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Normal and abnormal heme biosynthesis. Part 7. Synthesis and metabolism of coproporphyrinogen-III analogues with acetate or butyrate side chains on rings C and D. Development of a modified model for the active site of coproporphyrinogen oxidase ☆

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ABSTRACT

Analogues of coproporphyrinogen-III have been prepared with acetate or butyrate groups attached to the C and D pyrrolic subunits. The corresponding porphyrin methyl esters were synthesized by first generating a,c-biladienes by reacting a dipyrrylmethane with pyrrole aldehydes in the presence of HBr. Cyclization with copper(II) chloride in DMF, followed by demetalation with 15% H_2SO_4–TFA and reesterification, gave the required porphyrins in excellent yields. Hydrolysis with 25% hydrochloric acid and reduction with sodium-amalgam gave novel diacetate and dibutyrate porphyrinogens **9**. Diacetate **9a** was incubated with chicken red cell hemolysates (CRH), but gave complex results due to the combined action of two of the enzymes present in these preparations. Separation of uroporphyrinogen decarboxylase (URO-D) from coproporphyrinogen oxidase (CPO) allowed the effects of both enzymes on the diacetate substrate to be assessed. Porphyrinogen **9a** proved to be a relatively poor substrate for CPO compared to the natural substrate coproporphyrinogen-III, and only the A ring propionate moiety was processed to a significant extent. Similar results were obtained for incubations of **9a** with purified human recombinant CPO. Diacetate **9a** was also a substrate for URO-D and a porphyrinogen monoacetate was the major product in this case; however, some conversion of a second acetate unit was also evident. The dibutyrate porphyrinogen **9b** was only recognized by the enzyme CPO, but proved to be a modest substrate for incubations with CRH. However, **9b** was an excellent substrate for purified human recombinant CPO. The major product for these incubations was a monovinylporphyrinogen, but some divinyl product was also generated in incubations using purified recombinant human CPO. The incubation products were converted into the corresponding porphyrin methyl esters, and these were characterized by proton NMR spectroscopy and mass spectrometry. The results extend our understanding of substrate recognition and catalysis for this intriguing enzyme and have allowed us to extend the active site model for CPO. In addition, the competitive action of both URO-D and CPO on the same diacetate porphyrinogen substrate provides additional perspectives on the potential existence of abnormal pathways for heme biosynthesis.

1. Introduction

In vertebrates, uroporphyrinogen-III (uro'gen-III, Scheme 1) is generated in four enzyme mediated steps from glycine and succinyl CoA.[1,2] Uro'gen-III is then acted upon by the cytoplasmic enzyme uroporphyrinogen decarboxylase (URO-D, EC 4.1.1.37) to produce the tetracarboxylate species coproporphyrinogen-III (copro'gen-III).[3,4] The four decarboxylations occur sequentially and hepta-, hexa-, and pentacarboxylate intermediates are formed during this conversion.[3] Under physiological conditions, this process occurs regioselectively starting with the acetate group on ring D and then proceeding to ring A, then B and finally ring C (the 'clockwise' decarboxylation pathway).[3,5–7] However, there are four possible hepta-, six hexa- and four pentacarboxylate porphyrinogens that could be formed from uro'gen-III, and overall there are 24 possible pathways between uro'gen-III and copro'gen-III.[3,5–7] Interestingly, in enzyme incubation studies using uro'gen-III or specific hepta- or hexacarboxylate porphyrinogens, the process appears to be random and all of the possible porphyrinogen intermediates can be identified.[5–8] Moreover, all 14 possible type III intermediates are metabolized by URO-D and the type I, type II and type IV isomers of uro'gen are also substrates for this enzyme.[9–11] However, the clockwise decarboxylation pathway does appear to be dominant at very low substrate concentrations.[5,6]

☆ For part 6 in the series, see Ref. 33b.

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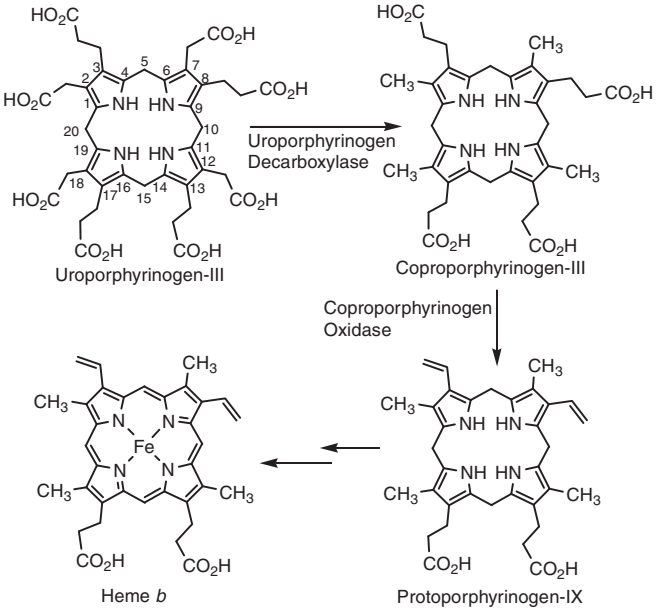
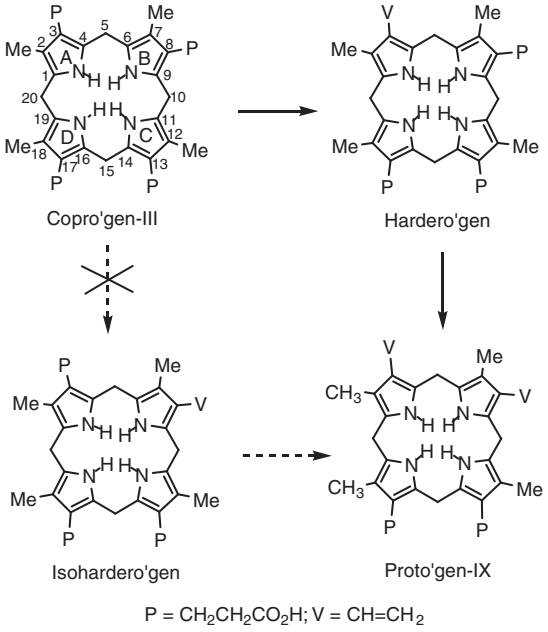
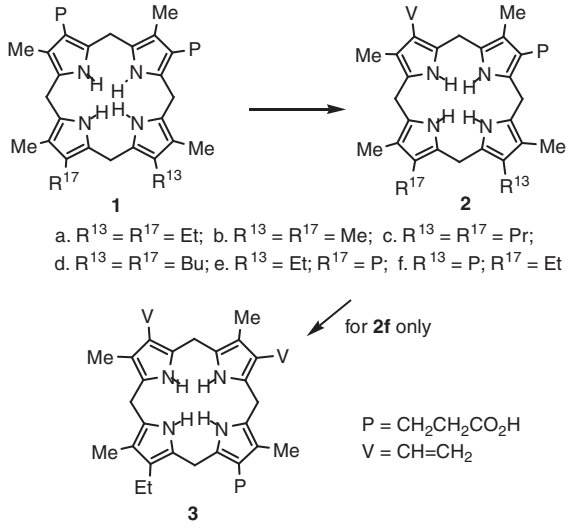


Scheme 1. Later stages of heme biosynthesis.



Scheme 2. Conversion of copro'gen-III to proto'gen-IX.



Scheme 3. Metabolism of substrate analogues by CPO.

Copro'gen-III is transferred into the mitochondria and undergoes two oxidative decarboxylations, promoted by coproporphyrinogen oxidase (CPO, EC 1.3.3.3) to produce proto'gen-IX (Scheme 1).[4b,12,13] Subsequent dehydrogenation by protoporphyrinogen oxidase affords the corresponding porphyrin and iron(II) insertion by ferrochelatase then gives heme *b* (Scheme 1). Protoporphyrin-IX is also the precursor to most of the other hemes and chlorophylls.[1,2,14] CPO remains one of the least well understood enzymes in the heme biosynthetic pathway, in part because the mechanism for the conversion of the A and B ring propionate groups to vinyl moieties remains controversial.[15] CPO is a more selective enzyme than URO-D and only metabolizes two of the four possible type isomers of coproporphyrinogen.[16–20] In addition, the two oxidative decarboxylations are known to take place sequentially, but only one of the two possible tricarboxylate intermediates is formed.[21–23] The corresponding porphyrin, harderoporphyrin, was first isolated and characterized from the harderian gland of rodents,[24] but this monovinylporphyrin and its metabolites can be observed in numerous other biological materials, including urine and feces.[25–27] Nevertheless, although isoharderoporphyrinogen (Scheme 2) is not formed from copro'gen-III, it is a substrate for CPO.[28] A number of synthetic porphyrinogens have also been shown to be metabolized by CPO using crude enzyme preparations from chicken red cell hemolysates (CRH), including mesoporphyrinogen-VI (**1a**; meso'gen-VI) where the C and D ring propionate units have been replaced with ethyl groups (Scheme 3).[29,30] Meso'gen-VI and related porphyrinogens **1b–d** with methyl, propyl or butyl groups at these positions are converted by CPO to the corresponding monovinyl products, but the second oxidative decarboxylation is not observed.[13,30,32] However, the specific alkyl residues at positions 13 and 17 do have an impact, as meso'gen-VI is the best substrate, followed by **1b** and **1c**. Porphyrinogen **1d** is a very poor substrate and only gives <10% conversion to **2d**. These data demonstrate that larger nonpolar residues are not easily accommodated by CPO. Two related tripropionate porphyrinogens were also assessed,[31,32] where only one of the propionate residues was replaced by an ethyl group. Porphyrinogen **1e**, with a 13-ethyl unit, was a good substrate for CPO and was again converted into a monvinylic product **2e**. However, the 17-ethylporphyrinogen **1f** was further metabolized by CPO and afforded the divinylporphyrinogen product **3f**. On the basis of these and related investigations, it was concluded that a porphyrinogen needs the sequence of peripheral substituents R Me-P Me-P, where R = Me, vinyl, Et or H and P = $CH_2CH_2CO_2H$, in order for it to be recognized as a good substrate for CPO.[12,13] These requirements were incorporated into a model for the substrate binding sites in CPO where three regions were designated (Fig. 1). Region Y corresponds to the catalytic site where the propionate group is converted into the vinyl moiety, region X designates a site that requires the presence of a second propionate side chain for substrate binding, and position Z represents a region that can accommodate the presence of small nonpolar groups such as H, Me, vinyl (V) or Et, but not propionate or acetate groupings. However, this model does not account for the influence of alkyl groups at positions 13 and 17 on the first oxidative decarboxylation, which is favored when the substituents are Et or Me (**1a** and **1b**), but less so for the dipropyl substrate **1c** and greatly inhibited for dibutyl porphyrinogen **1d**. The 8-butyrate derivative **4a** of copro'gen-III has been shown to be a substrate for CPO preparations from *Euglena gracilis*, affording 3-vinylporphyrinogen **5a**,[34] but does not appear to be metabolized by enzyme preparations from CRH.[35]

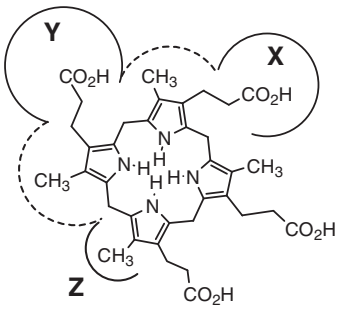
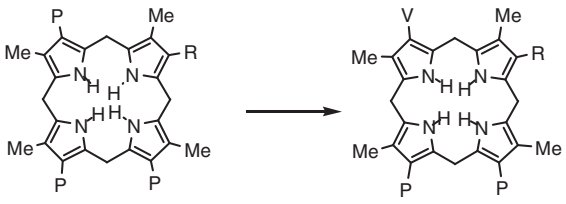


Figure 1. Active site model for substrate binding by coproporphyrinogen oxidase. Y is the catalytic site, X recognizes and binds a second propionate residue, and Z can accommodate only small nonpolar groups like Me, Et, V or H.

However, the branched substituents in porphyrinogens **4b** and **4c** did allow these species to be metabolized to a limited extent to give primarily the monovinyl products **5b** and **5c**, respectively (Scheme 4).[35] These data show that extended or branched carboxylate units can be accommodated at site X in the binding model,[12,35] but the modified porphyrinogens are far less effective substrates for CPO.[36]

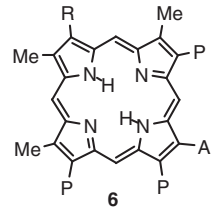
Further support for the substrate binding model can be discerned from the presence of abnormal isocoproporphyrin-type metabolites **6** (Fig. 2) in the urine and feces of humans suffering from porphyria cutanea tarda (PCT) and from rats that have been poisoned with hexachlorobenzene.[37] These metabolites were designated as isocoproporphyrins because the most common member of the series, 3-ethylporphyrin **6** (R = Et), has the same molecular weight as coproporphyrin-III.[38] The identity of these compounds was confirmed by total synthesis[39–41] and detailed spectroscopic investigations.[42] It was hypothesized that the usual pentacarboxylate intermediate, 5dab, formed by the previous enzyme URO-D, was metabolized out of sequence by CPO to generate dehydroisocoproporphyrinogen (**7**; Scheme 5),[43,44] and that this species gives rise to the observed porphyrins **6**. Pentacarboxylate porphyrinogen 5dab has been shown to be a substrate for rat liver CPO and highly purified human CPO, but only the A ring propionate residue undergoes oxidative decarboxylation to afford **7** and the hypothetical divinyl metabolite **8** is not seen (Scheme 5).[43,44] Synthetic isocoproporphyrinogen has also been shown to not be a substrate for the rat liver enzyme.[45] These data show that the acetate moiety inhibits further conversion by CPO. However, **7** is a substrate for URO-D and can be converted into hardero'gen, which in turn may be metabolized by CPO to produce proto'gen-IX (Scheme 5).[43] It has been proposed that this route (Scheme 5) may provide an alternative pathway for heme biosynthesis under abnormal conditions,[43,44] although evidence has recently been presented that suggests that the uncoupling of CPO within the mitochondrial intermembrane space may also be a factor.[37d] Evidence for this abnormal pathway has also been reported for mutants of *Saccharomyces cerevisiae*.[46]



a. R = B; b. R = $CH(CH_3)CH_2CO_2H$; c. R = $CH_2CH(CH_3)CO_2H$

B = $(CH_2)_3CO_2H$; P = $CH_2CH_2CO_2H$; V = $CH{=}CH_2$

Scheme 4. Metabolism of porphyrinogens with modified side chains on ring B.



A = CH_2CO_2H
P = $CH_2CH_2CO_2H$
V = $CH{=}CH_2$

R = V, Et, H, $CH(OH)CH_3$ or $COCH_3$

Figure 2. Isocoproporphyrin series.

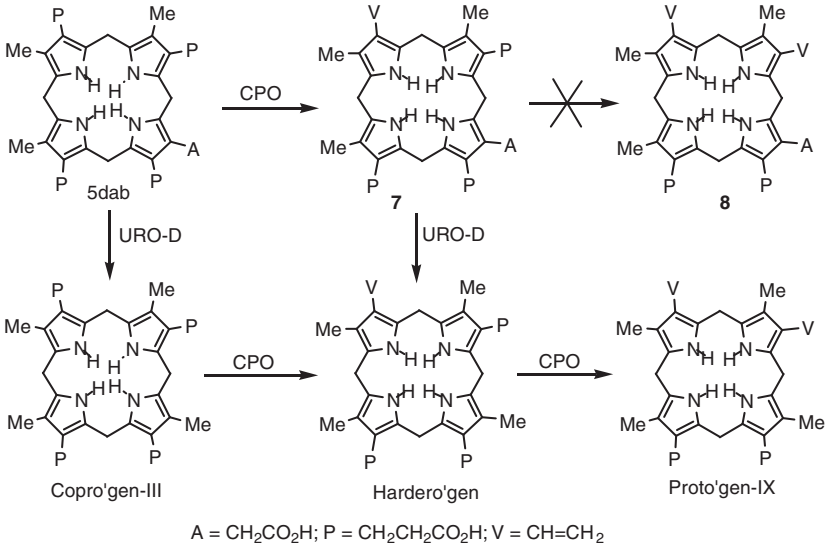
In aerobic organisms, CPO is an oxygen-dependent enzyme, although a structurally unrelated CPO is found in anaerobic bacteria.[47] Oxygen-dependent CPO catalyzes the oxidative decarboxylation of two propionate groups to vinyl moieties, but the presence of transition metal ions are not required and no cofactors are needed.[48] Although several mechanisms for this reaction have been proposed,[4] the most plausible explanation involves a base catalyzed addition of O_2 to generate a peroxide anion, followed by intramolecular deprotonation and elimination of H_2O_2 and CO_2.[15,49] Recently, a crystal structure for the oxygen-dependent form of CPO from *S. cerevisiae* was reported,[50] and a similar structure was also published for human recombinant CPO.[51] CPO was characterized as a homodimer[52] that generates a nonpolar cleft that has been tentatively assigned as the active site.[50] Subsequent site-directed mutagenesis studies demonstrated that the invariant amino acids aspartate 400, arginine 262 and arginine 401 were essential for significant catalytic activity,[53] and these residues may be involved in substrate binding and catalysis.[53] However, the details of substrate binding interactions and catalytic oxidative decarboxylation by CPO remains far from complete.

Considerable progress has been made over the last ten years on the structure and activity of URO-D[7,54] and CPO,[13,15,31,49–51,53] but a detailed understanding of the latter enzyme remains elusive. A full understanding of the later stages of heme biosynthesis is necessary to fully appreciate nature's primary pathways for metabolism, although this goal also has medicinal implications with regard to the treatment of porphyrias.[55] Defects in URO-D and CPO can result in porphyria cutanea tarda (PCT) and coproporphyria, respectively, and these diseases are associated with many pathological symptoms, including skin photosensitivity, liver damage and neurological problems.[55] In order to gain a better understanding of substrate binding and catalysis for CPO, two analogues of copro'gen-III with acetate or butyrate groups at rings C and D, **9a** and **9b** (Fig. 3), were targeted for study. As porphyrinogens are highly unstable, and prone to oxidations and acid catalyzed rearrangements, it was necessary to synthesize the corresponding porphyrins **10a** and **10b**. Full details on the synthesis of porphyrins **10a** and **10b**, and the action of CPO on porphyrinogens **9a** and **9b**, are described below.

2. Results and discussion

2.1. Synthesis of substrates as porphyrin methyl esters

Although porphyrin diacetate **10a** has not been reported previously, dibutyrate **10b** was synthesized some years ago.[56,57] This earlier work indicated that **9b** could be used to probe the enzyme CPO, but no further information was provided.[56] We elected to carry out the synthesis of **9a** and **9b** using the a,c-biladiene strategy.[58,59] A retrosynthetic analysis (Scheme 6) shows that the syntheses can be accomplished using dipyrrylmethane **11** and dialdehydes **12a** and **12b**. We have made extensive use of dipyrrylmethane **11** in our earlier studies,[13,31] so it only remained for us to prepare pyrrole aldehydes **12**. These were prepared from pyrrole esters that were in turn generated using Knorr-type chemistry.[60]



Scheme 5. Normal and abnormal pathways between 5dab and hardero'gen.

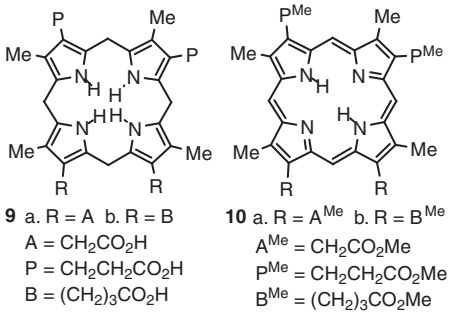
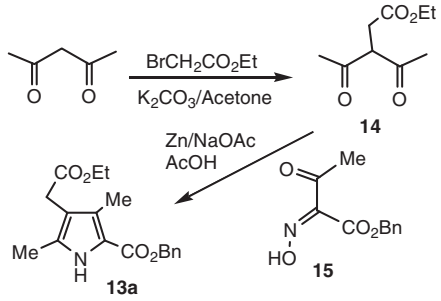
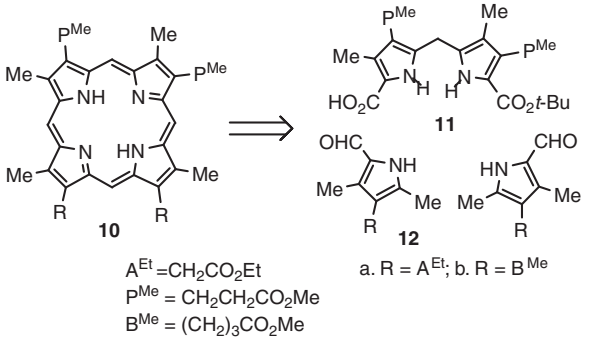


Figure 3. New substrate analogues for CPO and the related porphyrin tetramethyl esters.

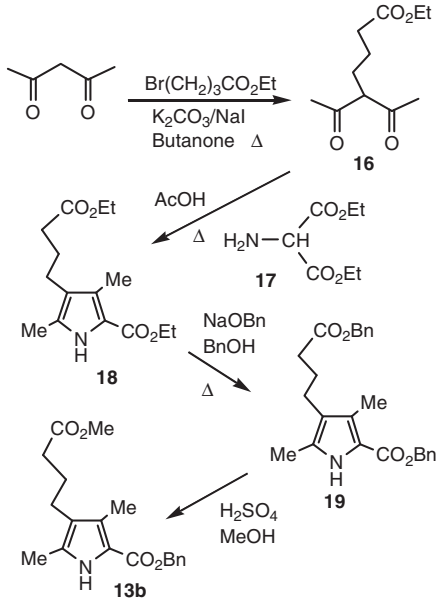


Scheme 7. Synthetic of a pyrrole with an acetate side chain.



Scheme 6. Retrosynthetic analysis of porphyrins **10a** and **10b**.

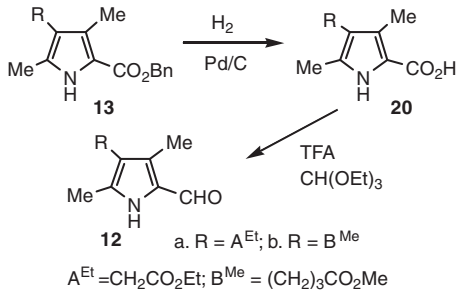
Pyrrole **13a** with an acetate side chain was prepared in two steps from 2,4-pentanedione (Scheme 7). Alkylation of the diketone with ethyl bromoacetate and potassium carbonate in refluxing acetone gave ester **14** in 69% yield and this was further reacted with oxime **15** and zinc dust in acetic acid to give **13a** in 33% yield. This relatively low yield is typical of classical Knorr chemistry,[60] but nevertheless still enables significant amounts of the intermediate to be prepared from reasonably inexpensive starting materials. The pyrrole is also obtained with a benzyl ester unit that can be selectively deprotected prior to the formation of the required pyrrole aldehyde. The corresponding pyrrole with a butyrate side chain was prepared by a longer but more efficient route, again starting with 2,4-pentanedione (Scheme 8). Reaction of 2,4-pentanedione with ethyl 4-bromobutanoate, sodium iodide



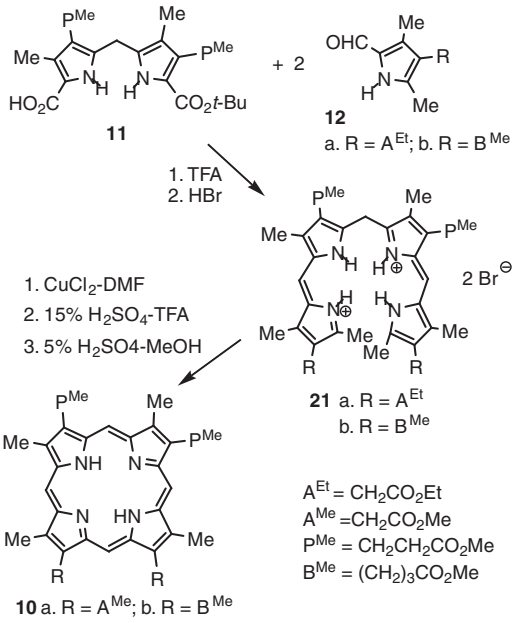
Scheme 8. Synthesis of pyrroles with butyrate side chains.

and potassium carbonate in refluxing methyl ethyl ketone gave the diketo ester **16** in 76% yield. This was reacted with diethyl aminomalonate (**17**)[61,62] under modified Kleinspehn conditions[61–63] to give the pyrrole diester **18** in 66% yield. It was then necessary to selectively introduce a benzyl ester at the α-position. Reaction of **18** with sodium benzyloxide in benzyl alcohol gave dibenzyl ester **19**, and this was treated with 5% sulfuric acid in methanol to give the required mixed ester **13b** in good overall yields (Scheme 8).

Pyrrole benzyl esters **13a** and **13b** were deprotected by hydrogenolysis over 10% Pd/C to give the related carboxylic acids **20** (Scheme 9). Treatment with TFA decarboxylated these pyrroles, and subsequent reaction with triethyl orthoformate introduced the required formyl units to give pyrroles **12** in >70% yield. Dipyrrole **11** (Scheme 10) was treated with TFA for 10 min to cleave the *tert*-butyl ester protective group and decarboxylate both terminal carboxylate units. Two equiv of pyrrole aldehyde **12a** or **12b** were then added, followed immediately by HBr in acetic acid, and the corresponding a,c-biladienes were subsequently precipitated with ether as the dihydrobromide salts **21** (Scheme 10). Cyclization with copper(II) chloride in DMF at room temperature[64] gave the porphyrin products as copper(II) complexes, but these could easily be demetalated with 15% H_2SO_4 in TFA and reesterified with 5% H_2SO_4 in methanol, and the tetramethyl esters **10** were isolated in excellent yields. It is worth noting that the acetate side chains were introduced in a,c-biladiene **21a** as the ethyl esters, but following the final treatment with H_2SO_4–MeOH the tetramethyl ester **10a** was



Scheme 9. Synthesis of pyrrole aldehydes.



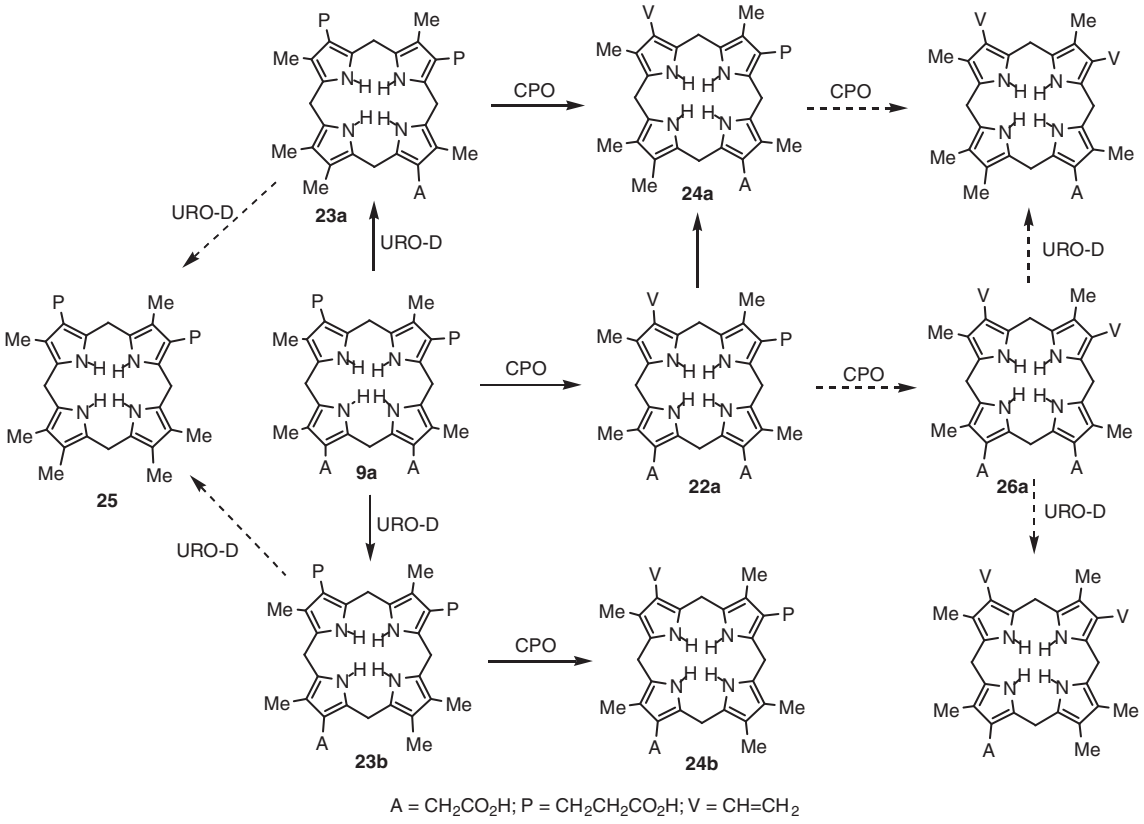
Scheme 10. Synthesis of porphyrins via a,c-biladienes.

the final product. Porphyrins **10a** and **10b** were characterized by UV–vis, proton NMR and carbon-13 NMR spectroscopy, and mass spectrometry. The UV–vis absorption spectra for the diacetate **10a** showed minor bathochromic shifts (0–2 nm) compared to **10b**. For the free base forms in 1% triethylamine-chloroform, **10a** gave a λ_{max} value for the Soret band at 401 nm, and Q bands at 499, 533, 569 and 623 nm; these values were 400, 499, 533, 567 and 621 nm, respectively for dibutyrate **10b**. In 1% TFA–chloroform, the corresponding diprotonated porphyrin dications **10**H_2^{2+} were formed and these gave sharper and more intense Soret bands at 400 and 401 nm for **10a**H_2^{2+} and **10b**H_2^{2+}, respectively.

The macrocycles were conveniently prepared as porphyrin methyl esters **10**, but needed to be hydrolyzed with 25% hydrochloric acid and reduced with 3% sodium amalgam to give the required porphyrinogen substrates **9**.[13,65] These were generated immediately prior to the biochemical studies. The products of metabolism were isolated as the fully conjugated porphyrins and converted back into the methyl ester derivatives prior to analysis.

2.2. Action of CPO and URO-D on the diacetate substrate 9a

Initial biochemical studies were conducted using chicken red cell hemolysates (CRH). CRH is a convenient source of active CPO and was used in many of our earlier investigations.[13,31–33,35] However, incubations of **9a** with CRH gave complex results that were not consistent with straightforward metabolism by CPO. CRH contains endogenous protoporphyrin-IX (proto-IX), but HPLC analysis of the incubation products for **9a** showed the formation of several additional products. CRH also contains URO-D and we speculated that porphyrinogen **9a** might also be a substrate for this enzyme. Indeed, this type of conversion would parallel the processes that are believed to lead to the generation of isocoproporphyrins (see earlier).[44] Although a peak could be identified as the expected monovinyl product **22a**, a larger slightly less polar peak was always present in these extracts. If URO-D can act on **9a**, two monoacetate porphyrinogens, **23a** and **23b**, could be formed (Scheme 11). These in turn could be further processed by CPO to give **24a** and **24b**, although the substrate binding model suggests that only **23a** should be a substrate, while further action by URO-D would give the hexamethylporphyrinogen **25**. Further action by either enzyme could lead to a number of other products (Scheme 11). The predominant porphyrin product was separated by flash chromatography. Although NMR data could not be obtained, the sample was successfully analyzed by FAB MS and gave a quasimolecular ion at *m/z* 625. FAB MS for porphyrins always gives the M+H peak, and this means that the sample has a molecular weight of 624. High resolution MS data gave a value for *m/z* of 625.3029, which is an excellent match for the formula $C_{36}H_{40}N_4O_6$+H (calcd *m/z* 625.3026), and corresponds to the expected porphyrin trimethyl esters derived from porphyrinogens **23a** and **23b**. Although the precise identity of this product cannot be discerned (**23a**, **23b**, or a mixture of both), these data strongly imply that URO-D is acting on this substrate. A second decarboxylation by URO-D to **25**, or the further metabolism of **23a** or **23b** by CPO, may be occurring, but these and related processes appear to be slow in comparison to the formation of the initial metabolites. In order to gain further insights, incubations were carried out in the presence of *N*-ethylmaleimide (NEM). NEM is known to be an inhibitor of URO-D[66] and it is notable that the incubations showed an increase in the peak for the monovinyl product compared to the monoacetate peak corresponding to **22a** and/or **22b**. Further support for this analysis was made by carrying out incubations with mitochondrial preparations derived from CRH. As URO-D is associated with the cytoplasm, and CPO is found in the mitochondria, these preparations would be expected to give **22a** but not **23a** or **23b**. Incubations of **9a** with these mitochondrial preparations did indeed primarily



Scheme 11. Multiple pathways for the metabolism of porphyrinogen **9a** due to the action of Uro-D and CPO.

show a product peak corresponding to the monovinyl porphyrin and the results were essentially unchanged when the experiments were repeated in the presence of NEM. However, the mitochondrial preparations retained some contamination with URO-D and the results were difficult to reproduce.

In order to obtain cleaner data and deconvolute the results for the two enzymes, CRH derived URO-D and CPO were separated using porphyrin affinity chromatography.[67] In addition, purified human recombinant CPO from *Escherichia coli* was isolated using the procedure reported by Medlock and Daily.[48,68] For kinetic studies, the incubation products were extracted using a microassay[69] and analyzed by HPLC. Incubations of **9a** with the URO-D containing fraction showed monoacetate **23** as the major product, but a small peak corresponding to the porphyrin derived from **25** can also be seen (Fig. 4). Incubations of **6a** with CRH-derived CPO or human recombinant CPO gave rise to a different product peak corresponding to the monovinyl porphyrin (Fig. 4), confirming our interpretations of the earlier data. Porphyrinogen **9a** is a reasonable substrate for URO-D but appears to be a comparatively poor substrate for CPO. This is surprising because meso'gen-VI (**1a**), and two related porphyrinogens **1b** and **1c** (Scheme 3), are much better substrates for CPO even though **9a** is structurally more closely related to the natural substrate copro'gen-III. Trace amounts (<1%) of a nonpolar porphyrin corresponding to divinyl product **26** appear to be generated in longer time incubations with human recombinant CPO (detected by TLC and HPLC), and the data indicate that the second oxidative decarboxylation step is virtually shut down for this substrate. This demonstrates that an acetate group is poorly tolerated at binding site X in the active site model (Fig. 1).

Kinetic studies were conducted in triplicate using URO-D and purified human CPO (Figs. 5 and 6). Incubations of URO-D with **9a** gave a maximum product formation of 49.5 ± 1.9% after

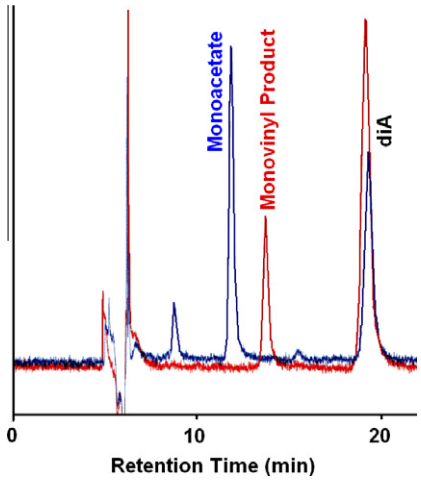


Figure 4. HPLC traces showing the porphyrin methyl ester products for a 30 min incubation of **9a** with purified recombinant human CPO (red line) and a 2 h incubation with URO-D (blue line). The URO-D fraction was isolated from CRH using porphyrin affinity chromatography.[67] The HPLC analysis was performed on a normal phase 5 μ partisil column (250 × 4.6 mm), using a 20 μL injection loop, with a mobile phase of 35/65 v/v ethyl acetate–cyclohexane. The UV–vis detector was set for a wavelength of 404 nm.

150 min (Fig. 5) while CPO produced a maximum product of 35.7 ± 1.2 after 30 min (Fig. 6). The metabolic conversions occurring for **9a** with the purified enzymes are summarized in Scheme 12. In both cases, two different side chains might be metabolized, but only the first conversion is favored. URO-D does appear to recognize and convert the first formed products **23**, but the second decarboxylation only occurs to a limited extent to form **25**.

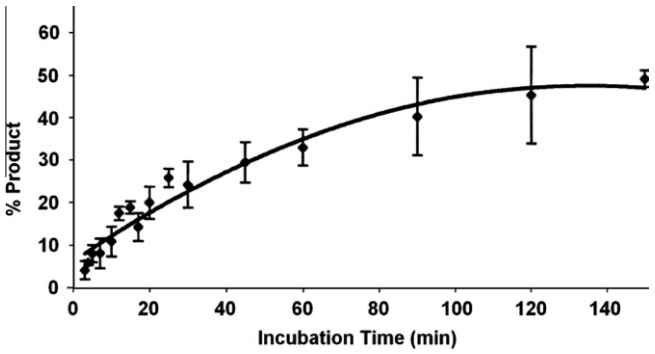


Figure 5. Kinetic study for incubations of **9a** with the CRH URO-D fraction showing % product formed with time in min.

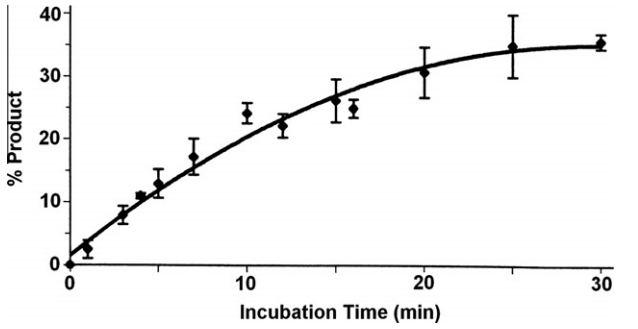
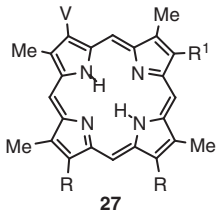


Figure 6. Kinetic study for incubations of **9a** with purified human CPO showing % product formed with time in min.

However, as noted above, virtually no conversion of **22a** to the divinyl product **26** by CPO is seen in these studies. Finally, a sample of the monovinylporphyrin **27a** (Fig. 7) derived from **22a** was isolated from incubations of **9a** with purified human CPO. Poor conversions were obtained in these preparative experiments but it was possible to obtain a low quality 400 MHz proton NMR spectrum for this product, and this allowed the presence of a vinyl moiety to be identified (Fig. 8). Electrospray ionization mass spectrometry gave an M+H peak at *m/z* 623. The high resolution



a. R = A^{Me}, R^1 = P^{Me}
b. R = B^{Me}, R^1 = P^{Me}
c. R = B^{Me}, R^1 = V

A^{Me} = CH_2CO_2Me
P^{Me} = $CH_2CH_2CO_2Me$
B^{Me} = $(CH_2)_3CO_2Me$
V = $CH=CH_2$

Figure 7. Porphyrin products isolated from incubations of porphyrinogens **9a** and **9b** with CPO.

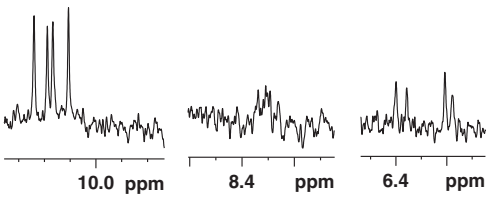
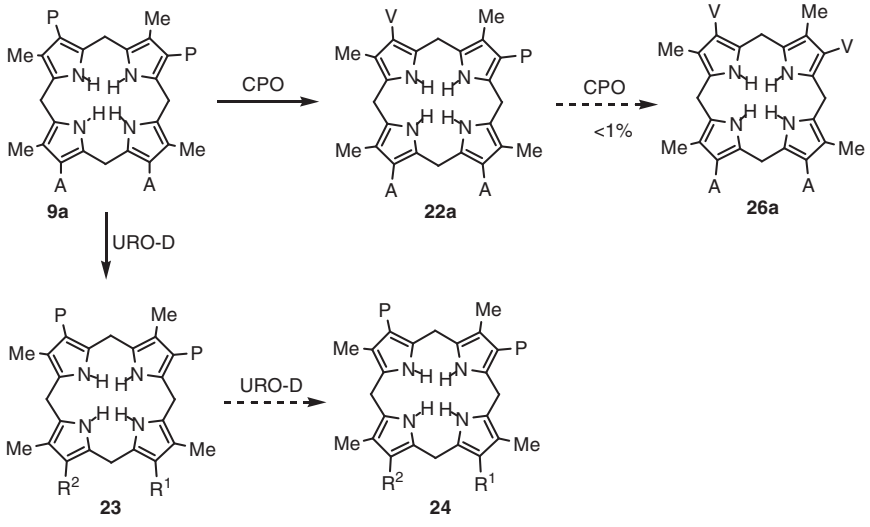


Figure 8. Partial 400 MHz proton NMR spectrum of the monovinylporphyrin product **27a** isolated from incubations of **9a** with purified recombinant human CPO. The figure shows the downfield region with four singlets for the *meso*-protons between 10.0 and 10.2 ppm and resonances for the vinyl moiety at 6.2, 6.4 and 8.3 ppm.

MS results gave a value of *m/z* 623.2870, which closely matches the expected value for $C_{36}H_{38}N_4O_6$+H.

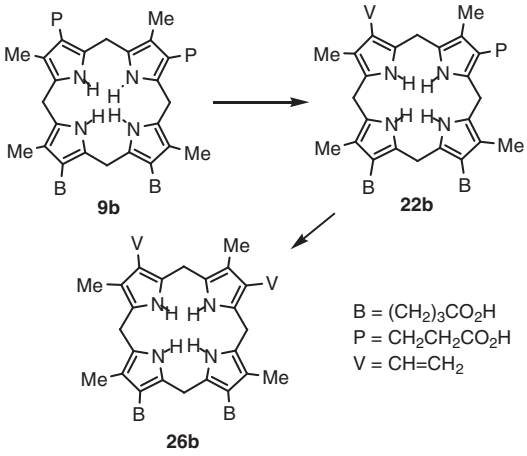
2.3. Action of CPO on the dibutyrate substrate 9b

The metabolism of the dibutyrate porphyrinogen **9b** was also investigated using CRH and purified recombinant human CPO. However, these studies were greatly simplified because **9b** is not affected by URO-D and even crude preparations of CRH only resulted in products due to the catalytic action of CPO. In the initial studies using CRH, it became clear that **9b** is also a mediocre substrate for CPO, although it gave better results than **9a**. However, porphyrinogen **9b** proved to be an excellent substrate for purified human CPO producing **22b** as the major metabolic product (Scheme 13). In the initial studies using CRH, a quasimolecular ion for the major porphyrin product was obtained using FAB MS



A = CH_2CO_2H; P = $CH_2CH_2CO_2H$; V = $CH=CH_2$

Scheme 12. Metabolism of porphyrinogen **9a** with partially purified CPO and Uro-D.



Scheme 13. Metabolism of porphyrinogen **9b** with CPO.

at *m/z* 679. This gave a high resolution peak at *m/z* 679.3499, which closely matches the calculated value for $C_{40}H_{46}N_4O_6$+H (*m/z* calcd 679.3496). A time course study for incubations of **9b** with CRH gave only ca. 20% metabolism after 30 min (Fig. 9), but incubations with purified recombinant human CPO showed much better conversions (Fig. 10). This shows the rapid generation of **22b** and the slow formation of the divinyl product **26b** (Scheme 13). The maximum% product formation (**22b** + **26b**) exceeded 80%, although about 5% conversion to **26b** was observed after 30 min. These results indicate that unlike the acetate units in **9a**, the butyrate side chains in **9b** are a good fit for the first oxidative decarboxylation by CPO. However, butyrate groups are not very effective in replacing the propionate residues in binding to site X, and this limits metabolism of the second propionate moiety.

Table 1 shows the kinetic constants calculated for **9a**, **9b** and the natural substrate copro'gen-III using human recombinant

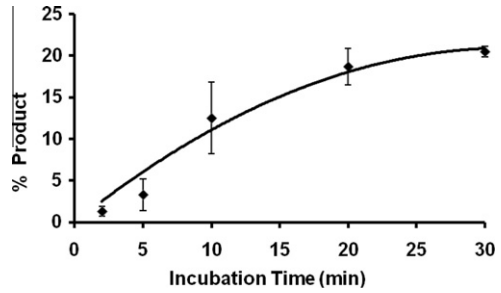


Figure 9. Kinetic study for incubations of **9b** with CRH showing % product **22b** (monovinyl) formed with time in min. No more than a trace of **26b** (divinyl) was formed in these experiments.

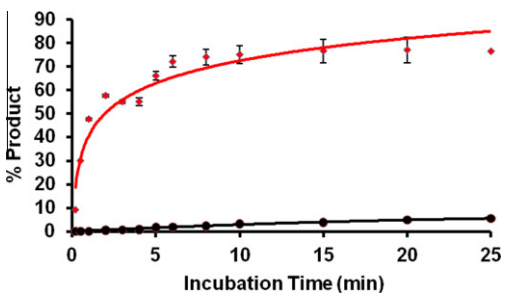


Figure 10. Kinetic study for incubations of **9b** with purified human CPO showing % products **22b** (monovinyl ♦--♦--♦) and **26b** (divinyl ●–●–●) formed with time.

Table 1
Kinetic values obtained with human recombinant CPO using three different substrates under initial velocity conditions

Substrate	K_m (mM)	K_{cat}	K_{cat}/K_m
Copro'gen-III[68a]	0.68	3.6	5.3
9b	0.76	0.90	1.2
9a	6.8	0.004	0.00059

CPO and porphyrinogen substrates at concentrations ranging from 0.1 to 10 μM obtained under initial velocity conditions. The K_m values using copro'gen-III and **9b** were essentially the same, while the value for **9a** was about 10 fold higher indicating that the diacetate substituents fit less well into the active site. The catalytic efficiencies (K_{cat}/K_m values) using the three substrates indicate that the authentic substrate is processed most efficiently and that **9b** is almost as well processed for the first catalytic step. However, **9a** showed a substantially increased K_m value and a reduced K_{cat}/K_m value, results that strongly suggest that the substrate fit of **9a** is compromised as well as the enzyme's ability to perform the oxidative decarboxylation reaction.

A preparative experiment was carried out by incubating **9b** with purified recombinant human CPO and the porphyrin products **27b** and **27c** (Fig. 8) were separated by flash chromatography. The major porphyrin product **27b** was characterized by 400 MHz proton NMR spectroscopy (Fig. 11). The spectrum showed four 1H singlets for the *meso*-protons between 10.1 and 10.3 ppm, and three 1H resonances for the vinyl group at 6.18, 6.38 and 8.32 ppm. The four porphyrin methyl groups and the three ester methoxy units gave rise to five 3H singlets and one 6H singlet between 3.6 and 3.8 ppm, and the remaining peaks were also consistent with the proposed structure **27b**. High resolution electron impact mass spectrometry gave a value for the molecular ion of *m/z* 678.3415, closely matching the expected value for $C_{40}H_{46}N_4O_6$ (*m/z* calcd 678.3417). A minor fraction corresponding to the divinylporphyrin **27c** was also isolated. It was possible to obtain a low quality proton NMR spectrum for **27c** in $CDCl_3$ that showed the presence of two vinyl moieties. In addition, high resolution electrospray ionization mass spectrometry gave a value for the M+H species of *m/z* 619.3303. This was a good match for the expected molecular formula $C_{38}H_{42}N_4O_6$+H (*m/z* calcd 619.3284).

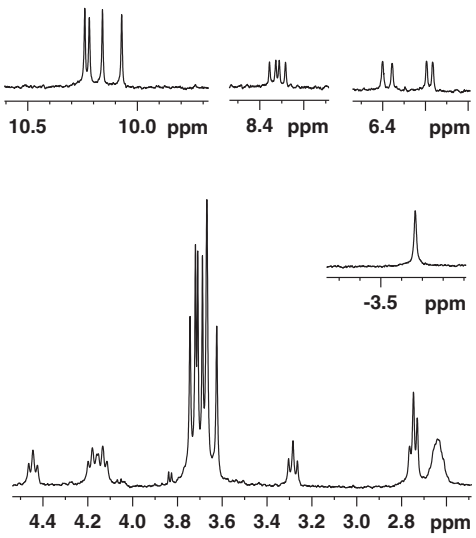


Figure 11. 400 MHz proton NMR spectrum of the monovinylporphyrin product isolated from incubations of **9b** with purified human CPO in $CDCl_3$.

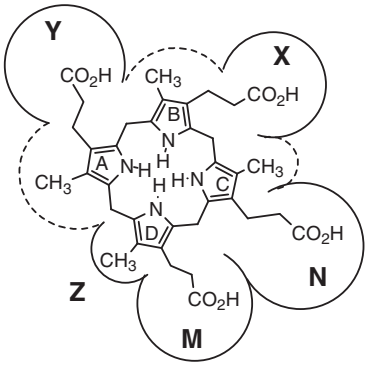


Figure 12. Modified model for the active site of CPO showing two new regions (M and N). These sites accommodate propionate, butyrate, methyl, ethyl and to a lesser extent propyl groups, but show less tolerance for acetate units.

3. Conclusions

Replacement of the C and D ring propionate residues of copro'gen-III with acetate groups greatly reduces substrate recognition and catalysis by either chicken or human CPO, and gives a comparatively low rate of conversion to a monovinylporphyrinogen. Although copro'gen-III undergoes two oxidative decarboxylations, the diacetate porphyrinogen **9a** gives no more than trace amounts of the divinyl product. A related dibutyrate **9b** was a much better substrate and was processed at a similar rate to the natural substrate copro'gen-III by human recombinant CPO. Incubations with CRH gave poorer conversions, possibly due in part to minor differences in the binding requirements for the avian and human enzymes. Porphyrinogen **9b** again gave mostly the monovinyl product, but in this case a more significant amount of divinyl product was generated, albeit with a maximum product conversion of less than 10%. Previously, porphyrinogens with alkyl groups (Me, Et, Pr or Bu) at positions 13 and 17 were shown to be substrates, affording the corresponding monovinyl porphyrinogens.[13,32] In those studies, the rates of conversions decreased as the alkyl group size increased from ethyl to propyl to butyl. This is probably not due to steric factors, as butyl groups are no larger than the propionate groups found in copro'gen-III, but this indicates that larger nonpolar groups cannot be tolerated at these positions. Figure 12 shows a modified model for the active site, designating M and N as the substrate binding sites that would accommodate the 13 and 17-substituents. The new data demonstrate that the larger butyrate group still fits into these sites very easily but the smaller acetate groups in **6a** do not. This suggests that these sites favor nonpolar chains close to the macrocycle, but a polar carboxylate species is needed for longer chains to be a good fit. This would make sense if the environment immediately next to the porphyrinogen was lipophilic while a more polar environment is present further into the pockets described by M and N. The second oxidative decarboxylation requires the substrate to be rotated through 90° so that the B ring propionate unit can be placed at the catalytic site Y.[13] In copro'gen-III, the C ring propionate binds to site X and facilitates further metabolism.[13] The results in this paper show that butyrate groups can still fit into site X, in accord with earlier results for *E. gracilis*,[34] but the unit considerably reduces conversion to the divinyl product. An acetate group at this position has an even larger negative effect and allows at best only trace amounts of divinyl porphyrinogen to be formed. These data demonstrate the substrate binding at position X shows the following preferences, although propionate groups are highly preferred.

$$CH_2CH_2CO_2H \gg (CH_2)_3CO_2H \gg CH_2CO_2H \text{ or alkyl groups}$$

These results further extend our understanding of the substrate recognition requirements for CPO and may help to solve the issue of how these substrates associate with this enzyme. In addition, the observation that diacetate **9a** can act as a substrate for URO-D, as well as for CPO, provides additional insights into the viability of abnormal pathways in heme biosynthesis.[44]

4. Experimental section

4.1. General methods

Ethyl bromoacetate, ethyl 4-bromobutanoate, 2,4-pentanedione, benzyl acetoacetate, diethyl malonate, benzyl alcohol, 30% HBr-acetic acid, copper(II) chloride, triethylamine, *N*,*N*-dimethylformamide and 10% palladium/charcoal were purchased from Aldrich or Acros, and were used without further purification. Triethyl orthoformate was distilled immediately prior to use. Chromatography was performed using grade 3 neutral alumina or 70–230 mesh silica gel. Melting points were determined in open capillary tubes using a Mel-Temp apparatus and are uncorrected. UV–Vis absorption spectra were run on a Varian Cary Spectrophotometer. Proton and carbon-13 NMR data were obtained on a Varian Gemini 400 MHz FT NMR spectrometer or a 500 MHz Bruker NMR Avance III spectrometer. Mass spectral determinations were conducted at the Mass Spectral Laboratory, School of Chemical Sciences, University of Illinois at Urbana-Champaign, and elemental analyses were obtained from the School of Chemical Sciences Microanalysis Laboratory at the University of Illinois.

4.2. Synthetic procedures

4.2.1. Benzyl 4-ethoxycarbonylmethyl-3,5-dimethylpyrrole-2-carboxylate (13a)

Ethyl bromoacetate (183.4 g, 1.09 mol) was added to a stirred mixture of 2,4-pentanedione (150.0 g, 1.50 mol), anhydrous potassium carbonate (200 g) and acetone (200 mL) in a 1 L three necked round bottom flask equipped with a reflux condenser and a mechanical stirrer. The resulting mixture was stirred under reflux for 1.5 h. The inorganic salts were removed by suction filtration and the solvent evaporated under reduced pressure. The residue was distilled to give ethyl 3-acetyl-4-oxopentanoate (**14**, 140.7 g, 0.756 mol, 69%) as a pale yellow oil, bp 158–160 °C at 0.05 torr.

A solution of sodium nitrite (106 g) in water (250 mL) was added dropwise to a solution of benzyl acetoacetate (172 g, 0.896 mol) in acetic acid (300 mL), maintaining the temperature of the reaction mixture below 15 °C with the aid of a salt-ice bath. The resulting solution was stirred at room temperature overnight affording a solution of the corresponding oxime. Ethyl ester **11** (140.7 g, 0.756 mmol) in acetic acid (300 mL) was heated to 70 °C, and the oxime solution was added dropwise while simultaneously adding a mixture of zinc dust (200 g) and sodium acetate (96 g), adjusting the rate of addition so that the reaction temperature remained between 80 and 90 °C. After the addition was complete, the mixture was heated for 1 h on a boiling water bath. The mixture was cooled to 70 °C and poured into 4 L of ice-water. The precipitate was collected by suction filtration, and then taken up in hot ethanol and filtered to remove zinc and other inorganic materials. The solvent was removed under reduced pressure and the residue recrystallized twice from ethanol to give the title pyrrole (78.10 g, 0.248 mol, 33%) as off-white crystals, mp 81.5–83 °C (lit. mp[70] 79–80 °C); IR (Nujol mull): ν 3322 (s, NH str.), 1734 (s, acetate C=O str.), 1668 (s, pyrrole-C=O str.); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.24 (3H, t, J = 7.2 Hz), 2.22 (3H, s), 2.30 (3H, s), 3.36 (2H, s), 4.11 (2H, q, J = 7.2 Hz), 7.30–7.42 (5H, m), 8.80 (1H, br s);

^{13}C NMR ($CDCl_3$): δ 10.9, 11.8, 14.4, 30.5, 60.8, 65.7, 115.1, 117.1, 128.26, 128.30, 128.4, 128.7, 131.3, 136.9, 161.5, 171.8.

4.2.2. Ethyl 4-ethoxycarbonylpropyl-3,5-dimethylpyrrole-2-carboxylate (18)

Ethyl 4-bromobutanoate (25.00 g, 0.128 mol) was added to 2,4-pentanedione (12.8 g, 0.128 mol), anhydrous potassium carbonate (17.8 g), sodium iodide (19.2 g) and butanone (400 mL), and the resulting mixture was heated under reflux for 16 h while stirring vigorously with the aid of a mechanical stirrer. The inorganic salts were removed by suction filtration and the solvent evaporated under reduced pressure. The residue was distilled to give ethyl 5-acetyl-6-oxoheptanoate (**16**, 20.85 g, 97.4 mmol, 76%) as a pale yellow liquid, bp 97–105 °C at 0.5 torr.

A mixture of the foregoing diketone **16** (20.85 g, 97.4 mmol) and diethyl aminomalonate[62] (17.0 g, 97 mmol) were added in a steady stream to gently refluxing acetic acid (55 mL). The resulting solution was stirred under reflux for 2.5 h. The mixture was cooled to 70 °C and poured into ice-water (500 mL). The precipitate was allowed to stand overnight, and then was suction filtered and recrystallized from ethanol-water to give pyrrole **18** (18.09 g, 64.4 mmol, 66%) as off-white crystals, mp 63–64 °C; IR (Nujol mull): ν 3304 (s, NH str.), 1729 (s, butyrate C=O str.), 1650 cm^{-1} (s, pyrrole-C=O str.); ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.24 (3H, t, J = 7.1 Hz), 1.34 (3H, t, J = 7.1 Hz), 1.75 (2H, quintet, J = 7.5 Hz), 2.19 (3H, s), 2.26 (3H, s), 2.28 (2H, t, J = 7.4 Hz), 2.40 (2H, t, J = 7.6 Hz), 4.11 (2H, q, J = 7.1 Hz), 4.29 (2H, q, J = 7.1 Hz), 8.80 (1H, br s); ^{13}C NMR ($CDCl_3$): δ 10.8, 11.6, 14.4, 14.8, 23.5, 26.0, 33.8, 59.8, 60.4, 117.1, 121.1, 127.3, 130.0, 162.0, 173.8. Anal. Calcd for $C_{15}H_{23}NO_4$: C, 64.04; H, 8.24; N, 4.98. Found: C, 64.27; H, 7.91; N, 5.01.

4.2.3. Benzyl 4-benzyloxycarbonylpropyl-3,5-dimethylpyrrole-2-carboxylate (19)

A solution of sodium benzyloxide was prepared by reacting 0.20 g of sodium with 20 mL of benzyl alcohol. Diethyl ester **18** (42.15 g, 0.150 mol) and benzyl alcohol (90 mL) were placed in a 500 mL Erlenmeyer flask clamped in an oil bath. A thermometer was positioned ca. 5 cm above the surface of the solution and the temperature was gradually raised from room temperature to 240 °C over a period of 90 min while intermittently adding small portions of the sodium benzyloxide solution. When the vapor temperature reached 200 °C, a final portion of sodium benzyloxide was added and the mixture was stirred for an additional 5 min. The hot solution was poured into a stirred ice cooled mixture of methanol (240 mL), water (165 mL) and acetic acid (3 mL). The resulting precipitate was suction filtered and recrystallized from ethanol to give the dibenzyl ester (52.75 g, 0.130 mol, 87%) as off-white crystals, mp 64–65 °C; IR (Nujol mull): ν 3302 (s, NH str.), 1722 (s, butyrate C=O str.), 1658 cm^{-1} (s, pyrrole-C=O str.); ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.78 (2H, quintet, J = 7.5 Hz), 2.15 (3H, s), 2.26 (3H, s), 2.35 (2H, t, J = 7.4 Hz), 2.40 (2H, t, J = 7.5 Hz), 5.10 (2H, s), 5.29 (2H, s), 7.31–7.43 (10H, m), 8.64 (1H, br s); ^{13}C NMR ($CDCl_3$): δ 10.7, 11.5, 23.2, 25.7, 33.6, 65.4, 66.2, 116.5, 121.1, 128.0, 128.1, 128.2, 128.3, 128.5, 128.6, 130.1, 136.0, 136.6, 161.2, 173.4. Anal. Calcd for $C_{25}H_{27}NO_4 \cdot {}^1/_4H_2O$: C, 73.24; H, 6.76; N, 3.42. Found: C, 73.17; H, 6.57; N, 3.38.

4.2.4. Benzyl 4-methoxycarbonylpropyl-3,5-dimethylpyrrole-2-carboxylate (13b)

Conc. sulfuric acid (20 mL) was cautiously added to methanol (400 mL), dibenzyl ester **19** (50.0 g, 0.123 mol) was added, and the resulting mixture was stirred for 16 h at room temperature. The solution was diluted with chloroform, washed with water, 5% aqueous sodium bicarbonate, and water, and then dried over sodium sulfate. The drying agent was removed by suction filtration, the solvent evaporated, and the oily residue crystallized from 90% ethanol–water to give the mixed ester pyrrole (36.56 g, 0.111 mol, 90%) as white crystals, mp 66.5–67.5 °C (lit. mp[34] 66.5–67.5 °C); IR (Nujol mull): ν 3319 (s, NH str.), 1729 (s, butyrate C=O str.), 1666 cm^{-1} (s, pyrrole-C=O str.); ^{1}H NMR (500 MHz, $CDCl_3$): δ 176 (2H, quintet, J = 7.5 Hz), 2.18 (3H, s), 2.28 (3H, s), 2.30 (2H, t, J = 7.4 Hz), 2.40 (2H, t, J = 7.5 Hz), 3.66 (3H, s), 5.30 (2H, s), 7.30–7.43 (5H, m), 8.84 (1H, br s); ^{13}C NMR ($CDCl_3$): δ 10.9, 11.6, 23.4, 25.9, 33.5, 51.6, 65.6, 116.7, 121.2, 127.8, 128.18, 128.20, 128.7, 130.4, 136.8, 161.5, 174.2. Anal. Calcd for $C_{19}H_{23}NO_4$: C, 69.28; H, 7.04; N, 4.25. Found: C, 69.17; H, 6.55; N, 4.18.

4.2.5. 4-Ethoxycarbonylmethyl-3,5-dimethylpyrrole-2-carboxylic acid (20a)

Benzyl ester **13a** (10.00 g, 31.7 mmol) was placed in a hydrogenation vessel and dissolved in methanol (200 mL). Triethylamine (20 drops) was then added, the air was displaced by a stream of nitrogen, and 250 mg of 10% palladium-charcoal was added. The mixture was shaken at room temperature under an atmosphere of nitrogen at 30 psi for 16 h. The catalyst was removed by suction filtration, and the solvent removed under reduced pressure. The residue was taken up in 5% aqueous ammonia, cooled in a salt ice bath, and then neutralized with dilute hydrochloric acid while maintaining the temperature <10 °C. The resulting precipitate was suction filtered, washed well with water, and dried in a vacuum desicator overnight. The pyrrole carboxylic acid (6.35 g, 28.2 mmol, 89%) was isolated as a pink powder, mp 137 °C, dec; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.14 (3H, t, J = 7.2 Hz), 2.09 (3H, s), 2.13 (3H, s), 3.30 (2H, s), 4.01 (2H, q, J = 7.2 Hz), 11.05 (1H, br s), 11.84 (1H, br s); ^{13}C NMR (DMSO-d_6): δ 11.0, 11.5, 14.8, 30.4, 60.6, 114.4, 117.4, 126.7, 131.5, 162.9, 172.0. Anal. Calcd for $C_{11}H_{15}NO_4$: C, 58.66; H, 6.71; N, 6.22. Found: C, 58.89; H, 6.94; N, 6.12.

4.2.6. 4-Methoxycarbonylpropyl-3,5-dimethylpyrrole-2-carboxylic acid (20b)

Pyrrole benzyl ester **10b** (10.00 g, 30.4 mmol) was hydrogenolyzed using the foregoing procedure. The pyrrole carboxylic acid (6.93 g, 29.0 mmol, 95%) was isolated as a light red powder, mp 124–125 °C, dec; ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.59 (2H, quintet, J = 7.5 Hz), 2.07 (3H, s), 2.13 (3H, s), 2.23–2.29 (4H, two overlapping triplets), 3.56 (3H, s), 10.91 (1H, s), 11.73 (1H, br s); ^{13}C NMR (DMSO-d_6): δ 10.5, 10.9, 22.9, 25.7, 32.8, 51.3, 116.6, 119.9, 125.5, 129.9, 162.5, 173.5. Anal. Calcd for $C_{12}H_{17}NO_4$: C, 60.24; H, 7.16; N, 5.85. Found: C, 60.42; H, 6.67; N, 5.88.

4.2.7. 4-Ethoxycarbonylmethyl-3,5-dimethylpyrrole-2-carbaldehyde (12a)

Pyrrole carboxylic acid **20a** (1.40 g, 6.22 mmol) was stirred with TFA (5 mL) at room temperature for 10 min. The solution was cooled in an ice bath, freshly distilled triethyl orthoformate (3 mL) was added dropwise, and the mixture was stirred at 40 °C for a further 10 min. The mixture was then poured into water (50 mL) and extracted with chloroform (3 x 50 mL). The combined organic solutions were washed with 5% aqueous ammonia (50 mL) and water (50 mL), and dried over magnesium sulfate. The drying agent was removed by suction filtration, the solvent evaporated, and the residue recrystallized from ethanol to give the pyrrole aldehyde (0.920 g, 4.40 mmol, 71%) as pale green crystals, mp 114–115 °C (lit. mp[71] 115–117 °C); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.25 (3H, t, J = 7.2 Hz), 2.279 (3H, s), 2.284 (3H, s), 3.37 (2H, s), 4.13 (2H, q, J = 7.2 Hz), 9.48 (1H, s), 9.67 (1H, br s); ^{13}C NMR ($CDCl_3$): δ 9.0, 11.9, 14.4, 30.2, 61.0, 116.0, 128.3, 132.6, 136.3, 171.5, 176.3.

4.2.8. 4-Methoxycarbonylpropyl-3,5-dimethylpyrrole-2-carbaldehyde (12b)

Pyrrole carboxylic acid **20b** (2.00 g, 8.37 mmol) was reacted under the foregoing conditions. Recrystallization from chloroform–hexanes gave the aldehyde[72] (1.44 g, 6.46 mmol, 77%) as pale green needles, mp 64–65 °C. Further recrystallization from chloroform–hexanes gave an analytical sample as small fluffy off-white needles, mp 64–64.5 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.77 (2H, quintet, J = 7.5 Hz), 2.24 (3H, s), 2.26 (3H, s), 2.31 (2H, t, J = 7.4 Hz), 2.41 (2H, t, J = 7.5 Hz), 3.66 (3H, s), 9.27 (1H, br s), 9.47 (1H, s); ^{13}C NMR ($CDCl_3$): δ 8.9, 11.8, 23.3, 25.7, 33.5, 51.6, 122.1, 128.3, 132.3, 135.6, 174.0, 176.0. Anal. Calcd for $C_{12}H_{17}NO_3$: C, 64.55; H, 7.67; N, 6.27. Found: C, 64.35; H, 7.88; N, 6.33.

4.2.9. 2,18-Bis(ethoxycarbonylmethyl)-8,13-bis(2-methoxycarbonylethyl)-1,3,7,12,17,19-hexamethyl-20,23-dihydrobilin dihydrobromide (21a)

Dipyrrylmethane carboxylic acid **11**[13] (200.0 mg, 0.408 mmol) was stirred under nitrogen with trifluoroacetic acid (1 mL) for 10 min. A solution of pyrrole aldehyde **12a** (170.6 mg, 0.816 mmol) in methanol (3.6 mL) was added, followed immediately by the addition of 30% HBr-acetic acid (0.7 mL), and the mixture was stirred for 30 min. Ether (15 mL) was added dropwise and the mixture was stirred for a further 2 h. The precipitate was filtered off, washed thoroughly with ether and dried in vacuo overnight to give the title a,c-biladiene dihydrobromide (269 mg, 0.302 mmol, 74%) as an orange-red powder, mp 185–186 °C; UV–vis ($CHCl_3$): λ_{max} ($\log_{10}\varepsilon$) 368 (4.22), 457 (4.43), 523 nm (5.34); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.26 (6H, t, J = 7.1 Hz), 1.92 (2H, t, J = 7.9 Hz), 1.96 (3H, s), 2.26 (3H, s), 2.33 (3H, s), 2.38 (3H, s), 2.50 (2H, t, J = 7.0 Hz), 2.72 (6H, s), 2.79 (2H, t, J = 7.9 Hz), 2.95 (2H, t, J = 7.1 Hz), 3.41 (3H, s), 3.44 (2H, s), 3.45 (2H, s), 3.59 (3H, s), 4.14 (4H, q, J = 7.1 Hz), 5.25 (2H, s), 7.16 (1H, s), 7.40 (1H, s), 13.35 (1H, br s), 13.36 (1H, br s), 13.48 (1H, br s), 13.52 (1H, br s); ^{13}C NMR ($CDCl_3$): δ 9.5, 10.2, 10.58, 10.63, 13.3, 14.4, 19.8, 20.0, 26.0, 30.3, 33.9, 34.6, 51.5, 52.0, 61.5, 120.6, 121.7, 122.5, 122.6, 125.4, 125.9, 126.1, 127.1, 127.5, 128.6, 143.3, 144.56, 144.64, 145.4, 149.2, 149.9, 156.7, 157.2, 170.0, 172.78, 178.84. Anal. Calcd for $C_{41}H_{54}N_4O_8Br_2$: C, 55.29; H, 6.11; N, 6.29. Found: C, 55.56; H, 6.25; N, 6.22.

4.2.10. 8,13-Bis(2-methoxycarbonylethyl)-2,18-bis(3-methoxycarbonylpropyl)-1,3,7,12,17,19-hexamethyl-20,23-dihydrobilin dihydrobromide (21b)

Dipyrrylmethane **11**[13] (200.0 mg, 0.408 mmol) was stirred under nitrogen with trifluoroacetic acid (1 mL) for 10 min. A solution of pyrrole aldehyde **12b** (182 mg, 0.816 mmol) in methanol (3.6 mL) was added, followed immediately by the addition of 30% HBr-acetic acid (0.7 mL), and the mixture was stirred for 30 min. Ether (15 mL) was added dropwise and the mixture was stirred for a further 2 h. The precipitate was filtered off, washed thoroughly with ether and dried in vacuo overnight to give the title a,c-biladiene dihydrobromide (295 mg, 0.321 mmol, 78%) as a red powder, mp 197–198 °C; UV–vis ($CHCl_3$): λ_{max} ($\log_{10}\varepsilon$) 368 (4.19), 458 (4.44), 525 nm (5.32); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.78 (4H, quintet, J = 7.4 Hz), 1.92 (2H, t, J = 7.9 Hz), 1.96 (3H, s), 2.24 (3H, s), 2.30 (3H, s), 2.35 (3H, s), 2.32–2.37 (4H, m), 2.46–2.51 (6H, m), 2.71 (6H, s), 2.78 (2H, t, J = 7.9 Hz), 2.94 (2H, t, J = 7.1 Hz), 3.42 (3H, s), 3.60 (3H, s), 3.683 (3H, s), 3.686 (3H, s), 5.22 (2H, s), 7.10 (1H, s), 7.33 (1H, s), 13.27 (2H, br s), 13.36 (1H, br s), 13.40 (1H, br s); ^{13}C NMR ($CDCl_3$): δ 9.5, 10.2, 10.38, 10.40, 13.3, 19.3, 20.0, 23.5, 25.0, 26.0, 33.4, 33.9, 34.7, 51.5, 51.8, 52.0, 120.0, 121.0, 125.1, 125.5, 125.8, 127.4, 127.9, 128.3, 129.2, 129.3, 142.6, 143.6, 144.0, 144.4, 148.5, 149.1, 157.0, 157.5, 172.9, 173.0, 173.6. Anal. Calcd for $C_{43}H_{58}N_4O_8Br_2{\cdot}H_2O$: C, 55.10; H, 6.52; N, 5.98. Found: C, 55.04; H, 6.48; N, 5.95.

4.2.11. 3,8-Bis(2-methoxycarbonylethyl)-13,17-bis(methoxycarbonylmethyl)-2,7,12,18-tetramethylporphyrin (10a)

a,c-Biladiene dihydrobromide **21a** (250 mg, 0.281 mmol) was added to a stirred solution of copper(II) chloride (0.74 g) in DMF (95 mL), and the resulting mixture was stirred in the dark for 2 h. The dark red solution was diluted with dichloromethane (100 mL) and washed with water (3 x 100 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 any remaining DMF. The solid residue was taken up in 15% v/v sulfuric acid–TFA (35 mL) and stirred in the dark at room temperature for 45 min. The reaction mixture was diluted with dichloromethane (150 mL), and then washed with water (2 x 100 mL) and 5% aqueous sodium bicarbonate solution (100 mL). The aqueous layers were back extracted with dichloromethane and the combined organic layers were dried over sodium sulfate and the solvent was evaporated under reduced pressure. The porphyrin was then reesterified by dissolving the residue in 5% sulfuric acid–methanol (35 mL) and stirring the mixture in the dark overnight. The mixture was diluted with dichloromethane, washed with water and then with 5% aqueous sodium bicarbonate solution. The aqueous layers were back extracted with dichloromethane at each stage, and the combined organic layers were dried over sodium sulfate and the solvent was evaporated under reduced pressure. The residue was chromatographed on a grade 3 alumina column, eluting with dichloromethane. A dark violet product fraction was collected and the solvent removed under reduced pressure. The residue was recrystallized from chloroform–methanol to give the title porphyrin (121 mg, 0.177 mmol, 63%) as a maroon solid, mp 209–210 °C; UV–vis (1% Et_3N–$CHCl_3$): λ_{max} ($\log_{10}\varepsilon$) 401 (5.27), 499 (4.20), 533 (4.02), 569 (3.88), 623 nm (3.73); UV–vis (1% TFA–$CHCl_3$): λ_{max} ($\log_{10}\varepsilon$) 408 (5.57), 552 (4.19), 594 nm (3.82); ^{1}H NMR (400 MHz, $CDCl_3$): δ −3.75 (2H, br s), 3.27 (4H, t, J = 7.8 Hz), 3.63 (3H, s), 3.65 (3H, s), 3.66 (3H, s), 3.682 (3H, s), 3.685 (3H, s), 3.691 (3H, s), 3.769 (3H, s), 3.774 (3H, s), 4.37–4.43 (4H, m), 5.07 (2H, s), 5.08 (2H, s), 10.05 (1H, s), 10.09 (2H, s), 10.14 (1H, s); ^{1}H NMR (400 MHz, TFA–$CDCl_3$): δ −3.42 (4H, br s), 3.14–3.18 (4H, 2 overlapping triplets), 3.67 (3H, s), 3.68 (6H, s), 3.69 (6H, s), 3.696 (3H, s), 3.698 (3H, s), 3.71 (3H, s), 4.47 (4H, t, J = 7.6 Hz), 5.19 (4H, s), 10.70 (1H, s), 10.83 (1H, s), 10.85 (2H, s); ^{13}C NMR (TFA–$CDCl_3$): δ 12.10, 12.13, 12.32, 12.34, 21.9, 32.9, 35.67, 35.70, 52.9, 53.5, 99.6, 99.68, 99.75, 99.9, 133.6, 133.7, 139.4, 139.8, 140.5, 140.8, 141.9, 142.1, 142.5, 142.7, 171.7, 174.9. HRMS (EI), m/z Calcd for $C_{38}H_{42}N_4O_8$: 682.3002. Found: 682.3013. Anal. Calcd for $C_{38}H_{42}N_4O_8$: C, 66.85; H, 6.20; N, 8.20. Found: C, 66.61; H, 6.12; N, 8.09.

4.2.12. 3,8-Bis(2-methoxycarbonylethyl)-13,17-bis(methoxycarbonylpropyl)-2,7,12,18-tetramethylporphyrin (10b)

Using the previous procedure, a,c-biladiene **21b** (258 mg, 0.281 mmol) was cyclized with $CuCl_2$ (0.74 g), demetalated and reesterified. Chromatography on a grade 3 alumina column, eluting with dichloromethane, gave a dark violet band. Recrystallization from chloroform–methanol gave the dibutyrate (142 mg, 0.192 mmol, 68%) as a maroon solid, mp 140.5–141.5 °C (lit. mp[56] 138–140 °C); UV–vis (1% Et_3N-$CHCl_3$): λ_{max} ($\log_{10}\varepsilon$) 400 (5.25), 499 (4.14), 533 (3.98), 567 (3.81), 621 nm (3.68); UV-vis (1% TFA–$CHCl_3$): λ_{max} ($\log_{10}\varepsilon$) 407 (5.56), 551 (4.21), 592 nm (3.88); ^{1}H NMR (400 MHz, $CDCl_3$): δ −3.76 (2H, br s), 2.64 (4H, quintet, J = 7.0 Hz), 2.72–2.77 (H, m), 3.25–3.30 (4H, 2 overlapping triplets), 3.63 (3H, s), 3.645 (3H, s), 3.649 (3H, s), 3.66 (3H, s), 3.68 (3H, s), 3.70 (3H, s), 3.71 (3H, s), 3.72 (3H, s), 4.15 (2H, t, J = 7.6 Hz), 4.39–4.45 (4H, 2 overlapping triplets), 10.08 (2H, s), 10.09 (1H, s), 10.22 (1H, s); ^{1}H NMR (400 MHz, TFA–$CDCl_3$): δ −3.93 (4H, br s), 2.40–2.46 (4H, m), 2.70–2.76 (4H, 2 overlapping triplets), 3.18

(4H, t, J = 7.6 Hz), 3.67 (3H, s), 3.688 (3H, s), 3.694 (3H, s), 3.70 (3H, s), 3.72 (3H, s), 3.73 (3H, s), 3.78 (3H, s), 3.79 (3H, s), 4.21 (4H, t, J = 8.0 Hz), 4.50 (4H, t, J = 8.0 Hz), 10.72 (1H, s), 10.83 (1H, s), 10.84 (1H, s), 10.95 (1H, s); ^{13}C NMR (TFA–$CDCl_3$): δ 11.89, 11.94, 11.98, 12.04, 21.9, 26.0, 27.2, 33.97, 34.00, 35.77, 52.9, 53.2, 99.2, 99.47, 99.52, 139.4, 139.58, 139.64, 140.0, 140.3, 140.4, 141.4, 141.8, 142.2, 142.3, 142.4, 142.8, 175.9, 176.9. HRMS (EI), m/z Calcd for $C_{42}H_{50}N_4O_8$: 738.3628. Found: 738.3626.

4.3. Enzyme assays

4.3.1. Enzyme incubation studies using chicken red cell hemolysates (CRH)

Enzyme incubations and analyses of metabolic products were carried out as described elsewhere. For kinetic studies, the incubation products were isolated using a previously developed microassay technique[69] and analyzed by TLC and/or HPLC. Incubations of the diacetate porphyrinogen were carried out in the presence or absence of *N*-ethylmaleimide, which is reported to be an inhibitor for Uro-D.[66] The HPLC analyses were performed using normal phase columns (5 m partisil silica, Alltech) eluting with appropriate ratios of ethyl acetate and cyclohexane. Kinetic data are reported as mean ± standard deviation for three replicate experiments and compared statistically using analysis of variance (ANOVA) following Fisher's LSD post Test. Values are considered different at $p < 0.05$.

4.3.2. Separation of CPO and URO-D from CRH

Uro-D and CPO were separated using porphyrin-affinity chromatography following a published procedure.[67]

4.3.3. Cloned human CPO

Purified human recombinant CPO from *E. coli* was isolated following the procedure of Medlock and Daily.[48]

4.4. Porphyrins isolated from preparative enzymic studies

These metabolites were obtained by reducing the tetracarboxylic acids derived from **10a** and **10b**, respectively, with 3% sodium amalgam and incubating the resulting porphyrinogens with human recombinant CPO. The products were purified as their methyl esters by flash chromatography, eluting with 10% ethyl acetate–toluene, and characterized by EI and/or ESI MS, and 400 MHz proton NMR spectroscopy. The diacetate porphyrinogen **9a** gave monovinylporphyrin **27a** and only trace amounts of a divinyl species. Dibutyrate **6b** also gave mostly the monovinylporphyrin **27b** but sufficient divinyl derivative **27c** could be isolated in this case to obtain a low quality proton NMR spectrum as well as MS data.

4.4.1. 8-(2-Methoxycarbonylethyl)-13,17-bis(methoxycarbonylmethyl)-2,7,12,18-tetramethyl-3-vinylporphyrin (27a)

^{1}H NMR (400 MHz, $CDCl_3$): δ −3.60 (2H, br s), 3.28 (2H, t, J = 8.1 Hz), 3.66 (3H, s), 3.678 (3H, s), 3.684 (3H, s), 3.71 (3H, s), 3.74 (3H, s), 3.768 (3H, s), 3.775 (3H, s), 4.1–4.2 (2H, obscured by impurity peaks), 5.08 (2H, s), 5.12 (2H, s), 6.19 (1H, d, J = 11.7 Hz), 6.38 (1H, d, J = 16.9 Hz), 8.26–8.34 (1H, m), 10.11 (1H, s), 10.17 (1H, s), 10.19 (1H, s), 10.24 (1H, s). HRMS (ESI), m/z Calcd for $C_{36}H_{38}N_4O_6$+H: 623.2870. Found: 623.2870.

4.4.2. 8-(2-Methoxycarbonylethyl)-13,17-bis(3-methoxycarbonylpropyl)-2,7,12,18-tetramethyl-3-vinylporphyrin (27b)

^{1}H NMR (400 MHz, $CDCl_3$): δ −3.66 (2H, br s), 2.60–2.68 (4H, m), 2.75 (4H, t, J = 7.4 Hz), 3.28 (2H, t, J = 7.6 Hz), 3.62 (3H, s), 3.67 (6H, s), 3.69 (3H, s), 3.71 (3H, s), 3.72 (3H, s), 3.74 (3H, s), 4.11–4.20 (4H, m), 4.44 (2H, t, J = 8.0 Hz), 6.18 (1H, dd, J = 1.4, 11.5 Hz), 6.38 (1H, d, J = 1.4, 17.8 Hz), 8.32 (1H, dd, J = 11.5, 17.8 Hz), 10.11 (1H, s), 10.17 (1H, s), 10.19 (1H, s), 10.24 (1H, s). HRMS (ESI), m/z Calcd for $C_{40}H_{46}N_4O_6$+H: 679.3496. Found: 679.3486. HRMS (EI), m/z Calcd for $C_{40}H_{46}N_4O_6$: 678.3417. Found: 678.3415.

4.4.3. 13,17-Bis(3-methoxycarbonylpropyl)-2,7,12,18-tetramethyl-3,8-divinylporphyrin (27c)

^{1}H NMR (400 MHz, $CDCl_3$; downfield region only): δ 6.16–6.22 (2H, m), 6.35–6.42 (2H, m), 8.26–8.36 (2H, m), 10.14 (1H, s), 10.20 (1H, s), 10.22 (1H, s), 10.29 (1H, s). HRMS (ESI), m/z Calcd for $C_{38}H_{42}N_4O_6$+H: 619.3284. Found: 619.3303.

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