Biosynthesis of Vitamin B_{12}: the Structure of Factor IV, the Oxidized Form of Precorrin-4

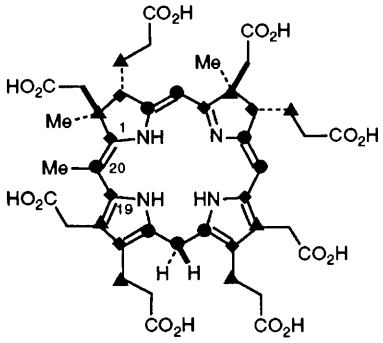
Denis Thibaut, Laurent Debussche, Denise Fréchet, Frédéric Herman, Marc Vuilhorgne and Francis Blanche*

Département Analyse, Centre de Recherche de Vitry-Alfortville, Rhône-Poulenc Rorer S.A., 13, Quai Jules Guesde, BP14, F-94403 Vitry-sur-Seine Cedex, France

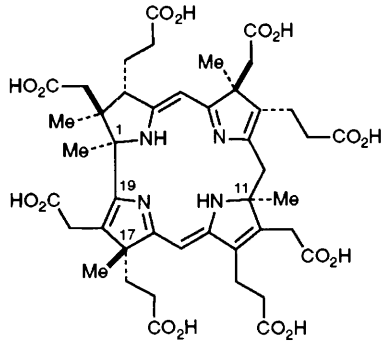
^{13}C Labelling and NMR experiments establish the structure of factor IV, the oxidized form of the tetramethylated intermediate on the biosynthetic pathway to precorrin-6x **2** in *Pseudomonas denitrificans*, thus demonstrating that the contraction of the macrocycle leads to an acetyl derivative at C-1.

Precorrin-6x **2**, a hexamethylated biosynthetic precursor of vitamin B_{12} recently identified in *Pseudomonas denitrificans*,[1–4] is formed from the trimethylated intermediate precorrin-3[1,5,6] **1a**. **1a** is routinely isolated in its aromatized form, *i.e.* the trimethylisobacteriochlorin **3**, which can be reduced back to **1a** in the cell-free enzyme system from *P. denitrificans*.[1] Available evidence[1,7] indicates that the conversion of **1a** into **2** involves the stepwise delivery of three methyl groups from *S*-adenosylmethionine (SAM) onto carbon centres at C-17, C-11 and C-1 (in this order), contraction of the macrocycle, elimination of C-20 with its attached methyl group, and adjustment of the oxidation level of the macrocycle (introduction of an additional double-bond). However, so far, no intermediate in this process has been discovered.

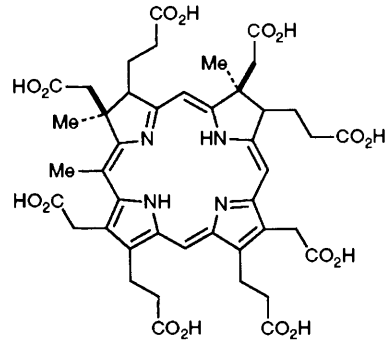
We have found recently[8] that cell-free protein preparations from *P. denitrificans cobM* mutants efficiently convert **1a** into a previously undescribed intermediate, precorrin-4, which was isolated in its oxidized form, named factor IV. Factor IV could be resolved by HPLC into two closely related compounds, named A and B (A, *ca.* 3 parts; B, *ca.* 2 parts). Both compounds are octaacids, showing an M + H^+ at 907[8] (fast atom bombardment mass spectrometry; Gly/SGly) corresponding to a molecular ion $C_{44}H_{50}N_4O_{17}$ (M 906), and carry four *C*-methyl groups[8] as determined from double-labelling experiments (with ^{14}C and ^{3}H). Moreover, the additional methyl group introduced from **1a** appears at C-17 of the precorrin-6x **2** eventually formed enzymically from factor IV (A or B)[8]. Factor IV (compound A) is a blue pigment and shows λ_{max}/nm [H_2O–0.1% trifluoroacetic acid (TFA)] 369 (ε_{rel} 1.00), 467 (0.11), 498 (0.11), 590 (0.34) and 629 (0.42).



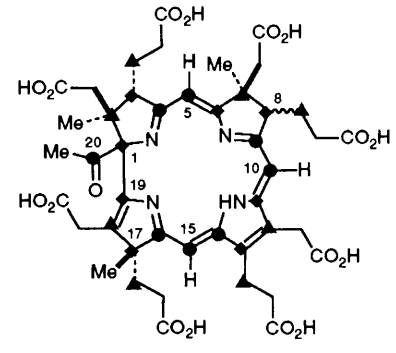
1a; ▲ = ◆ = ● = ^{12}C
1b; ▲ = ^{13}C, ◆ = ● = ^{12}C
1c; ◆ = ^{13}C, ▲ = ● = ^{12}C
1d; ● = ^{13}C, ◆ = ▲ = ^{12}C



2



3



4a; ▲ = ◆ = ● = ^{12}C
4b; ▲ = ^{13}C, ◆ = ● = ^{12}C
4c; ◆ = ^{13}C, ▲ = ● = ^{12}C
4d; ● = ^{13}C, ◆ = ▲ = ^{12}C

Compound B exhibits the same UV–VIS spectrum, except for a slight 3 nm longwave-shift of the secondary maximum to 632 nm. Purified A and B are not converted into each other when they are reextracted and repurified by HPLC. From all the data reported above, it is likely that A and B are two epimeric species generated during the isolation process, presumably because of the oxidation of precorrin-4 to factor IV. We now describe ^{13}C labelling and NMR experiments which establish structure **4a** for the major component of factor IV (isomer A).

^{13}C-Labelled factor IV **4c** was produced biosynthetically from ^{13}C-labelled precorrin-3 **1c**, which in turn had been generated from 5-amino[4-^{13}C]laevulinic acid,[1] [4-^{13}C]ALA. The ^{1}H NMR spectrum of **4c** (400 MHz, 0.1% TFA in D_2O, 4 °C) showed the presence of three ethylenic protons (5-H, 10-H and 15-H) as three singlets (δ 5.9, 6.25 and 6.75), while three methyl groups (bound to three quaternary carbons at C-2, C-7 and C-17) appeared at δ 0.9, 1.18 and 1.35. A fourth signal due to the acetyl group (see below) was found at δ 2.15. The ^{13}C NMR spectrum of **4c** (processed by maximum entropy[9]) exhibited four sp^2 carbons (δ 193.5, 158.2, 146.5 and 146.1) and four sp^3 carbons at δ 82.8, 66.5, 53.4 and 50.1. A 55 Hz coupling constant was observed between carbons at δ 82.8 and 146.5, providing firm evidence for ring contraction and direct linkage of C-1 to C-19.

The ^{1}H NMR spectrum of **4d** produced from [5-^{13}C]ALA showed that the three ethylenic protons (5-H, 10-H and 15-H) are directly coupled to ^{13}C centres (each one appeared as a doublet with a $J^{1}H$–^{13}C value between 160 and 164 Hz). The ^{13}C NMR spectrum of **4d** provided evidence for a carbonyl resonance (δ 210.8), two pairs of coupled sp^2 carbons [δ 183.5 (or 179.0) and 94.1, *J* 66 Hz; δ 191.8 and 95.9, *J* 68 Hz] and three carbons linked to each other [δ 179.0 (or 183.5), *J* 66 Hz; 102.8, *J* 66 and 76 Hz; 148.5, *J* 76 Hz]. Because of the low signal-to-noise ratio, these coupling constants were measured after a maximum entropy processing of the free induction decay. As expected the broad band decoupled ^{13}C spectrum of **4b** produced from [3-^{13}C]ALA showed the presence of eight singlet carbon resonances. Two of them (δ 139.5 and 135.1) which arise from sp^2 centres, are unambiguously assigned to C-12 and C-18. Therefore, the carbon linked to C-18 by a double-bond is necessarily C-19 since C-17 is sp^3, as evidenced from the NMR study of **4c** (see above). This establishes that the acetyl group generated by the contraction of the macrocycle is linked to C-1.

The above spectroscopic evidence, summarized in Table 1 with tentative assignments, is self-consistent and leads to structure **4a** (or possibly one of its double-bond tautomers) for factor IV. Factor IV isomer B, biosynthesised with the same three labelling patterns as isomer A, was also subjected to NMR study. ^{1}H and ^{13}C NMR spectra thus obtained were very similar to those of isomer A. Only a downfield shift of one of the methyl groups (from 1.18 to 0.95) in the ^{1}H spectrum and few changes in the ^{13}C spectra were observed (Table 1). The stereochemistry at each asymmetric centre, apart from C-1, has been set as illustrated because **4a** is enzymically formed from **1a** and is converted in 25% yield into **2** (in our standard cell-free system[1] with NADH as the reducing cofactor), both of firmly established structures. However, bearing in mind that (*i*) 3-H is retained whereas 8-H is lost during the biosynthetic conversion of **1a** to the corrin ring system,[10] (*ii*) A and B are not converted into each other upon repurification and (*iii*) factor IV is a dehydrogenated form of precorrin-4, it is likely that compounds A and B are epimeric at C-8, this asymmetric centre arising from double-bond migration during the oxidative process of purification. Due to the small amount of factor IV obtained in each labelling experiment (0.3–0.4 mg), to its relatively low stability, even at 4 °C, and to the broadness of the signals thus obtained, no further NMR experiments were attempted.

In conclusion, the structure of factor IV reveals two important features of the biosynthetic pathway to precorrin-

Table 1 ^{13}C NMR data[a] for factor IV (isomer A)[b] as labelled forms **4b**, **4c** and **4d**, and tentative assignments

4b			**4c**			**4d**		
δ_c	Coupling	Assignment	δ	Coupling *J*/Hz	Assignment	δ_c	Coupling *J*/Hz	Assignment
139.5 (140.5)	s	C-12 and	193.5	s	C-6	210.8 (209.0)	s	C-20
135.1	s	C-18	158.2	s	C-11	191.8	d, 68	C-9
57.9 (55.7)	s	C-7	146.5	d, 55	C-19	183.5	d, 66	C-4
52.1	s	C-2	146.1	s	C-13	179.0 (178.1)	d, 66	C-16
31.1	s		82.8	d, 55	C-1	148.5	d, 76	C-14
24.5	s	4 CH_2	66.5	s	C-17	102.8	dd, 66, 76	C-15
22.5(25.9)	s	propionates	53.4	s	C-3	95.9	d, 68	C-10
21.5	s		50.1 (56.2)	s	C-8	94.1 (95.5)	d, 66	C-5

[a] δ_{CD_3OD} 50 external reference, pH value not adjusted. [b] The significant changes in the spectra obtained with isomer B are given in brackets.

6x, *i.e.* the contraction of the macrocycle is an early process and, quite unexpectedly, this process leads to an acetyl derivative at C-1, and not at C-19 as previously assumed.

B. Monegier Du Sorbier and M. Danzer are warmly acknowledged for obtaining mass spectra.

Received, 2nd December 1992; Com. 2/06447B

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 L. Debussche, M. Couder, D. Thibaut, B. Cameron, J. Crouzet and F. Blanche, *J. Bacteriol.*, 1992, **174**, 7445.
4 F. Blanche, M. Kodera, M. Couder, F. J. Leeper, D. Thibaut and A. R. Battersby, *J. Chem. Soc., Chem. Commun.*, 1992, 138.
5 For reviews of earlier literature, see F. J. Leeper, *Nat. Prod. Rep.*, 1989, **6**, 171 and references cited therein.
6 M. J. Warren, C. A. Roessner, S.-I. Ozaki, N. J. Stolowich, P. J. Santander and A. I. Scott, *Biochemistry*, 1992, **31**, 603.
7 F. Blanche, D. Thibaut, D. Frechet, M. Vuilhorgne, J. Crouzet, B. Cameron, K. Hlineny, U. Traub-Eberhard, M. Zboron and G. Müller, *Angew. Chem., Int. Ed. Engl.*, 1990, **29**, 884.
8 L. Debussche, D. Thibaut, B. Cameron, J. Crouzet and F. Blanche, manuscript in preparation.
9 M. A. Delsuc, in *Maximum Entropy and Bayersian Methods*, 1989, ed. J. Skilling, Kluwer Academic Publishers, Dordrecht, The Netherlands.
10 M. Kodera, F. J. Leeper and A. R. Battersby, *J. Chem. Soc., Chem. Commun.*, 1992, 835.