Biosynthesis of porphyrins and related macrocycles. Part 51.[1,2] Proof that a reductive step occurs during the biosynthesis of vitamin B_{12} by the microaerophilic organism, *Propionibacterium shermanii*

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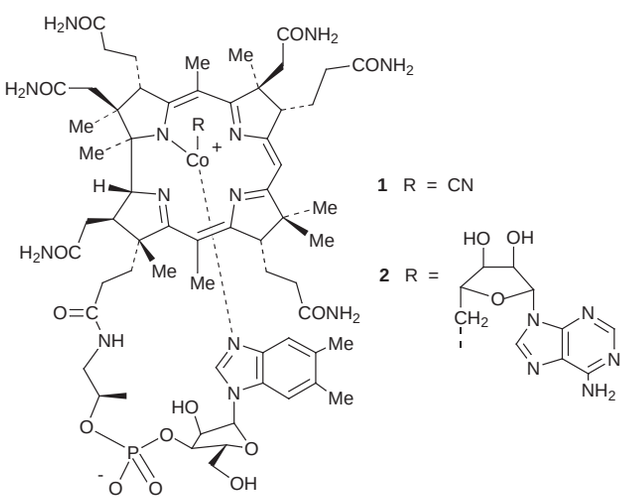
5-Amino[4-^{13}C]laevulinic acid has been synthesised for enzymic conversion into ^{13}C-labelled precorrin-2 **12a**. This was incubated with an enzyme system from *Propionibacterium shermanii* in the presence of [4-$^{2}H_2$]NADH **5a** and [4-$^{2}H_2$]NADPH **6a** to yield cobyrinic acid **15a**, shown to carry ^{2}H at C-19 by appropriate ^{13}C-NMR studies. The same reducing cofactors but now stereospecifically labelled at C-4 with tritium were similarly used to biosynthesise cobyrinic acid which was ^{3}H-labelled (**15b**) from the 4(*R*)-cofactors but carried no ^{3}H (**15c**) when the 4(*S*)-cofactors were used. Suitable degradation of the cobyrinic acid **15b** after conversion into its ester **16b** proved ^{3}H-labelling at C-19. These results establish that the biosynthesis of vitamin B_{12} in the microaerophilic organism *Pr. shermanii* involves a reductive step in which a reductase enzyme transfers 4-H_R of the cofactor to C-19 of the macrocycle.

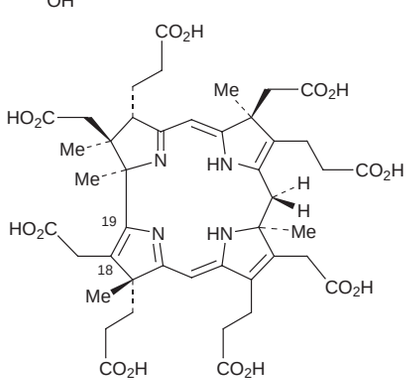
Introduction

The entire biosynthetic pathway used by the aerobic organism, *Pseudomonas denitrificans* for construction of vitamin B_{12} **1** and the B_{12}-coenzyme **2** has been elucidated in essentially complete detail.[3] This pathway was not what workers in the field, including those in the Cambridge group, had expected and several steps were very surprising ones. For example, it turned out that an enzymic reduction step[4] was needed in which the C-18–C-19 double bond of the hexamethylated intermediate, precorrin-6A **3** was reduced to form precorrin-6B **4**.[4,5] Reduction at this stage showed that oxidation must have occurred at an earlier one, not difficult to envisage for a biosynthesis occurring in an aerobic organism. This is not the case for an anaerobic organism and so there was, at the time of the work[2] reported fully here, great interest in knowing whether a similar reduction occurred during B_{12}-biosynthesis in an anaerobe. Fortunately, the organism used for much of the first two decades of research on the biosynthesis of vitamin B_{12} was *Propionibacterium shermanii* which is grown under essentially anaerobic conditions (strictly speaking, microaerophilic conditions). Accordingly, this was the bacterium chosen for our work on the question concerning reduction.



1 R = CN

2 R =

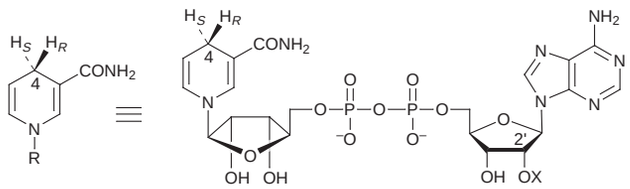


3

4 18,19-dihydro

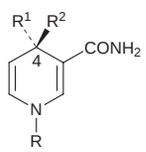
Studies of the reduction of precorrin-6A **3** to precorrin-6B **4** in *Ps. denitrificans* mentioned above were simplified by having the reductase, specific for this step, available in quantity (by overproduction) and of substantially enriched purity; this enzyme is dependent on reduced nicotinamide adenine dinucleotide phosphate, NADPH, **6**. Use of the isolated reductase with the [4-$^{2}H_2$]labelled form of NADPH, part structure **6a**, for conversion of **3** into **4** followed by appropriate analysis by ^{1}H-NMR gave proof[6] that the hydride equivalent is transferred from C-4 of the cofactor to C-19 of precorrin-6A **3**. Similar experiments based on NADPH labelled stereospecifically with ^{2}H at C-4 then showed[7] that H_R is transferred from C-4 of NADPH (see **6**) to precorrin-6A **3**.

For the work with *Pr. shermanii*, we faced greater experimental difficulties: (a) the enzymes from this organism were only available at the natural unenhanced levels so biosynthetic conversions were less efficient; (b) if a reductase is required for the biosynthesis of vitamin B_{12} in *Pr. shermanii*, it was not known whether its cofactor was NADH **5** or NADPH **6** or some other reductant such as a flavin; (c) even during the foregoing studies with enriched, partly purified reductase, there was substantial loss of the ^{2}H-label due to the known exchange with the medium, *via* flavins, of hydrogen at C-4 of reduced



5 X = H (NADH)

6 X = (NADPH)



5a $R^1 = R^2 = {}^2H$
6a 2'-Phosphate of **5a**
7 $R^1 = H$, $R^2 = {}^3H$
8 2'-Phosphate of **7**
9 $R^1 = {}^3H$, $R^2 = H$
10 2'-Phosphate of **9**

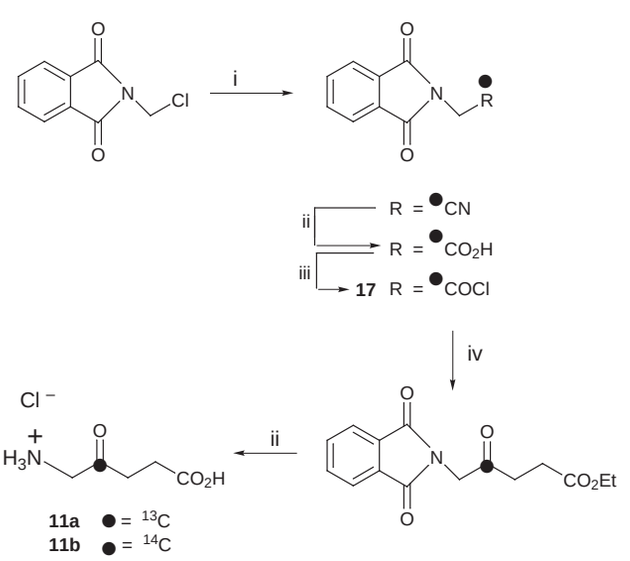
nicotinamide cofactors. This exchange could be expected to be greater with the crude enzyme system from *Pr. shermanii* leading to a still smaller level of labelling in the product. Clearly, rapid total exchange would result in failure. A different approach was needed based on ^{13}C-labelling.

Results and discussion

Experiments using deuterium

The plan was to biosynthesise precorrin-2 **12a**, an established precursor[8] of cobyrinic acid **15** *en route* to vitamin B_{12} **1**, from 5-amino[4-^{13}C]laevulinic acid (ALA) **11a** to set up the illustrated labelling pattern having a ^{13}C-label at C-1. If during the biosynthesis of cobyrinic acid from precorrin-2 **12a**, ^2H is transferred from a reducing cofactor to C-19 to form **15a** then the ^{13}C-NMR signal from C-1 of the isolated ester **16a** will show a shifted signal, probably small, *ca*. 0.07 ppm upfield of the larger unshifted signal due to a β-^2H isotopic shift.[9] The greater sensitivity of this approach is because a positive result leads to a signal shifted away from the major one; were ^1H-NMR to be used, a small change in the size of a large single signal would have to be detected.

The synthesis of [4-^{13}C]ALA **11a** is shown in Scheme 1. This

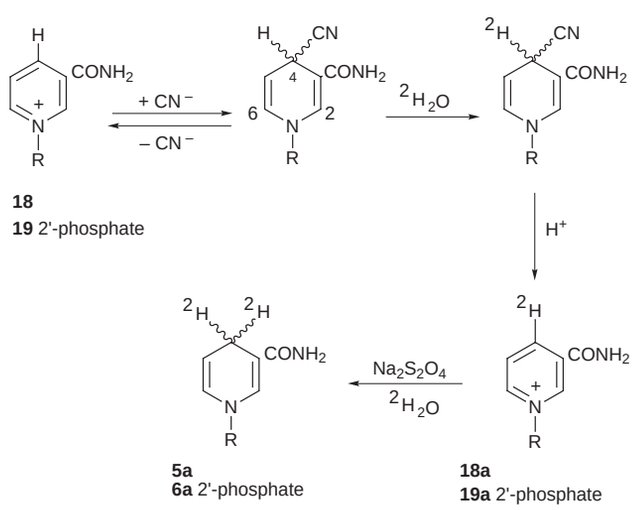


Scheme 1 *Reagents and conditions*: i, $K^{13}CN$; ii, HCl, HOAc, heat; iii, $(COCl)_2$; iv, $ICH_2CH_2CO_2Et$, Zn–Cu then $(Ph_3P)_4Pd^0$ then **17**.

essentially followed the route developed by Campbell and Johnston[10] for the preparation of [4-^{14}C]ALA **11b**, a key step being the palladium-mediated coupling of 2-(ethoxycarbonyl)ethylzinc iodide with an acid chloride.[11] We also carried out the first run with ^{14}C-labelled material; the product is used below and just the yields are reported in the Experimental section. The overall yield from potassium [^{14}C]cyanide to [4-^{14}C]ALA **11b** was 53%, confirming that reported[10] of 56%. [4-^{13}C]ALA **11a** was then synthesised in the same way from potassium [^{13}C]-cyanide in 49% overall yield.

The required ^{13}C-labelled precorrin-2 **12a** was prepared enzymically by incubating [4-^{13}C]ALA **11a** with a cell suspension of *Propionibacterium technicum* that had been grown throughout in cobalt-free media.[12] Precorrin-2 **12a** rapidly undergoes oxidation during handling in air, so it is isolated and purified as the octamethyl ester **14a** of its didehydro aromatised form, ^{13}C-labelled sirohydrochlorin.[13] This product was shown by ^1H-NMR to carry *ca*. 20 atom% ^{13}C at the labelled sites. The recovered *Pr. technicum* cells from this first preparation were then incubated again with more [4-^{13}C]ALA to yield a second batch of ^{13}C-labelled sirohydrochlorin ester **14a** carrying *ca*. 40 atom% ^{13}C at each labelled site. The sample with the higher labelling level was used for the subsequent experiment and was stored while the ^2H-labelled cofactors were prepared.

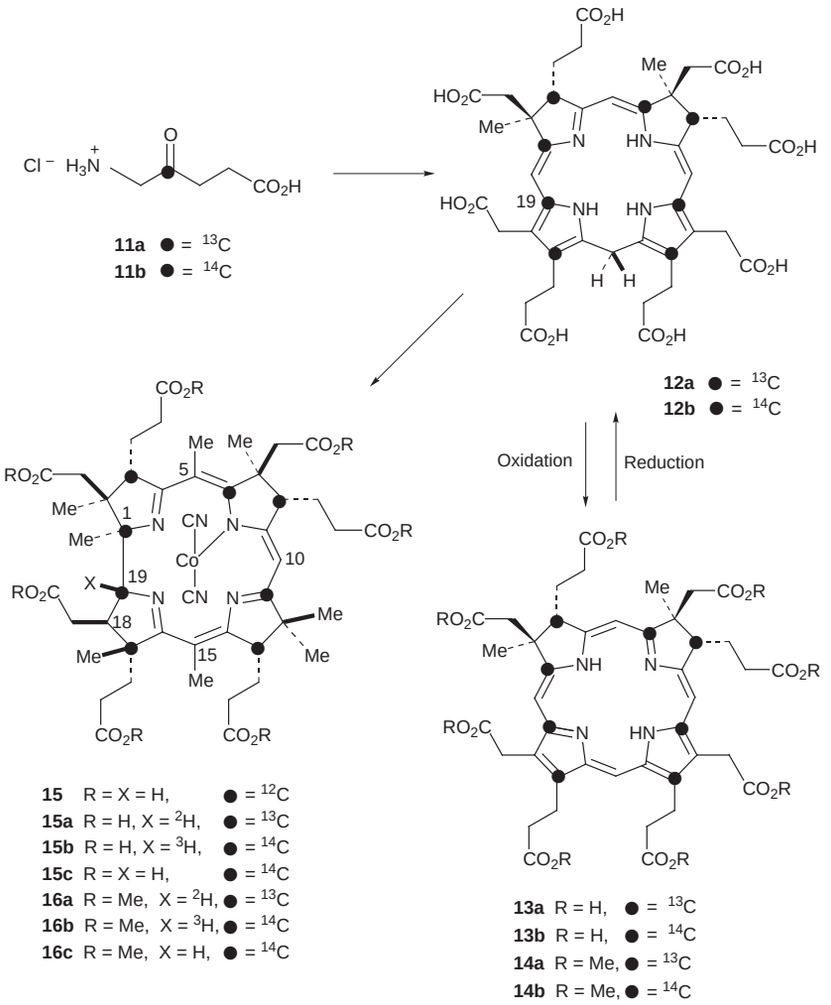
As mentioned above, it was not known which reducing cofactor might be used for the possible reductive step during B_{12}-biosynthesis in *Pr. shermanii*. Accordingly, [4-$^2H_2$]NADH **5a** and [4-$^2H_2$]NADPH **6a** were to be added in admixture to the crucial incubations. Both were prepared by first treating separate solutions of unlabelled NAD^+ **18** and $NADP^+$ **19** in deuterium oxide (99.9 atom% ^2H) with potassium cyanide,[14,15] Scheme 2. The accelerated exchange of hydrogen at C-4 to form



Scheme 2 Preparation of [4-$^2H_2$] labelled cofactors.

[4-^2H]NAD^+ **18a** and [4-^2H]$NADP^+$ **19a** occurs[14] by reversible formation of the illustrated cyanide complex. Our ^1H-NMR data on **18a** and **19a** confirm the earlier conclusion,[14] based on non-spectroscopic methods, that the most rapid exchange occurs at the 4-position. However there had been 28% and 16% exchange at C-2 for **18a** and **19a** respectively, but none detectable at C-6. These [4-^2H]cofactors were then reduced using sodium dithionite in deuterium oxide to generate [4-$^2H_2$]-NADH **5a** and [4-$^2H_2$]NADPH **6a**. They were isolated by ion exchange chromatography eluting with aqueous ammonium hydrogen carbonate; the latter was then removed by lyophilisation. This is a considerable improvement over earlier methods[15] for isolation.

All the labelled materials were now available for the key experiments. The ^{13}C-labelled sirohydrochlorin octamethyl ester **14a** was hydrolysed to afford the octaacid **13a**. This was incubated with a cell-free enzyme system prepared from *Pr. shermanii* cells that had been grown in a cobalt-containing lactate medium. All the required cofactors were added to this



incubation, including [4-$^2H_2$]NADH **5a** and [4-$^2H_2$]NADPH **6a**. It is well known that this enzyme system is able to reduce sirohydrochlorin **13a** back to precorrin-2 **12a** and the biosynthesis then runs forward to form cobyrinic acid **15a**. The reduction involves delivery of a hydride equivalent to C-15 of **13a** and, importantly, this proton (deuteron) is lost before cobyrinic acid is formed with its fully substituted position-15. The preparation of the enzyme system and the procedure for the incubation were similar to those described earlier;[16] the changes needed for the present study are outlined in the Experimental section.

The cobyrinic acid **15a** biosynthesised in this experiment was isolated with the minimal addition of unlabelled cobyrinic acid **15** normally used as carrier material to assist the handling of otherwise small amounts of product. Clearly, the more unlabelled cobyrinic acid added, the larger the ^{13}C-signal from natural abundance ^{13}C at C-1 of this carrier material. Finally, the isolated **15a** was esterified to afford the crystalline cobester (heptamethyl dicyanocobyrinate) **16a** which was carefully purified.

The ^{13}C-NMR spectrum of cobester at natural abundance has been assigned.[17,18] The signal from C-1 of the present sample **16a** was a doublet due to coupling to ^{13}C at C-19 superimposed on a larger singlet from ^{13}C at C-1 bonded to ^{12}C at C-19. Both the singlet and doublet showed additional signals shifted upfield, Fig. 1. The shifted signals are marked with flat-topped signs whilst the doublet from ^{13}C–^{13}C coupling is indicated by the normal roof-shaped mark. The shifted signal for the low field leg of the doublet perhaps requires the eye of faith but the others are clear and the observation that the upfield shifts for all three are 0.07 ppm fits well for a β-^2H isotopic shift,[9] so supporting the presence of ^2H in a β-position to C-1.

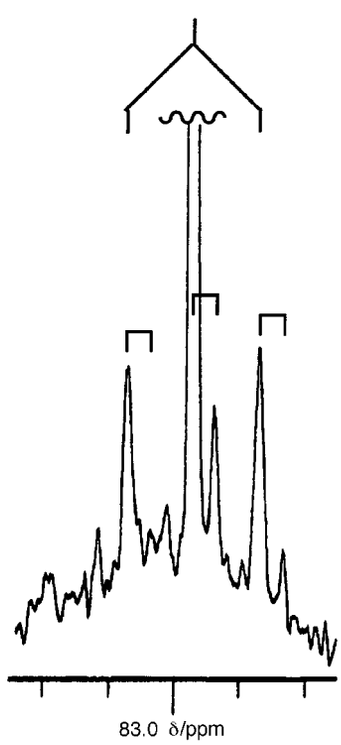


Fig. 1 The peaks for C-1 from the ^{13}C NMR spectrum of cobester **16a** derived from ^{13}C labelled sirohydrochlorin **13a** and [4,4-$^2H_2$]NAD(P)H **5a/6a**. The large central singlet has been truncated, the flanking doublet indicated by the roof-shaped mark is due to coupling to ^{13}C at C-19 and the shifted peaks marked by the three flat-topped signs are due to ^2H at C-19.

This places ^{2}H at C-19 since this is the only centre carrying a β-hydrogen relative to C-1, a fact that led to the choice of the original ^{13}C-labelling pattern.

It is evident from Fig. 1 that the level of ^{2}H-labelling in the foregoing cobester **16a** was low (*ca.* 10%) so, as expected, there had been serious loss of ^{2}H by exchange from the cofactor **5a** or **6a** in the crude enzyme system. Nevertheless, this first result indicated that complete exchange had been avoided and further, it encouraged a second series of more sensitive experiments based on labelling with tritium to provide rigorous evidence.

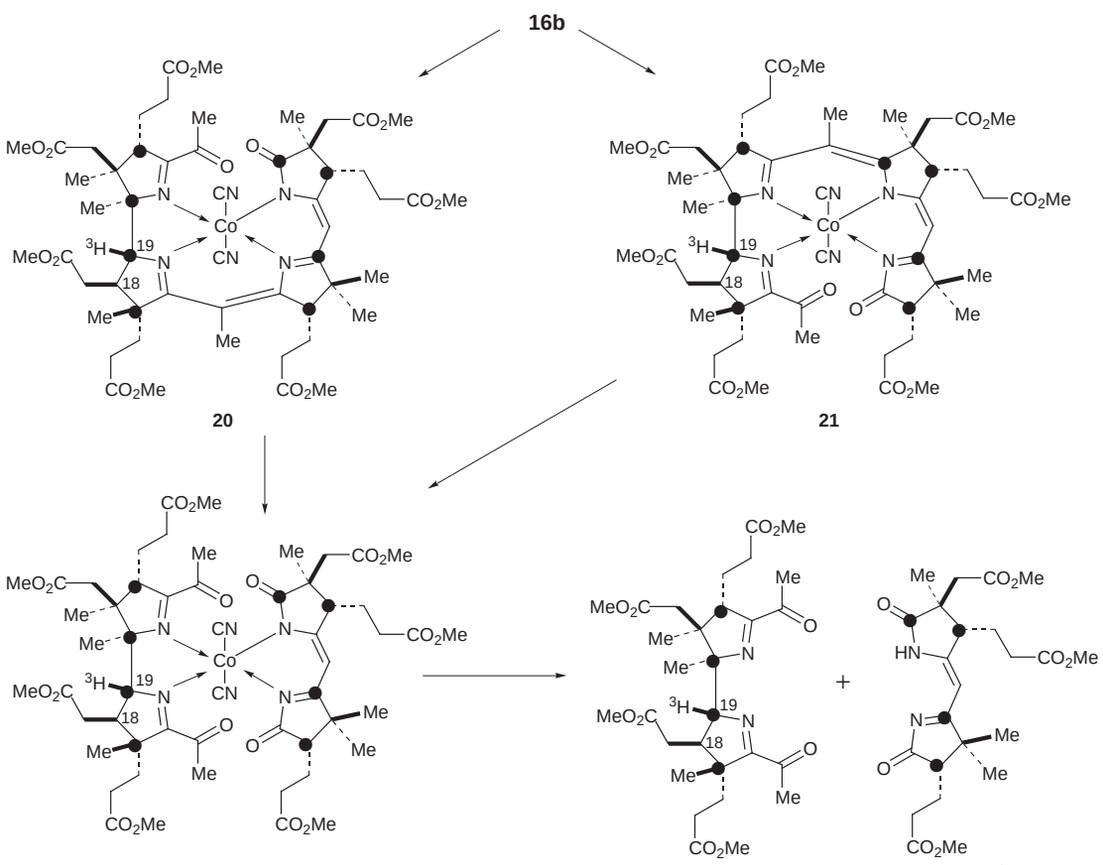
Experiments using tritium

It was noted in the Introduction that the reduction of precorrin-6A **3** in *Ps. denitrificans* involved the stereospecific transfer of H_R from NADPH **6**. The reductase that by now we felt rather confidently was present among the enzymes from *Pr. shermanii* would also be expected to function stereospecifically. Therefore, if one experiment is based on a mixture of [4(*R*)-$^{3}H_1$]NADH **7** with [4(*R*)-$^{3}H_1$]NADPH **8** (the *R*-experiment) and a second one uses [4(*S*)-$^{3}H_1$]NADH **9** with [4(*S*)-$^{3}H_1$]-NADPH **10** (the *S*-experiment), the cobacid **16b** finally formed should be ^{3}H-labelled from one experiment but not from the other. This stereochemical test adds great strength because if the outcome were as predicted, there could be little doubt that an enzymic reduction was being observed.

The synthesis of the four stereospecifically labelled cofactors **7**–**10** has been described in detail[19] and the other required material, ^{14}C-labelled sirohydrochlorin **13b**, was prepared from [4-^{14}C]ALA **11b** as for the earlier ^{13}C-labelled sample **13a**. As before, it was isolated and purified as its octamethyl ester **14b**.

The incubation of ^{14}C-sirohydrochlorin **13b** (as the source of precorrin-2 **12b**) with the cell-free enzyme system from *Pr. shermanii* containing the two 4*R*-cofactors **7** and **8** was carried out with the quantities and the conditions used for the study with deuterium. However, the switch to ^{3}H, as is often the case, necessitated one change for the following reason. The ^{2}H-labelled cofactors **5a** and **6a** carried 99.9 atom% ^{2}H and the necessary substantial quantity of each was used without dilution in the earlier incubation. In contrast, the ^{3}H-labelled cofactors **7** and **8** were available in lower quantity at high specific activity; used in this state, there would have been insufficient material for a successful biosynthesis. Thus dilution was necessary to provide the right quantity of material. Importantly, the dilution was made with the [4-$^{2}H_2$]-labelled cofactors **5a** and **6a** because of kinetic isotope effects. The aim was to enhance the transfer of ^{3}H by making the competition ^{3}H *versus* ^{2}H rather than ^{3}H *versus* ^{1}H, had unlabelled diluents been used. A parallel run *using the same enzyme preparation* was carried out in an identical way apart from replacing the two [4(*R*)]$^{3}H_1$]-cofactors by the two [4(*S*)-$^{3}H_1$]isomers **9** and **10**.

The cobyrinic acid biosynthesised in each incubation was isolated after addition of unlabelled **15** as carrier, both products were purified and then esterified to form two samples of cobester. These were extensively purified by chromatography and multiple recrystallisation. The product from the *S*-experiment carried ^{14}C-activity corresponding to a high incorporation (35%) of precorrin-2 **12b** into cobyrinic acid but the ^{3}H-level was so low that it could not be accurately determined (see Table 1). Thus the cobyrinic acid from the *S*-experiment carried ^{1}H not ^{3}H at C-19, *i.e.* it was **15c** and the corresponding ester was **16c**. In contrast, the cobyrinic acid from the *R*-experiment again showed a high incorporation (27%) of the ^{14}C-precorrin-2 **12b** and in addition, this product was strongly labelled with ^{3}H. The changes in the ^{14}C and ^{3}H activity levels during the purification of this product from the *R*-experiment showed that a



Scheme 3 Degradation of cobester from the *R*-experiment.

Table 1 ^{3}H and ^{14}C activities after incorporation of labelled NAD(P)H and sirohydrochlorin

	Amount/ μmol		Total activity/ dpm	Specific activity/ dpm μmol^{-1}
(*S*)-Series				
Substrates				
[^{14}C]Sirohydrochlorin octamethyl ester **14b**	1.36		1.51×10^{6}	1.11×10^{6}
[4(*S*)-^{3}H]NADH **9**	41.0		1.64×10^{8}	4.00×10^{6}
[4(*S*)^{3}H]NADPH **10**	44.8		2.30×10^{8}	5.13×10^{6}
Products				
Dicyanocobyrinic acid[a] **15c**	33.3	^{14}C	6.59×10^{5}	
		^{3}H	$<1.1 \times 10^{4}$	
Heptamethyl dicyanocobyrinate **16c**	22.4	^{14}C	4.44×10^{5}	1.98×10^{4}
		^{3}H	$<7.1 \times 10^{3}$	$<3.2 \times 10^{2}$
(*R*)-Series				
Substrates				
[^{14}C]Sirohydrochlorin octamethyl ester **14b**	1.36		3.50×10^{6}	2.57×10^{6}
[4(*R*)-^{3}H]NADH **7**	41.0		1.82×10^{8}	4.45×10^{6}
[4(*R*)-^{3}H]NADPH **8**	44.8		2.28×10^{8}	5.09×10^{6}
Products				
Dicyanocobyrinic acid[a] **15b**	91.1	^{14}C	9.22×10^{5}	
		^{3}H	7.29×10^{4}	
Heptamethyl dicyanocobyrinate **16b**	30.1	^{14}C	8.34×10^{5}	2.77×10^{4}
		^{3}H	6.59×10^{4}	2.19×10^{3}
Degradation				
Heptamethyl dicyanocobyrinate **16b** (after dilution)	91.1	^{14}C	4.30×10^{5}	4.72×10^{3}
		^{3}H	3.40×10^{4}	3.73×10^{2}
Heptamethyl dicyanodisecocobyrinate **22**	41.7	^{14}C	1.63×10^{5}	3.91×10^{3}
		^{3}H	1.05×10^{4}	2.52×10^{2}
AD-Fragment **23**	27.8	^{14}C	5.61×10^{4}	2.02×10^{3}
		^{3}H	8.14×10^{3}	2.93×10^{2}
BC-Fragment **24**	33.8	^{14}C	5.84×10^{4}	1.73×10^{3}
		^{3}H	0	0

[a] The dicyanocobyrinic acid **15b**,**c** was not isolated as such but instead converted into its heptamethyl ester **16b**,**c**. The total activity given for **15b**,**c** has been calculated from the specific activity measured for **16b**,**c** and the total quantity of **15b**,**c**, which was assumed to be 33 mg (32 mg was added and *ca*. 1 mg from the incubation).

stage had been reached where both activities were at almost constant values. It was decided not to seek perfect constancy at this stage. The reason was that whilst further recrystallisation of the ^{3}H,^{14}C-cobester would have achieved exact constancy, the amount of material left would have been insufficient to carry out the degradation needed to pinpoint the site of ^{3}H-labelling. Moreover, it will become evident that during this degradation, three new substances are generated each of which was to be purified. The chances of carrying a radiochemical impurity through such a sequence are vanishingly small.

The cobester **16b** from the *R*-experiment was degraded by Kräutler's photochemical method[20] which relies on singlet oxygen generated under strong irradiation using methylene blue as photosensitiser, Scheme 3. It is necessary to use a specially designed cell.[20] One initial product **20**, 45%, results from cleavage at the 5,6-double bond and a second product **21**, 54%, has been cleaved at the 14,15-double bond. These were purified and then separately subjected once more to the photochemical oxidation to afford two samples, in 42 and 49% yields respectively, of the heptamethyl tetraoxodisecocobyrinate **22**. After purification of both samples, they were combined for palladium-catalysed reduction in the presence of ethylenediaminetetraacetic acid to liberate the A/D **23** and B/C **24** fragments. The yields in the final step from **22** were 67% of **23** and 82% of **24**. Two of the foregoing materials, **20** and **23**, had also been prepared previously by a different method.[21]

Radio-assay of these fragments **23** and **24** showed that the [^{3}H,^{14}C]cobyrinic acid **15b** biosynthesised using the [4(*R*)-^{3}H]cofactors and isolated as cobester **16b** carried its ^{3}H-label entirely in the A/D fragment **23**.† The only hydrogen atoms that are introduced during the conversion of precorrin-2 **12b** into cobyrinic acid **15b** are those present in the *C*-methyl groups and the two at C-18 and C-19. The *C*-methyl groups derive from *S*-adenosylmethionine (SAM), which was supplied in the incubation mixture, and so will not be ^{3}H-labelled from the reducing cofactors. This is confirmed by the absence of ^{3}H-activity in the B/C fragment **24** which possesses two SAM-derived methyl groups. Since H-18 has been shown (see below) to be derived from the medium, it follows that the ^{3}H-label of the A/D fragment **23** resides on C-19. This specific transfer of ^{3}H from one or other of the reducing [4(*R*)^{3}H]cofactors **7** or **8**, eventually to appear at C-19 of **15b**, fully confirms the earlier result based on ^{2}H-labelling. The combined evidence thus demonstrates that a reductive step is necessary for the biosynthesis of vitamin B_{12} in the microaerophilic organism *Pr. shermanii*, just as it is in the aerobe *Ps. denitrificans*. It is significant that the reductase in the aerobic organism also transferred H_R specifically from its cofactor, NADPH.[7]

† The Table in fact shows that the ^{3}H specific activity *increased* slightly from 252 to 293 dpm μmol^{-1} in going from the diseco compound **22** to the A/D fragment **23**. However, in view of the errors inevitably involved in counting ^{3}H radioactivity in the presence of a higher activity of ^{14}C, these two values are considered to be within experimental error of each other.

Earlier mention was made of the exchange that the C-4 hydrogen atoms of the reducing cofactors **5** and **6** can undergo with hydrogen of the medium. It is therefore important to eliminate the possibility that the ^{3}H-labelling of the foregoing cobyrinic acid **15b** might have come by way of the medium rather than by direct transfer; such indirect transfer would undermine our evidence that a reductive step is necessary for the biosynthesis of vitamin B_{12} as discussed above. This can be done by first calculating the extent of dilution of the biosynthesised cobyrinic acid **15b** by endogenous **15** and by the unlabelled **15** added as carrier. The ratio of the molar ^{14}C specific activity of the precursor, precorrin-2 **12b**, to that of the final cobester **16b** gives this value. Knowing the molar ^{3}H specific activity of the isolated cobester **16b** and the foregoing dilution factor, the molar ^{3}H specific activity of the biosynthesised cobyrinic acid **15b** before dilution can be calculated. That value was over 500 times greater than the *maximum* specific activity that could be reached by exchange of all the ^{3}H from both cofactors **7** and **8** into the medium followed by transfer from the ^{3}HO^{1}H to C-19. This is because of the massive dilution of ^{3}H by ^{1}H that occurs in the aqueous medium. It was thus certain that direct not indirect transfer of ^{3}H had occurred.

Earlier experiments[22] had run the biosynthesis of cobyrinic acid using the enzymes from *Pr. shermanii* in ^2H_2O containing 6–13% of H_2O. The results clearly showed that 18-H is derived from the medium but that ^{2}H-labelling at C-19 does also occur, although at an appreciably lower level than that at C-18; both results were later confirmed.[23] The reason for this difference is now clear from the findings in the present paper. Initially, ^{1}H will be transferred from the unlabelled cofactor **5** or **6** but also the cofactor starts to undergo exchange of its C-4 hydrogens with the medium during the incubation. So a period follows when 4-^{1}H and 4-^{2}H species will be in competition. Finally, when the exchange process has been completed, only transfer of ^{2}H will occur. This process would explain how ^{2}H from ^2H_2O ends up at C-19 but at a level which is necessarily lower than that at C-18.

Recently, it was found that ^{3}H,^{14}C-doubly labelled precorrin-6A **3** and its reduction product precorrin-6B **4** (isolated from *Ps. denitrificans*) were both incorporated into cobyrinic acid (as **15**) with an unchanged ^{3}H:^{14}C ratio by a cell-free enzyme system from *Pr. shermanii*.[24] The incorporation levels of 0.88 and 2.7%, though low, were reasonable bearing in mind that **3** and **4** need to sequester cobalt from the medium to enter the biosynthetic pathway in this organism. Further, a cobalto tetramethylated macrocycle has been isolated from *Pr. shermanii*[25] which, though not itself a biosynthetic intermediate, points strongly to the involvement of the cobalt complex of precorrin-6A **3** as a B_{12}-precursor in this system. These experiments interlock with the foregoing proof that B_{12}-biosynthesis in *Pr. shermanii* requires a reductive step to reduce a C-18–C-19 double bond carried out by a reductase that specifically transfers 4-H_R from the cofactor.

Experimental

General

General directions are given in Parts 41[16] and 42.[26] The abbreviation 'dpm' denotes disintegrations per min.

5-Amino[4-^{13}C]laevulinic acid 11a

Potassium [^{13}C]cyanide (5 g, 90 atom% ^{13}C) was used as in the following synthesis to afford the hydrochloride of [4-^{13}C]ALA (7.7 g, 49% overall).

5-Amino[4-^{14}C]laevulinic acid 11b

This was prepared from potassium [^{14}C]cyanide (60 mg, 0.92 mmol, 2.1 mCi mmol^{-1}) essentially as described[10] to yield the hydrochloride of [4-^{14}C]ALA (82 mg, 0.49 mmol, 2.1 mCi mmol^{-1}, 53% from the [^{14}C]cyanide).

[1,3,6,8,11,13,17,19-^{13}C]Sirohydrochlorin octamethyl ester 14a

Propionibacterium technicum cells were grown as previously described for *Pr. shermanii*[27] save that cobalt was excluded from the initial growth media and cobalt-free peptone medium was used for the final growth stage. The cells were harvested by centrifugation before the aeration step. These moist cells (150 g) were suspended in a solution made up in water (1.5 dm^3) containing the foregoing [4-^{13}C]ALA hydrochloride (20 mg, 90 atom%), KH_2PO_4 (3 g), K_2HPO_4 (1.5 g), Na_2HPO_4 (1.7 g), $(NH_4)_2HPO_4$ (9.1 g), $MgSO_4{\cdot}7H_2O$ (310 mg), glucose (31 g), glutathione (860 mg), L-methionine (30 mg), chloramphenicol (30 mg) and a few milligrams each of NaCl and $MnSO_4{\cdot}4H_2O$. Argon was passed through this stirred suspension at 18 °C in the dark for 1.5 h and the mixture was then incubated with stirring at 30 °C under argon for 3 days, again with protection against light. The pH of the suspension fell continuously and was adjusted back to pH 6.8 twice each day using 5 M aqueous sodium hydroxide. After collecting the cells (Cells A) by centrifugation (9000 rpm, 30 min at 5 °C), DEAE-cellulose‡ (DE-52, 50 g) was added to the supernatant which was stirred under argon for 30 min. The cellulose, now red-violet coloured, was filtered off, washed first with water (3 × 500 cm^3) then methanol (3 × 500 cm^3) and dried at high vacuum overnight. The dry cellulose was washed with deoxygenated methanol–H_2SO_4 (40:1 v/v) to remove all the pigments (total volume *ca*. 500 cm^3) and trimethyl orthoformate (25 cm^3) was added to the methanolic solution. After being kept at 18 °C under argon for 16 h in the dark, the solution was partitioned between water (500 cm^3) and dichloromethane (125 cm^3) and the organic solution was washed successively with saturated aqueous sodium hydrogen carbonate, water and saturated brine. The dried solution was evaporated and the residue was purified by PLC on silica gel using chloroform–diethyl ether (1:1) to yield the ^{13}C-labelled sirohydrochlorin octamethyl ester **14a** (4.5 mg), identified by spectroscopic and chromatographic comparison with authentic material. The ^{1}H-NMR signal intensity from the protons at positions 3 and 8 of this product, which appear slightly overlapped between δ 4.02 and 4.13, was decreased due to ^{1}H–^{13}C splitting which showed that the ^{13}C-level was *ca*. 20 atom%.

The recovered Cells A above were kept cold and for as short a time as possible before being suspended again in an incubation medium of the same composition used the first time (only lacking the [4-^{13}C]ALA) that had been deoxygenated by argon bubbling for 2 h. [4-^{13}C]ALA (20 mg) was then added and incubation was continued at 30 °C for 2 days as before. The rest of the work-up was as above to yield a second sample of ^{13}C-labelled sirohydrochlorin octamethyl ester **14a** (3 mg). ^{1}H-NMR as above showed the ^{13}C-level to be *ca*. 40 atom%.

[1,3,6,8,11,13,17,19-^{14}C]Sirohydrochlorin octamethyl ester 14b

The method described for the foregoing preparation was repeated for the ^{14}C-labelled material. The same quantity of *Pr. technicum* cells was used in the same volume of solution though a few of the components differed somewhat (but not importantly) in amount. These were KH_2PO_4 (2.3 g), K_2HPO_4 (760 mg), Na_2HPO_4 (1.8 g), glutathione (760 mg) and chloramphenicol (15 mg); the rest were unchanged. [4-^{14}C]ALA (21.1 mg, 0.3 mCi total activity, specific activity 2.1 mCi mmol^{-1}) was incubated with this cell suspension as before for 45 h to afford, by the same isolation procedure, ^{14}C-labelled sirohydrochlorin

‡ DEAE = diethylaminoethyl.

octamethyl ester **14b** (5.9 mg, 19.6 μCi total activity, specific activity 3.3 mCi $mmol^{-1}$). Had there been no dilution of the labelled material by endogenous ALA and other intermediates, the specific activity of the sirohydrochlorin ester would have been 8 × 2.1 = 16.8 mCi $mmol^{-1}$. The observed value of 3.3 mCi $mmol^{-1}$ showed that dilution by *ca*. four parts of unlabelled material had occurred during the biosynthesis (*cf*. the result from the first incubation using [4-^{13}C]ALA).

Deuteriation of NAD^+ and $NADP^+$

In preliminary experiments the exchange of 4-H of $NADP^+$ was followed by ^{1}H NMR spectroscopy in D_2O. Upon addition of KCN to a solution of $NADP^+$ in D_2O there was an immediate change in the spectrum, thought to be due to formation of the two diastereoisomers of the adduct shown in Scheme 2. After 24 h a reduction in the size of the doublets at δ 4.34 and 4.52 was observed and after 80 h they had almost disappeared. A sample removed after 24 h was worked-up as below and the recovered $NADP^+$ showed exchange (*ca*. 40%) only at C-4. For $NADP^+$: δ_H (D_2O, 400 MHz) 9.38 (1 H, s, 2-H), 9.23 (1 H, d, *J* 6, 6-H), 8.89 (1 H, d, *J* 8, 4-H), 8.53 and 8.32 (each 1 H, s, adenine-H), 8.25 (1 H, dd, *J* 8 and 6, 5-H), 6.22 and 6.13 (each 1 H, d, *J* 5, 2 × 1′-H), 5.05 (1 H, m, adenosine 2′-H), 4.61–4.18 (9 H, m, other ribose H). For the diastereoisomeric NADP–CN complexes (ratio *ca*. 3:2; where distinguishable, the signals for the minor isomer are given in brackets): δ_H (D_2O, 400 MHz) 8.47 (8.48) and 8.20 (8.23) (each 1 H, s, adenine-H), 7.20 (7.22) (1 H, s, 2-H), 6.32 (1 H, d, *J* 8, 6-H), 6.225 (6.213) (1 H, d, *J* 5, adenosine 1′-H), 4.99–4.86 (3 H, m, 5-H, nicotinamide 1′-H and adenosine 2′-H), 4.34 (4.52) (1 H, d, *J* 4.5, 4-H) and 4.64 (1 H, q, *J* 5), 4.43 (1 H, br s) and 4.32–4.09 (7 H, m, other ribose H).

Deuterium oxide (10 cm^3; 99.9 atom% D) was degassed by bubbling argon through. NAD^+ sodium salt (0.38 g, 0.5 mmol) and potassium cyanide (0.38 g, 23.2 mmol) were added and the degassing continued for 5 min. The flask was then sealed and left in the dark at room temperature for 4 days. Dowex 50-X8 (H^+ form; 2 g) was then added and argon bubbled through the mixture for 30 min [**CAUTION**: toxic HCN evolved]. The mixture was filtered and freeze-dried. The product was dissolved in water (2 cm^3) and purified on a column (2 × 20 cm) of DEAE cellulose (Whatman DE-52; 70 g), which had previously been equilibrated with aqueous ammonium hydrogen carbonate (0.5 M and then 10 mM). The column was eluted with a gradient of 10–100 mM aqueous ammonium hydrogen carbonate (1000 cm^3 total volume). NAD^+ ammonium salt **18a** eluted between 350 and 500 cm^3 (by UV absorbance at λ 258 nm) and these fractions were combined, concentrated to *ca*. 5 cm^3 on a high vacuum rotary evaporator and then freeze-dried. The yield (0.24 g, 0.32 mmol, 64%) was determined by UV absorbance before freeze-drying. The ^{1}H NMR spectrum in D_2O showed complete disappearance of the peak for 4-H, with the peak for 5-H becoming a doublet instead of a triplet. The integration of the peak for 2-H showed that 28% exchange had occurred but there was no reduction in size of the peak for 6-H.

Exchange at C-4 of the $NADP^+$ sodium salt (1.52 g) was performed by the same procedure using D_2O (40 cm^3) and KCN (1.52 g) at room temperature for 7 days. The product was purified in the same way and the [4-^{2}H]$NADP^+$ diammonium salt **19a** recovered (890 mg, 59%) also showed complete exchange at C-4, a small amount of exchange (16%) at C-2 and no exchange at C-6.

[4,4-$^2H_2$]NADH 5a and [4,4-$^2H_2$]NADPH 6a

[4-^{2}H]NAD^+ ammonium salt **18a** (913 mg, 1.4 mmol) and sodium hydrogen carbonate (1.0 g, 11.9 mmol) were dissolved in D_2O (20 cm^3) and then evaporated to dryness on a high vacuum rotary evaporator. This procedure was then repeated to ensure complete exchange of all exchangeable protons. The residue was dissolved in D_2O (30 cm^3; 99.9atom% D) and degassed by evacuation and refilling the flask with argon five times. The solution was stirred under a flow of argon in the dark at room temperature and sodium dithionite (0.6 g, 3.4 mmol) was added, producing a bright yellow colour. After 2 h the colour had faded to pale yellow and the solution was applied to a column (4 × 30 cm) of DEAE cellulose (Whatman DE-52), which had previously been equilibrated with aqueous ammonium hydrogen carbonate (30 mM). The column was eluted with a gradient of 30–300 mM aqueous ammonium hydrogen carbonate (1800 cm^3 total volume). NADH eluted between 1300 and 1650 cm^3 (by UV absorbance) and the fractions having an A_{259}/A_{338} ratio less than 2.3 were combined, treated with sodium hydrogen carbonate (0.22 g), concentrated to *ca*. 10 cm^3 on a high vacuum rotary evaporator (at < 30 °C) and then freeze-dried to give [4,4-$^2H_2$]NADH sodium salt **5a** (842 mg, 86% by UV spectroscopy). ^{1}H NMR spectroscopy showed very small peaks at δ 2.72 and 2.60 corresponding to 4.4 and 3.4% residual protium respectively at the two diastereotopic positions at C-4.

[4-^{2}H]$NADP^+$ diammonium salt **19a** (880 mg, 1.08 mmol) was reduced by the same procedure to give [4,4-$^2H_2$]NADPH disodium salt **6a** (890 mg, 95%). ^{1}H NMR spectroscopy showed very small peaks at δ 2.77 and 2.67 corresponding to 4.3 and 3.5% residual protium respectively at the two diastereotopic positions at C-4. For unlabelled NADPH: δ (400 MHz, D_2O) 8.42 and 8.18 (each 1 H, s, adenine-H), 6.87 (1 H, s, 2-H), 6.16 (1 H, d, *J* 5, adenosine 1′-H), 5.89 (1 H, d, *J* 8, 6-H), 4.92 (1 H, br q, *J* 5.5, adenosine 2′-H), 4.73 (2 H, m, 5-H and nicotinamide 1′-H), 4.56 (1 H, t, *J* 4.5), 4.36 (1 H, br s) and 4.26–3.99 (7 H, m, other ribose H) and 2.76 and 2.65 (each 1 H, br d, *J* 18, 4-H_2).

Biosynthesis of cobyrinic acid 15a using [4-$^2H_2$]cofactors 5a and 6a

Pr. shermanii cells were grown in a lactate medium containing cobalt as earlier[27] and were harvested after 3 days, before the aeration step. Each litre of the medium contained the following: yeast extract (10 g), KH_2PO_4 (3 g), Na_2HPO_4 (3 g), 70% aqueous sodium lactate (40 cm^3) and $CoCl_2 \cdot 6H_2O$ (80 mg). The moist cells (100 g) were suspended in 0.05 M phosphate buffer (pH 7.7, 115 cm^3) containing ethylenediaminetetraacetic acid (0.1 mM) and dithiothreitol (0.6 mM). The cells were broken under argon by three passes through a French press and the resultant suspension was centrifuged at 18000 rpm for 1 h at 4 °C. The supernatant was passed using argon pressure through a column of DEAE-Sephadex A-25 (10 g), which had previously been equilibrated for 1 day with the foregoing phosphate buffer, and more buffer (50 cm^3) was then passed through. The eluate and washings were combined to provide the cell-free enzyme system used below.

In parallel with the foregoing work, the ^{13}C-labelled sirohydrochlorin octamethyl ester **14a** prepared above (1 mg, 40 atom% ^{13}C) was hydrolysed with piperidine–water (3:7, 0.5 cm^3) in the dark for 2 days in a glove box at < 5 ppm O_2. At this point the cell-free enzyme system above was mixed with adenosine triphosphate (220 mg), glutathione (105 mg), SAM (105 mg), $MgCl_2 \cdot 6H_2O$ (85 mg), $CoCl_2 \cdot 6H_2O$ (35 mg), cysteine (25 mg), [4-$^2H_2$]NADH (200 mg) and [4-$^2H_2$]NADPH (250 mg). The resultant enzyme system was incubated under argon with the sirohydrochlorin piperidinium salt prepared above (including the excess piperidine) for 20 h at 30 °C in the dark. Further portions of the two [4-$^2H_2$]cofactors (227 mg, and 325 mg, respectively) were added after 8 h. The pH was maintained at 7.66 throughout the incubation by means of an automatic titrator using aqueous 1 M sodium hydroxide (6.3 cm^3 consumed). Dicyanocobyrinic acid (7.2 mg) in water (6 cm^3) containing potassium cyanide (24 mg) was then added, the mixture was stirred under argon at 30 °C for 30 min, then cooled on ice and treated with more dicyanocobyrinic acid (6 mg).

DEAE-cellulose (5 g) was added to the mixture and filtered off again after being stirred for 5 min; this treatment was repeated three times with fresh DEAE-cellulose each time. The combined DEAE-cellulose was washed with water, methanol and acetone then dried at high vacuum for 16 h (Cellulose A1). The filtrate from the cellulose was adjusted to pH 2.5 with hydrochloric acid and passed through a column (3 × 18 cm) of Amberlite XAD-4 resin which had been pre-washed with methanol, water and finally with degassed water of pH 2.5. The adsorbed cobyrinic acid was washed with water of pH 2.5, then eluted with degassed methanol and the eluate was evaporated to remove the methanol. The resultant aqueous solution was stirred for 5 min with DEAE-cellulose (5 g), the cellulose was collected and washed with water, methanol and acetone before being dried at high vacuum (Cellulose B1). The filtrate (after filtering off Cellulose B1) was evaporated to dryness (Yellow pigments C1).

The foregoing work was repeated on the same scale to give Cellulose A2, Cellulose B2 and Yellow pigments C2.

The Yellow pigments C1 and C2 were combined in dry methanol (5 cm^3) and insoluble material was removed by filtration, the filter pad of Celite being washed with methanol (5 cm^3). The residue from evaporation of the methanolic solution and washings was dissolved in dry methanol (25 cm^3) containing sulfuric acid (1.2 cm^3) and trimethyl orthoformate (2.4 cm^3). This solution was heated under reflux in the dark for 20 h under argon, cooled and mixed with saturated aqueous sodium hydrogen carbonate (70 cm^3) containing potassium cyanide (40 mg). Extraction with carbon tetrachloride (3 × 30 cm^3) removed a purple pigment which was recovered by evaporation and purified by PLC on silica using benzene–methanol (9 : 1) to give cobester **16a** (Sample A).

The aqueous layer after extraction with carbon tetrachloride was still purple; extraction with dichloromethane (3 × 30 cm^3) removed these pigments which were re-esterified as above, a process that was repeated until all the purple material was extractable by carbon tetrachloride. The combined crude pigment obtained in this way was purified by PLC as above to give more cobester **16a** (Sample B).

Methanol–sulfuric acid (97.5 : 2.5 v/v) was degassed with an argon stream for 30 min and then used to elute the pigments from the combined Celluloses A1, A2, B1 and B2 packed into a column. This operation was run under argon in the dark. The eluate finally ran colourless and the total amount (200 cm^3) was treated with trimethyl orthoformate (10 cm^3) and kept under argon for 48 h at 18 °C. The resultant cobester was isolated as above (Sample C) and the two bands that ran slower than cobester on the PLC plates were eluted and re-esterified in methanol–sulfuric acid–trimethyl orthoformate (24 cm^3 : 1.2 cm^3 : 2.4 cm^3) but now under reflux for 48 h. Work-up as before gave more cobester (Sample D).

The cobester Samples A, B, C and D were combined (total 12.9 mg), diluted with unlabelled crystalline cobester (5 mg) and recrystallised twice from methyl acetate–hexane to yield pure cobester **16a** (14 mg). This material was used for the ^{13}C-NMR spectrum shown in Fig. 1.

Biosynthesis of cobyrinic acid 15b using ^{3}H-labelled cofactors

(a) The (*R*)-experiment. The cell-free enzyme system used for this study was prepared from the same batch of moist *Pr. shermanii* cells (85 g) exactly as described for the foregoing work with ^{2}H-labelled cofactors. The amounts of the various cofactors added were in exact proportion to the lower quantity of cells used. ^{14}C-Labelled sirohydrochlorin **13b** was prepared as before by hydrolysis, using aqueous piperidine, of the corresponding octamethyl ester **14b** (1.17 mg), described above, which had been mixed with a small amount of unlabelled **14** to give a specific activity of 1.16 mCi $mmol^{-1}$. [4(*R*)-$^3H_1$]NADH[19] was diluted with [4-$^2H_2$]NADH synthesised above to give 41 μmol of material having specific activity of 2.00 mCi $mmol^{-1}$. Similarly, [4(*R*)-$^3H_1$]NADPH[19] was diluted with [4-$^2H_2$]NADPH from above to give 44.8 μmol of material with specific activity of 2.29 mCi $mmol^{-1}$. The enzyme system, the ^{14}C-labelled sirohydrochlorin and both reducing cofactors were then incubated as for the ^{2}H-series for 16 h after which dicyanocobyrinic acid (32 mg) and potassium cyanide (39 mg) were added. The mixture was adjusted to pH 2.5 with 3 M hydrochloric acid and centrifuged at 12000 rpm for 15 min at 4 °C. The supernatant A was stored at 4 °C while the pellet (*ca.* 27 g) was ground in a mortar with sodium chloride (27 g). After the solids had been extracted with acetone–pH 2.5 water (4 : 1), the suspension was centrifuged and the pellet was treated again in the same way. This afforded two pigmented supernatants which were combined and evaporated and the residue, dissolved in degassed pH 2.5 water, was combined with supernatant A. This solution was carried through the steps described for the ^{2}H-series using the same amount of Amberlite XAD-4 resin but here the methanolic eluate from the resin column was evaporated to dryness.

The residue in dry methanol containing potassium cyanide (5 mg) was filtered through a plug of Celite, the filtrate was evaporated to dryness and the residue was treated with dry methanol (24 cm^3), trimethyl orthoformate (1.2 cm^3), and conc. sulfuric acid (0.3 cm^3). The mixture was heated under reflux under argon in the dark for 16 h and then cooled. Potassium cyanide (25 mg) and saturated aqueous sodium hydrogen carbonate (50 cm^3) were added and the mixture was extracted with carbon tetrachloride (4 × 15 cm^3). The combined extracts were washed with saturated aqueous sodium hydrogen carbonate (10 cm^3) and then water (10 cm^3) and evaporated to dryness. The residue was purified twice by PLC first using chloroform–methanol (95 : 5) and then with benzene–methanol (85 : 15) to give cobester **16b** as a purple solid (32.8 mg). The specific activity and ^{3}H : ^{14}C ratio were almost constant after three recrystallisations from methyl acetate–hexane and the total ^{14}C-activity (8.34×10^5 dpm) corresponded to an incorporation of **12b** into **16b** of 26.7%.

Unlabelled cobester was added to slightly more than half of the labelled material and recrystallised as above to give the diluted sample (99.2 mg, 91 mmol; total activity: ^{14}C, 4.3×10^5 dpm; ^{3}H, 3.4×10^4 dpm) ready for degradation.

(b) The (*S*)-experiment. The incubation was carried out as for the (*R*)-experiment with essentially the same quantities of materials which had slightly lower specific activities. These were: ^{14}C-labelled sirohydrochlorin (0.5 mCi $mmol^{-1}$), [4(*S*)-$^3H_1$]NADH[19] (1.8 mCi $mmol^{-1}$) and [4(*S*)-$^3H_1$]NADPH[19] (2.31 mCi $mmol^{-1}$). The enzyme system (from 85 g of moist cells) was identical to that used for the foregoing experiment (170 g of moist cells were broken as one batch and half the final solution was used for each of the two parallel experiments). The final cobester **16c** (24.4 mg) showed total activities of ^{14}C 4.44×10^5 dpm (corresponding to an incorporation of 35%) and $^3H < 7.1 \times 10^3$ dpm, the latter value being too low for accurate determination.

Photooxygenation of cobester 16b

The diluted sample of cobester from the (*R*)-experiment in CD_3CN (3 cm^3) was oxidised in a specially designed photoreaction cell[20] under the published conditions,[20] though our halogen lamp was somewhat more powerful (20 V, 240 W). At the end of the oxidation, the solution was evaporated and the residue was purified by PLC in benzene–methyl acetate (1 : 4) to give the dioxo-secocobyrinate esters **20** (46.3 mg, 45%) and **21** (55.6 mg, 54%). They were eluted from the plates with methanol–dichloromethane (1 : 3). Both the lower R_f 5,6-seco-product[21] **20** and the higher R_f 14,15-seco-isomer[20] **21** have previously been thoroughly characterised. The spectroscopic data below on our products closely match those reported[20,21] and left no doubt

that the same substances were in hand. For **20**:§ δ_H ($CDCl_3$, 400 MHz) 0.93, 1.13, 1.17, 1.19, 1.23, 2.04 (each 3 H, s, Me), 2.15 (3 H, s, 15-Me), 2.62 (3 H, s, MeCO), 1.6–3.0 (m, side chain CH_2's), 3.20 (1 H, dd, *J* 3.3 and 8.4, 3-H), 3.51, 3.60, 3.63, 3.65, 3.66, 3.67, 3.71 (each 3 H, s, OMe), 5.49 (1 H, s, 10-H). For **21**§: δ_H ($CDCl_3$, 400 MHz) 1.24 (6 H, s, 2 × Me), 1.29, 1.30, 1.32 and 1.48 (each 3 H, s, Me), 2.10 (3 H, s, 5-Me), 2.80 (3 H, s, MeCO), 1.6–2.9 (m, side chain CH_2's), 2.32 and 3.31 (each 1 H, d, *J* 15, CH_2CO_2), 3.22 (1 H, dd, *J* 4.5 and 9.3, 13-H), 3.62 (3 H, s), 3.65 (6 H, s), 3.68 (6 H, s), 3.71 (3 H, s) and 3.72 (3 H, s, 7 × OMe plus 1 H obscured), 3.82 (1 H, d, *J* 9, 3- or 8-H), 4.93 (1 H, d, *J* 8.3, 19-H), 5.58 (1 H, s, 10-H).

Heptamethyl (*Coα*, *Coβ*-dicyano)5,6,14,15-tetraoxo-5,6:14,15-disecocobyrinate 22

Each of the singly cleaved products **20** and **21** were photo-oxygenated by the same procedure as described above save that CD_3OD was used as solvent for the latter substance. The work-up and purification methods were also the same except that PLC purification of the product from **20** used dichloro-methane–methanol (19:1). The yields were: from **20**, 42% of **22** (19.9 mg) and 25% of recovered starting material (11.8 mg); from **21**, 49% of **22** (28.2 mg) and 14% of recovered starting material (7.7 mg). The two samples of **22** were combined and an aliquot was assayed showing total activities of ^{14}C 1.63×10^5 and ^3H 1.05×10^4 dpm. This product had previously been fully characterised[20] and the NMR data below§ agree closely with those reported earlier; δ_H ($CDCl_3$, 400 MHz) 1.02, 1.15, 1.19, 1.21, 1.23, 1.28 (each 3 H, s, Me at C-1, 2, 7, 12*a*, 12*β* and 17), 1.65–3.05 (29 H, m, 18-H, -CH_2CH_2 and -CH_2CO), 2.47 and 2.85 (each 3 H, s, MeCO), 3.21 (1 H, dd, *J* 5 and 10, 13-H), 3.50, 3.63, 3.64, 3.66, 3.69, 3.70, 3.71 (each 3 H, s, 7 × OMe + 8-H obscured), 3.99 (1 H, br d, *J* 9, 3-H), 5.85 (1 H, s, 10-H) and 6.05 (1 H, d, *J* 6.4, 19-H); δ_C ($CDCl_3$, 100 MHz) 15.41, 16.93, 19.49, 20.72, 20.86, 21.80, 23.67, 26.64, 27.03, 29.19, 30.30, 31.58, 31.95, 32.18, 33.07, 33.25, 34.61, 38.93, 40.57, 42.84, 44.00, 45.57, 48.01, 50.06, 50.97, 51.51 (2 C), 51.65, 51.79 (2 C), 52.07, 52.42, 59.03, 62.01, 82.83, 88.28, 102.7, 170.6, 171.9, 171.9, 172.3, 172.4, 173.7, 173.9, 183.9, 184.1, 186.9, 189.1, 189.5, 190.5, 192.7 and 199.0.

Cleavage of heptamethyl disecocobyrinate 22 by hydrogenation

This step was carried out as previously described[20] using the foregoing ^3H,^{14}C-labelled sample (47.6 mg) and 10% palladium on charcoal (7.5 mg). The only difference was that the products were isolated by PLC on silica using methyl acetate–diethyl ether (1:2) to give (a) the A/D fragment **23** (16.1 mg, 67%), total activity ^{14}C 5.61×10^4 and ^3H 8.14×10^3 dpm and (b) the B/C fragment **24** (15.7 mg, 82%), total activity ^{14}C 5.84×10^4 dpm and ^3H, no activity.

Standard samples of **23** and **24** were prepared for characterisation purposes by repeating the whole degradative sequence on unlabelled cobester. The A/D fragment **23**§ showed: δ_H ($CDCl_3$, 400 MHz) 0.95, 1.13 and 1.50 (each 3 H, s, 1-, 2- and 17-Me), 1.66–1.86 (4 H, m) and 2.10 (1 H, ddd, *J* 13.5, 12 and 5, 18-H and 2 × CH_2CH_2CO_2), 2.25 and 2.40 (each 3 H, s, MeCO), 2.40–2.65 (5 H, m) and 2.97 (1 H, ddd, *J* 10.7, 7.5 and 3.3, 18-CH_2 and 2 × CH_2CH_2CO_2), 2.69 and 3.24 (each 1 H, d, *J* 15, 2-CH_2), 3.38 (1 H, dd, *J* 7.7 and 4.7, 3-H), 3.60, 3.65, 3.67 and 3.69 (each 3 H, s, OMe) and 4.37 (1 H, d, *J* 7.5, H-19); *m/z* (FAB) 579 ($M^+ + 1$), $C_{29}H_{43}N_2O_{12}$ requires 579. The B/C fragment **24**§ showed: δ_H ($CDCl_3$, 400 MHz) 1.13, 1.21, 1.37 (each 3 H, s, 7-, 12*a* and 12*β*-Me), 1.8–2.8 (m), 3.24 (1 H, t, *J* 8, 8-H), 3.60, 3.65 and 3.70 (each 3 H, s, OMe) and 5.39 (1 H, s, C=CH). These data agree closely with those published[20,21] for these products.

§ The numbering used in the assignments corresponds to that of cobyrinic acid **15**.

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