Biosynthesis of Vitamin B_{12}: When is the 12β-Methyl Group of the Vitamin Generated by Acetate Decarboxylation?

Francis Blanche,*[a] Sheetal Handa,[b] Denis Thibaut,[a] Colin L. Gibson,[b] Finian J. Leeper,[b] and Alan R. Battersby*[b]

[a] *Department de Chimie Analytique, Centre de Recherche de Vitry, Rhône-Poulenc Santé, BP14, F-94403 Vitry-sur-Seine Cedex, France*

[b] *University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, U.K.*

The 12-methyl analogue (**13**) of the aromatised form of precorrin-3 (**5**) has been prepared by enzymic *C*-methylation of 12-decarboxy-uro'gen-III (**2**) and is used for incorporation experiments which indicate that decarboxylation of the 12-acetate residue in B_{12}-biosynthesis occurs *after* C-17 methylation.

The biosynthesis[1] of vitamin B_{12} goes *via* cobyrinic acid (**7**) which in turn is built from uro'gen-III (**1**) by a multi-step sequence involving *inter alia* eight *C*-methylations. The last isolated intermediate[2] beyond uro'gen-III (**1**) is the di-*C*-methylated precorrin-2†(**3**) but isolation[4] of the aromatised system (**11**) and appropriate incorporation studies[5] (see also below) make it essentially certain that the next B_{12}-precursor is the tri-*C*-methylated precorrin-3 (**5**). No tetra-, penta-*C*-methylated, or later biosynthetic intermediates have so far been isolated but the development of pulse-labelling conditions established[6] in 1982 that the fourth *C*-methyl group is introduced at C-17. This gave the important knowledge that the tetramethylated intermediate on the biosynthetic pathway is based on a fully conjugated macrocycle called a pyrrocorphin[7] [see *e.g.*, (**14**)]. More recently, the pulse-labelling approach has been further developed to give strong support to the view that the remaining four *C*-methyl groups are introduced into vitamin B_{12} in one specific sequence.[3,8,9]

Inspection of the structures of precorrin-3 (**5**) and cobyrinic acid (**7**) shows that decarboxylation of the C-12 acetate residue still present in (**5**) must occur at some stage on the pathway and there are good reasons why this probably occurs before the fifth[3,8,9] *C*-methylation at C-12. On this basis, the candidates for decarboxylation are precorrin-3 (**5**) and the pyrrocorphin (**14**). Decarboxylation of the former would yield (**6**) and the aromatised form of this material (**13**) has now been prepared for biosynthetic studies.

It was known[10] that the crude methyltransferase preparation from *Propionibacterium shermanii* was sufficiently non-specific to transform synthetic[11] 12-decarboxylated uro'gen-III (**2**) into the 12-methyl analogue (**4**) of precorrin-2 (**3**). Similarly, the transmethylases from *Pseudomonas denitrificans* transformed this substrate (**2**) into a mixture of mono-, di-, and tri-methylated products. Aromatisation of these (by air), esterification, and separation by over-pressure t.l.c. afforded a chlorin (1%), the known[10] isobacteriochlorin (**9**; 0.5%) and the desired trimethylated system (**12**; 2.5%), the last being produced on a preparative scale in 3.6% yield.

The structure of (**12**) follows from (a) accurate mass (*m/z* 930.4269; $C_{49}H_{62}N_4O_{14}$ requires 930.4263); (b) u.v. absorption which closely matches that of (**10**); (c) ^{1}H-n.m.r. giving the key signals in Table 1; those from the 12-acetate system (**10**) are recorded for comparison. Importantly, the three low-field signals establish that the *meso*-methyl group of (**12**) is at C-20; (d) nuclear Overhauser effect (n.O.e.) difference, decoupling ^{1}H-n.m.r., and COSY experiments which establish the connectivities arrowed on (**16**) and give unequivocal evidence for the structure.

The preparation of a [2,7,20-*methyl*-^{14}C]-labelled sample of

† For nomenclature see ref. 3.

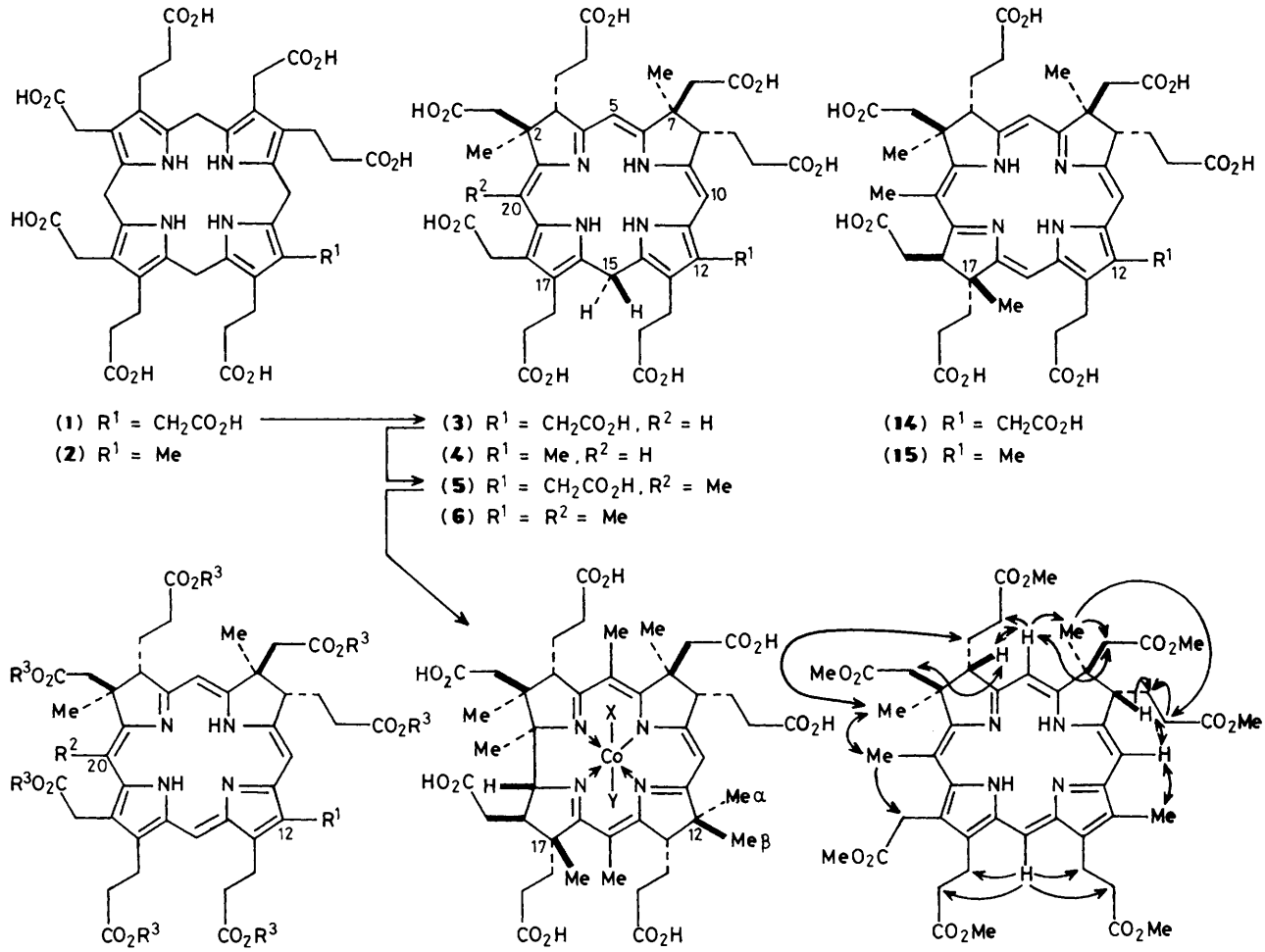

Table 1. Important ^{1}H-n.m.r. signals from (**10**) and (**12**) determined in C_6D_6 at 400 MHz.

Assignment	12-Acetate system (**10**)	12-Methyl system (**12**)
H-15	8.74, s	8.82, s
H-10	7.51, d	7.42, d
H-5	6.52, s	6.65, s
C-18 CH_2	4.22, ABq	4.28, ABq
C-12 CH_2	4.07, ABq	—
H-8	Overlapping	4.20, t
H-3	4.1—4.05	4.13, ABq
C-20 CH_3	2.78, s	2.85, s
C-12 CH_3	—	2.55, s
C-2 CH_3	1.67, s	1.69, s
C-7 CH_3	1.41, s	1.50, s

the 12-methyl system (**12**) at high specific activity using [*methyl*-^{14}C]SAM demanded purified enzymes *viz.* *S*-adenosylmethionine (SAM) : uro'gen-III methyltransferase[12] which normally generates precorrin-2 (**3**) and SAM : precorrin-2 methyltransferase[13] which normally converts precorrin-2 (**3**) into precorrin-3 (**5**). Special conditions were devised to overcome the observed strong inhibition of the enzymes by *S*-adenosylhomocysteine (the by-product of methyl transfer from SAM); these conditions will be described in our full paper. The [2,7,20-*methyl*-^{14}C]-labelled product (**12**) was purified as above to radiochemical purity. The crucial incorporation experiments were then carried out.

Table 2. Incorporations into cobyrinic acid[a] (**7**) from enzymic incubations of (**11**) and (**13**).

Expt. No.	Incorporation (%) into (**7**) From 12-acetate system (**11**)	From 12-methyl system (**13**)
1	10.1	0.02
2	9.2	0.025
3	4.8	0.01

[a] Isolated chromatographically and purified, after dilution, by esterification and crystallisation of the resultant cobester to constant activity.

Essentially all earlier studies[14] of incorporation of the di- and tri-methylated systems into cobyrinic acid (**7**) have

involved incubation of the labelled *aromatic* materials (**8**) and (**11**), usually 100—200 μg, with a cell-free enzyme preparation from a B_{12}-producing organism. The crude enzyme system contains a large excess of added NADH and thiols and the incubations are run anaerobically for 18 h at pH 7.45. The currently accepted view is that the isobacteriochlorins (**8**) and (**11**) are reduced‡ to the dihydro state during the incubation before incorporation into cobyrinic acid (**7**). Accordingly, we compared in a strictly parallel way incubation of the [*methyl*-^{3}H,^{14}C]-12-acetate system§ (**11**) with incubation of the above [2,7,20-*methyl*-^{14}C]-12-methyl system (**13**) in the cell-free system[11] from *P. shermanii*; both precursor acids (**11**) and (**13**) were obtained from the corresponding esters (**10**) and (**12**) using aqueous 2 M piperidine. The results in Table 2 show high incorporations into cobyrinic acid (**7**) from incubation of the 12-acetate system (**11**) but negligibly low incorporations from the 12-methyl system (**13**).

These results indicate that (a) precorrin-3 (**5**) does not undergo decarboxylation; (b) the 12-methyl system (**6**) is not an intermediate on the B_{12}-pathway; (c) decarboxylation occurs *after* methylation at C-17; (d) if this decarboxylation has to occur before the fifth *C*-methylation at position-12, as appears probable (see above), then two pyrrocorphins should exist with structures (**14**) and (**15**).

Grateful acknowledgement is made to Christ's College for the award of the Dow Senior Research Fellowship (to C. L. G.), and to the S.E.R.C. for financial support. We also thank J. Crouzet and B. Cameron for providing organisms and for helpful discussions, M. Couder and M. Chenu for excellent technical assistance, and Dr. J. Lunel for his valuable contribution in the development of scientific collaboration between the Cambridge and Rhône-Poulenc groups.

Received, 2nd March 1988; Com. 8/01121D

‡ How this reduction occurs is not known, but interpretation of the incorporation results presented in this paper would only be in doubt if the reduction of (**8**) and (**11**) during incubation is not by the reducing medium but catalysed by enzymes so highly specific that they will not accept the close relative (**13**). Since the dihydro intermediates (**3**) and (**5**) are produced directly in the biosynthesis and *not* by a reductive process, the existence of very specific enzymes to catalyse a reduction which is not normally needed seems unlikely.

§ This material was biosynthesised from [4-^{14}C]aminolaevulinic acid and [*methyl*-^{3}H]SAM, ratio ^{14}C:^{3}H = 2.8.

References

1 Reviewed by A. R. Battersby in *Acc. Chem. Res.*, 1986, **19**, 147.
2 A. R. Battersby, K. Frobel, F. Hammerschmidt, and C. Jones, *J. Chem. Soc., Chem. Commun.*, 1982, 455.
3 H. C. Uzar, A. R. Battersby, T. A. Carpenter, and F. J. Leeper; *J. Chem. Soc., Perkin Trans. 1*, 1987, 1689.
4 K.-H. Bergmann, R. Deeg, K. D. Gneuss, H.-P. Kriemler, and G. Müller, *Z. Physiol. Chem.*, 1977, **358**, 1315; A. R. Battersby and E. McDonald, *Bioorg. Chem.*, 1978, **7**, 161.
5 N. G. Lewis, R. Neier, G. W. J. Matcham, E. McDonald, and A. R. Battersby, *J. Chem. Soc., Chem. Commun.*, 1979, 541; G. Müller, K. D. Gneuss, H.-P. Kriemler, A. I. Scott, and A. J. Irwin, *J. Am. Chem. Soc.*, 1979, **101**, 3655.
6 H. C. Uzar and A. R. Battersby, *J. Chem. Soc., Chem. Commun.*, 1982, 1204.
7 For pyrrocorphin chemistry, see *e.g.*, J. E. Johnson, V. Piermattie, Ch. Angst, E. Diener, Ch. Kratky, and A. Eschenmoser, *Angew. Chem., Int. Ed. Engl.*, 1981, **20**, 261.
8 H. C. Uzar and A. R. Battersby, *J. Chem. Soc., Chem. Commun.*, 1985, 585.
9 A. I. Scott, N. E. Mackenzie, P. J. Santander, P. E. Fagerness, G. Müller, E. Schneider, R. Sedlmeier, and G. Wörner, *Bioorg. Chem.*, 1984, **12**, 356.
10 A. R. Battersby, C. Bladon, J.-J Huang, and S. Seo, *J. Chem. Soc., Chem. Commun.*, 1986, 1492.
11 A. R. Battersby, E. McDonald, R. Hollenstein, M. Ihara, F. Satoh, and D. C. Williams, *J. Chem. Soc., Perkin Trans. 1*, 1977, 166.
12 F. Blanche, L. Debussche, D. Thibaut, J. Crouzet, and B. Cameron, in preparation.
13 D. Thibaut and F. Blanche, unpublished work.
14 Reviewed by A. R. Battersby and E. McDonald, in 'B_{12},' ed. D. Dolphin, Wiley, New York, 1982, vol. 1, p. 107.