Biosynthesis of Vitamin B$_{12}$: Structure of Precorrin-3B, the Trimethylated Substrate of the Enzyme catalysing Ring Contraction

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FAB-MS, FTIR spectroscopy, ^{13}C labelling and NMR experiments establish the structure of precorrin-3B, a further trimethylated intermediate along the vitamin B$_{12}$ pathway, thus demonstrating that precorrin-3B is synthesised from precorrin-3 (called precorrin-3A from now) by a complex oxidative reaction involving C-20 hydroxylation and γ-lactone formation from ring-A acetate to C-1, catalysed by the CobG protein in *Pseudomonas denitrificans*.

It has been demonstrated recently[1] that the sequence of reactions from precorrin-3A[2]† 1a to precorrin-6x[3] 2 is catalysed in *Pseudomonas denitrificans* by four enzymes operating in the following order: CobG (an iron–sulfur protein), CobJ, CobM and CobF, the last three introducing a methyl group from S-adenosylmethionine (SAM) at C-17, C-11 and C-1, respectively. Precorrin-4, the product of the CobJ-catalysed reaction, was found to be accumulated in a cell-free system from a strain lacking CobM, and the structure of its oxidised form factor-IV[4] 3, was established. This study revealed that precorrin-4 (5 or one of its double-bond tautomers) has undergone ring contraction and still carries an acetyl group at C-1, which is lost during precorrin-6x formation. It has also been found[1] that cell-free protein preparations from *P. denitrificans* strains overexpressing CobG convert in high yield (*ca.* 40%) 1a into a previously undescribed intermediate named precorrin-3B,† which was isolated by chromatography under argon atmosphere. Precorrin-3B could be subsequently resolved by reverse-phase high performance liquid chromatography into two closely related isomers (see below) named α and β (*ca.* 1/1).

Both isomers tested at a concentration of 1 µmol dm^{-3} are converted at the same rate into precorrin-4 (identified as its oxidised form 3; yield *ca.* 80%) when incubated with pure CobJ and SAM under an argon atmosphere.[1] They exhibit the same [M + H]$^+$ ion at *m/z* 895 [fast atom bombardment mass spectrometry (FAB-MS); Gly/SGly] corresponding to the molecular formula C$_{43}$H$_{50}$N$_4$O$_{17}$ (*M* 894). Therefore, precor-
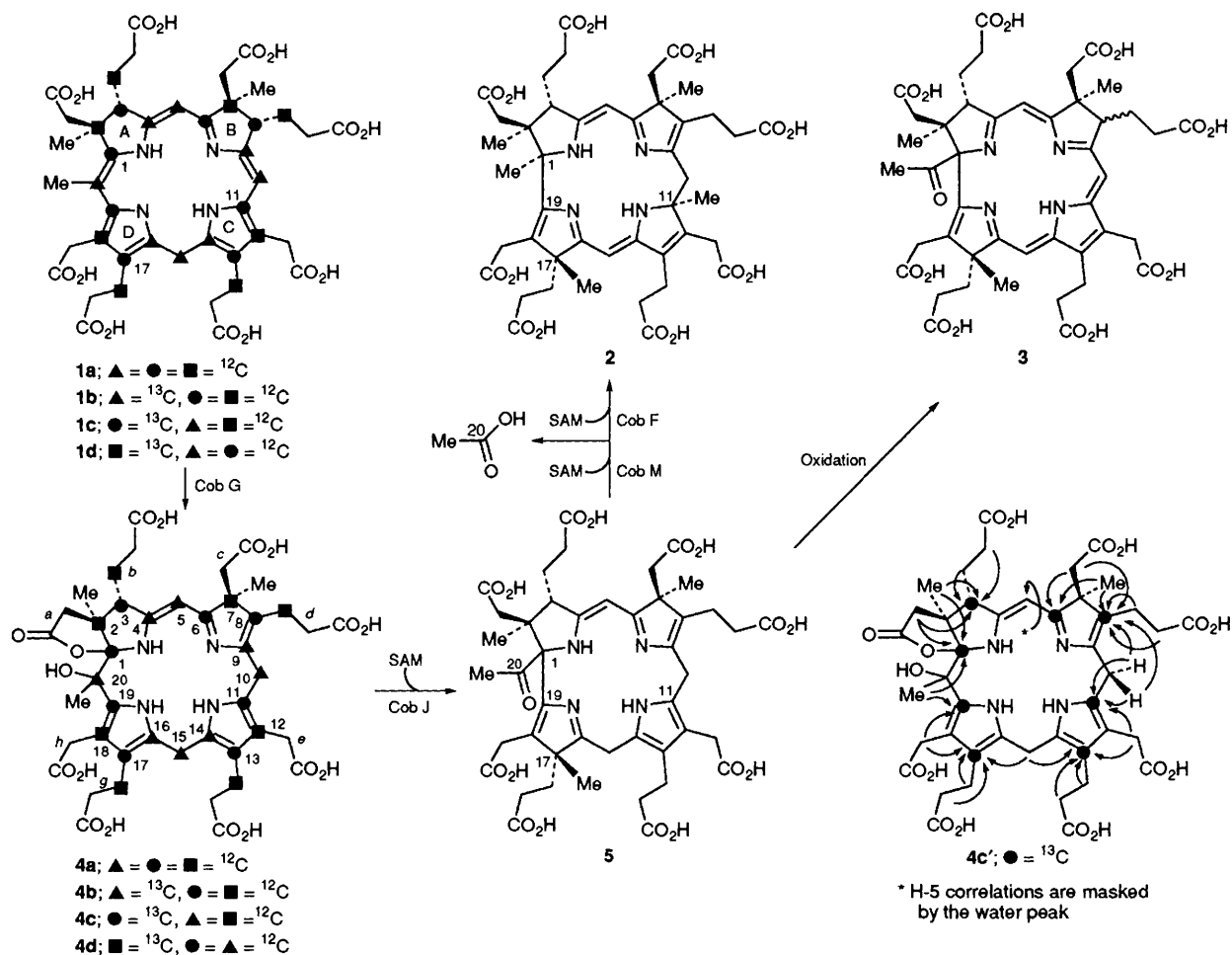
rin-3B has one more oxygen atom than 1a. It is consequently at the same level of oxidation as 2 plus acetic acid, the fragment extruded during the biosynthesis of the corrin chromophore.[5,6] This shows that CobG carries out the oxidation which is known to occur[3,7] during the conversion of 1a into 2. Isomer α is a pale-yellow compound and shows λ_{max}/nm [H$_2$O–0.1% trifluoroacetic acid (TFA)] 376 (ε 17 000 $\pm$ 2000 dm^3 mol^{-1} cm^{-1}). Isomer β exhibits the same UV–VIS spectrum but is 3 nm longwave-shifted, suggesting that α and β are epimers. We now describe Fourier-transformed infrared (FTIR) spectra, ^{13}C labelling and NMR experiments that establish structure 4a for precorrin-3B isomers α and β in acidic medium.

FTIR spectra of α and β reveal a characteristic γ-lactone band (FTIR; α: 1799 cm^{-1} and β: 1792 cm^{-1}). To carry out FTIR spectroscopy, α and β were adsorbed on LiChroprep RP18 (Merck, Darmstadt, Germany), washed with degassed hydrochloric acid (10 mmol dm^{-3}), eluted in methanol and dried on the KRS-5 crystal under argon atmosphere. After acquisition of FTIR spectra, the recovered material (>90%) exhibited the same characteristics as freshly prepared precorrin-3B (UV–VIS, ^{1}H NMR and FAB-MS spectra, chromatographic behaviour and conversion into precorrin-4).

For NMR studies, three ^{13}C-labelled precorrin-3B (4b, 4c and 4d) were biosynthesised from the corresponding precorrin-3A (1b, 1c and 1d) derived from ^{13}C-labelled 5-aminolaevulinic acid (ALA) in position 5, 4 and 3, respectively. 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.[8]

The ^{1}H NMR spectrum of 4b (isomer α) shows three methyl groups at δ 1.8; 1.5 and 1.05. The methyl centred at δ 1.8 is assigned as the one attached to C-20 because it is coupled to a ^{13}C nucleus (doublet, ^2J 4 Hz). The broad band (BB) [13]C

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† We report presently that precorrin-3 is converted into a further trimethylated biogenetic precursor of vitamin B$_{12}$. Consequently, precorrin-3 must be renamed precorrin-3A and the new precursor is called precorrin-3B according to the current nomenclature, (see ref. 2).

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

| Cob G

Me—$\overset{20}{\underset{\parallel O}{C}}$—OH ⟵SAM⟶| Cob F
                    ⟵SAM⟶| Cob M

Oxidation

2

3

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

⟶ SAM, Cob J ⟶

5

4c'; ● = ^{13}C

* H-5 correlations are masked
by the water peak

Table 1 ^{13}C NMR data^a for precorrin-3B isomer α (isomer β)^b as labelled forms 4b, 4c and 4d

4b					4c					4d				
	Coupling					Coupling					Coupling			
δ_c	BB	J/Hz	OR	Parity	Assignment	δ_c	BB	OR	Parity	Assignment	δ_c	BB	OR	Parity	Assignment
169.1	d	74	d	C	C-4	176.8 (177.3)	s	s	C	C-6	110.9	s	s	C	C-12/C-18
135.6	d	49	d	C	C-9	132.4 (131.9)	s	s	C	C-19	108.9	s	s	C	C-12/C-18
126.2	d	51	d	C	C-14/C-16	126.4 (126.9)	s	s	C	C-8	56.3	s	s	C	C-2/C-7
123.6	d	51	d	C	C-14/C-16	121.4 (121.4)	s	s	C	C-11	51.5	s	s	C	C-2/C-7
78.5	d	74	dd	CH	C-5	120.8 (120.5)	s	s	C	C-17	21.7	s	t	CH$_2$	Propionate
74.8	s	—	s	C	C-20	118.3 (118.3)	s	s	C	C-13	18.7	s	t	CH$_2$	Propionate
20.8	d	49	m	CH$_2$	C-10	106.2 (108.3)	s	s	C	C-1	18.6	s	t	CH$_2$	Propionate
20.6	dd	51^c	m	CH$_2$	C-15	53.0 (54.6)	s	d	CH	C-3	17.6	s	t	CH$_2$	Propionate

^{a} 100.6 MHz; 4°C; D$_2$O, 0.1% TFA, ≈ 10% CD$_3$CN; pH value not adjusted; δ_{CD_3CN} (CD$_3$) 0, internal reference. ^{b} Chemical shifts of 4c isomer β are given in brackets. ^{c} Both coupling constants are identical.

NMR spectrum of 4b (isomer α) exhibits eight signals [five quaternary carbons, two methylenes and one methine, these parities being supported by an off-resonance (OR) ^{13}C spectrum]. Seven of these carbons are coupled to one or two other nuclei and considering the expected biosynthetic pathway most of these signals can be unambiguously assigned (see Table 1). The eighth ^{13}C signal, a quaternary carbon at δ 74.8 is not coupled and arises from C-20. The chemical shift of this carbon surprisingly proves that it is not a carbonyl group as in 3,[4] but probably a tertiary alcohol.

The ^{1}H NMR spectrum of 4c (isomer α) has been assigned using a 2D COSY experiment. The three methyl groups

appear as three singlets indicating no ^{1}H–^{13}C coupling. ^{13}C NMR spectra of 4c (isomer α; BB and OR) demonstrates that seven out of the eight labelled carbons derived from [4-^{13}C]ALA are not coupled. The last one is coupled to one hydrogen. Therefore, there is no direct link between C-1 and C-19. The methine (δ 53.0) is assigned to C-3. Six out of the seven quaternary carbons display chemical shifts characteristic of sp^2 centres (δ 176.8; 132.4; 126.4; 121.4; 120.8 and 118.3) and can be tentatively assigned to C-6, C-19, C-8, C-17 or C-11, and C-13. The remaining signal (δ 106.2) would thus correspond to C-1. Therefore, C-1 cannot be an sp^2 atom forming a double bond with the flanked nitrogen, but it should

Table 2 ^{1}H NMR data^a for precorrin-3B isomers α and β as labelled form **4c**

4c isomer α				4c isomer β			
δ_H		Coupling	Assignment	δ_H		Coupling	Assignment
4.95^b	(1H)	s	H-5	≈ 5^c			H-5
3.85	(1H)	bd	H-10	3.85	(1H)	bd	H-10
3.8	(2H)	bq	AB	2 × H-15	3.8	(2H)	bq	AB	2 × H-15
3.7	(2H)	bq	AB	2 × H$_h$	3.7	(2H)	bq	AB	2 × H$_h$
3.5	(2H)	vbs	AA′	2 × H$_e$	3.5	(2H)	vbs	AA′	2 × H$_e$
3.3	(1H)	bd	H-10	3.3	(1H)	bd	H-10
≈ 3.2^d	(1H)	vb signal	H-3	≈3.2^d	(1H)	vb signal	H-3
3.0	(2H)	bdd	AB	2 × H$_c$	3.0	(2H)	bdd	AB	2 × H$_c$
2.8	(1H)	bd	H$_a$				
2.6	(1H)	bd	H$_a$	2.8 to 2.35	(≈ 16H)	m	8 × CH$_2$ lateral chains
2.7 to 2.35	(≈ 14H)	m	7 × CH$_2$ lateral chains				
2.0	(1H)	vb signal	H$_b$	2.1	(2H)	vbs	2 × H$_b$
1.8	(1H)	vb signal	H$_b$				
1.8	(3H)	s	20-Me	1.75	(3H)	s	20-Me
1.5	(3H)	s	2-Me	1.4	(3H)	s	2-Me
1.05	(3H)	s	7-Me	1.05	(3H)	s	7-Me

^{a} 400.13 MHz; 4 °C; D$_2$O, 0.1% TFA, ≈10% CD$_3$CN; pH value not adjusted; δ_{MeCN} 1.90, internal reference. ^{b} This signal was observed at 45 °C because it is masked by the water peak at 4 °C. ^{c} Under the water peak. ^{d} This ^{1}H–^{12}C residual signal is very weak. It appears as a singlet at δ 3.15 in the ^{1}H NMR spectra of **4b** and **4d** (isomer α).

be involved in the γ-lactone ring. To confirm these assignments, an HMBC experiments has been performed with a delay of 50 ms set to the optimum for couplings of 10 Hz. The arrows on structure **4c′** show the long-range ^{1}H–^{13}C couplings detected. Most of these correlations confirmed our preliminary ^{13}C interpretation. Both methyls at δ 1.8 and 1.5 show long range cross peaks to the ^{13}C centred at δ 106.2, thus establishing that this signal originates from C-1. The ^{1}H assignments deduced from the 2D COSY and HMBC experiments are given in Table 2.

4d (isomer α) exhibits the expected ^{1}H and ^{13}C (BB and OR) spectra. The two methyl groups at δ 1.5 and 1.05 are coupled to ^{13}C nuclei (^2J 4 Hz). The four quaternary carbons (δ 110.9; 108.9; 56.3 and 51.5) are the carbons of the macrocycle skeleton and the remaining four methylenes (δ 21.7; 18.7; 18.6 and 17.6) are those located on propionate lateral chains. Assignments are given in Table 1.

With all these assignments in hand, and taking into consideration the spectroscopic evidence for γ-lactone in the molecule, we conclude that precorrin-3B exhibits seven double bonds located as shown in structure **4a** (a second structure with the other tautomeric arrangement of the two double bonds near C-5 is also compatible). Precorrin-3B thus contains only one lactone. This lactone has to link the ring-A acetate chain to C-1.‡ Finally the additional oxygen atom of the molecule must be attached to C-20 to form an alcohol function. The proposed structure **4a** is consistent with all above spectroscopic evidence. **4b** (Isomer β) was also studied by ^{1}H and ^{13}C NMR spectroscopies. The spectra obtained are very similar to those of isomer α (see Tables 1 and 2). These data prove that α and β are epimeric forms. The carbon and hydrogen atoms presenting the largest chemical shift differences for the two epimers all belong to ring-A and its attached groups. Since the reaction catalysed by the CobG enzyme is surely stereoselective, the site of epimerisation is most probably C-3 but not C-1 or C-20. Partial epimerisation at C-3 has also been observed for other B$_{12}$ intermediates.[8,9] The configuration of C-1 and C-20 has not been established and the

configuration of the asymmetric C-2, C-3 and C-7 centres is set as illustrated because **4a** is on the pathway from **1a** to **2**, both of firmly established stereochemistry.[10,11] This study does not allow us to determine which one, between α and β, has the configuration of **4a** at C-3.

The structure of precorrin-3B is reminiscent of the structure of the so-called stable yellow compound obtained by transforming cobester under the conditions of the Udenfriend reaction.[12] This compound, which has been shown to be a 5-hydroxy derivative of the corrin template with a γ-lactone between the ring-B acetate group and C-6, exhibits chemical shifts for carbons and hydrogens in the vicinity of the lactone which are quite similar to the shifts found for the corresponding centres in precorrin-3B. It has been shown that the lactone prevents the formation of a 5-oxo-derivative with methyl migration from C-5 to C-6 by a pinacol-pinacolone type rearrangement.[13] The lactone of precorrin-3B may play the same stabilisation role.

Noting that the site of ring contraction is the *meso* position between rings-A and -D with two flanking acetic side chains which may serve as a clamp, Eschenmoser has suggested[14] hypothetical dilactonic intermediates along the pathway to rationalise the biosynthetic contraction of the porphyrinoid to the corrinoid macrocycle. Carbonyl oxygen label exchange has been shown[15,16] to occur at the acetamido group of ring-A during vitamin B$_{12}$ biosynthesis in *Propionibacterium shermanii*. This regiospecific oxygen exchange has been interpreted as resulting from the hydrolysis of the hypothetical δ-lactone (between ring-A acetate and C-20) during the ring contraction reaction, thus providing the first experimental support for Eschenmoser's hypothesis. The present study establishes that a γ-lactone between ring-A acetate and C-1 is indeed put in place, setting the stage for the subsequent ring contraction. Unexpectedly, the ring contraction is actually effected by the CobJ protein, the methyltransferase catalysing, probably by a concerted mechanism, both a methyl transfer at C-17 and the ring contraction process.

Studies on the fate of the carbonyl oxygen atom of the ring-A acetate group during the conversion of precorrin-3A to precorrin-4 and on the origin of the oxygen atom borne by C-20 in these two intermediates will be of utmost interest now to elucidate the mechanism of ring contraction in *P. denitrificans*, in which the insertion of cobalt occurs at a later stage than in *P. shermanii*.[17–19]

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‡ It is possible that precorrin-3B is generated first as a δ-lactone at C-20. This isomer will probably interconvert readily with **4a**. Appropriate experiments are in progress.

References

1 L. Debussche, D. Thibaut, B. Cameron, J. Crouzet and F. Blanche, manuscript in preparation.
2 H. C. Uzar, A. R. Battersby, T. A. Carpenter and F. J. Leeper, *J. Chem. Soc., Perkin. Trans. 1*, 1987, 1689.
3 D. Thibaut, L. Debussche and F. Blanche, *Proc. Natl. Acad. Sci. USA*, 1990, **87**, 8795.
4 D. Thibaut, L. Debussche, D. Fréchet, F. Herman, M. Vuilhorgne, and F. Blanche, *J. Chem. Soc., Chem. Commun.*, 1993, 513.
5 L. Mombelli, C. Nussbaumer, H. Weber, G. Müller and D. Arigoni, *Proc. Natl. Acad. Sci. USA*, 1981, **78**, 11.
6 A. R. Battersby, M. J. Bushell, C. Jones, N. G. Lewis and P. Pfenniger, *Proc. Natl. Acad. Sci. USA*, 1981, **78**, 13.
7 F. Blanche, D. Thibaut, A. Famechon, L. Debussche, B. Cameron and J. Crouzet, *J. Bacteriol.*, 1992, **174**, 1036.
8 A. R., Battersby, E. McDonald, R. Neier and M. Thompson, *J. Chem. Soc., Chem. Commun.*, 1979, 960.
9 D. Thibaut, F. Kiuchi, L. Debussche, F. J. Leeper, F. Blanche and A. R. Battersby, *J. Chem. Soc., Chem. Commun.*, 1992, 139.
10 A. R. Battersby and F. J. Leeper, *Chem. Rev.*, 1990, **90**, 1261.
11 D. Thibaut, F. Blanche, L. Debussche, F. J. Leeper and A. R. Battersby, *Proc. Natl. Acad. Sci. USA*, 1990, **87**, 8800.
12 A. Gossauer, B. Grüning, L. Ernst, W. Becker and W. S. Sheldrick, *Angew. Chem. Int. Ed. Engl.*, 1977, **16**, 481.
13 B. Grüning and A. Gossauer, in *Vitamin B_{12}*, ed. B. Zagalak and W. Friedrich, Walter de Gruyter & Co, Berlin, New York, p. 141 1979.
14 A. Eschenmoser, *Angew. Chem., Int., Ed. Engl.*, 1988, **27**, 5.
15 K. Kurumaya, T. Okazaki and M. Kajiwara, *Chem. Pharm. Bull.*, 1989, **37**, 1151.
16 A. I. Scott, N. J. Stolowich, B. P. Atshaves, P. Karuso, M. J. Warren, H. J. Williams, M. Kajiwara, K. Kurumaya and T. Okazaki, *J. Am. Chem. Soc.*, 1991, **113**, 9891.
17 L. Debussche, M. Couder, D. Thibaut, B. Cameron, J. Crouzet and F. Blanche, *J. Bacteriol.*, 1992, **174**, 7445.
18 G. Müller, K. Hlineny, E. Savvidis, F. Zipfel, J. Schmiedl and E. Schneider, in *Chemical Aspects of Enzyme Biotechnology*, ed. T. O. Baldwin, Plenum Publishing Corp., New York, p. 281, 1990.
19 G. Müller, F. Zipfel, K. Hlineny, E. Savvidis, R. Hertle, U. Traub-Eberhard, A. I. Scott, H. J. Williams, N. J. Stolowich, P. J. Santander, M. J. Warren, F. Blanche and D. Thibaut, *J. Am. Chem. Soc.*, 1991, **113**, 9893.