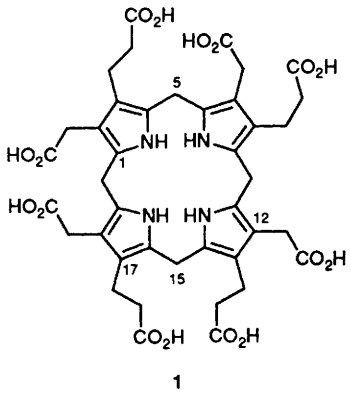
On the Putative Role of Some Norcorrins in the Biosynthesis of Cobyrinic Acid

Jan Kulka, Cornelius Nussbaumer and Duilio Arigoni*

Laboratorium für Organische Chemie, ETH-Zentrum, CH-8092 Zürich, Switzerland

It is shown that none of the three truncated corrins 5^1-norcobyrinic acid, 15^1-norcobyrinic acid and $5^1,15^1$-dinorcobyrinic acid plays a role as a precursor of cobyrinic acid.

In spite of considerable efforts by different groups only three intermediates, the precorrins **2–4**, have been identified to date in the complex biosynthetic pathway which leads from uro'gen III **1** to cobyrinic acid **5a**, the precursor of the B_{12} family of cofactors.[1] However, it is known from pulse-feeding experiments that the five additional methylation steps that follow formation of **4** must involve sequentially positions C-17, C-12 and C-1, followed by C-15 and C-5 in an order that seems to vary in different organisms.[2] Derivatives of cobyrinic acid lacking one or both of the methyl groups at C-5 and C-15 are



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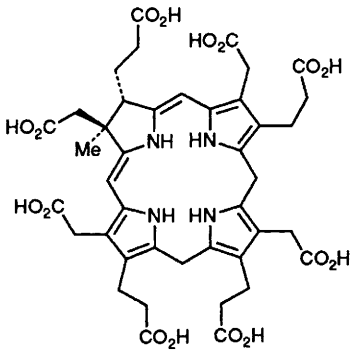

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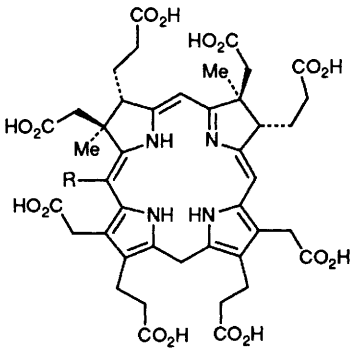
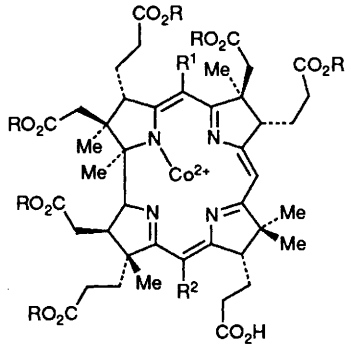


3 R = H
4 R = Me



Set a: R = H
Set b: R = Me

5 $R^1 = R^2 = Me$
6 $R^1 = H, R^2 = Me$
7 $R^1 = Me, R^2 = H$
8 $R^1 = R^2 = H$
9 $R^1 = H, R^2 = CH_2OH$
10 $R^1 = CH_2OH, R^2 = H$
11 $R^1 = R^2 = CH_2OCH_3$

known to display the necessary chemical reactivity at these centres[3] and represent, therefore, possible candidates for the late methylation steps. The ready availability of the three truncated cobesters **6b–8b**[4] has now been exploited for an evaluation of the biochemical role of the corresponding acids.

Table 1 Incorporation experiments with cell-free system from *P. shermanii*

Experiment no.	Addition(s) to normal system	Activity in purified cobester fraction (%)
1	[5-^{14}C]-δ-Ala	22
2	[5^1-^{3}H]-15^1-noracid **7a**	<0.08
3	[15^1-^{3}H]-5^1-noracid **6a**	4.7
4	[15^1-^{3}H]-5^1-noracid **6a**	4.4
5	[15^1-^{3}H]-5^1-noracid **6a**	10.7
6	[15^1-^{3}H]-5^1-noracid **6a**	17.2
7	[15^1-^{3}H]-5^1-noracid **6a** + [methyl-^{13}C]-SAM	11
8	[methyl-^{14}C]-SAM + δ-Ala	12.5
9	[methyl-^{14}C]-SAM + 5^1,15^1-dinoracid **8a**	0.2
10	[methyl-^{14}C]-SAM	0.23
11	[methyl-^{14}C]-SAM; boiled preparation	<0.004

Regiospecifically tritiated samples of **6b** and **7b** were conveniently prepared as follows: oxidation of 5^1-norcobester **6b**[4] with lead tetraacetate in benzene followed by mild hydrolysis of the resulting 15^1-acetoxy intermediate gave the 15^1-hydroxy derivative **9b**, which upon treatment with cyanoborotritiide in DMF and in the presence of two mole equivalents of methanesulphonic acid was cleanly reduced to the desired [15^1-^{3}H] **6b**. The expected regiospecific location of the label was checked by NMR for the product of an experiment in which sodium cyanoborodeuteride was used as a reducing agent. By a similar procedure [5^1-^{3}H$_1$] **7b** was prepared from **7b**[4] *via* the 5^1-hydroxy derivative **10b**.

For the biological experiments HPLC purified specimens of the tritiated norcobesters were hydrolysed to the corresponding heptaacids by exposure to 2 M aqueous piperidine (NMR control) and the resulting Co(III)-aquo-complexes treated with H_2/Pt or, more conveniently, with an excess of dithiothreitol to generate the Co(II)-aquo species (UV control), which were then incubated with a cell-free system from *Propionibacterium shermanii*[5] (A.T.C.C. Nr. 9614) in the presence of an excess of unlabelled *S*-adenosyl-methionine (SAM). At the end of the incubation, cold cobyrinic acid was added and the product mixture obtained after re-esterification (5% conc. H_2SO_4 in methanol, room temp., 20–48 h) submitted first to TLC and then to HPLC purification. The isolated cobester fraction was admixed with an equivalent amount of the relevant norcobester and resubmitted to HPLC; this dilution–separation process was repeated until the radioactivity of the cobester fraction had reached a constant value. The results of several incubation experiments are summarized in the Table 1. The biological activity of the cell-free preparation was checked in each case by using either [5-^{14}C]-δ-Ala or [methyl-^{14}C]-SAM as a source of label (*cf.* experiments 1 and 8 for typical values).

Comparison of the results from experiments 1 and 2 makes it clear that an enzyme system that is quite active in catalysing the formation of cobyrinic acid from δ-Ala has virtually no effect on 15^1-norcobyrinic acid **7a**, which is, therefore, ruled out as precursor of **5a**. In contrast, the outcome of experiments 3 to 6 seemed at first to indicate that the 5^1-noracid **6a** is a fairly efficient precursor of cobyrinic acid. A subsequent attempt to corroborate this conclusion by independent means led, however, to a surprising result. The products from experiment 7, in which the radioactive 5^1-noracid had been incubated with the cell-free system in the presence of [methyl-^{13}C]-SAM, were purified by TLC to give a *ca.* 1:1 mixture of 5^1-norcobester **6b** and cobester **5b**. The ^{13}C NMR spectrum of this mixture disclosed the presence of strongly enriched signals corresponding to the cobester methyl group

at C-1, C-5, C-12, C-15 and C-17 and of lower intensity signals for the methyl groups at C-2 and C-7, but failed to reveal the specific accumulation of the ^{13}C label predicted for the 5^1-methyl on the basis of the apparent 11% incorporation of radioactivity observed for the HPLC purified cobester fraction from the same experiment. As, in addition, no ^{13}C-enrichment can be detected in the spectrum of the mixture for the methyl groups of the 5^1-norcobester component, it must be concluded that: (i) formation of ^{13}C-labelled cobyrinic acid in experiments 3–7 is entirely due to processing of endogenous precursors of type **1–4**; (ii) the cell-free system is not capable of generating 5^1-norcobyrinic acid **6a** from endogenous precursors, nor can it convert it into cobyrinic acid **5a**; (iii) the apparent incorporation of tritium observed for the cobester fraction in experiments 3–7 must be due to the presence of an impurity which differs from 5^1-norcobester **6b** and coincidentally displays the same HPLC behaviour as cobester **5b**.† In keeping with this last conclusion it was subsequently shown that treatment of the radioactive cobester fractions from experiments 3–6 with lead tetraacetate followed by methanolysis resulted in each case in the formation of probes of $5^1,15^1$-dimethoxycobester **11b**[4] containing only negligible residual activity.

Finally, an active role of the $5^1,15^1$-dinoracid **8a** in the formation of cobyrinic acid can also be excluded on the basis of experiments 9 and 10. Addition of unlabelled **8a**, obtained as above by hydrolysis of **8b**,[4] to the incubation mixture (experiment 9) fails to raise the percentage incorporation of label from ^{14}C-SAM into cobyrinic acid with respect to the blank of experiment 10. Nevertheless, the low incorporation values of the two experiments must be significant as evidenced by comparison with the outcome of experiment 11, in which a boiled enzyme preparation was used. Subsequent chemical degradation of the cobester fraction from experiment 9 gave a $5^1,15^1$-dinorcobester **8b**[4] retaining 61% of the original radioactivity. This matches the value expected on the basis of the hypothesis that the low incorporation represents the outcome of the methylation of endogenous precursors mainly of type **3** and **4**.

The observation that none of the three truncated corrins **6a**, **7a** and **8a** can act as precursor of cobyrinic acid in *P. shermanii* makes it most likely that the last two methylation steps in the biosynthesis of **5a** must precede the contraction step responsible for the formation of the corrin ring system.

It had been agreed with Professor Battersby that an independent investigation of this problem would be carried out by his team and the results of the Cambridge group are presented in the accompanying communication.[7] The matching outcome of these synchronized efforts casts serious doubts on the validity of a previous paper,[8] in which it was claimed without convincing analytical support that cobinamide, a close relative of **5a**, can be generated efficiently from its 5^1, 15^1-dinor and 15^1-nor counterparts.

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† The disturbing artefact has now been identified as the 8′-epi-isomer of **6b**, generated from the latter by non-enzymic means in the course of the hydrolysis–re-esterification procedure.[6]

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