Biosynthesis of Porphyrins and Related Macrocycles. Part 41.[1] Fate of Oxygen Atoms as Precorrin-2 Carrying Eight Labelled Carboxyl Groups ($^{13}C^{18}O_2H$) is Enzymatically Converted into Cobyrinic Acid

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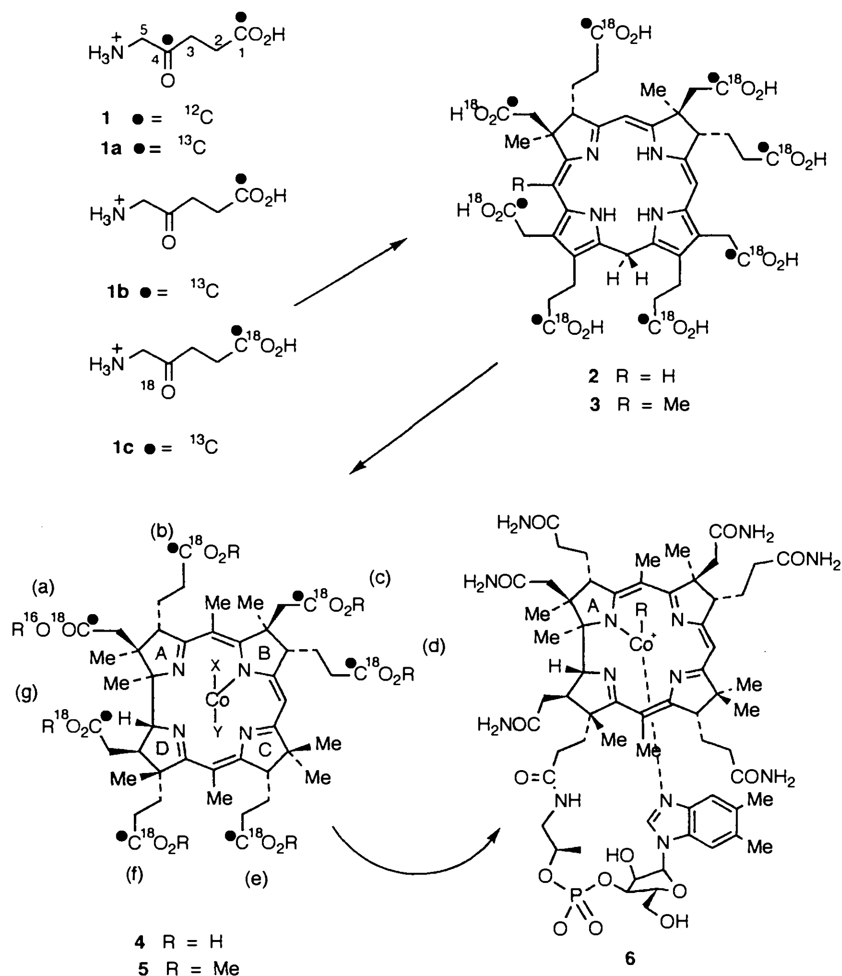
5-Amino[1,4-$^{13}C_2$]laevulinic acid and 5-amino[1-^{13}C]laevulinic acid are synthesised and all three ^{16}O atoms of the latter are exchanged for ^{18}O. The ^{13}C,^{18}O-labelled material is then converted *in vitro* into precorrin-2 **2** by the combined action of four genetically overproduced enzymes. The product is isolated in its aromatised form, sirohydrochlorin **10** and ^{13}C-NMR shows that all 8 carboxyl groups of **10** retain both oxygen atoms throughout the biosynthesis. A cell-free enzyme preparation from *Propionibacterium shermanii* converts the ^{13}C,^{18}O-labelled sirohydrochlorin **10** *via* **2** into cobyrinic acid **4**, a late precursor of vitamin B_{12}. ^{13}C-NMR proves that 6 carboxyl groups of cobyrinic acid (b–g, inclusive) retain both oxygen atoms whereas the a-carboxyl group undergoes specific loss of one labelled oxygen atom.

In 1989, Kajiwara's group[2] reported the incorporation of 5-amino[1-^{13}C,1,1,4-$^{18}O_3$]laevulinic acid (ALA) **1b** into vitamin B_{12} **6** using growing *Propionibacterium shermanii* cells. They found that substantial loss of ^{18}O had occurred from just one of the seven carboxamide groups of isolated vitamin B_{12} presumably by exchange with the aqueous medium. On the basis of earlier literature, this particular carboxamide group was assigned as the acetamido residue on ring-A. Initially, there was some doubt as to whether the literature assignment was entirely secure but its correctness has been confirmed.[3]

It is clearly of great interest to know at which step in the long biosynthetic sequence leading to vitamin B_{12}[4] **6** (Scheme 1) this specific loss of labelled oxygen occurs. Our aim is to use ^{18}O : ^{13}C double-labelling to study several earlier intermediates

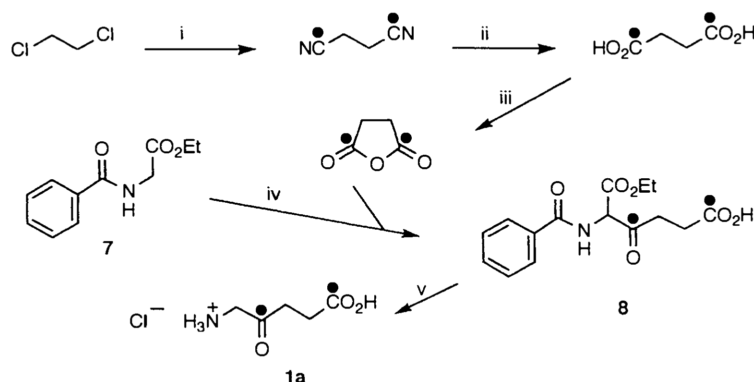


Scheme 1

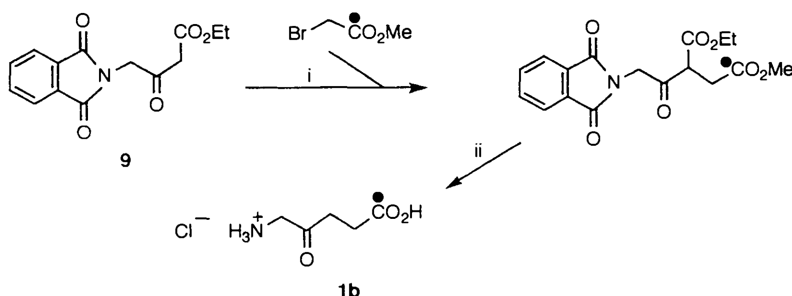
Table 1 Assignments of ^{13}C signals (100.6 MHz) for the carbonyl groups (and δ_H for OMe groups) of cobester **5** in C_6D_6

Carbonyl groups	a	b	c	d	e	f*	g
δ_C Values	172.12	172.41	170.77	173.22	173.86	172.86	171.75
δ_H Values of coupled α- and β-protons	2.28, 2.94	1.98, 2.52	2.55	1.81, 2.10 2.37, 2.64	2.38, 2.74	—	2.22, 2.36
δ_H for OMe	3.28	3.47	3.27	3.385 or 3.388	3.34	3.40	3.388 or 3.385

* This CO signal only showed a significant correlation peak to the ester Me protons at δ_H 3.40 but since all the other ^{13}CO resonances are firmly assigned, δ_C 172.86 is from the f-carbonyl.



Scheme 2 *Reagents and conditions:* $K^{13}CN$, 18-crown-6; ii, reflux with conc. hydrochloric acid; iii, Ac_2O, heat; iv, LDA; v, acetic acid, conc. hydrochloric acid at reflux



Scheme 3 *Reagents and conditions:* i, $NaN(SiMe_3)_2$; ii, acetic acid, conc. hydrochloric acid at reflux

on the pathway to see whether they have or have not undergone ^{18}O-exchange from a specific carboxyl group. In this way, one will be able to pin-point the exact stage where the ^{18}O-loss occurs. The present paper makes the first leap backwards down the pathway to cobyrinic acid **4** chosen because (a) the corrin ring has been constructed at this point, (b) the ^{13}C-signals from the seven ester carbonyl groups of cobyrinic acid heptamethyl ester (cobester) **5** are well separated, (c) ^1H-NMR spectrum of cobester is fully assigned[5,6] so opening the way to assignment of the carboxyl ^{13}C-resonances by a ^{13}C-^1H COSY experiment and, importantly, (d) all 14 peripheral oxygens remain for study in **4** in contrast to vitamin B_{12} **6** where seven of these oxygens have been eliminated during amidation, $CO_2H \rightarrow CONH_2$.

Assignment of the carbonyl ^{13}C-resonances of cobester **5** used ^{13}C-^1H COSY to detect long-range (2–3 bond) couplings with the sample concentration the same as had been used earlier[5] for the complete ^1H-signal assignments. Good sensitivity was achieved by using the ^1H-detection mode and the results are collected in Table 1. This work incidentally provided ^1H-signal assignments for the OMe groups of **5**, included in Table 1, and confirmed several earlier assignments[5,6] of signals from sp^2 carbons. The latter are given in the Experimental section.

We now required ALA ^{13}C-labelled at the carboxyl group **1b** but initially it seemed that the synthesis would be simplified by building [1,4-$^{13}C_2$]ALA **1a** rather than [1-^{13}C]ALA **1b**. By so doing, a short route[7] to ALA using ethyl hippurate **7** and succinic anhydride could be used (see Scheme 2). Accordingly, [1,4-$^{13}C_2$]succinic anhydride was prepared in high yield starting from $K^{13}CN$ as in Scheme 2. The final steps **7**→**8**→**1a** worked well and the overall yield from $K^{13}CN$ was excellent at 54%. The ^{13}C NMR spectrum at 100.6 MHz of [1,4-$^{13}C_2$]ALA **1a** immediately revealed a problem; there was significant long-range coupling (*J* 1.7 Hz) between the labelled centres. Since the final experiment depends on small ^{18}O-induced shifts on ^{13}C-signals, it was clear that a similar coupling in the biosynthetically derived cobester **5** would make observation of the expected shifts difficult and therefore this approach was not satisfactory.

[1-^{13}C]ALA **1b** was therefore prepared from the keto ester[8] **9**, kindly provided by Dr. C. Lambert, and methyl [1-^{13}C]-bromoacetate at 99 atom% ^{13}C as shown in Scheme 3. The oxygens in this product were then exchanged by two sequential treatments with $H_2{}^{18}O$ (97 atom% ^{18}O) using acid catalysis at 120 °C.[9] The ^{13}C NMR showed that the exchanged product **1c** contained *ca.* 75% of $^{13}C^{18}O_2H$ together with *ca.* 25% of $^{13}C^{16}O^{18}OH$ and only a trace of $^{13}C^{16}O_2H$. The latter two species correspond to the two small signals downfield relative to the major signal from $^{13}C^{18}O_2H$ (Fig. 1).

Methods have been developed in Cambridge for the preparative enzymic synthesis of precorrin-2 **2** and precorrin-3 **3** from ALA **1**; **2** and **3** are early precursors on the pathway[4]

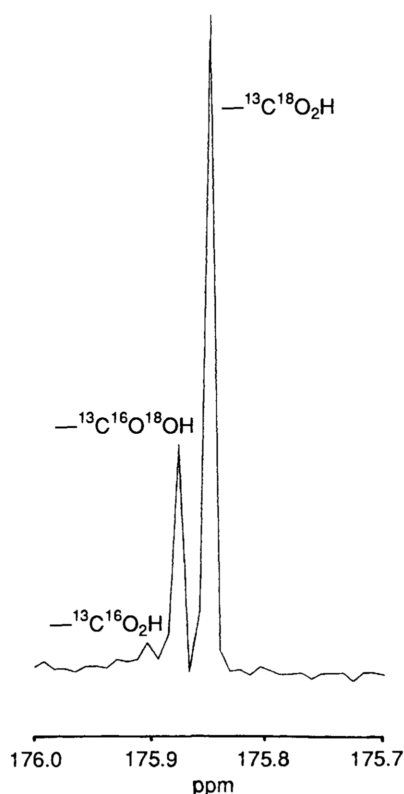


Fig. 1 ^{13}C NMR (100.6 MHz) signals from the carboxyl carbonyl of 5-amino[1-^{13}C,1,1,4-$^{18}O_3$] laevulinic acid hydrochloride **1c** in CD_3OD acquired over spectral width of 20120 Hz and 64K datapoints using CPD (Waltz-16) decoupling

after it has branched towards vitamin B_{12}. These syntheses depend on molecular biological methods for overproduction of all the necessary enzymes which are then used together *in vitro*. For the production of precorrin-2 **2** needed for the present work, four enzymes have been over-produced, *viz* ALA dehydratase, hydroxymethylbilane synthase (porphobilinogen deaminase), uroporphyrinogen III synthase (cosynthetase) and *S*-adenosyl-L-methionine: uroporphyrinogen III methyltransferase (SUMT), with valuable help for the last enzyme from Dr. J. Crouzet.[10] Details of this major effort on molecular biology and enzymology are being published separately.[11] The [1-^{13}C,1,1,4-$^{18}O_3$]ALA **1c** was converted into precorrin-2 **2** by incubation with the four enzymes above and the product was isolated as its aromatised form (by ready oxidation in air), part as the acid (sirohydrochlorin) **10**, for later use as a precursor of cobyrinic acid, and part as the ester **11** for ^{13}C NMR spectroscopy. It was important to make the ester under basic conditions using trimethyloxonium tetrafluoroborate (Meerwein's reagent) to avoid loss of ^{18}O from the carboxyl groups. The ^{13}C NMR spectrum of the octamethyl ester **11** was measured after adding a little unlabelled **11** to provide a slightly larger unshifted reference signal (natural abundance) for the 8 methoxycarbonyl groups; this was because the proportion of carboxyl groups carrying no ^{18}O in the original ALA **1c** was so low (see Fig. 1). All 8 signals from the ester groups of **11** were resolved, with one family of 4 signals (δ 170.8–171.8) corresponding to the acetate esters and the other set of 4 (δ 173.0–173.3) arising from the propionate esters. Importantly, by far the major signal (*ca.* 75% of total) for all 8 ester groups of **11** was the fully shifted one corresponding to $^{13}C^{18}O_2Me$ (all shifts 5.0–5.2 Hz at 100.6 MHz). The carbonyl groups which gave sufficiently well-separated signals also showed small peaks (*ca.* 10–12% of total) corresponding to $^{13}C(=^{16}O)-^{18}OMe$ and $^{13}C(=^{18}O)-^{16}OMe$. Fig. 2 shows the ^{13}C NMR spectrum of

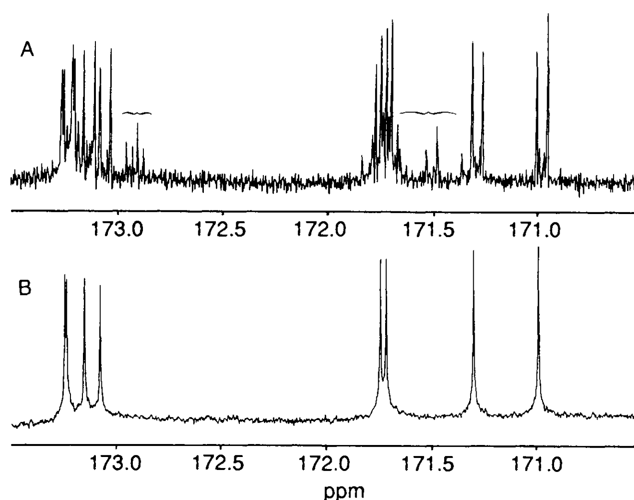
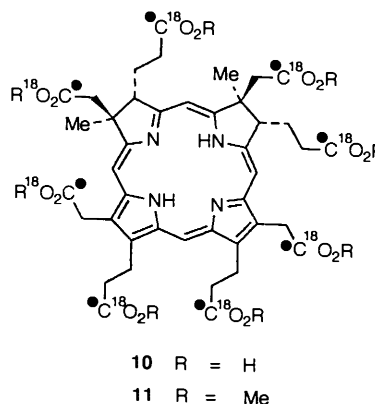
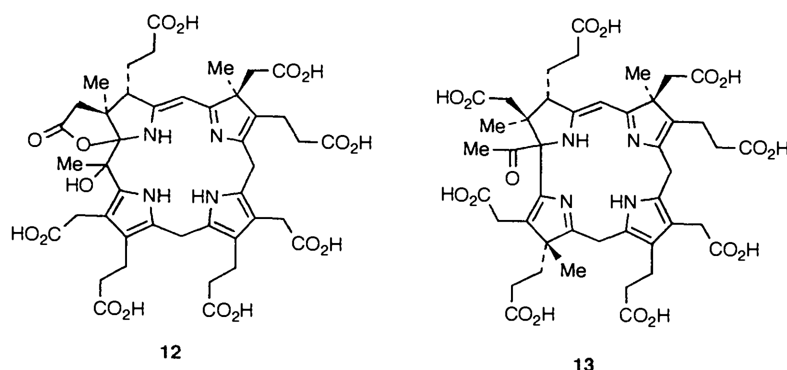


Fig. 2 (A) ^{13}C NMR (100.6 MHz) spectrum of ^{13}C,^{18}O-labelled sirohydrochlorin octamethyl ester **11** in C_6D_6 after substantial dilution with unlabelled material. The data were acquired over spectral width 1400 Hz and 16K datapoints using CPD (Waltz-16) decoupling with 9538 scans, pulse angle 45°, acquisition time 5.8 s; for resolution enhancement, a line broadening factor of −0.7 was applied together with Gaussian multiplier of 0.15, 0.085 Hz/datapoint. FID zero-filled to 32K prior to FT. The signals enclosed in brackets are largely from the 3-epimer of **11** as shown by comparison with the spectrum from a standard sample. (B) ^{13}C NMR spectrum of sirohydrochlorin octamethyl ester (as **11**) labelled with ^{13}C only at each ester carbonyl group.



this labelled sample of **11** after further addition of a substantial amount of natural abundance material which simply increases the size of the unshifted signals. These results gained directly from the dimethylated ester **11** show that no loss of labelled oxygen occurs from any of the carboxyl groups over the entire biosynthetic pathway from ALA **1c** through to precorrin-2 **2**, a result in agreement with another study which used a different stage for the analysis.[3]

The labelled sirohydrochlorin **10** isolated above and now proven to have all 8 carboxyl groups largely as $^{13}C^{18}O_2H$ was incubated with the cell-free enzyme system[12] from the *P. shermanii*. It is well known that **10** is reduced back to precorrin-2 **2** under these conditions and the biosynthesis was then run forward to yield cobyrinic acid **4**. This was isolated by reversed-phase chromatography, then with minimal dilution with unlabelled carrier material, was purified by HPLC and finally esterified with Meerwein's reagent as above to afford labelled cobester **5**. The ^{13}C NMR spectrum of this product is shown as the upper spectrum (A) in Fig. 3 and below it is the natural abundance ^{13}C spectrum (B) of unlabelled cobester (as **5**) with the earlier signal assignments marked. Six methoxycarbonyl groups (b–g, inclusive) show a large fully shifted signal (5.2 Hz)



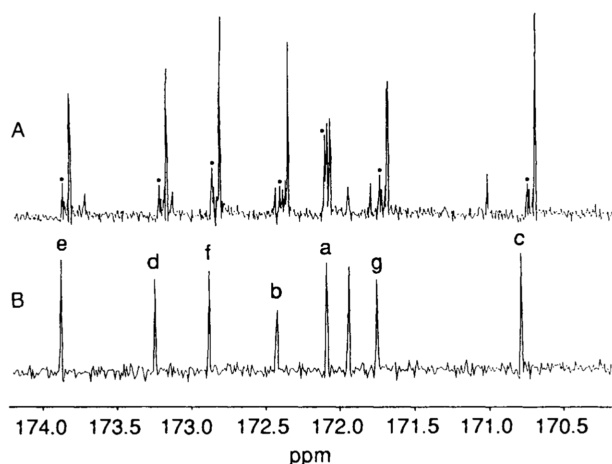


Fig. 3 (A) ^{13}C-NMR (100.6 MHz) spectrum of $^{13}C,^{18}O$-labelled cobester **5** in C_6D_6. Data acquired over spectral width 20120.2 Hz and 64K datapoints using CPD (Waltz-16) decoupling with 36510 scans, acquisition time 0.8 s, line broadening factor −0.7 together with Gaussian multiplier 0.2 and 0.61 Hz/datapoint. The signals marked by a dot are the unshifted ones which act as internal standards since they arise mainly from added natural abundance carrier material. (B) ^{13}C NMR spectrum at natural abundance from unlabelled cobester (as **5**); the slight differences in chemical shift for some signals between those dotted in (A) and the signals in (B) are due to the far higher concentration used for acquisition of (B). The unmarked signal at δ 171.9 is from C-9.[5,6]

corresponding[9,13] to $^{13}C^{18}O_2Me$; three small signals at lower field can also be seen in each case arising from $^{13}C^{16}O_2Me$, $^{13}C(=^{16}O)–^{18}OMe$ (1.4 Hz) and $^{13}C(=^{18}O)–^{16}OMe$ (3.6 Hz), all shifts measured at 100.6 MHz.

The signal from the a-carboxyl group is strikingly different. Here there is no signal derived from $^{13}C^{18}O_2Me$ and in its place are two strong signals of about equal intensity from $^{13}C(=^{16}O)–^{18}OMe$ and $^{13}C(=^{18}O)–^{16}OMe$. These two strong signals show the appropriate upfield shifts of 1.6 and 3.7 Hz, respectively, relative to the unshifted natural abundance signal from the $^{13}C^{16}O_2Me$ group. These results demonstrate that both oxygens of the carboxyl groups b–g, inclusive, of cobyrinic acid **4** are retained during its biosynthesis from precorrin-2 **2**. However, the a-carboxyl group undergoes exchange of *one* of its oxygen atoms as by far the main process. Comparison of the pattern of signals from the a-carboxyl group with those from the other six shows there is some possibility that a very minor amount of $C^{16}O_2H$ species *may* have been generated at the a-carboxyl group from the original $^{13}C^{18}O_2H$ system, thus adding a little intensity to the standard natural abundance signal. However, this may be more apparent than real because the large increase in size of the next very close signal upfield adds to the height of the small unshifted signal. So there is no firm evidence for any loss of both labelled oxygens from the a-carboxyl group and the major effect at this centre is exchange of *one* oxygen atom.

Discussion

The loss of one of the original two oxygen atoms from the ring-A acetate group at some stage during the biosynthesis of cobyrinic acid **4** is in keeping with Eschenmoser's proposal[14] that the ring-contraction process leading eventually to the corrin ring might involve lactone formation between the ring-A acetate and the macrocyclic framework. Subsequent opening of such a lactone by attack at the carbonyl group would lead to loss of one of the original oxygen atoms of the initial acetate function. Recent advances in knowledge of the early stages of the biosynthetic pathway[15,16] mean that the details of Eschenmoser's proposal need to be changed but the principle remains entirely valid. It is probable that the single oxygen loss occurs at that part of the pathway occupied by precorrin-3A[4] **3**, precorrin-3B[15] (**12** or an isomer) and precorrin-4[16] **13**. The origin of the oxygen atoms around ring-A and C-20 of these two intermediates **12** and **13** is currently being studied.

The final point concerns the ^{13}C:^{18}O-labelling studies,[2,3] mentioned earlier, on vitamin B_{12} **6**. In this case, it is stated[3] that almost complete $^{18}O/^{16}O$ exchange of the carbonyl oxygen of the ring-A acetamido group had occurred. Since only one has been lost at the stage of cobyrinic acid **4**, it will be interesting to learn at what stage the second is removed.

Experimental

General Directions.—Except where stated otherwise, the following procedures were adopted. Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Electronic spectra were recorded on Kontron Uvikon spectrophotometer. All NMR spectra were recorded on a Bruker WH-400 instrument operating at 400.1 MHz (^1H) or 100.6 MHz (^{13}C), and the solvent signal was used as internal reference. All samples were dissolved in $CDCl_3$ except cobester **5** and sirohydrochlorin octamethyl ester **11** were in C_6D_6 and ALA hydrochloride **1** in CD_3OD and few others specifically stated. *J* Values are given in Hz.

The cells and cell-free systems were cooled in ice throughout their preparation and protected against light and oxygen as much as possible; all water used in the biological work was glass-distilled. Centrifugations were performed on a Europa 24M centrifuge with 8 × 50 cm^3 rotor at 18 000 rpm at 4 °C. The bacterial cells were broken by three passes through a French-Press (American Instruments Company) at a pressure of 10 000–16 000 psi* (gauge pressure 500–1000 psi at the high ratio setting) under argon. Enzymic incorporation experiments

* psi ≈ 6.894 757 × 10^3 Pa.

were carried out in the dark under argon in a system equipped with a pH electrode and an automatic pH titration system consisting of a PH 82 standard pH meter, TTT 80 titrator and AUB80 autoburette (Radiometer, Copenhagen).

HPLC purifications were carried out using a Waters 600E multisolvent delivery system, the pigments being detected by a CE 272 linear readout UV spectrometer (Cecil Instruments) at 365 nm (sensitivity range 0.1 to 1.0) equipped with RE 511.20 recorder (Venture, 10 mV range). The reversed phase column was Hichrom S 5 ODS 1 (4.6 × 250 mm) and before injecting, samples were filtered through Acrodisc^R 3 (0.45 μm, Gelman Sciences). Preparative TLC was on plates coated to 2 mm with Kieselgel H60 and all solvent systems used for chromatography of cobester were saturated with KCN.

ATP, NAD^+ and NADP were from Boehringer Mannheim; glutathione, DL-cysteine, *S*-adenosyl-L-methionine, DL-dithiothreitol, DEAE-Sephadex A-25, bromo[1-^{13}C]acetic acid, $H_2{}^{18}O$, $K^{13}CN$ from Sigma; $CoCl_2{\cdot}6H_2O$, EDTA $Na_2{\cdot}H_2O$ from Fisons; DEAE-Cellulose (DE-52) from Whatman; trimethyloxonium tetrafluoroborate from Lancaster, and Sep-Pak C-18 cartridges from Waters. Acetonitrile, 1,2-dichloroethane and tetramethylethylenediamine were distilled from calcium hydride. Acetic anhydride was fractionally distilled before use and THF distilled from sodium–benzophenone. $K^{13}CN$ was finely ground and dried *in vacuo* at 100 °C for 8 h. Wet organic solutions were dried over anhydrous Na_2SO_4 or $MgSO_4$. Solvents were evaporated on a Buchi rotary evaporator at reduced pressure.

[1,4-$^{13}C_2$]*Succinonitrile*.[17]—A mixture of 1,2-dichloroethane (1.9 g, 19.1 mmol), $K^{13}CN$ (90 atom%; 2.5 g, 37.8 mmol), 18-crown-6 (292 mg, 1.1 mmol) and acetonitrile (10 cm^3) was heated at reflux with vigorous stirring under argon for 4 h. The cooled solution was filtered and the solids were washed with dichloromethane. The filtrate was evaporated and the residue was purified on a silica gel column (23 × 80 mm), eluting with dichloromethane, to give [1,4-$^{13}C_2$]succinonitrile (1.43 g, 92%), m.p. 48–50 °C (Found: M^+, 82.0440. $^{12}C_2{}^{13}C_2H_4N_2$ requires *M*, 82.0442); δ_H 2.75 (mainly d, *J* 4.2); δ_C 14.60 (dd, *J* 59.5 and 2.7, C-2 and 3), 14.62 (d, *J* 2.8, C-3 for [1-$^{13}C_1$]succinonitrile), 116.1 (enriched s, C-1 and -4), peak height ratio (δ_C 14.60: δ_C 14.62) = 6.6:1.

[1,4-$^{13}C_2$]*Succinic Acid*.—[1,4-$^{13}C_2$]Succinonitrile (2.87 g, 35 mmol) was heated at reflux with conc. hydrochloric acid (12 cm^3) under argon for 4 h after which the cooled mixture was freeze-dried. The residue was extracted continuously with diethyl ether in a Soxhlet extractor for 24 h. The extract was concentrated at 0 °C to give [1,4-$^{13}C_2$]succinic acid (4.11 g, 97.8%), m.p. 189 °C (Found: $M^+ - H_2O$, 102.0228. $^{12}C_2{}^{13}C_2H_6O_4$ requires $M - H_2O$, 102.0228); $\delta_H(CD_3OD + CDCl_3)$ 2.56 (mainly d, *J* 1.9); $\delta_C(CD_3OD + CDCl_3)$ 175.7 (s, enriched carbons, C-1 and -4).

[1,4-$^{13}C_2$]*Succinic Anhydride*.—[1,4-$^{13}C_2$]Succinic acid (1.77 g, 14.7 mmol) was heated with acetic anhydride (4 cm^3) at 95 °C under argon for 1.5 h after which the mixture was cooled, diluted with diethyl ether and held at 0 °C to give [1,4-$^{13}C_2$]-succinic anhydride (1.38 g, 91.7%), m.p. 109–110 °C (Found: M^+, 102.0224. $^{12}C_2{}^{13}C_2H_4O_3$ requires *M*, 102.0228); δ_H 2.99 (mainly d, *J* 2.9); δ_C 170.6 (s, enriched signal, C-1 and - 4).

1-*Ethyl* 2-*Benzoylamino*-3-*oxo*[3,6-$^{13}C_2$]*hexanedioate* (cf. *ref.* 7) **8**.—A mixture of diisopropylamine (3 g, 30 mmol), tetramethylethylenediamine (3.52 g, 30 mmol) and THF (60 cm^3) was cooled to −78 °C and treated with butyllithium (15% solution in hexane; 19 cm^3, 30 mmol) whilst being stirred for 20 min. Ethyl hippurate[7] (3.1 g, 15 mmol) in THF (20 cm^3) was added dropwise at −78 °C to the mixture and the resulting yellow suspension was stirred for 1.5 h. [1,4-$^{13}C_2$]Succinic anhydride (1.01 g, 9.9 mmol) in THF (35 cm^3) was added to it and the mixture was stirred at −78 °C for 1.5 h; it was then allowed to warm to 0 °C when it was poured into ice–water containing conc. hydrochloric acid (12 cm^3). The water layer was extracted with diethyl ether (4 × 100 cm^3) and the combined extracts were washed with brine (50 cm^3), dried and evaporated. The residue was crystallised from diethyl ether to give the acid **8** as needles (1.58 g, 51.5%); m.p. 104–106 °C (lit.,[7] 114–116 °C). The mother liquor was evaporated and the residue chromatographed on silica gel (100 cm^3) with chloroform–methanol (19:1). The residue from the appropriate fractions was recrystallised from diethyl ether to give a second crop (0.46 g, total 2.04 g, 66.5%) (Found: M^+, 309.1148. $^{12}C_{13}{}^{13}C_2H_{17}NO_6$ requires *M*, 309.1123); δ_H 1.31 (3 H, t, *J* 7.1, CH_3), 2.72 (2 H, m, 5-H), 2.99 (1 H, m, 4-H), 3.18 (1 H, m, 4-H), 4.30 (2 H, q, *J* 7.1, CH_3CH_2), 5.47 (1 H, t, *J* 6.1, 2-H), 7.34 (1 H, d, *J* 6.1, NH), 7.44 (2 H, t, *J* 7.5, 3'-H and 5'-H), 7.51 (1 H, t, *J* 7.5, 4'-H) and 7.83 (2 H, d, *J* 7.5, 2'-H and 6'-H); δ_C 13.9 (CH_3), 27.6 (d, *J* 38, C-5), 35.2 (d, *J* 42, C-4), 62.8 (d, *J* 38, C-2), 62.9 (s, CH_3CH_2), 127.2 (s, C-3' and C-5'), 128.6 (s, C-2' and C-6'), 132.2 (s, C-4'), 132.7 (s, C-1'), 165.9 and 167.0 (each s, C-1 and C-7'), 177.2 (s, enriched signal, C-6) and 199.6 (s, enriched signal, C-3).

5-*Amino*[1,4-$^{13}C_2$]*laevulinic Acid Hydrochloride* **1a**.—The acid **8** (309 mg, 1 mmol) was heated with conc. hydrochloric acid (5 cm^3) and acetic acid (5 cm^3) in an oil-bath at 110 °C under argon for 23 h and then evaporated. The residue was redissolved in water, the solution evaporated, and the residue then transferred to a continuous extractor with water and diethyl ether; the water layer was extracted with diethyl ether for 24 h. The water layer was separated and evaporated (below 40 °C) to give [1,4-$^{13}C_2$]ALA hydrochloride **1a** (165 mg, 97.7%), m.p. 139–141 °C, identical by TLC (butanol–water–acetic acid, 12:5:3) with an authentic sample: $\delta_H(D_2O)$ 2.72 (2 H, br quintet, *J* 6, 2-H_2), 2.90 (2 H, br quintet, *J* 6, 3-H_2) and 4.13 (2 H, d, *J* 4.2, 5-H_2); δ_C(D_2O with CH_3CN as internal standard, δ 1.2) 27.7 (d, *J* 52.8, C-2), 34.6 (d, *J* 42.1, C-3), 47.4 (d, *J* 39.8, C-5), 177.2 (d, *J* 1.7, enriched signal C-1) and 204.5 (d, *J* 1.7, enriched signal C-4); *m*/*z* (+FAB): 134 ($M^+ + H$, 100%).

5-*Amino*[1-^{13}C]*laevulinic Acid Hydrochloride* **1b** (*with Dr. C. Lambert*).—To a solution of ethyl 4-phthalimidoacetoacetate[8] (302 mg, 1.1 mmol) in THF (30 cm^3) was added a solution of sodium hexamethyldisilazide (1 mol dm^{-3} solution in THF; 1.1 cm^3). The reaction mixture was stirred at room temperature for 45 min and then methyl bromo[1-^{13}C]acetate (154 mg, 1 mmol, 99 atom%) was added to it. After 16 h, the solution was evaporated and the residue was dissolved in CH_2Cl_2 (20 cm^3) and the solution washed with 1 mol dm^{-3} hydrochloric acid (30 cm^3), dried and evaporated to give methyl 3-ethoxycarbonyl-5-phthalimido[1-^{13}C]laevulinate (340 mg, 89%).

The phthalimido ester was dissolved in glacial acetic acid (10 cm^3) and conc. hydrochloric acid (10 cm^3) and heated at 120 °C under argon for 24 h. Work-up as in the previous case with recrystallisation from ethanol–diethyl ether gave 5-amino[1-^{13}C]laevulinic acid hydrochloride (114 mg, 69%).

5-*Amino*[1-^{13}C,1,1,4-$^{18}O_3$]*laevulinic Acid Hydrochloride* **1c**.—5-Amino[1-^{13}C]laevulinic acid hydrochloride (120 mg) in [^{18}O]water (97 atom% $H_2{}^{18}O$; 0.25 cm^3) and 0.3 mol dm^{-3} hydrochloric acid (0.007 cm^3) was heated in a sealed tube at 120 °C for 2 h and the [^{18}O]water was recovered by vacuum transfer. The exchange reaction was repeated with an equal quantity of fresh [^{18}O]water to give >90% exchange of the

oxygen atoms in the carboxyl group. This was shown by NMR (see Fig. 1).

Assignment of ^{13}C Signals from Carbonyl Groups of Cobester **5**.—The data on cobester were recorded in C_6D_6 saturated with KCN and the solvent signal (δ_H 7.20 or δ_C 128.02 for centre line) was used as internal reference. The ^1H-detected ^{13}C–^1H-correlation spectrum of cobester was recorded over 2081 Hz (0.8–6.0 ppm) with 4K data points in F2 frequency and 1611 Hz (162–178 ppm) for F1 frequency. A ^{13}C-^1H COSY experiment was carried out on unlabelled cobester (as **5**) at the same concentration used for earlier assignments [5] (40 mg in 1 cm^3). Good sensitivity was achieved by using the ^1H-detection mode and only the carbonyl region (162–178 ppm) was observed. The delay D 2 was set at 50 ms, optimum for ^{13}C–^1H coupling constants of 10 Hz. The signals from the carbonyl groups could be unambiguously distinguished from the expected folded-in signals since all the former gave clear correlation peaks with the ester *OMe* protons. Sections in the ^1H dimension through the peaks allowed correlations with the already assigned α- and β-protons and thus assignment of all the carbonyl groups as shown in Table 1.

The previous assignments [5] of some of the sp^2 carbons were also confirmed. The carbon at δ_C 163.5 showed correlation peaks with the 15-Me (δ_H 2.24) while that at δ_C 175.5 showed correlation peaks with the 17-Me (δ_H 1.01), 15-Me, and 19-H (δ_H 4.01). Therefore, the carbon at δ_C 175.5 is C-16 and that at δ_C 163.5 is C-14. The carbon at δ_C 164.0 is C-6 since it showed correlation peaks with the 7-Me (δ_H 1.39), 5-Me (δ_H 2.10), c′ protons (δ_H 2.53) and 8-H (δ_H 3.27). The carbon at δ_C 175.3 showed correlation peaks with the 5-Me, b′ protons (δ_H 1.98) and 3-H (δ_H 3.93), thus it is C-4. The carbon at δ_C 176.5 is C-11 as it is coupled to the 12β-Me (δ_H 0.98), 12α-Me (δ_H 1.12), 13-H (δ_H 2.81) and 10-H (δ_H 5.74).

Enzymic Synthesis of ^{13}C,^{18}O-Labelled Sirohydrochlorin **10**.—Full details are being published separately [11] covering the production and use of the four overproduced enzymes (see text) for the enzymic synthesis of ^{13}C : ^{18}O labelled sirohydrochlorin **10** from labelled ALA **1c**; the yields of **10** are in the range 40–45%.

For the present work, [1-^{13}C,1,1,4-$^{18}O_3$]ALA hydrochloride **1c** (8 mg) was enzymatically converted into **10** (2.3 mg) and 60% of the product mixture was directly incubated with the enzyme system from *P. shermanii* as described in the next section. The remainder of the mixture was freeze-dried and the residue was suspended in MeOH (3 cm^3) and $CHCl_3$ (20 cm^3) and treated with trimethyloxonium tetrafluoroborate (350 mg) and diisopropylethylamine (0.5 cm^3). The slurry was sonicated for 15 min under argon and then treated with more trimethyloxonium tetrafluoroborate (350 mg) and diisopropylethylamine (0.5 cm^3). After being stirred for 20 min, the mixture was partitioned between water (200 cm^3) and dichloromethane (3 × 20 cm^3) and the organic solution was washed successively with aqueous sodium hydrogen carbonate, water and brine (each 40 cm^3) and then dried and evaporated. Purification by PLC under argon using chloroform–diethyl ether (1 : 1, pre-filtered through basic alumina) as eluent, gave ^{13}C,^{18}O-labelled sirohydrochlorin octamethyl ester **11** containing a little of the 3-epimer (total 0.4 mg by UV assay). This was identified by direct comparison with authentic material and the ^{13}C NMR spectrum was determined after dilution with *ca.* 1 mg of unlabelled **11**. The ^{13}C NMR spectrum of this labelled sample to which 7.7 mg of the unlabelled form was added, was recorded in C_6D_6 at 100.6 MHz (see Fig. 2).

Incorporation of ^{13}C,^{18}O-Labelled Sirohydrochlorin **10** *into Cobyrinic Acid* **4**.—Frozen cells of *P. shermanii* (grown in a yeast medium containing cobalt chloride; 120 g) were partially thawed and suspended in phosphate buffer (50 mmol dm^{-3} potassium phosphate, pH 7.7, 0.2 mmol dm^{-3} dithiothreitol and ethylenediamine tetraacetic acid, degassed with argon; 120 cm^3). The cells were broken by three passes through a French Press and the suspension was centrifuged at 27 000 *g* (18 000 rpm) for 60 min at 4 °C. The supernatent was passed through a column of DEAE-Sephadex A-25 (5.4 g, 18 × 20 mm) at 4 °C. This cell-free extract (240–250 cm^3) was placed in a three-necked flask equipped with a pH electrode and automatic pH titrator system in a constant temperature bath at 30 °C. The foregoing labelled sirohydrochlorin (which is reduced *in situ* to precorrin-2) was added together with the requisite cofactors; ATP (272 mg), NAD^+ (132 mg), NADH (142 mg), glutathione (130 mg), DL-cysteine (30 mg), *S*-adenosyl-L-methionine (108 mg), $CoCl_2·6H_2O$ (41 mg) and $MgCl_2·6H_2O$ (101 mg). The mixture was incubated at pH 7.7 for 19 h in the dark under argon. Then pyridinium acetate buffer (27 mmol dm^{-3} pyridine containing 10 mmol dm^{-3} KCN, adjusted to pH 4.1 by addition of acetic acid) was added to the mixture which was then adjusted to pH 4 with 2 mol dm^{-3} hydrochloric acid. The precipitated proteins were removed by centrifugation (27 000 *g*, 4 °C). The supernatent was passed through reversed phase C-18 Sep-Pak cartridges which were washed with 5% acetonitrile in the above pyridinium acetate buffer and the cobyrinic acid was then eluted with 20% acetonitrile–pyridinium acetate buffer. The eluent was freeze-dried and the residue was redissolved in phosphate buffer, filtered, diluted with unlabelled cobyrinic acid (0.4 mg) and purified by HPLC on a reversed phase (ODS, C-18) column eluting with a gradient over 75 min from potassium phosphate buffer (0.1 mol dm^{-3}, pH 5, containing 10 mmol dm^{-3} KCN) to 20% acetonitrile in the same buffer solution. The fractions containing cobacid (eluted at *ca.* 28 min) were collected, desalted by absorption on a C-18 Sep-Pak and eluted with 20% acetonitrile–pyridinium acetate buffer and freeze-dried to give pure cobacid which was further diluted with unlabelled carrier cobacid (1.4 mg).

Basic Esterification of Cobyrinic acid to yield Cobester **5**.—All the foregoing cobyrinic acid was dissolved in MeOH–$CHCl_3$ (1 : 9; 10 cm^3) and treated with trimethyloxonium tetrafluoroborate (500 mg), and diisopropylethylamine (0.5 cm^3). The mixture was stirred for 15 min under argon after which further trimethyloxonium tetrafluoroborate (300 mg) and diisopropylethylamine (0.3 cm^3) were added to it. The mixture was stirred for 15 min and then washed with water followed by saturated brine containing KCN. The dried organic layer was evaporated to give pure heptamethyl cobyrinate (cobester) (1.9 mg). Spectroscopic data derived from this sample are given in the text.

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