Biosynthesis of Vitamin B_{12}: Incorporation of (11*S*)-[11-$^2H_1$]-, and (11*R*)-[11-$^2H_1$]Porphobilinogen into Sirohydrochlorin and 2,7,20-Trimethylisobacteriochlorin

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

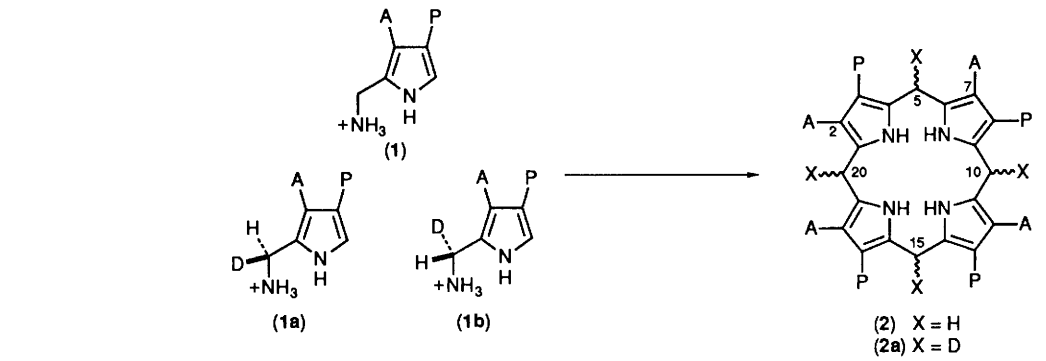
[a] *University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, UK*
[b] *Department de Chimie Analytique, Centre de Recherche de Vitry, Rhône Poulenc Santé, BP 14, F-94403 Vitry-sur-Seine Cedex, France*

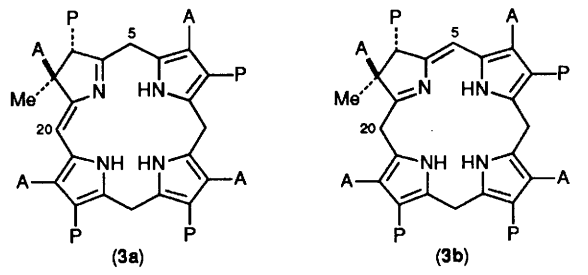
(11*S*)-[11-$^2H_1$]Porphobilinogen (**1a**) and the (11*R*)-isomer (**1b**) are incorporated biosynthetically into precorrin-2 and precorrin-3 which are isolated as the aromatised esters (**7**) and (**9**) for ^1H NMR studies; the unexpected results are discussed.

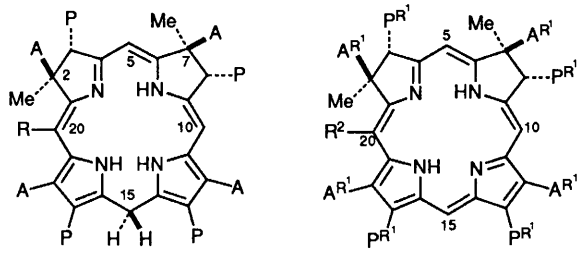
The biosynthesis of vitamin B_{12} involves the stepwise introduction of eight *C*-methyl groups into the macrocycle of uro'gen-III (**2**) which is built from four molecules of porphobilinogen (**1**), PBG, by a largely understood sequence.[1] The first *C*-methyl group is inserted at C-2, the second at C-7, and the third at C-20;[2] the two initial *C*-methylations generate, respectively, precorrin-1[3] (**3a** or **3b**) and precorrin-2[4] (**4**). It is highly probable, by analogy with precorrin-2 (**4**), that the third biosynthetic intermediate, precorrin-3, is formed and further transformed enzymically at the dihydro-aromatic oxidation level (**5**). These three intermediates are all readily oxidised by air, the di- and tri-methylated products being, respectively, sirohydrochlorin (**6**) and 2,7,20-trimethylisobacteriochlorin (**8**).

The aim of the experiments here outlined was to study the incorporation of PBG (**1**), stereoselectively labelled with deuterium[5] at C-11, into precorrin-2 (**4**) and precorrin-3 (**5**) and to isolate them after aromatisation as the octamethyl esters (**7**) and (**9**), respectively, for analysis by NMR spectroscopy. Previous analyses by degradation and NMR[5] had shown that the (11*S*)-[11-$^2H_1$]PBG contained 83% *S* (**1a**) and 17% *R* whilst the (11*R*)-sample contained 83% *R* (**1b**) and 17% *S*. These values were confirmed, within experimental error, by NMR with a chiral shift reagent† on the *R*- and *S*-PBG lactam esters from which the samples of PBG are derived

† Tris[3-(Heptafluoropropylhydroxymethylene)-(+)-camphorato]-europium(III).







A = CH_2CO_2H
P = $CH_2CH_2CO_2H$
A^{R^1} = $CH_2CO_2R^1$
P^{R^1} = $CH_2CH_2CO_2R^1$

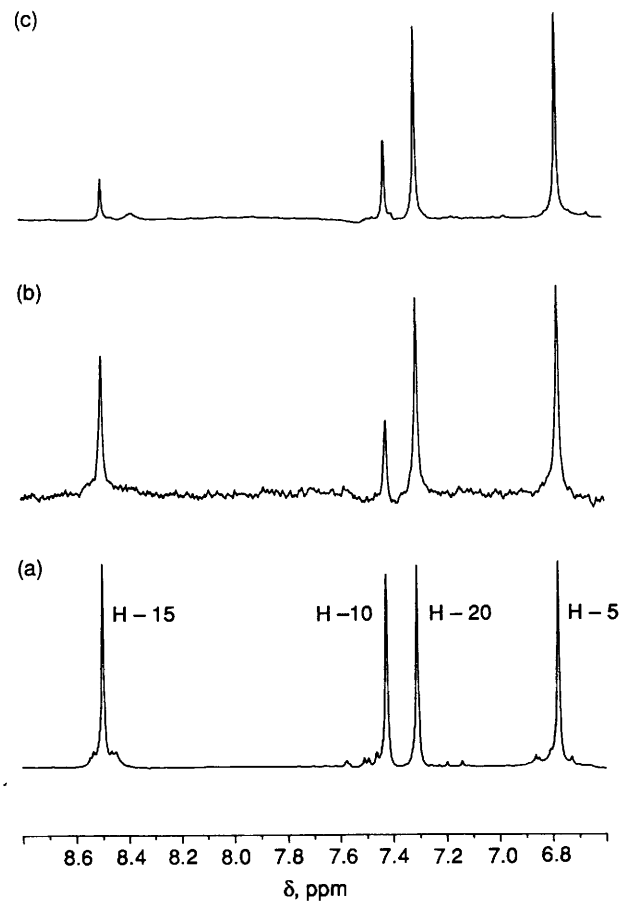


Figure 1. Low-field ^1H NMR signals at 400 MHz using CD_2Cl_2 from (a) unlabelled (**7**); (b) (**7**) derived from (11*R*)-[11-$^2H_1$]PBG (**1b**); (c) (**7**) derived from (11*S*)-[11-$^2H_1$]PBG (**1a**).

by hydrolysis. Earlier work had shown[6] that PBG (**1a, b**) stereoselectively labelled at C-11 is converted enzymically into stereoselectively labelled uro'gen-III (**2a**) but the configurations at the labelled sites are unknown. The simplest results from the present study should be obtained if the hydrogen loss from C-5, C-10, and C-20 to generate (**4**) and (**5**) is stereospecific. In that case, the (11*S*)-precursor (**1a**) should give mainly retention or mainly loss of deuterium at each site and the (11*R*)-PBG (**1b**) should lead to a set of complementary results. The removal of hydrogen from C-15 by air oxidation was expected to be non-stereospecific leading to a large retention of deuterium from both *S* and *R* precursors, (**1a**) and (**1b**), due to the expected significant kinetic isotope effect.

(11*S*)-[11-$^2H_1$]PBG (**1a**) and, separately, (11*R*)-[11-$^2H_1$]-PBG (**1b**) were incubated anaerobically with an enzyme preparation containing hydroxymethylbilane synthase, uroporphyrinogen III synthase, the appropriate methylase enzyme(s),[7] and all necessary cofactors to form in two separate experiments di-, and in a third experiment, tri-methylated products. These products were aromatised in air before the pigments (**6**) and (**8**) were trapped on a basic ion-exchanger, eluted with aqueous piperidine and esterified under basic conditions using trimethyloxonium tetrafluoroborate.[4] The purified esters (**7**) and (**9**) gave ^1H NMR signals from positions 5, 10, 15, and 20 which appear uncluttered over the region δ 6.4—8.6, Figure 1, and the amounts of protium they represent were determined. Table 1 gives the averaged values which were not as expected. However, some clear deductions can be made and reasonable rationalisations suggested.

Table 1. Low-field ^1H NMR signals from (**7**) and (**9**) derived from (11*S*)- and (11*R*)-[11-$^2H_1$]PBG (**1a** and **1b**).

	Signal intensities relative to standard[a]			
Position	(**7**) from *S*(**1a**)	(**7**) from *R*(**1b**)	(**9**) from *S*(**1a**)	(**9**) from *R*(**1b**)
H-5	1.05, 0.95	1.0, 0.95	1.05	0.8
H-10	0.35, 0.6	0.2, 0.4	1.0	0.75
H-15	0.15, 0.25	0.6, 0.4	0.3	0.2
H-20	0.85, 1.0	0.8, 0.85	—	—

[a] The integrated signal intensities from H-5, 10, 15, and 20 for the unlabelled sample of (**7**) were standardised against signals from H-3 and H-8 plus the acetate methylenes at C-2, 7, 12, and 18 together with the b-methylenes of the C-13 and 17 propionates for a total of 14H. The signals used as standards for unlabelled (**9**) were this same set plus those from the C-20 methyl and the b-methylene of the C-8 propionate for a total of 19H. The ^1H signals from the labelled samples were integrated and standardised in exactly the same way. The Table gives the ratios of standardised intensities from labelled relative to unlabelled samples rounded to closest 0.05.

(a) Almost complete loss of deuterium had occurred from C-5 and C-20 of the isolated sirohydrochlorin ester (**7**) from both (11*S*)-(**1a**), and (11*R*)-[11-$^2H_1$]PBG (**1b**) showing exchange with the medium at these positions. Control experiments with [5,10,20-$^2H_3$]sirohydrochlorin (see **6**), prepared by acid-catalysed exchange,[8] proved that the work-up and esterification procedures caused no significant exchange at positions 5, 10, or 20. This loss of deuterium from C-5 and C-20 could occur by equilibration on the enzyme of two possible forms,[3] (**3a**) and (**3b**), of precorrin-1 or at C-20 *via* imine–enamine tautomerism of the initial product from *C*-methylation at C-2 and subsequently at C-5 in the same way after *C*-methylation at C-7.

(b) The deuterium retention values at C-10 of (**7**) from both (11*S*)- and (11*R*)- precursors point to largely non-stereospecific loss of hydrogen from this position as the main process with only a small preference for loss of deuterium from (**1a**) compared with (**1b**).

(c) Surprisingly more protium was retained at C-15 of (**7**) in the *R* than in the *S* series. A possible explanation is based on the known rapid air-oxidation of precorrin-2 (**4**). If this occurs whilst a substantial part of the precorrin-2 is still bound to protein, one face could be preferentially protected.

(d) Loss of almost all the deuterium from C-5 of (**9**) in both *S*-, and *R*-series interlocks with the foregoing results. Now, though, there was also substantial exchange with the medium at C-10 and mainly deuterium retention at C-15, in each case from both labelled precursors (**1a**) and (**1b**). A possible rationalisation of the results at C-10 and C-15 is that they are both a reflection of the long life of precorrin-3 (**5**); experience in our two laboratories shows that (**5**) is much more stable to oxidation than (**4**). This stability (i) gives time for slower exchange with the medium at C-10 *via* enamine–imine tautomerisation before aromatisation and (ii) could lead to the aromatisation of precorrin-3 (**5**) occurring after leaving the protein when it is reasonable to expect the kinetic isotope effect to operate.

We thank the SERC for financial support, Dr. J. Lunel (Rhône Poulenc) for his interest and help and M. K. Rosen for preliminary experiments.

Received, 30th April 1990; Com. 0/01908I

References

1 A. R. Battersby, C. J. R. Fookes, G. W. J. Matcham, and E. McDonald, *Nature*, 1980, **285**, 17; F. J. Leeper, *Nat. Prod. Rep.*, 1989, **6**, 171.
2 Reviewed by A. R. Battersby and E. McDonald, in 'B_{12},' ed. D. Dolphin, Wiley, New York, 1982, p. 107.
3 A. R. Battersby, R. D. Brunt, F. J. Leeper, and I. Grgurina, *J. Chem. Soc., Chem. Commun.*, 1989, 428.
4 A. R. Battersby, K. Frobel, F. Hammerschmidt, and C. Jones, *J. Chem. Soc., Chem. Commun.*, 1982, 455.
5 A. R. Battersby, W. Neidhart, P. C. Anderson, and G. J. Hart, *J. Chem. Soc., Chem. Commun.*, 1985, 924.
6 C. Jones, P. M. Jordan, and M. Akhtar, *J. Chem. Soc., Perkin Trans. 1*, 1984, 2625; A. H. Jackson, W. Lertwanawatana, G. Procter, and S. G. Smith, *Experientia*, 1987, **43**, 892.
7 F. Blanche, L. Debussche, D. Thibaut, J. Crouzet, and B. Cameron, *J. Bacteriol.*, 1989, **171**, 4222; D. Thibaut, F. Blanche, L. Debussche, J. Crouzet, and B. Cameron, *ibid.*, 1990, (submitted).
8 R. Bonnett, I. A. D. Gale, and G. F. Stephenson, *J. Chem. Soc. C*, 1967, 1168; H.-H. Inhoffen, J. W. Buchler, and R. Thomas, *Tetrahedron Lett.*, 1969, 1141.