Biosynthesis of Porphyrins and Related Macrocycles. Part 16.[1,2] Proof that the Single Intramolecular Rearrangement leading to Natural Porphyrins (Type-III) occurs at the Tetrapyrrole Level

By **Alan R. Battersby,* Christopher J. R. Fookes, Mary J. Meegan, Edward McDonald,** and **Hanns K. W. Wurziger,** University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW

The unrearranged aminomethylbilane (2) is synthesised by a rational route and is proved to be converted by the enzymes deaminase and cosynthetase, working co-operatively, into uro'gen-III (3). The single rearrangement step established earlier is thus proved to take place at the tetrapyrrole level. Synthesis of singly ^{13}C-labelled bilane (2) followed by its enzymic conversion into uro'gen-III serves to register each of the pyrrole rings of the product relative to the initial bilane. Finally, methods for synthesis of the bilane are developed in two different doubly ^{13}C-labelled forms to allow the following key points to be established largely by ^{13}C n.m.r. spectroscopy: (*a*) as the bilane system is converted into uro'gen-III, intramolecular rearrangement of the terminal ring D occurs, and (*b*) the linear tetrapyrrole is converted intact into uro'gen-III.

Syntheses of [15-^{13}C]-, and [20-^{13}C]-uroporphyrin octamethyl ester are described together with improved h.p.l.c. conditions for the separation of isomeric coproporphyrin tetramethyl esters.

THE curious and puzzling results[3] from biosynthetic studies with the unrearranged aminomethylpyrromethane (1) eventually led to the hypothesis[1] (Scheme 1) that *chemical* dimerisation of (1) might be generating the aminomethylbilane (2) *in situ* which could undergo enzymic conversion into uro'gen-III (3). Strong circumstantial evidence was presented in Part 15[1] supporting this hypothesis but before these studies were complete we had embarked on a rational synthesis of the unrearranged aminomethylbilane (2).

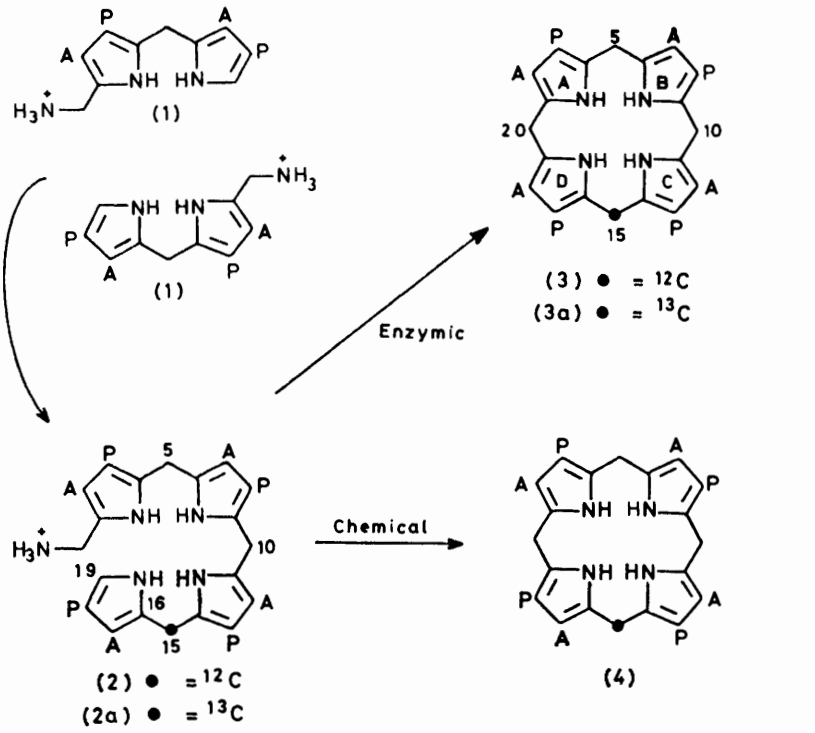
Experience of related pyrromethanes[4] and bilanes[5] led to selection of the lactam ester (5) as the immediate synthetic target and this was to be approached[6] *via* the *a,c*-biladiene (6) (Scheme 2).

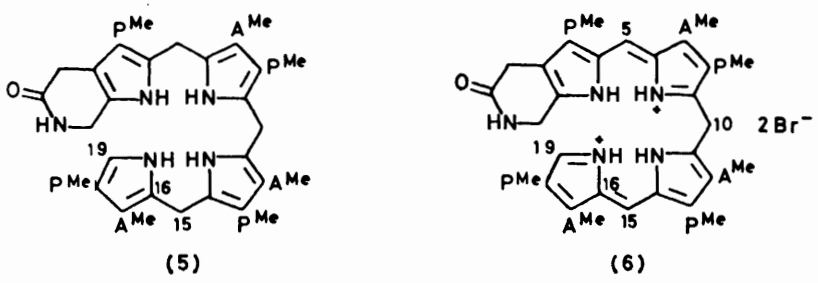
Reaction of the formylpyrromethane[5] (8) with PBG lactam ester (7) catalysed by hydrobromic acid gave the tripyrrene hydrobromide (9) in 92% yield. The CO_2Bu^t group was removed by trifluoroacetic acid in the presence of the formylpyrrole (10) to yield the crystalline biladiene salt (6). After extensive development work, good procedures were devised for the reduction of (6) to the bilane (5) using either catalytic hydrogenation or sodium borohydride (see Experimental section). The bilane (5) was shown to be chromatographically homogeneous and was characterised by n.m.r. and mass spectrometry. Alkaline hydrolysis of the lactam ester (5) gave an aqueous solution of the aminomethylbilane (2) which cyclised at 55 °C and pH 7.2 to uroporphyrinogen, which was isolated, after aromatisation, as the corresponding porphyrin ester. Hot acid hydrolysed the esters in this product and decarboxylated the acetate side-chains to give coproporphyrin which was analysed as its tetramethyl ester by high pressure liquid chromatography (h.p.l.c.).[7] The product was found to be essentially pure type-I isomer [96% tetramethyl coproporphyrin-I (11)] showing that the macrocycle formed chemically from the aminomethylbilane (2) was uro'gen-I (4). This confirms that the synthesis had indeed yielded the unrearranged tetrapyrrole system (2) and that this ring closes chemically without significant rearrangement.

In striking contrast, incubation of bilane (2) (4 mg) at pH 7.2, 37 °C for 4 h with the enzymes deaminase and cosynthetase from *Euglena gracilis*[8] (*ca.* 40 000 units) gave a mixture of uro'gens shown by h.p.l.c.-type analysis of the derived coproporphyrin esters (11) and (12) to contain mainly type-III (3) (70%) with only 30% of the type-I (4) isomer.[2a] At a late stage of our work, Müller *et al.*[9] reported a different synthesis of the bilane (2) which was converted by deaminase and cosynthetase from *P. shermanii* into uro'gens comprising type-III (14—18%) and type-I (82—86%) isomers. Both sets of results show that the aminomethylbilane (2) can be converted by deaminase and cosynthetase into uro'gen-III (3) and, in agreement with the earlier work,[1] they strongly point to the conclusion that the rearrangement step in the biosynthesis of type-III porphyrins occurs at the tetrapyrrole level.

To eliminate the possibility that the bilane (2) might be degraded prior to its enzymic conversion into uro'gen-III (3), a specifically labelled specimen was required, and the [15-^{13}C]bilane (2a) was therefore prepared using the [*formyl*-^{13}C]aldehyde (10a). The labelled aldehyde (10a) was synthesised by the route outlined in Scheme 3 which is essentially that used earlier[5] except that the debenzylation step (13) ⟶ (14) was effected more reliably under strongly acidic conditions than by hydrogenolysis (which always resulted in partial reduction of the aldehyde function).

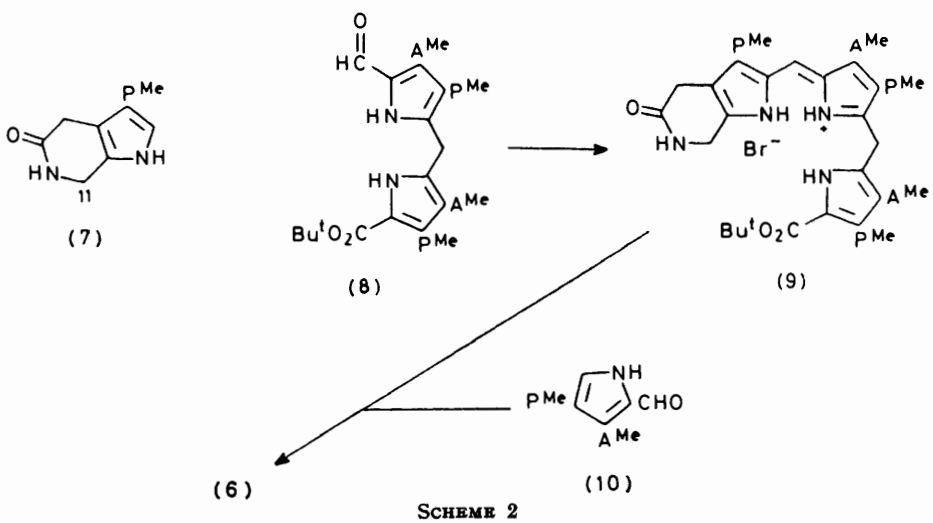
Incubation of the [15-^{13}C]bilane (2a), at *ca.* 90 atom % ^{13}C, with deaminase and cosynthetase gave, after aromatisation and esterification, uroporphyrin ester in 34% yield. Decarboxylation afforded coproporphyrin ester which was shown to contain 73% type-III (12) and 27% type-I (11) isomers (h.p.l.c. analysis). The earlier h.p.l.c. methods[7] were improved and details are given in the Experimental section. Preparative h.p.l.c. afforded a pure sample of coproporphyrin-III ester [as (12)] and the site of labelling was determined by ^{13}C n.m.r. spectroscopy. In the presence of $Pr([^2H_9]fod)_3$, most of the ^{13}C signal at δ 96.3 (arising from carbons 5, 10, 15, and 20) moved massively upfield to δ 87.8. Since there



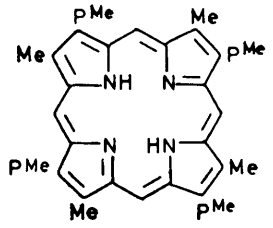


$A = CH_2CO_2H$ $P = CH_2CH_2CO_2H$
$A^{Me} = CH_2CO_2Me$ $P^{Me} = CH_2CH_2CO_2Me$

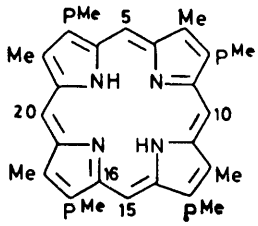
SCHEME 1



SCHEME 2



(11)



(12)

is strong evidence [8,10] that the shifted signal corresponds to C-15 of coproporphyrin-III ester [labelled (12)] and the carbon so affected is obviously the labelled one, it follows that the coproporphyrin-III is specifically labelled at C-15. Support came from the ^{1}H n.m.r. spectrum by showing that the doublet, J 154 Hz, moved considerably downfield in the presence of Eu([$^2H_9$]fod)$_3$.[10] However, rigorous proof came from unambiguous synthesis of [15-^{13}C]coproporphyrin-III tetramethyl ester, using MacDonald's approach [6] followed by the appropriate n.m.r. studies (see Figure 1). These results clearly demonstrated [2a] the specific enzymic conversion of bilane (2a) into uro'gen-III (3a) and also served to register each of the four pyrrole rings of the unrearranged bilane (2) relative to the rearranged product uro'gen-III (3) as shown in structures (2) and (3).

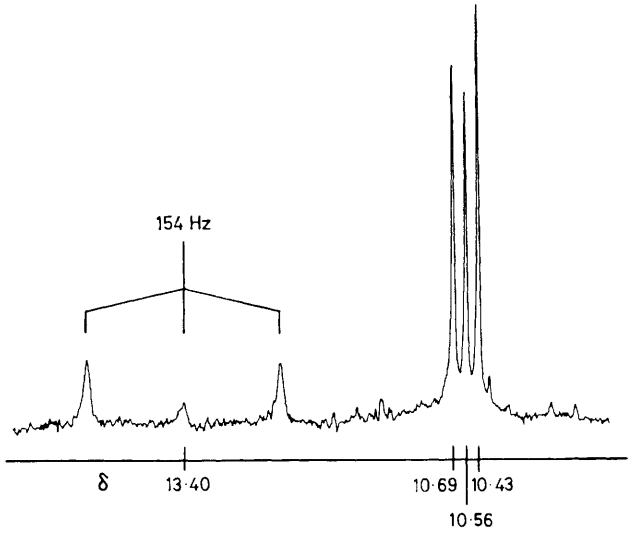
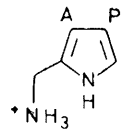


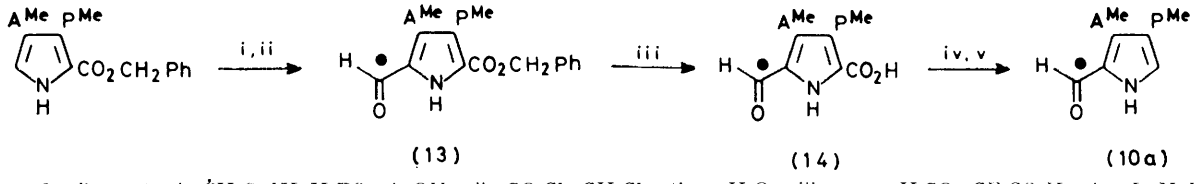
FIGURE 1 Low-field portion of ^{1}H n.m.r. spectrum of synthetic [15-^{13}C]coproporphyrin-III tetramethyl ester [as (12)] in $CDCl_3$ containing Eu([$^2H_9$]fod)$_3$

When porphobilinogen, PBG (15), is the substrate for deaminase and cosynthetase, the rearrangement step *en route* to uro'gen-III (3) involves only that PBG molecule which is built into ring D with C-20.[8] It was thus important to test whether the rearrangement of the bilane (2) to uro'gen-III (3) has the same characteristics.

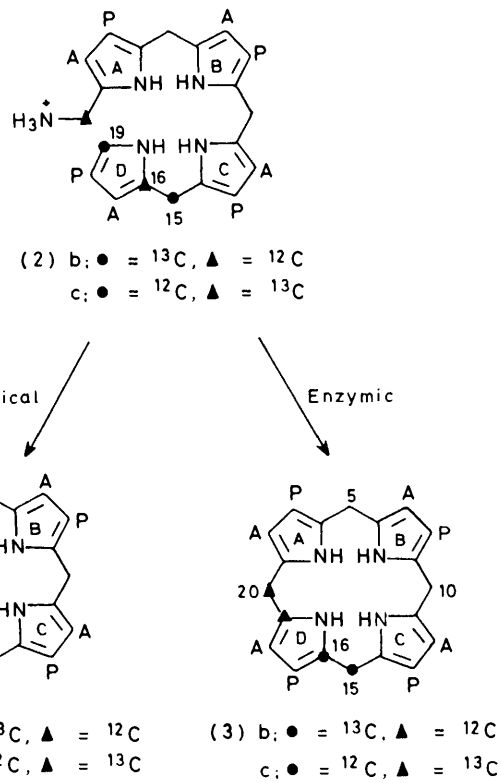


(15)

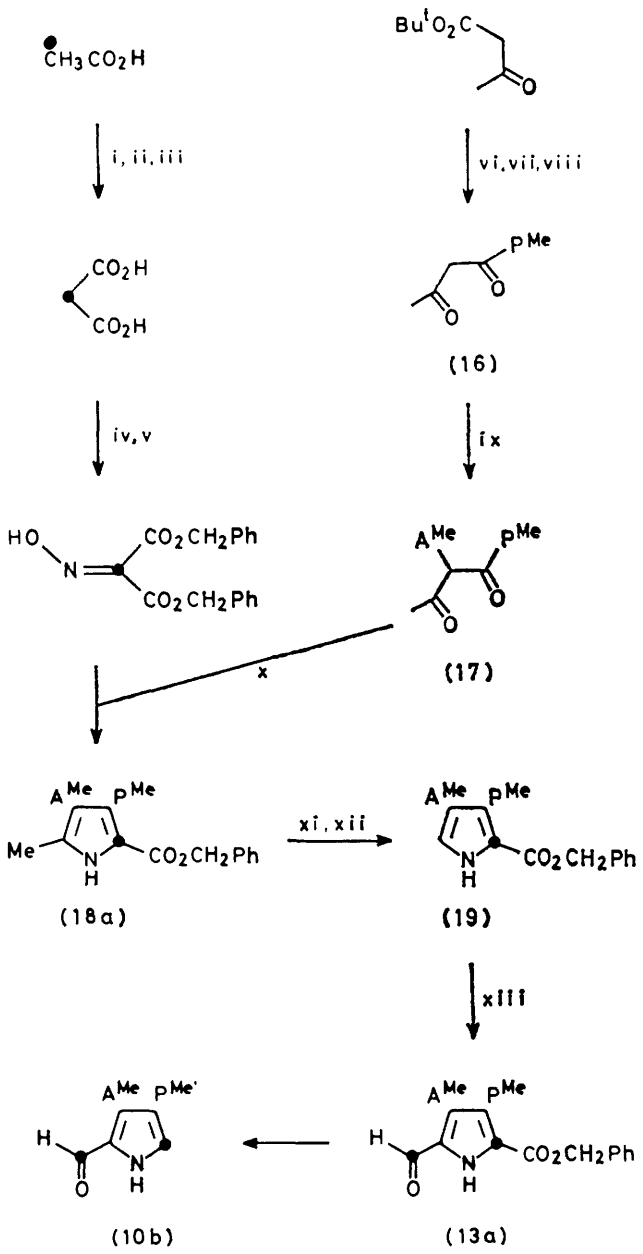
A doubly ^{13}C-labelled specimen of (2) was required for this purpose and, knowing how the bilane (2) registers with respect to uro'gen-III (3) (see above), the [15,19-$^{13}C_2$]bilane (2b) (Scheme 4) was chosen. The doubly labelled aldehyde (10b) required for the synthesis of (2b) was prepared by the route outlined in Scheme 5 which starts with the construction of pyrrole (18a) by Clezy's method.[11] Because one of the labels is introduced at a very early stage, it was important to achieve high yields throughout the synthesis, and the introduction of the



SCHEME 3 *Reagents:* i, CH_2O–HI–H_3PO_2–AcOH; ii, SO_2Cl_2–CH_2Cl_2, then H_2O; iii, conc. H_2SO_4–CF_3CO_2H; iv, I_2–$NaHCO_3$; v, H_2–Pt–NaOAc



SCHEME 4



SCHEME 5 *Reagents:* i, Br_2–P; ii, KCN–H_2O; iii, HCl–H_2O, 100 °C; iv, $PhCH_2OH$–*p*-TsOH–C_6H_6; v, $NaNO_2$–HOAc; vi, $Mg(OMe)_2$–Et_2O, vii, $MeO_2CCH_2CH_2COCl$–Et_2O; viii, *p*-TsOH, heat; ix, MeO_2CCH_2Cl–KI–K_2CO_3; x, Zn–HOAc–NaOAc; xi, SO_2Cl_2–CH_2Cl_2; xii, $(Ph_3P)_3RhCl$–PhMe; xiii, Me_2NCHO–$POCl_3$–MeCN

second label by direct formylation of the α-free pyrrole (19) is a considerable improvement on the earlier method outlined in Scheme 3.

The synthesis of the aldehyde (10b) was carried out from 90 atom % [2-^{13}C]acetic acid and 90 atom % [*formyl*-^{13}C]dimethylformamide without dilution, so that 81% of the aldehydic molecules (10b) carried two ^{13}C-atoms. This doubly labelled aldehyde was diluted with 3 parts of unenriched aldehyde (10) before conversion by synthesis as before into the bilane (2) which then contained 20.3% of doubly labelled molecules. The diluted [15,19-$^{13}C_2$]bilane (2b) was incubated with deaminase and cosynthetase to give uroporphyrinogen which was isolated, after aromatisation, as the corresponding uroporphyrin ester in 45% yield. Decarboxylation of the acetate side-chains gave coproporphyrin ester which was found by h.p.l.c. to contain 75% type-III and 25% type-I isomers. These were separated by taking advantage of the greater solubility of the type-III isomer in diethyl ether. Analysis [12] of the molecular ion peaks in the mass spectrum of the separated coproporphyrin-III ester showed it to be 20.6 ± 2.0% doubly ^{13}C-labelled. Its ^{13}C n.m.r. spectrum showed at δ 96.3 a 70 Hz doublet centred on a smaller singlet [Figure 2(a)]. On addition of Pr([$^2H_9$]-fod)$_3$, the doublet moved 4.7 p.p.m. upfield [Figure 2(b)]

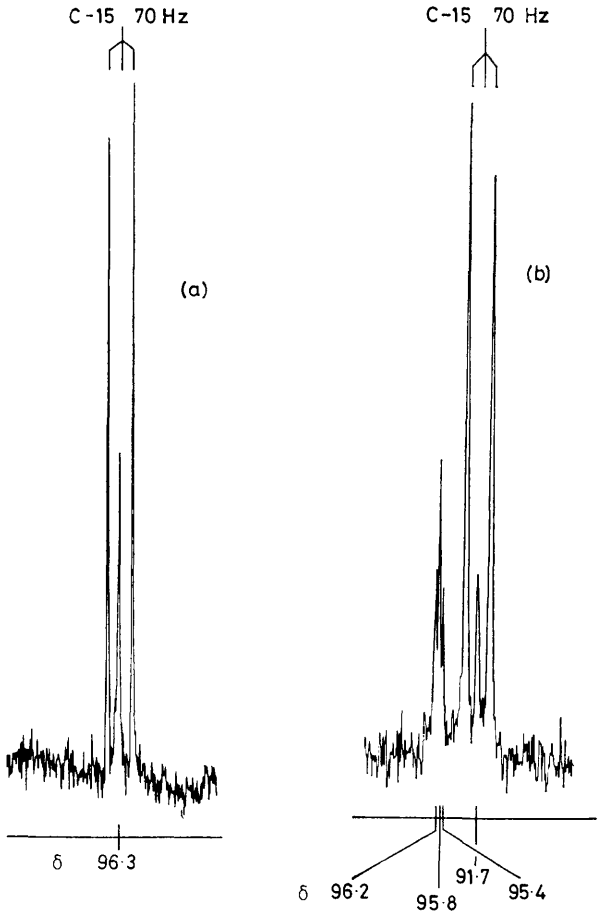


FIGURE 2 ^{13}C N.m.r. signals from *meso*-bridges of [15,16-$^{13}C_2$]-coproporphyrin-III tetramethyl ester [as (12)] (a) in $CDCl_3$ and (b) in $CDCl_3$ containing Pr([$^2H_9$]fod)$_3$

showing [8] that this signal arises from ^{13}C-nuclei at C-15 which are directly bonded (and therefore coupled) to ^{13}C at C-16. The relative sizes of the doublet and singlet were within experimental error, those calculated for complete *intramolecular rearrangement* of ring D of the bilane (2b). There was no detectable intermolecular reaction with the 3-fold excess of unenriched bilane (2). This result clearly demonstrated [2b] that enzymic rearrangement of the bilane (2b) to give uro'gen-III (3b)

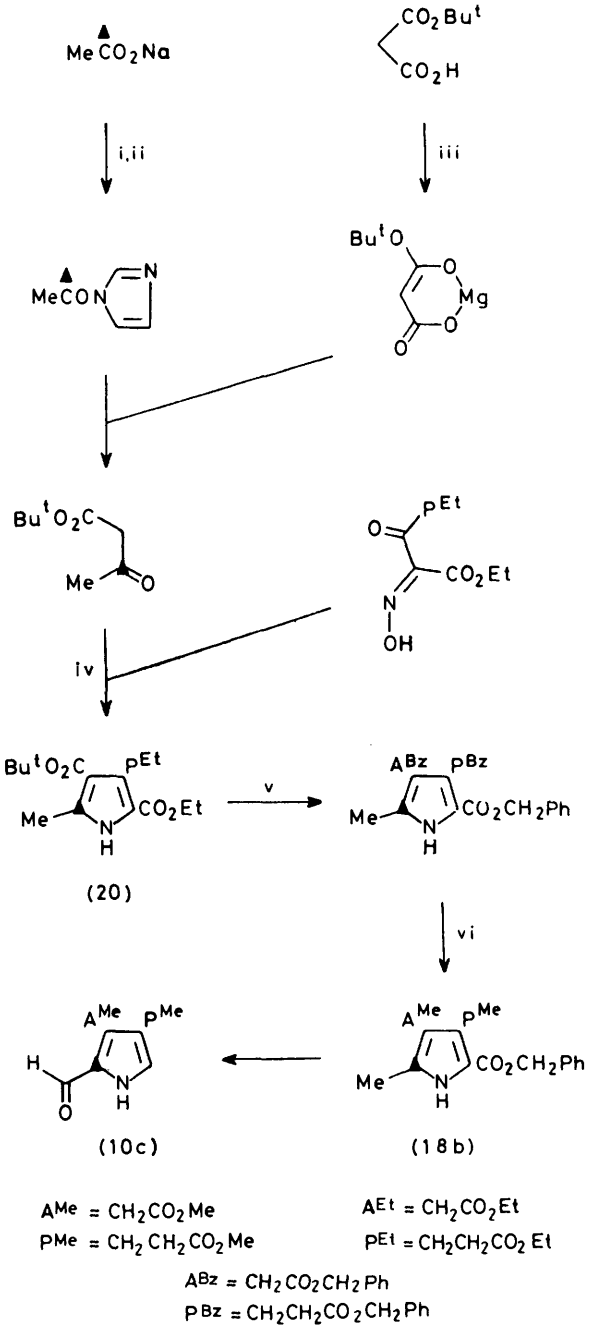
has the same characteristics as the single rearrangement step found earlier [9] when PBG (15) is enzymically converted into uro'gen-III (3) (Scheme 4).

Chemical ring-closure of the bilane (2b) at pH 7 and 37° C gave essentially pure (96%) uro'gen-I (4b) [*i.e.* no rearrangement] and mass spectroscopy on the derived coproporphyrin-I ester [as (11)] showed that the content of doubly labelled $^{13}C_2$-molecules had not significantly changed (20.2 ± 1.5%). The ^{13}C n.m.r. spectrum of this coproporphyrin-I ester showed a 6 Hz doublet for the signal from the *meso*-bridge carbon which corresponds to a 3-bond coupling; [13] this demonstrates the unchanged location of the two labels in the original uro'gen-I as shown in (4c).

In the foregoing experiments rings A, B, and C of the bilane (2b) were unlabelled, and so *intact* incorporation of the bilane (2) had not yet been established. To obtain proof of this, the [*aminomethylene*,16-$^{13}C_2$]bilane (2c) was synthesised as earlier (Scheme 1) from [11-^{13}C]PBG lactam ester (7) (90 atom %) which was obtained by treatment of the corresponding acid [14] with diazomethane. The other two components required were the unlabelled pyrromethane (8) and the [2-^{13}C]aldehyde (10c). Synthesis of the latter from sodium [1-^{13}C]acetate (90 atom %) is outlined in Scheme 6.

The synthesis was carried out without dilution as far as the crystalline biladiene salt [as (6)] and this compound, with 81% of its molecules carrying two ^{13}C-atoms, was mixed with 3 parts of unenriched salt (6) before reduction and hydrolysis to give the diluted [*aminomethylene*,16-$^{13}C_2$]bilane (2c) now with 20% doubly labelled molecules. Incubation of this bilane with deaminase and cosynthetase gave uroporphyrinogens and the derived uroporphyrins (68%) were decarboxylated to give coproporphyrins. The corresponding methyl esters were shown as above to contain 89% type-III and 11% type-I isomers (by h.p.l.c.).

The ^{13}C n.m.r. spectrum of the isolated coproporphyrin-III ester [as (12)] showed a strong doublet



SCHEME 6 *Reagents:* i, $(COCl)_2$–DMF–C_6H_6; ii, imidazole; iii, $Me_2CHMgBr$–THF; iv, Zn–HOAc–NH_4OAc; v, see ref. 15; vi, MeOH–H_2SO_4–$(MeO)_3CH$

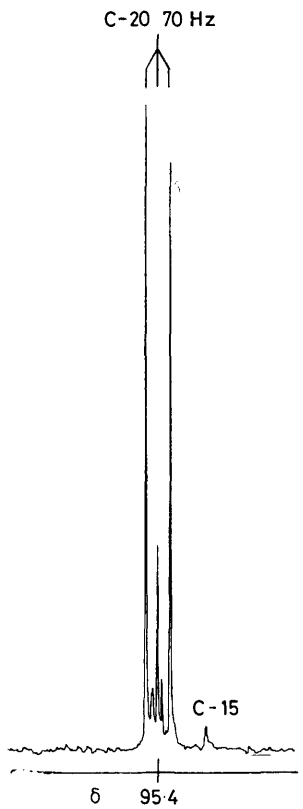
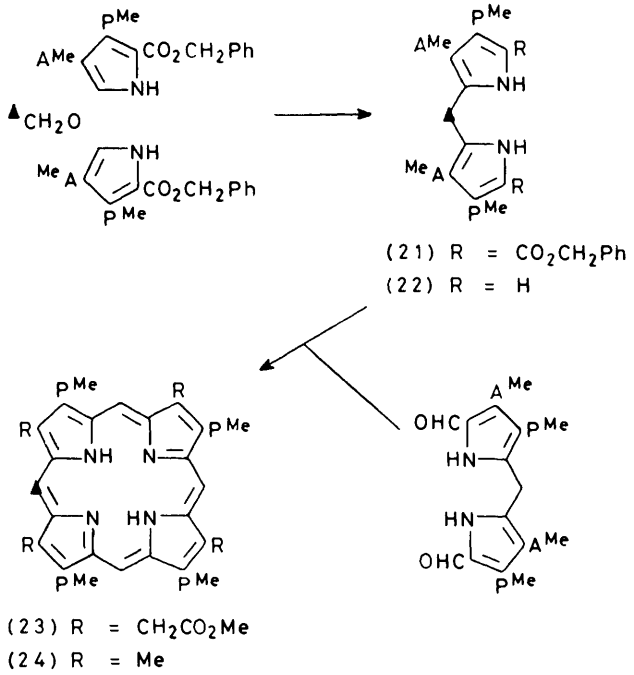


FIGURE 3 ^{13}C N.m.r. signals from *meso*-bridges of [19,20-$^{13}C_2$]-coproporphyrin-III tetramethyl ester [as (12)] in $CDCl_3$ containing Pr([$^2H_9$])fod)$_3$

(J 71 Hz) at δ 96.3 centred on a small singlet which, as hoped, only moved to δ 95.4 on addition of $Pf(fod)_3$ (Figure 3). This proves that the two ^{13}C-atoms have become directly bonded in the initially formed uro'gen-III (3c) and therefore that *intact* incorporation of the whole carbon skeleton of bilane (2c) into uro'gen-III (3c) has occurred; the results are also in agreement with labelling at C-20. Here again the relative sizes of the doublet and singlet showed that the rearrangement process was, within experimental error, entirely intramolecular.[2b] Confirmation came from mass spectrometry (22.8 ± 1.7% doubly labelled).

The precise location of the *meso*-^{13}C atom in the labelled coproporphyrin ester was proved by the ^1H n.m.r. spectrum. In the presence of $Eu(fod)_3$, the resonances corresponding to the four *meso*-hydrogens of coproporphyrin-III ester [as (12)] separate. As proven above, the signal from H-15 moves furthest downfield, relative to its normal position, since it is flanked by two ester side-chains.[10] For the ^{13}C-labelled sample, the signal from the hydrogen atom bonded to ^{13}C was clearly visible as a doublet (J 154 Hz) superimposed on a singlet and this pattern was shifted least of the four ^1H-resonances as expected for H-20 which is flanked by two methyl groups. This assignment was rigorously confirmed by synthesising [*via* the corresponding uroporphyrin (23)] [20-^{13}C]coproporphyrin-III tetramethyl ester (24) (Scheme 7) and comparing the ^1H n.m.r. spectra of



SCHEME 7

this product, in the absence and presence of $Eu(fod)_3$, with those from the foregoing biosynthetically derived sample (see Figure 4).

The blank run involving chemical ring-closure of the bilane (2c) in the absence of enzyme gave, as expected

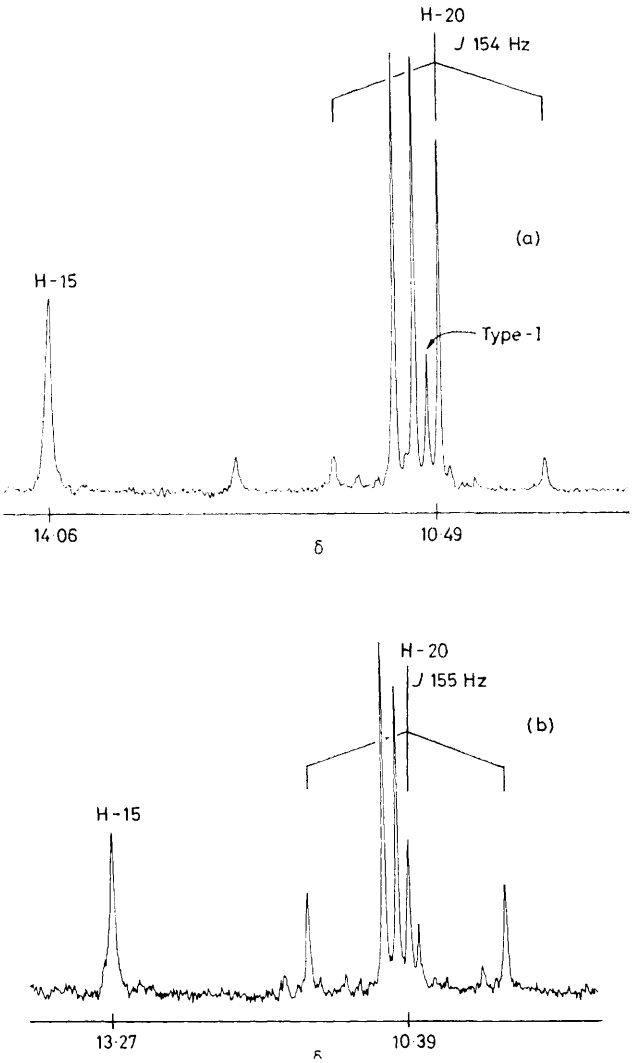
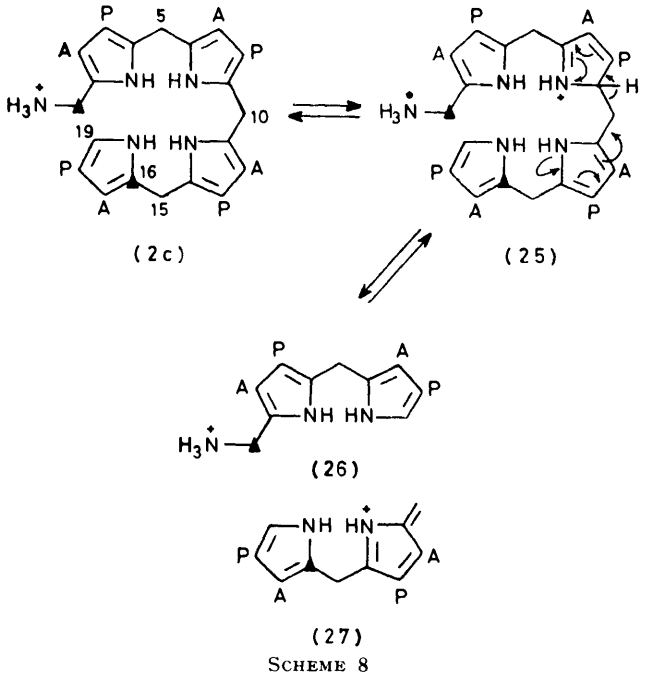


FIGURE 4 ^1H N.m.r. signals from *meso*-bridges of (a) biosynthetic [19,20-$^{13}C_2$]coproporphyrin-III tetramethyl ester [as (12)] and (b) synthetic [20-^{13}C]coproporphyrin-III tetramethyl ester [as (12)]. Both spectra run in $CDCl_3$ containing $Eu([^2H_9]fod)_3$

by this stage, virtually pure (96%) uro'gen-I (4c). The derived coproporphyrin-I ester [as (11)] showed in its ^{13}C n.m.r. spectrum a 6 Hz doublet for the labelled *meso*-carbon confirming three-bond separation of the two ^{13}C-atoms. An interesting difference from the experiment with the alternative double-labelling pattern, *viz.* (2b) ⟶ (4b) was that for (2c) ⟶ (4c) there was a small but significant fall in the content of doubly labelled molecules (16.8 ± 1.5%). Breakdown must occur, *e.g.* (2c) ⟶ (26) and (27) (Scheme 8), which allows mixing with unlabelled fragments before recombination. This is detectable in the slower chemical ring-closure (longer reaction period) but is an insignificant competitor in the much faster enzymic reaction.[16]

One site of protonation is illustrated as (25) in Scheme 8. There are, however, three such α-pyrrolic sites adjacent to propionate substituents which for the bilane (2c) would allow fragmentation separating the two labels. Similar separation of the labels for the bilane

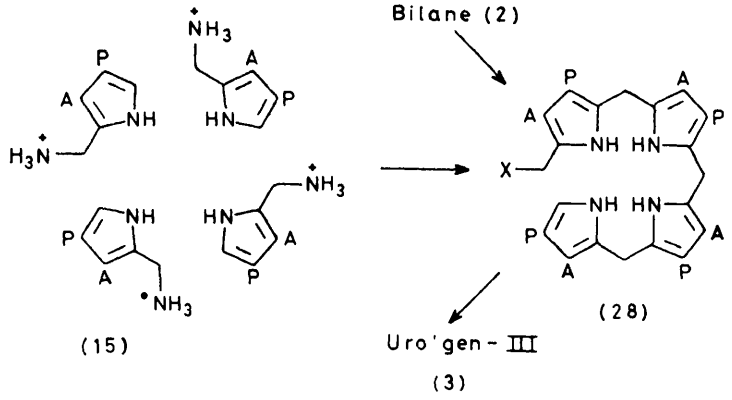


SCHEME 8

(2b), in contrast, requires specific protonation at C-16 (see Scheme 4) which would be expected to be disfavoured, relative to protonation at sites β to a propionate group, by being β to the more electron-withdrawing acetate residue. This combination of statistical and electronic factors can explain why the chemical conversion (2b) ⟶ (3b) did not lead to a detectable diminution in the content of doubly labelled molecules.

The experiments described above show consistently and reproducibly that the aminomethylbilane (2) is efficiently converted by deaminase with cosynthetase into uro'gen-III (3),* that the bilane is incorporated with its carbon skeleton intact registering rings A, B, C, and D of the bilane with rings A, B, C, and D of uro'gen-III (as in Scheme 4), and that the terminal ring D of the bilane undergoes intramolecular rearrangement exactly as found [8] for PBG (15).

It is thus firmly established that deaminase with cosynthetase brings about head-to-tail assembly of four PBG units (15) to form the unrearranged bilane (28) (Scheme 9). A group X is illustrated because it was clear that the $\overset{+}{N}H_3$ group of PBG (15) and similarly the $\overset{+}{N}H_3$ group of the aminomethylbilane (2) might be enzymically replaced before final ring-closure with rearrangement. It was envisaged at this stage that the group X could be some nucleophilic group on the enzyme or an external nucleophile. The nature of this nucleophile and the precise function and mode of action of the enzymes deaminase and cosynthetase will be considered in subsequent papers [17] which build on the foundations laid by the experiments described above.



SCHEME 9

EXPERIMENTAL

Most general methods are given in ref. 4. In addition, silica for column chromatography was Merck Kieselgel H60 and alumina refers to Woelm neutral grade III. High pressure liquid chromatography (h.p.l.c.) of coproporphyrin tetramethyl esters was carried out on two Waters μCN Bondapak columns connected in series using hexane–toluene–acetonitrile (62 : 10 : 3 v/v/v) as eluant at a flow rate of 1.5 ml min^{-1}. The four isomers were eluted in the order type-II, type-III plus type-IV, then type-I.

Synthesis of Labelled Pyrroles

Methyl 4,6-Dioxoheptanoate (16). Carbon tetrachloride (1 ml) was added to magnesium turnings (35 g) in dry methanol (100 ml) and the mixture was warmed until evolution of hydrogen commenced. Additional methanol (500 ml) was added slowly, and heating was continued until all magnesium had dissolved. t-Butyl acetoacetate (224 g) was then added with stirring over 10 min and the mixture was refluxed for 1 h. The crystalline magnesium complex (297 g) was collected from the cooled mixture, washed with dry methanol, and dried *in vacuo*.

3-Methoxycarbonylpropionyl chloride [18] (220 g) was added over 15 min to a stirred suspension of the foregoing product (297 g) in dry ether (500 ml). The thick mixture was heated under reflux for 30 min, cooled on ice, and acidified with 2M-sulphuric acid. After separation, the aqueous

* The recent failure to observe enzymic conversion of the aminomethylbilane (2) into uro'gen-III (3) (L. Diaz, R. B. Frydman, A. Vlasinas, and B. Frydman, *J. Am. Chem. Soc.*, 1979, **101**, 2710) is a result of difficulties with enzymic rates. Since a subsequent paper [16] (Part 17) deals with kinetics and rates, fuller comment will be made there.

phase was extracted with fresh ether (2 × 300 ml) and the combined ethereal solutions were washed with water (4 × 200 ml). Toluene-*p*-sulphonic acid (1.5 g) was added to the pale yellow oil left after evaporation of the ether and the mixture was heated under reflux, over 30 min, to 180 °C. By this time, evolution of carbon dioxide and isobutene had ceased and the mixture was cooled to 20 °C. A solution of the product in ether (1 l) was extracted as quickly as possible with ice-cold 2M-sodium hydroxide solution (350, 180, 100, and 70 ml). Each extract was run directly into ice-cold 1.8M-sulphuric acid (500 ml), the liberated dione was extracted into dichloromethane (3 × 300 ml), and the combined organic solutions were washed with dilute aqueous sodium hydrogencarbonate. Distillation of the product gave *methyl* 4,6-*dioxoheptanoate* (136.7 g, 56.8%) as an oil, b.p. 81—83 °C at 0.05 Torr (Found: C, 55.8; H, 7.2. $C_8H_{12}O_4$ requires C, 55.8; H, 7.0%); M^+, 172; $\nu_{max.}$ 1 739, 1 707, and 1 618 cm^{-1}; $\lambda_{max.}$ 273 nm; δ (enol tautomer) 2.01 (s, $CH_3C{=}C$), 2.63 (s, CH_2CH_2), 3.68 (s, OCH_3), 5.50 (s, C–CH), 15.1br (OH); (keto-tautomer) 2.23 (s, CH_3CO), 2.67 (m, CH_2CH_2), 3.62 (s, $COCH_2CO$), and 3.68 (s, OCH_3). The keto : enol ratio was estimated to be 1 : 2.7.

Dimethyl 3-*Acetyl*-4-*oxoheptanedioate* (17).—A mixture of the foregoing dione (16) (138 g, 0.8 mol), methyl chloroacetate (72 g, 0.66 mol), potassium carbonate (91 g, 0.66 mol), potassium iodide (30 g), and dry acetone (400 ml) was stirred and heated under reflux for 5 h. The cooled mixture was filtered, the filter cake was dissolved in the minimum volume of water, and this solution was extracted with ether (3 × 200 ml). The filtrate above was evaporated, the residue was dissolved in the ethereal extracts, and the solution was washed with water (4 × 100 ml). The product was distilled and the fraction boiling at 125—140 °C and 0.05 Torr collected as *dimethyl* 3-*acetyl*-4-*oxoheptanedioate* (123.7 g, 76.8%) (Found: C, 54.3; H, 6.7. $C_{11}H_{16}O_6$ requires C, 54.1; H, 6.6%); M^+, 244; $\nu_{max.}$ 1 734, 1 707, and 1 600 cm^{-1}; $\lambda_{max.}$ 282 nm; δ 2.27 (3 H, s, CH_3CO), 2.75 (4 H, m, CH_2CH_2), 2.90 (2 H, d, *J* 7 Hz, $CHCH_2$); 3.67 (6 H, s, 2 × OCH_3), and 4.18 (1 H, t, *J* 7 Hz, $CHCH_2$).

[2-^{13}C]*Malonic Acid*.—Thionyl chloride (2 drops) and red phosphorus (5 mg) were added to [2-^{13}C]acetic acid (90 atom %, 1.00 g) and the mixture was stirred and heated under reflux (anhydrous conditions) to 80 °C. Bromine (3.3 g, 1.25 mol equiv.) was then added slowly and the bath temperature was raised to 125—135 °C for 2 h. The temperature was reduced to 60 °C and dry nitrogen was passed through the liquid to remove the excess of bromine and hydrogen bromide. The residual colourless oil (bromoacetic acid) crystallised on cooling (2.39 g).

The product was dissolved in water (8 ml) and the pH adjusted to 8 with sodium carbonate. Potassium cyanide (1.62 g, 1.5 mol equiv.) was then added at such a rate that the temperature remained below 70 °C. After being stirred for 15 min, the solution was heated on a steam-bath for 45 min and evaporated under reduced pressure. The brown residue was treated with 10M-hydrochloric acid (3 ml), evaporated again, and then heated in a Carius tube with 10M-hydrochloric acid (40 ml) at 100 °C for 2.5 h. The solution was evaporated once more and the residue, after drying to constant weight *in vacuo* over solid potassium hydroxide, was powdered and extracted with dry ether (100 ml and 3 × 50 ml). Evaporation of the filtered extracts gave [2-^{13}C]malonic acid (1.53 g, 88.4%) as a white, crystalline solid, m.p. 132—135 °C (lit.,[19] 135 °C) which was used without further purification in the next step.

Dibenzyl Hydroxyimino[2-^{13}C]*malonate*.—Redistilled benzyl alcohol (7.36 g, 2.4 mol equiv.), benzene (10 ml), and sulphuric acid (50 mg) were added to the foregoing [2-^{13}C]-malonic acid (from 2 preparations, 3.00 g) and the mixture was heated under reflux with a Dean–Stark trap until separation of water ceased (1 h). The cooled solution was diluted with ether (300 ml) and washed (water, 50 ml; saturated aqueous sodium hydrogencarbonate, 50 ml; water, 2 × 50 ml). Successive washings were back-extracted with ether (150 ml) and the combined ethereal solutions were evaporated, finally at 90 °C and 0.1 Torr to remove the excess of benzyl alcohol. The residual dibenzyl [2-^{13}C]malonate (8.00 g) was *ca.* 95% pure (t.l.c., n.m.r.); $\nu_{max.}$ 1 745 cm^{-1}; $\lambda_{max.}$ 220 nm; δ 3.37 (1.8 H, d, *J* 133 Hz, $CO^{13}CH_2CO$, and 0.2 H, s, $CO^{12}CH_2CO$), 5.10 (4 H, s, 2 × CH_2Ph), and 7.28 (10 H, s, 2 × C_6H_5).

Sodium nitrite (21 g) in water (30 ml) was added over 5 h to a stirred solution of the foregoing crude ester (8.00 g) in acetic acid (20 g) and stirring continued for 16 h. Solid sodium nitrite (2 g) was added, and after being stirred for an additional 4 h, the mixture was partitioned between water (200 ml) and dichloromethane (100 ml) and neutralised by cautious addition of sodium hydrogencarbonate. The organic layer was separated and the aqueous phase extracted with fresh dichloromethane (4 × 50 ml). The organic solutions were washed with dilute brine (150 ml) and evaporated, finally at 70 °C and 0.1 Torr, to give the oxime (8.26 g) as a viscous yellow oil, used as such in the following step; $\nu_{max.}$ 3 330br and 1 744 cm^{-1}; $\lambda_{max.}$ 216 nm; δ 5.16 and 5.24 (each 2 H, s, 2 × CH_2), 7.20 (10 H, s, 2 × C_6H_5), and 10.9br (1 H, OH).

Benzyl 3-(2-*Methoxycarbonylethyl*)-4-*methoxycarbonylmethyl*-5-*methyl*[2-^{13}C]*pyrrole*-2-*carboxylate* (18a).—A stirred mixture of dimethyl 3-acetyl-4-oxoheptanedioate (17) (6.1 g) and acetic acid (15 ml) was heated to 100 °C. One half of the foregoing crude oxime in acetic acid (4 ml) and water (1.3 ml) was added dropwise at the same time as a mixture of zinc dust (10 g) and anhydrous sodium acetate (4 g) was added in portions. The additions were regulated such that zinc was always in excess and the temperature remained at 110 ± 5 °C. The remaining oxime solution was washed in with more acetic acid (5 ml) and the mixture was boiled gently for 45 min. The supernatant was decanted from the excess of zinc, which was washed with acetic acid (2 × 10 ml) and ether (5 × 30 ml). The combined solutions were partitioned between water (100 ml) and ether (200 ml) and the aqueous phase was extracted with fresh ether (150 ml). The ethereal solutions were washed (water, 2 × 50 ml; 5% aqueous sodium hydroxide, 4 × 50 ml; water, 2 × 50 ml), combined and evaporated, finally at 90 °C and 0.1 Torr. The residual oil (3.75 g) was chromatographed on silica (18 g) using light petroleum–dichloromethane (4 : 1—0 : 1 v/v) and methanol in dichloromethane (0.5 and 1% v/v). Appropriate fractions from this and a second, identical preparation were combined and recrystallised from toluene–cyclohexane to give the pyrrole (2.64 g) as needles, m.p. 80.5—81.5 °C (lit.,[15] 78.5—79.5 °C). The yield was increased to 2.97 g (24.1% from [2-^{13}C]acetic acid) by rechromatography of the mother-liquors and mixed fractions (Found: M^+, 374. Calc. for $^{13}C^{12}C_{19}H_{23}NO_6$: *M*, 374); $\nu_{max.}$ 3 293, 1 741, 1 736, and 1 671 cm^{-1}; $\lambda_{max.}$ 248 and 282 nm; δ 2.20 (3 H, s, pyrrole-CH_3), 2.51 (2 H, m, $CH_2CH_2CO_2$), 2.99 (2 H, m, $CH_2CH_2CO_2$), 3.42 (2 H, s, CH_2CO_2), 3.61 and 3.65 (each 3 H, s, 2 × OCH_3), 5.27 (2 H, s, CH_2Ph), 7.36 (5 H, s, C_6H_5), and 8.70br (1 H, NH).

Benzyl 5-([^{13}C]Formyl)-3-(2-*methoxycarbonylethyl*)-4-*methoxycarbonylmethyl*[2-^{13}C]*pyrrole*-2-*carboxylate* (13a).—The foregoing pyrrole (18a) (1.50 g) was converted into the corresponding aldehyde (1.48 g, 95.4%) by the method used in the sequel for pyrrole (18b), m.p. 78.5—79.5 °C (lit.,[13] m.p. 80—82 °C) (Found: M^+, 388. Calc. for $^{13}C^{12}C_{19}N_{21}NO_7$: *M*, 388).

This aldehyde (1.40 g) was decarbonylated with tris(triphenylphosphine)rhodium(I) chloride as earlier [13] to give the α-free pyrrole (1.03 g, 79.2%), m.p. 55.0—56.5 °C (lit.,[13] 60—63 °C) (Found: M^+, 360. Calc. for $^{13}C^{12}C_{18}H_{21}NO_6$: *M*, 360).

[*formyl*-^{13}C]Dimethylformamide (90 atom %, 297 mg, 4.00 mmol) was stirred at 5 °C under nitrogen and treated with freshly distilled phosphoryl chloride (615 mg, 4.00 mmol) added dropwise over 2 min. Dry acetonitrile (2 ml) was added followed by the foregoing α-free pyrrole (988 mg, 2.74 mmol), then more acetonitrile (2 ml), and the solution was kept at 20 °C for 48 h before being poured into methanol–water (2 : 1 v/v; 30 ml). This solution was warmed to 40 °C for 15 min, aqueous potassium carbonate (2%, 100 ml) was then added, and the product was extracted into dichloromethane (80 ml and 4 × 50 ml). Chromatography of the product on silica (4 g), eluant dichloromethane, and recrystallisation from dichloromethane–ether–hexane gave the doubly labelled aldehyde (916 mg) as prisms, m.p. 79.0—79.5 °C (lit.,[13] 80—82 °C). Preparative t.l.c. of the mother-liquor gave recovered α-free pyrrole (38 mg) and more aldehyde (35 mg) (Found: M^+, 389. Calc. for $^{13}C_2{}^{12}C_{18}H_{21}NO_7$: *M*, 389); $\nu_{max.}$ 3 250, 1 740, 1 700, and 1 640 cm^{-1}; $\lambda_{max.}$ 233 and 302 nm; δ 2.54 (2 H, m, $CH_2CH_2CO_2$), 2.99 (2 H, m, $CH_2CH_2CO_2$), 3.61 and 3.69 (each 3 H, s, 2 × OCH_3), 3.82 (2 H, s, CH_2CO_2), 5.33 (2 H, s, CH_2Ph), 7.38 (5 H, s, C_6H_5), 9.60br (1 H, NH), 9.75 (0.9 H, d, *J* 179 Hz, ^{13}CHO and 0.1 H, s, ^{12}CHO).

t-Butyl [3-^{13}C]*Acetoacetate*.—Sodium [1-^{13}C]acetate (90 atom %, 4.08 g) was suspended in dry benzene (30 ml) containing dimethylformamide (3 drops) at 5 °C. Oxalyl chloride (6.37 g, 1.02 mol equiv.) was added over 5 min, with stirring and, 30 min later, the mixture was heated under reflux for 30 min. More benzene (60 ml) was added, the cooled mixture treated with imidazole (7.02 g, 2.10 mol equiv.) and then stirred vigorously for 5 min and heated under reflux for 1 h. The precipitate was filtered off, washed with benzene, and the filtrate was evaporated to give the imidazolide (4.97 g) as needles for immediate use below; δ 2.52 (3 H, d, *J* 7.5 Hz, $^{13}COCH_3$), and 7.07, 7.45, and 8.12 (each 1 H, br, 3 × ring-H).

A Grignard reagent was prepared from magnesium (3.0 g) and isopropyl bromide (12.30 g) in dry tetrahydrofuran (100 ml). Mono-t-butyl malonate [20] (8.00 g) in dry tetrahydrofuran (20 ml) was stirred under nitrogen at 5 °C and the above Grignard solution was added over 15 min. This solution was heated under reflux for 1 h, then cooled on ice, and a solution of the foregoing imidazolide (4.97 g) in dry tetrahydrofuran (50 ml) was added over 5 min. The mixture was stirred at 20 °C for 30 min, heated under reflux for 2 h, and the solvent was removed *in vacuo*. Ice-cold 3M-hydrochloric acid was added at 0 °C to the residue and when pH 2 was reached, the product was extracted into dichloromethane (5 × 50 ml). The extracts were washed with saturated aqueous sodium hydrogencarbonate and evaporated to constant weight at 30 °C and 20 Torr to give the labelled β-ketoester (7.00 g, 89.5% from sodium [1-^{13}C]-acetate) as an oil, pure by n.m.r.; δ 1.49 [9 H, s, $C(CH_3)_3$], 2.25 (3 H, d, *J* 6 Hz, $^{13}COCH_3$), and 3.36 (2 H, d, *J* 6 Hz, $^{13}COCH_2$).

Ethyl 4-*t-Butoxycarbonyl*-3-(2-*ethoxycarbonylethyl*)-5-*methyl*[5-^{13}C]*pyrrole*-2-*carboxylate* (20).—The foregoing β-ketoester (7.00 g) and diethyl 3-oxohexanedioate [15] (9.60 g) were converted [21] into the title pyrrole (8.51 g, 54.6%), m.p. 109—111.5 °C (lit.,[21] 112.5 °C) (Found: M^+, 354.1879. Calc. for $^{13}C^{12}C_{17}H_{27}NO_6$: *M*, 354.1872); δ 1.25 (3 H, t, *J* 7 Hz, side-chain OCH_2CH_3), 1.36 (3 H, t, *J* 7 Hz, ring OCH_2CH_3), 1.57 [9 H, s, $C(CH_3)_3$], 2.52 (3 H, d, *J* 7 Hz, $^{13}CCH_3$), 2.53 (2 H, m, $CH_2CH_2CO_2$), 3.40 (2 H, m, $CH_2CH_2CO_2$), 4.14 (2 H, q, *J* 7 Hz, side-chain OCH_2CH_3), 4.34 (2 H, g, *J* 7 Hz, ring OCH_2CH_3), and 10.00br (1 H, NH).

Ethyl 3-(2-*Ethoxycarbonylethyl*)-4-*iodo*-5-*methyl*[5-^{13}C]-*pyrrole*-2-*carboxylate*.—The above t-butyl ester (8.50 g) was converted as usual [15] into the iodopyrrole (9.13 g, 100%), m.p. 106—108 °C (lit.,[15] 109—110.5 °C) (Found: M^+, 380.0326. Calc. for $^{13}C^{12}C_{12}H_{18}INO_4$: *M*, 380.0314); δ 1.27 (3 H, t, *J* 7 Hz, side-chain OCH_2CH_3), 1.37 (3 H, t, *J* 7 Hz, ring OCH_2CH_3), 2.30 (3 H, d, *J* 7 Hz, $^{13}CCH_3$), 2.52 (2 H, m, $CH_2CH_2CO_2$), 3.05 (2 H, m, $CH_2CH_2CO_2$), 4.16 (2 H, g, *J* 7 Hz, side-chain OCH_2CH_2), 4.34 (2 H, q, *J* 7 Hz, ring OCH_2CH_3), and 10.07br (1 H, NH).

Ethyl 3-(2-*Ethoxycarbonylethyl*)-4-*ethoxycarbonylmethyl*-5-*methyl*[5-^{13}C]*pyrrole*-2-*carboxylate*.—An acetate side-chain was built onto the foregoing iodopyrrole (9.13 g) as earlier [15] to give the title pyrrole (7.04 g, 86.1%), needles, from ether–hexane, m.p. 63—65 °C (lit.,[15] 63—65 °C) (Found: M^+, 340.1708. Calc. for $^{13}C^{12}C_{16}H_{25}NO_6$: *M*, 340.1715); δ 1.24 (6 H, t, *J* 7 Hz, 2 × side-chain OCH_2CH_3), 1.35 (3 H, t, *J* 7 Hz, ring OCH_2CH_3), 2.23 (3 H, d, *J* 7 Hz, $^{13}CCH_3$), 2.55 (2 H, m, $CH_2CH_2CO_2$), 3.04 (2 H, m, $CH_2CH_2CO_2$), 3.41 (2 H, d, *J* 5.5 Hz, CH_2CO_2), 4.12 (4 H, q, *J* 7 Hz, 2 × side-chain OCH_2CH_3), 4.30 (2 H, q, *J* 7 Hz, ring OCH_2CH_3), and 9.11br (1 H, NH).

Benzyl 3-(2-*Methoxycarbonylethyl*)-4-*methoxycarbonylmethyl*-5-*methyl*[5-^{13}C]*pyrrole*-2-*carboxylate* (18b).—The foregoing ester (7.03 g) was converted into the tribenzyl ester as earlier [15] and the crude product (no OEt by n.m.r.) was stirred for 16 h with methanol (300 ml) containing sulphuric acid (15 ml) and trimethyl orthoformate (30 ml). The mixture was partitioned between aqueous ammonia and dichloromethane, and chromatography of the product on alumina, eluant ether, and recrystallisation from ether–hexane gave the pyrrole (18b) (6.63 g, 85.7%) as needles, m.p. 78—79 °C (lit.,[15] 78.5—79.5 °C) (Found: M^+, 374.1576. Calc. for $^{13}C^{12}C_{19}H_{23}NO_6$: *M*, 374.1559); δ 2.20 (3 H, d, *J* 6.5 Hz, $^{13}CCH_3$), 2.53 (2 H, m, $CH_2CH_2CO_2$), 3.02 (2 H, m, $CH_2CH_2CO_2$), 3.43 (2 H, d, *J* 5.5 Hz, CH_2CO_2), 3.61 and 3.66 (each 3 H, s, 2 × OCH_3), 5.28 (2 H, s, CH_2Ph), 7.35 (5 H, s, C_6H_5), and 9.11br (1 H, NH).

Benzyl 5-*Formyl*-3-(2-*methoxycarbonylethyl*)-4-*methoxycarbonylmethyl*[5-^{13}C]*pyrrole*-2-*carboxylate*.—Freshly distilled sulphuryl chloride (767 mg, 2.1 mol equiv.) in dichloromethane (5 ml) was added over 1 min to a solution of the foregoing pyrrole (1.05 g) in dichloromethane (15 ml) and the mixture was stirred 20 °C for 2 h and then evaporated. Fresh dichloromethane (20 ml) was added and again evaporated. The resultant oil in acetone (25 ml) and water (15 ml) was kept for 30 min at 20 °C and then was partitioned between dichloromethane (50 ml) and dilute brine containing sufficient sodium hydrogencarbonate to keep it basic. The organic phase was combined with dichloromethane washings (4 × 20 ml) of the aqueous layer and the product was purified on silica (5 g) using ether (0—30%) in

dichloromethane as eluant. Recrystallisation from dichloromethane–ether–hexane gave the formylpyrrole (1.053 g, 96.7%) as prisms, m.p. 78—79 °C (lit.,[13] 80—82 °C) (Found: M^+, 388. $^{13}C^{12}C_{19}H_{21}NO_7$ requires M, 388); δ 2.55 (2 H, m, $CH_2CH_2CO_2$), 3.05 (2 H, m, $CH_2CH_2CO_2$), 3.60 and 3.68 (each 3 H, s, 2 × OCH_3), 3.83 (2 H, d, J 4.5 Hz, CH_2CO_2), 5.33 (2 H, s, CH_2Ph), 7.38 (5 H, s, C_6H_5), 9.75 (0.9 H, d, J 27 Hz, $^{13}CCHO$, and 0.1 H, s, $^{12}CCHO$), and 10.20br (1 H, NH).

The [*formyl*-^{13}C]-compound (13) was prepared from the corresponding 5-([^{13}C]methyl)pyrrole described later, by the same procedure; it showed δ 9.7 (1 H, d, J 174 Hz, ^{13}CHO).

2-Formyl-4-(2-methoxycarbonylethyl)-3-methoxycarbonylmethyl[2-^{13}C]*pyrrole* (10c) *and the* [formyl-^{13}C] (10a) *and* [formyl,5-$^{13}C_2$] (10b) *Analogues*.—A mixture of 18M-sulphuric acid (30 drops) and trifluoroacetic acid (4 ml) was added to a stirred solution of the above benzyl ester (1.05 g) in trifluoroacetic acid (2 ml). After 30 min, the trifluoroacetic acid was evaporated at 25 °C and the residue was partitioned between aqueous sodium carbonate (5%; 50 ml) and ethyl acetate (150 ml). The aqueous layer, together with sodium carbonate washings (5%, 3 × 10 ml) of the ethyl acetate layer, were washed with ethyl acetate (100 ml) and acidified (pH < 1), with 5M-sulphuric acid (ice cooling). The product, extracted into ethyl acetate (2 × 150 ml and 50 ml) was washed with dilute brine (3 × 50 ml). Evaporation below 50 °C and removal of residual trifluoroacetic acid by co-distillation with toluene (2 × 50 ml) left a solid which in boiling water (50 ml) was treated with a little decolourising charcoal and the filtered solution was evaporated. A solution of the residue in methanol (100 ml) was filtered, diluted with water (50 ml), and concentrated to give 5-formyl-3-(2-methoxycarbonylethyl)-4-methoxycarbonylmethyl[5-^{13}C]pyrrole-2-carboxylic acid (747 mg, 92.6%) as flakes, m.p. 133—135.5 °C (Found: M^+, 298. $^{13}C^{12}C_{12}H_{15}NO_7$ requires M, 298). *Unlabelled material*, m.p. 133.5—135.5 °C, was prepared as above (Found: M^+, 297.0853. $C_{13}H_{15}NO_7$ requires M, 297.0848): $\nu_{max.}$ 3 160br, 1 730, 1 700, and 1 646 cm^{-1}; $\lambda_{max.}$ 231 and 302 nm; δ (CD_3OD) 2.53 (2 H, m, $CH_2CH_2CO_2$), 2.99 (2 H, m, $CH_2CH_2CO_2$), 3.60 and 3.65 (each 3 H, s, 2 × OCH_3), 3.87 (2 H, s, CH_2CO_2), and 9.67 (1 H, s, CHO).

In the [*formyl*-^{13}C] series (10a), the carboxylic acid showed δ 9.8 (1 H, d, J 179 Hz, ^{13}CHO).

For the [*formyl*,5-$^{13}C_2$] compound (10b), the corresponding benzyl ester (13a) (945 mg) gave 652 mg (89.8%) of carboxylic acid, m.p. 133.5—135.5 °C (Found: M^+, 299. $^{13}C_2{}^{12}C_{11}H_{15}NO_7$ requires M, 299).

A mixture of the foregoing [5-^{13}C]carboxylic acid (746 mg), sodium hydrogencarbonate (600 mg), chloroform (ethanol-free; 9 ml), and water (6 ml) was stirred vigorously and heated rapidly under reflux. As soon as boiling commenced, a solution of iodine (730 mg) and potassium iodide (900 mg) in water (9 ml) was added over 1 min, and heating and stirring were continued for 5 min. Sufficient aqueous sodium hydrogensulphite to neutralise the excess of iodine was added and the mixture was partitioned between dilute brine (100 ml) and dichloromethane (50 ml). The organic layer, together with dichloromethane washings (4 × 30 ml) of the aqueous phase was evaporated and the product was chromatographed on silica (8 g) using dichloromethane–light petroleum (1 : 1 and 2 : 1 v/v) and methanol (0—0.5% v/v) in dichloromethane as eluant. Recrystallisation from dichloromethane–ether–hexane gave 2-formyl-5-iodo-4-(2-methoxycarbonylethyl)-3-methoxycarbonylmethyl[2-^{13}C]-pyrrole (829 mg, 87.2%) as needles, m.p. 103—104.5 °C (Found: M^+, 380. $^{13}C^{12}C_{11}H_{14}INO_5$ requires M, 380). *Unlabelled material*, prepared by the same procedure, was characterised (Found: C, 38.1; H, 3.7; N, 3.4%; M^+, 378.9916. $C_{12}H_{14}INO_5$ requires C, 38.0; H, 3.7; N, 3.7%; M, 378.9916). $\nu_{max.}$ 3 320, 1 729, 1 720, and 1 633 cm^{-1}; $\lambda_{max.}$ 269sh and 312 nm; δ 2.64 (4 H, m, CH_2CH_2), 3.68 and 3.71 (each 3 H, s, 2 × OCH_3), 3.82 (2 H, s, CH_2CO_2), 9.41 (1 H, s, CHO), and 9.99br (1 H, NH).

[*formyl*,5-$^{13}C_2$]Iodopyrrole (645 mg, 77.9%), similarly prepared from the corresponding carboxylic acid (650 mg), had m.p. 103.5—105 °C (Found: M^+, 381. $^{13}C_2{}^{12}C_{10}H_{14}INO_5$ requires M, 381).

The above [2-^{13}C]iodopyrrole (828 mg) in methanol (50 ml) containing sodium acetate (800 mg) and Adams catalyst (100 mg) was stirred under hydrogen until uptake ceased (1 h). The catalyst was removed (Celite) and after addition of sodium hydrogencarbonate (200 mg), the filtrate was evaporated. Partition of the product between water (150 ml) and dichloromethane (5 × 50 ml) was followed by chromatography on silica (8 g), eluant dichloromethane–light petroleum (1 : 1 and 2 : 1 v/v) and ether (0—20% v/v) in dichloromethane. Recrystallisation from dichloromethane–ether–hexane gave the α-free pyrrole (10c) (468 mg) as needles, m.p. 97.5—98.5 °C (lit.,[15] 97—98 °C). Preparative t.l.c. of the mother-liquors and mixed fractions increased the yield to 510 mg (92.2%) (Found: M^+, 254.0980. Calc. for $^{13}C^{12}C_{11}H_{15}NO_5$: M, 254.0984); δ 2.48—2.92 (4 H, m, CH_2CH_2), 3.67 and 3.70 (each 3 H, s, 2 × OCH_3), 3.77 (2 H, d, J 4 Hz, CH_2CO_2), 6.92 (1 H, dd, J 3 and 6.5 Hz, pyrrole H), 9.58 (0.9 H, d, J 27 Hz, $^{13}CCHO$, and 0.1 H, s, $^{12}CCHO$), and 9.96br (1 H, NH).

In the $^{13}C_2$-labelled series, the iodopyrrole (643 mg) gave 2-([^{13}C]formyl)-4-(2-methoxycarbonylethyl)-3-methoxycarbonylmethyl[5-^{13}C]pyrrole (10b) (394 mg, 91.6%) (Found: M^+, 255. $^{13}C_2{}^{12}C_{10}H_{15}NO_5$ requires M, 255); $\nu_{max.}$ 3 252, 1 744, 1 729, and 1 590 cm^{-1}; δ (partial spectrum) 3.78 (2 H, s, CH_2CO_2), 6.90 (0.9 H, dd, J 184 and 3 Hz, $N^{13}CH$, and 0.1 H, d, J 3 Hz, $N^{12}CH$), and 9.62 (0.9 H, dd, J 175 and 1 Hz, ^{13}CHO, and 0.1 H, d, J 1 Hz, ^{12}CHO); δ_C 123.2 (d, J 2.5 Hz, 5-C) and 177.5 (d, J 2.5 Hz, CHO).

Benzyl 3-(2-Methoxycarbonylethyl)-4-methoxycarbonylmethyl-5-([^{13}C]*methyl)pyrrole-2-carboxylate* (with Dr. A. Fenninger).—Hydriodic acid (*d* 1.94; 13 ml) was treated at 0 °C with acetic anhydride (13 ml), followed by hypophosphorous acid (50%; 5.5 ml), acetic acid (10 ml), and [^{13}C]paraformaldehyde (90 atom %, 72.1 mg). A solution of benzyl 3-(2-methoxycarbonylethyl)-4-methoxycarbonylmethylpyrrole-2-carboxylate [5] (821 mg) in acetic acid (10 ml) was then added over 10 min and stirring was continued at 0 °C for 30 min. The solution was then warmed to 20 °C. Saturated aqueous sodium carbonate (60 ml) was added, and the product was extracted into ether (2 × 40 ml) and washed with aqueous sodium carbonate, aqueous sodium thiosulphate, and water. Chromatography of the pyrrole on silica (20 g), eluant ethyl acetate–hexane (1 : 1 v/v), gave the labelled pyrrole (620 mg, 72%), needles from ether–hexane, m.p. 80—81.5 °C. The n.m.r. spectrum was identical to that of unlabelled material [15] except for δ 2.20 (3 H, d, J 128 Hz, $^{13}CH_3$).

Tripyrrenes

t-Butyl 2,7,12-Tris-(2-methoxycarbonylethyl)-3,8-bis-methoxycarbonylmethyl-6′-oxo-1′,2′,5′,6′-tetrahydropyrido-[3′,4′-m]*tripyrrene-b-1-carboxylate Hydrobromide* (9) *and*

[aminomethylene-^{13}C] *Material*.—A sample of the required formylpyrromethane (8), previously an oil,[5] was found to have crystallised. Recrystallisation from ether afforded needles, m.p. 108—109.5 °C.

Hydrogen bromide gas was passed through a stirred suspension of porphobilinogen lactam methyl ester (320 mg) in methanol (2 ml) until a clear solution was obtained (30 s). The above pyrromethane (850 mg) in methanol (2 ml) was then added and the mixture was stirred at 20 °C. After 1 min, dry ether (8 ml) was added and the mixture was cooled on ice for 5 min. The product was collected, washed with ether–methanol (3 : 1 v/v) and dried to give the *tripyrrene hydrobromide* (9) (1.157 g, 91.8%) as red needles, m.p. 133—135.5 °C, raised by recrystallisation from methyl acetate–hexane to 134—136 °C (Found: C, 54.9; H, 5.8; Br, 8.9; N, 6.6. $C_{40}H_{51}BrN_4O_{13}$ requires C, 54.9; H, 5.9; Br, 9.1; N, 6.4%); *m/e* 794 (*M* − HBr); $\nu_{max.}$ 3 400, 2 940, 1 730, 1 670, 1 515, 1 420, 1 360, 1 280, 1 200, 1 140, 1 100, 1 065, and 920 cm^{-1}; $\lambda_{max.}$ 275, 371, and 501 nm (ε 16 541, 5 838, and 62 277); δ 1.53 [9 H, s, $C(CH_3)_3$], 2.15—3.15 (12 H, m, 3 × CH_2CH_2), 3.56 (4 H) 3.81 (2 H) (s, 2 × CH_2CO_2, CH_2CONH), 3.61 (6 H) 3.64 (6 H) 3.74 (3 H) (s, 5 × OCH_3), 4.51 (2 H, s, methane CH_2), 4.81 (2 H, s, CH_2NH), 6.27br (1 H, CH_2NH), 7.65 (1 H, s, methene CH), 10.54br (1 H, pyrrole NH), and 13.46 and 13.54 (each 1 H, br, 2 × pyrromethene NH).

The [11-^{13}C]porphobilinogen lactam methyl ester [as (7)] required for the labelled tripyrrene was prepared by dissolving [11-^{13}C]porphobilinogen lactam [14] (216 mg) in hexamethylphosphoramide (3 ml) and carefully overlayering the solution with an excess of ethereal diazomethane. After 10 min, the mixture was homogenised and volatile materials were removed *in vacuo*. The remaining solution was re-treated with diazomethane and concentrated as before, after which water (20 ml) was added slowly, with stirring. The mixture was kept at 5 °C for 18 h and the crystalline product was then collected and washed with water to give the methyl ester [as (7)] (196 mg, 84.9%) as prisms, m.p. 248—249 °C (decomp.) (lit.,[22] 240—242 °C) (Found: M^+, 223.1040. Calc. for $^{13}C_1{}^{12}C_{10}H_{14}N_2O_3$: *M*, 223.1038); δ 2.38—2.76 (4 H, m, CH_2CH_2), 3.33 (2 H, t, *J* 3.3 Hz, CH_2CONH), 3.61 (3 H, s, OCH_3), 4.39 (1.8 H, td, *J* 3.3 and 150 Hz, $^{13}CH_2NH$, and 0.2 H, t, *J* 3.3 Hz, $^{12}CH_2NH$), 6.47 (1 H, pyrrole H), and 8.81br (1 H, pyrrole NH).

Condensation of this material (111.5 mg) and the formylpyrromethane (8) (295 mg), as above, gave the labelled tripyrrene [as (9)] (383 mg, 87.4%), m.p. 136.5—138.5 °C; the n.m.r. spectrum was identical with the unlabelled material except for δ 4.81br (1.8 H, d, *J* 145 Hz, $^{13}CH_2NH$ and 0.2 H, s, $^{12}CH_2NH$).

Biladienes and Bilanes

3,8,13,18-*Tetrakis*-(2-*methoxycarbonylethyl*)-7,12,17-*tris*-*methoxycarbonylmethyl*-6′-*oxo*-1′,2′,5′,6′-*tetrahydropyrido*-[3′,4′-a]*biladiene-a,c Dihydrobromide* (6) *together with Singly and Doubly* ^{13}C-*Labelled Samples*.—The tripyrrene hydrobromide (9) (235 mg) and α-free aldehyde (10) (78 mg) were dissolved in acetic acid (1 ml) and trifluoroacetic acid (2 ml) and hydrogen bromide in acetic acid (45%; 0.3 ml) was added. The solution was kept in the dark, under nitrogen, for 1.5 h after which dry ether (15 ml) was added. The precipitated solid was collected by centrifugation, washed several times with dry ether, and dried *in vacuo* to give the red *biladiene dihydrobromide* (6) (279 mg, 95%), m.p. 151—154 °C (from methyl acetate) (Found: C, 51.4; H, 5.2; N, 6.1. $C_{47}H_{57}Br_2N_5O_{15}$ requires C, 51.7; H, 5.3; N, 6.4%); *m/e* 929 (*M* − 2HBr); $\nu_{max.}$ 3 400, 2 900, 1 735, 1 610, 1 430, 1 260, 1 160, 1 140, 1 100, and 1 025 cm^{-1}; $\lambda_{max.}$ ($CHCl_3$–HBr_g) 373, 464, and 528 nm (ε 12 646, 26 979, and 115 926); δ 2.0—3.2 (16 H, m, 4 × CH_2CH_2), 3.30 and 3.37 (each 2 H, s, CH_2CO_2), 3.48br (2 H, CH_2CONH), 3.61, 3.68, and 3.72 (21 H, each s, 7 × OCH_3), 3.86 (2 H, s, CH_2CO_2), 5.00 (2 H, s, CH_2NH), 5.30 (2 H, s, methane CH_2), 7.56 (1 H, s, CH_2NH), 7.76 (3 H, 2 × methene CH and 19-H), and 13.5br and 13.9br (each 2 H, 4 × NH).

The [15-^{13}C]biladiene dihydrobromide [as (6)] (40 mg, 93%) was prepared similarly from the tripyrrene hydrobromide (9) (33.5 mg) and the labelled aldehyde (10a) (10 mg).

By the same procedure, the [*aminomethylene*,16-$^{13}C_2$]-biladiene dihydrobromide [as (6)] (43.7 mg, quantitative) was obtained from [*aminomethylene*-^{13}C]tripyrrene hydrobromide [as (9)] (35.0 mg) and the formyl[2-^{13}C]pyrrole (10c) (10.2 mg).

The doubly labelled aldehyde (10b) (5.10 mg, 0.020 mmol), diluted with unlabelled aldehyde (10) (15.18 mg, 0.060 mmol), reacted with the unlabelled tripyrrene hydrobromide (9) (70.00 mg, 0.080 mmol) to afford diluted [15,19-$^{13}C_2$]biladiene dihydrobromide [as (6)] (79.0 mg, 90.5%), 20.3% doubly labelled.

3,8,13,18-*Tetrakis*(2-*methoxycarbonylethyl*)-7,12,17-*tris*-*methoxycarbonylmethyl*-6′-*oxo*-1′,2′,5′,6′-*tetrahydropyrido*-[3′,4′-a]*bilane* (5) *together with Singly and Doubly* ^{13}C-*Labelled Samples*.—All solvents used in these preparations were degassed *in vacuo* and saturated with nitrogen. Light was excluded whenever possible.

Method A. Sodium acetate (100 mg) was added to a solution of the foregoing diluted [15,19-$^{13}C_2$]biladiene dihydrobromide [as (6)] (79.0 mg) in methanol (20 ml) containing pre-reduced Adams catalyst (100 mg) and the mixture was stirred under hydrogen for 18 h when the solution had become colourless and the bilane had precipitated. The solid was collected on Celite, washed with methanol (10 ml), and the product washed off the catalyst with methanol–dichloromethane (1 : 9 v/v; 30 ml). The residue from this solution was chromatographed in methanol–dichloromethane (1 : 9 v/v; total 8 ml) on alumina (Grade III; 1 g). The eluates were concentrated (to 0.2 ml) under nitrogen, methanol (3 ml) was added, the precipitate was collected by centrifugation, and, after two methanol washes, the solid was dried *in vacuo* to afford the *bilane* (31.6 mg, 46.8%) as a pale yellow powder, m.p. 225—228 °C (decomp.); it was used directly for hydrolysis. Unlabelled material, prepared by the same procedure, was characterised (Found: M^+, 933.3950. $C_{47}H_{59}N_5O_{15}$ requires *M*, 933.4003); $\nu_{max.}$ 3 265, 2 910, 2 840, 1 715, 1 655, 1 420, 1 340, 1 290, 1 260, 1 150, 1 080, and 1 000 cm^{-1}; δ 2.1—2.8 (16 H, m, 4 × CH_2CH_2), 3.26 (2 H, s, CH_2CONH), 3.37 (6 H, s, 3 × CH_2CO_2), 3.5—3.7 (27 H, m, 7 × OCH_3 and 3 × methane-CH_2), 4.34br (2 H, CH_2NH), 5.86br (1 H, CH_2NH), 6.35br (1 H, 19-H), and 8.45br, 8.85br, and 9.04br (4 H, 4 × NH).

The [15-^{13}C]bilane [as (5)] (7.8 mg, 54%) was prepared in a similar manner from the corresponding [15-^{13}C]biladiene dihydrobromide [as (6)] (17 mg).

Method B. Sodium borohydride (300 mg) was added during 5 min to a stirred solution of the [*aminomethylene*,-16-$^{13}C_2$]biladiene dihydrobromide [as (6)] (32.8 mg, 0.030 mmol) plus unlabelled material (6) (98.2 mg, 0.090 mmol) in methanol (8 ml), cooled on ice. Water (2 ml) was added to the pale yellow mixture which was then kept at 5 °C for

1 h. The product was collected by centrifugation, washed [methanol (8 ml); water (5 ml); methanol (2 × 5 ml)] and dried *in vacuo* to give 60.4 mg (54.0%) of the diluted [*aminomethylene*,16-$^{13}C_2$]bilane [as (5)] as a pale yellow powder, 20.3% doubly labelled. Identity with material prepared by method A was established by n.m.r., t.l.c., and m.p.

Hydrolysis of Bilanes and Incubation of Aminomethylbilanes (2), (2a), (2b), *and* (2c) *with Deaminase–Cosynthetase from* E.gracilis.—The diluted [15,19-$^{13}C_2$]bilane lactam ester [as (5)] (31.6 mg) was stirred in the dark under nitrogen with degassed, nitrogen-saturated aqueous 2M-potassium hydroxide (1.5 ml) until the solid dissolved (4 h). After 22 h, the solution was adjusted to pH 8 with aqueous 1M-potassium dihydrogenphosphate (4.0 ml) and exactly two thirds of this solution containing the bilane (2b) was added to deaminase–cosynthetase (127 000 units from *E. gracilis*) in 0.1M-phosphate buffer (pH 7.0, 88 ml). The mixture was sealed under nitrogen and incubated in the dark at 37 °C for 17 h.

The incubate was treated with aqueous 1% iodine in potassium iodide (1.0 ml) and 5 min later, the excess of iodine was destroyed with sodium hydrogensulphite. The residue, from evaporation, after drying *in vacuo*, was stirred for 16 h with methanolic sulphuric acid (5%; 40 ml) containing trimethyl orthoformate (0.3 ml). Water (200 ml) was then added, the porphyrin ester was extracted into dichloromethane (4 × 30 ml), washed with water (2 × 100 ml), and recovered by evaporation. Chromatography on silica (6 g) with methanol (1%) in dichloromethane as eluant gave uroporphyrin esters (9.65 mg, 45.3%).

This material (9.65 mg) in 7.5M-hydrochloric acid (6 ml) was distributed equally among 6 Carius tubes (250 × 25 mm) and water (24 ml) was added to each. The solutions were cooled on ice, evacuated (0.1 Torr) for 15 min, sealed *in vacuo* and heated at 180 °C for 4 h. The combined contents of the tubes were evaporated to dryness and esterified as above. Purification of the product by chromatography on silica (3 g) using methanol (0.5%) in dichloromethane afforded coproporphyrin tetramethyl ester (6.09 mg, 83.8%). Analytical h.p.l.c. gave an isomer composition of type-III (12), 75%, and type-I (11), 25%.

To separate the type-III isomer, the product was dissolved in a few drops of dichloromethane, ether (2 ml) was added and the solution was concentrated to about 0.1 ml with a stream of nitrogen, thereby producing a fine suspension. More ether (8 ml) was then added, and the mixture was stirred for 30 min and filtered. The insoluble material was submitted to the same procedure and the resulting solid was set aside. The two filtrates were combined, evaporated to small bulk, and extracted with ether (2 × 8 ml) as before to give a solution which contained 3.45 mg (by u.v.) of coproporphyrin esters, shown (h.p.l.c.) to contain 95.5% type-III, 4.5% type-I. The less soluble material from both extraction cycles was recrystallised twice from dichloromethane–ether to afford 0.98 mg of needles, shown (h.p.l.c.) to contain 90% type-I. Preparative h.p.l.c. of the mother-liquors led to the isolation of more type-III material (0.72 mg, 95% pure) and a type-I fraction which was recrystallised as above to give material of 90% purity (0.33 mg). Finally, the coproporphyrin-III ester (4.17 mg) was purified on silica (3 g) as usual for spectroscopy; δ_C (0.0065M) 11.7 (ring CH_3), 21.9 ($CH_2CH_2CO_2$), 36.9 and 37.0 ($CH_2CH_2CO_2$), 51.5 (OCH_3), 96.2 (d, *J* 70 Hz, 15-C coupled to 16-C in doubly labelled molecules), 96.3 (15-C in singly labelled molecules, 5-, 10-, 15-, 20-C at natural abundance and signal from *ca.* 4% of coproporphyrin-I ester; after the addition of $Pr(fod)_3$ (0.008M) 91.5 (d, *J* 70 Hz, 15-C coupled to 16-C in doubly labelled molecules), 91.6 (s, 15-C in singly-labelled molecules and natural abundance signal from 'diluent'), 95.4 (10-C at natural abundance), 95.8 (5, 20-C at natural abundance), 96.2 (d, *J* 6 Hz, signal from *ca.* 4% of coproporphyrin-I ester). Ratio of last 3 signals, *ca.* 1 : 2 : 1. Ratio of signals at 91.5 and 91.6, 6.31 ± 0.25 : 1 which corresponds to 0—1% crossover between doubly labelled and unlabelled molecules; *m/e* 710 (100), 711 (54.0 ± 1.9), 712 (43.3 ± 1.8) which indicates [12] that the material is 20.6 ± 2.0% doubly ^{13}C-labelled.

The coproporphyrin-I tetramethyl ester from this experiment had *m/e* 710 (100), 711 (51.3 ± 1.8), 712 (43.0 ± 1.6), corresponding to 21.6 ± 1.8% double labelling. Its ^{13}C n.m.r. spectrum was identical with that of material from chemical cyclisation (see below).

The [15-^{13}C]bilane lactam ester [as (5)] (10.8 mg) was hydrolysed and treated with deaminase–cosynthetase (50 000 units) as above (pH 7.1, 37 °C, 16 h) to give uroporphrin esters (3.7 mg, 34%). This material (3.6 mg) was converted as before into coproporphyrin tetramethyl ester (2.35 mg, 86.7%), which contained (h.p.l.c.) 73% type-III (12) and 27% type-I (11). Approximately half this product was separated by preparative h.p.l.c. to give coproporphyrin-III tetramethyl ester of 94% purity, δ_H (partial spectrum) 10.06 (s, 5-, 10-, and 20-H plus d, *J* 153 Hz, 15-H); after addition of Eu([$^2H_9$]fod)$_3$ (*ca.* 6 mol equiv.) 10.58 (20-H), 10.79 (5-H), 10.95 (10-H), and 14.60 (d, *J* 154 Hz, 15-H); δ_C (partial spectrum) 96.3 (15-C plus 5-, 10-, and 20-C at natural abundance), after the addition of $Pr(fod)_3$ (*ca.* 2 mol equiv.) major signal at δ 87.8 (15-C).

When mixed with authentic unlabelled coproporhyrin-III tetramethyl ester, the shifted ^1H spectrum showed no additional peaks except for a large singlet from the added unlabelled material centred on the 15-H singlet and doublet from the labelled sample.

An improved incubation and isolation procedure was used for the [*aminomethylene*,16-$^{13}C_2$]bilane. The diluted lactam ester [as (5)] (27 mg) was hydrolysed as above and the pH adjusted to 8 with aqueous potassium dihydrogenphosphate. This solution (12 ml) was kept on ice while it was added dropwise, over 100 min to a gently stirred solution of deaminase–cosynthetase (160 000 units) in 0.1M-phosphate buffer (pH 7.5, 78 ml), kept at 38 °C. After 20 min, uro'gen formation was complete and iodine oxidation as before was followed by addition of Whatman DE52 slurry (10 ml). The resin was collected (the filtrate contained no porphyrin) and washed with water, and the porphyrin was washed off with 3M-hydrochloric acid (20 ml). The residue from this solution was esterified and purified as above to give uroporphyrin octamethyl esters (18.6 mg, 68.1%).

This material (18.6 mg) was converted into coproporphyrin tetramethyl esters (13.0 mg, 92.8%) as before (2 tubes used for the decarboxylation step) and analysis by h.p.l.c. showed that it contained type-III (12), 89%, and type-I (11), 11%. After the ether separation procedure as earlier, coproporphyrin-III ester (95% pure; 9.5 mg) was obtained; δ (partial spectrum) [plus Eu([$^2H_9$]fod)$_3$, 1.1 mol equiv.] 10.49 (d, *J* 154 Hz, 20-^{13}CH, plus s, 20-^{12}CH), 10.72 (s, 5-H), 10.91 (s, 10-H), 14.06 (s, 15-H); δ_C (partial spectrum) [with $Pr(fod)_3$] 90.1 (15-C at natural abundance), 95.0 (5, 10-C at natural abundance), 95.4 (d, *J* 70 Hz, 20-C coupled to 19-C in doubly labelled molecules), 95.5 (s, 20-C

in singly labelled molecules and natural abundance signal from diluent); ratio of last two signals, 6.34 : 1 which corresponds to 0.5% crossover between doubly labelled and unlabelled molecules; *m/e* 710 (100%), 711 (53.0 ± 1.5), 712 (46.4 ± 1.6) corresponding to 22.8 ± 1.7% doubly ^{13}C-labelled species.

Chemical Cyclisation of Aminomethylbilanes (2a), (2b), *and* (2c).—The remaining hydrolysed [15,19-$^{13}C_2$]bilane (2b) from the previous section (from 10.5 mg lactam ester) was incubated in 0.1M-phosphate buffer (pH 7.1, 44 ml) for 17 h at 37 °C, as for the enzyme run, to give 4.98 mg (46.8%) of uroporphyrin ester. This was converted into coproporphyrin ester (3.31 mg, 88.3%) as above and h.p.l.c. showed it to contain type-I, 96%, type-III, 3%, and type-IV, 1%; δ_C 11.7 (ring CH_3), 21.9 ($CH_2CH_2CO_2$), 37.0 ($CH_2CH_2CO_2$), 51.6 (OCH_3), 96.5 (d, *J* 6 Hz, ' 15-C ' coupled to ' 19-C ' in doubly labelled molecules); after addition of Pr(fod)$_3$ (1.6 mol equiv.), the signal originally at δ 96.5 moved to 95.8; *m/e* 710 (100), 711 (54.6 ± 0.5), 712 (43.0 ± 1.8) which corresponds to 20.2 ± 1.5% double ^{13}C-labelling.

The diluted [*aminomethylene*,16-$^{13}C_2$]bilane lactam ester [as (5)] (60.4 mg) was hydrolysed in the usual way and incubated in 0.1M-phosphate buffer (pH 7.5, 240 ml), as above to give uroporphyrin ester (30.2 mg, 49.5%). From this material (29.0 mg) was obtained 20.6 mg (94.3%) of coproporphyrin-I tetramethyl ester, 96% pure by h.p.l.c. δ_C 96.5 (d, *J* 6 Hz, ' 20-C ' coupled to ' 16-C ' in doubly labelled molecules and s, ' 20-C ' in singly labelled molecules and other *meso*-carbons at natural abundance); ratio of doublet to singlet, *ca.* 2 : 1; *m/e* 710 (100), 711 (57.3 ± 1.3), 712 (39.4 ± 1.4) corresponding to 16.8 ± 1.5% doubly labelled and 7.6 ± 1.0% singly labelled molecules.

Dibenzyl 4,4′-*Bis*-(2-*methoxycarbonylethyl*)-3,3′-*bismethoxycarbonylmethyl*-2,2′-([^{13}C]methylene)*dipyrrole*-5,5′-*dicarboxylate* (21).—[^{13}C]Paraformaldehyde (90 atom %, 4.3 mg) was added at 0 °C to a stirred solution of benzyl 3-(2-methoxycarbonylethyl)-4-methoxycarbonylmethylpyrrole-2-carboxylate [5] (100 mg) in trifluoroacetic acid (1.5 ml). After 15 min at 0 °C and 1 h at 20 °C, the solution was evaporated and the residue in ethyl acetate (10 ml) was stirred with sodium hydrogencarbonate (400 mg). The filtered solution was then evaporated and the residue was recrystallised from methanol to give the pyrromethane (21) (98 mg, 96%), identical with a previously prepared sample; [13] *m/e* 731 (M^+, 3%) and 640 (100); δ 2.20—3.20 (8 H, m, 2 × CH_2CH_2), 3.50 (4 H, s, 2 × CH_2CO_2), 3.45 and 3.55 (each 6 H, s, 4 × OCH_3), 3.80 (2 H, d, *J* 128 Hz, $^{13}CH_2$), 5.20 (4 H, s, CH_2Ph), and 7.30 (10 H, s, C_6H_5).

[20-^{13}C]*Uroporphyrin-III Octamethyl Ester and the* [15-^{13}C]*Uroporphyrin-III Octamethyl Ester.*—The foregoing pyrromethane (98 mg) was hydrogenated over 10% palladium–charcoal (50 mg) in tetrahydrofuran (20 ml) containing triethylamine (2 drops). After 2 h, the solution was filtered through Celite and the pad was washed with hot tetrahydrofuran–triethylamine (100 : 1 v/v). The filtrate was concentrated (to *ca.* 1 ml), water (3 ml) was added followed by acetic acid (to pH 3), and the mixture was cooled to 0 °C for 3 h. The precipitate was collected and dried to give the pyrromethane-5,5′-dicarboxylic acid (68 mg, 92%), which was used directly in the next stage; *m/e* 463 (M^+ − 88, 58%) and 390 (100).

The [*methylene*-^{13}C]diacid (25.1 mg) was decarboxylated in hot acetic acid and condensed with 3,4′-bis-(2-methoxycarbonylethyl)-4,3′-bismethoxycarbonylmethyl-2,2′-methylenedipyrrole-5,5′-dicarbaldehyde [13] (21.4 mg) by an established procedure [13] to give [20-^{13}C]uroporphyrin-III octamethyl ester (14.7 mg, 37.7%); δ − 3.59br (2 H, 2 × NH), 3.38 (8 H, t, 4 × $CH_2CH_2CO_2$), 3.71 and 3.83 (each 12 H, s, 8 × OCH_3), 4.47 (8 H, t, 4 × $CH_2CH_2CO_2$), 5.12 (8 H, s, 4 × CH_2CO_2), 10.23 (d, *J* 158 Hz, 20-H, and s, 5-, 10-, and 15-H); *m/e* 943 (M^+).

For the [15-^{13}C] compound, dibenzyl 3,3′-bis-(2-methoxycarbonylethyl)-4,4′-bismethoxycarbonylmethyl-2,2′([^{13}C]-methylene)dipyrrole-5,5′-dicarboxylate [13] was converted into the diacid, as above and condensed with the same dialdehyde to give [15-^{13}C]uroporphyrin-III octamethyl ester (34%) as needles, m.p. 260.5—261.5 °C (lit.,[23] 255—258, 258—260 °C) (from dichloromethane–methanol). N.m.r. spectrum as above, except for δ 10.16 (d, *J* 157 Hz, 15-H, and s, 5-, 10-, and 20-H).

[15-^{13}C]*Coproporphyrin-III Tetramethyl Ester and the* [20-^{13}C]*Coproporphyrin-III Tetramethyl Ester.*—[15-^{13}C]-Uroporphyrin-III octamethyl ester (2.06 mg) was decarboxylated as above and the product was esterified to give 1.33 mg (86%) (u.v. assay) of [15-^{13}C]coproporphyrin-III ester. Analytical h.p.l.c. indicated an isomeric purity of 95%, the only contaminant being the type-I isomer; δ (0.012M) − 3.70br (2 H, 2 × NH), 3.30 (8 H, t, 4 × $CH_2CH_2CO_2$), 3.67 and 3.70 (24 H, 4 × OCH_3 and 4 × ring CH_3), 4.43 (8 H, t, 4 × $CH_2CH_2CO_2$), 10.05 (3 H, s, 5-, 10-, and 20-H), 10.07 (*ca.* 0.9 H, d, *J* 154 Hz, 15-^{13}CH, and *ca.* 0.1 H, s, 15-^{12}CH); after the addition of Eu([$^2H_9$]fod)$_3$ (0.016M), 10.43 (1 H, s, 20-H), 10.56 (1 H, s, 5-H), 10.69 (1 H, s, 10-H), 13.40 (*ca.* 0.9 H, d, *J* 154 Hz, 15-^{13}CH, and *ca.* 0.1 H, s, 15-^{12}CH); δ_C (0.004M) 96.3 (15-C), shifted by the addition of Pr(fod)$_3$ (0.008M) to 87.8.

Decarboxylation of [20-^{13}C]uroporphyrin-III ester similarly gave the corresponding labelled coproporphyrin-III ester; δ (plus Eu([$^2H_9$]fod)$_3$) (partial spectrum) 10.38 (d, *J* 155 Hz, 20-^{13}CH, and s, 20-^{12}CH), 10.49 (s, 5-H), 10.62 (s, 10-H), 13.27 (s, 15-H); *m/e* 711 (M^+).

Grateful acknowledgement is made to the Nuffield Foundation, the S.R.C., and Roche Products Ltd. for financial support, to the British Council for an Award (to H. K. W. W.) and to the Commissioners for the Exhibition of 1851 for a Scholarship (to M. J. M.). We also thank Dr. A. Pfenninger for the indicated preparation and Drs. D. C. Williams and G. W. J. Matcham for providing the *Euglena* enzyme systems.

[1/241 *Received*, 13*th February*, 1981]

REFERENCES

[1] Part 15, A. R. Battersby, D. G. Buckley, D. W. Johnson, L. N. Mander, E. McDonald, and D. C. Williams, preceding paper.

[2] Preliminary accounts in part, (*a*) A. R. Battersby, E. McDonald, D. C. Williams, and H. K. W. Wurziger, *J. Chem. Soc., Chem. Commun.*, 1977, 113; (*b*) A. R. Battersby, C. J. R. Fookes, E. McDonald, and M. J. Meegan, *ibid.*, 1978, 185.

[3] Reviews: (*a*) B. Frydman and R. B. Frydman, *Acc. Chem. Res.*, 1975, **8**, 201 and references therein; (*b*) A. R. Battersby and E. McDonald, *ibid.*, 1979, **12**, 14 and references therein.

[4] (*a*) A. R. Battersby, D. A. Evans, K. H. Gibson, E. McDonald, and L. Nixon, *J. Chem. Soc., Perkin Trans. 1*, 1973, 1546; (*b*) A. R. Battersby, J. F. Beck, and E. McDonald, *ibid.*, 1974, 160.

[5] A. R. Battersby, S. Kishimoto, E. McDonald, F. Satoh, and H. K. W. Wurziger, *J. Chem. Soc., Perkin Trans. 1*, 1979, 1927.

[6] K. M. Smith, in ' Porphyrins and Metalloporphyrins,' ed. K. M. Smith, Elsevier, Amsterdam, 1975, p. 29.

[7] A. R. Battersby, D. G. Buckley, G. L. Hodgson, E. McDonald, and R. E. Markwell in ' High Pressure Liquid Chromatography in Clinical Chemistry,' eds. P. F. Nixon, C. H. Gray, C. K. Lim, and M. S. Stoll, Academic Press, London, 1976, p. 63.

[8] A. R. Battersby, G. L. Hodgson, E. Hunt, E. McDonald, and J. Saunders, *J. Chem. Soc., Perkin Trans. 1*, 1976, 273.
[9] H.-O. Dauner, G. Gunzer, I. Heger, and G. Müller, *Hoppe-Seyler's Z. Physiol. Chem.*, 1976, **357**, 147.
[10] M. S. Stoll, G. H. Elder, D. E. Games, P. O'Hanlon, D. S. Millington, and A. H. Jackson, *Biochem. J.*, 1973, **131**, 429.
[11] P. S. Clezy and V. Diakiw, *Aust. J. Chem.*, 1973, **26**, 2697.
[12] J. H. Beynon, 'Mass Spectrometry and Its Applications to Organic Chemistry,' Elsevier, Amsterdam, 1960, p. 296.
[13] A. R. Battersby, M. Ihara, E. McDonald, J. Saunders, and R. J. Wells, *J. Chem. Soc., Perkin Trans. 1*, 1976, 283.
[14] A. R. Battersby, E. Hunt, E. McDonald, and J. Moron, *J. Chem. Soc., Perkin Trans. 1*, 1973, 2917.
[15] A. R. Battersby, E. Hunt, E. McDonald, J. B. Paine III, and J. Saunders, *J. Chem. Soc., Perkin Trans. 1*, 1976, 1008.
[16] A. R. Battersby, C. J. R. Fookes, K. Gustafson-Potter, E. McDonald, and G. W. J. Matcham, in preparation.
[17] Briefly reviewed by A. R. Battersby, C. J. R. Fookes, G. W. J. Matcham, and E. McDonald, *Nature*, 1980, **285**, 17.
[18] J. Cason, *Org. Synth.*, Coll. vol. 3, 1955, p. 169.
[19] 'Handbook of Physics and Chemistry,' ed. R. C. Weast, Chemical Rubber Company, Cleveland, 1975, p. C–359.
[20] D. M. Brunwin, G. Lowe, and J. Parker, *J. Chem. Soc. C*, 1971, 3756.
[21] A. Treibs and K. Hintermeier, *Chem. Ber.*, 1954, **87**, 1167.
[22] A. H. Jackson and S. F. MacDonald, *Can. J. Chem.*, 1957, **35**, 715.
[23] E. J. Tarlton, S. F. MacDonald, and E. Baltazzi, *J. Am. Chem. Soc.*, 1960, **82**, 4389.