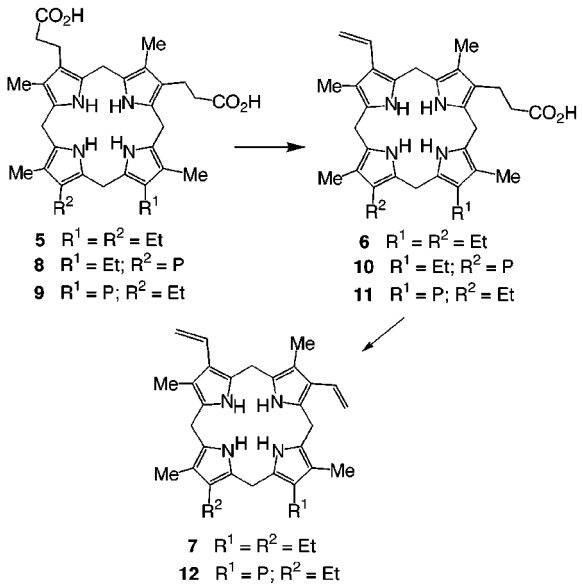


Figure 1. Model for the active site of coproporphyrinogen oxidase.

derived enzyme.[11] To gain further insights into the nature of the active site for copro'gen oxidase, the metabolism of synthetic substrate analogues has been investigated,[4,13−16] and these studies have led to a proposed model for binding at the active site of this important enzyme (Figure 1).[4,16] In this model, position Y represents the catalytic site where oxidative decarboxylation of a propionate residue takes place, X corresponds to a binding site that recognizes a second propionate unit and Z represents a region with steric requirements for a small nonpolar unit such as H, methyl, vinyl, or ethyl. In essence, the enzyme−substrate recognition requires a sequence R Me-P Me-P (where P = $CH_2CH_2CO_2H$) around the periphery of the porphyrinogen macrocycle.[4] In early studies of this type, mesoporphyrinogen-VI (**5**)

Scheme 2. Metabolism of Di- and Tricarboxylate Porphyrinogens



(Scheme 2) was reported to be a good substrate for copro'gen oxidase derived from chicken red cell hemolysates (CRH).[15] In these studies, **5** initially gave a monovinylic porphyrinogen **6**, but this species was further metabolized to give a divinyl product protoaetio'gen-IX (**7**).[15] In our hands, meso'gen-VI was also an excellent substrate for copro'gen oxidase derived from avian red cell hemolysates (chicken, duck, and turkey blood all gave similar results), but only the monovinylic product was formed in our studies.[4] Indeed, synthetic samples of **6** were not metabolized by copro'gen oxidase.[4] In the proposed model for substrate binding, **5** has the correct sequence of substituents (R Me-P Me-P) but the metabolic product **6** does not. The absence of a second propionate moiety for binding at region X suggests that **6** should not be a substrate for copro'gen oxidase, and this is born out to a certain extent in studies of related substrate analogues. However, these conflicting data are cause for some concern, and it seemed prudent to test this model with tripropionate species **8** and **9** (Scheme 2) that are structurally midway between the natural substrate copro'gen-III (**1**) and meso'gen-VI (**5**). If the model holds true, one would predict that tripropionate porphyrinogen **8** should be metabolized by copro'gen oxidase to give the hardero'gen analogue **10**, but this compound should not be further recognized by the enzyme. On the other hand, isomer **9** should be metabolized to initially give monovinylic product **11**, but this species still has the correct sequence for substrate recognition and therefore should be further converted to the proto'gen-IX analogue **12**. Hence, the rather different expected outcomes for these isomeric substrate analogues provides a stringent test for the proposed model for substrate binding (Figure 1).

Results and Discussion

Synthesis of Tripropionate Porphyrins. To carry out these studies, synthetic samples of the required substrate analogues were required. Porphyrinogens are unstable compounds, and for this reason the required materials were synthesized as the corresponding porphyrin methyl esters. The porphyrinogen carboxylic acids could then be prepared as needed.[4] Porphyrinogens **8** and

(8) Sano, S. *J. Biol. Chem.* **1966**, *241*, 5276. Yoshinaga, T.; Sano, S. *J. Biol. Chem.* **1980**, *255*, 4722; 4727. Battle, A. M. del C.; Benson, A.; Rimington, C. *Biochem. J.* **1965**, *97*, 731. Poulson, R.; Polglase, W. J. *J. Biol. Chem.* **1974**, *249*, 6367. Bogard, M.; Camadro, J.-M.; Nordmann, Y.; Labbe, P. *Eur. J. Biochem.* **1989**, *181*, 417. Camadro, J.-M.; Chambon, H.; Joles, J.; Labbe, P. *Eur. J. Biochem.* **1986**, *156*, 579.

(9) Martesek, P.; Camadro, J.-M.; Raman, C. S.; Lecomte, M. C.; Le Caer, J. P.; Demeler, B.; Grandchamp, B.; Labbe, P. *Cell. Mol. Biol. (Paris)* **1997**, *43*, 47. Taketani, S.; Kohno, H.; Furukawa, T.; Yoshinaga, T.; Tokunaga, R. *Biochim. Biophys. Acta* **1994**, *1183*, 547. Martasek, P.; Camadro, J. M.; Delfau-Larue, M.-H.; Dumas, J.-B.; Montagne, J. J.; de Verneuil, H.; Labbe, P.; Grandchamp, B. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 3024. Troup, B.; Jahn, M.; Hungerer, C.; Jahn, D. *J. Bacteriol.* **1994**, *176*, 673. Xu, K.; Elliott, T. *J. Bacteriol.* **1993**, *175*, 4990. Coomber, S. A.; Jones, R. M.; Jordan, P. M.; Hunter, C. N. *Mol. Microbiol.* **1992**, *6*, 3159. Zagorec, M.; Buhler, J.-M.; Treich, I.; Keng, T.; Guarente, L.; Labbe-Bois, R. *J. Biol. Chem.* **1988**, *263*, 9718.

(10) Kohno, H.; Furukawa, T.; Tokunaga, R.; Taketani, S.; Yoshinaga, T. *Biochim. Biophys. Acta* **1996**, *1292*, 156.

(11) Medlock, A. E.; Dailey, H. A. *J. Biol. Chem.* **1996**, *271*, 32507.

(12) Jones, M. A.; Hamilton, M. L.; Lash, T. D. *Prep. Biochem. Biotechnol.* **1997**, *27*, 47.

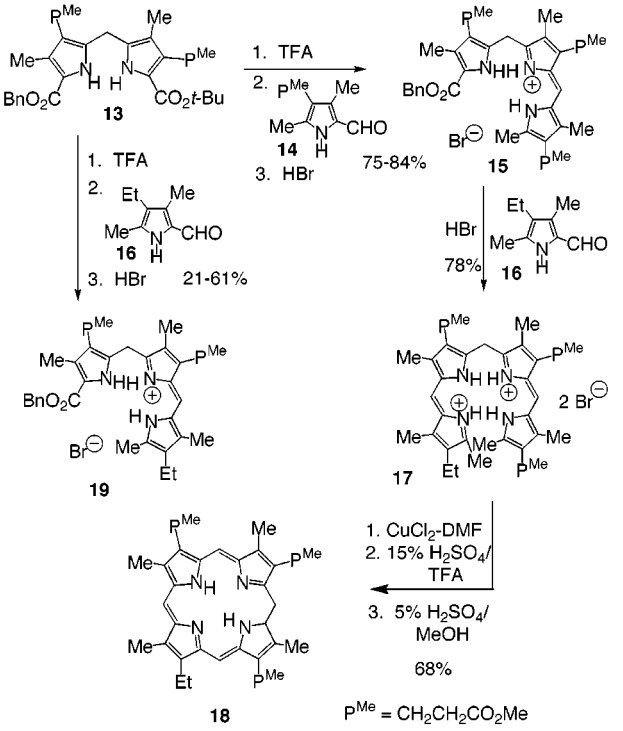
(13) Porra, P. J.; Falk, J. E. *Biochem. J.* **1964**, *90*, 69. Jackson, A. H.; Elder, G. H.; Smith, S. G. *Int. J. Biochem.* **1978**, *9*, 877. Elder, G. H.; Evans, J. O.; Jackson, J. R.; Jackson, A. H. *Biochem. J.* **1978**, *169*, 215. Al-Hazimi, H. M. G.; Jackson, A. H.; Ryder, D. J.; Elder, G. H.; Smith, S. G. *J. Chem. Soc., Chem. Commun.* **1976**, 188. Al-Hazimi, H. M. G.; Jackson, A. H.; Knight, D. W.; Lash, T. D. *J. Chem. Soc., Perkin Trans. 1* **1987**, 265. Frydman, R. B.; Frydman, B. *FEBS Lett.* **1975**, *52*, 317. Buldain, G.; Hurst, J.; Frydman, R. B.; Frydman, B. *J. Org. Chem.* **1977**, *42*, 2953. Buldain, G.; Diaz, L.; Frydman, B. *J. Org. Chem.* **1977**, *42*, 2957. Battersby, A. R.; Hamilton, A. D.; McDonald, E.; Mombelli, L.; Wong, O.-H. *J. Chem. Soc., Perkin Trans. 1* **1980**, 1283. Robertson, J. A.; McDonald, E.; Battersby, A. R. *J. Chem. Soc., Perkin Trans. 1* **1985**, 1699.

(14) Elder, G. H.; Evans, J. O. *Biochem. J.* **1978**, *169*, 205. Jackson, A. H.; Lash, T. D.; Ryder, D. J.; Smith, S. G. *Int. J. Biochem.* **1980**, *12*, 775. Jackson, A. H.; Lash, T. D.; Ryder, D. J. *J. Chem. Soc., Perkin Trans. 1* **1987**, 287.

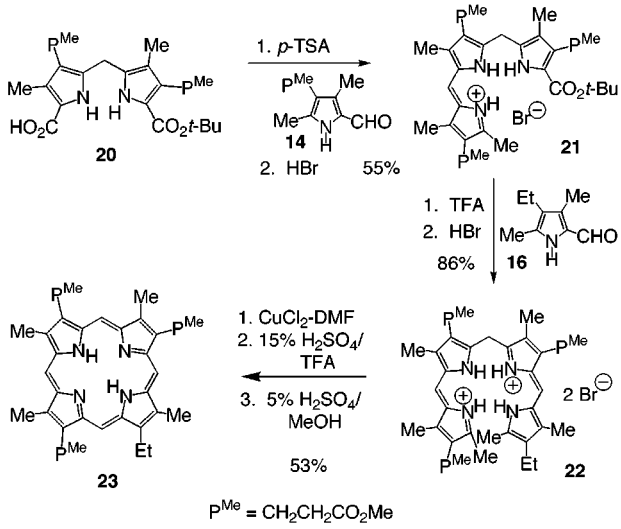
(15) Jackson, A. H.; Jones, D. M.; Smith, S. G.; Elder, G. H. *J. Chem. Soc., Chem. Commun.* **1976**, 322.

(16) Lash, T. D.; Drinan, M. A.; Zhen, C.; Mani, U. N.; Jones, M. A. *BioMed. Chem. Lett.* **1994**, *4*, 1607.

Scheme 3



Scheme 4



9 have no symmetry elements, and this necessitates a stepwise approach to the synthesis of the corresponding porphyrins. In this work, we favored the use of a tripyrrene-a,c-biladiene route for porphyrin synthesis[17−21] starting from the previously synthesized dipyrrylmethane **13**[4] that bears benzyl and *tert*-butyl protective groups (Scheme 3). Methodologies have been reported where the benzyl ester moiety is cleaved first, and following acid-catalyzed condensation with a pyrrole aldehyde a tripyrrene *tert*-butyl ester is produced.[18] The *tert*-butyl ester can then be cleaved with TFA and further condensed with a second pyrrole aldehyde to produce the required tetrapyrrole.[18] However, difficulties are sometimes encountered where the *tert*-butyl ester is prematurely cleaved,[19] and for this reason, we favored an alternative strategy where the *tert*-butyl ester protective group is removed first.[19] Hence, **13** was treated with TFA and further reacted with aldehyde **14** in the presence of hydrobromic acid to give the tripyrrene benzyl ester **15** (Scheme 3). This was treated with HBr in TFA at room temperature for 6 h to cleave the benzyl ester, and further reaction with aldehyde **16** gave the required a,c-biladiene **17** in excellent overall yields. Cyclization with copper(II) chloride in DMF at room temperature,[4,21] followed by demetalation with 15% sulfuric acid/TFA and reesterification with 5% H_2SO_4/methanol, afforded porphyrin trimethyl ester **18** in 68% yield (Scheme 3). This corresponds to the porphyrinogen substrate analogue **9**.

Initially, we had planned to synthesize the isomeric system using the same approach. However, treatment of **13** with TFA and condensation with pyrrole aldehyde **16** gave the required tripyrrene **19** in relatively low, albeit variable, yields (Scheme 3). While it may have been possible to further modify the reaction conditions to give more satisfactory results, we decided to instead investigate the alternative strategy[18] as detailed in Scheme 4. The benzyl ester of **13** was cleaved with hydrogen over 10% palladium−charcoal to give the carboxylic acid **20**, and this was reacted with pyrrole aldehyde **14** in the presence of *p*-toluenesulfonic acid in methanol−dichloromethane to afford the tripyrrene *tert*-butyl ester **21**, which was isolated as the hydrobromide salt in good yields. This was further treated with TFA and condensed with pyrrole aldehyde **16** in the presence of HBr to give the a,c-biladiene dihydrobromide salt **22**. Further cyclization with copper(II) chloride, demetalation, and reesterification gave the porphyrin trimethyl ester **23** corresponding to porphyrinogen **8** in 54% yield.

Biochemical Studies and Characterization of Metabolites. Treatment of porphyrin methyl esters **18** and **23** with 25% hydrochloric acid at room temperature for 16 h gave the corresponding carboxylic acids, and further reduction with 3% sodium-amalgam in aqueous potassium hydroxide solutions afforded the required porphyrinogens.[4] Following addition of Tris−HCl buffer (pH 7), these solutions were incubated with CRH for varying periods of time and the metabolites extracted and converted into the corresponding porphyrin methyl esters. The products were initially analyzed by TLC and HPLC. HPLC was carried out on normal-phase 5 μm Partisil eluting with 30% ethyl acetate−cyclohexane. Figure 2 shows a chromatogram of a standard mixture with the least polar monoester eluting first, followed by dicarboxylate porphyrin esters and then triesters **18** and **23**. Indeed, the retention times provide a useful preliminary indication as to the nature of the metabolic products (e.g., diesters vs monoesters). In addition, the two tripropionate esters are well resolved on the chromatogram, and the dimethyl ester of protoporphyrin-IX gives baseline separation from the dimethyl ester of mesoporphyrin-VI. Incubations of porphyrinogen **8** with CRH showed the formation of a single product that was chromatographically consistent with the monovinylic product **24** (Figure 3A, Chart 1). However, as anticipated, incubation of **9** with CRH produced a less polar product that was consistent with a monomethyl ester product **25** (Figure

(17) Smith, K. M. In *The Porphyrin Handbook*; Kadish, K. M., Smith, K. M., Guilard, R., Eds.; Academic Press: San Diego, 2000; Vol. 1, pp 119−148.
(18) Baptista de Almeida, J. A. P.; Kenner, G. W.; Rimmer, R.; Smith, K. M. *Tetrahedron* **1976**, *32*, 1793.
(19) Smith, K. M.; Craig G. W. *J. Org. Chem.* **1983**, *48*, 4302.
(20) Grigg, R.; Johnson, A. W.; Kenyon, R.; Math, V. B.; Richardson, K. *J. Chem. Soc. C* **1969**, 176.
(21) Smith, K. M.; Minnetian, O. M. *J. Chem. Soc., Perkin Trans. 1* **1986**, 277.

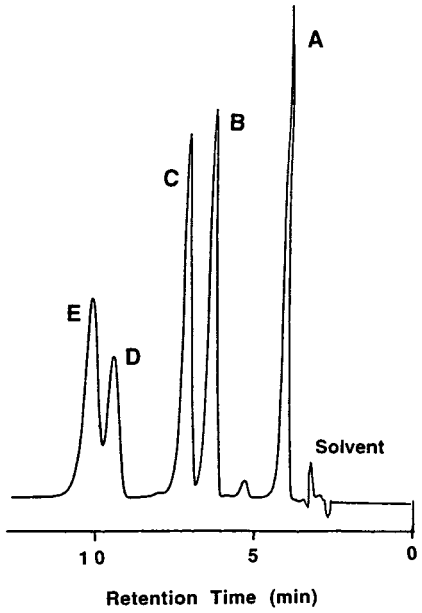


Figure 2. HPLC of a series of porphyrin standards on a 5 μm Partisil column (25 cm × 5 mm i.d.) eluting with 30% ethyl acetate−cyclohexane (v/v) at a flow rate of 1 mL/min: A = porphyrin monomethyl ester corresponding to **6**; B = mesoporphyrin-VI dimethyl ester; C = protoporphyrin-IX dimethyl ester; D = porphyrin trimethyl ester **23**; E = porphyrin trimethyl ester **18**.

3B, Chart 1), although a small amount of an intermediary monovinylic product was also evident in the chromatograms. Time course studies showed that **8** was an excellent substrate for copro'gen oxidase (Figure 4), and it was metabolized to give the monovinylic product at the same rate as the natural substrate copro'gen-III. Surprisingly, while **9** underwent both oxidative decarboxylations, it proved to be a poorer substrate (Figure 5) giving a lower overall conversion to the metabolic products **11** and **12** (Scheme 2). These differences were also evident in the apparent initial velocities for the two porphyrinogen substrates. Porphyrinogen **8** gave an initial velocity of 3.4 ± 0.81% product/min, a value that is comparable to copro'gen-III (3.4 ± 0.15% product/min), while the initial velocity of **9** was calculated as 1.4 ± 0.57% product/min. These differences are not well understood at the present time, although it may be that product inhibition plays a role for substrate **9**.

The structures of the metabolic products were confirmed by proton NMR spectroscopy and mass spectrometry. The products from preparative experiments were purified by flash chromatography on silica. The metabolite isolated from incubations of **8** with CRH, following air oxidation and Fischer esterification, gave an $[M + H]^+$ peak at *m*/*z* 593 by fast atom bombardment mass spectrometry, and high-resolution MS confirmed the molecular formula of the proposed metabolic product **24** as $C_{36}H_{40}N_4O_4$. The 300 MHz proton NMR spectrum of **24** showed the presence of two propionate side chains (triplets for 4H each at 3.3 and 4.4 ppm) and one vinyl group. In addition, six 3H units were evident as singlets between 3.6 and 3.8 ppm, corresponding to the two methyl esters and four porphyrin methyls (the was latter highly deshielded by the aromatic ring current). The product from incubations of **9** with CRH gave an $[M + H]^+$ peak at *m*/*z* 533 by fast atom bombardment MS, and high-resolution MS data gave the expected molecular

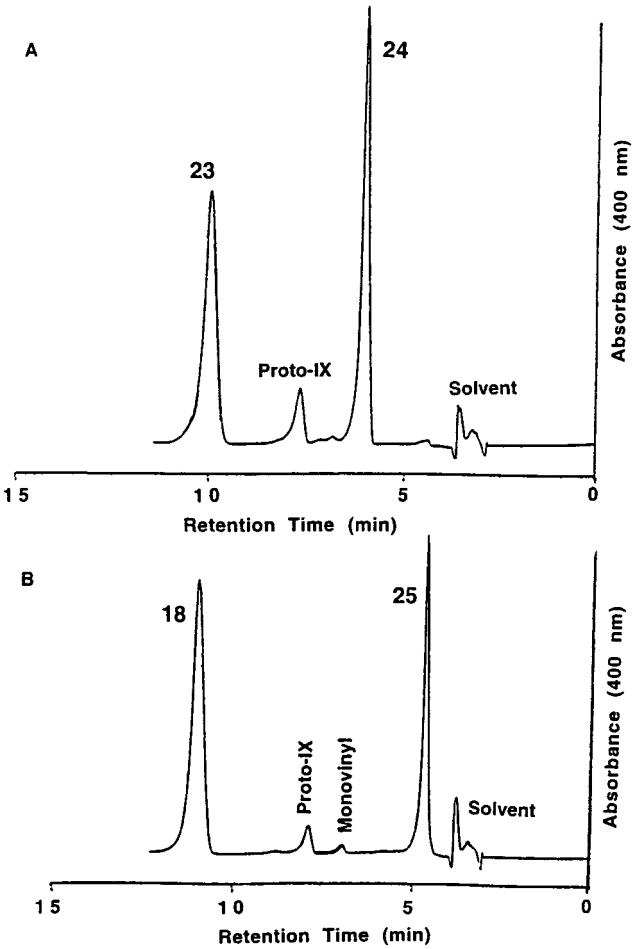
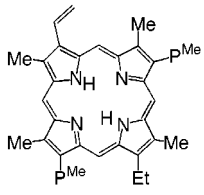


Figure 3. (A) HPLC of the esterified porphyrin extract from a 20 min incubation of **8** with chicken red cell hemolysates showing the formation of a monovinylic product **24**. (B) HPLC of the esterified porphyrin extract from a 20 min incubation of **9** with chicken red cell hemolysates. In this case, a divinyl product **25** predominates, although trace amounts of a monovinylic intermediate can be discerned. HPLC analyses were performed on a 5 μM Partisil column (25 cm × 5 mm i.d.) eluting with 30% ethyl acetate−cyclohexane (v/v) at a flow rate of 1 mL/min. In both chromatograms, a peak due to endogenous protoporphyrin-IX is also present. This is present in the crude enzyme preparations and is not a de novo product.

Chart 1



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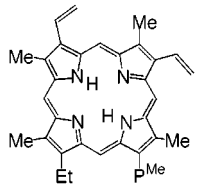

25

formula of **25** as $C_{34}H_{36}N_4O_2$. The proton NMR spectrum (Figure 6) was also consistent with structure **25** showing the presence of two vinyl moieties, one propionate side chain, and five methyl units resonating between 3.6 and 3.8 ppm.

Conclusion

Incubation of the isomeric substrate analogues **8** and **9** with crude preparations of copro'gen oxidase gave monovinylic and divinylic products, respectively. Al-

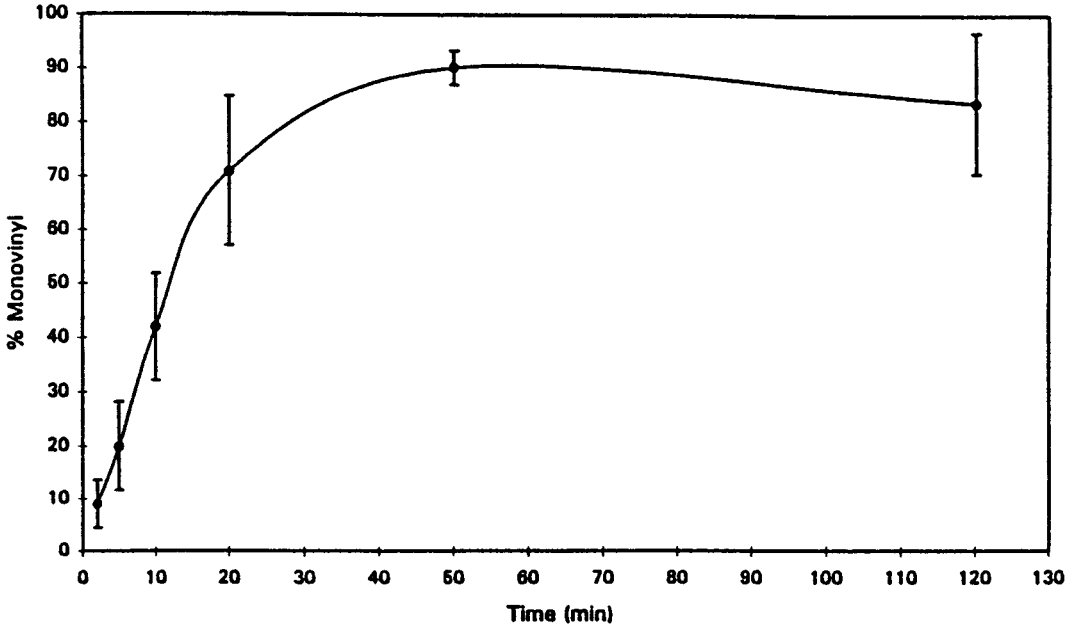

Figure 4. Time course study for the incubation of tripropionate porphyrinogen **8** with chicken red cell hemolysates showing the % monovinylic porphyrin product formation vs time.

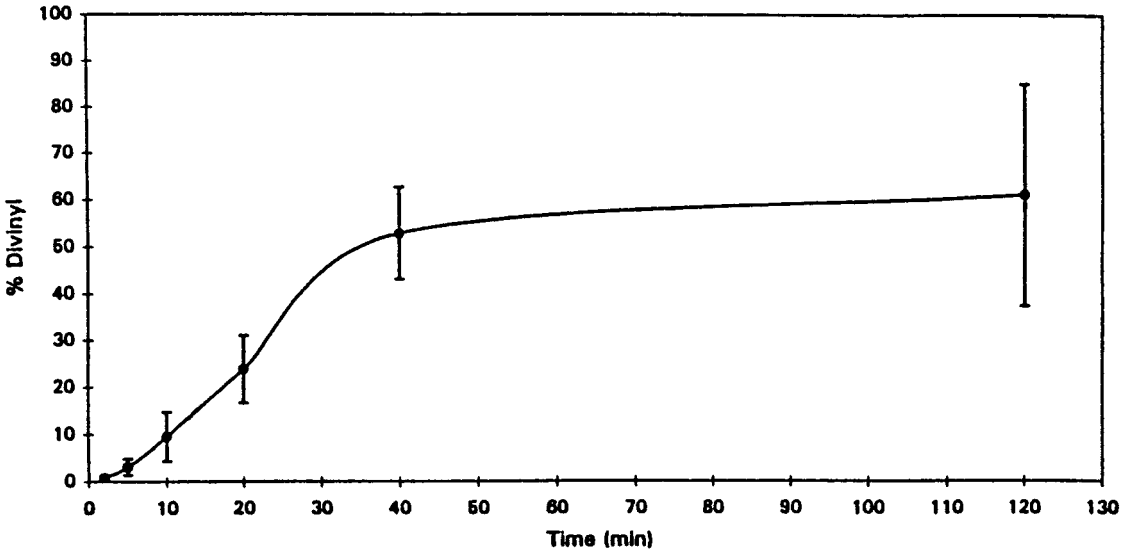

Figure 5. Time course study for the incubation of tripropionate porphyrinogen **9** with chicken red cell hemolysates showing the % final divinyl product formation vs time.

though only one propionate side chain is metabolized for **8**, two are converted into vinyl groups for porphyrinogen **9**, the former species is a far better substrate than the latter as evidenced by faster initial rates and larger amounts of total product formed. Nonetheless, the qualitative results of this study are in excellent agreement with the proposed model[4] for the active site of this poorly understood enzyme.

Experimental Section

Enzyme incubations and analyses of metabolic products were carried out as described previously.[4,22] HPLC analyses were performed using normal-phase columns (5 μm Partisil silica, Alltech) eluting with appropriate ratios of ethyl acetate and cyclohexane. Kinetic data are reported as mean ± standard deviation for four replicate experiments and compared statistically using analysis of variance (ANOVA) following Fisher's LSD Post Test. Values are considered significantly different at $p < 0.05$. Column 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.[23] Unless otherwise indicated, reagents were purchased from Aldrich Chemical Co. and were not further purified. 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). Elemental analyses were obtained from Micro-Analysis, Inc., Wilmington, DE 19808, or the School of Chemical Sciences Microanalysis Laboratory at the University of Illinois.

Benzyl 3,8,13-Tris(2-methoxycarbonylethyl)-2,7,12,14-tetramethyl-5,16-dihydrotripyrrin-1-carboxylate Hydrobromide (15). *tert*-Butyl 5′-benzyloxycarbonyl-3′,4-bis(2-methoxycarbonylethyl)-3,4′-dimethyl-2,2′-dipyrrylmethane-5-

(22) See also: Dresel, E. I. B.; Falk, J. E. *Biochem. J.* **1956**, *63*, 388.

(23) Vogel, A. I.; Furniss, B. S.; Hannaford, A. J.; Rogers, V.; Smith, P. W. G.; Tatchell, A. R. *Vogel's Textbook of Practical Organic Chemistry*, 4th ed.; Longman: Harlow, U.K., 1986; pp 314−315.

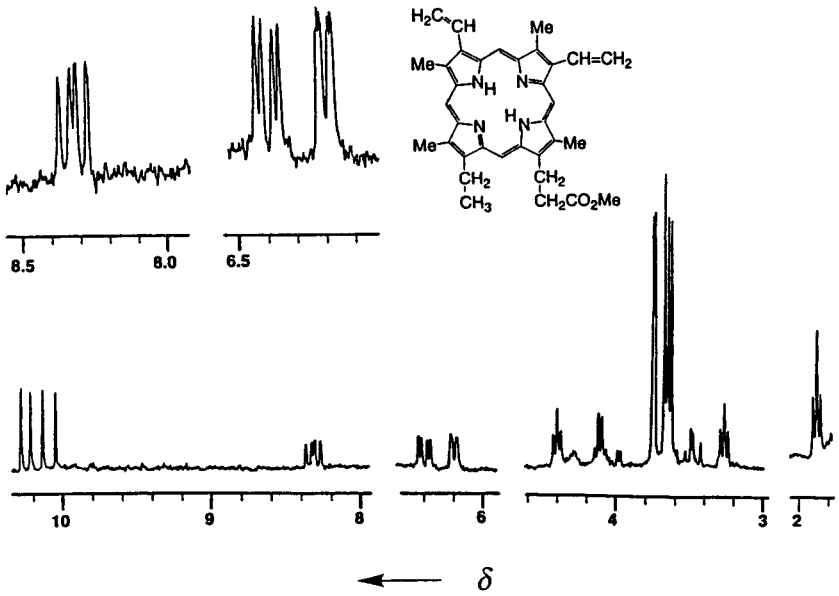

Figure 6. 300 MHz proton NMR spectrum of the esterified divinylporphyrin product **25** from incubations of **9** with chicken red cell hemolysates in $CDCl_3$.

carboxylate (**13**)[4] (0.725 g) was treated with trifluoroacetic acid (6.0 mL) for 5 min with stirring at room temperature. A solution of 4-(2-methoxycarbonylethyl)-3,5-dimethylpyrrole-2-carboxaldehyde (**14**)[1] (0.261 g) in methanol (30 mL) was added all at once, and the brownish orange solution was stirred for 1.5 h. Further addition of a mixture of aqueous HBr (48%) and acetic acid (1:2.33 v/v, 5 drops), followed by anhydrous ether (60 mL), and continued stirring for an additional 15 min led to the formation of a yellowish-orange precipitate. The precipitate was filtered, washed with ether, and dried in vacuo overnight to give the title tripyrrene hydrobromide (0.795 g; 85%) as orange crystals: mp 166.5−167.5 °C; UV−vis ($CHCl_3$) λ_{max} (log ϵ) 493 nm (4.88); ^{1}H NMR ($CDCl_3$) δ 2.05 (3H, s), 2.25 (3H, s), 2.35 (3H, s), 2.38 (2H, t), 2.45−2.53 (4H, m), 2.68 (3H, s), 2.74−2.82 (4H, m), 2.95 (2H, t, J = 7.0 Hz), 3.61 (3H, s), 3.67 (6H, s), 4.35 (2H, s), 5.31 (2H, s), 7.2−7.33 (3H, m), 7.30 (1H, s), 7.51 (2H, d), 10.64 (1H, br s), 13.11 (2H, br). Anal. Calcd for $C_{38}H_{46}N_3O_8Br$: C, 60.64; H, 6.16; N, 5.58. Found: C, 60.47; H, 6.15; N, 5.46.

Benzyl 13-Ethyl-3,8-bis(2-methoxycarbonylethyl)-2,7,-12,14-tetramethyl-5,16-dihydrotripyrrin-1-carboxylate Hydrobromide (19). Using the previous procedure, **13** (503 mg) and 4-ethyl-3,5-dimethylpyrrole-2-carboxaldehyde (**16**)[4] (132 mg) gave the tripyrrene (370 mg; 61%) as orange crystals, mp 153−154 °C. However, in two other runs yields of 21% and 31% were obtained: UV−vis ($CHCl_3$) λ_{max} (log ϵ) 495 nm (4.91); ^{1}H NMR ($CDCl_3$) δ 1.10 (3H, t), 2.07 (3H, s), 2.27 (3H, s), 2.34 (3H, s), 2.35−2.55 (6H, m), 2.66 (3H, s), 2.82 (2H, t), 2.95 (2H, t), 3.63 (3H, s), 3.67 (3H, s), 4.35 (2H, s), 5.33 (2H, s), 7.2−7.35 (3H, m), 7.27 (1H, s), 7.51 (2H, d), 10.66 (1H, br s), 13.10 (1H, br s), 13.18 (1H, br s). Anal. Calcd for $C_{36}H_{44}N_3O_6Br$: C, 62.24; H, 6.38; N, 6.05. Found: C, 61.78; H, 6.24; N, 6.00.

***tert*-Butyl 2,7,13-Tris(2-methoxycarbonylethyl)-3,8,12,-14-tetramethyl-5,16-dihydrotripyrrin-1-carboxylate Hydrobromide (21).** *p*-Toluenesulfonic acid monohydrate (388 mg; 2.5 equiv) in methanol (5.0 mL) was added all at once to a stirred mixture of 5′-*tert*-butoxycarbonyl-3,4′-bis-(2-methoxycarbonylethyl)-3′,4-dimethyl-2,2′-dipyrrylmethane-5-carboxylic acid (**20**)[4] (400 mg) and pyrrole aldehyde **14**[1] (0.170 g) in dichloromethane (100 mL), and the resulting mixture stirred at room temperature for 40 min. The brownish orange solution was washed with water (100 mL), saturated sodium bicarbonate (100 mL), and water (100 mL). The combined organic layers were dried over sodium sulfate and filtered, and the solvent was evaporated under reduced pressure. The residue was taken up in dichloromethane (50 mL), HBr gas was bubbled through the solution for 5 s, and the solvent was immediately flash evaporated under reduced pressure. The residue was dissolved in toluene (2 × 40 mL) and evaporated to azeotrope out traces of water and hydrobromic acid. The residue was then dissolved in ether (25 mL) and reevaporated. The residue was taken up in minimal amount of ether (15 mL) and left overnight in the freezer. The resulting precipitate were filtered, washed with ether, and dried in vacuo to give the tripyrrene (0.318 g; 55%) as red crystals with a green tinge: mp 183.5−184.5 °C; UV−vis ($CHCl_3$) λ_{max} (log ϵ) 493 (4.80); ^{1}H NMR ($CDCl_3$) δ 1.55 (9H, s), 2.04 (3H, s), 2.25 (3H, s), 2.31 (3H, s), 2.28 (2H, t), 2.46−2.51 (4H, m), 2.70 (3H, s), 2.68−2.79 (4H, m), 2.96 (2H, t), 3.65 (3H, s), 3.67 (6H, s), 4.35 (2H, s), 7.08 (1H, s), 10.23 (1H, br s), 13.19 (1H, br s), 13.24 (1H, br s). Anal. Calcd for $C_{35}H_{48}N_3O_8Br$: C, 58.49; H, 6.73; N, 5.85. Found: C, 58.40; H, 6.96; N, 5.63.

2-Ethyl-8,13,18-tris(2-methoxycarbonylethyl)-1,3,7,12,-17,19-hexamethyl-10,23-dihydrobilin Dihydrobromide (17). Tripyrrene hydrobromide **15** (302 mg) was treated with a mixture of trifluoroacetic acid (5.8 mL) and aqueous HBr (1.2 mL, prepared from aqueous HBr (48%) and acetic acid (1:2.33 v/v)) with stirring at room temperature for 6 h. A solution of 4-ethyl-3,5-dimethylpyrrole-2-carboxaldehyde[4] (**16**) (60.4 mg) in methanol (25 mL) was added all at once and the mixture stirred for a further 30 min. The brownish orange solution immediately turned dark red in color. Rapid but dropwise addition of ether (60 mL) gave a precipitate that upon suction filtration and drying under vacuum gave the title a,c-biladiene (261 mg; 78%) as very fine red crystals with a greenish tinge: mp 176.5−178.5 °C; UV−vis ($CHCl_3$) λ_{max} (log ϵ) 455 (4.46), 525 (5.23); ^{1}H NMR ($CDCl_3$) δ 1.08 (3H, t), 1.90−2.01 (2H, m), 1.96 (3H, s), 2.24 (3H, s), 2.29 (3H, s), 2.37 (3H, s), 2.4−2.5 (6H, m), 2.71 (3H, s), 2.73 (3H, s), 2.67−2.78 (4H, m), 2.94 (2H, t), 3.42 (3H, s), 3.60 (3H, s), 3.67 (3H, s), 5.21 (2H, s), 7.10 (1H, s), 7.33 (1H, s), 13.21 (1H, br s), 13.26 (1H, br s), 13.32 (1H, br s), 13.42 (1H, br s). Anal. Calcd for $C_{39}H_{52}N_4O_6Br_2 \cdot {}^3/_2H_2O$: C, 54.49; H, 6.45; N, 6.52. Found: C, 54.37; H, 6.08; N, 6.50.

2-Ethyl-7,12,18-tris(2-methoxycarbonylethyl)-1,3,8,13,-17,19-hexamethyl-20,23-dihydrobilin Dihydrobromide (22). *tert*-Butyl 2,7,13-tris(2-methoxycarbonylethyl)-3,8,12,14-tetramethyl-5,16-dihydrotripyrrin-1-carboxylate hydrobromide (**21**) (288 mg) was treated with trifluoroacetic acid (1.0 mL) for 5 min. A solution of pyrrole aldehyde **16** (60.4 mg) in methanol (4.0 mL) was added all at once, immediately followed by the addition of a mixture of aqueous HBr (48%) and acetic acid (1:2.33 v/v, 0.80 mL), and the resulting solution was stirred further for 30 min. Ether (30 mL) was added dropwise, and the resulting mixture was stirred for 2 h. The deep red precipitate was filtered, washed with ether, and dried in vacuo

overnight to give the title a,c-biladiene dihydrobromide (285 mg; 86%) as dark red crystals: mp 151.5−152.5 °C; UV−vis ($CHCl_3$) λ_{max} (log ϵ) 454 (4.42), 524 (5.13); ^{1}H NMR ($CDCl_3$) δ 1.09 (3H, t), 1.92 (2H, m), 1.96 (3H, s), 2.24 (3H, s), 2.32 (3H, s), 2.35 (3H, s), 2.48 (6H, m), 2.71−2.81 (4H, m), 2.74 (6H, s), 2.94 (2H, t), 3.42 (3H, s), 3.60 (3H, s), 3.67 (3H, s), 5.21 (2H, s), 7.11 (1H, s), 7.31 (1H, s), 13.22 (1H, br s), 13.26 (1H, br s), 13.33 (1H, br s), 13.38 (1H, br s). Anal. Calcd for $C_{39}H_{52}N_4O_6$-Br_2·$2H_2O$: C 53.92; H, 6.49; N, 6.45. Found: C, 53.52; H, 6.07; N, 6.49.

17-Ethyl-3,8,13-tris-(2-methoxycarbonylethyl)-2,7,12,-18-tetramethylporphyrin (18). a,c-Biladiene dihydrobromide **17** (234 mg) was added to a stirred solution of copper(II) chloride (0.74 g) in DMF (95 mL) and the resulting mixture stirred at room temperature in the dark for 2 h. The mixture was diluted with dichloromethane (150 mL) and washed with water (2 × 150 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 for removal of residual DMF. The solid residue was taken up in 15% v/v sulfuric acid/trifluoroacetic acid (35 mL) and stirred in the dark at room temperature for 45 min. The reaction mixture was diluted with dichloromethane (125 mL) and washed with water (2 × 150 mL) and 5% aqueous sodium bicarbonate solution (100 mL). The organic layer was dried over sodium sulfate and the solvent evaporated under reduced pressure. The residue was then dissolved in 5% sulfuric acid−methanol (35 mL) and stirred in the dark overnight for reesterification. The reaction mixture was diluted with dichloromethane and washed with water and then 5% aqueous sodium bicarbonate solution. The organic layer was dried over sodium sulfate and the solvent evaporated under reduced pressure. The residue was chromatographed on grade III alumina column (2.2 i.d. × 16 cm), eluting with dichloromethane. A deep violet fraction was collected and evaporated under pressure, and the residue recrystallized from chloroform−methanol. The crystals were filtered and dried in vacuo overnight to give the title porphyrin (125 mg; 68%) as purple crystals, mp 199.5−201 °C; UV-vis ($CHCl_3$): λ_{max} (log ϵ) 400 (5.21), 499 (4.12), 532 (3.97), 567 (3.80), 621 (3.64); ^{1}H NMR ($CDCl_3$) δ −3.82 (2H, s), 1.88 (3H, t, J = 7.6 Hz), 3.24−3.30 (6H, m), 3.62 (3H, s), 3.63 (3H, s), 3.65 (3H, s), 3.66 (3H, s), 3.68 (3H, s), 3.688 (3H, s), 3.693 (3H, s), 4.11 (2H, q, 7.6 Hz), 4.35−4.45 (6H, m), 10.06 (1H, s), 10.07 (3H, s); FAB-MS 653.3 [$(M + H)^+$]; HRFAB-MS calcd for $C_{38}H_{44}N_4O_6$ + H 653.3339, found 653.3343. Anal. Calcd for $C_{38}H_{44}N_4O_6$: C, 69.92; H, 6.79; N, 8.58. Found: C, 69.62; H, 6.76; N, 8.56.

13-Ethyl-3,8,17-tris(2-methoxycarbonylethyl)-2,7,12,18-tetramethylporphyrin (23). Prepared from a,c-biladiene dihydrobromide **22** (235 mg) by the procedure described above. Recrystallization from chloroform−methanol gave the title porphyrin (100 mg; 54%) as violet crystals: mp 182−183 °C; UV−vis ($CHCl_3$) λ_{max} (log ϵ) 399 (5.15), 498 (4.03), 532 (3.88), 567 (3.71), 620 nm (3.57); ^{1}H NMR ($CDCl_3$) δ −3.81 (2H, s), 1.88 (3H, t, J = 7.6 Hz), 3.24−3.29 (6H, m), 3.62 (3H, s), 3.64 (3H, s), 3.65 (3H, s), 3.67 (3H, s), 3.68 (6H, s), 3.70 (3H, s), 4.11 (2H, q, J = 7.6 Hz), 4.37−4.43 (6H, m), 10.07 (4H, s); FAB-MS 653.3 [$(M + H)^+$]; HR FAB-MS calcd for $C_{38}H_{44}N_4O_6$ + H 653.3339, found 653.3343. Anal. Calcd for $C_{38}H_{44}N_4O_6$: C, 69.92; H, 6.79; N, 8.58. Found: C, 69.63; H, 6.80; N, 8.46.

Porphyrins Isolated from Preparative Enzymic Studies. These products were purified as the methyl esters by flash chromatography, eluting with 10% ethyl acetate−toluene, and characterized by FAB MS and 300 MHz proton NMR spectroscopy.

13-Ethyl-8,17-bis(2-methoxycarbonylethyl)-2,7,12,18-tetramethyl-3-vinylporphyrin (24): ^{1}H NMR ($CDCl_3$) δ −3.65 (2H, s), 1.87 (3H, t, J = 7.5 Hz), 3.28 (4H, t, J = 8 Hz), 3.63 (3H, s), 3.65 (3H, s), 3.67 (6H, s), 3.69 (3H, s), 3.75 (3H, s), 4.09 (2H, q, J = 7.5 Hz), 4.43 (4H, t, J = 8 Hz), 6.19 (1H, d, J = 11.4 Hz), 6.39 (1H, d, J = 17.5 Hz), 8.32 (1H, dd, J = 12, 17.5 Hz), 10.07 (2H, s), 10.16 (1H, s), 10.24 (1H, s); HR FAB-MS calcd for $C_{36}H_{40}N_4O_4$ + H 593.3128, found 593.3126.

17-Ethyl-13-(2-methoxycarbonylethyl)-2,7,12,18-tetramethyl-3,8-divinylporphyrin (25): ^{1}H NMR ($CDCl_3$) δ −3.56 (2H, s), 1.88 (3H, t, J = 7.5 Hz), 3.27 (2H, t, J = 7.6 Hz), 3.63 (3H, s), 3.65 (3H, s), 3.67 (3H, s), 3.74 (3H, s), 3.75 (3H, s), 4.11 (2H, q, J = 7.5 Hz), 4.40 (2H, t, J = 7.6 Hz), 6.18−6.23 (2H, m), 6.38 (1H, d, J = 17.7 Hz), 6.41 (1H, d, J = 17.7 Hz), 8.31 (2H, dd), 10.05 (1H, s), 10.14 (1H, s), 10.22 (1H, s), 10.29 (1H, s); HR FAB-MS calcd for $C_{34}H_{36}N_4O_2$ + H 533.2916, found 533.2917.

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Supporting Information Available: Copies of 300 MHz ^{1}H NMR spectra for compounds **15**, **17**−**19**, and **21**−**23** and for the metabolic products **24** and **25**, together with the FAB MS for **18** and **23**−**25**. This material is available free of charge via the Internet at http://pubs.acs.org.

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