Biosynthesis of Vitamin B_{12}: the Fate of 8-H as Precorrin-2 is Enzymically converted into Cobyrinic Acid

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The dimethylated B_{12}-precursor, precorrin-2 **2**, is prepared strongly labelled with tritium at 3-H and 8-H to allow proof that the 3-H label is retained whereas the 8-H label is lost during the biosynthetic conversion of precorrin-2 into cobyrinic acid **3**.

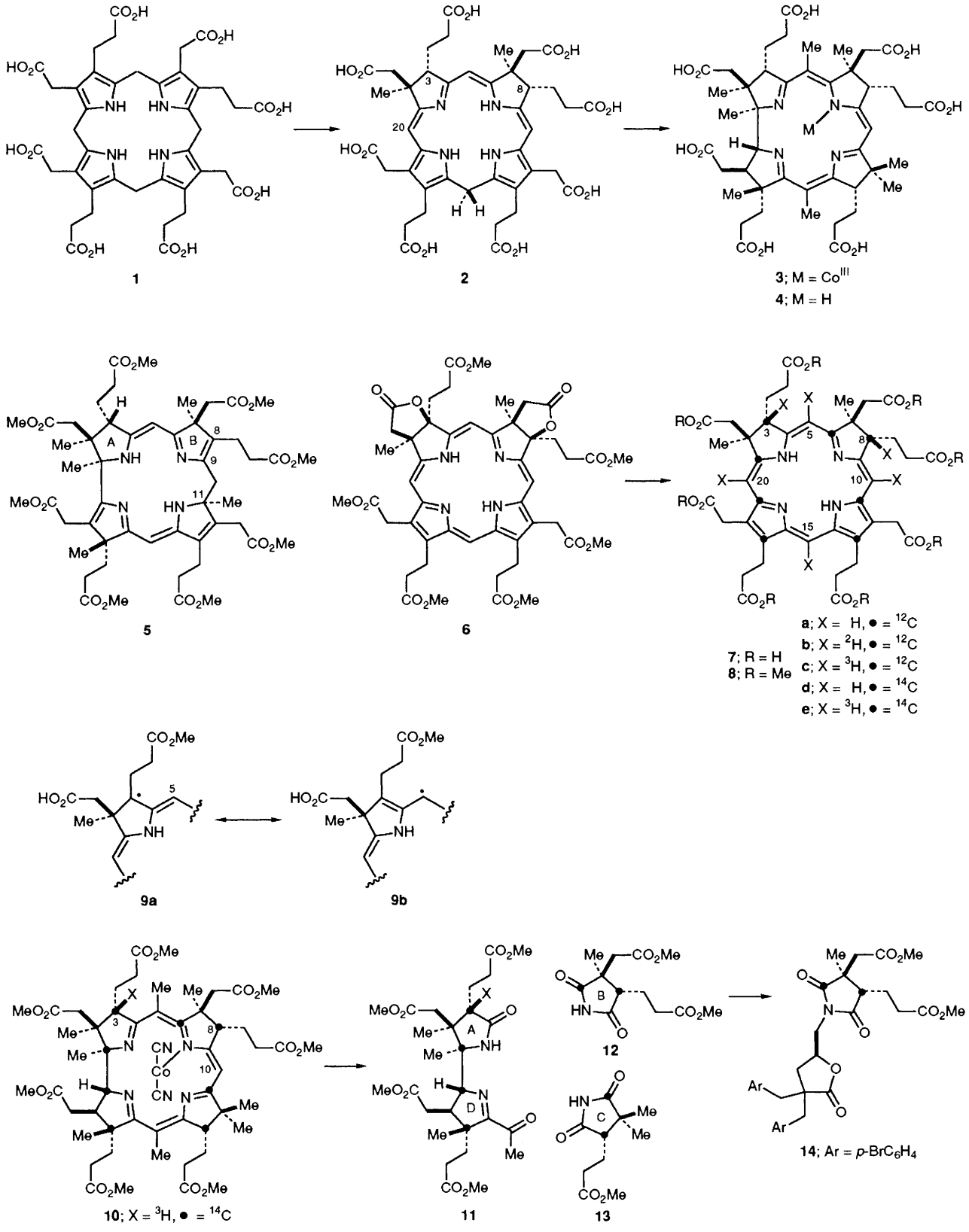
The corrin, cobyrinic acid **3**, is a late biosynthetic precursor of vitamin B_{12} in *Propionibacterium shermanii*[1] and the related metal-free form, hydrogenobyrinic acid **4**, is on the biosynthetic pathway to B_{12} in *Pseudomonas denitrificans*.[2] Both **3** and **4** are produced *in vivo* from uro'gen III **1** by a sequence of steps which includes eight *C*-methylations.[1] The first two *C*-methylations are carried out by one enzyme[3,4] which is well characterised[4] and the structure of the product **2** named[5] precorrin-2 was fully established by direct isolation.[6]

Precorrin-6x, the product of six *C*-methylations from **1**, was recently isolated[7] from a *P. denitrificans* strain, an event that changed the direction of research on the biosynthesis of vitamin B_{12} and related corrinoids. The reasons can be seen in the structure **5** established[7,8] for the octamethyl ester of precorrin-6x which reveals several unexpected features, especially the C-11 methyl group. The quaternary centre at C-11 generates two separated chromophores (*cf.* the conjugated systems **2** and **3**). The chromophore of ring A–ring B *in the ester of precorrin-6x* has a double bond at C-8 to C-9 which may or may not be located there in the original enzymic product, precorrin-6x itself. Because the dimethylated precursor **2** is the same for both the anaerobic *P. shermanii* and the aerobic *P. denitrificans* and the corrins **3** and **4** produced differ only in the presence or absence of cobalt, and bearing in mind the C-8 to C-9 double bond in **5**, it was of considerable interest to study the fate of 8-H of precorrin-2 **2** as it is enzymically converted into cobyrinic acid **3** in *P. shermanii*.

Precorrin-2 **2** can be generated *in situ* from sirohydrochlorin **7a** in the enzymic incubation mixture[1] and the method for labelling at C-8 of the corresponding ester **8a** was first developed as follows using deuterium. The lactone rings of the bis-lactone[9] **6** were reductively cleaved[10] with chromium(II) chloride in a mixture of deuterium oxide and tetrahydrofuran (THF) and the resultant dicarboxylic acid was esterified to afford ^{2}H-labelled sirohydrochlorin octamethyl ester **8b**, 60% yield. Repeated integration of the ^{1}H-NMR spectrum of this product allowed the extent of ^{2}H-labelling to be determined at the positions listed in Table 1. Two points need comment: (*a*) heavy labelling has been achieved at positions 3 and 8 but not complete deuteriation, (*b*) positions 5, 10, 15 and 20 are significantly labelled. We believe both (*a*) and (*b*) are due to the radical nature of chromium(II) reductions.[11] Thus, an initial radical **9a** can abstract deuterium from D_2O or protium from the THF whilst reaction through the alternative limiting form **9b** labels C-5; the labelling at 10, 15 and 20 is explained in the same way.

Table 1 Distribution of ^{2}H in **8b** and of ^{3}H in **8c**

Site	**8b** % ^{2}H at each site	**8c** % of total ^{3}H
3	66	29
5	46	20
8	66	29
10	7	3
15	28	12
20	15	7



The entire process was then repeated under the same conditions save that a small amount of high activity tritiated water was added to the D_2O–THF mixture at the outset. Note that D_2O was used as solvent rather than H_2O because transfer of tritium from DOT is more efficient than from HOT due to the smaller isotope effect in the former case. The ^{3}H-labelled sirohydrochlorin octamethyl ester **8c** was isolated in 50% yield, total activity 6.7 μCi, specific activity 0.93 mCi mmol^{-1}. Finally, the expected distribution of ^{3}H-activity at the various labelled sites† of the ^{3}H-sample **8c** was calculated from the deuterium values in Table 1.

† Because tritium is used at tracer level, the sample is an assembly of six singly ^{3}H-labelled species which together are equivalent to the multiply labelled molecule illustrated. Each labelled site also carries deuterium which for simplicity is not shown.

Table 2 Enzymic formation and subsequent degradation of labelled cobester **10**

		^3H : ^{14}C ratio		^3H : ^{14}C ratio calc. from Table 1		^{14}C-Specific activity /dpm mmol^{-1}		^3H-Specific activity /dpm mmol^{-1}	
Experiment		1	2	1	2	1	2	1	2
Precorrin-2 **2**		0.57	5.6	—	—	2.44×10^9	4.22×10^8	1.39×10^9	2.38×10^9
Cobester **10**	(i)[a]	0.17	1.8	0.17	1.6	1.16×10^7	2.06×10^7	2.03×10^6	3.63×10^7
	(ii)[a]	0.17	1.7	0.17	1.6	2.46×10^5	4.51×10^5	4.30×10^4	7.74×10^5
Ring-A/ring-D **11**		0.33	[b]	0.34	—	1.19×10^5	—	3.93×10^4	—
Ring-B imide **12**		<0.01	<0.01	0	0	5.57×10^4	1.14×10^5	[c]	[c]
Derivative **14**		<0.01	<0.01	0	0	5.23×10^4	—	[c]	[c]
Ring-C imide **13**		<0.01	<0.01	0	0	5.34×10^4	1.11×10^5	[c]	[c]

[a] Cobester (i) before and (ii) after substantial dilution for degradation. [b] The yield of ring-A/ring-D fragment from this degradation was too low to allow rigorous purification of a sufficiently active sample. [c] ^3H-Activity below detection level.

^{14}C-Labelled **7d** was biosynthesised[12] from 5-amino[4-^{14}C]laevulinic acid and was isolated as its ester **8d**, specific activity 3.3 mCi mmol^{-1}. This was mixed with an appropriate amount of the ^3H-sample **8c** and the radioactivity values are given in Table 2. Hydrolysis of this ^3H : ^{14}C-labelled sample **8e** and enzymic conversion of the resultant octa-acid into cobyrinic acid (as **3**) was carried out as usual using the cell-free enzyme system prepared[13] from *P. shermanii* cells. Unlabelled cobyrinic acid was then added as carrier and the derived cobester **10** was recrystallised to constant specific activity and ^3H : ^{14}C ratio (incorporation 18.3%). This product was further diluted with unlabelled cobester for degradation by ozonolysis[14] to yield the ring-B imide **12**, the ring-C imide **13** and the ring-A/ring-D fragment[15] **11** which were carefully purified for assay of radioactivity. Since it was known[16] that the ring-B imide **12** produced in this way contains some of the imide derived from ring-D of **10** and **11**, the ring-B imide was converted into the derivative **14** which is readily separated from the corresponding derivative of the ring-D imide[17]; the derivative **14** also serves to characterise further the ring-B fragment **12**. Table 2 collects the results for the set of labelled materials. All the foregoing studies were then repeated starting with a sample of **8e** having a higher ^3H : ^{14}C ratio especially to check the foregoing findings for the ring-B imide **12**; the incorporation was 27.5% and the activity values from this independent study are also in Table 2.

The key results from Table 2 are (*a*) the ring-C imide **13** carries the appropriate ^{14}C-activity but, as expected, no ^3H, (*b*) the ring-A/ring-D fragment **11** carries the required ^{14}C- and ^3H-activity corresponding to retention of 3-H but (necessarily) loss of 20-H of precorrin-2 **2**, (*c*) the ring-B imide **12** shows that all the ^3H has been lost from C-8 of **2** but the ^{14}C-activity of **12** matches the expected value, (*d*) the ^3H : ^{14}C ratio in the original cobester **10** interlocks with the results under (*b*) and (*c*) by agreeing with the loss of ^3H from C-5, C-8, C-10, C-15 and C-20 of precorrin-2 **2** but with retention of ^3H at C-3.

Loss of the small amount of ^3H at C-10 of cobester **10** is included in (*d*) above because the acidic methanol used for esterification of cobyrinic acid to give **10** is known to cause complete exchange at this position.[18] Possible similar exchange at C-8 was examined by using CD_3OD in a control esterification of **3** and NMR of the ester confirmed that there was no significant exchange at C-8.

Thus, the facts are that as precorrin-2 **2** is converted enzymically into cobyrinic acid **3**, 3-H is retained whereas 8-H is lost. These findings are consistent with there being a C-8 to C-9 double bond (as in **5**) at some stage of the biosynthesis but it remains to be established whether this double bond position is a requirement of one of the transforming enzymes. The present results will form part of the total picture when the structures are determined for the presently unknown intermediates on the pathway to cobyrinic acid **3**.

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References

1 F. J. Leeper, *Nat. Prod. Rep.*, 1989, **6**, 171 and references cited therein.
2 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.
3 G. Müller, R. Deeg, K. D. Gneuss, G. Gunzer and H.-P. Kriemler, in *Vitamin B_{12}*, ed. B. Zagalak and W. Friedrich, de Gruyter, Berlin, 1979, p. 279.
4 F. Blanche, L. Debussche, D. Thibaut, J. Crouzet and B. Cameron, *J. Bacteriol.*, 1989, **171**, 4222.
5 H. C. Uzar, A. R. Battersby, T. A. Carpenter and F. J. Leeper, *J. Chem. Soc., Perkin Trans. 1*, 1987, 1689.
6 A. R. Battersby, K. Frobel, F. Hammerschmidt and C. Jones, *J. Chem. Soc., Chem. Commun.*, 1982, 455.
7 D. Thibaut, L. Debussche and F. Blanche, *Proc. Natl. Acad. Sci. USA*, 1990, **87**, 8795.
8 D. Thibaut, F. Blanche, L. Debussche, F. J. Leeper and A. R. Battersby, *Proc. Natl. Acad. Sci. USA*, 1990, **87**, 8800; F. Blanche, M. Kodera, M. Couder, F. J. Leeper, D. Thibaut and A. R. Battersby, *J. Chem. Soc., Chem. Commun.*, 1992, 138.
9 A. R. Battersby, K. Jones, E. McDonald, J. A. Robinson and H. R. Morris, *Tetrahedron Lett.*, 1977, 2213; A. R. Battersby, E. McDonald, H. R. Morris, M. Thompson, D. C. Williams, V. Ya Bykhovsky, N. I. Zaitseva and V. N. Bukin, *Tetrahedron Lett.*, 1977, 2217.
10 C. Bladon and A. R. Battersby, Research Report, Cambridge, 1984.
11 J. R. Hanson and E. Premuzic, *Angew. Chem., Int. Ed. Engl.*, 1968, **7**, 247.
12 R. Deeg, H.-P. Kriemler, K.-H. Bergmann and G. Müller, *Hoppe Seyler's Z. Physiol. Chem.*, 1977, **358**, 339; A. R. Battersby, E. McDonald, M. Thompson and V. Ya Bykhovsky, *J. Chem. Soc., Chem. Commun.*, 1978, 150.
13 A. R. Battersby, E. McDonald, R. Hollenstein, M. Ihara, F. Satoh and D. C. Williams, *J. Chem. Soc., Perkin Trans. 1*, 1977, 166; *cf.* ref. 5.
14 T. L. Bogard and A. Eschenmoser, unpublished work.
15 D. Arigoni and M. Imfeld, unpublished work; M. Imfeld, PhD Thesis, ETH Zürich, 1980, No. 6565.
16 A. D. Carpenter and A. R. Battersby, Research Report, Cambridge, 1984.
17 S. P. Conway, PhD Thesis, Cambridge, 1992.
18 A. R. Battersby, C. Edington, C. J. R. Fookes and J. M. Hook, *J. Chem. Soc., Perkin Trans. 1*, 1982, 2265.