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# Metabolism of Analogues of Coproporphyrinogen-III with Modified Side Chains: Implications for Binding at the Active Site of Coproporphyrinogen Oxidase

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**Abstract**—Porphyrinogens with modified propionate side chains bearing methyl substituents were found to be modest substrates for coproporphyrinogen oxidase; the results indicate that alteration of the substituents involved in secondary binding interactions has a comparable affect to modifying the side chain that undergoes degradation at the catalytic site. 

Coproporphyrinogen oxidase remains one of the most poorly understood enzymes involved in primary metabolism.[1] Coproporphyrinogen-III, the natural substrate for coproporphyrinogen oxidase, undergoes sequential oxidative decarboxylations at the A and B rings to afford protoporphyrinogen-IX, a precursor to heme, via a unique tricarboxylate intermediate harderoporphyrinogen (Scheme 1).[1–4] Although the sequence of degradative steps, and the overall stereochemistry of this process, have been known for many years the mechanism for the oxidative decarboxylation remains unknown.[4] For aerobic organisms, oxygen is required for enzymic activity, but otherwise no cofactors have been identified.[4] Furthermore, the enzyme does not appear to incorporate any metal ions that could be involved in redox chemistry.[5] Although the intermediacy of hydroxypropionate intermediates (e.g., porphyrinogen **1**) has been postulated,[6] this possibility has been questioned by others.[7]

Although copro'gen oxidase selectively acts on two of the four propionate residues present in the natural substrate,[1] it is capable of metabolizing a number of synthetic substrates including copro'gen-IV and meso'gen-VI (Chart 1).[4,8] The results from a series of substrate analogue studies have been used to generate a model for

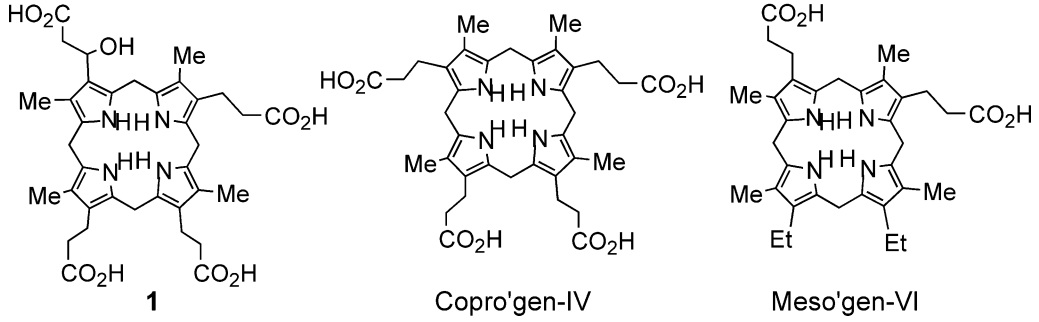


**Chart 1.**

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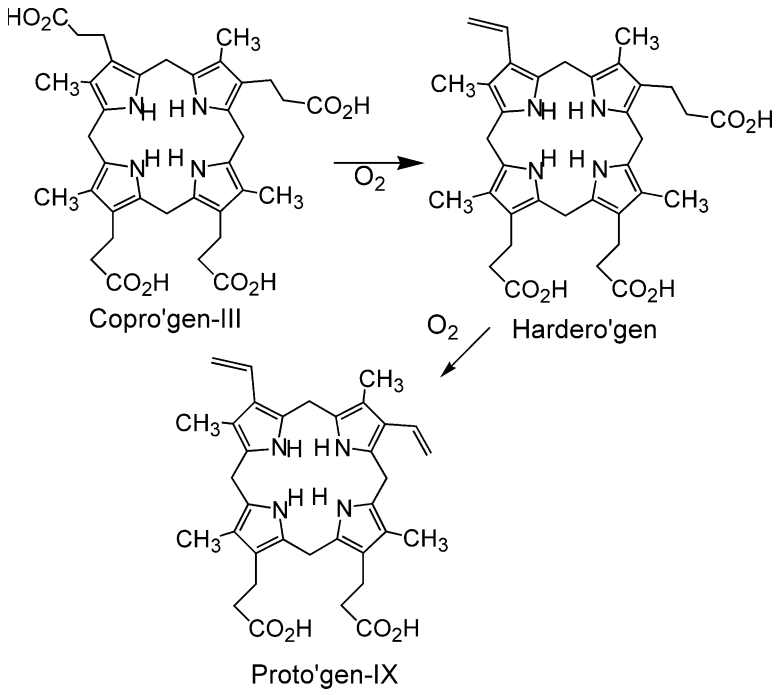



**Scheme 1.** Metabolism of coproporphyrinogen-III.

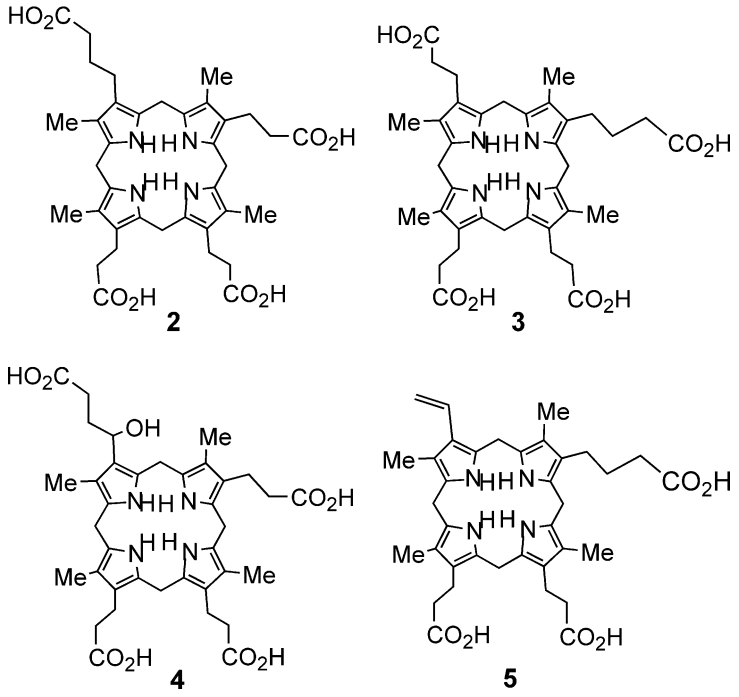
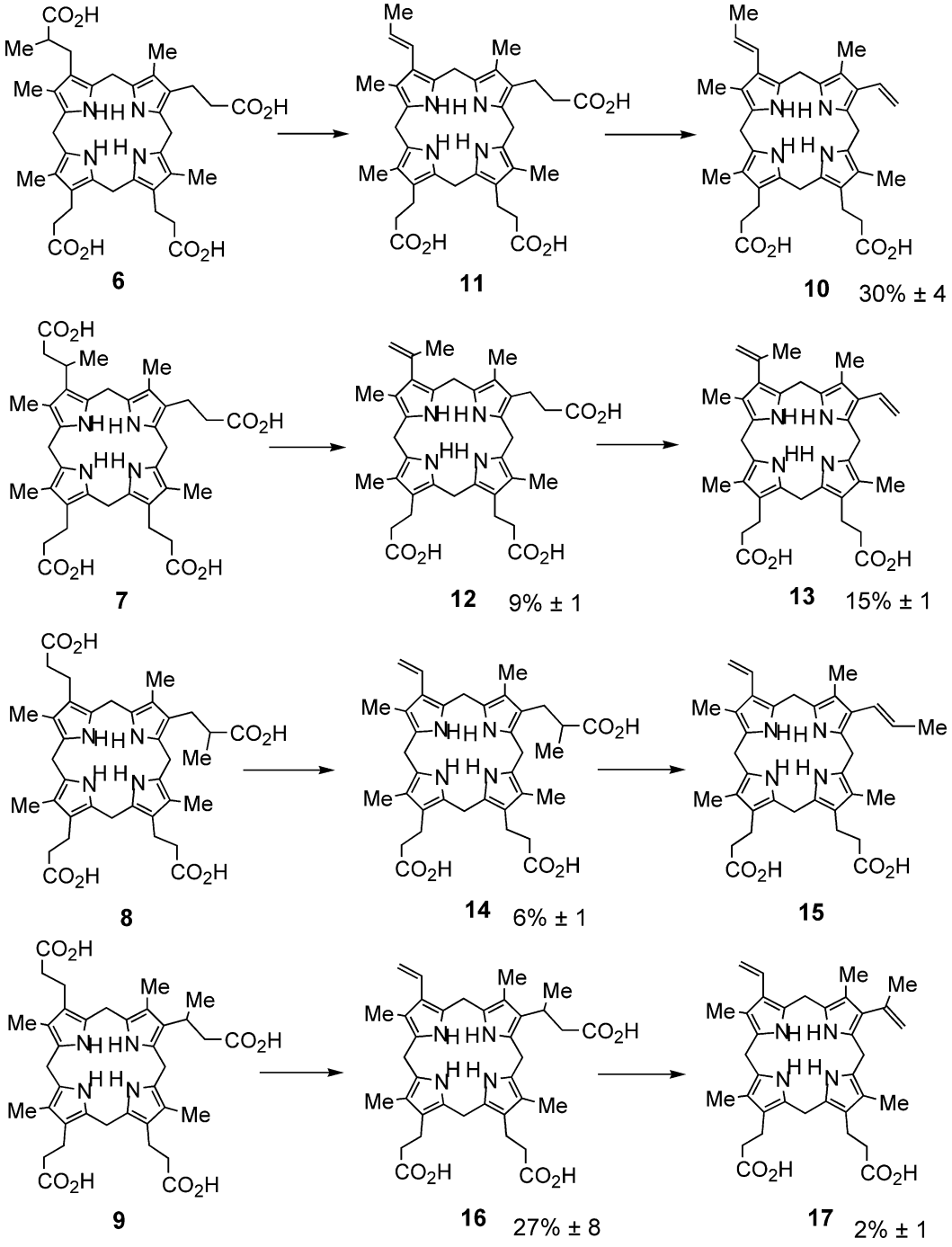



**Chart 2.**

the active site of copro'gen oxidase (Fig. 1).[4,8] In this model, three important regions are identified: region X is implicated in binding to a propionic acid side chain; region Y is the catalytic site where oxidative decarboxylation of a different propionate unit occurs; and region Z has steric constraints where H, Me, vinyl, or Et can be accommodated, but not a more bulky propionate group. In essence, the porphyrinogen must bear a peripheral sequence of substituents R Me-P Me-P where R = H, Me, V, or Et and P = $CH_2CH_2CO_2H$.

In an earlier investigation, Battersby and co-workers investigated the metabolism of copro'gen analogues **2** and **3** (Chart 2) with a four-carbon butyrate unit replacing either the A or B ring propionate moieties.[9] The modifed side chain could not give rise to a vinyl unit but it was speculated that this might undergo oxidation without decarboxylation to afford hydroxybutyrate products (e.g., **4**).[9] However, incubations of **2** with preparations of copro'gen oxidase derived from *Euglena gracilis* failed to give any new products.[9] The B ring




**Scheme 2.**

modified analogue **3** was a poor substrate for copro'gen oxidase and afforded the hardero'gen analogue **5**, although the butyrate side chain was again left untouched in these studies.[9] This result demonstrated that the butyrate unit can participate in binding to region X in the *E. gracilis* derived enzyme, but butyrate cannot be recognized at the catalytic site Y.

We speculated that porphyrinogens with methyl substituted propionate side chains (**6**–**9**; Scheme 2) might have a better chance of being metabolized by copro'gen oxidase and possibly producing products, such as hydroxylated species, that could offer insights into the mechanism of oxidative decarboxylation. With this in mind, the corresponding porphyrin methyl esters for **6**–**9** and the butyrate analogues **2** and **3** were synthesized via *a,c*-biladiene intermediates.[4,10] The biologically active porphyrinogen carboxylic acids are unstable and are generated as needed from the porphyrin esters by treatment with 25% HCl (to cleave the methyl esters) and reduction with 3% sodium amalgam in an aqueous buffer.[4] Chicken red cell hemolysates (CRH) were used as crude enzyme preparations and copro'gen oxidase activity was demonstrated in control experiments using

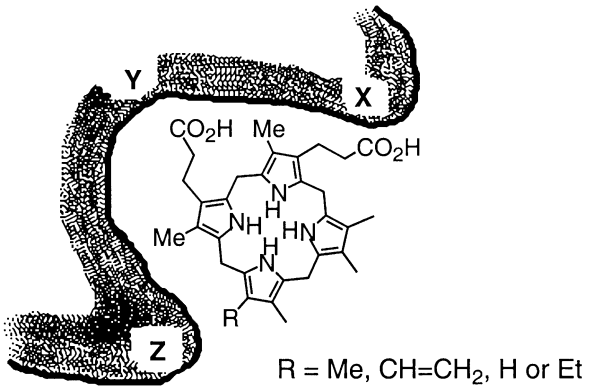



**Figure 1.** Model for the active site of coproporphyrinogen oxidase.

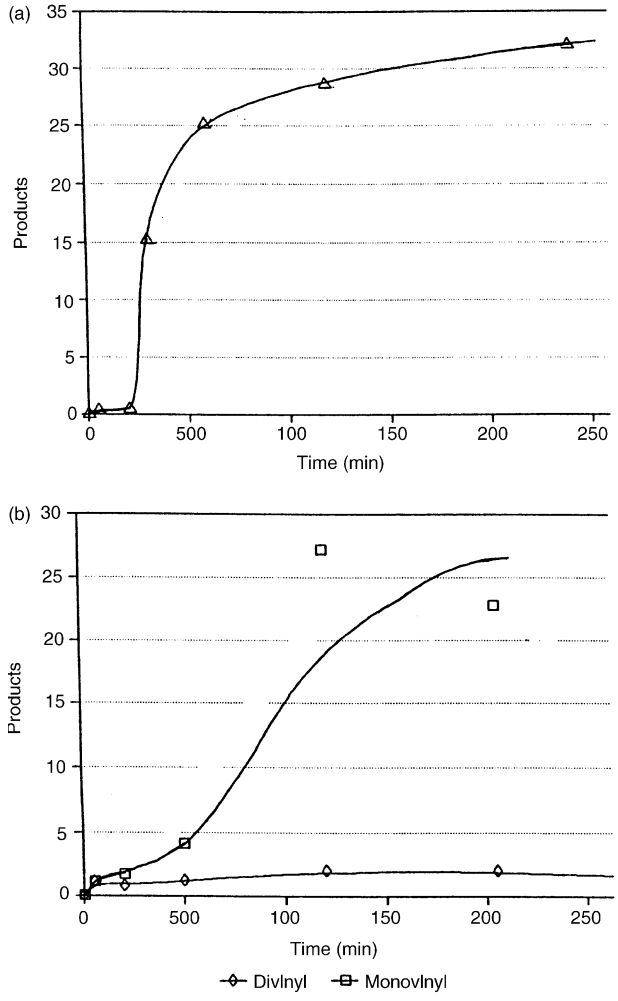



**Figure 2.** (a) Time course study for the incubation of porphyrinogen **6** with chicken red cell hemolysates showing the % divinylic porphyrin product formation versus time. (b) Time course study for the incubation of porphyrinogen **9** with chicken red cell hemolysates showing the % monovinylic and divinylic porphyrin product formation versus time.

the natural substrate copro'gen-III.[4] Product formation was assessed qualitatively by TLC on the corresponding porphyrin methyl esters or quantitively by HPLC as described elsewhere.[4,8]

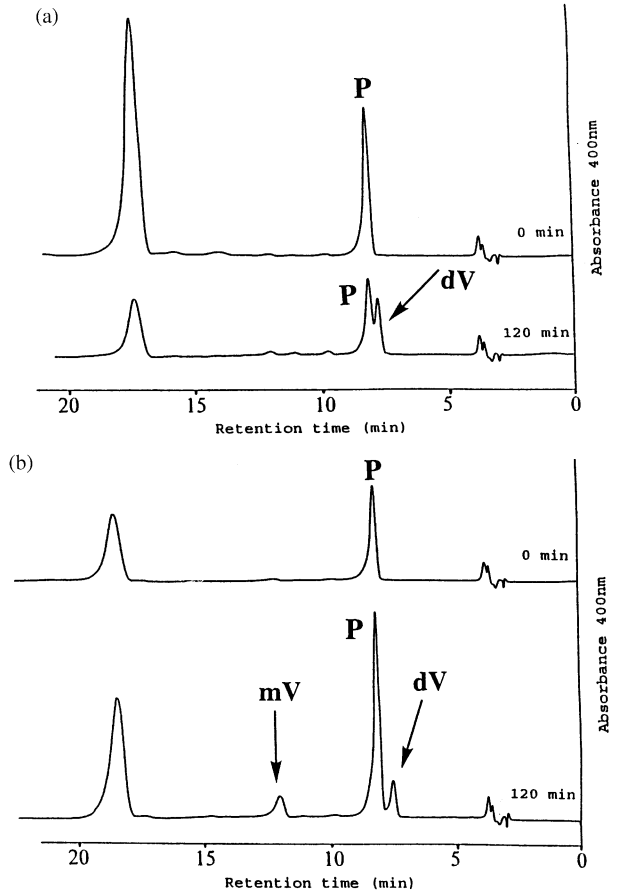



**Figure 3.** (a) HPLC of the esterified porphyrin extract from 0 min (upper) and 120 min (lower) incubations of **6** with chicken red cell hemolysates showing the formation of a divinylic product **10**. (b) HPLC of the esterified porphyrin extract from 0-min (upper) and 120-min (lower) incubations of **7** with chicken red cell hemolysates. In this case, monovinylic and divinylic products are evident. HPLC analyses were performed on a 5μ partisil column (25 cm × 4.6 mm i.d.) eluting with 30% ethyl acetate–cyclohexane (v/v) at a flow rate of 1 mL/min. Peak P is due to endogenous protoporphyrin-IX. mV, monovinylic product; dV, divinylic product.

The new substrate analogues all have one additional methyl group attached to the propionic acid side chain. This is attached at either the first or second carbon of the propionate group, and at either the A or B ring where metabolism usually occurs. This led to four different combinations being investigated. Initially, we had expected the more drastic effects to occur when the A ring residue was modified as this is the grouping that undergoes initial oxidative decarboxylation. However, this did not prove to be the case.

In incubations with CRH, porphyrinogens **2** and **3** were not metabolized but the new analogues **6**–**9** were all converted to some extent. It is notable that **3** was a substrate for the *E. gracilis* enzyme[9] and presumably the binding requirements at region X must differ somewhat from the chicken blood derived enzyme. The best substrate was porphyrinogen **6**, which was converted to the corresponding divinylic product **10**, presumably via the tricarboxylate species **11** (Figs 2a and 3a). Following a lag phase, **6** was metabolized to give approximately 25% **10** after 60 min and leveled off at 30–35% at longer

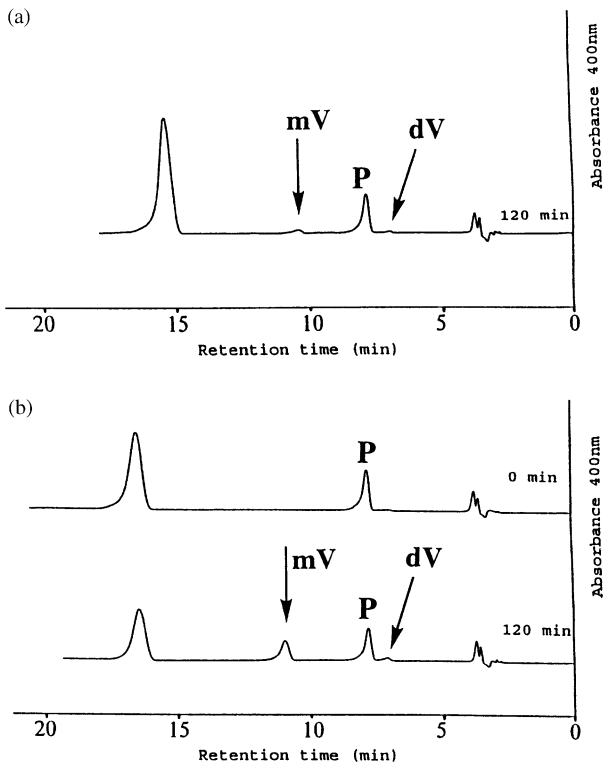


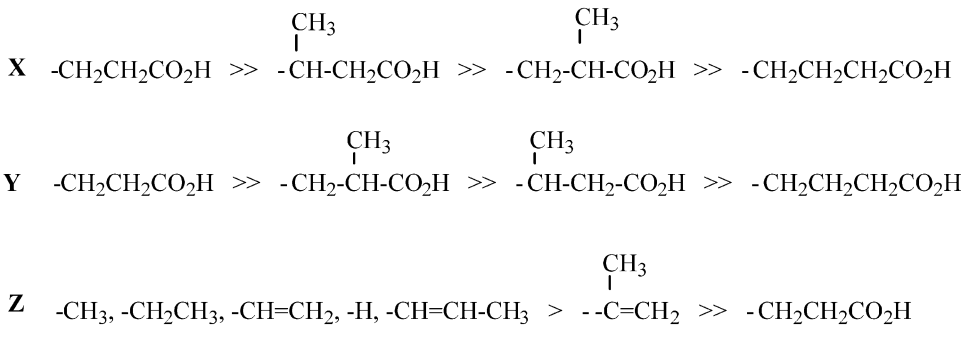

**Figure 4.** (a) HPLC of the esterified porphyrin extract from a 120-min incubation of **8** with chicken red cell hemolysates showing the formation of trace amounts of mono- and divinylic products **14** and **15**. (b) HPLC of the esterified porphyrin extract from 0- and 120-min incubations of **9** with chicken red cell hemolysates. In this case, the monovinylic product **16** predominates. Peak P is due to endogenous protoporphyrin-IX. mV, monovinylic product; dV, divinylic product.

time periods. Under the same conditions, the natural substrate afforded $>80\%$ product after only 10 min incubation with CRH. Porphyrinogen **6** is therefore a much poorer substrate than copro'gen-III. Interestingly, the hardero'gen analogue **11** does not accumulate at all, suggesting that this species is a much better substrate for copro'gen oxidase than **6**. The new product eluted very close to endogenous protoporphyrin-IX in the HPLC chromatograms (a small amount of protoporphyrin-IX is always present in these enzyme preparations). Nonetheless, the product fractions gave good evidence by proton NMR spectroscopy for product formation.[11] In addition, high resolution EI mass spectrometry gave the expected molecular ion for the esterified porphyrin product at $m/z$ 604.3062 (calcd for $C_{37}H_{40}N_4O_4$ 604.3050).

The second A ring modified porphyrinogen **7** was a somewhat poorer substrate giving $9\pm1\%$ of the monovinyl product **12** and $15\pm1\%$ of divinylic product **13** after 120 min (Fig. 3b). In this case, the second oxidative decarboxylation must occur at approximately the same rate as the first degradation, implying that the initially formed $-C(CH_3)=CH_2$ unit is a poor fit for region Z in the binding site. B ring modified porphyrinogen **8** proved to be the poorest substrate, giving only trace amounts of monovinyl **14** and divinylic products **15** in prolonged incubations (Fig. 4a). This shows that the $CH_2CH(CH_3)CO_2H$ unit is a very poor fit for region X. Finally, porphyrinogen **9** was a modest substrate (Fig. 2b), affording primarily the monovinyl porphyrinogen **16** ($27\pm8\%$ in 120-min incubations), although a trace of divinylic product **17** ($2\pm1\%$) can be discerned (Fig. 4b). In this case, the $CH(CH_3)CH_2CO_2H$ unit must be doing a far better job of binding at site X, although this is greatly inferior to a propionate substituent. The second step is also clearly inhibited, confirming that this unit is only slowly degraded at the catalytic site Y.

These data provide further surprises about the substrate specificity of copro'gen oxidase and indicate that the modified propionate side chain can just as easily disrupt binding at region X in the model as interfere with oxidative decarboxylation at the catalytic site Y. On the basis of these and related studies, we can deduce that the best order of fit for various substituents at positions X, Y and Z in our model are as shown in Scheme 3.

While no hydroxylated products could be detected in these experiments, the new results demonstrate that the oxidative decarboxylation mechanism can still occur on substrate analogues with modified side chains. The kinetic data for these studies also give some interesting indications. In particular, the lag phase observed for the metabolism of **6** may indicate that the enzyme needs to go through a conformational change (induced fit) to accomodate the extra methyl group. The hitherto unexplored ability of this enzyme to process these unusual substrates is intriguing and it is anticipated that these insights will allow the development of novel probes for the active site of this poorly understood enzyme.




**Scheme 3.**

## Acknowledgements

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11. **10**: $^1$H NMR ($CDCl_3$): δ −3.6 (2H, br s), 2.45 (3H, d, $J=6$ Hz), 3.29 (4H, t), 3.65 (3H, s), 3.66 (6H, s), 3.72 (3H, s), 3.74 (6H, s), 4.43 (4H, m), 5.4 (1H, m), 6.20 (1H, d, $J=11$ Hz), 6.40 (1H, $J=16$ Hz), 7.98 (1H, d, $J=16$ Hz), 8.26–8.36 (1H, m), 10.12 (1H, s), 10.14 (2H, s), 10.25 (1H, s).