Biosynthesis of Vitamin B_{12}: Structure of the Ester of a New Biosynthetic Intermediate, Precorrin-6y

Denis Thibaut,[a] Fumiyuki Kiuchi,[b] Laurent Debussche,[a] Finian J. Leeper,[b] Francis Blanche*[a] and Alan R. Battersby*[b]

[a] *Departement Analyse, Centre de Recherche de Vitry-Alfortville, Rhône-Poulenc Rorer, BP14, F-94403 Vitry-sur-Seine Cedex, France*
[b] *University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, UK*

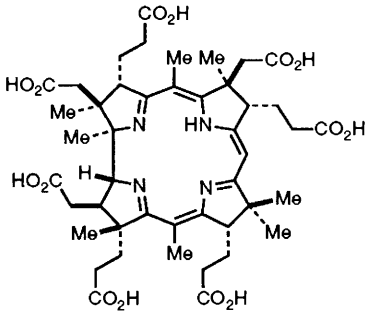
^{13}C Labelling and NMR experiments establish the structure of precorrin-6y octamethyl ester, the corresponding octa-acid being a further new intermediate on the biosynthetic pathway to hydrogenobyrinic acid **1**.

The enzymic formation of hydrogenobyrinic acid **1** from precorrin-6x[1,2] **2a**, a new intermediate which was highly informative about the biosynthetic pathway to **1**, first involves a reduction step specifically dependent[1] on NADPH (reduced nicotinamide adenine dinucleotide). Hydrogenobyrinic acid **1** is the cobalt-free form of cobyrinic acid, a direct biosynthetic precursor of vitamin B_{12}. Labelling experiments have demonstrated[3] that the hydride equivalent transferred from NADPH is delivered to C-19 of precorrin-6x. The product of this reduction is the next intermediate[4] on the biosynthetic pathway to **1** and it is named precorrin-6y† since it directly follows precorrin-6x **2a**. Precorrin-6y has been isolated and shown to be converted in 90% yield[4] into **1** by the complete cell-free enzyme system from *Pseudomonas dentrificans*[1] in the presence of *S*-adenosylmethionine (SAM).

† The letter y will eventually be replaced by a capital letter *e.g.* C or D, when it is known whether 2 or 3 intermediates, also at the hexamethylated level, precede precorrin-6y on the pathway.

The structure of precorrin-6x octamethyl ester has been rigorously established[1,2,5] to be **2b** so the actual biosynthetic intermediate, precorrin-6x octa-acid, is **2a** or possibly a double-bond tautomer[2] of that structure. Since in the reduction process the hydride equivalent is transferred to C-19 of precorrin-6x **2a**, structure **3a** can be considered for precorrin-6y provided only reduction and no rearrangements have occurred. We now describe ^{13}C labelling and NMR experiments which fully support structure **3b** for precorrin-6y octamethyl ester.

It had already been shown[4] that the very pale yellow precorrin-6y has a UV–VIS spectrum very similar to that of precorrin-6x **2a** but with the peaks shifted *ca.* 20 nm to shorter wavelength. Thus precorrin-6y has separated chromophores (*cf.* **2a**) and its molecular weight by FAB-MS (fast atom bombardment mass spectrometry) was 896 whilst the corresponding methyl ester showed *m/z* 1008.[4] Both values are 2 units higher than found[1] for precorrin-6x **2a** and its ester **2b**, respectively, thus indicating that only a single reduction step has occurred. Further, the mass change from precorrin-6y to



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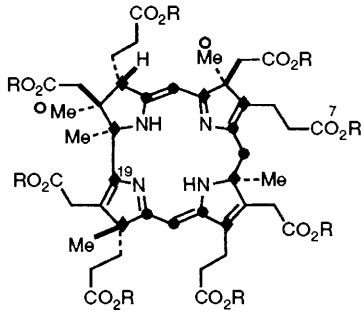
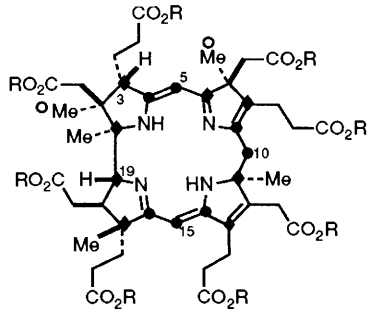


2a R = H, ◆ = ● = ○ = ^{12}C
2b R = Me, ◆ = ● = ○ = ^{12}C
2c R = H, ◆ = ^{13}C, ● = ○ = ^{12}C
2d R = H, ◆ = ^{12}C, ● = ○ = ^{13}C



3a R = H, ◆ = ● = ○ = ^{12}C
3b R = Me, ◆ = ● = ○ = ^{12}C
3c R = Me, ◆ = ^{13}C, ● = ○ = ^{12}C
3d R = Me, ◆ = ^{12}C, ● = ○ = ^{13}C

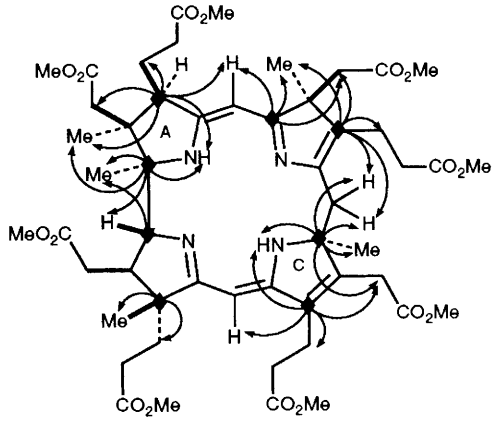
its ester showed that eight carboxy groups are still present. This intermediate, like precorrin-6x, is thus a member of the precorrin-6 family.[6] The esterified precorrin-6y proved to be a mixture of two separable isomers (major, *ca.* 3 parts, *m/z* 1008.4941; minor, *ca.* 2 parts *m/z* 1008.4931; $C_{52}H_{72}N_4O_{16}$ requires 1008.4943) shown below to be epimeric at C-3.

The structural work started with the enzymic production as previously[1,2] of ^{13}C labelled precorrin-6x **2c** from 5-amino-[4-^{13}C]laevulinic acid. This was incubated with a cell-free extract from the engineered strain[4] SC510 Rif^r (pXL253) to afford labelled precorrin-6y which was isolated as the methyl ester. A second preparation of precorrin-6y was carried out from 5-amino[5-^{13}C]laevulinic acid *via* precorrin-6x **2d** and differing from the previous one in the use of [*methyl*-^{13}C]SAM in the early stages[1,2] so that the *C*-methyl groups at C-2 and C-7 were ^{13}C labelled. In both preparations, the two epimeric esters were separated.

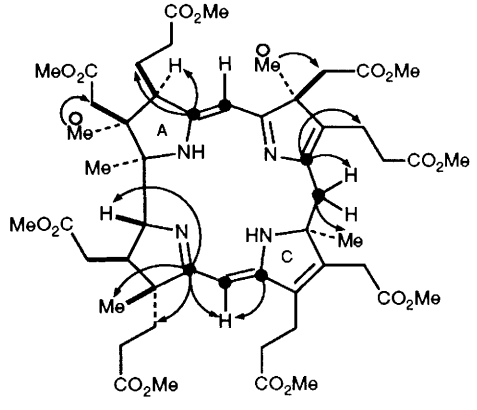
The four labelled esters above were examined by ^{13}C NMR and by ^1H–^{13}C correlation experiments with the delay set to the optimum for couplings of 10 Hz to reveal long-range ^{13}C–^1H couplings through up to three bonds. Table 1 collects the ^{13}C chemical shifts and couplings and the arrows on structures **4a** and **4b** show the long-range ^{13}C–^1H couplings detected in the two samples of 3-*epi*-precorrin-6y ester which were used for the main COSY experiments because more material was available. It should be emphasised that the various ^{13}C labels arrive unambiguously at the illustrated sites as a result of the early steps of building the tetrapyrrolic macrocycle which are firmly established.[7]

It is not intended in this brief account to set every piece of the data into place; rather the simplest main line of the structural argument for 3-*epi*-precorrin-6y ester (**4a**,**b**) will be given. Every other observation collected in Table 1 and illustrated on structures **4a** and **4b** reinforces that argument by being fully self-consistent.

(*i*) C-15, C-1 and C-19 can be directly assigned since C-15 in **4b** is the only centre coupled to two other carbons and C-1 and C-19 in **4a** are the two directly coupled carbons; (*ii*) this allows assignment of the 1-Me, 19-H and 15-H; (*iii*) there is a connection from 15-H to C-16 and on to 17-Me in **4b** and finally between the 17-Me and C-17 in **4a**; (*iv*) C-14 is located by being the remaining carbon coupled to C-15; (*v*) 15-H in **4a** is coupled to C-13 and this to 12-CH_2 which is coupled to a carbon giving a signal at δ 69.6. Though the latter was not resolved from the C-1 signal in the 2D-spectrum, C-1 cannot be coupled to the 12-CH_2 so the coupling partner must be C-11; (*vi*) C-1 or C-11 is coupled to a CH_2 group in **4a** whose carbon was labelled in **4b** and only C-5 and C-10 remain as candidates for this group. Neither C-1 nor C-11 is close enough to C-5 so C-11 must be coupling to 10-H which locates this CH_2 group at C-10. Interlocking evidence comes from the coupling of the carbon on this CH_2 group in **4b** to a methyl;



4a ◆ = ^{13}C



4b ● = ○ = ^{13}C

C-5 is not close enough to any methyl and so the CH_2 group must be C-10 which is coupled to 11-Me. Pinpointing the C-10 methylene immediately allows assignment of C-10, C-9 and C-8 from the couplings shown on **4a** and **4b**. C-8 in **4a** connects to 7-Me and this allows assignment of C-6; (*vii*) the remaining

Table 1 ^{13}C NMR data for precorrin-6y octamethyl ester **3c,d** and its C-3 epimer **4a,b**

3c			**3d**			**4a**			**4b**		
δ_C	Coupling, *J*/Hz	Assignment	δ_C	Coupling, *J*/Hz	Assignment	δ_C	Coupling, *J*/Hz	Assignment	δ_C	Coupling, *J*/Hz	Assignment
53.3	s	C-3	17.6	s	2-Me	54.6	s	C-3	20.4	s	2-Me
56.0	s	C-17	23.5	s	7-Me	56.0	s	C-17	23.1	s	7-Me
70.0	s	C-11	35.7	d, 50.8	C-10	69.6	s	C-11	35.6	d, 50.4	C-10
71.6	d, 40.5	C-1	76.0	dd, 66.4, 71.7	C-15	69.9	d, 41.8	C-1	76.2	dd, 66.2, 72.0	C-15
72.8	d, 39.5	C-19	80.8	dd, 6.5, 70.1	C-5	72.4	d, 41.0	C-19	78.1	dd, 5.6, 71.7	C-5
129.6	s	C-8	146.9	dd, 6.0, 50.0	C-9	130.5	s	C-8	147.3	dd, 5.6, 50.5	C-9
135.8	s	C-13	160.0	d, 72.3	C-14	135.8	s	C-13	159.3	d, 72.3	C-14
181.8	s	C-6	160.8	d, 70.0	C-4	182.4	s	C-6	159.6	d, 71.4	C-4
			176.5	d, 66.4	C-16				176.9	d, 66.1	C-16

meso-H must be 5-H from which C-5 and C-4 can be assigned using **4b**; (*viii*) a proton coupled to either C-4 or C-14 in **4b** is also directly attached to ^{13}C in **4a** so this proton must be 3-H and hence C-3 and 2-Me can be assigned; (*ix*) the couplings from 2-Me to C-3 and on to 5-H in **4a** confirm that the assignments of C-5 and C-10 are correct.

Finally the NH groups can be securely placed on rings *A* and *C* because they couple in **4a** to C-1 and C-3 and to C-11 and C-13 and not to other carbons. In support, the chemical shifts for C-6 and C-16 (δ *ca.* 180) fit N=**C**–C whereas those for C-4 and C-14 (δ *ca.* 160 ppm) agree with N–**C**=C.

The stereochemical difference between the major and minor isomers of precorrin-6y ester which has appeared in structures **3b** and **4a,b** without justification can now be considered. That C-3 was the site of epimerisation was clear by comparing the ^{13}C and ^{1}H chemical shifts of the two isomers. The largest differences were all from carbon and hydrogen atoms of ring-*A* and its attached groups. For 3-H, δ_H(minor) − δ_H(major) was +0.59 ppm and δ_C(minor) − δ_C(major) was +2.7 ppm for C-5 and −2.8 ppm for 2-Me. It is the *downfield* shift of the 2-Me signal in the major isomer relative to the minor one (loss of γ-effect) that leads to the major isomer being assigned the 3-*epi* structure **4a,b**; current work aims to provide additional evidence on this point. Partial epimerisation at C-3 has also occurred for all earlier B_{12} intermediates which have been isolated (*e.g.* see ref. 8).

To all the independent structural evidence summarised above must be added the additional strength from the mass of data on precorrin-6x ester **2b** since precorrin-6y **3a** is derived from precorrin-6x. The stereochemistry at C-19 of **3a** is set as illustrated because **3a** is enzymically converted in high yield into hydrogenobyrinic acid **1** where the β-H at C-19 is beyond doubt. Finally, it should be stressed that though the double-bond positions are secure for the esters **3b** and **4a,b**, it is possible that precorrin-6y itself may be a double-bond tautomer of **3a** exactly as for precorrin-6x **2a**.

In conclusion, the structure **3b** has been established for the ester of another intermediate, precorrin-6y, on the pathway to hydrogenobyrinic acid **1**. This has opened the way to studies on the final steps going forward from **3a** which involve methylation at C-5 and C-15, decarboxylation of the 12-acetate group and migration of the methyl group from C-11 to C-12.

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References

1 D. Thibaut, L. Debussche and F. Blanche, *Proc. Natl. Acad. Sci. USA*, 1990, **87** 8795.
2 D. Thibaut, F. Blanche, L. Debussche, F. J. Leeper and A. R. Battersby, *Proc. Natl. Acad. Sci. USA*, 1990, **87**, 8800.
3 G. W. Weaver, F. J. Leeper, A. R. Battersby, F. Blanche, D. Thibaut and L. Debussche, *J. Chem. Soc., Chem. Commun.*, 1991, 976.
4 F. Blanche, D. Thibaut, A. Famechon, L. Debussche, B. Cameron and J. Crouzet, *J. Bacteriol.*, submitted for publication.
5 F. Blanche, M. Kodera, M. Couder, F. J. Leeper, D. Thibaut and A. R. Battersby, *J. Chem. Soc., Chem. Commun.*, 1992, preceding communication.
6 H. C. Uzar, A. R. Battersby, T. A. Carpenter and F. J. Leeper, *J. Chem. Soc., Perkin Trans. 1*, 1987, 1689.
7 A. R. Battersby and F. J. Leeper, *Chem. Rev.*, 1990, **90**, 1261.
8 A. R. Battersby, E. McDonald, R. Neier and M. Thompson, *J. Chem. Soc., Chem. Commun.*, 1979, 960.