Biosynthesis of Vitamin B_{12}: Does 5^1-Norcobyrinic Acid or 15^1-Norcobyrinic Acid Act as a Precursor of Cobyrinic Acid?

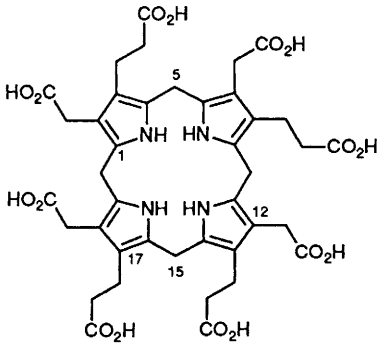
Ingeborg Grgurina, Sheetal Handa, George Weaver, Philip A. Cole and Alan R. Battersby*

University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, UK

5^1-Norcobyrinic acid and 15^1-norcobyrinic acid are prepared in labelled form for enzymic experiments that show that neither one acts as a biosynthetic precursor of cobyrinic acid, a late intermediate *en route* to vitamin B_{12}.

The late stages of the biosynthetic pathway to vitamin B_{12} involve further elaboration of a well-characterised intermediate, cobyrinic acid **4a**, which in turn is built from uro'gen III **1** by a multi-step process requiring *inter alia* eight *C*-methylations.[1] The order of introduction of the first three *C*-methyl groups was known[1] by 1979 and pulse labelling[2] then showed that the fourth *C*-methyl group is inserted at C-17. Extension of the pulse labelling approach[3–5] indicated that the C-12α methyl group is introduced next at C-1 and the methyls at C-5 and C-15 are the last ones with still some doubt about the order of introduction in different organisms.[3–5]

This knowledge still leaves open many alternative late biosynthetic sequences *en route* to cobyrinic acid **4a** but one possibility is that either 5^1-norcobyrinic acid **5a** nor the 15^1-nor



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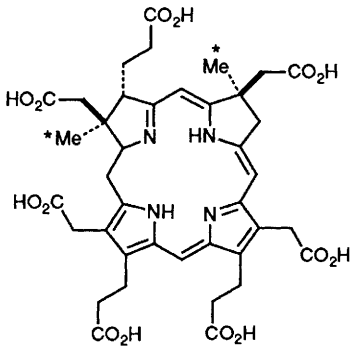

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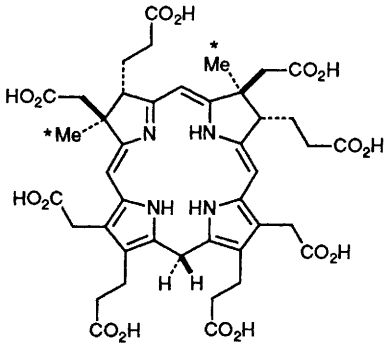

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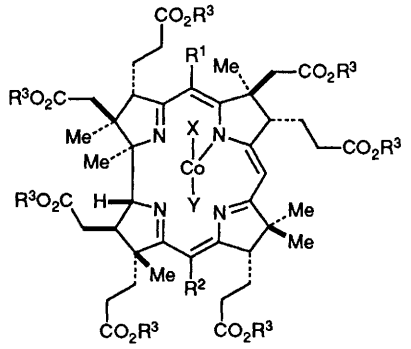

Set **a**: R^3 = H
Set **b**: R^3 = Me

4 $R^1 = R^2$ = Me
5 R^1 = H, R^2 = Me
6 R^1 = Me, R^2 = H
7 R^1 = H, R^2 = CH_2OH
8 R^1 = H, R^2 = CH_2T
9 R^1 = CH_2T, R^2 = H

isomer **6a** undergoes *C*-methylation to generate **4a**. The recent availability of the corresponding heptamethyl esters **5b** and **6b** of these corrins[6] allowed the necessary enzymic experiments to be carried out. It will be seen that rigour in experimental design was of crucial importance for these studies.

Heptamethyl 5[1]-norcobyrinate[6] **5b** was oxidised by lead tetracetate[6] and the resultant 15[1]-acetoxymethyl derivative was mildly hydrolysed to yield heptamethyl 15[1]-hydroxymethyl-5[1]-norcobyrinate **7b**. This was reduced either by zinc and $CH_3CO_2{}^3H$-tetrahydrofuran[6] or by cyanoborotritiide and acid (conditions for the latter kindly provided by Professor Arigoni) to afford heptamethyl [15-^{3}H-methyl]-5[1]-norcobyrinate **8b**. Heptamethyl [5-^{3}H-methyl]-15[1]-norcobyrinate **9b** was prepared analogously from the 15[1]-nor ester[6] **6b**.

At each stage of both preparations, HPLC conditions were developed to separate quantitatively the 5[1]-nor series from the 15[1]-nor series and also both nor-compounds from heptamethyl cobyrinate (cobester) itself **4b**. In this way, it was rigorously established that neither series contained any detectable quantity of the other and importantly, that both nor-series **8b** and **9b** were free of labelled cobester (as **4b**). Unlabelled samples of the 5[1]-nor ester **5b** and the 15[1]-nor ester **6b** were also prepared for use below and the latter was crystallised in suitable form for structure confirmation by X-ray analysis.[7]

A single preparation of the cell-free enzyme system from *C. tetanomorphum*[5] was divided into three equal parts which were used in set a, Table 1, for three experiments. Experiment 1 involved incubation with [2,7-^{14}C-methyl]sirohydrochlorin **2**. This material **2** is known to be reduced *in situ* to precorrin-2 **3**, which is the true biosynthetic intermediate[8] and thus acts as a standard substrate to check the efficacy of the enzyme system. Experiment 2, set a, involved the same amount of labelled **2** as in experiment 1 together with unlabelled 15[1]-nor acid **6a** prepared by hydrolysis of its ester (2 M aqueous piperidine) and in experiment 3, set a, ^{3}H-labelled 15[1]-nor acid **9a** was incubated alone in the same quantity used for experiment 2. Set b, Table 1, shows the results from the equivalent set of three runs carried out with the 5[1]-nor acid, **5a** and **8a**.

The cobyrinic acid samples isolated by ion-exchange HPLC from experiments 1 and 2 in sets a and b, Table 1, were separately esterified (5% conc. H_2SO_4 in methanol, room temp., 16–24 h), each was diluted with unlabelled cobester **4b** and then multiply recrystallised. The cobester similarly isolated from experiment 3, set a was mixed with a substantial quantity of unlabelled 15[1]-nor ester **6b** and the cobester was then re-isolated using the foregoing efficient HPLC methodology. This mixing with unlabelled 15[1]-nor ester **6b** and re-isolation of the cobester by HPLC was carried out 2–3 times to ensure complete removal of the ester **6b** of any residual labelled precursor from the cobester. Finally, the cobester so isolated was diluted with highly purified unlabelled cobester **4b** and then multiply recrystallised. The cobester from experimental 3, set b, Table 1 was rigorously purified and crystallised in an analogous way but this time using unlabelled 5[1]-nor ester **5b** at the HPLC stage.

Experiment 1, set a gave a good incorporation value, thus demonstrating the effectiveness of the enzyme preparation. Interestingly, experiment 2, set a established that the 15[1]-nor acid **6a** strongly inhibits the incorporation of precorrin-2 **3** into cobyrinic acid **4a**. Finally the low level of radioactivity found in cobester **4b** from experiment 3, set a showed that the 15[1]-nor acid **9a** is not a precursor of cobyrinic acid **4a**.

The experiments in set b, Table 1, also involved enzymes from *C. tetanomorphum* but from a different growth. Even though the enzyme preparation was less effective than for set a, the results from experiments 1 and 2 demonstrated that the 5[1]-nor acid **5a** does not inhibit the incorporation of precorrin-2 **3** into cobyrinic acid **4a**. Experiment 3, set b suggested by comparison with experiments 1 and 2 that the 5[1]-nor acid **8a** does not act as a precursor of cobyrinic acid **4a** but this result was not clear cut and required confirmation.

Table 1 Incorporation experiments

Experiment no.	Material(s) incubated[a]	Fraction (%) of original radioactivity in cobester **4b**		
		Set a 15[1]-Nor Acid **6a** and **9a**	Set b 5[1]-Nor Acid **5a** and **8a**	Set c 5[1]-Nor Acid **5a** and **8a**
1	[2,7-^{14}C-methyl] **2**	10.5	1.7	11.9
2	[2,7-^{14}C-methyl] **2** plus unlabelled nor acid	<0.18[a]	2.0	—
3	The indicated [^{3}H-methyl] nor acid	<0.6[a]	<0.7[b]	<0.11[a]

[a] The quantities of **2** used in experiments 1 and 2 and of nor acid in experiments 2 and 3 were the same for each set but varied somewhat from one set to another. [b] The specific activity was still slowly falling from the penultimate to the last crystallisation; these values are, thus, maximal ones.

Accordingly, set c, Table 1 was carried out now using *Propionibacterium shermanii* cells as source of the enzyme system.[9] The results in Table 1 made it certain that the 5[1]-nor acid **8a** fails to act as a biological precursor of cobyrinic acid **4a**.

It should be noted that the levels of radioactivity present in cobester **4b** after the HPLC purification from experiment 3 of sets a, b and c, Table 1, corresponded in each case to an apparent 'incorporation' of 1–1.5%. These values only fell to those recorded after multiple recrystallisation of the cobester. Knowing that the nor-esters **5b** and **6b** and cobester **4b** are all cleanly separable by HPLC, we suspected that the labelled nor-acids **8a** and **9a** used as precursors must have contained *ca.* 1–1.5% of an epimer, which as its ester is inseparable by HPLC from cobester **4b**, but which is removable by recrystallisation. Professor Arigoni's group[10] has directly proved the presence of such an epimer.

It was recognised at the outset that testing the nor-acids as possible precursors of cobyrinic acid was experimentally very demanding and by agreement with Professor Arigoni, independent studies were, therefore, made simultaneously by the two groups. Our results are in full agreement with those from the Zürich team[10] and they place further limitations on the possible sequences for the late stages of biosynthesis of cobyrinic acid **4a**. Additional work is necessary to pin-point the one nature uses. Finally, these interlocking results cast doubt on an earlier report[11] that nor-corrins can be enzymically converted into a 5,15-dimethylated corrin.

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