Biosynthesis of Vitamin B_{12}: Mechanistic Studies on the Transfer of a Methyl Group from C-11 to C-12 and Incorporation of ^{18}O

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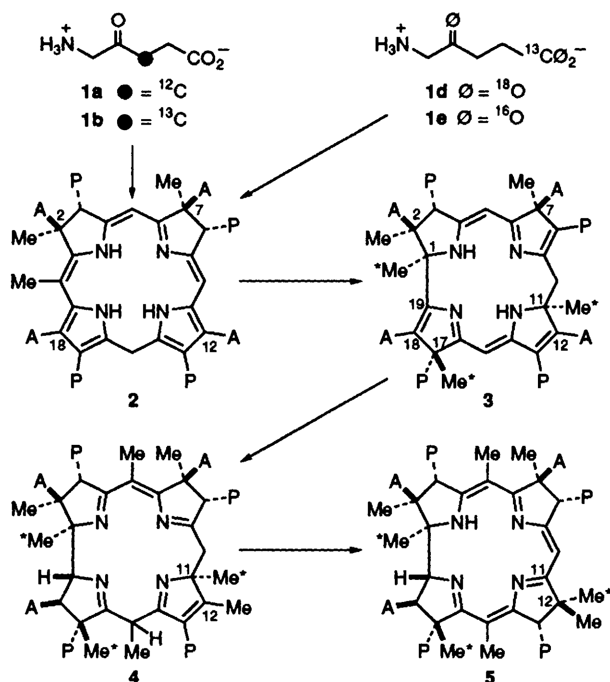
The transfer of a methyl group from C-11 to C-12 in vitamin B_{12} biosynthesis is shown, by a crossover experiment involving [1,11,17-$(CD_3)_3$]-labelled precursors, to occur in an intramolecular fashion; it is found that in *Pseudomonas denitrificans*, unlike *Propionibacterium shermanii*, no exchange of the ring A acetate oxygen atoms occurs during formation of the corrin ring.

The elucidation of the entire biosynthetic pathway to vitamin B_{12} has recently been completed.[1,2] However, some mechanistic questions remain to be answered[1] and two are considered here.

One of many surprising features of the pathway to B_{12} was *C*-methylation at C-11, first recognised in the intermediate precorrin-6A[3,4] **3**. This was unexpected because B_{12} and its precursor in *Pseudomonas denitrificans*,[5] hydrogenobyrinic acid **5** (Hby), are both methylated at C-12 not at C-11. The C-11 methyl group remains in place for the next two intermediates, precorrin-6B[6] (18,19-dihydro-**3**) and precorrin-8x[7] **4**, before the latter is transformed into Hby **5** by Hby synthase (CobH).[8] In principle the change of **4** into **5** could be achieved in three ways: (i) removal of the methyl group at C-11 and replacement by another at C-12; (ii) by some intermolecular transfer; (iii) by an intramolecular suprafacial [1,5]sigmatropic rearrangement. Mechanism (i) would be difficult to reconcile with the many pulse-labelling and other incorporation experiments that have been carried out[1,2] but both (ii) and (iii) are possible. The 11α-stereochemistry of precorrin-6A **3** was proposed[4] on the assumption that the more attractive mechanism (iii) is correct. However, there has been no direct supporting evidence, hence the present work.

The labelled compounds required for this work were [$^{13}C_8$]precorrin-6A **3b** and its analogue **3c** having CD_3 groups at C-1, C-11 and C-17. First [$^{13}C_8$]precorrin-3A **2b** was prepared enzymically[9] from 5-amino[3-^{13}C]laevulinic acid **1b**



Scheme 1 Part of the biosynthetic pathway to vitamin B_{12}. A = CH_2CO_2H, P = $CH_2CH_2CO_2H$; for compounds **2–5**: **a**, unlabelled; **b**, [$^{13}C_8$]-labelled (at C-2, 7, 12 and 18 and on the first CH_2 of each P side-chain; from **1b**); **c**, [$^{13}C_8$]-labelled and Me* = CD_3; **d**, [carboxyl-$^{13}C^{18}O_2$]; **e**, [carboxyl-^{13}C].

(ALA). Part of this precorrin-3A **2b** was converted enzymically[3,4] into [$^{13}C_8$]precorrin-6A **3b** using *S*-adenosylmethionine (SAM) and the rest was converted similarly into [$^{13}C_8$,$(CD_3)_3$]precorrin-6A **3c** using [*methyl*-$^2H_3$]SAM. Equal quantities of the two labelled samples of precorrin-6A were mixed and converted into Hby **5**, using enzymes from *Pseudomonas denitrificans* and unlabelled SAM. The Hby was purified by HPLC and analysed by positive ion electrospray mass spectrometry (Fig. 1). Peak a corresponds to [$^{13}C_8$,$(CD_3)_3$]Hby **5c** and peak b to [$^{13}C_7$,$(CD_3)_3$]Hby, which arises because the initial [3-^{13}C]ALA was only *ca.* 95 atom% ^{13}C; peaks c and d are from [$^{13}C_8$]Hby **5b** and [$^{13}C_7$]Hby. The ^{13}C label was included in this experiment so as to ensure that the biosynthesised Hby **5b** could be distinguished in the mass spectrum from endogenous Hby **5a**, which appears eight mass units lower, peak x.

If mechanism (i) above had been correct, the CD_3 group at C-11 of precorrin-6A **3c** would be lost and so the higher mass set of peaks would not appear at a and b but three mass units lower at e and f. For the intermolecular mechanism (ii), crossover of CD_3 and Me groups would occur, giving rise to Hby with two CD_3 groups arising from **3c** and with one CD_3 group from **3b**; as a result there would be sets of peaks at e and f and at g and h of equal height to those at a–d. This is clearly not so. In fact, the observed pattern of peaks from 894 to 900 closely matches the pattern of parent ions observed for the original [$^{13}C_8$,$(CD_3)_3$]precorrin-6A **3c**, which demonstrates that the conversion of precorrin-8x **4** to Hby **5** is very largely (and probably entirely) intramolecular. Our results thus

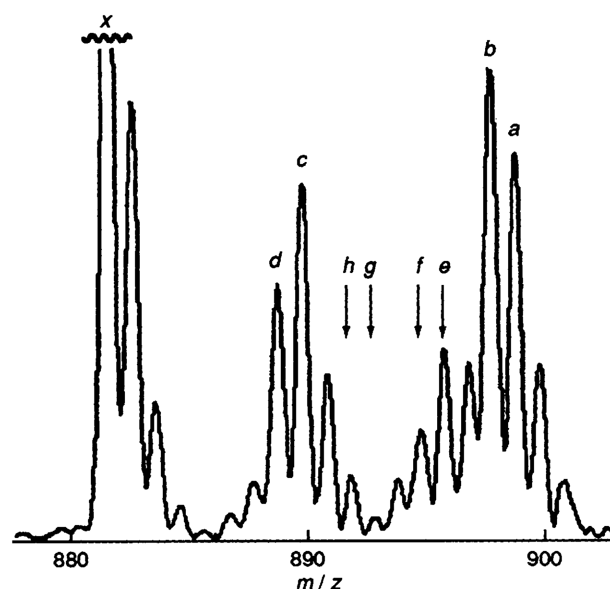


Fig. 1 Electrospray mass spectrum of Hby **5** derived from the labelled precorrin-6A (**3b** + **c**). Peaks above *m/z* 886 are expanded 3-fold. Peaks a and b are due to **5c**, peaks c and d are due to **5b** and peak x is due to endogenous unlabelled Hby **5a**. The small peaks at e and f are due to incorporation of some precorrin-6A bearing only two CD_3 groups, which mass spectrometry showed was present in the precursor.

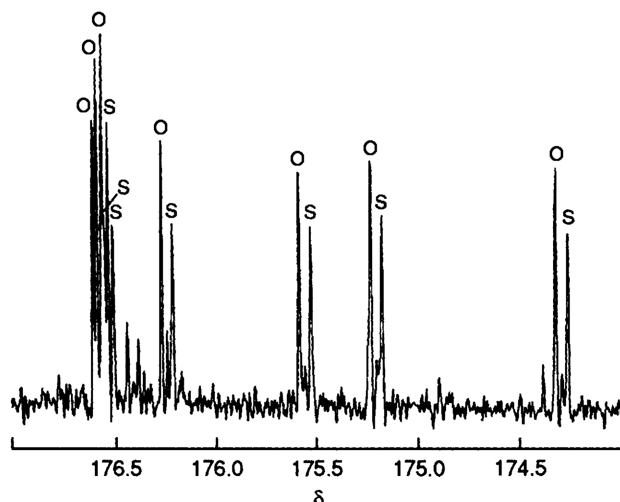


Fig. 2 Carboxyl region of the ^{13}C NMR spectrum of Hby **5** derived from precorrin-3A **2d** and **2e** (100 MHz, CD_3OD). The three well separated sets of signals to lower frequency are the acetate carboxyls and the four others are propionate signals. Peaks marked o are the unshifted ones due to $^{13}CO_2H$, peaks marked s are the shifted ones due to $^{13}C^{18}O_2H$ and the small peaks in between are due to $^{13}CO^{18}OH$.

directly support the [1,5]sigmatropic rearrangement and hence also the α stereochemical assignment at C-11 of precorrin-6A **3**.

In *Propionibacterium shermanii* there is exchange of oxygen from the C-2 acetate carboxyl with the medium as B_{12} is biosynthesised[10] and one oxygen has cleanly exchanged in going from precorrin-3A **2** to cobyrinic acid[11] (Co^{III} complex of Hby **5**). However in *Ps. denitrificans*, no such loss occurs from the C-2 acetate up to precorrin-4[12] or precorrin-5.[13] We now examine whether there has been oxygen exchange by the time Hby **5** has been formed.

Precorrin-3A **2d** was prepared from [1-^{13}C,1,1,4-$^{18}O_3$]ALA **1d** (*ca.* 90 atom% ^{18}O) and precorrin-3A **2e** was similarly prepared from [1-^{13}C]ALA **1e**. The two samples were separately converted into Hby using the necessary seven overexpressed enzymes from *Ps. denitrificans*.[3] This 'two-pot' synthesis of Hby **5** from ALA **1** repeats preparatively the genetically engineered (12 enzymes) synthesis of this complex molecule first carried out in 1990.[3] The pure major epimer of Hby **5** was isolated by HPLC (two minor epimers were also detected) for each sample and its isotopic constitution was studied both by electrospray mass spectrometry and ^{13}C NMR spectroscopy. The ^{18}O sample showed a series of peaks due to molecules with 10 to 14 ^{18}O atoms, the latter being at *m/z* 916.4996 ($C_{38}{}^{13}C_7H_{61}N_4{}^{18}O_{14}$ requires 916.5014), whereas the sample without ^{18}O gave *m/z* 888.4404 ($C_{38}{}^{13}C_7H_{61}N_4O_{14}$ requires 888.4419). The shift of up to 28 mass units indicates retention of both ^{18}O atoms in all seven carboxyl groups. For the ^{13}C NMR spectrum (Fig. 2), the two samples were mixed in approximately equivalent proportions so that a reference unshifted peak (marked o) would be seen for each carboxyl. The presence of the large peak shifted to lower frequency[14] (marked s, Δδ 0.055–0.058 ppm) for each of the seven carboxyl groups proves that none has exchanged any significant proportion of its oxygen during the biosynthesis of Hby **5**. This is in contrast to the results with *Pr. shermanii* and indicates a difference in mechanism between the pathways in these two organisms (see ref. 12 for a brief discussion).

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