BIOORGANIC CHEMISTRY 12, 356–362 (1984)

COMMUNICATION

Biosynthesis of Vitamin B_{12}: Timing of the Methylation Steps between Uro'gen III and Cobyrinic Acid

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Received July 2, 1984

The sequence of methylation between uro'gen III and cobyrinic acid has been defined by applying ^{13}C pulse-labeling methods to a cell-free system from *Propionibacterium shermanii*. Feeding experiments using unenriched S-adenosyl methionine ($^{12}CH_3$-SAM) followed by ^{13}C-enriched SAM ($^{13}CH_3$-SAM) (or vice versa) at various intervals caused differentiation in the ^{13}C NMR signals of the SAM-derived methyl groups in cobyrinic acid (isolated as cobester). Unenriched uro'gen III and sirohydrochlorin as substrates led to cobyrinic acid containing seven and five enriched methyl groups, respectively, which on NMR analysis gave as a sequence of methylation C-2 > C-7 > C-20 > C-17 > C-12α > C-1 > C-5 ≈ C-15.

The details of the pathway of corrin biosynthesis beyond the key intermediate uro'gen III (*1*) (**1**) are still incomplete. The first three enzymatic steps consist of successive C-methylations of uro'gen III at C-2 (*2*), C-7 (*3*), and C-20 (*4*), and the corresponding intermediates have been isolated in their stable aromatized forms (*5*), e.g., the 2,7-dimethyl isobacteriochlorin, sirohydrochlorin (**2**) and its 20-methyl derivative, Factor III (**3**). In spite of an intensive search spanning the last 10 years (*6*), the remaining steps are unknown with respect to isolation of intermediates containing four or more methyl groups (up to a possible of 8) but, as noted previously (*1*), the proven biochemical conversion of Factor III (**3**) to cobryinic acid (**4**) must involve the following events (see Scheme 1), not necessarily in the order indicated: (i) The successive addition of five methyls derived from *S*-adeno-

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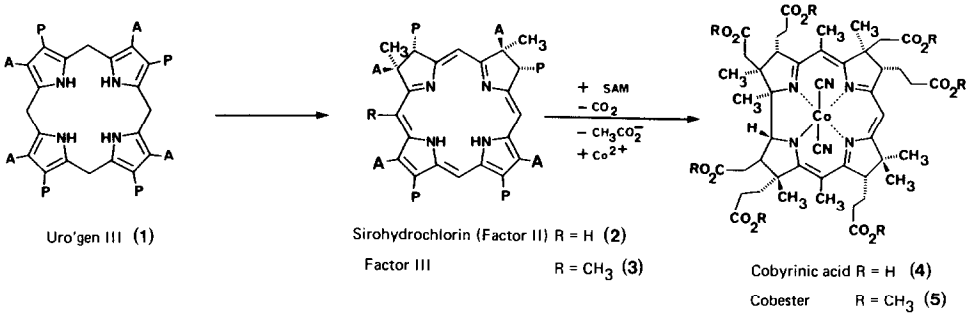


SCHEME 1

syl methionine (SAM) to Factor III (**3**); (ii) the contraction of the permethylated (seven or eight methyls) macrocycle to corrin; (iii) the extrusion of C-20 and its attached methyl group leading to the isolation of acetic acid (*7, 8, 4b*); (iv) decarboxylation of the acetic acid side-chain in ring C (C-12); and (v) insertion of Co^{2+}. In order to justify the continuation of our search for such intermediates whose inherent lability is predictable, we have applied ^{13}C pulse-labeling methods to the cell-free system from *Propionibacterium shermanii* (*9*), which converts uro'gen III (**1**) (*1, 9*) and sirohydrochlorin (**2**) (*3, 10*) to cobyrinic acid (**4**), a technique used previously in our laboratory to detect the flux of ^{14}C label through the intermediates of alkaloid biosynthesis in higher plants (*11*).

It was argued that the full methylation cascade could be differentiated in time, provided that enzyme-free intermediates accumulated in sufficient pool sizes to affect the resultant methyl signals in the ^{13}C NMR spectrum of the target molecule, cobyrinic acid (**4**), when the cell-free system is challenged with a pulse of $^{12}CH_3$-SAM followed by a second pulse of $^{13}CH_3$-SAM (or vice versa) at carefully chosen intervals in the total incubation time (9–10 hr). By this approach it should be possible to "read" the biochemical history of the methylation sequences as reflected in the dilution (or enhancement in the reverse experiment) of $^{13}CH_3$ label in the seven methionine-derived methyl groups of cobyrinic acid after conversion to cobester (**5**), whose ^{13}C NMR spectrum has been completely assigned (*1, 12, 13*).

The validity of the method was tested in a preliminary experiment using a two-phase system. $^{13}CH_3$-Methionine (90% ^{13}C; 30 mg) was added to a whole-cell (100 g) suspension of *P. shermanii* in phosphate buffer containing δ-aminolevulinic acid (20 mg), in the absence of Co^{2+}. Under these conditions the cells produce uro'gen III (**1**), sirohydrochlorin (**2**), and Factor III (**3**), but no corrin. Cell disruption and incubation of the extract (*10*) with added Co^{2+} and pulses of $^{12}CH_2$-SAM at varied time intervals was monitored for differential methylation by isolation of cobyrinic acid, conversion to cobester (**5**), extensive purification, and finally ^{13}C NMR analysis of the enriched samples (normally 50–150 μg). Optimization of these conditions led to the spectrum shown in Fig. 1, which reveals a clear distinction between the peak heights of the seven methyl groups of cobester when compared with a spectrum obtained by adding $^{13}CH_3$-SAM at the outset. The high

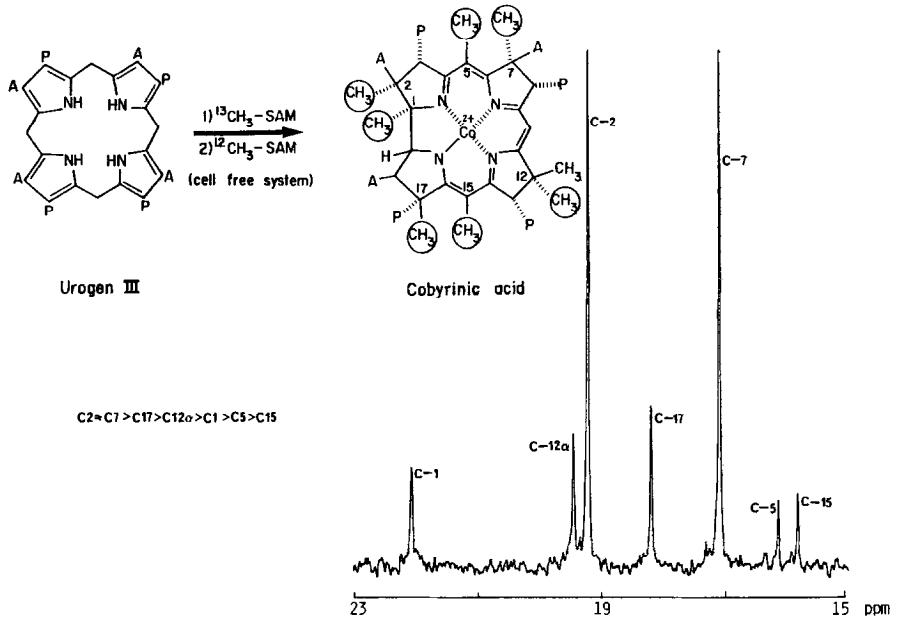


FIG. 1. 125-MHz, broad band, ^{1}H-decoupled (1 w), ^{13}C NMR spectrum of ^{13}C-enriched cobester (**5**) (270 μg in 0.5 ml KCN saturated benzene-d_6) isolated from *P. shermanii* (see text for incubation details). Chemical shifts are with respect to tetramethylsilane (TMS) at 0 ppm. Spectral parameters: spectral width, 30 ppm; pulse repetition rate, 0.7 sec; α, 65°. Spectrum represents the sum of 51,382 free-induction decays (FIDs) with 2.0-Hz line broadening.

relative intensities of the C-2 and C-7 signals bear testimony to the initial formation in whole cells of the 2,7-dimethyl isobacteriochlorin, sirohydrochlorin (**2**) in the absence of Co^{2+}. The differential dilution of methyl intensity in cobester not only suggests that free intermediates have accumulated, but that even in this qualitative experiment, the sequence of methylation is revealed as C-2 ≈ C-7 > C-17 > C-12α > C-1 > C-5 ≈ C-15 since the timing of the addition of $^{12}CH_3$-SAM dilutes the pool sizes of $^{13}CH_3$-enriched methylated intermediates in the order in which they are formed. Thus C-17 is the site of the fourth, C-12α the fifth, and C-1 the sixth methylation. Note that the experiment does not distinguish C-5 from C-15 (the seventh and eighth methylations) (*14*).

Finer tuning of the experiment was achieved in the reversed pulse mode using a porphyrin-free, cell-free system and unlabeled sirohydrochlorin (900 μg) (**2**) as substrate. Incubation with $^{12}CH_3$-SAM was followed by a pulse of $^{13}CH_3$-SAM (*15*) after an interval (ca. 6 hr) which allowed the accumulation of unlabeled intermediates. The resultant cobester (**5**) will bear only five enriched methyls, at C-1, C-5, C-12α, C-15, and C-17. The spectrum shown in Fig. 2a provides striking confirmation and extension of the preliminary experiment in that the relative intensities of C-12α and C-17 are much lower than that of C-1 which, in turn, is differentiated from C-5 and C-15 (*14*), i.e. the pulse of $^{13}CH_3$-SAM is diluted by an accumulated pool of unlabeled tetra- and pentamethyl intermediates, and becomes available for more efficient labeling of the hexa- → octamethyl

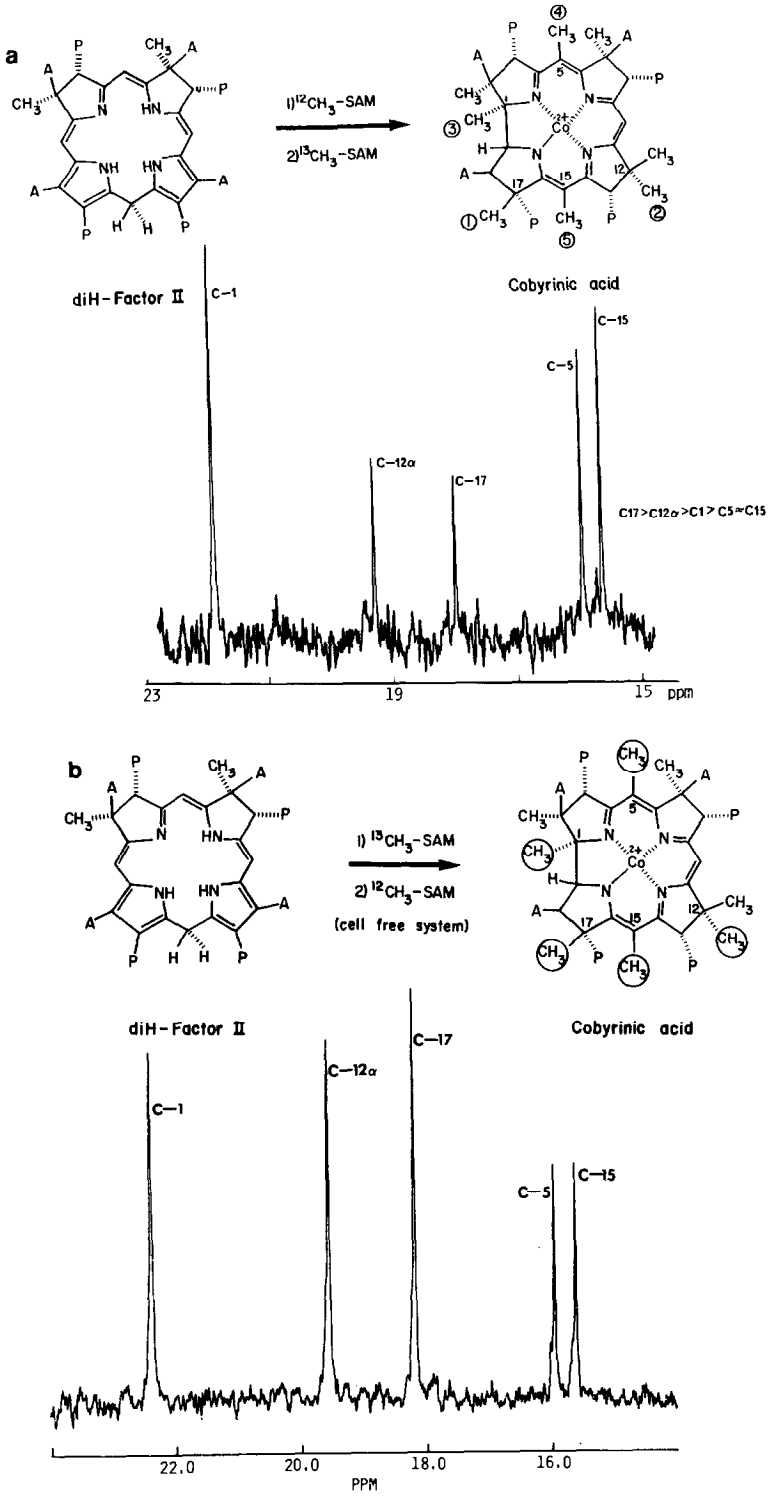
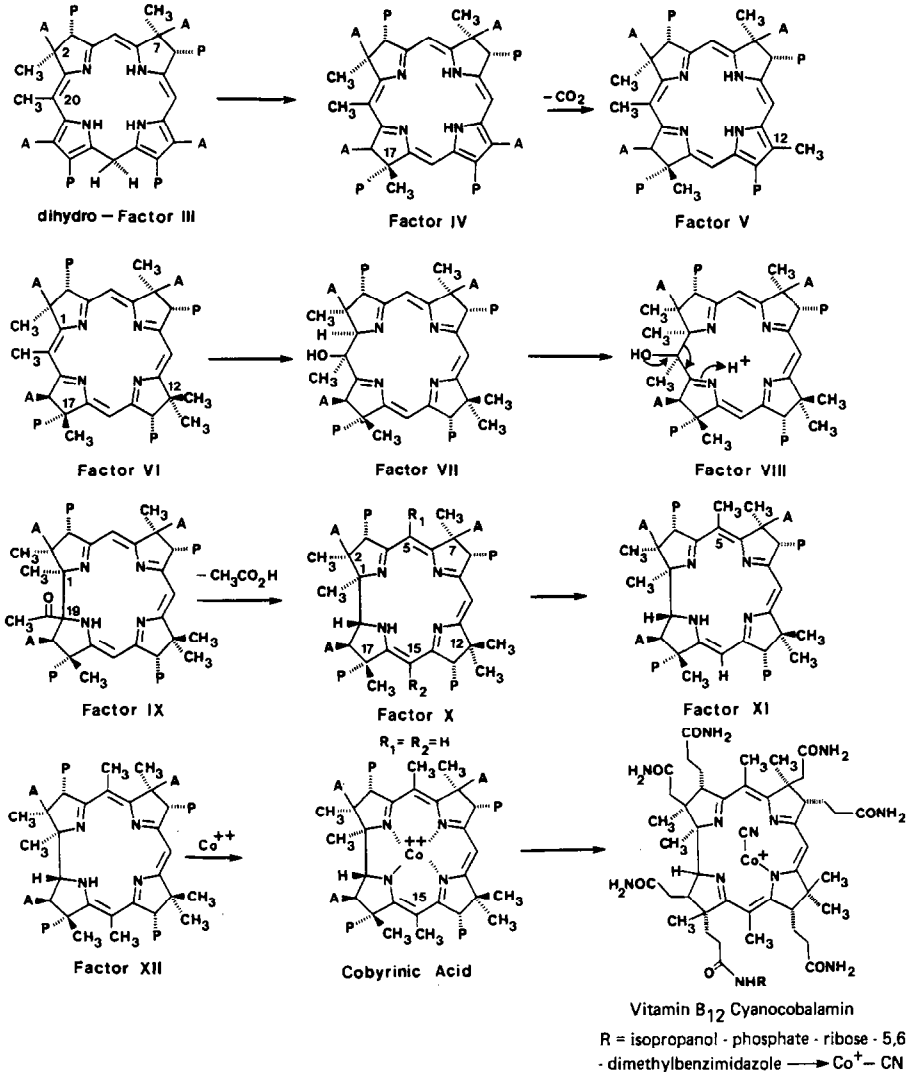


FIG. 2. (a) Spectrum of enriched cobester (5) (46 μg), accumulated as in Fig. 1, with 112,340 FIDs. See text for incubation conditions. (b) Spectrum of enriched cobester (5) (105 μg), accumulated as in Fig. 1, with 79,080 FIDs. See text for incubation conditions.



SCHEME 2

intermediates. Control experiments show that very early pulsing (0–60 min) leads to a spectrum of cobester showing approximately equal intensity of all five methyls after "normalization" for C-5 and C-15 (*14*) (Fig. 2b), whilst late addition (~9 hr) affords unlabeled cobester. Thus, the complete sequence of methylation of uro'gen III can be described as C-2 > C-7 > C-20 > C-17 > C-12α > C-1 > C-5 ≈ C-15, a result which complements and extends a report on the differentiation of C-17 as the fourth methylation site in a pulse experiment with extracts of a different bacterium, *Clostridium tetanomorphum* (*16*). On the basis of this sequence, we propose the structures shown in Scheme 2 as the targets of the search for the "missing" intermediates of corrin biosynthesis. For example, the pyrrocorphin structures suggested for Factors V and VI take into account the sequence C-17

methylation, decarboxylation of the C-12 acetate side-chain, and C-12α methylation. Similarly, the structures of Factors VII and VIII are proposed to rationalize the methylation of C-1 and the hydration of C-20 as a prelude to the ring contraction/acetic acid extrusion (→ Factor X) for which we have used Eschenmoser's *in vitro* analogy (*17*). The final methyl functionalization at C-5 and C-15 (Factors XI, XII) is suggested to precede Co^{2+} insertion (*1, 18*), although the NMR experiments do not reveal the timing of acetate extrusion or cobalt incorporation. However, the most important implications of the differential incorporations of SAM-derived methyl groups into cobester as a function of time are as follows: (a) Several stable enzyme-free intermediates must accumulate. (b) There appear to be *at least* four "methylases" responsible for the uro'gen–corrin connection. Methylase I (C-2, C-7), which has already been shown (*10*) to transform uro'gen III as far as the mono- and dimethyl intermediates Factors I and II (**2**), methylase II (C-20), methylase III (C-17, C-12α)[1], and methylase IV (C-1, C-5, C-15). (c) The fact that cobalt-deficient cells produce substantial amounts of C-2-, C-7, and C-20 methylated intermediates [as **(2)** and **(3)**] but insufficient tetra- → octamethyl intermediates to perturb the ratio of methyl labeling in cobester (Fig. 1) suggests that similar pulse experiments with Co^{2+} should define the point at which cobalt insertion takes place.

In summary, the experiments described herein suggest that the search for compounds related to the structures suggested in Scheme 2 can now proceed with confidence.

ACKNOWLEDGMENTS

We (A.I.S., P.J.S., P.E.F., and N.E.M.) thank NIH (AM32034 and GM32596), and the Robert A. Welch Foundation, and (G.M.) DFG for support of this work, which was initiated by a grant from SERC (to A.I.S., N.E.M., and G.M.) at the University of Edinburgh (1980–81).

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[1] *Note added in proof.* Further refinement of the experiment illustrated in Fig. 2a, where the incubation was now pulsed with $^{13}CH_3$-SAM after an interval of only 3–4 hr, afforded a spectrum of cobester (*5*) in which the signal C-17 had almost disappeared, leading to complete differentiation between the methylation times for C-17 (3–4 hr) and C-12α (4–6 hr). This implies that discrete methylating enzymes are involved at these two centers and (as discussed in the text) at C-2/7, C-20, C-1, and C-5/15. Thus, identification of a maximum of six discrete methylases and the possibility of isolating the corresponding intermediate structures shown in Scheme 2 must now be considered as viable targets of research in mapping the porphyrinogen → corrin pathway.

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