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LINEAR TETRAPYRROLIC INTERMEDIATES FOR BIOSYNTHESIS OF THE NATURAL PORPHYRINS

EXPERIMENTS WITH MODIFIED SUBSTRATES†

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Abstract A brief survey is given of earlier researches on the involvement of linear tetrapyrroles in the biosynthesis of natural porphyrins and their relatives. This brings out the importance of probing the effect of chemically modifying the terminal ring of the linear tetrapyrrolic system. Two such modified hydroxymethylbilanes have been synthesised and the way they interact with the enzyme cosynthetase allows conclusions about the enzymic ring-closure step which generates the uroporphyrinogen III macrocycle.

LIVING systems depend on a variety of metal ions held as complexes within tetrapyrrolic macrocycles to carry out many vitally important functions. Haemoglobin and the cytochromes (Fe^{II} complexes), chlorophylls (Mg^{II} complexes) and vitamin B_{12} (Co^{III} complex) are examples which bring out the central role played by these systems. Remarkably, the organic macrocycles for all these materials are biosynthetically derived from a single intermediate, uroporphyrinogen III (**3**), shortened to uro'gen III. This is enzymically modified to produce the final pigments, and the modifications may be extensive as occurs in the biosynthesis of vitamin B_{12}.[1] An understanding of the biosynthesis of uro'gen III (**3**) is obviously of key importance in this area.

Uro'gen III (**3**) is produced from four molecules of porphobilinogen (**1**), PBG, by the cooperative action of two enzymes, deaminase and cosynthetase[2] (Scheme 1). Cosynthetase is easily destroyed by heat and deaminase alone acts on PBG (**1**) eventually to produce uroporphyrinogen I (**4**) (uro'gen I), an unnatural isomer. Since uro'gen I (**4**) is not enzymically isomerised to uro'gen III[2] (**3**), it follows that cosynthetase must act on an intermediate produced by deaminase, or it must interact with deaminase in some way to modify the manner in which the PBG units are linked together. The original studies in this Laboratory[3] established that the biosynthesis of uro'gen III (**3**) involves a single intramolecular rearrangement affecting only that PBG unit which becomes C-15 of structure (**3**) and the pyrrole ring which appears in (**3**) as ring D. The PBG units corresponding to ring A with C-20, ring B with C-5 and ring C with C-10 were shown to be incorporated intact and without rearrangement.[3]

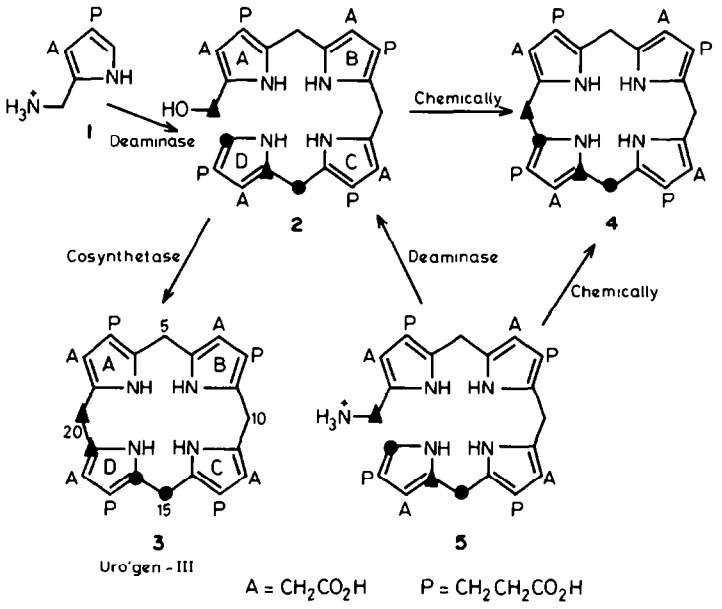
In principle, the single rearrangement step could occur at any stage of the building process, i.e. at the monopyrrole, dipyrrole, tripyrrole or tetrapyrrole levels. Proof that rearrangement occurs at the tetrapyrrole level came from synthesis of the unrearranged linear aminomethylbilane[4] (**5**) which corresponds to head-to-tail assembly of PBG units (**1**). Two samples of the bilane (**5**) were synthesised, one carrying 90 atom % ^{13}C at the sites marked ● and the other similarly labelled at the sites marked ▲. This bilane was shown to be a substrate for deaminase-cosynthetase and these enzymes converted it into uro'gen III (**3**) essentially free from isomers; the labelling pattern of the uro'gen III produced from the two ^{13}C-labelled samples of bilane (**5**) showed that a single intramolecular rearrangement had occurred (both experiments are summarised in Scheme 1).

Ring-closure of the aminomethylbilane (**5**) was strongly accelerated by the enzyme system relative to the non-enzymic rate of ring-closure which produced uro'gen I (**4**). However, kinetic studies revealed that this enzymic rate was slower by a factor of *ca* 15 than the rate at which uro'gen III (**3**) was produced enzymically from the natural substrate, PBG (**1**). Clearly the aminomethylbilane (**5**) cannot be, in that exact form, a true intermediate on the biosynthetic pathway from PBG (**1**) to uro'gen III (**3**).

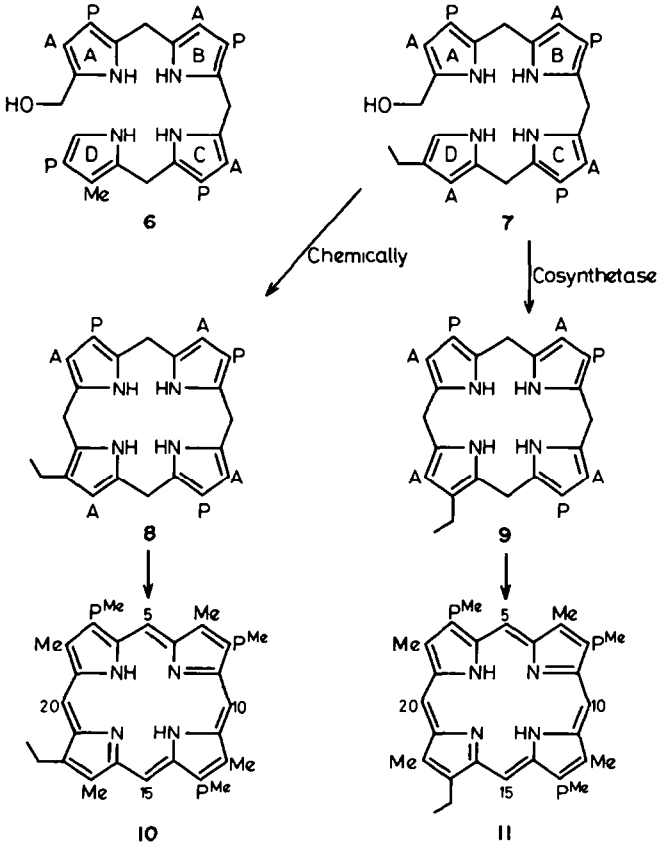
This problem of rate was further explored by examining the action of deaminase alone (no cosynthetase) on the aminomethylbilane (**5**). It was found[5] that enzymic acceleration of ring-closure again occurred but there was a clear lag over the first few minutes of the run before formation of uro'gen I (**4**) reached the maximum rate. This observation was quite different from the results when deaminase and cosynthetase were used together; then uro'gen III (**3**) was formed without any detectable lag.

This lag was even more apparent when a large amount of deaminase acted on PBG (**1**); when almost all of the PBG had been used, uro'gen I formation had reached only *ca* 10% of its eventual maximum.[5] Extensive studies were carried out, especially by ^{13}C-NMR, to determine the structure of the intermediate accumulated during the lag phase. This work proved[5] that the labile intermediate released when deaminase acted alone on PBG (**1**) or on the unrearranged aminomethylbilane (**5**) was the linear hydroxymethylbilane (**2**); this ring-closed chemically, without assistance from deaminase, to form uro'gen I (**4**). Rigorous confirmation of the structure (**2**) came from total synthesis.[6] The synthetic and natural samples of the hydroxymethylbilane (**2**) were excellent and identical

†This paper is regarded as Part 19 of our series on "Biosynthesis of Porphyrins and Related Macrocycles"; Part 18 appeared in *J. Chem. Soc.*, Perkin Trans. 1, 2427 (1982).



Scheme 1.



Scheme 2.

substrates for cosynthetase (free from deaminase) which catalysed extremely rapid formation of uro'gen III (**3**). These structural and synthetic studies also eliminated a cyclic structure (the so-called preuro'gen) for the intermediate which had been erroneously claimed by others.[7]

The foregoing knowledge allowed definition of the roles of deaminase and cosynthetase in the biosynthesis of the natural porphyrins, chlorins and corrins. It is clear that deaminase joins four PBG units (**1**) head-to-tail and in the absence of cosynthetase releases the unrearranged hydroxymethylbilane (**2**) into solution; *deaminase is the assembly enzyme.* Cosynthetase then converts the bilane (**2**) by intramolecular rearrangement of ring D, into uro'gen III (**3**); *cosynthetase is the rearranging and ring-closing enzyme.*

At this stage, the way was open to probe the substrate specificity of cosynthetase. By synthesising a set of isomeric hydroxymethylbilanes which differed in the arrangement of the acetate and propionate side-chains, it was found[8] (among other results) that the natural, unrearranged bilane (**2**) was by far the best substrate for cosynthetase. In addition, the isomer of bilane (**2**), which was synthesised with ring D reversed, was also a moderately good substrate for cosynthetase and in the process of ring-closing this bilane, the enzyme brought about appreciable inversion of ring D to form uro'gen I (as **4**, unlabelled).

It was then decided that a fascinating expansion of such studies would be to restrict the structural changes in the hydroxymethylbilanes to the side-chains on ring D, since this is the one involved in the intramolecular rearrangement process. The present paper covers the results obtained from this work.

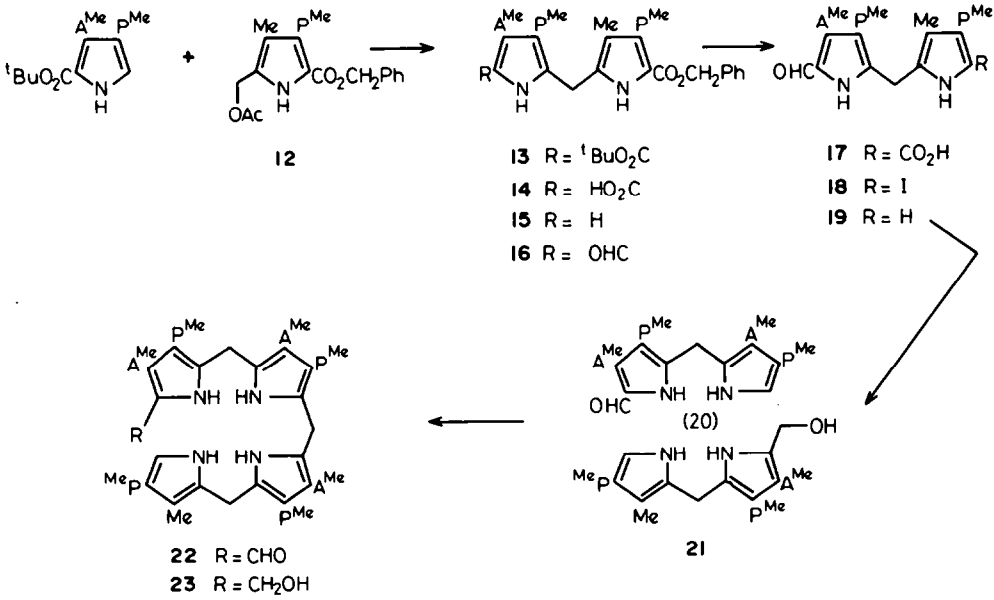
The plan was to synthesise the bilanes (**6**) and (**7**), Scheme 2. The former differs from the natural substrate (**2**) for cosynthetase only by lacking the acetate carboxyl group on ring D. Similarly, the latter differs from the natural material (**2**) by lacking the propionate carboxyl group on that ring.

Schemes 3 and 4 show the synthetic routes to the corresponding heptamethyl esters (**23**) and (**36**) of the required bilanes; these routes were based on the general approach which had been successfully used for constructing other hydroxymethylbilanes.[6,8,9] The formyl esters (**22**) and (**35**) were carefully purified by preparative h.p.l.c. and characterised by NMR (400 MHz) and mass spectrometry (field desorption).

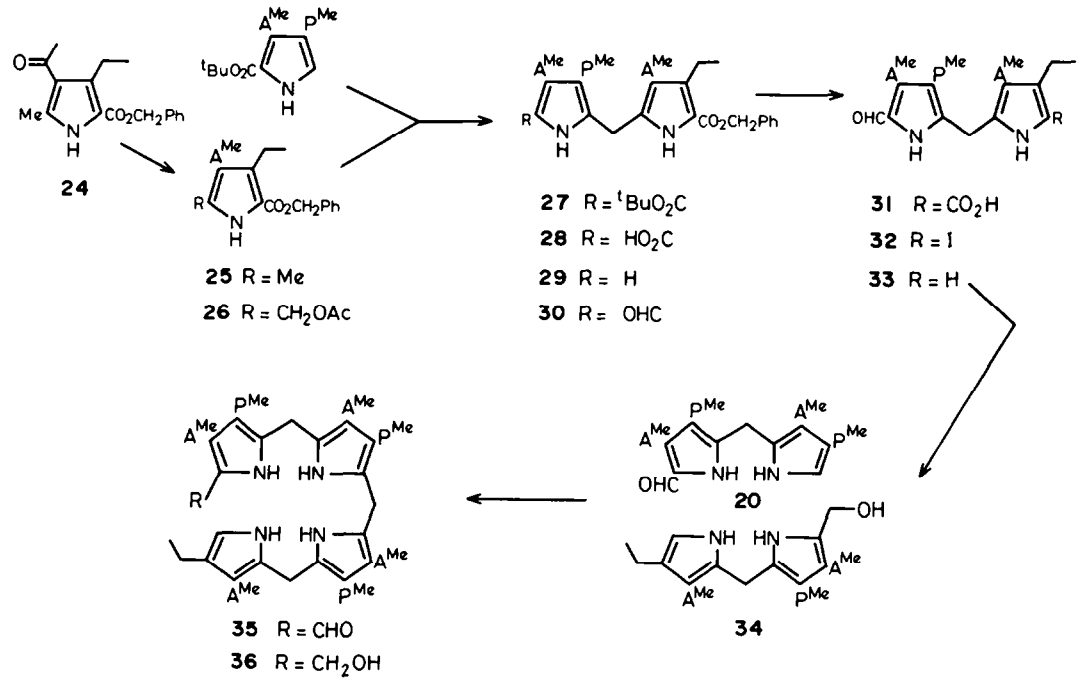
Alkaline hydrolysis of the esters (**23**) and (**36**) afforded the required substrates (**6**) and (**7**). These were incubated separately with cosynthetase, free from deaminase, which had been isolated from *Euglena gracilis*; non-enzymic ("chemical") ring-closure of each bilane was carried out at the same concentration and pH. Finally, the unrearranged hydroxymethylbilane (**2**) was included in the set of experiments as the standard.

The hydroxymethylbilane (**6**) lacking an acetate residue on ring D proved to be a very poor substrate for cosynthetase. There was only slight acceleration of the ring-closure of (**6**) relative to the rate of chemical cyclisation and the enzymic rate was about seventy times less than the rate at which cosynthetase ring-closed the natural hydroxymethylbilane (**2**). This result points to the importance of the acetate function on ring D of the natural substrate (**2**) for full interaction with cosynthetase. Strikingly, the other bilane (**7**), lacking the propionate carboxyl group on ring D, was the best modified substrate for cosynthetase so far examined (Fig. 1); we will refer to bilane (**7**) as the "ring-D A/Et bilane". The rate of enzymic ring-closure was about one quarter that of the natural bilane (**2**) in a parallel run. So the ring D propionate carboxyl can be removed from bilane (**2**), (as in **7**) without the dramatic effect observed when the acetate group on ring D of bilane (**2**) was replaced by a methyl group (as in **6**).

The next step was to determine the nature of the products formed by chemical and enzymic cyclisation of the ring-D A/Et bilane (**7**). The structures of interest are the macrocycles (**8**) and (**9**); the former is the result of straightforward ring-closure of bilane (**7**)



Scheme 3.



Scheme 4.

whilst the latter is formed if ring D of (**7**) undergoes reversal during the enzymic cyclisation. The actual products (**8**) and (**9**) formed were studied by oxidising them to the corresponding porphyrins which were decarboxylated at the acetate residues (cf. ref. 4[b]); the spectroscopic work was then carried out on the corresponding trimethyl esters (**10**) and (**11**).

The final product so obtained from chemical ring-closure of ring-D A/Et bilane (**7**) had the expected molecular weight (652) and its ^{1}H-NMR spectrum at 400 MHz supported homogeneity. It can be assigned structure (**10**) since many chemical ring-closures of hydroxymethyl and aminomethylbilanes have always occurred without significant rearrangement.[4-6,8,9] In contrast, the final product from the action of cosynthetase on the ring-D A/Et bilane (**7**) was clearly shown by ^{1}H-NMR to be a mixture of the porphyrins (**10**) and (**11**). This was expected because, as in all earlier cases studied,[8,9] some chemical ring-closure occurs during preparation of the labile hydroxymethylbilane and in this case an appreciable amount of such cyclised product (**8**) was shown to be present at the start of the treatment with cosynthetase. It has not been possible to separate the isomers (**10**) and (**11**) despite many trials using our most effective h.p.l.c. methods.[10] However, NMR studies allowed the amounts of (**10**) and (**11**) in the mixture to be determined.

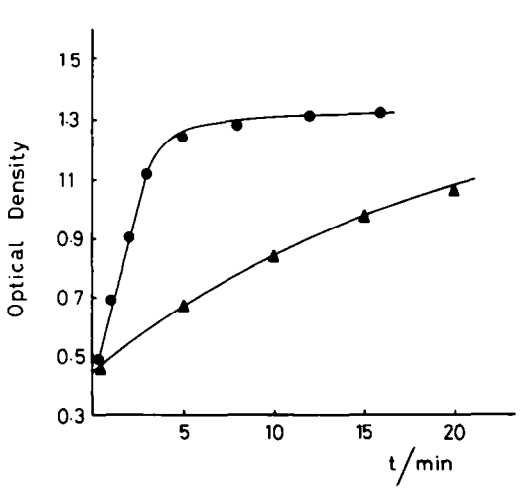


Fig. 1. Conversion of ring-D A/Et bilane (**7**) into porphyrinogens at pH 8.25, chemically ▲ and enzymically using cosynthetase ●.

First, the ^{1}H-signals from the *meso*-positions of the pure porphyrin (**10**) were recorded in the presence of shift reagent,[11] Eu([$^2H_9$]fod)$_3$. In confirmation of the assigned structure, the signals from the ethyl group of porphyrin (**10**) were barely shifted; also, H-20 was little affected (no adjacent ester group), H-15 was appreciably shifted (one adjacent ester group) and H-5 and H-10 were shifted most (one adjacent and one close ester group), see Fig. 2a. This same set of signals from the *meso*-protons (set A) was also apparent in the shifted ^{1}H-NMR spectrum of the mixture of porphyrins from the enzymic run and this set was enhanced when more pure porphyrin (**10**) was added to the sample. The original shifted spectrum of the mixture also showed a second set of *meso*-H signals (set B) as well as a smaller pair of *shifted* signals from the Et group of the porphyrin ester (**11**). In set B, the signal from H-20 was shifted slightly more and that from H-15 slightly less than in set A (Fig. 2b). It is a reasonable view that because of the steric bulk of the ethyl group, the shift reagent can interact more closely with H-15 for **10** than for **11** and, in a complementary way, that the interaction will be closer with H-20 in porphyrin (**11**) than in **10**. Careful measurement of the size of these *meso*-signals and correction for the amount

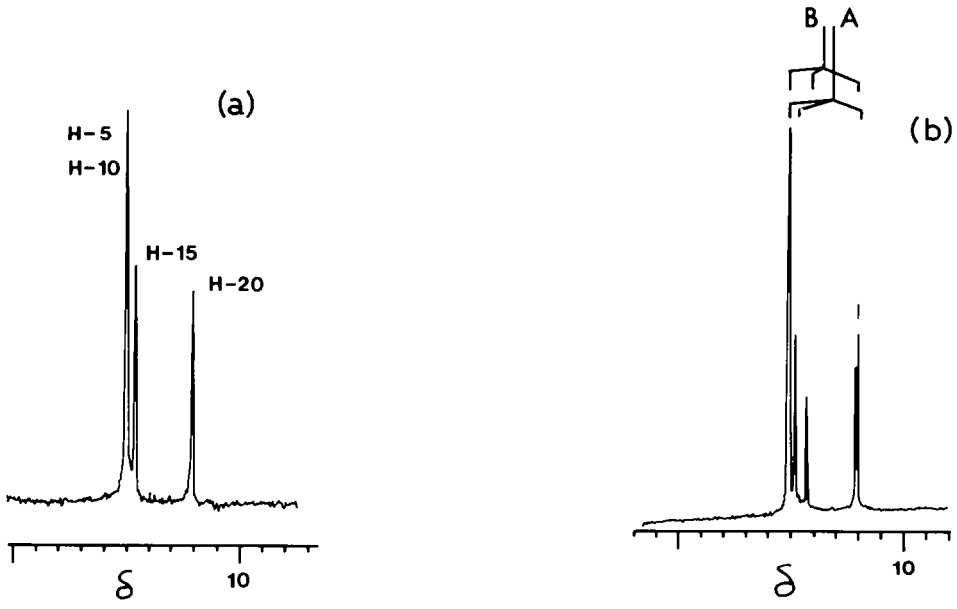


Fig. 2. ^{1}H-NMR signals from H-5, H-10, H-15 and H-20 of porphyrin (**10**) (Fig. 2a) and of the mixture of porphyrins (**10**) and (**11**) (Fig. 2b). Both spectra were recorded in the presence of Eu([$^2H_9$]fod)$_3$.

of the porphyrin (**10**) from the chemically ring-closed product (**8**) present at the start of the enzymic run and that produced chemically during the incubation leads to the following result; of the ring-D A/Et bilane (**7**) which was ring-closed by cosynthetase, 65 ± 5% underwent inversion of ring D.

In summary, these studies lead to the following conclusions: (*a*) when cosynthetase acts on the natural hydroxymethylbilane (**2**), the ring D acetate carboxyl group must have an important function; the analogous bilane (**6**) lacking only this carboxyl group is an extremely poor substrate for the enzyme; (*b*) the ring D propionate carboxyl group of the natural bilane (**2**) apparently plays a less critical role; the modified bilane (**7**) is still a good substrate for cosynthetase; (*c*) however, the ring D propionate carboxyl group is not totally uninvolved; cosynthetase ring-closes the natural bilane (**2**) with quantitative inversion of ring D whereas for the analogue (**7**) lacking the propionate carboxyl group, the efficiency of ring D inversion falls to *ca* 65%

EXPERIMENTAL

The general directions given in ref. 10 were followed. In addition, preparative and analytical TLC for sensitive products (e.g. bilanes) was carried out in the dark under argon. The ^{1}H-NMR spectra were run on Varian EM 360 or Bruker WH-400 spectrometers and refer to solns in $CDCl_3$ unless otherwise stated.

Benzyl 4-acetyl-3-ethyl-5-methylpyrrole-2-carboxylate (**24**)

Isoamyl nitrite (30 g) was added slowly to a mixture of benzyl 3-oxopentanoate[12] (40 g) and conc. HCl (1 ml) at 0°, and the mixture was stirred at room temp overnight. It was then added dropwise during 1 hr to a vigorously stirred soln of acetylacetone (19 g) in AcOH (190 ml) containing NH_4OAc (30 g), CCl_4 (1 ml) and Zn powder (11 g). During the addition period, more Zn powder (22 g) was added in portions. The mixture was then heated at 90–100° for 3 hr, cooled and poured into ice-water (2L). The product was collected after 4 hr, washed with water and recrystallised from EtOH-water to give the title pyrrole as needles (22 g, 40%), m.p. 148° (Found: C, 71.4; H, 7.0; N, 5.0. $C_{17}H_{19}NO_3$ requires: C, 71.5; H, 6.7; N, 4.9%). δ 1.17 (3H, t, J = 7 Hz, CH_2CH_3), 2.45 (3H, s, $COCH_3$), 2.50 (3H, s, pyrrole-CH_3), 3.10 (2H, q, J = 7 Hz, CH_2CH_3), 5.27 (2H, s, CH_2Ph), 7.30 (5H, s, Ph), 9.45 (1H, br, NH).

Benzyl 3-ethyl-4-methoxycarbonylmethyl-5-methylpyrrole-2-carboxylate (**25**)

A soln of thallium(III) nitrate trihydrate (19.7 g, 45 mmol) in MeOH (50 ml) containing conc HNO_3 (2 ml) was added at room temp to a soln of the foregoing acetylpyrrole (11.4 g, 40 mmol) in MeOH (60 ml) and CH_2Cl_2 (60 ml). The mixture was stored for 24 hr and then trimethylorthoformate (15 ml) was added. Next day, the thallium(I) nitrate was filtered off, washed with MeOH and the filtrate was diluted with water (200 ml). The product was extracted into CH_2Cl_2 (50 ml, 3 × 25 ml), the combined organic layers were washed with water, then filtered through alumina (50 g, neutral, grade I) and the soln and column-washings were evaporated. The residue was crystallised from diethyl ether–n-hexane to give the title pyrrole (9.45 g, 75%) as needles, m.p. 82–83° (Found: C, 68.4; H, 6.9; N, 4.7. $C_{18}H_{21}NO_4$ requires: C, 68.5; H, 6.7; N, 4.4%). δ 1.10 (3H, t, J = 7 Hz, $CH_2\underline{CH_3}$), 2.20 (3H, s, pyrrole-CH_3), 2.75 (2H, q, J = 7 Hz, $C\underline{H_2}CH_3$), 3.35 (3H, s, CH_2CO_2), 3.65 (3H, s, CO_2CH_3), 5.30 (2H, s, CH_2Ph), 7.33 5H, s, Ph), 9.25 (1H, br, NH).

Benzyl 5-acetoxymethyl-3-ethyl-4-methoxycarbonylmethylpyrrole-2-carboxylate (**26**)

A stirred soln of the foregoing pyrrole (6.3 g, 20 mmol) in glacial AcOH (60 ml) and Ac_2O (10 ml) was treated with lead tetraacetate (10.5 g, 20 mmol) in one portion. The mixture was heated to 80° until TLC showed no starting material (45 min). Ethylene glycol (10 ml) was added, then water (400 ml) and the product was collected, washed with water, dried and recrystallised from diethyl ether–n-hexane to give plates of the acetoxymethylpyrrole (6.5 g, 87.8%), m.p. 129–130° (Found: C, 64.0; H, 6.2; N, 4.0. $C_{20}H_{23}NO_6$ requires: C, 64.3; H, 6.2; N, 3.7%). δ 1.10 (3H, t, J = 7 Hz, CH_2CH_3), 2.03 (3H, s, $COCH_3$), 2.75 (2H, q, J = 7 Hz, $C\underline{H_2}CH_3$), 3.50 (2H, s, CH_2CO_2), 3.65 (3H, s, CO_2CH_3), 5.05 (2H, s, CH_2OCO), 5.30 (2H, s, CH_2Ph), 7.33 (5H, s, Ph), 9.50 (1H, br, NH).

t-Butyl 5'-benzyloxycarbonyl-4'-ethyl-3-(2-methoxycarbonylethyl)-4,3'-bismethoxycarbonylmethyl-2,2'-methylenedipyrrole-5-carboxylate (**27**)

The foregoing acetoxymethylpyrrole (5.74 g, 15.4 mmol) in CH_2Cl_2 (150 ml) was added dropwise under N_2 to a stirred soln of t-butyl 4-(2-methoxycarbonylethyl)-3-methoxycarbonylmethylpyrrole-2-carboxylate[10] (5 g, 15.4 mmol) and *p*-toluenesulphonic acid (240 mg, 1.4 mmol) in CH_2Cl_2 (20 ml) and MeOH (5 drops). After 3 hr, the resulting red soln was treated with $NaBH_4$ (10 mg) in MeOH (2 ml) until it became pale yellow and then it was passed through alumina (50 g, neutral, grade I), the elution being continued with diethyl ether. Appropriate fractions were combined and the residue from evaporation was chromatographed on silica (300 g) using diethyl ether : n-hexane (3 : 2) to give the pyrromethane as a gum (7 g, 71%) (Found: M^+, 638.2837; $C_{34}H_{42}N_2O_{10}$ requires: M, 638.2839). δ 1.03 (3H, t, J = 7 Hz, CH_2CH_3), 1.50 (9H, s, $C(CH_3)_3$), 2.40–2.90 (6H, m, $CH_2CH_2CO_2$ and CH_2CH_3), 3.45 and 3.75 (each 2H, s, $2 \times CH_2CO_2$), 3.50, 3.60 and 3.70 (each 3H, s, $3 \times CO_2CH_3$), 3.85 (2H, s, pyrrole-CH_2-pyrrole), 5.20 (2H, s, CH_2Ph), 7.25 (5H, s, Ph), 9.75 and 9.90 (each 1H, br, $2 \times NH$).

5'-Benzyloxycarbonyl-4'-ethyl-3-(2-methoxycarbonylethyl)-4,3'-bismethoxycarbonylmethyl-2,2'-methylenedipyrrole-5-carboxylic acid (**28**)

The foregoing dipyrromethane (6.4 g) in dry nitromethane (20 ml) was treated with BF_3-etherate (3 ml) for 2 hr at −15° under N_2. Acidified water (20 ml) was then added and the product was extracted into CH_2Cl_2 (2×20 ml), and the residue from the extracts was triturated with diethyl ether. The resultant solid was recrystallised from CH_2Cl_2-ether-hexane to give flakes of the title dipyrromethane (3.78 g, 65%), m.p. 168–169°. Found: $M^+ - CO_2$, 538.2315; $C_{30}H_{34}N_2O_{10} - CO_2$ requires: 538.2315. (Found: C, 61.8; H, 5.8; N, 5.1. $C_{30}H_{34}N_2O_{10}$ requires: C, 61.8; H, 5.9; N, 4.8%). δ 1.03 (3H, t, J = 7 Hz, CH_2CH_3), 2.40–2.90 (6H, m, $CH_2CH_2CO_2$ and CH_2CH_3), 3.45 and 3.75 (each 2H, s, $2 \times CH_2CO_2$), 3.50, 3.60 and 3.67 (each 3H, s, $3 \times CO_2CH_3$), 3.83 (2H, s, pyrrole-CH_2-pyrrole), 5.17 (2H, s, CH_2Ph), 7.30 (5H, s, Ph), 10.00 (2H, br, $2 \times NH$).

Benzyl 4-ethyl-5'-formyl-3'-(2-methoxycarbonylethyl)-3,4'-bismethoxycarbonylmethyl-2,2'-methylenedipyrrole-5-carboxylate (**30**)

A mixture of foregoing acid (3.2 g, 5.5 mmol) and *p*-toluenesulphonic acid (1.72 g, 10 mmol) in CH_2Cl_2 (100 ml) and MeOH (10 ml) was stirred under N_2 in the dark and at room temp for 15 hr. The red soln was treated with $NaBH_4$ (300 mg) until pale yellow, then made alkaline with concentrated ammonia and the product was extracted into CH_2Cl_2 (2×60 ml). The recovered **29** was an oil (2.7 g, 91%) from which a pure sample was prepared by PLC on silica using diethyl ether : n-hexane (3 : 1) (Found: M^+, 538.2353; $C_{29}H_{34}N_2O_8$ requires: 538.2315). δ 1.10 (3H, t, J = 7 Hz, CH_2CH_3), 2.40–2.90 (6H, m, $CH_2CH_2CO_2$ and CH_2CH_3), 3.45 and 3.53 (each 2H, s, $2 \times CH_2CO_2$), 3.53, 3.65 and 3.73 (each 3H, s, $3 \times CO_2CH_3$), 3.90 (2H, s, pyrrole-CH_2-pyrrole), 5.25 (2H, s, CH_2Ph), 6.55 (1H, d, J = 2 Hz, pyrrole-H), 7.33 (5H, s, CH_2Ph), 9.33 and 9.70 (each 1H, br, $2 \times NH$).

This product (2.2 g) in dry N,N-dimethylformamide (15 ml) was stirred for 2 hr with benzoyl chloride (1.5 ml) at room temp under N_2 and the mixture was then poured into a soln of NaOAc (5 g) in water (20 ml). This mixture was stirred at room temp for 1 hr and the product was extracted into CH_2Cl_2 (2×20 ml), washed with 10% Na_2CO_3aq, and recovered by evaporation. The residue was chromatographed on silica (100 g) using CH_2Cl_2 : MeOH (95 : 5) to give the formylpyrromethane as a gum (2 g, 87%) (Found: M^+, 566.2271; $C_{30}H_{34}N_2O_9$ requires: 566.2264). δ 1.10 (3H, t, J = 7 Hz, CH_2CH_3), 2.40–2.90 (6H, m, $CH_2CH_2CO_2$ and CH_2CH_3), 3.50 and 3.70 (each 2H, s, $2 \times CH_2CO_2$), 3.53, 3.67 and 3.75 (each 3H, s, $3 \times CO_2CH_3$), 3.95 (2H, s, pyrrole-CH_2-pyrrole), 5.26 (2H, s, CH_2Ph), 7.30 (5H, s, Ph), 9.50 (1H, s, CHO), 9.90 and 10.50 (each 1H, br, $2 \times NH$).

4-Ethyl-5'-formyl-3'-(2-methoxycarbonylethyl)-3,4'-bismethoxycarbonylmethyl-2,2'-methylenedipyrrole-5-carboxylic acid (**31**)

A soln of the foregoing product (1.7 g) in THF (20 ml) and Et_3N (1 ml) was hydrogenated over 10% Pd-C (50 mg) at room temp for 3 hr. The filtered soln was evaporated and the residue crystallised from CH_2Cl_2-ether to give the title acid as needles (1.15 g, 80%), m.p. 183–185° (dec) Found: $M^+ - CO_2$ 432.1888, $C_{23}H_{28}N_2O_9 - CO_2$ requires: 432.1896. (Found: C, 58.0; H, 5.8; N, 6.0 $C_{23}H_{28}N_2O_9$ requires: C, 58.0; H, 5.9; N, 5.9%). δ ($CDCl_3 - CD_3OD$) 1.10 (3H, t, J = 7 Hz, CH_2CH_3), 2.40–2.90 (6H, m, $CH_2CH_2CO_2$ and CH_2CH_3), 3.47 (2H, s, CH_2CO_2), 3.69 (11H, br s, $3 \times CO_2CH_3$ and CH_2CO_2), 4.01 (2H, s, pyrrole-CH_2-pyrrole), 9.38 (1H, s, CHO).

4'-Ethyl-5-formyl-5'-iodo-3-(2-methoxycarbonylethyl)-4,3'-bismethoxycarbonylmethyl-2,2'-methylenedipyrrole (**32**)

A soln of I_2 (0.55 g, 2 mmol) and KI (0.56 g) in water (5 ml) was added dropwise at 20° over 2 min to a vigorously stirred mixture of the foregoing acid (0.8 g, 1.68 mmol), $NaHCO_3$ (1 g), CH_2Cl_2 (15 ml) and water (15 ml). After 15 min, sat aqueous sodium metabisulphite was added to destroy excess I_2 and the product was extracted into CH_2Cl_2 (2×20 ml). The extracts were chromatographed on silica (30 g) elution being continued with CH_2Cl_2 : diethyl ether (1 : 9). The product from appropriate fractions was recrystallised from CH_2Cl_2–ether–n-hexane to give the iodide as needles (0.65 g, 70%), m.p. 140° (dec) (Found: C, 47.6; H, 4.8; N, 4.9. $C_{22}H_{27}N_2O_7I$ requires: C, 47.3; H, 4.9; N, 5.0%). δ 1.05 (3H, t, J = 7 Hz, CH_2CH_3), 2.40–2.90 (6H, m, $CH_2CH_2CO_2$ and CH_2CH_3), 3.55 (2H, s, CH_2CO_2), 3.69 and 3.75 (11H, s, $3 \times CO_2CH_3$ and CH_2CO_2), 3.80 (2H, s, pyrrole-CH_2-pyrrole), 9.50 (1H, s, CHO), 9.40 and 10.30 (each 1H, br, $2 \times NH$).

4'-Ethyl-5-formyl-3-(2-methoxycarbonylethyl)-4,3'-bismethoxycarbonylmethyl-2,2'-methylenedipyrrole (**33**)

A mixture of the foregoing iodide (0.5 g), PtO_2 (0.1 g) and Et_3N (2 ml) in MeOH (20 ml) was hydrogenated at 20° for 45 min. The filtered soln was diluted with water (100 ml) and the product was extracted into CH_2Cl_2 (3×25 ml). The combined extracts were washed with 10% Na_2CO_3aq and the residue from them was recrystallised from CH_2Cl_2–diethyl ether–n-hexane to give the pyrromethane (260 mg, 67%) as needles, m.p. 112° (Found: C, 61.2; H, 6.6; N, 6.5. M^+ 432.1862. $C_{22}H_{28}N_2O_7$ requires: C, 61.1; H, 6.5; N, 6.5% M^+ 432.1896). δ 1.16 (3H, t, J = 7 Hz, CH_2CH_3), 2.30–2.90 (6H, m, $CH_2CH_2CO_2$ and CH_2CH_3), 3.50 and 3.75 (each 2H, s, $2 \times CH_2CO_2$), 3.65, 3.69 and 3.80 (each 3H, s, $3 \times CO_2CH_3$), 3.91 (2H, s, pyrrole-CH_2-pyrrole), 6.48 (1H, d, J = 2 Hz, pyrrole-H), 9.55 (1H, s, CHO), 8.68 and 10.20 (each 1H, br, $2 \times NH$).

18-Ethyl-1-formyl-3,8,13-tri-(2-methoxycarbonylethyl)-2,7,12,17-tetrakismethoxycarbonylmethylbilane (**35**)

$NaBH_4$ (20 mg) was added portionwise over 5 min to a stirred soln of **33** (20 mg) in MeOH (0.5 ml), $CHCl_3$ (0.5 ml) and Et_3N (3 drops). After 10 min, $CHCl_3$ (5 ml) was added, the soln was washed with brine (3×2 ml) and evaporated to yield **34**. This in CH_2Cl_2 (2 ml) containing Et_3N (2 drops) was added dropwise over 5 min under argon to a stirred soln of **20**[9] (60 mg) in CH_2Cl_2 (5 ml) and AcOH (1 ml). After 15 min, the soln was washed with brine and carefully neutralised with solid $NaHCO_3$; the organic layer was again washed with dilute brine (2×5 ml), dried and evaporated. The residue was partly purified by PLC on silica in MeOH (5%) and Et_3N (0.1%) in $CHCl_3$ and the product was 80% pure (HPLC). It was purified by HPLC on a preparative μCN Bondapak column with hexane (30 ml), toluene (14 ml) and acetonitrile (6 ml) to give pure **35** as a pale yellow solid (14 mg, 33%) (Found: M^+ 906.3929; $C_{46}H_{58}N_4O_{15}$ requires: 906.3898). λ_{max} 310 nm. δ 1.13 (3H, t, J = 7 Hz, CH_2CH_3), 2.30–2.90 (14H, m, $3 \times CH_2CH_2CO_2$ and

CH_2CH_3), 3.37, 3.38, 3.43, 3.67, 3.72, 3.75 and 3.79 (each 2H, s, 3 × pyrrole-CH_2-pyrrole and 4 × CH_2CO_2), 3.56, 3.60, 3.61, 3.63, 3.65, 3.67 and 3.70 (each 3H, s, 7 × CO_2CH_3), 6.42 (1H, d, J = 2 Hz, pyrrole-H), 9.50 (1H, s, CHO), 8.56, 9.13, 9.28 and 9.80 (each 1H, br, 4 × NH).

3,8,13-Tri-(2-carboxyethyl)-2,7,12,17-tetrakiscarboxymethyl-18-ethyl-1-hydroxymethylbilane (**7**) *and the corresponding heptamethyl ester* (**36**)

$NaBH_4$ (10 mg) was added over 2 min to a stirred soln of **35** (6 mg) in $CHCl_3$ (0.5 ml), MeOH (0.5 ml) and Et_3N (3 drops). After 5 min, $CHCl_3$ (1 ml) was added, the soln was washed with dilute brine (3 × 1 ml), dried and concentrated with argon before dilution with MeOH (2 ml) and concentration. The residue was resuspended in MeOH (0.5 ml) containing Et_3N (2 drops); and the resultant solid was collected by centrifugation to afford **36** (5.25 mg). The foregoing **36** (2.9 mg) was hydrolysed under argon in 2 M KOH (80 μl) for 16 hr at 25°. This alkaline soln of hydroxymethylbilane was used directly for the kinetic studies with cosynthetase described later.

t-Butyl 5'-benzyloxycarbonyl-3,4'-di-(2-methoxycarbonylethyl)-4-methoxycarbonylmethyl-3'-methyl-2,2'-methylenedipyrrole-5-carboxylate (**13**)

Compound **12**[1,3] (5.74 g) was converted into **13** exactly as for **27** above. It was obtained as a gum (6 g, 61%) (Found: M^+ 638.2801; $C_{34}H_{42}N_2O_{10}$ requires: 638.2839). δ 1.50 (9H, s, $C(CH_3)_3$), 2.00 (3H, s, pyrrole-CH_3), 2.30–3.15 (8H, m, 2 × $CH_2CH_2CO_2$), 3.57, 3.63 and 3.69 (each 3H, s, 3 × CO_2CH_3), 3.75 (2H, s, CH_2CO_2), 3.90 (2H, s, pyrrole-CH_2-pyrrole), 5.30 (2H, s, CH_2Ph), 7.35 (5H, s, Ph), 9.05 and 9.60 (each 1H, br, 2 × NH).

5'-Benzyloxycarbonyl-3,4'-di-(2-methoxycarbonylethyl)-4-methoxycarbonylmethyl-3'-methyl-2,2'-methylenedipyrrole-5-carboxylic acid (**14**)

The foregoing **13**, (5.5 g) was converted into the corresponding **14** as for **28** above. It crystallised from ether as needles (3.6 g, 72%), m.p. 152–155° (Found: C, 61.9; H, 5.9; N, 4.8. $C_{30}H_{34}N_2O_{10}$ requires: C, 61.8; H, 5.9; N, 4.8%). δ ($CDCl_3$–CD_3OD) 2.00 (3H, s, pyrrole-CH_3), 2.25–3.00 8H, m, 2 × $CH_2CH_2CO_2$), 3.59, 3.67 and 3.69 (each 3H, s, 3 × CO_2CH_3), 3.95 (2H, s, CH_2CO_2), 4.05 (2H, s, pyrrole-CH_2-pyrrole), 5.25 (2H, s, CH_2Ph), 7.25 (5H, s, Ph).

Benzyl 5'-formyl-3',4-di-(2-methoxycarbonylethyl)-4'-methoxycarbonylmethyl-3-methyl-2,2'-methylenedipyrrole-5-carboxylate (**16**)

The foregoing acid (3 g) was decarboxylated as for **28** above to yield **15** as a gum. This was directly formylated as for **29** to yield **16** (1.7 g, 58%) (Found: M^+ 566.2271. $C_{30}H_{34}N_2O_9$ requires: 566.2264). δ 2.02 (3H, s, pyrrole-CH_3), 2.20–3.00 (8H, m, 2 × $CH_2CH_2CO_2$), 3.60, 3.64 and 3.67 (11H, s, 3 × CO_2CH_3 and CH_2CO_2), 3.93 (2H, s, pyrrole-CH_2-pyrrole), 5.20 (2H, s, CH_2Ph), 7.24 (5H, s, Ph), 9.45 (1H, s, CHO), 10.02 and 10.46 (each 1H, br, 2 × NH).

5'-Formyl-3',4-di-(2-methoxycarbonylethyl)-4'-methoxycarbonylmethyl-3-methyl-2,2'-methylenedipyrrole-5-carboxylic acid (**17**)

This was prepared from the foregoing product (1.5 g) as for **31** earlier; it crystallised from CH_2Cl_2–diethyl ether–n-hexane as needles (0.95 g, 75%), m.p. 160–165° (dec) (Found: C, 58.1; H, 6.0; N, 5.9. $C_{23}H_{28}N_2O_9$ requires: C, 58.0; H, 5.9; N, 5.9%). δ 2.03 (3H, s, pyrrole-CH_3), 2.30–3.20 (8H, m, 2 × $CH_2CH_2CO_2$), 3.63 and 3.67 (11H, s, CH_2CO_2 and 3 × CO_2CH_3), 3.96 (2H, s, pyrrole-CH_2-pyrrole), 9.33 (1H, s, CHO), 10.77 (2H), 11.06 (1H) (br, 2 × NH and CO_2H).

5-Formyl-5'-iodo-3,4'-di-(2-methoxycarbonylethyl)-4-methoxycarbonylmethyl-3'-methyl-2,2'-methylenedipyrrole (**18**)

The foregoing acid (0.7 g) was iodinated as for **31** to yield **18** (0.5 g, 60%) (Found: M^+ 558.0855. $C_{22}H_{27}IN_2O_7$ requires: 558.0865). δ 2.00 (3H, s, pyrrole-CH_3), 2.25–2.75 (8H, m, 2 × $CH_2CH_2CO_2$), 3.65 (11H, br s, 3 × CO_2CH_3 and CH_2CO_2), 3.80 (2H, s, pyrrole-CH_2-pyrrole 9.40 (1H, s, CHO), 9.00 and 10.10 (each 1H, br, 2 × NH).

5-Formyl-3,4'-di-(2-methoxycarbonylethyl)-4-methoxycarbonylmethyl-3'-methyl-2,2'-methylenedipyrrole (**19**)

The foregoing iodide (0.4 g) was hydrogenated as for **32** to yield **19** as a gum (150 mg, 48%) (Found: M^+ 432.1924. $C_{22}H_{28}N_2O_7$ requires: 432.1896). δ 2.01 (3H, s, pyrrole-CH_3), 2.30–3.00 (8H, m, 2 × CH_2CH_2), 3.65, 3.67 and 3.69 (11H, s, CH_2CO_2 and 3 × CO_2CH_3), 3.89 (2H, s, pyrrole-CH_2-pyrrole), 6.42 (1H, d, J = 2 Hz, pyrrole-H), 9.43 (1H, s, CHO), 8.69 and 10.09 (each 1H, br, 2 × NH)

1-Formyl-3,8,13,18-tetra-(2-methoxycarbonylethyl)-2,7,12-trismethoxycarbonylmethyl-17-methylbilane (**22**)

The foregoing pyrromethane (20 mg) was reduced as for **33** and **21** was condensed with **20** (60 mg) as for the preparation of **35**. The work up and extensive purification also followed the earlier preparation exactly to yield the amorphous **22** (8 mg, 19%) (Found: M^+ 906.3865. $C_{46}H_{58}N_4O_{15}$ requires: 906.3898). λ_{max} 310 nm. δ 1.94 (3H, s, pyrrole-CH_3), 2.30–2.90 (16H, m, 4 × CH_2CH_2), 3.39, 3.41, 3.60, 3.63, 3.68 and 3.80 (each 2H, s, 3 × CH_2CO_2 and 3 × pyrrole-CH_2-pyrrole), 3.49, 3.59, 3.61, 3.65, 3.66, 3.67 and 3.71 (each 3H, s, 7 × CO_2CH_3), 6.33 (1H, d, J = 2 Hz, pyrrole-H), 9.49 (1H, s, CHO), 8.14 (1H), 8.51 (1H) and 9.38 (2H) (br, 4 × NH).

3,8,13,18-Tetra-(2-carboxyethyl)-2,7,12-triscarboxymethyl-1-hydroxymethyl-17-methylbilane (**6**) *and the corresponding heptamethyl ester* (**23**)

The foregoing formylbilane (5 mg) was reduced as for **35** to yield **23**. This ester was hydrolysed in 2 M KOH (100 μl) under argon for 16 hr at 25° to yield a soln of **6** which was used directly for the enzymic experiments with cosynthetase.

Enzymic experiments

The alkaline soln of **7** prepared as above from **36** (2.9 mg) was diluted to 1.45 ml with water. Part (0.87 ml, 1.74 mg of bilane) of the above soln was incubated at 25° and pH 8.25 in 0.25 M-Tris-HCl buffer (4.3 ml) containing 1 M HCl (96 μl) with cosynthetase[9] (73,500 units). A similar soln was used for the parallel chemical cyclisation but without cosynthetase. Aliquots (50 μl) of each soln were withdrawn at appropriate times and the porphyrinogen produced was converted into the corresponding porphyrin and measured by optical density at 406 nm (scanned) in a standard 3 ml assay[10] (Fig. 1).

At the end of the kinetic experiments, the porphyrinogens in the main soln were oxidised with iodine as usual, the resulting porphyrins were isolated on DEAE-cellulose and decarboxylated in the usual way. The products were esterified and the esters were chromatographed on silica using $CHCl_3$: MeOAc (93 : 3). The porphyrin esters from the enzymic run were examined by ^{1}H-NMR (Fig. 2). The pure porphyrin (**10**) from the chemical ring-closure showed M^+ 652.3263. $C_{38}H_{44}N_4O_6$ requires M 652.3261. λ_{max} ($CHCl_3$) 400, 496, 532, 566, 620 nm. δ 1.87 (3H, t, J = 7 Hz, CH_2CH_3), 3.22–3.30 (6H, m, 3 × CH_2CO_2), 3.65, 3.77 and 3.80 (21H, s, 3 × CO_2CH_3 and 4 × porphyrin-CH_3), 4.10 (2H, q, J = 7 Hz, CH_2CH_3), 4.38–4.47 (6H, m, 3 × porphyrin-CH_2), 10.10 (4H, s, 4 × porphyrin-H).

The same conditions on one tenth the scale were used throughout for kinetic studies on **6**.

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