Biosynthesis of Vitamin B_{12}: Use of Specific ^{13}C-Labelling for Structural Studies on Factor IV

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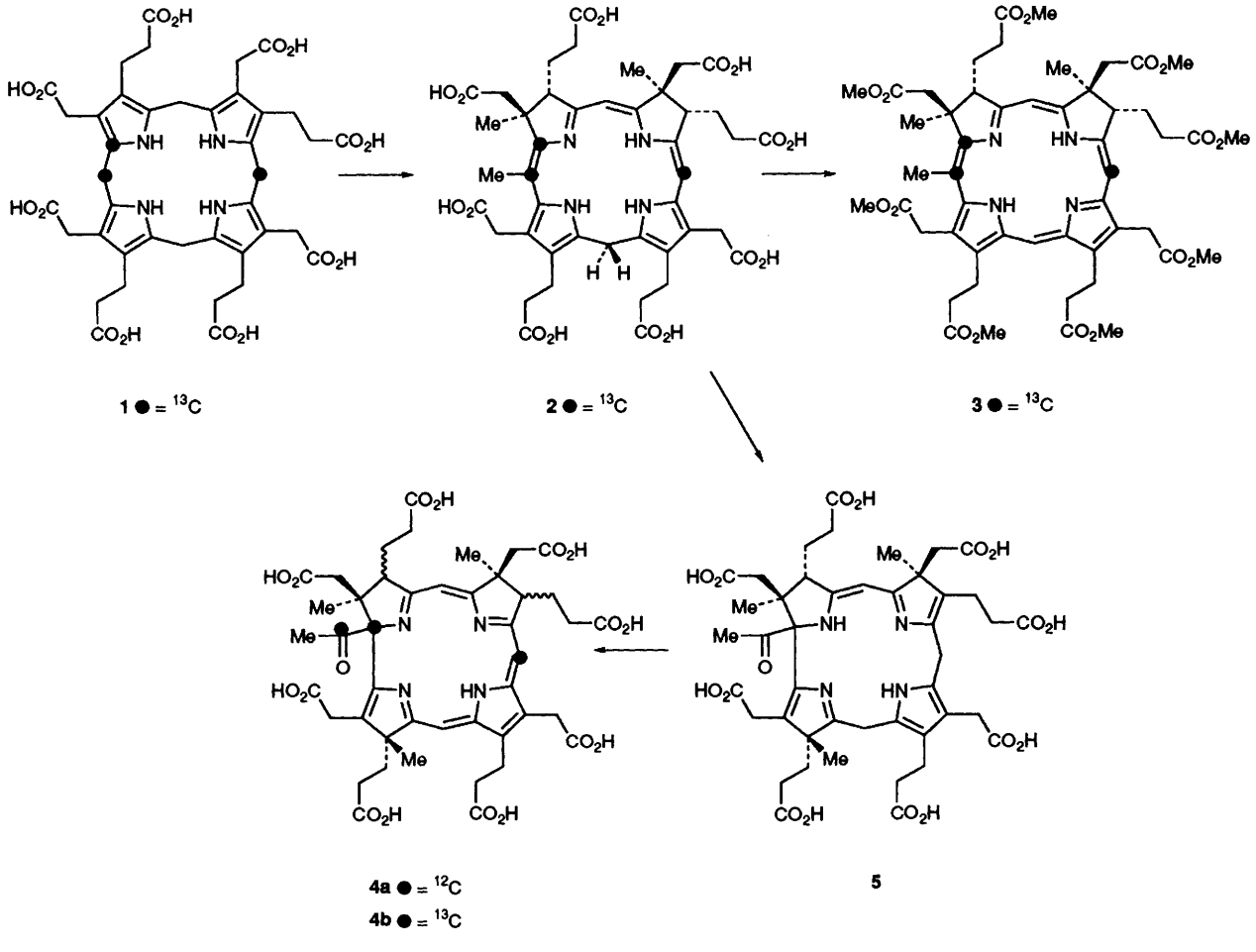
[1,10,20-$^{13}C_3$]Uro'gen III is unambiguously synthesised for enzymic conversion into precorrin-4 isolated after aerial oxidation as two epimers of Factor IV, the ^{13}C NMR spectra of which rigorously confirm the presence of a C-1 acetyl group in precorrin-4; attachment of the fourth methyl group at C-17 of precorrin-4 is also confirmed by related ^{13}C-labelling experiments.

The availability of mutants of *Pseudomonas denitrificans* in which the *cobM* gene had been inactivated[1] allowed the biosynthetic pathway to vitamin B_{12} to be interrupted at the stage of the tetramethylated intermediate, precorrin-4. This was isolated[2] as its oxidised form called Factor IV which could be separated into two isomers, A and B. There is good evidence that Factor IVA and Factor IVB are epimers and the formation of such epimers is a familiar feature of research on vitamin B_{12} intermediates.

Factor IVA was produced in different ^{13}C-labelled forms from ^{13}C-labelled samples of 5-aminolaevulinic acid (ALA) as in earlier structure determinations in this area.[3–6] ^{13}C NMR then led to structure **4a** for Factor IV and tentative signal assignments were made, the evidence as a whole being self-consistent. The aim of the present work was to provide unimpeachable evidence especially for the placement of the acetyl group at C-1 but also for the C-methylation at C-17.

The former studies depended on the unambiguous synthesis of [1,10,20-$^{13}C_3$]uroporphyrin III octamethyl ester **15** as outlined in Scheme 1. The [5-^{13}C]pyrrole[7] **6**, 90 atom% ^{13}C, was alkylated with the pyrrole **7** under acid-catalysed conditions. Surprisingly, the required dipyrromethane **8** was accompanied by an appreciable amount of a rearrangement product **12**, the ^1H NMR spectrum of which showed the aldehydic proton as a singlet not split by adjacent ^{13}C whereas the corresponding signal from the desired product **8** was a doublet as expected. The mechanism of formation of **12** deserves attention.

Pure unrearranged material **8** was isolated from the mixture, deformylated using a rhodium(I) catalyst[8] and finally the benzyloxycarbonyl group was removed from **9** by the standard steps **9** → **10** → **11** in Scheme 1. The product **11** was immediately condensed with the [*formyl*-$^{13}C_2$]diformyl dipyrromethane **13** prepared in turn by Vilsmeier formylation of the dicarboxylic acid[9] **14** using 99.5 atom% dimethyl[*formyl*-^{13}C]formamide. [1,10,20-$^{13}C_3$]Uroporphyrin III octamethyl ester **15** was isolated in 40–45% yield and a small part was converted into its zinc complex for NMR spectroscopy. The



signals (Table 1) confirmed specific ^{13}C-labelling entirely at the required sites.

The remaining porphyrin **15** was hydrolysed and the product was reduced with sodium amalgam to yield [1,10,20-$^{13}C_3$]uroporphyrinogen III **1** which was converted into [1,10,20-$^{13}C_3$]precorrin-3A† **2** by enzymic methylation using isolated, over-produced enzymes.[10] The product **2** was isolated after aerial oxidation as the isobacteriochlorin and the corresponding octamethyl ester **3** was purified. Its ^{13}C NMR spectrum showed only three signals (Table 1) so confirming specific labelling. After the methyl ester groups of **3** had been hydrolysed, the derived acid was incubated with the enzyme system from the *cobM* mutant of *P. denitrificans* where *in situ* reduction occurs to form [1,10,20-$^{13}C_3$]precorrin-3A **2** which was further converted into precorrin-4. Aerial oxidation and isolation as above gave Factor IVA and Factor IVB.

The ^{13}C NMR spectrum of Factor IVA (Table 1) gave the important result that the signals from both the ketonic carbonyl group (originally C-20) at δ 210.9 and from C-1, δ 84.2, were doublets (*J* 39 Hz) thus rigorously confirming that the acetyl group in Factor IVA, and therefore also in precorrin-4, **5** or a tautomer, is attached at C-1. The signal from C-10 was a singlet as expected. The NMR spectrum also showed that the chromatographic fraction composed mainly of Factor IVA also contained *ca.* 20% of Factor IVB.

Factor IVB gave a ^{13}C NMR spectrum (Table 1) in full agreement with that above in that the signals from the ketonic carbonyl group and C-1 were both doublets (*J* 39 Hz).

It had earlier seemed likely[2] that the A and B isomers of Factor IV are related as epimers at C-8. However, the additional data in Table 1 showing that the change A to B substantially affects the chemical shift of the signal from the ketonic carbonyl group whereas that from C-10 is much less affected indicates that epimerisation at C-3 must also be considered. In addition, a small amount of another isomer Factor IVC is formed which runs chromatographically with

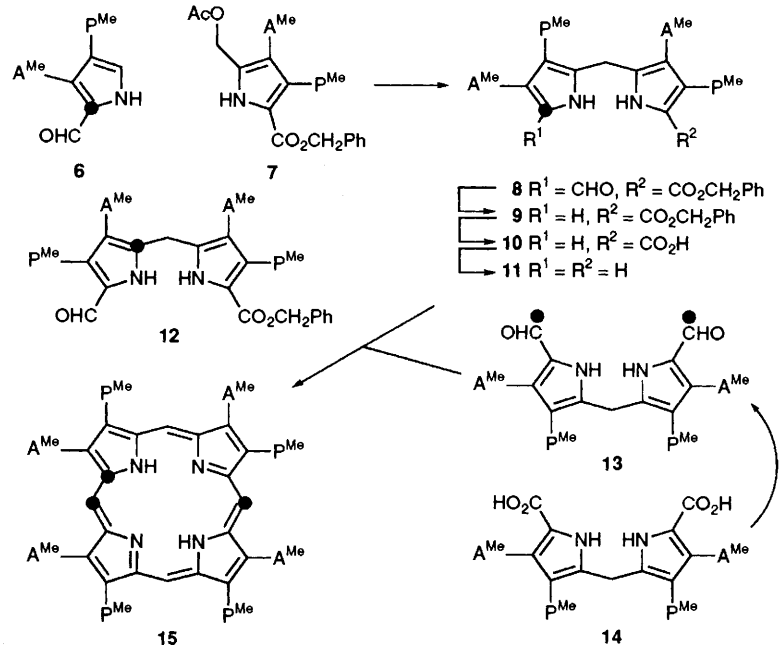
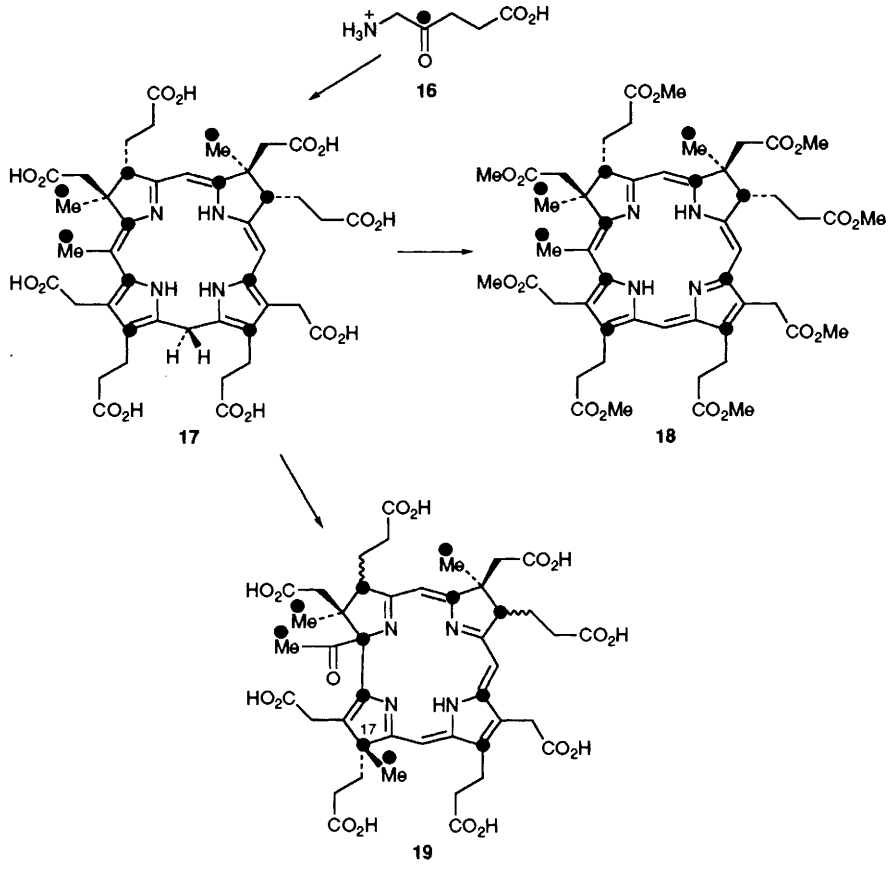


Table 1 Relevant ^{13}C NMR data at 100.6 MHz for Factor IV **4** and its precursors[a]

	Chemical shifts, δ, and coupling, *J*/Hz					
Substance	C-1	C-10	C-17	C-20	CO of MeCO	Me of MeCO
Uroporphyrin III ester[b] **15** (as Zn^{II} complex)	147.3 d, 70	98.4 s	—	98.5 d, 70	—	—
Isobacteriochlorin[c] **3**	153.0 d, 79	95.5 s	—	104.8 d, 79	—	—
Factor IVA[d] **4b**	84.2 d, 39	95.9 s	—	—	210.9 d, 39	—
Factor IVB[d] **4b**	83.97 d, 39	96.3 s	—	—	208.8 d, 39	—
Factor IVC[d] **4b**	84.06 d, 38	98.2 s	—	—	208.7 d, 38	—
Factor IVA[d] **19**	83.5 d, 51	—	66.7 d, 31	—	—	30.6 s
Factor IVB[d] **19**	83.7 d, 50	—	67.4 d, 31	—	—	29.4 s

[a] For best consistency, the δ values for the isomers of Factor IV have been set by matching one sharp signal in our spectra, *e.g.* from C-10, with the corresponding signal reported earlier.[2] However, due to the small scale involved, all the sample from each preparation was used for NMR and the concentration varied which contributes to the small differences in the δ values in the two pairs of samples. [b] In $CDCl_3$. [c] In C_6D_6. [d] In D_2O–0.1% CF_3CO_2D.



Factor IVB. This gives a second set of much smaller signals alongside those from Factor IVB showing exactly the same pattern of doublets for the ketonic carbonyl group and C-1 and a singlet for C-10. In this case the signal from C-10 is substantially affected whilst the other two were almost identical to those from Factor IVB (Table 1). A considerable effort by NOE and ^{13}C-labelling will be required to clarify these lower priority stereochemical points.

Evidence by ^{13}C-labelling for methylation at C-17 of Factor IV, and so also in precorrin-4, was gained by preparing multiply ^{13}C-labelled precorrin-3A **17** from [4-^{13}C]ALA **16** and [*methyl*-^{13}C]-*S*-adenosyl-L-methionine (SAM) by using the necessary set of five isolated overproduced enzymes,[10] *viz.* ALA dehydratase, hydroxymethylbilane synthase, uro'gen III synthase and the two methylases.[11] The product was isolated as **18** after aromatisation and esterification. The ester **18** was hydrolysed and the octa-acid was converted into precorrin-4, again using [*methyl*-^{13}C]SAM, by the foregoing enzyme system from *P. denitrificans*. As previously, this was isolated after aerial oxidation in the form of the isomeric Factors IVA and IVB **19**. Importantly, the signal from C-17 in both isomers was a doublet (*J* 31 Hz) due to its direct connection to the newly introduced ^{13}C-labelled fourth methyl group; 17-Me in Factor IVB gave a doublet (δ 20.5, *J* 31 Hz) and one leg of the corresponding doublet for Factor IVA was resolved from the overlapping 2-Me and 7-Me signals. This confirms that the fourth methyl group of precorrin-4 is indeed at C-17 and eliminates any possibility of rearrangement as precorrin-6x is formed from it. In addition the methyl group of the acetyl residue of **19** appeared as a strong singlet (Table 1).

The foregoing experiments provide rigorous additional evidence concerning key features of the structures of Factor IV **4a** and so also of precorrin-4 **5**. Taken in combination with the earlier studies,[2] these leave only such details as the stereochemistry at C-3 and C-8 in the various epimers to be elucidated by future work.

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Footnote

† Precorrin-3A was previously referred to as simply precorrin-3 but since the discovery that the subsequent intermediate is also at the trimethylated stage, these two intermediates should be called precorrin-3A and precorrin-3B respectively.[6]

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