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BIOSYNTHESIS OF PORPHYRINS AND RELATED MACROCYCLES. PART 27[1]. SYNTHESES OF MODIFIED HYDROXYMETHYLBILANES AND STUDIES OF THEIR CHEMICAL AND BIOLOGICAL PROPERTIES.†

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Abstract: The biosynthetic pathway to uroporphyrinogen-III, the parent macrocycle for all the pigments of life, involves the formation and ring-closure of an hydroxymethylbilane. The non-enzymic ring-closure of this bilane is studied under different pH conditions. Also octamethyl esters of related bilanes are synthesised which have either a cyano or a methyl group blocking position-19 which is free on the terminal ring-D of the natural bilane. Studies are made of the ring-closure of these substituted bilanes under acidic conditions. The conclusion is reached that there is a strong preference for non-enzymic ring-closure of an hydroxymethylbilane to occur at the terminal carbon atom (position-19).

The octa-acids derived from the cyano and methyl substituted bilanes inhibit the action of cosynthetase on the natural hydroxymethylbilane.

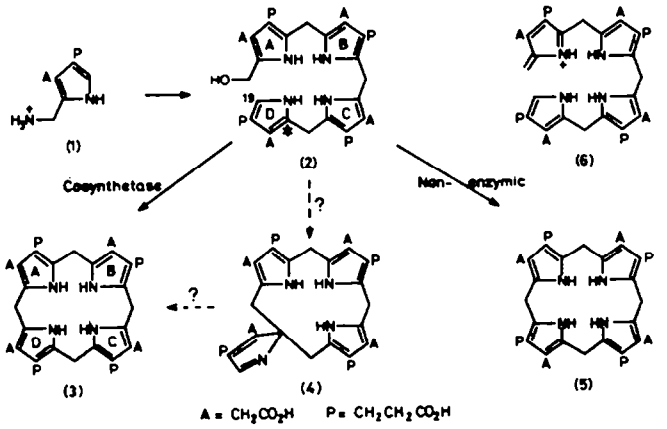
Uroporphyrinogen-III (3), shortened to uro'gen-III, is the biosynthetic parent of all the pigments of life such as protohaem, the chlorophylls and vitamin B_{12}. This central position held by uro'gen-III has led to extensive studies being made of the biosynthesis of the macrocycle (3) starting in the 1950's and continuing, with increasing scope and sophistication, to the present day; the progress made has been reviewed.[2] For our purpose in this paper, it is necessary to concentrate only on the terminal stages shown in Scheme 1. These stages require two enzymes, hydroxymethylbilane synthase (E.C. 4.3.1.8) and uroporphyrinogen-III synthase (E.C. 4.2.1.75); the latter is normally referred to as cosynthetase.

Hydroxymethylbilane synthase builds the unrearranged bilane[3,4] (2) from four molecules of porphobilinogen (1) and the bilane (2) is then ring-closed by cosynthetase, with a single intramolecular rearrangement of ring-D[5,6], to generate uro'gen-III (3). Scheme 1 also shows a possible mechanism for the rearrangement process via the spiro-system[7] (4) and there is evidence from synthetic studies[8,9] that this mechanism is chemically feasible. An alternative mechanistic proposal, which also fits all the experimental data, has been made[4] but has been less studied than the spiro-mechanism. Finally, the hydroxymethylbilane (2) rapidly ring-closes non-enzymically under very mild conditions (ca. pH 8) to form essentially pure uro'gen-I (5), that is, ring-closure without rearrangement[4]. It is probable that ring-closure of the bilane (2) both enzymically and non-enzymically involves the azafulvene (6) (or the unprotonated form) as an intermediate.

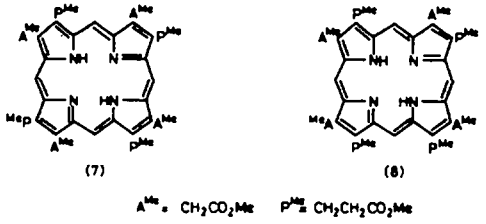
Ring-closure of the Natural Hydroxymethylbilane

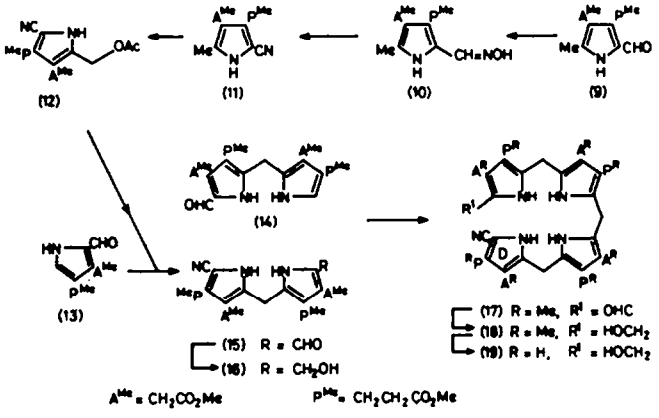
Our first aim was to examine the effect of ring-closing the natural bilane (2) under conditions more acidic than pH 8. At pH 8, many if not all of the acidic side-chains of the bilane (2) will be ionised. So it was conceivable that by

†Dedicated to Ralph Raphael on his 65th birthday.

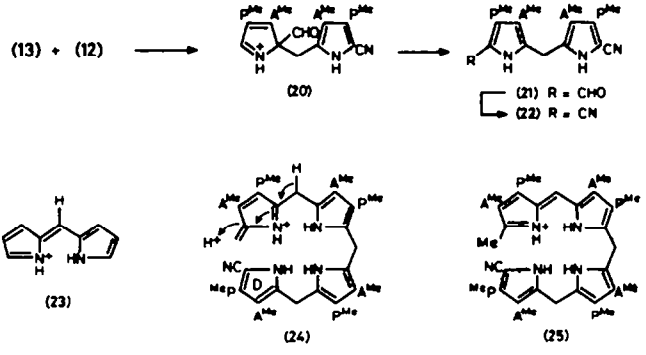


SCHEME 1





SCHEME 2



lowering the pH, thus altering the charges on the side-chains, the preferred conformation of the bilane in solution might be changed. There would then be a chance for ring-closure to occur at the alternative starred site on ring-D [see (2) Scheme 1] finally to produce some uro'gen-III (3) via the spiro system (4).

Accordingly, the hydroxymethylbilane (2) was synthesised essentially as earlier[4]; details of improvements are given in the Experimental part. Separate portions were then treated for 5 mins in the appropriate buffers at pH's 2, 4, 6 and also at pH 8 as standard. The uro'gen(s) thus formed were aromatised by oxidation with iodine and the esters of the resultant porphyrin(s) were fractionated under conditions which separate the type-I (7) and type-III (8) systems[10]. All four experiments gave the same result viz. that the product of acid-catalysed ring-closure of bilane (2) was essentially pure uro'gen-I (5). So there is a pronounced preference for ring-closure onto the terminal α-free position of ring-D in bilane (2) to produce the larger macrocycle (5) rather than the smaller one (4).

Ring-closure of Bilanes Carrying C-19 Substituents

A possible alternative way to affect the direction of ring-closure of an hydroxymethylbilane could be to block the α-free position on ring-D [i.e. position-19, see (2)]. Our second aim therefore was to determine the effect of treating the 19-cyanobilane (18) and the 19-methylbilane (36a) with acid.

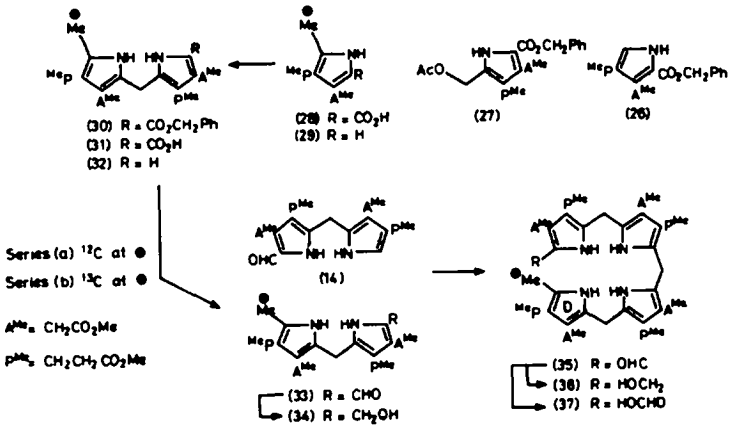
The 19-cyanobilane (18) was synthesised from the pyrromethanes (14) and (16). The former was built as earlier[4] and the route to the latter is shown in Scheme 2. The aldehyde[11] (9) reacted with hydroxylamine to yield a mixture of the syn and anti oximes (10) which, without separation, were converted into the nitrile (11) by treatment with phosphorous oxychloride and dimethylformamide[12]. Finally, the acetoxymethylpyrrole (12) was prepared from the nitrile (11) using lead tetraacetate.

Normally, an acetoxymethylpyrrole is coupled with an α-free pyrrole (e.g. 13) using a catalytic quantity of p.-toluene sulphonic acid but only a very slow reaction occurred under these conditions with the cyanopyrrole (12). One equivalent of acid was needed and then two isomeric pyrromethanes were formed. One was the required pyrromethane (15). The formyl group of the other isomer was converted into the oxime for dehydration as above and the product was shown by n.m.r. to be the symmetrical dicyanopyrromethane (22). It follows that the second intial coupling product was the pyrromethane (21).

This unexpected product presumably arises by attack at the formyl-bearing carbon of pyrrole (13) to yield the protonated pyrrolenine (20) followed by a series of 1,5-sigmatropic rearrangements; related migrations of alkyl groups on similar systems have been previously observed[13].

With the required nitrile (15) in hand, steps analogous to those used for synthesis of the unsubstituted bilane (2) were carried out to yield the 19-cyanobilane (18); see Scheme 2.

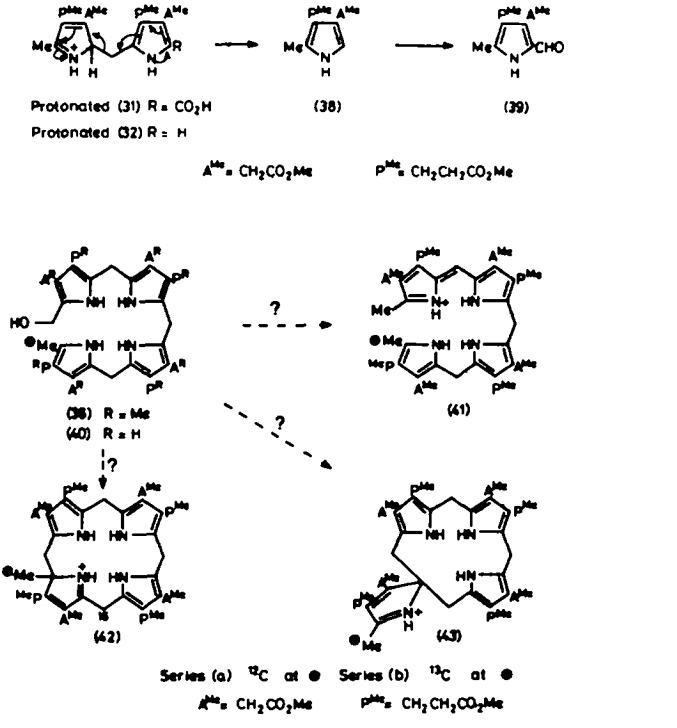
In comparison to the properties of the unsubstituted bilane (2), the 19-cyanobilane (18) was remarkably stable. It was substantially transformed only by treatment with trifluoroacetic acid (TFA) in dichloromethane. Under these conditions, the n.m.r. signal for the $HOCH_2$-group was rapidly lost and the solution became red-orange (strong absorbance at 487 nm). Pyrromethenes (see 23) show absorbance around 480-490 nm and that shown by the red-orange solution corresponded to 25-30% of the original bilane having yielded this chromophore. Also new n.m.r. signals appeared in the region δ 7.4-7.8 where methine protons of pyrromethenes (23) appear[14]. Finally, pyrromethenes are readily reduced to colourless pyrromethanes by borohydride and the colour of the red-orange solution was rapidly discharged by borohydride.



SCHEME 3

It thus seems that ring-closure of the initially formed azafulvene (24) onto the severely deactivated ring-D is so slow that an alternative pathway for stabilisation is followed at least in part. Prototropic rearrangement could yield the bilene (25) and isomers; this contains the pyrromethene chromophore (23). A full investigation was not made because the product of acid treatment of the 19-cyanobilane (18) was obviously complex. So the foregoing conclusion remains a reasonable interpretation rather than established fact. Our hope was that clearer results would come from a different bilane and so it proved in our studies of the 19-methylbilane (36a). This structure retains the blockage at position-19 but avoids the deactivation so evident in the 19-cyano series.

The synthesis of the 19-methylbilane (36a) is shown in Scheme 3 and the required building blocks are the known[4] pyrromethane (14) and the system (34a). Initially, the latter was built from the pyrroles (27) and (29) via (33) but it was found that pyrrole (29) could advantageously be replaced by its precursor (28). Decarboxylation and coupling then occurred under the same conditions to yield the product (30). Hydrogenolysis gave the acid (31) which was thermally decarboxylated



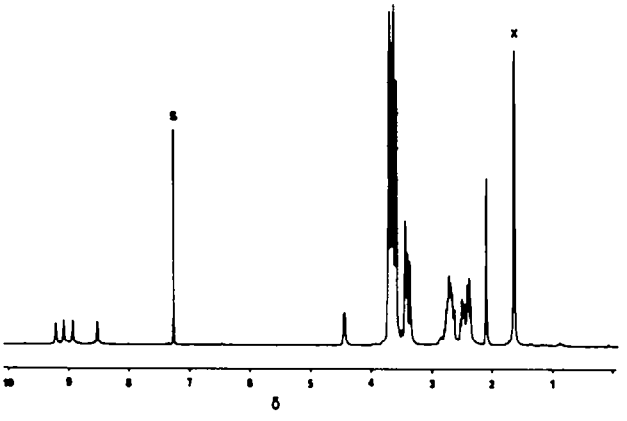


Figure 1a. 250 MHz ^{1}H-n.m.r. of 19-methylbilane (36a) in $CDCl_3$; signal S is from solvent and X from H_2O.

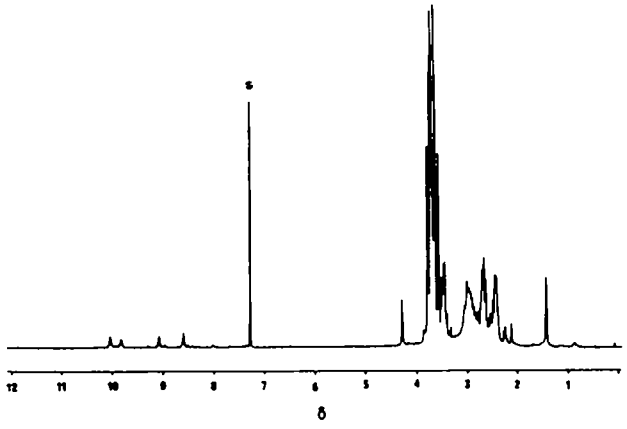


Figure 1b. 250 MHz ^{1}H-n.m.r. of 19-methylbilane (36a) treated with 1 equiv. of TFA in $CDCl_3$; signal S is from solvent.

in refluxing diethylformamide and the product (32) was formylated to provide the desired aldehyde (33). Interestingly, attempted direct formylation of acid (31) with TFA and trimethylorthoformate gave the monopyrrole (39) as the only isolable product. This probably arises by acid-catalysed fragmentation of the protonated pyrromethane (31) or of its decarboxylated product (32) to yield pyrrole (38) which is trapped by formylation.

The remaining steps to the 19-methylbilane (36a) essentially followed those used above for the unsubstituted and 19-cyanobilanes (2) and (18).

Three tetrapyrrolic products can be envisaged from treatment of the 19-methylbilane (36a) with acid. The open-chain bilene (41a) and isomers could be formed analogously to what was observed above for the 19-cyanobilane (19). Alternatively, the two possible modes of ring-closure could yield the spiro-system (43a) or the larger macrocycle (42a). These should all be distinguishable by n.m.r. so a solution (in $CDCl_3$) of the 19-methylbilane (36a) was treated in the n.m.r. tube with one equivalent of TFA. The ^{1}H-n.m.r. spectrum of this solution is shown in Fig. 1b which should be compared with the spectrum (Fig. 1a) from the starting bilane (36a). The important observations are: (a) Fig. 1b still shows 4 NH signals in keeping with there being one major product; (b) the signal at δ 4.42 for the $HOCH_2$-pyrrole residue in Fig. 1a is absent in the product, Fig. 1b; (c) a new signal appears at δ 4.25 from the product which is barely resolved in Fig. 1b but is an AB double doublet and was clearly observed to be so with the slightly different concentrations used for later labelling experiments (e.g. Fig. 2b). This low field signal is one of the two crucially important signals and is assigned to the diastereotopic hydrogens at C-15 of structure (42a); the chemical shift and the AB-pattern are as expected for this environment. We can be sure that the spiro-system (43a) would not show such a low-field signal by having closely related synthetic models available[9]; (d) the second crucial signal was that from the C-

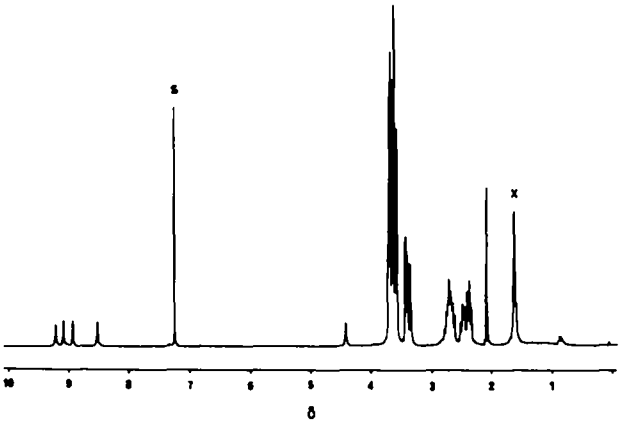


Figure 2a. 250 ^{1}H-n.m.r. of 19-methyl-[$^{2}H_{1}$-hydroxymethyl]bilane (37a) in $CDCl_3$; signal S is from solvent and X from H_2O.

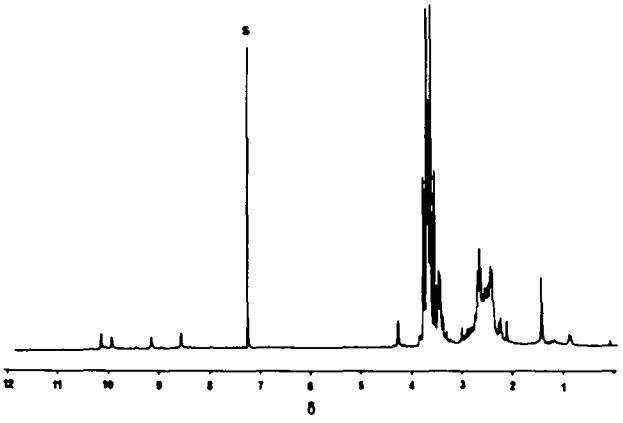


Figure 2b. 250 MHz ^{1}H-n.m.r. of 19-methyl-[$^{2}H_{1}$-hydroxymethyl]bilane (37a) treated with 1 equiv. TFA in $CDCl_3$; signal S is from solvent.

methyl group which moved upfield from δ2.08 in Fig. 1a to δ1.41 in the product, Fig. 1b. The latter position corresponds well with what is expected for structure (**42a**) on the basis of synthetic standards[8] containing a 2-methyl-2H-pyrrolenine system.

The crucial signal assignments above were confirmed by the appropriate labelling experiments. Thus, [^{13}C-methyl]-19-methylbilane (**36b**) was synthesised from the [^{13}C-methyl]pyrrole (**28a**). The latter was obtained by reductive methylation[15], using 90 atom % ^{13}C-paraformaldehyde, of the pyrrole[c.f.][3] (**26**) followed by reductive cleavage of the benzyl ester; the product was diluted with unlabelled material so that the final mixture contained ca. 40 atom % ^{13}C. This was used for synthesis of the [^{13}C-methyl]-19-methylbilane (**36b**) which was ring-closed with TFA as above. The signal at δ1.41 was now a doublet (J=130Hz) superimposed on a singlet (from ^{12}C-material) thus confirming its assignment to the C-methyl group in the product.

In addition, the [$^{2}H_{1}$-hydroxymethyl]bilane (**37a**) was prepared by reduction of the aldehyde (**35a**) with borodeuteride and it showed only 1H at δ4.42, Fig. 2a. This product was treated with TFA as above and the ^{1}H-n.m.r. spectrum of the solution still showed the 2H AB double doublet at δ4.25 (Fig. 2b) in keeping with the earlier assignement of this signal to the CH_2 at C-15.

Finally, because of the results obtained with the 19-cyanobilane (**18**), we checked for the formation of bilenes (e.g. **41a**) from the 19-methylbilane (**36a**). The absence of significant signals in Fig. 1b around δ7-8 pointed against significant quantities of such bilenes being formed and measurement of u.v. absorption at 488 nm. confirmed that conclusion.

The sum of all this evidence shows that the major product from acidic ring-closure of the 19-methylbilene (**36a**) is the macrocycle (**42a**) and not the spiro-

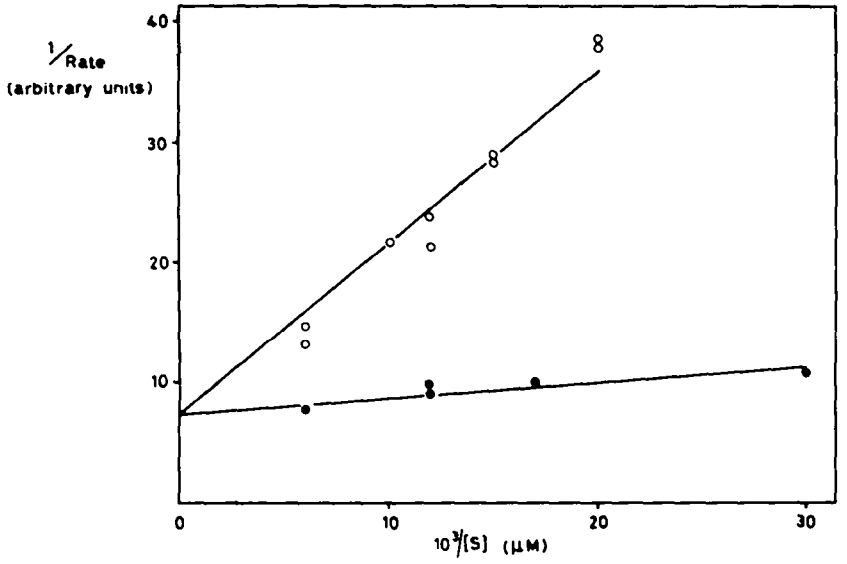


Figure 3. Inhibition of cosynthetase by the 19-methylbilane (40a) Double-reciprocal plots are shown for the cosynthetase-catalysed conversion of hydroxymethylbilane (2) into uroporphyrinogen-III (3) in the presence (O) and absence (●) of 19-methylbilane (40a), 102μM). Assays were performed in 0.2 M-Tris/HCl buffer, pH 8.25, at 25 °C. Duplicate assays were made in each case and both results are shown where these were not identical.

system (43a).

Thus, both the natural bilane (2) and the 19-methylbilane (36a) undergo non-enzymic acid-catalysed ring-closure to the larger macrocycles (5) and (42a), respectively, rather than to the smaller spiro systems (4) and (43a). This can be understood on grounds of steric strain. Examples of the smaller macrocycle present in the spiro system (4) have been synthesised[9] and X-ray analysis of one of these showed the macrocycle to be so tight that considerable puckering occurred to relieve the steric pressures.

Enzymic Studies

Our main interest (see below) was to determine whether the hydrolysed 19-methyl or 19-cyanobilanes (40a) and (19) acted as inhibitors of cosynthetase. Initially, however, we sought for signs as to whether the two bilanes were themselves affected by cosynthetase. In neither case could any evidence for this be found; one example of these experiments will suffice. A solution of the hydrolysed 19-methylbilane (40a) slowly developed absorption at 490 nm (bilene formation?) and the rate of this process was unaffected by addition of cosynthetase; exactly parallel results were found for the hydrolysed 19-cyanobilane (19). It was important in the case of the 19-cyanobilane (19) to check that basic hydrolysis had not destroyed the cyano function. Accordingly, the more readily available 5-cyanopyrromethane (16) was hydrolysed under the same conditions and the i.r. spectrum of the resultant acid showed that the cyano function was intact.

Addition of hydrolysed 19-methylbilane (40a) to cosynthetase assays which use the hydroxymethylbilane (2) as substrate[1] showed that the 19-methylbilane was an effective inhibitor. The kinetic results (Fig. 3) are consistent with the inhibition being competitive with respect to the hydroxymethylbilane (2). The K_i determined from Fig. 3 was 11μM. An alternative approach using a Dixon plot[16] in which the concentration of 19-methylbilane (40a) was varied at a fixed concentration of hydroxymethylbilane (2) showed the inhibition to be linear over the concentrations of 19-methylbilane (40a) used (0-153 μM). The K_i derived from this second plot was 8μM in good agreement with the value from Fig. 3.

The 19-cyanobilane (19) was similarly studied but with less precision. What was clear, however, was that this bilane also inhibits cosynthetase, the results were consistent with the inhibition being competitive with respect to hydroxymethylbilane (2), and the K_i value was ca. 10μM.

EXPERIMENTAL

General Directions

In addition to the directions given in ref. 3, the following should be noted. Anhydrous $MgSO_4$ was also used for drying organic solutions. For substances which were distilled in a Kugelrohr apparatus, the b.p. quoted is the oven temperature. Most ^{1}H-n.m.r. spectra were run at 90 MHz on an EM-390 spectrometer unless otherwise stated. I.r. and u.v. spectra were measured in $CHCl_3$. All solvents were redistilled before use.

Studies on the Hydroxymethylbilane (2)

Benzyl 5-Acetoxymethyl-3-(2-methoxycarbonylethyl)-4-methoxy-carbonylmethylpyrrole-2-carboxylate

Freshly distilled sulphuryl chloride (2.9 g, 21.5 mmol) was added dropwise to a stirred solution of benzyl 5-methyl-3-(2-methoxycarbonylethy)-4-methoxycarbonylmethylpyrrole-2-carboxylate (7.8 g, 20.9 mmol) in CH_2Cl_2 (50 ml). The solution was stirred at 18 °C for 1.5 h then evaporated and the residue was mixed with NaOAc (5 g, 60.9 mmol) in HOAc (150 ml). The mixture was stirred at 60 °C for 20 min then evaporated and the residue was slurried with water (50 ml). The solid was collected by filtration, washed with water (2 x 50 ml) and dried. The product was dissolved in CH_2Cl_2 and filtered through silica gel (2 x 2 cm), washing with more CH_2Cl_2–ether–hexane to give the title acetoxymethylpyrrole (7.44 g, 83%) m.p. 106.5–109 °C (lit.[17], m.p. 107–108 °C).

Benzyl 5'-Formyl-4,3'-di(2-methoxycarbonylethyl)-3,4'-bismethoxy-carbonylmethyl-2,2'-methylenedipyrrole-5-carboxylate

A solution of benzyl 5'-t.butyloxycarbonyl-4,3'-di(2-methoxycarbonylethyl)-3,4'-bismethoxycarbonylmethyl-2,2'-methylenedipyrrole-5-carboxylate (2.94 g, 4.22 mmol) in TFA (20 ml) was stirred at 18 °C in the dark, under argon for 2 h, then cooled in ice and treated with freshly distilled $HC(OMe)_3$ (20 ml). After 20 min, the solution was poured into aqueous Na_2CO_3 (10%, 100 ml) and the product was extracted into CH_2Cl_2 (3 x 50 ml). The residue from the organic extracts was purified by flash chromatography on silica (15 x 3 cm) using 6:4 EtOAc:hexane and crystallisation from CH_2Cl_2–ether–hexane to give the formylpyrromethane (2.24 g, 85%), m.p. 119–121 °C (lit.[4] 119–120.5 °C).

Non-enzymic Ring-closure of Hydroxymethylbilane (2)

The octamethyl ester of (2) (25.9 mg) was stirred in the dark under argon, with argon saturated aqueous KOH (2N, 1 ml). Aliquots (0.10 ml) of this solution were added 0.05M pH 2.0 (KCl–HCl), 4.0 (Phthalate–HCl), 6.0 (KH_2PO_4–NaOH) and 8.0 (KH_2PO_4–NaOH) buffers.[18] Aqueous HCl (0.2N, 1 ml) was added to each buffer with the alkaline solution to neutralise the KOH. After 5 min, the solutions were adjusted to pH 8 with aqueous NaOH (0.1N) and then mixed with I_2 (0.5%) in aqueous KI (1.0%, 1 ml). Excess I_2 was destroyed after 5 min with aqueous $NaHSO_3$ (5%, 1ml) and a slurry of DEAE cellulose (DE52) (5 g) was added. After being stirred for 15 min, the resin was collected and washed with water (100 ml). For the pH 2 run, the DEAE cellulose treatment was repeated. The porphyrin was eluted from the cellulose with methanolic HCl (100 ml) and the dried residues from evaporation were treated with MeOH–18M H_2SO_4–$HC(MeO)_3$ (20:1:2, 20 ml). After 16 h, the mixtures were poured into water (100 ml), neutralised with aqueous NH_3 (d0.88) and extracted with CH_2Cl_2 (50 ml, 3 x 25 ml). Chromatography of the products on silica (1 g in a Pasteur pipet eluting with 1:99 CH_2Cl_2) gave uroporphyrin octamethyl esters as shown below.

pH	Yield of uroporphyrin ester	% Yield
2.0	2.1 mg	82
4.0	1.6 mg	63
6.0	1.1 mg	44
8.0	0.74 mg	29

Each product was fractionated by h.p.l.c.[10] on Spherosorb 55W with 1:1 hexane: EtOAc at 1 ml/min^{-1} with chart speed 120 mm/h^{-1} and the detector set at 400 nm. These conditions were demonstrated to give a good separation of authentic samples uroporphyrin-I and uroporphyrin-III octamethyl esters. All the above products proved to be essentially pure type-I esters.

Studies on the 19-Cyanobilane (18)

2-Acetoxymethyl-5-cyano-4-(2-methoxycarbonylethyl)-3-methoxy-carbonylmethylpyrrole (12)

A mixture of the formylpyrrole[11] (9) (2.68 g, 10 mmol), $NH_2OH.HCl$ (0.78 g, 11 mmol) and NaOAc (0.82 g, 10 mmol) in MeOH (10 ml) was heated under reflux for 45 min, then evaporated. The residue was dissolved in CH_2Cl_2 (25 ml), washed with 5% aqueous $NaHCO_3$ (25 ml), dried and evaporated to yield syn and anti forms of the oxime (10) as an oil; δ 2.14 (0.9H, s, Ar-CH_3), 2.20 (2.1H, s, Ar-CH_3), 2.39-2.57 (2H, m, $CH_2\underline{CH_2}CO_2Me$), 2.73-2.97 (2H, m, $\underline{CH_2}CH_2CO_2Me$), 3.39 (0.6H, s, $\underline{CH_2}CO_2Me$), 3.40 (1.4H, s, $\underline{CH_2}CO_2Me$), 3.63 and 3.67 (each 3H, s, 2 X CO_2CH_3), 7.33 (0.7H, s, $\underline{CH}NOH$), 8.09 (0.3H, s, $\underline{CH}NOH$), 8.42 (0.7H, br, NH), 8.62 (0.3H, br, NH), 9.63 (0.3H, br, OH) and 10.00 (0.7H, br, OH).

$POCl_3$ (1.5 ml) was added to a solution of all the oxime in dry DMF (8 ml) at -20 °C. After 15 min at -20 °C, the mixture was warmed to room temperature over 1 h and then treated with 15% aqueous NaOAc (25 ml). Stirring was continued for 5 min and then the mixture was neutralized with saturated aqueous K_2CO_3, diluted with water (40 ml) and extracted with CH_2Cl_2 (3 x 20 ml). The combined extracts were washed with water (3 X 20 ml) and yielded the cyanopyrrole as an oil; δ 2.13 (3H, s, Ar-CH_3), 2.48-2.66 (2H, m, $CH_2\underline{CH_2}CO_2Me$), 2.78-2.97 (2H, m, $\underline{CH_2}CH_2CO_2Me$), 3.40 (2H, s, $\underline{CH_2}CO_2Me$), 3.68 (6H, s, 2 X CO_2CH_3) and 9.71 (1H, br, NH).

All the cyanopyrrole in HOAc (20 ml) and Ac_2O (4 ml) at 80 °C was treated with $Pb(OAc)_4$ (7.7 g) until t.l.c. analysis showed no remaining starting material. The cool mixture was treated with $(CH_2OH)_2$ (5 ml) then diluted with water (20 ml) and extracted with CH_2Cl_2 (50 and 3 X 20 ml). The combined extracts were washed with 5% aqueous $NaHCO_3$ (2 X 100 ml) and preparative t.l.c. (eluent ether-hexane, 4:1) of the product yielded the acetoxymethylpyrrole (1.89 g) as an oil (Found: M^+, 322.1155. $C_{15}H_{18}N_2O_6$ requires M, 322.1160); υ_{max} 3 420, 3 300br, 2 220 and 1 730 cm^{-1}; δ 2.04 (3H, s, CH_3CO_2), 2.47-2.67 (2H, m, $CH_2\underline{CH_2}CO_2Me$), 2.79-2.98 (2H, m, $\underline{CH_2}CH_2CO_2Me$), 3.50 (2H, s, $\underline{CH_2}CO_2Me$), 3.67 (6H, s, 2 X CO_2CH_3), 5.01 (2H, s, $\underline{CH_2}OAc$) and 9.78 (1H, br, NH); $m/_z$ 322 (21%, M^+), 271 (27), 262 (75, $M^+ - CH_3CO_2H$), 230 (77), 220 (65), 171 (100) and 143 (85).

5-Cyano-5'-formyl-4,3'-di-(2-methoxycarbonylethyl)-3,4'-bismethoxy carbonylmethyl-2,2'-methylenedipyrrole (15)

A mixture of the foregoing acetoxymethylpyrrole (320 mg, 1 mmol), the formylpyrrole[19] (13) (250 mg, 1 mmol) and anhydrous p-TosOH (170 mg, 1 mmol) in dry CH_2Cl_2 (0.5 ml) was stirred under nitrogen for 4 h then diluted with CH_2Cl_2 (10 ml). After washing the solution with 5% aqueous $NaHCO_3$ (20 ml), the residue from evaporation gave by p.l.c. (eluent ether) a mixture of two compounds which were separated by p.l.c. (eluted twice with CH_2Cl_2-ether, 1:11, containing 1% MeOH). The higher R_f product was 5-cyano-5'-formyl-4,4'-di-(2-methoxycarbonylethyl)-3,3'-bismethoxycarbonylmethyl-2,2'-methylenedipyrrole (21) (74 mg, 14%), see below, m.p. 132-134 °C from EtOAc. (Found: C, 58.0; H, 5.7; N, 8.0. $C_{25}H_{29}N_3O_9$ requires C, 58.2; H, 5.7; N, 8.15%); υ_{max} 3 300br, 2 220, 1 735 and 1 650 cm^{-1}; δ 2.54 (2H, t, J 7.3 Hz, $CH_2\underline{CH_2}CO_2Me$), 2.57 (2H, t, J 7.6 Hz, $CH_2\underline{CH_2}CO_2Me$), 2.86 (2H, t, J 7.6 Hz, $\underline{CH_2}CH_2CO_2Me$), 3.01 (2H, t, J 7.3 Hz, $\underline{CH_2}CO_2Me$), 3.56 and 3.58 (each 2H, s, 2 X $\underline{CH_2}CO_2Me$), 3.63, 3.66, 3.80 and 3.82 (each 3H, s, 4 X CO_2CH_3), 3.83 (2H, s, methane CH_2), 9.58 (1H, s, CHO), 10.30 and 10.43 (each 1H, br, 2 X NH); $m/_z$ 515 (M^+).

The lower R_f product was the desired pyrromethane (15) (159 mg, 31%), m.p. 109-110 °C from EtOAc. (Found: C, 58.3; H, 5.6; N, 8.3. $C_{25}H_{29}N_3O_9$ requires C, 58.25; H, 5.7; N, 8.15%); υ_{max} 3

300br, 2 225, 1 740 and 1 650 cm^{-1}; δ2.54 (2H, t, J 7.6 Hz, $CH_2\underline{CH_2}CO_2Me$), 2.58 (2H, t, J 6.5 Hz, $CH_2\underline{CH_2}CO_2Me$), 2.76 (2H, t, J 6.5 Hz, $\underline{CH_2}CH_2CO_2Me$), 2.83 (2H, t, J 7.6 Hz, $\underline{CH_2}CH_2CO_2Me$), 3.51 (2H, s, $\underline{CH_2}CO_2Me$), 3.63, 3.64 and 3.66 (each 3H, s, 3 X CO_2CH_3), 3.71 (2H, s, $\underline{CH_2}CO_2Me$), 3.73 (3H, s, CO_2CH_3), 3.92 (2H, s, methane CH_2), 9.48 (1H, s, CHO), 10.32 and 10.53 (each 1H, br, 2 X NH); $m/_z$ 515 (M^+).

5,5'-Dicyano-4,4'-di-(2-methoxycarbonylethyl)-3,3'-bismethoxycarbonylmethyl-2,2'-methylenedipyrrole (22)

A solution of the 5-cyano-5'-formylpyrromethane (21) (52 mg) in MeOH (0.2 ml) was heated under reflux for 1.5 h with $NH_2OH.HCl$ (8 mg) and NaOAc (9 mg) and then evaporated. The residue in CH_2Cl_2 (5 ml) was washed with 5% aqueous $NaHCO_3$ (10 ml) and the product from the dried CH_2Cl_2 solution was treated at -20 °C in dry DMF (0.2 ml) with $POCl_3$ (15 μl). After 15 min, the mixture was warmed during 30 min to 18 °C, then diluted with water (2 ml), neutralised with saturated aqueous K_2CO_3 and extracted with CH_2Cl_2 (3 X 5 ml). The extracted material was purified by p.l.c. on silica with ether to give the dicyanopyrromethane (22) (34 mg, 65%); δ (400MHz) 2.56 (4H, t, 2 X $CH_2\underline{CH_2}CO_2$), 2.85 (4H, t, 2 X $\underline{CH_2}CH_2CO_2$), 3.56 (4H, s, 2 X CH_2CO_2), 3.65 and 3.79 (each 6H, s, 4 X OCH_3), 3.77 (2H, s, methane-CH_2), 10.47 (2H, br, s, 2 X NH); $m/_z$ Found: 512.1915 $C_{25}H_{28}N_4O_8$ requires 512.1907.

1-Cyano-19-formyl-2,7,12,17-tetra(2-methoxycarbonylethyl)-3,8,13,18-tetrakismethyoxycarbonylmethylbilane (17)

$NaBH_4$ (40 mg) was added in several portions to a stirred solution of the pyrromethane (15) (42 mg), in dry MeOH (1 ml) containing NEt_3 (6 drops). After 10 min, the mixture was diluted with CH_2Cl_2 (10 ml), washed with brine (3 X 4 ml), and the product from evaporation, [which was the hydroxymethylpyrromethane] was dissolved in CH_2Cl_2 (4 ml) containing NEt_3 (2 drops) and added dropwise over 5 min to a stirred solution of the formylpyrromethane[4] (14) (40 mg) in CH_2Cl_2 (3 ml) and HOAc (1 ml) under argon in the dark. After 15 min, the mixture was washed with 5% aqueous $NaHCO_3$ (50 ml), dried and evaporated to ca. 0.5 ml under argon. MeOH (3 ml) was added to the residue and the mixture was again evaporated. This procedure was repeated and the resulting solid was collected by centriguation and purified by p.l.c. (eluent $CHCl_3$:MeOH, 19:1, containing 0.1% i-$PrNEt_2$). The formylbilane (17) (25 mg, 31%) was recovered with minimum exposure to air and light as a powder, m.p. 140-145 °C (decomp.); δ (CD_2Cl_2) 2.40-2.60 (8H, m, 4 X $CH_2\underline{CH_2}CO_2Me$), 2.69-2.88 (8H, m, 4 X $\underline{CH_2}CH_2CO_2Me$), 3.45-3.86 (38H, each s, 4 X $\underline{CH_2}CO_2Me$, 3 X methane CH_2 and 8 X CO_2CH_3), 9.20 and 9.38 (each 1H, br, 2 X NH), 9.52 (1H, s, CHO), 9.82 and 9.98 (each 1H, br, 2 X NH); $m/_z$ 990 (M^+ + 1).

19-Cyano-1-hydroxymethyl-3,8,13,18-tetra-2(2-methoxycarbonylethyl)-2,7,12,17-tetrakismethoxycarbonylmethylbilane (18)

$NaBH_4$ (60 mg) was added in one portion to a stirred solution of the foregoing formylbilane (15 mg) in $CHCl_3$ (1.5 ml) containing MeOH (0.6 ml) and NEt_3 (5 drops). After 5 min, the mixture was diluted with $CHCl_3$ (5 ml), washed with brine (3 X 2 ml), dried and evaporated with a stream of argon. The residue was purified by p.l.c. (eluent $CHCl_3$:MeOH, 19:1 containing 0.1% NEt_3), the plate being run in the dark under argon. The product was recovered with minimum exposure to air and light by extraction from the plate with $CHCl_3$:MeOH, 19:1, containing 0.1% NEt_3 (10 ml). The extract was concentrated to ca. 0.2 ml under argon, then diluted with MeOH (2 ml) and concentrated as before. The residue was suspended in MeOH (0.5 ml) containing NEt_3 (3 drops) and the resulting solid was collected by centrifugation to yield the cyano-hydroxymethylbilane (18) (6 mg, 40%) as a powder; δ (CD_2Cl_2) 2.30-2.61 (9H, m 4 X $CH_2\underline{CH_2}CO_2Me$ and OH), 2.71-2.87 (8H, m, 4 X $\underline{CH_2}CH_2CO_2Me$), 3.43, 3.46, 3.45, 3.52, 3.58, 3.62, 3.67, 3.68, 3.69, 3.69, 3.70, 3.72, 3.74, 3.75 and 3.82 (38H, each s, 4 X $\underline{CH_2}CO_2Me$, 3 X methane CH_2 and 8 X CO_2CH_3), 4.44 (2H, s, $\underline{CH_2}OH$), 9.16 (2H, br, 2 X NH), 9.35 and 10.09 (each 1 H, br, 2 X NH); $m/_z$ 992 (M^+ + 1), 991 (M^+), 976, 975, 974 and 973.

Studies on the 19-Methylbilane (36a)

3,4'-Di(2-methoxycarbonylethyl)-4,3'-bismethoxycarbonylmethyl-5'-methyl-2,2'-methylenedipyrrole-5-carboxylic acid (29a)

A solution of the benzyl ester of (28a) (6 g) in MeOH (500 ml) was hydrogenated at room temperature over Pd-C (10%, 0.5 g). When hydrogen uptake ceased, the solution was filtered and the residue from evaporation was recrystallized from CH_2Cl_2-ether-hexane to give pyrrolecarboxylic acid

(28a) (4.13 g, 91%), m.p. 133-138 °C (decomp.) δ 2.23 (3 H, s, CH_3), 2.30-2.87 (4H, m, $CH_2CH_2CO_2$), 3.70 and 3.73 (each 3H, s, 2 X OCH_3), 3.86 (2H, s, CH_2CO_2), 5.20 (1H, br, COOH), 9.00 (1H, br, NH); m/z 283 (M^+).

A solution of the foregoing acid (28a) (1 g) in TFA (20 ml) was stirred at room temperature for 30 min. Evaporation of the solvent and flash chromatography of the residue on silica (15 X 1 cm) with 3:2 ether:hexane gave the unstable pyrrole (29a) (730 mg, 86%) as an oil. δ 2.15 (3 H, s, CH_3), 2.23-2.90 (4H, m, $CH_2CH_2CO_2$), 3.45 (2H, s, CH_2CO_2), 3.67 and 3.69 (each 3H, s, 2 X OCH_3), 6.53 (1H, d, J=3Hz, pyrrole-H), 8.07 (1H, br, NH); m/z 239 (M^+). This product could be converted into the desired material but the following method was simpler.

p-TosOH (0.72 g) was added to a stirred solution of acid (28a) (1.5 g, 5.3 mmol) and acetoxymethylpyrrole (27) (1.52 g, 3.52 mmol) in CH_2Cl_2 (20 ml). The solution was stirred in the dark for 1 h at 18 °C under argon and then evaporated. Flash chromatography of the residue on silica (15 X 3 cm) with 2:3 EtOAc:hexane, then 1:1 EtOAc:hexane gave benzyl 3,4'-di(2-methoxycarbonylethyl)-4,3'-bismethoxycarbonylmethyl-5'-methyl-2,2'-methylenedipyrrole-5-carboxylate (30a) as an oil. (Found: 610.2515 $C_{32}H_{38}N_2O_{10}$ requires 610.2526). δ 2.15 (3H, s, CH_3), 2.30-3.00 (8H, m, 2 X $CH_2CH_2CO_2$), 3.53 (2H, s, CH_2CO_2), 3.67, 3.68,, 3.69 and 3.71 (each 3H, s, 4 X OCH_3), 3.90 (4H, s, CH_2CO_2 and methane-CH_2), 5.30 (2H, s, OCH_2Ph), 7.45 (5H, m, PH), 8.70 and 9.05 (each 1H, br, 2 X NH).

All the foregoing product in MeOH (15 ml) was hydrogenated at 18 °C over Pd-C (10%, 100 mg). After hydrogen uptake ceased, the solution was filtered (Celite) and the residue from evaporation was recrystallised from CH_2Cl_2-hexane to give the pyrromethanecarboxylic acid (29a) (695 mg, 38% over two steps), m.p. 150-154 °C (decomp.) (Found: C, 57.6; H, 6.1; N, 5.4. $C_{25}H_{32}N_2O_{10}$ requires C, 57.7; H, 6.2; N, 5.4%); ν_{max} 3 660-2450 br, 3420m, 3010m, 1730s, 1665s cm^{-1}; δ (250 MHz) 2.10 (3H, s, CH_3), 2.39 (2H, dd J = 8.5, 6.1 Hz, $CH_2CH_2CO_2$), 2.57 (2H, t, J=6.2 Hz, $CH_2CH_2CO_2$), 2.68 (2H, dd, J=9.7, 7.1 Hz, $CH_2CH_2CO_2$), 2.79 (2H, t, J=7.2 Hz, $CH_2CH_2CO_2$), 3.47 (2H, s, CH_2CO_2), 3.63, 3.65, 3.67 and 3.74 (each 3H, s, 4 X OCH_3), 3.82 and 3.83 (each 2H, s, CH_2CO_2 and methane-CH_2), 8.55 and 9.82 (each 1H, br, 2 X NH); m/z 520 (M^+).

5-Formyl-3,4'-di(2-methoxycarbonylethyl)-4,3'-bismethoxycarbonylmethyl-5'-methyl-2,2'-methylenedipyrrole (33a)

A solution of acid (31a) (294 mg, 0.565 mmol) in freshly distilled Et_2NCHO (3 ml) was heated at reflux for 3 h. The solvent was removed by Kugelrohr distillation (100 °C, 19 mm) then the residue (32a) in DMF (2 ml) was stirred at 18 °C under argon with PhCOCl (167 mg, 1.19 mmol) for 16 h. The solution was then poured into aqueous Na_2CO_3 (10%, 50 ml) and extracted with CH_2Cl_2 (1 X 50 ml, 3 X 25 ml) the extracted product being taken up in ether (100 ml) and washed with water (4 x 25 ml). Preparative t.l.c. of the product from the ether with 7:3 EtOAc:hexane and recrystalisation from CH_2Cl_2-ether-hexane gave aldehyde (33a) (133 mg, 47%), m.p. 126-127.5 °C. (Found: C, 60.0; H, 6.4; N, 5.5. $C_{25}H_{32}N_2O_9$ requires C, 59.5; H, 6.4; N, 5.5%). ν_{max} 3410w, 3310w, 3010, 1730s, 1645 cm^{-1}; λ_{max} 331 nm; δ (250 MHz) 2.11 (3H, s, CH_3), 2.40 (2H, m, $CH_2CH_2CO_2$), 2.65 (4H, m, $CH_2CH_2CO_2$, 2.83 (2H, m, $CH_2CH_2CO_2$), 3.48 (2H, s, CH_2CO_2), 3.64, 3.66, 3.68 and 3.80 (each 3H, s, 4 X OCH_3), 3.74 and 3.85 (each 2H, s, CH_2CO_2 and methane-CH_2), 8.58 (1H, br, NH), 9.55 (1H, s, CHO), 10.30 (1H, br, NH); m/z Found: 504.2105; $C_{25}H_{32}N_2O_9$ requires 504.2105.

1-Formyl-3,8,13,18-tetra(2-methoxycarbonylethyl)-2,7,12,17-tetrakismethoxy-carbonylmethyl-19-methylbilane (35a)

$NaBH_4$ (20.2 mg, 0.53 mmol) was added to a stirred solution of formylpyrromethane (33a) (19.1 mg, 0.038 mmol) in MeOH (1 ml) containing NEt_3 (3 drops). After 10 min, CH_2Cl_2 (10 ml) was added and the solution was washed with brine (3 X 5 ml). The combined aqueous solution was extracted with CH_2Cl_2 (5 ml) and the product from the combined CH_2Cl_2 solutions in CH_2Cl_2 containing NEt_3 (3 drops) was added dropwise to a stirred solution of aldehyde (14) (41.8 mg, 0.085 mmol) in CH_2Cl_2 (5 ml) containing HOAc (1 ml) under argon. After 10 min at 18 °C, the solution was washed with brine (20 ml) and neutralized with solid $NaHCO_3$. The organic phase was washed with water (2 X 10 ml) and the residue therefrom was triturated with MeOH (2 ml) and evaporated again with an argon stream. The residue was resuspended in MeOH (2 ml) and centrifued. The supernatant (containing excess pyrromethane (14) was removed and replaced by fresh MeOH. This trituration–centrifugation

procedure was repeated twice more and the final solid residue was further purified by preparative t.l.c. on silica under argon eluting with 1:19 MeOH:$CHCl_3$. The product was made solid using MeOH as above to give formylbilane (35a) (12.2 mg, 33%). (Found: m/z 978.4109. $C_{49}H_{62}N_4O_{17}$ requires 978.4110; υ_{max} 3 390 m, 3000 m, 1730s, 1650s cm^{-1}; λ_{max} 311 nm; δ (400 MHz) 2.08 (3H, s, CH_3), 2.35–2.51 (8H, m, 4 X $CH_2CH_2CO_2$), 2.62–2.81 (8H, m, 4 X $CH_2CH_2CO_2$), 3.379, 3.383 and 3.42 (each 2H, s, 3 X CH_2CO_2), 3.58, 3.60, 3.62, 3.64, 3.647, 3.654, 3.67 and 3.70 (each 3H, s, 8 X OCH_3), 3.628, 3.637, 3.72 and 3.80 (each 2H, s, CH_2CO_2 and 3 X methane-CH_2), 8.47, 9.17 and 9.29 (each 1H, br, 3 X NH), 9.50 (1H, s, CHO), 9.85 (1H, br, NH).

1-Hydroxymethyl-3,8,13,18-tetra(2-methoxycarbonylethyl)-2,7,12,17-tetrakismethoxycarbonylmethyl-19-methylbilane (36a)

$NaBH_4$ (13.8 mg) was added to a stirred solution of formylbilane (35a) (6.5 mg) in 5:2 $CHCl_3$:MeOH (0.7 ml) at 18 °C. After 15 min, the solution was diluted with $CHCl_3$ (2 ml) and extracted with brine (3 X 1 ml). The residue from the organic solution was triturated with MeOH and the MeOH was evaporated with nitrogen. The residue was resuspended in MeOH (2 ml) and centrifuged and the solid was dried in vacuo to give hydroxymethylbilane (36a) (5.6 mg, 86%). (Found: 980.4248. $C_{49}H_{64}N_4O_{17}$ requires 980.4266); υ_{max} 3440 br, 3030m, 1770s cm^{-1}; λ_{max} 244 nm; δ(400 MHz) 2.08 (3H, s, CH_3), 2.30–2.90 (16H, m, 4 X $CH_2CH_2CO_2$), 3.35, 3.39, 3.42 and 3.43 (each 2H, s, 4 X CH_2CO_2), 3.56, 3.59, 3.61, 3.63, 3.64, 3.67 and 3.68 (each 3H s, 8 X OCH_3), 3.58, 3.69 and 3.71 (each 2H, s, 3 X methane-CH_2), 4.43 (2H, d, J=5.4Hz, CH_2OH), 8.52, 8.92, 9.07 and 9.20 (each 1H, br, 4 X NH).

^{13}C-Labelled and ^{2}H-Labelled Series

4-(2-Methoxycarbonylethyl)-3-methoxycarbonylmethyl-5-[^{13}C]methylpyrrole-2-carboxylic acid (28b)

The benzyl ester of the title acid was prepared as earlier[6] from [^{13}C] paraformaldehyde (90 atom %) and part (112 mg) was diluted with unlabelled material (112 mg). This was hydrogenolysed as for unlabelled material above to yield the acid (148 mg, 92%) having an n.m.r. spectrum identical to that of the unlabelled sample save that the signal at δ 2.23 was a doublet $^{13}CH_3$ (J=128Hz) superimposed on a singlet $^{12}CH_3$; δ_C 11.67 ($^{13}CH_3$). m/z 284 ($^{13}C_1{}^{12}C_{12}H_{17}NO_6$) and 283 ($^{12}C_{13}H_{17}NO_6$).

Hydroxymethyl-3,8,13,18-tetra(2-methoxycarbonylethyl)-2,7,12,17-tetrakismethoxy-carbonylmethyl-19-[^{13}C]methylbilane (36b)

The synthesis of this labelled bilane followed the unlabelled series exactly; the data for the intermediates and final bilane were as follows.

Acid (28b) (268 mg) and pyrrole (27) (407 mg) gave the [^{13}C-methyl] methylenedipyrrole (31b) (128 mg, 26%), m.p. 148–153 °C (decomp.). The signal at δ 2.10 was a doublet $^{13}CH_3$ (J=127Hz) superimposed on a singlet $^{12}CH_3$; δ_C 11.12 ($^{13}CH_3$). m/z 521 ($^{13}C_1{}^{12}C_{24}H_{32}N_2O_{10}$), 520 ($^{12}C_{25}H_{32}N_2O_{10}$).

This product (120 mg) yielded the 5-formyl-[^{13}C-methyl]methylenedipyrrole (33b) (34 mg, 29%), m.p. 124–126 °C; δ 2.11 doublet $^{13}CH_3$ (J=127Hz) superimposed on a singlet $^{12}CH_3$; δ_C 11.12 ($^{13}CH_3$). m/z 505 ($^{13}C_1{}^{12}C_{24}H_{32}N_2O_9$), 504 ($^{12}C_{25}H_{32}N_2O_9$).

The foregoing product (19 mg) and pyrromethane (14) (47 mg) gave the formyl-19-[^{13}C-methyl]bilane (35b) (12.3 mg, 33%); δ 2.08 (3H, d, J=127Hz, $^{13}CH_3$ superimposed on s, $^{12}CH_3$) δ_C 11.09 ($^{13}CH_3$).

The labelled formylbilane (8.2 mg) was reduced to the hydroxymethyl-19-[^{13}C-methyl]bilane (36b) (5.8 mg, 71%); δ 2.08 (3H, d, J=127Hz, $^{13}CH_3$ superimposed on s, $^{12}CH_3$).

The unlabelled formylbilane (35a) (5.1 mg) was reduced as earlier with $NaBD_4$ (10 mg) to yield 1-([$^{2}H_1$]hydroxymethyl)-3,8,13,18-tetra(2-methoxycarbonylethyl)-2,7,12,17-tetrakismethoxycarbonylmethyl-19-methylbilane (37a), (4.7 mg, 92%); ^{1}H-n.m.r. identical with unlabelled material except for δ4.43 (1H, br, HOCHD).

Acid catalysed ring-closure of unlabelled and labelled forms of 19-methylbilane (36)

The details of these experiments and the n.m.r. spectra obtained are given in the discussion section.

Enzymic experiments

The bilane esters (18) and (36a) were hydrolysed at 18–20 °C with aqueous 2N-KOH (100 l/mg of

bilane) for 16-18 h under argon or nitrogen and and the final alkaline solution was carefully adjusted to pH 8-9 with HCl. Assays for cosynthetase activity were performed as earlier[1] using various concentrations (range 20-166 μM) of hydroxymethylbilane (2) without and with 19-methylbilane (**40a**) (102 μM) or 19-cyanobilane (**19**) (13.5 μM). For the Dixon plot, the concentration of 19-methylbilane (**40a**) was varied over the range 0-153 μM and that of the hydroxymethylbilane (2) was held at 50 μM.

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