Biosynthesis of Vitamin B_{12}: Order of the Later *C*-Methylation Steps

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Pulse labelling experiments with [*methyl*-^{13}C]*S*-adenosylmethionine (SAM) had previously shown that during the biosynthesis of vitamin B_{12}, the fourth *C*-methylation site is C-17; new inverted pulse studies show that the remaining four SAM-derived methyl groups are introduced in the order 12, 1, 15, 5.

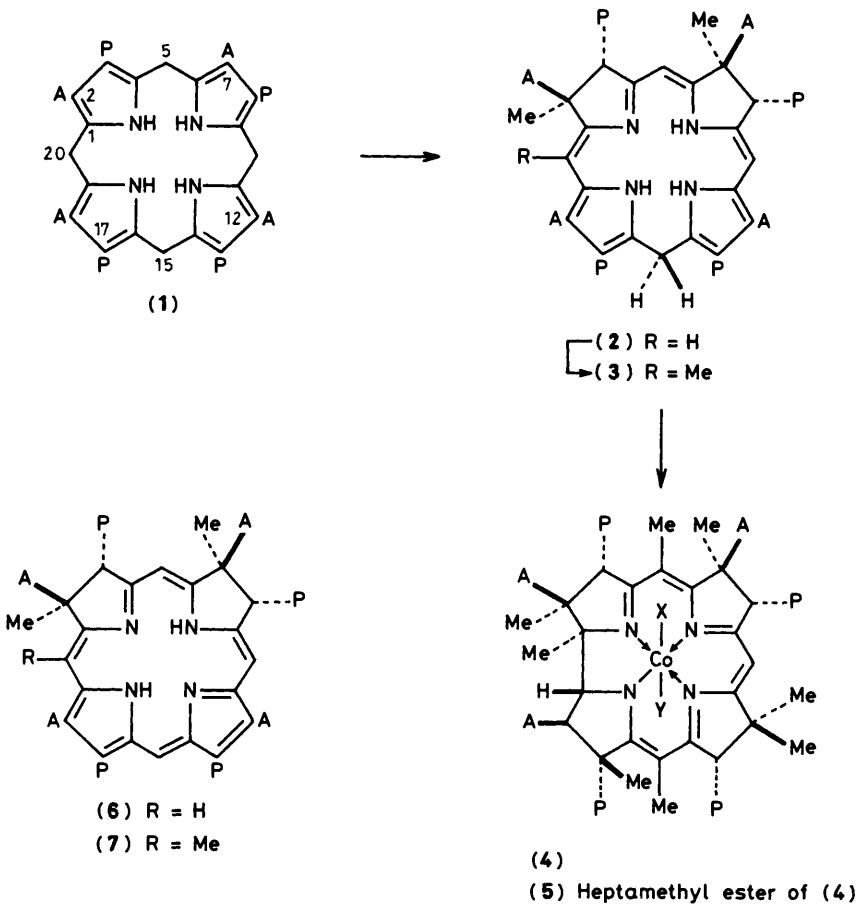
Biosynthesis of cobyrinic acid (**4**), the precursor of vitamin B_{12}, from uro'gen-III (**1**) involves the introduction of eight *C*-methyl groups by transfer from *S*-adenosylmethionine (SAM); these *C*-methylations occur at positions 1, 2, 5, 7, 12, 15, 17, and 20[1] [see structure (**1**)]. Earlier work based on isolation of intermediates and structure determination had shown[1] that the first three methylations occur in the order C-2, followed by C-7 and then C-20. The dimethylated intermediate was proved by isolation to be the dihydroisobacteriochlorin[2] (**2**) and since *C*-methylation does not affect the oxidation state, it is reasonable to assume that the trimethylated system is also formed at this same dihydro level (**3**). Under normal conditions of work-up (aerobic), the dimethylated and trimethylated intermediates are isolated as the aromatised macrocycles (**6**) and (**7**), respectively, but these can evidently be reduced again to the dihydro level by enzyme preparations from B_{12}-producing organisms.[1]

So far, no intermediates on the pathway to cobyrinic acid (**4**) have been isolated carrying 4, 5, or more *C*-methyl groups. Accordingly, we developed an approach based on pulse labelling which established[3,4] that the fourth *C*-methyl group is introduced at C-17 of the trimethyl system (**3**). It must be borne in mind that enzymic decarboxylation of the C-12 acetate residue in (**3**), which is necessary for the formation of

Table 1. Relative ^{13}C-incorporations[a] into *C*-methyl groups of cobyrinic acid (**4**).

	Position of methyl group				
	1	5	12	15	17
Cobester (**5**) from Inverted Pulse (IP)	100	95	106	102	155
Cobester (**5**) from Normal Pulse[b] (NP)	100	109	93	107	72
Difference[c] (IP − NP)	0	−14	13	−5	83

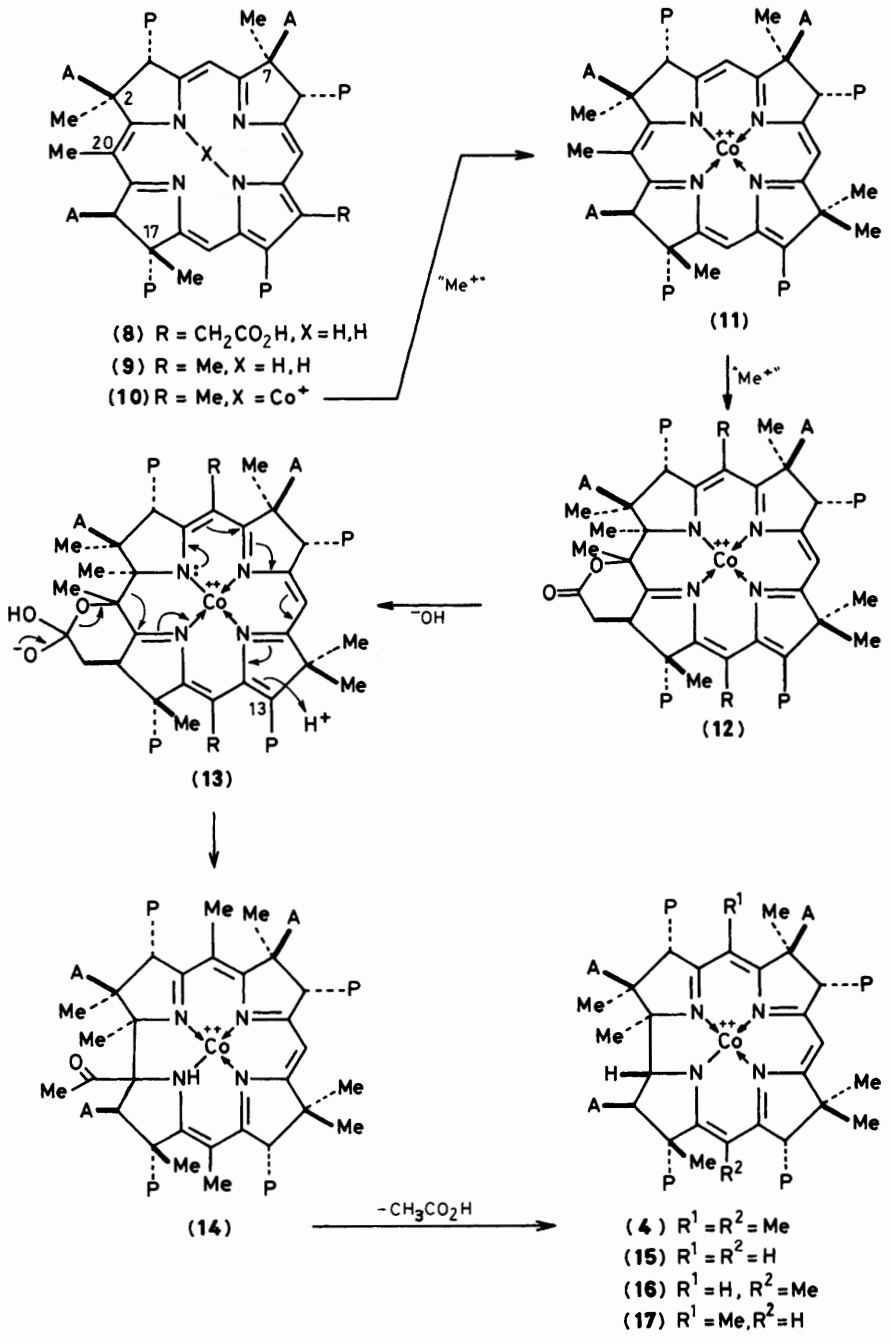
[a] The relative amounts of ^{13}C at each methyl group were obtained by comparison of the peak areas (obtained by line-fitting) with those from a standard sample of (**5**) uniformly labelled at all the SAM-derived methyl groups. The results are expressed as percentages of the ^{13}C relative value for the C-1 methyl group. Statistical analysis of many intensity determinations gave the worst error for each peak as <2%, mostly *ca.* 1%. [b] The samples for the Normal Pulse and the standard are those described in ref. 3. For each set of ^{13}C-determinations, the 3 spectra (IP, NP, and standard) were recorded consecutively under identical conditions. [c] The more positive this number, the earlier the corresponding methyl group is added in the biosynthetic sequence.



(**4**), may occur before the C-17 methylation. So the pulse labelling work showed that the tetramethylated intermediate is either the 12-acetate pyrrocorphin (**8**) or the 12-methyl pyrrocorphin (**9**).

The foregoing experiments made use of a cell-free enzyme system from *Clostridium tetanomorphum* which converts the dimethylated system (**6**) into cobyrinic acid (**4**). The enzyme preparation containing (**6**) was incubated with a deficiency of unlabelled SAM and after a period to allow *C*-methylated intermediates to be produced, a large excess of [*methyl*-^{13}C]-SAM was added (this is the Normal Pulse). Further incubation then generated cobyrinic acid (**4**) in which the *C*-methyl groups introduced early in the sequence could be detected by their having a lower ^{13}C-content (assay by ^{13}C n.m.r.[3] or by ^{1}H n.m.r. spectroscopy using a heteronuclear spin echo method[4]).

These studies have now been extended by performing the pulse labelling experiments in the inverted sense, *i.e.* adding the [*methyl*-^{13}C]SAM first and then, after an incubation period to generate intermediates, completing the biosynthesis of cobyrinic acid (**4**) by further incubation with a large excess of unlabelled SAM (Inverted Pulse). This approach requires the amount of endogenous SAM to be small relative to the amount of added [*methyl*-^{13}C]SAM and this was established first by dilution analysis. Now the *C*-methyl groups which are enzymically transferred early in the methylation sequence will have a higher ^{13}C-content and the later ones will have lower levels. The labelled product was isolated as cobester (**5**) and assayed by ^{13}C n.m.r. spectroscopy (see Figure 1). The high ^{13}C-content in the C-17 methyl group is clear by inspection, a result in agreement with our earlier finding[3] that the fourth methylation site is C-17. Such inspection is only sufficient for large differences in ^{13}C-content; for medium sized or smaller differences, it is essential to carry out accurate determinations of signal intensities and to compare them with those from a standard sample of (**5**) uniformly labelled at the SAM-derived methyl groups. Accordingly, the intensities of the signals from the other *C*-methyl groups were measured by several methods (*e.g.* integration, cutting and weighing, line fitting). The Lorentzian line fitting programme consistently gave values very close (within 1—2%) to the average from the other methods. Four long-term ^{13}C n.m.r. runs were carried out on each labelled sample followed by many determinations of peak area for each signal in every spectrum. The data in Table 1 are from one such set of determinations and the key values are the differences between the values from the Inverse and Normal Pulses since these increase the discrimination of the approach. The more positive this difference value, the earlier the corresponding methyl group is added in the biosynthetic sequence. It should be emphasised that the same methylation sequence emerged from the difference values for each of the 4 separate ^{13}C n.m.r. runs. The results extend our earlier



Scheme 1

findings beyond the fourth *C*-methylation at C-17[3] to showing the fifth is at 12, then C-1, 15, and 5.†

This knowledge allows some important features of the biosynthetic pathway beyond the tetramethylated intermediate (**8**) or (**9**) to be briefly picked out to help future research (Scheme 1). The stage on the pathway where cobalt is inserted is unknown but recent experiments using *Propionibacterium shermanii* indicate that this step is not the last one.[5] Also, Nussbaumer and Arigoni[6] found that 5,15-bisnorcobyrinic acid (**15**) and the 5-nor (**16**) and 15-nor (**17**) analogues are not incorporated into cobyrinic acid (**4**) by an active enzyme preparation from *P. shermanii*. These two studies together suggest that cobalt is inserted at some earlier stage. As one possibility, Scheme 1 shows cobalt insertion at the pyrrocorphin stage (**9**) but it is emphasised that the chemistry shown could also proceed on the metal-free macrocycles which at present must not be excluded.[7] Methylation of the pyrrocorphin (**10**) at C-12 yields the corphin[8] (**11**) and subsequent methylation at C-1 sets up the system (**12**) for ring-

† In the unlikely event that errors accumulated in one direction for all 4 runs, then the discrimination between the methylation order at C-1 and C-15 would become doubtful.

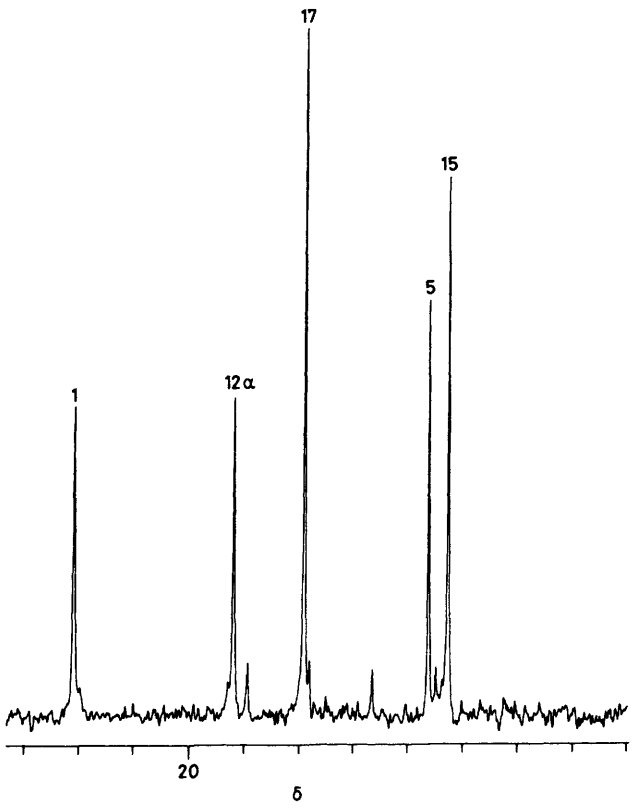


Figure 1. Proton-noise decoupled ^{13}C-spectrum showing enhanced signals from labelled cobester [as (**5**)] from the Inverse Pulse experiment with ^{13}C[SAM]. Spectrum recorded in C_6D_6 at 100.6 MHz with 1.5 Hz line-broadening.

contraction. The illustrated mechanism for the contraction (**13**)→(**14**) is based on Eschenmoser's important biomimetic model studies[9] together with the firm knowledge that during ring-contraction, C-20 and its attached methyl group are extruded as acetic acid.[10,11] It is not yet possible to indicate the methylation state at C-5 and C-15 over the stages (**12**) and (**13**) but by having both methyls in place on the 19-acetylcorrin (**14**), then the deacetylation step directly yields cobyrinic acid (**4**) ready for conversion finally into vitamin B_{12}.[1] Experiments to prepare and/or detect several of the key intermediates in Scheme 1‡ are in hand.

The pulse labelling approach has recently been adopted elsewhere[12] and used with our signal assignments for (**5**)[13] to confirm our earlier finding[3] that the fourth methylation site is at C-17 and to extend this to indicate the later order as 12, then 1 followed by 5 and 15 in unknown sequence.

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‡ Scheme 1 revised (March 1985) to include results in ref. 5.