Biosynthesis of Porphyrins and Related Macrocycles. Part 9.[1,2] Biosynthesis of the Corrin Macrocycle of Vitamin B_{12} Including the Stereochemistry of *C*-Methylation in Ring c

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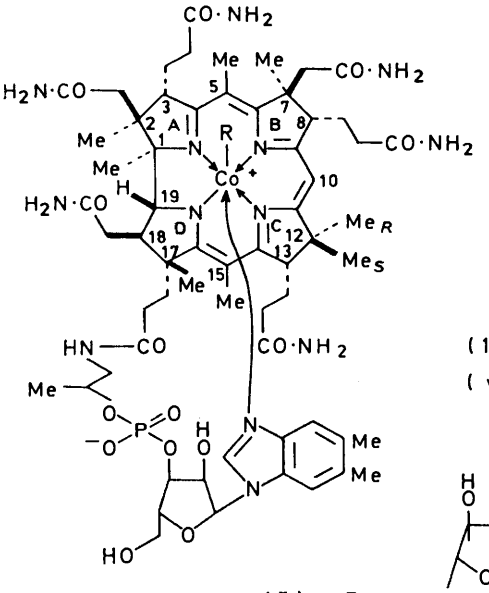
Earlier work on the biosynthesis of vitamin B_{12} is outlined; carbon-13 labelling coupled with n.m.r. spectroscopy is used for the current studies with *Propionibacterium shermanii*. Growth of this organism, administration of precursors, and isolation of vitamin B_{12} are described. Incorporation experiments with 5-amino[5-^{13}C]laevulinic acid and with [*methyl*-^{13}C]methionine confirm that the corrin macrocycle is constructed from eight molecules of 5-aminolaevulinic acid (one of which loses C-5) and seven methyl groups derived from methionine.

Degradation of the labelled vitamin B_{12} derived from [*methyl*-^{13}C]methionine proves that the *pro-R* methyl group at C-12 of ring c is the one derived from methionine. This result is confirmed by experiments with [*methyl*-$^{2}H_3$]-methionine which also establish that *C*-methylation at C-7 and C-12 of vitamin B_{12} occurs by intact transfer of the *S*-methyl group from methionine.

CRYSTALS OF vitamin B_{12} (cyanocobalamin) (1) were isolated in 1948 [3,4] as the culmination of a search for the anti-pernicious anaemia factor. Later the cyanide ligand of vitamin B_{12} (1) was found to be an artefact of the isolation process, the true coenzyme forms being [5] adenosylcobalamin (5a) and methylcobalamin (5b). The parent corrin nucleus (2) of the vitamin resembles the macrocycles of haem (3) and chlorophyll *a* (4) but with several differences, a striking one being the highly chiral nature of vitamin B_{12} itself. The unique structural features shown by the molecules (1) and (5a) were revealed by a classical series of studies which combined the chemical approach of Johnson and Todd [6] and Folkers [7] with the *X*-ray studies of Hodgkin.[8] This knowledge stimulated new research in inorganic chemistry [9] and gave the target for Eschenmoser and Woodward's brilliant synthesis [10,11] of vitamin B_{12}. With the challenges of structure and synthesis now met, there remain those of

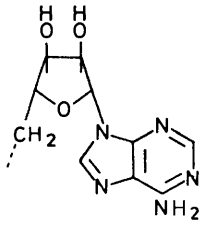
[1] Part 8, A. R. Battersby, E. Hunt, E. McDonald, and J. B. Paine, III, *J.C.S. Perkin I*, 1976, 1008.
[2] Preliminary accounts, A. R. Battersby, M. Ihara, E. McDonald, J. R. Stephenson, and B. T. Golding, *J.C.S. Chem. Comm.*, 1973, 404; 1974, 458.
[3] E. L. Rickes, N. G. Brink, F. R. Koniuszy, and K. Folkers, *Science*, 1948, **107**, 396.
[4] E. L. Smith and L. F. J. Parker, *Biochem. J.*, 1948, **43**, viii; K. H. Fantes, J. E. Page, L. F. J. Parker, and E. L. Smith, *Proc. Roy. Soc.*, 1949, *B*, **136**, 592.
[5] 'Cobalamin: Biochemistry and Physiology,' ed. B. M. Babior, Wiley–Interscience, New York, 1975.
[6] R. Bonnett, J. R. Cannon, V. M. Clark, A. W. Johnson, L. F. J. Parker, E. L. Smith, and A. R. Todd, *J. Chem. Soc.*, 1957, 1158, and references therein; A. W. Johnson and A. R. Todd, *Vitamins and Hormones*, 1957, **15**, 1.
[7] K. Folkers and D. E. Wolf, *Vitamins and Hormones*, 1954, **12**, 1.
[8] D. C. Hodgkin, A. W. Johnson, and A. R. Todd, Chem. Soc. Special Publ., No. 3, 1955, p. 109; D. C. Hodgkin, J. Kamper, M. MacKay, J. Pickworth, K. N. Trueblood, and J. G. White, *Nature*, 1956, **178**, 64 and references therein; D. C. Hodgkin and P. G. Lenhert, *Nature*, 1961, **192**, 937.
[9] Reviewed by D. G. Brown, 'Current Research Topics in Bioinorganic Chemistry,' ed. S. J. Lippard, Interscience, New York, 1973, p. 177.
[10] R. B. Woodward, *Pure Appl. Chem.*, 1973, **33**, 145.
[11] A. Eschenmoser, 23rd International Congress of Pure and Applied Chemistry, Special Lectures, 1971, vol. 2, 69; *Naturwiss.*, 1974, **61**, 513; A. Pfaltz, B. Hardegger, P. M. Müller, S. Farooq, B. Kräutler, and A. Eschenmoser, *Helv Chim. Acta*, 1975, **58**, 1444.

understanding the biosynthesis of vitamin B_{12} and its mechanism of action; see ref. 5 for a review of the bio-



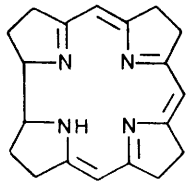
(1) R = CN (vitamin B_{12})

(5) a; R =

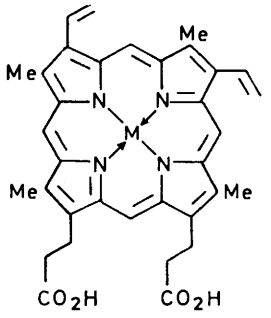


b; R = Me

(coenzyme forms of vitamin B_{12})



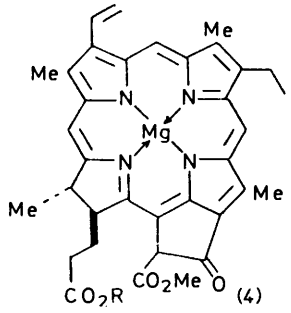
(2)



(3) M = Fe^{II} (haem)

(10) M = H,H

(protoporphyrin IX)



(R = phytyl; chlorophyll *a*)

chemistry of vitamin B_{12} and refs. 12 and 13, respectively, for mechanistic studies and *in vitro* model systems.

The starting point for the research in Cambridge on the biosynthesis of vitamin B_{12} was selected on the basis of knowledge available in 1969—1970, which in outline was as follows. Bernhauer's group had established [14] that cobyrinic acid (6) is a biosynthetic precursor of coenzyme B_{12}, and they and others had also investigated the sequential amidation of the side chains.[14,15] The enzymes responsible for these amidations seem to have a rather low substrate specificity and several sequences were found to be possible.

The structural relation of vitamin B_{12} to the porphyrin macrocycle has already been remarked, and Shemin's group [16] had tested 5-aminolaevulinic acid (7) (ALA), a primary building block for porphyrins,[17] as a precursor of vitamin B_{12}. [5-^{14}C]ALA and [2,3-$^{14}C_2$]ALA [see (7)] were used and [*methyl*-^{14}C]methionine (8) was also tested in separate experiments using an actinomycete (ATCC 11072). The labelling patterns in the three samples of vitamin B_{12} so obtained were partly determined by oxidative degradation leading to the ring c imide (9) and by Kuhn–Roth oxidation of both cobyrinic acid (6) and the imide (9). The results [16] showed that (*a*) ALA (7) is built into cobyrinic acid (6) in essentially the same way as it is into protoporphyrin IX (10), (*b*) approximately six of the eight *C*-methyl groups of cobyrinic acid are derived from methionine, (*c*) one of the geminal methyl groups at C-12 of ring c is derived from [2,3-$^{14}C_2$]ALA, and the other is inserted from methionine, and (*d*) a small amount of radioactivity from [5-^{14}C]ALA is incorporated into one or more of the *C*-methyl groups. Shemin tentatively regarded the last result as supporting a biosynthetic pathway in which the C-1 methyl group of cobyrinic acid (6) is derived from C-5 of ALA, as is C-20 of the porphyrins.[17] At about the same time, Schwartz [18] reported briefly that [2,11-$^{14}C_2$]PBG (11) is incorporated into vitamin B_{12}, but no degradations were described.

The foregoing results were important in establishing that the natural porphyrins and corrins are constructed from the same early building blocks, but degradative techniques were not (and are not) able to locate precisely all the labelled atoms in such radioactive samples of

[12] Recent reviews: B. M. Babior, *Accounts Chem. Res.*, 1975, **8**, 376; R. H. Abeles and D. Dolphin, *ibid.*, 1976, **9**, 114; for summarising collection of additional references see *J. Amer. Chem. Soc.*, 1975, **97**, 4754. Also B. T. Golding and L. Radom, *J. Amer. Chem. Soc.*, 1976, **98**, 6331.

[13] *Inter alia* R. B. Silverman, D. Dolphin, and B. M. Babior, *J. Amer. Chem. Soc.*, 1972, **94**, 4028; B. T. Golding and S. Sakrikar, *J.C.S. Chem. Comm.*, 1972, 1183; G. N. Schrauzer, *Fortschr. Chem. org. Naturstoffe*, 1974, **31**, 583; B. T. Golding, T. J. Kemp, E. Nocchi, and W. P. Watson, *Angew. Chem. Internat. Edn.*, 1975, **14**, 813; G. Bidlingmaier, H. Flohr, U. M. Kempe T. Krebs, and J. Rétey, *ibid.*, p. 822; P. Dowd, M. Shapiro, and K. Kang, *J. Amer. Chem. Soc.*, 1975, **97**, 4754 and references quoted in these papers.

[14] K. Bernhauer, F. Wagner, H. Michna, P. Rapp, and H. Vogelmann, *Z. physiol. Chem.*, 1968, **349**, 1297; see also F. Wagner, *Ann. Rev. Biochem.*, 1966, **35/1**, 405.

[15] R. Rapp, *Z. physiol. Chem.*, 1973, **354**, 136; W. Friedrich, *Biochem. Z.*, 1965, **342**, 143; A. DiMarco and C. Spalla, *Giorn. Microbiol.*, 1961, **9**, 237.

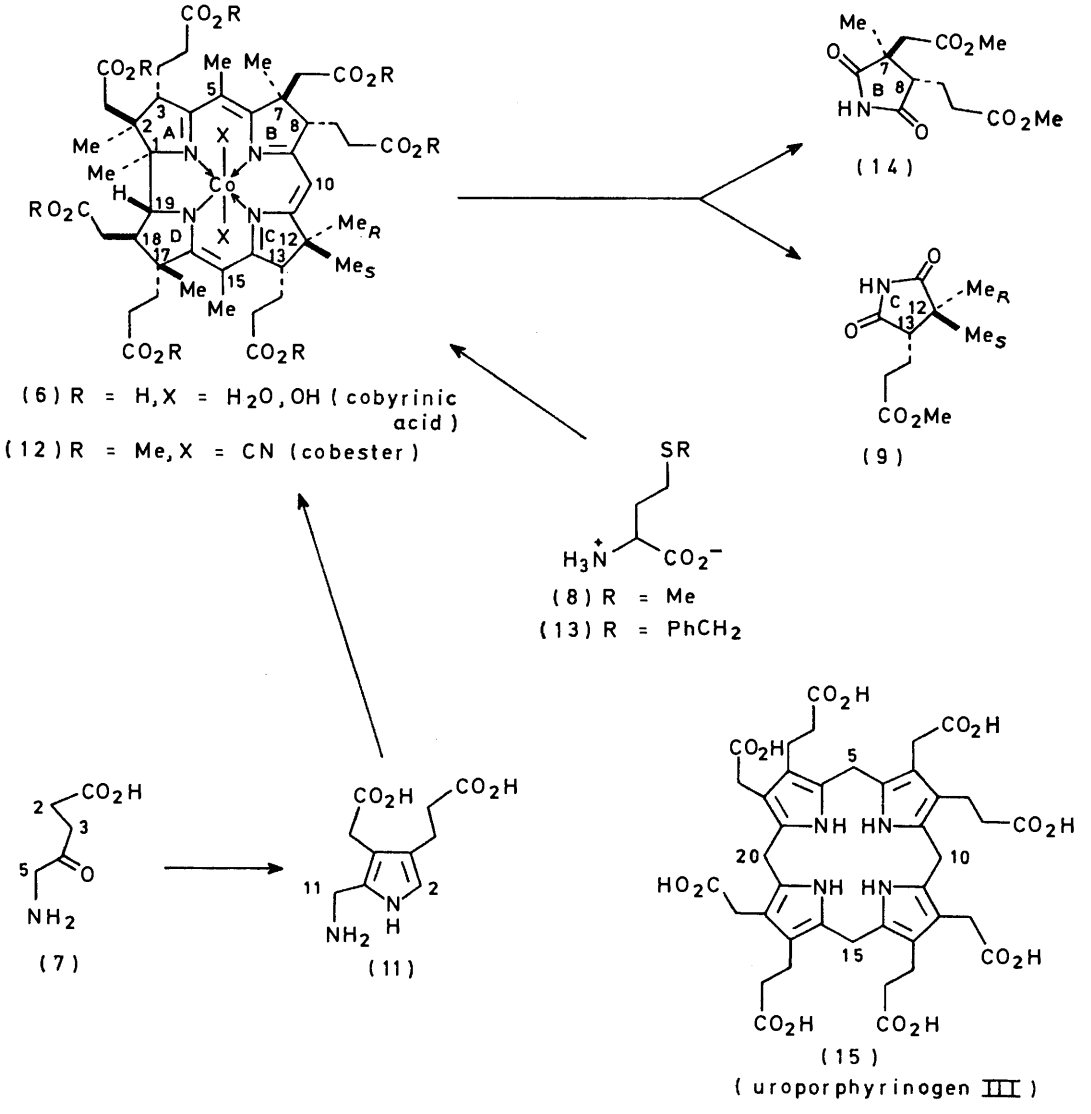
[16] R. C. Bray and D. Shemin, *J. Biol. Chem.*, 1963, **238**, 1501.

[17] A. R. Battersby and E. McDonald in 'Falk's Porphyrins and Metalloporphyrins,' 2nd edn., ed. K. M. Smith, Elsevier, Amsterdam, 1975.

[18] S. Schwartz, K. Ikeda, I. M. Miller, and C. J. Watson, *Science*, 1959, **129**, 40.

vitamin B_{12}. Accordingly, we decided to base our biosynthetic studies of vitamin B_{12} on ^{13}C-labelled precursors, as far as possible, since the sites of labelling could in principle be determined precisely by ^{13}C n.m.r. spectroscopy. As it turned out, Shemin's and Scott's groups had independently decided upon a similar approach and their results and ours will be correlated at the appropriate points below.

Propionibacterium shermanii (NCIB 10585) was chosen as the organism since it was reputed [19] to synthesise relatively large quantities of corrins and had been used in biosynthetic studies.[20] The bacteria grow readily under anaerobic conditions, excreting propionic acid into the medium, and they synthesise cobyrinic acid (6) and some of its simple amides. The nucleotide loop however is constructed only in the presence of oxygen.[21] An aerobic stage was therefore included in the growing regime since we wished in the early stages of this work to isolate and crystallise vitamin B_{12} (1) itself. After considerable experimentation, satisfactory results were obtained when a culture of *P. shermanii* was grown anaerobically for 3 days, the pH being kept at 6.8 by automatic titration of the propionic acid with ammonia, then aerobically for 3 days, again with automatic titration with ammonia. The harvested cells were broken by heating a suspension in an autoclave in the presence of cyanide. Vitamin B_{12} (1) was then isolated from the supernatant by phenol extraction followed by chromatography first on diethylaminoethyl (DEAE) cellulose and then on carboxymethyl (CM) cellulose with final recrystallisation from aqueous acetone. A typical yield of pure vitamin B_{12} from a 3.5 l fermentation was 17 mg. This phase of our work was greatly helped by the advice of Dr. W. F. J. Cuthbertson, Dr. L. Mervyn, and their co-workers (Glaxo Research Ltd.), whom we thank.



[19] L. Mervyn and E. L. Smith, *Progr. Ind. Microbiol.*, 1964, **5**, 151.

[20] *E.g.* P. Renz, *Methods Enzym.*, 1971, **18C**, 82.

[21] D. Perlman, *Methods Enzym.*, 1971, **18C**, 75.

The natural abundance ^{13}C n.m.r. spectrum of vitamin B_{12} in deuterium oxide had been reported and partially assigned [22] but we decided to carry out our spectroscopic studies on a simpler derivative of vitamin B_{12}, *viz.* heptamethyldicyanocobyrinate (12), commonly called cobester; this is available from vitamin B_{12} in >90% yield by methanolysis using the procedure [23] of Keese, Werthemann, and Eschenmoser. The reasons were: (*a*) cobester (12) lacks the C_{17} nucleotide chain and, importantly has three fewer *C*-methyl groups than vitamin B_{12} itself, leading to greater clarity of signal assignments; (*b*) 43 of its 45 skeletal carbon atoms give fully resolved signals when its ^{13}C n.m.r. spectrum (Figure 1) is determined in hexadeuteriobenzene; (*c*) cobester is crystalline † and is soluble in organic solvents. The assignments in Table 1 were made on the basis of chemical shift values and off-resonance ^1H-decoupling. Single-frequency ^1H-

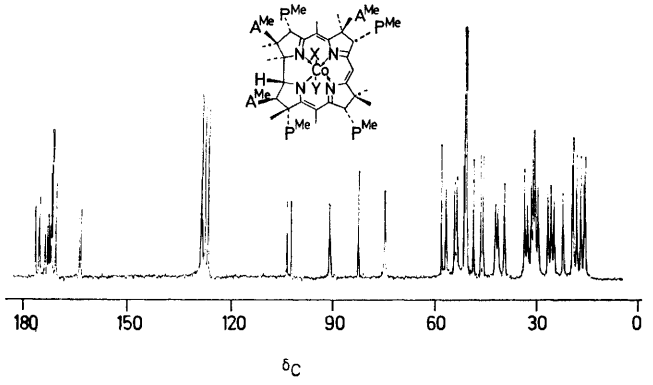


FIGURE 1 ^{13}C N.m.r. spectrum of heptamethyl dicyanocobyrinate (cobester) at natural abundance; X = Y = CN; A^{Me} = $CH_2{\cdot}CO_2Me$; P^{Me} = $CH_2{\cdot}CH_2{\cdot}CO_2Me$ [^1H-noise decoupled, in C_6D_6, δ_C values (p.p.m. downfield from Me_4Si)]

decoupling (Table 2) revealed that the low-field *C*-methyl resonance is at δ_C 30.7 or 30.9.

[5-^{13}C]ALA (7) was prepared from potassium cyanide (90 atom % ^{13}C) by our published route.[24] The product was diluted with unlabelled ALA to give an enrichment of 70 atom % and this sample (175 mg) was incubated with *P. shermanii* (3.5 l run) under the foregoing growth conditions. Crystalline vitamin B_{12} (18 mg) was isolated and its ^{13}C n.m.r. spectrum run in deuterium oxide showed signals only ‡ at δ_C 95.8, 105.1, 108.4, 166.7, 174.2, 179.6, and 180.7 (*cf.* refs. 25 and 26). The sample was diluted to 40 mg with vitamin B_{12} of natural abundance and methanolysis then gave cobester which showed

TABLE 1

Assignments of ^{13}C signals from heptamethyl dicyanocobyrinate based on chemical shifts, off-resonance decoupling, and biosynthetic ^{13}C enrichments; δ_C values (in p.p.m. from Me_4Si); solvent C_6D_6

Carbon type	Assignment	δ_C
sp		
2 C≡N		Not observed
sp^2		
7 C=O	7 CO_2Me	176.0, 173.1, 172.6, 172.3
2 	C-6, C-11	171.3,[a] 170.2, 162.7
4 	C-4, C-9, C-14, C-16	175.1,[b] 174.9,[b] 171.8,[b] 163.1 [b]
2	C-5, C-15	102.4,[b] 103.8 [b]
1	C-10	91.2 [b]
sp^3		
1	C-1	82.8
1	C-19	75.0
7 MeO	7 MeO	51.7, 51.3, 51.1 (overlapping signals)
4	C-2, C-7, CK12, CK17	58.5, 49.0, 46.9, 46.1
3	C-3, C-8, C-13	57.1, 54.5, 53.8
1	C-18	42.4, 41.7, 39.8, 33.9 33.2, 31.9, 31.4, 30.9 or 30.7, 30.0, 26.9, 26.0,
11 CH_2	Side chain CH_2	25.1
1 CH_3-C	*pro-S* at C-12	30.9 or 30.7
5 CH_3-C	At C-1, C-2, C-7, C-12 (*pro-R*), and C-17	22.3,[c] 19.6,[c] 19.3 [c] 18.3,[c] 17.0 [c]
2 CH_3-C	At C-5 and C-15	16.0,[c] 15.6 [c]

[a] Three overlapping signals. [b] These seven signals were greatly enhanced after incorporation of [5-^{13}C]ALA. [c] These seven signals were greatly enhanced after incorporation of [*methyl*-^{13}C]methionine.

TABLE 2

Correlation by decoupling studies of ^1H n.m.r. signals with corresponding ^{13}C signals for heptamethyl dicyanocobyrinate (in C_6D_6)

Irradiation at point below in ^1H n.m.r. spectrum (δ_H)	^{13}C Signals affected by irradiation (δ_C)
1.49	22.3, 19.3
1.40	22.3, 19.3, 17.0
1.15	*ca.* 30.9, 19.6, 18.3, 17.0
1.10	*ca.* 30.9, 19.6, 18.3
3.30	51.7, 51.3, 51.1

strong ^{13}C signals as single lines at δ_C 91.3, 102.5, 103.7, 163.1, 171.5, 174.7, and 175.0 (Figure 2). The first three of these signals arise from C-5, C-10, and C-15 and

† We thank Professor R. B. Woodward and Dr. M. Wuonola for a generous sample of the high m.p. crystalline form of cobester (m.p. 197–203°) and for information on the best solvent system for high-pressure liquid chromatography of cobester.

‡ The sample was too small to give significant signals from the natural abundance of carbon-13.

[22] D. Doddrell and A. Allerhand, *Proc. Nat. Acad. Sci. U.S.A.*, 1971, **68**, 1083; *Chem. Comm.*, 1971, 728.

[23] R. Keese, L. Werthemann, and A. Eschenmoser, unpublished results: *cf.* L. Werthemann, Diss. No. 4097, E.T.H., Zürich, 1968.

[24] A. R. Battersby, E. Hunt, E. McDonald, and J. Moron, *J.C.S. Perkin I*, 1973, 2917.

[25] (*a*) C. E. Brown, J. J. Katz, and D. Shemin, *Proc. Nat. Acad. Sci. U.S.A.*, 1972, **69**, 2585; (*b*) C. E. Brown, D. Shemin, and J. J. Katz, *J. Biol. Chem.*, 1973, **248**, 8015.

[26] A. I. Scott, C. A. Townsend, K. Okada, M. Kajiwara, P. J. Whitman, and R. J. Cushley, *J. Amer. Chem. Soc.*, 1972, **94**, 8267; 1974, **96**, 8069.

the other four correspond to C-4, C-9, C-14, and C-16. None of the *C*-methyl resonances was enhanced, and so in agreement with Shemin and Scott (see below), we deduce that the methyl group at C-1 is not derived from C-5 of ALA.

[*methyl*-^{13}C]Methionine (8) was prepared [27] from 90 atom % [^{13}C]methyl iodide and *S*-benzyl-L-homocysteine (13), and was incubated as before with *P.shermanii*. Methanolysis of the isolated vitamin B_{12} yielded cobester (12), the ^{13}C n.m.r. spectrum of which showed *seven* enhanced signals corresponding to seven *C*-methyl groups (Figure 3). The only *C*-methyl group not enhanced was

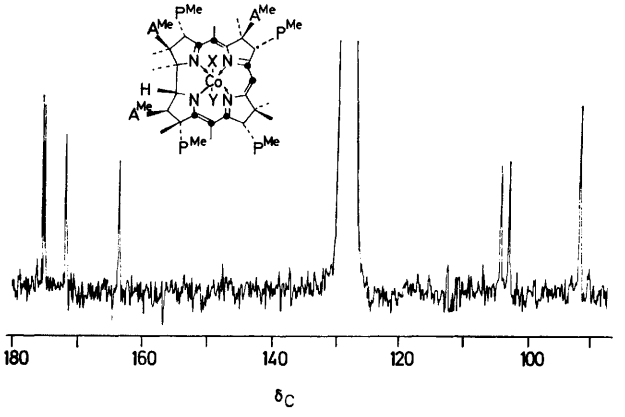


FIGURE 2 ^{13}C N.m.r. spectrum of cobester enriched by incorporation of [5-^{13}C]ALA; X = Y = CN; A^{Me} = CH_2·CO_2Me; P^{Me} = CH_2·CH_2·CO_2Me (^{1}H-noise decoupled, in C_6D_6, δ_C values)

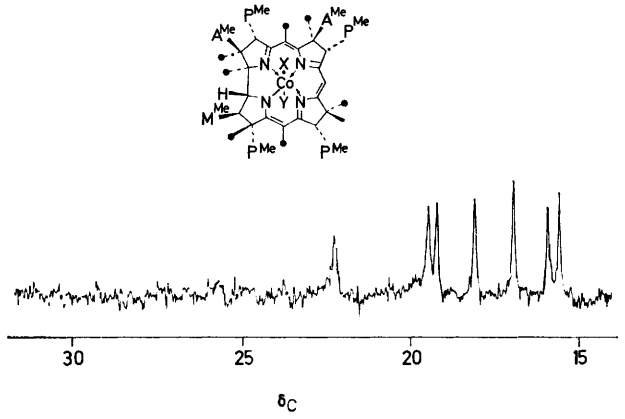


FIGURE 3 ^{13}C N.m.r. spectrum of cobester enriched by incorporation of [^{13}C-*methyl*]methionine; X = Y = CN; A^{Me} = CH_2·CO_2Me; P^{Me} = CH_2·CH_2·CO_2Me (^{1}H-noise decoupled, in C_6D_6; δ_C values)

that at δ_C *ca.* 30.8, and it follows that this signal corresponds to one of the two *C*-methyl groups at C-12 (ring c) which was already known [16,26] to be derived from C-2 of ALA. Thus, in agreement with the American workers,[25,26] we consider that the *C*-methyl groups at C-2, C-5, C-7, C-12 (one), C-15, C-17 and, importantly, C-1 are derived from methionine.

[27] *Cf.* D. B. Melville, J. R. Rachelle, and E. B. Keller, *J. Biol. Chem.*, 1947, **169**, 419.

[28] Ozonolysis and isolation of crystalline ring c imide: T. L. Bogard and A. Eschenmoser, unpublished work.

The foregoing work with ALA and methionine was in its final stages [2] when the preliminary account from Shemin's group [25] and then that from Scott's group [26] appeared. Their spectra were determined on vitamin B_{12} (cyanocobalamin) or on dicyanocobalamin and they were the first to show that the C-1 methyl group of vitamin B_{12} is derived from methionine rather than from C-5 of ALA. Our results to this point thus provide independent confirmation of theirs. It should be emphasised that for these studies where the *number* of enhanced signals is of crucial importance, our findings add strength by being based on spectroscopic measurements with a different corrin (cobester) under different conditions. The danger of overlapping signals leading to false conclusions is well brought out by both the Yale group [26] and ours finding only six enhanced signals for cyanocobalamin isolated from the experiment with [*methyl*-^{13}C]methionine; conversion of the vitamin into dicyanocobalamin [26] or, in Cambridge, into cobester revealed the full set of seven signals.

We can now turn to a further biosynthetically important problem which was to determine which of the geminal methyl groups at C-12 of vitamin B_{12} (1) is derived from methionine. Our approach was to oxidise the cobester, derived from [*methyl*-^{13}C]methionine, with ozone by Bogard and Eschenmoser's method [28] to form the ring c imide (9) and then to determine which of the diastereotopic methyl groups of the imide was ^{13}C-labelled by inspection of the ^{1}H n.m.r. spectrum. Clearly if all the vitamin B_{12} were biosynthesised without dilution from [*methyl*-^{13}C]methionine carrying 90 atom % carbon-13, then the ring c imide obtained from it *via* cobester would show 90% of the ^{1}H n.m.r. signal from the ^{13}C-labelled methyl group as a widely split doublet due to ^{1}H–^{13}C coupling (*J ca.* 125—130 Hz). This doublet would be centred on an unsplit signal representing 10% of the total signal. However, a considerable part of the vitamin B_{12} formed in the biosynthetic experiment will be unlabelled and so it was expected that the derived ring c imide would show two small satellites in its ^{1}H n.m.r. spectrum centred on a larger unsplit signal which would thus be identified as that of the ^{13}C-labelled *C*-methyl group.

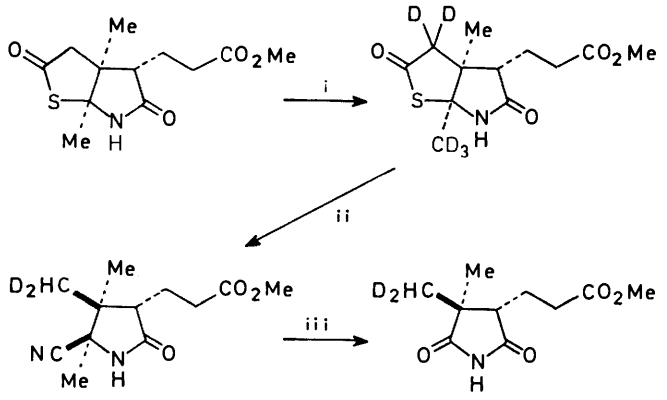
Eschenmoser and Dubs had unambiguously assigned [29] the two ^{1}H resonances from the geminal methyl groups of the imide (9) by the synthesis of deuterium-labelled material as outlined in the Scheme. The high-field resonance was found to correspond to the *C*-methyl group *cis* to the propionate side-chain, *i.e.* to the *pro-R* methyl group.

The foregoing approach is valid only if the stereochemical relationship between the diastereotopic methyl group and the propionate side chain is preserved throughout the reaction sequence from vitamin B_{12} (1) through cobester (12) to the ring c imide (9). It was essential to check this point thoroughly since it is known [30] that

[29] P. Dubs and A. Eschenmoser, unpublished results; *cf.* P. Dubs, Diss. No. 4297, E.T.H., Zürich, 1969.

[30] R. Bonnett, J. M. Godfrey, and V. B. Math, *J. Chem. Soc.* (*C*), 1971, 3736.

equilibration of the chiral centre at C-13 of corrins to give a mixture of 13-normal and 13-*epi*-isomers can occur under vigorous acidic conditions. However, the conditions for the preparation of cobester (12) do not affect C-13 and, in confirmation, methanolysis under these conditions of cyano-13-*epi*-cobalamin [as (12),



SCHEME Synthetic sequence of Eschenmoser and Dubs;[29] i, But-OD–But^{t}O$^-$; ii, $(Ph_3P)_3RhCl$ then KCN; iii, Ag salt of αα-dimethylsuccinimide, then O_3

C-13 inverted] yields heptamethyl dicyano-13-*epi*-cobyrinate [30] (13-*epi*-cobester). Thus, it was very unlikely that our sample of labelled vitamin B_{12} had suffered any stereochemical change on conversion exactly as above into labelled cobester. But rigorous proof was gained as follows. A sample of unlabelled cobester was treated with concentrated sulphuric acid under conditions known [30] to equilibrate C-13 and analysis by high pressure liquid chromatography (h.p.l.c.) showed the product to contain 35% cobester and 65% 13-*epi*-cobester. From the natural abundance ^{13}C spectrum of this mixture was subtracted the natural abundance spectrum of pure cobester, so giving the ^{13}C spectrum of 13-*epi*-cobester. The seven enhanced ^{13}C signals from the ^{13}C-enriched *C*-methyl groups of the biosynthetic sample of cobester exactly matched the corresponding signals of authentic cobester and differed from those shown by 13-*epi*-cobester. In addition, h.p.l.c. showed that the labelled cobester contained <2% of the 13-*epi*-isomer.

Ozonolysis of the labelled cobester (12) gave the ring B imide (14) and the ring C imide (9), isolated by preparative layer chromatography (p.l.c.); the former was amorphous and the latter was recrystallised several times. The optical activity of a larger unlabelled sample of ring C imide prepared under identical conditions corresponded to only 11% inversion of the propionate group during the oxidative degradation. When the ^1H n.m.r. spectrum of the labelled ring C imide (9) was run in deuteriochloroform, the high field *C*-methyl signal was of lowered intensity [*cf.* Figure 4(a)] and a satellite signal (*J* 128 Hz) was clearly visible at high field [Figure 4(b)]. The low-field satellite overlapped the signals from the methylenes of the propionate side chain and therefore the spectrum was run again in hexadeuteriobenzene. Both satellites of the high-field methyl signal were then clearly visible (see Experimental section). The absence of significant satellites centred on the low-field *C*-methyl resonance confirms that appreciable racemisation did not occur at C-13 during the ozonolysis.

These results conclusively prove that the methyl group *cis* to the propionate side chain (the *pro-R* methyl group) is the one derived from methionine. The biosynthetic process thus corresponds to the formal addition of Me and H to ring C in an overall *trans* manner, as for rings A, B, and D.

After our preliminary publication [2] of this work, Scott and his co-workers reported [31] the same conclusion based upon a ^{13}C spectroscopic argument. Also, Shemin and his co-workers [25b] correlated each enhanced ^{13}C methyl resonance of vitamin B_{12} derived from [*methyl*-^{13}C]methionine with the appropriate ^1H methyl resonance by ^1H-decoupling. The ^1H assignments [32] of Brodie and Poe were then applied and the conclusion reached [25b] was the opposite of that recorded above. We believe that the error lies in the literature ^1H assignments.

These degradative experiments also allowed us to determine whether or not the *C*-methyl groups are transferred *intact* as CH_3 units from methionine to rings B and C of vitamin B_{12} (1). [*methyl*-$^2H_3$]Methionine was incorporated as before into vitamin B_{12} and methanolysis gave the labelled cobester (12). The mass spectrum of unlabelled cobester has no molecular ion but the base peak X^+ appears [28] at *m/e* 962. The assignment [30] of this peak as $M^+ - (HCN + CN + CH_2{\cdot}CO_2Me)$] has

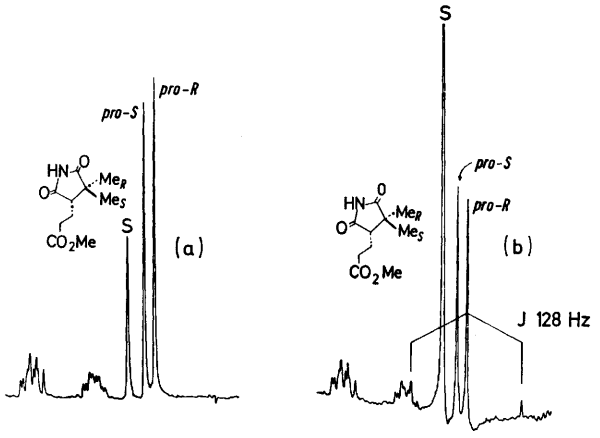


FIGURE 4 ^1H N.m.r. spectrum of ring C imide in $CDCl_3$: (a) derived from natural cobester; (b) derived from [^{13}C-*methyl*] methionine-enriched cobester; signal from solvent marked S

now been confirmed by accurate mass measurement. The mass spectrum of the deuteriated cobester revealed that a mixture of deuteriated species was present; strong peaks appeared at X^+ (*m/e* 962), $X^+ + 3$, $X^+ + 6$, $X^+ + 9$ and so on up to $X^+ + 21$, but the pattern was too complex to permit safe conclusions concerning the

[31] A. I. Scott, C. A. Townsend, and R. J. Cushley, *J. Amer. Chem. Soc.*, 1973, **95**, 5759.

[32] J. D. Brodie and M. Poe, *Biochemistry*, 1971, **10**, 914; 1972, **11**, 2534.

oxidation level of all seven methionine-derived *C*-methyl groups. However, ozonolysis of the deuteriated cobester gave the ring B (14) and ring C (9) imides and their mass spectra showed in both cases only M^+ and $M^+ + 3$ species, the latter corresponding to $26 \pm 1\%$ of the trideuterio-imides being present. We could detect no significant quantity of $^2H_1$ or $^2H_2$ species. Thus, *C*-methylation at C-7 and C-12 of vitamin B_{12} occurs by intact transfer of the *S*-methyl group from methionine. Furthermore, in the ^1H n.m.r. spectrum of the deuteriated ring C imide, the high-field *C*-methyl resonance was of reduced intensity, thereby confirming that the *pro-R* methyl group at C-12 of vitamin B_{12} is the one labelled from methionine as we had shown earlier by ^{13}C labelling.

Thus, from the foregoing incorporation studies using methionine and ALA enriched with heavy isotopes it has been possible to determine the resultant labelling patterns in the isolated vitamin B_{12} by ^{13}C and ^1H n.m.r., including precise stereochemical information. The results show that the corrin nucleus is built up from eight molecules of ALA (7) together with seven methyl groups donated by methionine. In the biosynthetic process, one of the ALA molecules loses C-5 whilst another loses C-1. These experiments were technically possible because the organism *Propionibacterium shermanii* can absorb methionine and ALA from the medium in which it is growing, leading to suitably high levels of specific incorporation. Trial incorporations with the same organism showed that [2,11-$^{14}C_2$]PBG (11) and [^{14}C]-uroporphyrinogen III † (15) were not incorporated to any appreciable extent under the same conditions into vitamin B_{12} (1). It was therefore necessary to develop a broken cell system for biosynthetic studies with these later precursors, and this work is described in the following paper.[33]

EXPERIMENTAL

General Directions.—Standard microbiological techniques were used for growing *Propionibacterium shermanii*. The media were sterilised in an autoclave at 15 lb in^{-2} for 15 min. Contamination during transfers of the organism was prevented by flaming the necks of opened flasks. For the final stages of growth, the air was filtered through sterile cotton wool before it entered the medium. Any emulsions formed during extractions were broken by centrifugation. Corrins were at all stages stored in the dark and handled in subdued light. AnalaR solvents were used in the final stages of purification. For other general directions, see ref. 1.

[methyl-^{13}C]-L-*Methionine* (8) (cf. *ref.* 27).—*S*-Benzyl-L-homocysteine was prepared from L-methionine as described in ref. 34 for the preparation of DL-material. The crude product after recrystallisation from water (2 g l^{-1}) showed $[\alpha]_D^{30}$ +21.6° (*c* 7.5 in aqueous N-HCl). A solution of *S*-benzyl-L-homocysteine (13) (1.58 g) in sodium-dried ammonia (30 ml) was cooled to −70 °C. Small pieces of sodium were added and the resulting suspension was stirred vigorously until a blue colour persisted for several min. The temperature of the cooling bath was raised to −50 °C and [^{13}C]methyl iodide (1.00 g; 90 atom % ^{13}C) was introduced in a stream of dry nitrogen directly beneath the surface of the mixture, the transfer from its ampoule being carried out entirely in a sealed system. The mixture was allowed to reach 20 °C and was kept for 16 h while a stream of nitrogen caused evaporation of the ammonia. The residue in water (30 ml) was treated with hydriodic acid (redistilled from red phosphorus) until acidic to litmus but still alkaline to Congo Red. After concentration of the filtered solution to *ca.* 5 ml, hot ethanol (130 ml) was added and the mixture kept at 0 °C overnight to yield [*methyl*-^{13}C]methionine (915 mg; 86%), m.p. 270—276°, δ (CF_3CO_2D) 4.67 (1 H, m, H-2), 3.10—2.35 (4 H, m, 2 × CH_2), 2.25 (0.9 × 3 H, d, *J* 138.7 Hz $^{13}CH_3$, and 0.1 × 3 H, s, $^{12}CH_3$). Runs with unlabelled material gave 79—82% yields of L-methionine which after one recrystallisation showed $[\alpha]_D$ +23.4° (*c* 12.9 in aqueous N-HCl) {lit.,[35] $[\alpha]_D^{25}$ +23.4° (*c* 3.1 in aqueous N-HCl)}.

Growth of Propionibacterium shermanii (NCIB 10585).—Krebs' yeast–lactate medium was used for maintaining the bacteria and for the early stages of growth. The medium (1 l) contains yeast extract (10 g), KH_2PO_4 (1 g), Na_2HPO_4 (3 g), sodium lactate (aqueous 70% solution) (40 ml), and cobalt(II) chloride (8 mg), and was adjusted to pH 7 before use. The bacteria were maintained on agar slopes of this medium and growth was initiated by adding fresh medium (5 ml) to a slope and incubating in still culture at 25 °C for 7 days with once daily agitation. The resultant cells were used to inoculate a larger batch of fresh medium (50 ml) and after a similar incubation these cells became the inoculum for a preparative run in a 4 l fermentation vessel charged with Shemin's medium [16] (3.5 l). After inoculation, the culture was deoxygenated by purging with nitrogen for 2 h; the vessel was then sealed and stirred for 3 days at 30 °C, the pH being maintained at 6.85 by automatic titration with ammonia (s.g. 0.880). The culture was then aerated continuously for 2 days at 37 °C and kept at pH 6.85 by automatic titration with 5N-ammonia. The cells (100—200 g wet weight) were harvested by centrifugation at 5 °C and 10 000 rev. min^{-1}.

Cell growth during the final incubation was monitored by measuring the optical density of samples at 800 nm. The logarithmic growth phase was complete after 2—3 days and the aeration was carried out during the stationary phase.

Isolation and Purification of Vitamin B_{12} (1).—Cells of *P. shermanii* prepared as above were suspended in 0.2M-sodium acetate buffer (200 ml) at pH 4.5. Potassium cyanide (600 mg) was added and the suspension was kept in an autoclave at 15 lb in^{-2} for 15 min. The cell debris was collected by centrifugation (10 000 rev. min^{-1}; 1 h; 5 °C) and washed with water. The combined supernatant was filtered through Celite, adjusted to pH 2 with concentrated hydrochloric acid, and extracted with phenol (1 × 50 ml; 1 × 40 ml; 1 × 10 ml). The phenol extract (115 ml), which contained the corrins, was washed with 0.01M-hydrochloric acid (3 × 60 ml) and was then mixed with ether (400 ml), 0.01M-potassium cyanide (200 ml) and acetone (100 ml) and shaken. The corrins passed into the aqueous layer which was washed with ether (5 × 135 ml), filtered, and evaporated at 30—40 °C.

† The ^{14}C label was placed specifically at the methylene carbon of the acetate residue on C-12.

[33] A. R. Battersby, E. McDonald, R. Hollenstein, M. Ihara, F. Satoh, and D. C. Williams, following paper.

[34] M. D. Armstrong, *Biochem. Preps.*, 1957, **5**, 91; C. A. Dekker and J. S. Fruton, *J. Biol. Chem.*, 1948, **173**, 471.

[35] G. P. Wheeler and A. W. Ingersoll, *J. Amer. Chem. Soc.*, 1951, **73**, 4604.

The crude corrin extract was chromatographed first on DEAE cellulose (Whatman DE23) and then on CM cellulose (Whatman CM23). The columns were first precycled with 0.5N-sodium hydroxide and 0.5N-hydrochloric acid in the appropriate order, and were then washed to neutrality with water. The corrin extract was added to the DEAE column in a small volume of 1.0M-potassium cyanide and vitamin B_{12} was eluted slowly with water. (Another red band, probably containing corrin acids, was eluted with aqueous 10% acetic acid.) The crude vitamin B_{12} was rechromatographed on the CM cellulose column in a similar manner and evaporation gave a residue (*ca.* 300 mg) containing the vitamin (*ca.* 20 mg). This was dissolved in sufficient aqueous 0.04% potassium cyanide (*ca.* 20 ml) to convert the vitamin completely into dicyanocobalamin and the solution was applied to a DEAE cellulose column which had been previously washed with aqueous 0.01% potassium cyanide. The column was washed with one column volume of 0.01% cyanide, followed by a similar volume of water; the dicyanocobalamin remained near the top of the column. Elution with aqueous 10% acetic acid then regenerated vitamin B_{12} and washed it from the column; the eluate was evaporated and the residue dried and dissolved in a small volume of aqueous methanol; some contaminating protein was precipitated by adding more methanol. The solution was filtered and evaporated and the residue was crystallised from water (0.4 ml) and acetone (1.8 ml) to give pure vitamin B_{12} (*ca.* 17 mg); t.l.c. on silica [in butyl alcohol (6 ml), water (1 ml), and conc. ammonia (0.5 ml)] revealed one spot, identical with that from authentic vitamin B_{12}; $\lambda_{max.}$ 276, 362, 515, and 549 nm.

Incorporation Experiments with ^{13}C- *and* ^{2}H-*Labelled Precursors.*—(a) *5-Aminolaevulinic acid.* [5-^{13}C]ALA (89.2 atom % ^{13}C, 137 mg) was mixed with normal ALA (38 mg) and the resultant sample (175 mg; 70 atom % ^{13}C) and natural L-methionine (175 mg) were added to Shemin's medium (3.5 l) freshly inoculated with *P. shermanii*. The cells were grown and harvested as above (140 g wet weight) and vitamin B_{12} (total 25 mg) was isolated from this and a duplicate run. The ^{13}C n.m.r. spectrum of this material (run in D_2O) showed strongly enhanced signals at δ_C 180.7 179.6, 174.2, 166.7, 108.4, 105.1, and 95.8.

(b) [^{13}C]*Methionine.* [*methyl*-^{13}C]Methionine (90 atom % ^{13}C; 175 mg) and natural ALA (175 mg) were added to fresh medium (3.5 l) freshly inoculated with *P. shermanii* as above. The wet cells from five such runs were extracted to give vitamin B_{12} (8 mg) and a corrin-acid fraction (38 mg). The n.m.r. spectrum of the former (run in D_2O) showed strongly enhanced signals at δ_C 17.9, 18.2, 18.6, 19.6, 21.8, and 22.0 (overlap of two signals). The pure vitamin B_{12} and the total corrin acids were separately methanolysed (see later) to give crystalline cobester (total 25 mg).

(c) [$^{2}H_3$]*Methionine.* [*methyl*-$^{2}H_3$]-L-Methionine (158 mg) and natural ALA (175 mg) were added as above to a suspension of *P. shermanii* in fresh medium (3.5 l). Three such incubations afforded 200 g, 200 g, and 120 g of wet cells, respectively, and extraction gave crystalline vitamin B_{12} (total 30 mg).

Four more incubations were carried out in the same way using 250 mg of deuteriated L-methionine in each experiment. Here, most of the corrin appeared in the acidic fraction and the yield of crystalline vitamin B_{12} was low (total 15 mg).

Heptamethyl Dicyanocobyrinate [23] (12).—Crystalline vitamin B_{12} (15 mg) in methanol (10 ml) was treated with a cold solution of concentrated sulphuric acid (1 ml) in methanol (5 ml). After the solution had been deoxygenated, it was heated under reflux in darkness under nitrogen for 5 days. The solution was evaporated to *ca.* 2 ml, diluted with cold water, and quickly neutralised with solid sodium carbonate. Potassium cyanide (70 mg) was added, and the solution was extracted with carbon tetrachloride (3 × 20 ml) and dichloromethane (3 × 20 ml). Both extracts were evaporated and the residue from the latter, which contained partially esterified corrins, was recycled as above. The combined material from the carbon tetrachloride extracts afforded the heptamethyl ester, which was normally of sufficient purity for direct crystallisation; yield 11 mg (91%); m.p. 193—198° (decomp.) (from benzene–hexane) [Found: *m/e*, 962.4052. $C_{49}H_{67}N_4O_{12}Co$ requires 962.4087; this fragment corresponds to $M^+ - (HCN + CN + CH_2CO_2Me)$]; $\lambda_{max.}$ 368, 510, 545, and 585 nm; $\nu_{max.}$ 1 735 cm^{-1}; δ_H (0.34mM in C_6D_6) 3.48 (3 H, s), 3.40 (9 H, s), 3.35 (3 H, s), and 3.29 (6 H, s) (7 × OMe), 2.25 and 2.19 (each 3 H, s, Me at C-5 and C-15), and 1.54, 1.41, 1.20, 1.15, and 1.02 (3 H, 3 H, 3 H, 3 H, and 6 H, 6 × Me); for δ_C see Tables 1 and 2.

In some cases the cobester was chromatographed on silica packed in cyclohexane. The column was washed with benzene, then 1 : 9 methyl acetate–benzene, and finally the cobester was eluted with 1 : 9 methanol–chloroform. Evaporation of the appropriate fractions gave a residue which was dissolved in carbon tetrachloride and shaken with aqueous 0.2% potassium cyanide. The organic phase was filtered through a cotton plug and evaporated, and the residue was crystallised as above.

The above procedure was also used to convert the isolated fraction containing mixtures of acidic corrins into cobester.

[^{13}C]*Cobester derived from* [5-^{13}C]*ALA.*—The biosynthetically derived vitamin was diluted with normal vitamin B_{12} and this sample (40 mg) was methanolysed as above. Part of the cobester (10 mg in 5 ml of C_6D_6) showed strongly enhanced signals at δ_C 175.0, 174.7, 171.5, 163.1, 103.7, 102.5, and 91.3.

[^{13}C]*Cobester derived from* [methyl-^{13}C]*Methionine.*—This ester showed strongly enhanced signals at δ_C (C_6D_6) 15.6, 16.0, 17.0, 18.3, 19.3, 19.6, and 22.3.

[$^{2}H_x$]*Cobester derived from* [methyl-$^{2}H_3$]*Methionine.*—Two samples were obtained from different incubations. The patterns in their mass spectra near to *m/e* 962, compared with that of normal cobester, are given in Table 3.

Ozonolysis of ^{13}C- *and* ^{2}H-*Labelled Samples of Cobester* [28] (12).—Ozone, generated from oxygen (30 l h^{-1}) at 210 V, was bubbled through a solution of cobester (24 mg) in methanol (7 ml) at −78 °C for 20 min. After the solution had then been kept at 20 °C for 10 min, 30% hydrogen peroxide (1.5 ml) was added; the mixture was heated under reflux for 15 min and was then evaporated to *ca.* 3 ml. Saturated brine (5 ml) was added and the solution was extracted with dichloromethane (6 × 6 ml). The combined extracts were washed with saturated sodium hydrogen carbonate, 0.1N-hydrochloric acid, and saturated brine, dried, and evaporated to give an oil (26 mg) which was fractionated by p.l.c. on silica with purified ether. Two bands, R_F 0.38 (fluorescent) and 0.31 (not fluorescent), containing the ring C imide (9) and ring B imide (14), respectively, were eluted with 15% methanol–chloroform.

The ring C imide (9) was further purified by distillation at 90—140 °C (bath) and 0.03—0.1 mmHg to give an oil, which was crystallised from ether–n-pentane (yield *ca.* 1 mg); m.p. 67—69°, $[\alpha]_D$ −48.6° (*c* 0.185 in $CHCl_3$) {ref. 28

quotes m.p. 71—72°, $[\alpha]_D$ −43° for imide obtained by the method described above, and m.p. 83—84° $[\alpha]_D$ −62° for synthetic imide (Scheme 2)} (Found: *m/e*, 213.1003. Calc. for $C_{10}H_{15}NO_4$: *M*, 213.1001); δ_H [$CDCl_3$ (values in C_6D_6 in parentheses)] 3.72 (3.42) (3 H, s, OMe), 2.6 (2.5) (2 H, m, CH_2CO), 2.6 (2.1) (1 H, m, ring CH), 1.9 (1.6) (2 H, m, CH_2), 1.36 (0.94) (3 H, s, *pro-S*-Me), and 1.25 (0.77) (3 H, s, *pro-R*-Me).

TABLE 3

m/e	Natural sample	Sample A (CD_3 content 16.7%)	Sample B (CD_3 content 33%)
960	0.11	0.25	
961	0	0.26	
962	1.00	0.79	1.95
963	0.57	0.85	2.55
964	0.18	0.70	2.45
965	0.13	1.00	5.4
966	0.05	0.98	5.2
967		0.70	4.25
968		0.54	6.45
969		0.38	5.9
970		0.30	4.25
971		0.26	4.6
972		0.15	3.4
973		0.12	2.2
974			2.1
975			1.55
976			1.05
977			1.3
978			0.9
979			0.9
980			1.1
981			0.9
982			0.75
983			0.85
984			0.6
985			0.5
986			0.5
987			0.4

For the enriched ring c imide (9) derived from [*methyl*-^{13}C]-methionine, the high field methyl signals at δ 1.25 (0.77) were of lowered intensity relative to the corresponding signals from an unlabelled sample, and satellites were present (*J* 128 Hz) (see Figure 4). The ^{13}C content was calculated to be 12—14 atom % from this spectrum and from the mass spectrum.

The enriched ring c imide (9) derived from [*methyl*-$^2H_3$]-methionine again showed the high field methyl resonance having lowered intensity and the mass spectrum of this sample showed strong peaks at *m/e* 213 and 216.1190 (calc. for $C_{10}H_{12}D_3NO_4$: 216.1189). The pattern of peaks in this region showed that no $^2H_1$ or $^2H_2$ species were present and that the proportion of [$^2H_3$]imide was 26% (average of 4 runs).

The ring B imide (14) from the above ozonolysis was further purified by distillation at 105—137 °C (bath) and 0.05 mmHg to afford a glass (*ca.* 1 mg) (Found: *m/e*, 271.1058. Calc. for $C_{12}H_{17}NO_6$: *M*, 271.1055), δ_H ($CDCl_3$) 1.22 (3 H, s, Me), 1.9 (2 H, m, CH_2), 2.7 (4 H, m, 2 × CH_2CO), 3.0 (1 H, m, ring CH), 3.66 (6 H, s, 2 × OMe), and 8.06br (1 H, s, NH).

The enriched ring B imide (14) derived from [*methyl*-$^2H_3$]-methionine showed its resonance at δ_H 2.60 clearly of lowered intensity, and the mass spectrum showed strong peaks at *m/e* 271 and 274.1249 (calc. for $C_{12}H_{14}D_3NO_6$: 274.1243). The mass spectrometric pattern in this region established that 27% of the [$^2H_3$]imide was present, with negligible amounts of $^2H_1$ or $^2H_2$ molecules (average results from 5 runs).

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