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Enzymatic Synthesis and Structure of Precorrin-3, a Trimethyldipyrrocorphin Intermediate in Vitamin B_{12} Biosynthesis†

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ABSTRACT: The trimethylated intermediate of vitamin B_{12} (corrin) biosynthesis, precorrin-3, was produced from various ^{13}C-enriched isotopomers of 5-aminolevulinic acid (ALA), using a multiple-enzyme system containing ALA dehydratase, porphobilinogen deaminase, uro'gen III synthetase, and the *S*-adenosyl-L-methionine- (SAM)-dependent uro'gen III methyltransferase (M-1) and precorrin-2 methyltransferase (M-2) in the presence of [^{13}C]SAM. Structural analysis of the resulting product, precorrin-3, reveals a close similarity to precorrin-2 but with several subtle differences in the conjugated array of C=C and C=N bonds which reflect the presence of the new *C*-methyl group at C20 and its influence on the electronic distribution in the dipyrrocorphin chromophore. The implications of this structure for corrin biosynthesis are discussed.

For the past 20 years, the elucidation of the biosynthetic pathway to vitamin B_{12} has proven to be a major experimental challenge to chemists and biochemists alike. The reasons for this are manifold but include the small number of known biosynthetic intermediates, which are mostly oxygen sensitive, and the complexity of their structures which had to be determined at the submilligram level. However, the recent localization of the genes required for the synthesis of B_{12} (Jeter & Roth, 1987; Crouzet et al., 1990a,b) has now paved the way for a more thorough understanding of approximately 20 enzyme-catalyzed steps involved in the formation of the corrinoids, which are among the most complex of natural products.

Cobyrinic acid (**6**), the simplest corrinoid precursor of B_{12}, is synthesized from 5-aminolevulinic acid (ALA)[1] by the shared pathway outlined in Scheme I via the first common

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[1] Abbreviations: ALA, 5-aminolevulinic acid; PBG, porphobilinogen; SAM, *S*-adenosyl-L-methionine; M-1 (or SUMT), uroporphyrinogen (uro'gen) III methyltransferase; M-2 (or SP_2MT), precorrin-2 methyltransferase; DTT, dithiothreitol; NMR, nuclear magnetic resonance.

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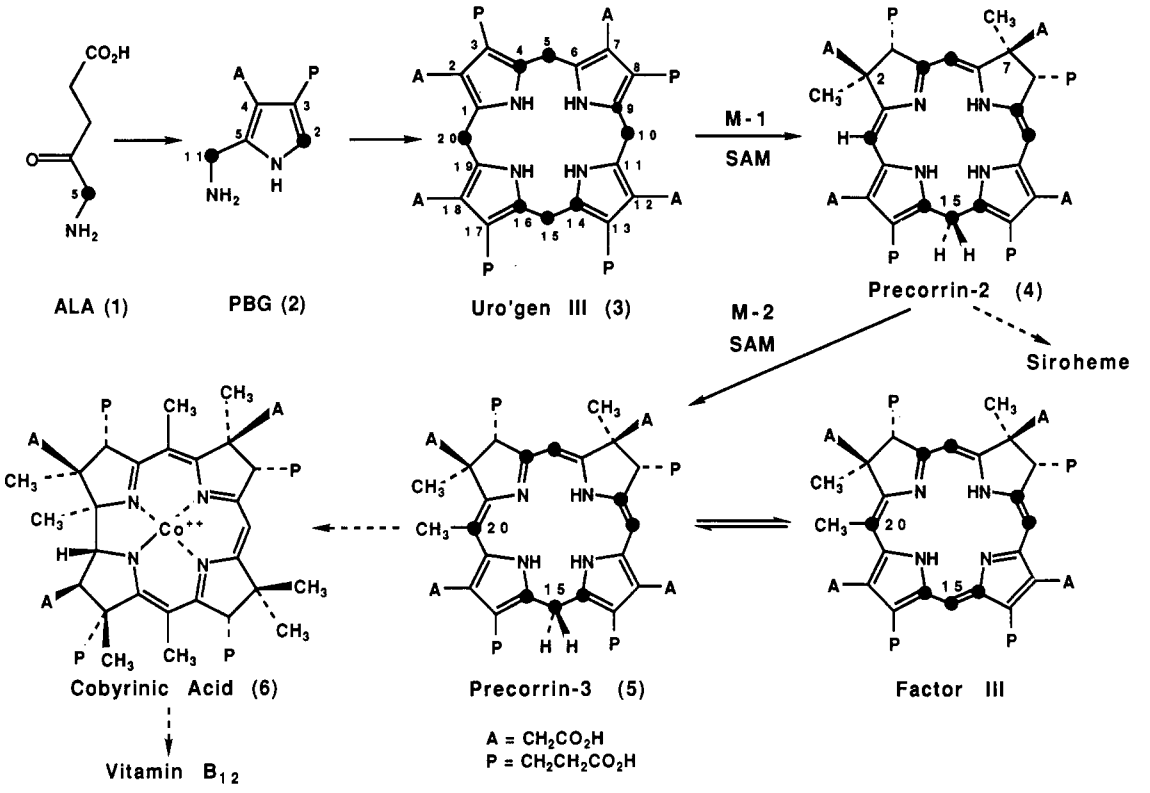
Scheme I



macrocyclic intermediate of all biologically derived tetrapyrroles, uroporphyrinogen III (**3**) [for a review, see Warren and Scott (1990)]. To divert this intermediate from the synthesis of heme and chlorophyll, the molecule is C-methylated at positions 2 and 7 by the *S*-adenosylmethionine-(SAM)-dependent uroporphyrinogen III methyltransferase (M-1 or SUMT) (Warren et al., 1990a; Blanche et al., 1989). This reaction has recently been studied by NMR (Warren et al., 1990b), which revealed the true tautomeric form of the product, the dimethylated dipyrrocorphin, precorrin-2 (Battersby et al., 1982). Methylation at C20 of precorrin-2 by the second methyltransferase enzyme, precorrin-2 methyltransferase (M-2 or SP_2MT) (Crouzet et al., 1990a) commits the pathway irreversibly toward cobyrinic acid by forming precorrin-3, which in principle can exist in several tautomeric forms including the trimethyldipyrrocorphin structure (**5**), which for simplicity is shown in Scheme I with the same double-bond arrangement as that of **4**. This intermediate has, until now, been studied only in its oxidized form, i.e., the trimethylisobacteriochlorin, factor III, which is converted to cobyrinic acid in cell-free systems presumably containing an enzyme capable of reducing factor III to precorrin-3.

Recent reports (Crouzet et al., 1990a; Thibaut et al., 1990a) have described the isolation and nucleotide sequence of the *cobI* gene, purification of the associated gene product from *Pseudomonas denitrificans*,[2] and the demonstration that the *cobI* gene product is precorrin-2 methyltransferase (M-2) without, however, defining the locus of the methyl transfer or the exact structure and oxidation level of the enzymatic product. This latter point is of importance since it is possible to transform oxidized precorrin-3 (the trimethylisobacteriochlorin, factor III) into cobyrinic acid with a cell-free system (Müller et al., 1981). This discrepancy in the oxidation level of the intermediate has been explained in the past by invoking the presence of an enzyme that is able to reduce the isobacteriochlorin in the crude enzymatic mixture (Scott et al., 1978; Battersby et al., 1982). To clarify the reaction catalyzed by M-2, we have performed NMR studies of the C20 methyl-transfer reaction in order to obtain direct, rigorous proof for the structure of precorrin-3 and the mechanism of its formation.

Material and Methods

Chemicals. [5-^{13}C]-, [4-^{13}C]-, and [3-^{13}C]-5-aminolevulinic acid (ALA) isotopomers were prepared as previously described (Pfaltz & Anwar, 1984; Kurumaya et al., 1989). Specimens of [2,11-$^{13}C_2$]-, [3,5-$^{13}C_2$]-, and [4,6-$^{13}C_2$]porphobilinogen (PBG) were prepared from the appropriately labeled ALA using highly purified ALA dehydratase (Jordan & Berry, 1982). *S*-Adenosyl-[*methyl*-^{13}C]-L-methionine was prepared as previously described (Hegazi et al., 1978). All other chemicals were purchased from Sigma Chemical Co. and were of the highest grade obtainable.

Enzyme Preparations. ALA dehydratase, PBG deaminase, uroporphyrinogen III synthase, and uroporphyrinogen III methyltransferase (M-1) were purified from recombinant *Escherichia coli* strains as previously described (Warren et al., 1990a). *S*-Adenosylhomocysteine hydrolase was purified from a strain harboring a plasmid containing the rat *S*-adenosylhomocysteine hydrolase gene as previously reported (Gomi et al., 1989). Adenosine deaminase, type VI from calf intestine, was purchased from Sigma Chemical Co.

Expression and Purification of Precorrin-2 Methyltransferase (M-2). Precorrin-2 methyltransferase was expressed by cloning the *P. denitrificans cobI* gene into an *E. coli* expression vector, pHN1+, using the expression cassette polymerase chain reaction (ECPCR) technique (MacFerrin et al., 1990). The PCR 5′ primer was synthesized to contain a *Bam*HI restriction enzyme recognition site, a strong *E. coli*

[2] We have also recently isolated precorrin-2 methyltransferase (M-2) (M_r 26 000) as the gene product of the 12th open reading frame (*cbiL*) of the cobinamide operon (CobI) of *S. typhimurium* (unpublished results). Full details of this work will be published elsewhere.

ribosome binding site, and the first 10 codons of the *cobI* gene. The 3′ PCR primer was synthesized to contain the last 10 codons of the *cobI* gene, a stop codon (TAA), and a *Sal*I restriction enzyme recognition site. The primers were incubated with *P. denitrificans* genomic DNA in a PCR reaction using a PCR kit purchased from Perkin Elmer-Cetus. The PCR product and pHN1+ plasmid DNA were cut with *Bam*HI and *Sal*I, ligated together, and transformed into *E. coli* strain TB1 (Baldwin et al., 1984). The resultant strain, CR333, was used to purify the *cobI* gene product.

For purification of the *cobI* gene product, LB medium containing 50 μg/mL ampicillin was inoculated with CR333 and incubated overnight in a 37 °C rotary shaker. The cells from 8 L of culture were collected by centrifugation, resuspended in 80 mL of PE buffer (100 mM KH_2PO_4, 2.0 mM EDTA, adjusted to pH 8.0 with NaOH) containing 50 μg/mL lysozyme, incubated at room temperature for 30 min, and lysed by sonication. The lysate was centrifuged at 12000*g* for 10 min, and the supernatant was adjusted to 50% ammonium sulfate. The precipitate was collected by centrifugation, dissolved in 20 mL of PE buffer, and loaded onto a Sephadex G-100 gel filtration column (5 × 80 cm). The column was washed with PE buffer, and the fractions containing the expressed protein (determined by SDS–PAGE) were pooled, concentrated by ultrafiltration, and loaded onto a MonoQ 10/10 anion-exchange column (Pharmacia). The column was developed with a linear gradient of 0–250 mM KCl in 25 mM potassium phosphate buffer, pH 8.0, using a Waters advanced protein purification system. Fractions that eluted with about 180 mM KCl contained the expressed protein and were pooled, concentrated, and stored at −20 °C in 50% glycerol.

Precorrin Synthesis. Precorrin-2 and precorrin-3 were synthesized by employing the following incubation conditions in either the absence or presence (10 mg) of the *cobI* gene product, respectively. A total of 100 mL of degassed buffer (50 mM Tris-HCl, pH 8.0, 5 mM DTT, 100 mM KCl), containing 3 mg of the appropriately labeled PBG, 5 mg of PBG deaminase, 2 mg of uroporphyrinogen III synthase, 4 mg of uro'gen III methyltransferase, 3 mg of *S*-adenosylhomocysteine hydrolase, 50 units of adenosine deaminase, and 20 mg of SAM was incubated under anaerobic conditions in an argon-purged glove box for 6–12 h. During the reaction period, the uroporphyrinogen III concentration was monitored by removing aliquots and analyzing by HPLC the amount of uroporphyrin present (Rideout et al., 1983). The reaction was stopped when the amount of uroporphyrin was <5% of the equivalent starting concentration of PBG. This is important not only to maximize the consumption of uro'gen III but also to prevent overmethylation of the resulting precorrins by uro'gen III methyltransferase (M-1) to their corresponding pyrrocorphins (Warren et al., 1990a,b). Samples of precorrin-2 or precorrin-3 were then isolated from the reaction mixture by binding to a small column (1 × 1.2 cm) of DEAE-Sephadex, followed by successive washes with H_2O and 0.2 M NaCl, and eluting the yellow precorrin fraction in a minimal volume (2–3 mL) of 2 M NaCl solution containing 20% D_2O.

Other Techniques. Factor III octamethyl ester was prepared by adsorbing precorrin-3 on DEAE-Sephadex A-25 and lyophilizing the support, followed by esterification in MeOH/H_2SO_4 (95:5). HPLC analysis was performed using a C_{18} reverse-phase silica column with an isocratic solvent system of methanol/water (85:15). Sirohydrochlorin and factor III were extracted from *Propionibacterium shermanii* as previously described (Müller et al., 1981). The methyl esters of these compounds were formed (MeOH/H_2SO_4 95:5) and reduced by catalytic hydrogenation (Pt) in ethyl acetate, and the corresponding dipyrrocorphins were hydrolyzed in 2 M piperidine (10 h). All procedures were performed anaerobically (<5 ppm O_2).

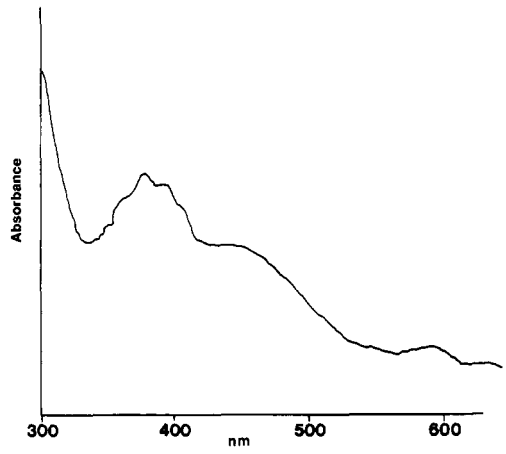


FIGURE 1: UV/visible spectrum of precorrin-3 obtained under anaerobic conditions from the multi-enzyme system containing precorrin-2 methyltransferase (M-2).

NMR Spectroscopy. ^{13}C NMR spectra were acquired at 75.47 MHz on a Bruker WM-300 wide-bore spectrometer equipped with a 10-mm selective probe head and an Aspect 2000 computer. Precorrin samples (in ~3 mL of 2 M NaCl containing 20% D_2O) were transferred to a 10-mm sample tube, and the tube was capped and tightly wrapped with parafilm before removal from the glove box. Spectra were recorded at ambient temperature using bilevel WALTZ proton decoupling to minimize sample heating. Approximately 45° pulse width, 1.5-s repetition delay, and 0.25-s acquisition time were used. In most cases, the 8K FID was subjected to 10-Hz exponential line broadening before Fourier transformation. When accurate coupling constants were required, the FID was first zero-filled to 16K and only 2.5-Hz line broadening applied.

RESULTS

Expression and Purification of the cobI Gene Product. Cloning of the *P. denitrificans cobI* gene into *E. coli* plasmid vectors resulted in the expression of a protein with M_r 26000, as visualized by SDS–PAGE analysis of a crude cell lysate of CR333 (data not shown). The molecular weight of the new protein is in close agreement with that reported for the purified protein SP_2MT (Thibaut et al., 1990a) and with the predicted subunit molecular weight derived from the sequence data (Crouzet et al., 1990b). The enzyme was purified to ca. 90% by a modification of the procedure reported by Thibaut et al. (1990a) and was free of any contaminating uroporphyrinogen decarboxylase activity.

Precorrin-3 Activity. The cell lysate of CR333 was tested for M-2 activity by incubation with radiolabeled [*methyl*-^3H]SAM and precorrin-2 (prepared by catalytic hydrogenation and hydrolysis of sirohydrochlorin octamethyl ester). These incubations led to incorporation of tritium into the product only with cell extracts containing the *cobI* gene product. Further confirmation of M-2 activity came from incubations with a coupled enzyme system, containing PBG, SAM, PBG deaminase, uroporphyrinogen III synthase, M-1, and M-2. A sample of the latter incubation mixture after reaction was transferred to a cuvette and sealed with parafilm prior to removal from the glove box. The UV/visible spectrum of the sample (Figure 1) is typical of that of a dipyrrocorphin

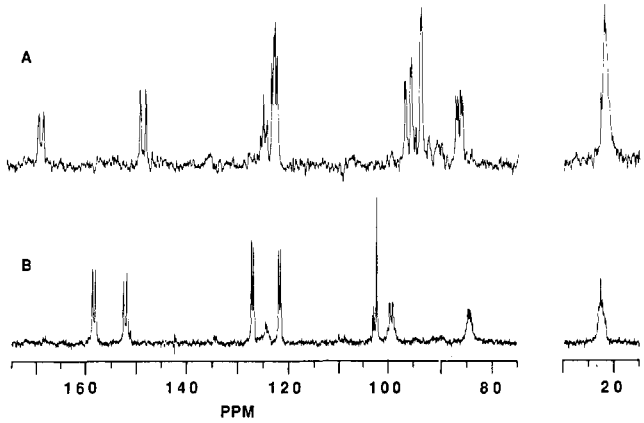


FIGURE 2: 75.47-MHz ^{13}C NMR spectra of (A) precorrin-2 (**4**) and (B) precorrin-3 (**5**) derived from [2,11-$^{13}C_2$]PBG and the multi-enzyme system (B) with, and (A) without, precorrin-2 methyltransferase (M-2). The precorrin samples were maintained in 2 M NaCl containing 20% D_2O under argon atmosphere (see text for complete details and Scheme I for labeling pattern).

(Leumann, 1986), as expected for precorrin-3. Oxidative esterification of the lyophilized DEAE mixture of these incubations afforded the product (80–85% yield based on PBG) which, when further characterized by TLC, HPLC, UV/visible spectroscopy, ^1H NMR spectroscopy, and mass spectrometry, was indistinguishable from the octamethyl ester of authentic factor III isolated from *P. shermanii*. These results confirm that the *cobI* gene had been successfully cloned and that the protein expressed in the heterologous *E. coli* system was active.

^{13}C NMR of Precorrin-2 and Precorrin-3 Derived from [2,11-$^{13}C_2$]PBG. To monitor the conversion of precorrin-2 (**4**) to precorrin-3 (**5**), samples derived from PBG (**2**) specifically ^{13}C-enriched at C2 and C11 (●, Scheme I), would yield the most diagnostic labeling pattern for the detection of methylation at C20. Accordingly, [2,11-$^{13}C_2$]PBG was incubated in the coupled-enzyme system described in the Materials and Methods section in either the presence or absence of M-2 and the products **4** and **5** compared by NMR. In the absence of M-2, the ^{13}C NMR spectrum of the resulting precorrin-2 (Figure 2A) displayed the coupling pattern characteristic of uro'gen III derived systems. The singlet carbon resonance at 94.3 ppm can be unambiguously assigned to C20, the only ^{13}C-enriched carbon position without an immediately adjacent enriched neighbor. The remaining two doublets in the sp^2 meso region (δ 86.8 and δ 96.8) can be assigned to meso positions C5 and C10, respectively, and are directly coupled to C4 (168.3 ppm, J = 69 Hz) and C9 (148.4 ppm, J = 81 Hz). The fourth meso carbon, C15, is observed upfield at 21.9 ppm as a broad triplet, due to direct coupling with both C14 and C16 (δ 123), thus confirming the structure of precorrin-2 as a 15-dihydroisobacteriochlorin with the dipyrrocorphin chromophore **4**.

When the above reaction was repeated in the presence of M-2, **4** was converted to a second dipyrrocorphin whose ^{13}C NMR spectrum is shown in Figure 2B. This spectrum also displays the uro'gen III coupling pattern; however, C20 is shifted 9 ppm downfield to δ 103.0, indicative of C-alkylation. A similar downfield chemical shift upon methylation of C20 was previously observed in factor III, the oxidized counterpart of **5** (Müller et al., 1981). Furthermore, when the experiment was repeated with [*methyl*-^{13}C]SAM, C20 was observed as a doublet centered at δ 17.6 (not shown) due to one bond coupling (J = 45 Hz) with the attached ^{13}C-enriched methyl group. Importantly, C15 is once again located in the sp^3 methylene region, indicating that the 15-dihydroisobacterio-

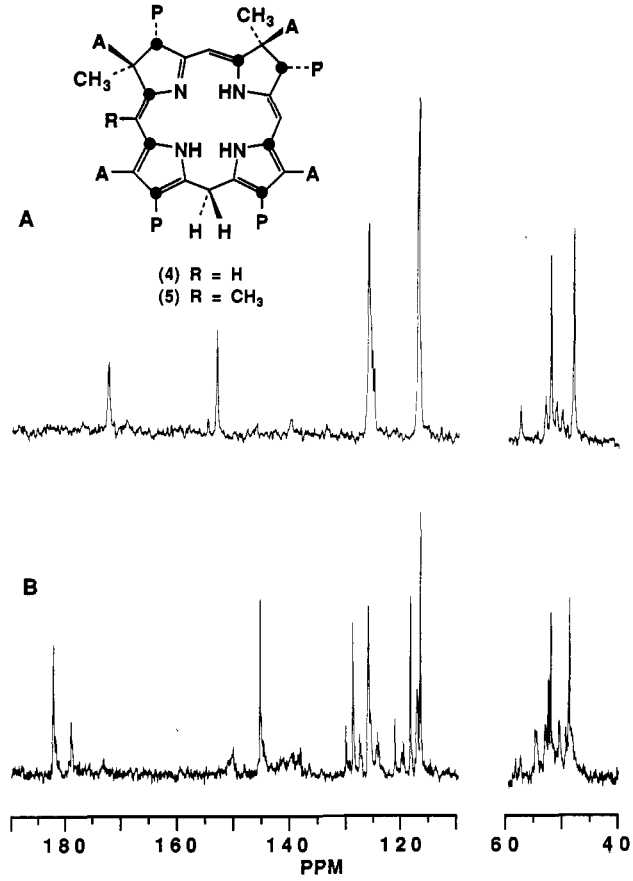


FIGURE 3: 75.47-MHz ^{13}C NMR spectra of (A) precorrin-2 (**4**) and (B) precorrin-3 (**5**) derived from [3,5-$^{13}C_2$]PBG, prepared as described in the legend of Figure 2.

chlorin structure of **4** is preserved in precorrin-3 (**5**). The chemical shifts of the remaining [2,11-$^{13}C_2$]PBG-derived carbons of **5** convey structural similarity to **4**; however, subtle differences are observed. Notable are the signals for C14 and C16, which are nearly equivalent in **4** but are separated by over 5 ppm in **5**, presumably due to the dissymmetry created by methylation at C20. On the other hand, C4 and C9, observed at distinctly different chemical shifts in **4**, display almost equivalent chemical shifts in **5**.

^{13}C NMR of Precorrin-2 and Precorrin-3 Derived from [3,5-$^{13}C_2$]- and [4,6-$^{13}C_2$]PBG. To complete the assignment of the remaining macrocyclic carbons, **4** and **5** were prepared from two other isotopomers of PBG. Comparison of the ^{13}C NMR spectra of **4** and **5** derived from [3,5-$^{13}C_2$]PBG (Figure 3) revealed that both structures are characterized by two sp^3 carbons (C3 and C8) and six sp^2 carbons, four of which display chemical shift values typical of α- and β-substituted pyrrolic carbons, lending further support to the dipyrrocorphin structure. In the case of **4**, the pairs of α-pyrrolic (C11 and C19) and the β-pyrrolic carbons (C13 and C18) were found to be equivalent (Figure 3A), giving rise to two signals located at 127 and 118 ppm, respectively, while in **5**, four separate signals are observed (Figure 3B). In addition, the remaining two sp^2 carbons (C1 and C6) of **5** display a much larger chemical shift spread in comparison to those of **4**. In **4**, the C1, C6 pair is observed at 173.5 and 154.1 ppm, while in **5** these carbons shift in opposite directions to the extreme values of 183.2 and 146.2 ppm. It is interesting to note that the changes observed in C1 and C6 between **4** and **5** are similar but are shifted in the opposite direction to those noted for C4 and C9 (Figure 2), suggesting that the origin of the chemical shift differences between **4** and **5** may result from different tautomeric forms.

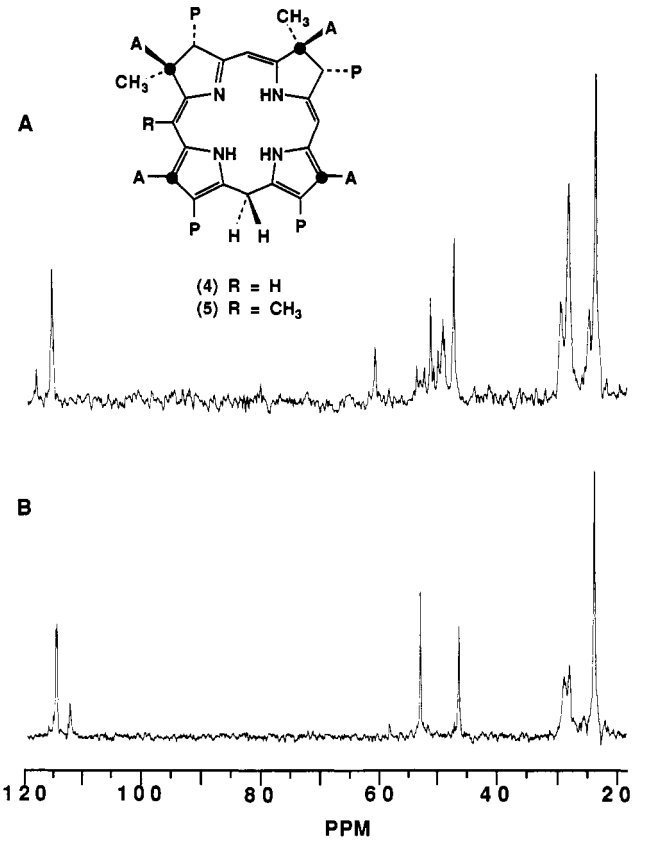


FIGURE 4: 75.47-MHz ^{13}C NMR spectra of (A) precorrin-2 (**4**) and (B) precorrin-3 (**5**) derived from [4,6-$^{13}C_2$]PBG, prepared as described in the legend of Figure 2.

Finally, when precorrin-3 (**5**) was synthesized from [4,6-$^{13}C_2$]PBG, two sp^2 (C2 and C7) and two sp^3 (C12 and C18) signals associated with the main macrocyclic structure (Figure 4B) were observed. The other signals arising from the peripheral sp^2 carbons in the propionate side chains can be seen in the 20–30 ppm region. This spectrum is very similar to the spectrum of precorrin-2 (Figure 4A) except that the two sp^2 signals are no longer quite superimposable and both have moved slightly downfield.[3]

Discussion

Although the structures of the *oxidized* C-methylated intermediates of B_{12} biosynthesis, viz., factors II and III, and the requirement for their reduction before bioconversion to corrin have been suggested for some years (Scott et al., 1978, Müller et al., 1981), it has only recently been established (Warren et al., 1990b) that reduced (dihydro) factor II exists as the direct product of C-methylation of uro'gen III (by M-1 or SUMT) as the dipyrrocorphin tautomer (**4**) in which C15 is reduced from methine (sp^2) to methylene (sp^3) as previously suggested by isolation experiments (Battersby et al., 1982). Although it has been assumed that precorrin-3 (**5**) exists in the same tautomeric form (as **4**), there is no direct evidence that this species is the true product of the action of M-2. The use of ^{13}C labeling has now allowed the solution of this important problem of structural definition together with the acquisition of the biosynthetic enzymes for each step, in high yield and purity, which allow the synthesis of precorrin-3 in over 80% yield. It has also been possible to overcome the major obstacle in the reaction catalyzed by these SAM-dependent methyltransferases, viz., the inhibitory action of the byproduct *S*-adenosylhomocysteine (Blanche et al., 1989), by converting the *S*-adenosylhomocysteine into homocysteine and inosine by the coupled enzyme system using *S*-adenosylhomocysteine hydrolase and adenosine deaminase. Also, in order to prevent overmethylation by M-1, incubations were monitored for a decrease in the concentration of uroporphyrinogen III. This precaution is vital because once uroporphyrinogen III has been utilized, M-1 is capable of further nonspecific C-methylation of precorrin-3 into tetramethylpyrrocorphin structures (unpublished results) just as M-1 overmethylates precorrin-2 (Warren et al., 1990a,b).

Table I: ^{13}C NMR Chemical Shifts[a] of Precorrin-2 and Precorrin-3 Derived from ^{13}C-Labeled PBG

	precorrin-2 (**4**)	precorrin-3 (**5**)
C15	21.90	21.88
C2,7[b]	49.03, 53.13	49.61, 52.9
C3,8[b]	46.28, 50.36	45.17, 51.93
C5	86.75 (J = 69 Hz)	84.53 (J = 74 Hz)
C10	96.75 (J = 81 Hz)	100.02 (J = 81 Hz)
C20	94.29	103.04
C12,18[b]	116.47[c]	115.46, 115.62
C13,17[b]	118.11[c]	117.55, 118.13
C14	122.62 (J = 49 Hz)	122.13 (J = 51 Hz)
C16	123.05 (J = 49 Hz)	127.48 (J = 51 Hz)
C11,19[b]	127.03[c]	126.90, 129.62
C9	148.42 (J = 81 Hz)	152.59 (J = 81 Hz)
C1	154.08	146.17
C4	168.31 (J = 69 Hz)	159.06 (J = 74 Hz)
C6	173.47	183.17

[a] Referenced to internal dioxane (δ 66.5). For numbering see Scheme I. [b] Not unambiguously assigned. [c] Represents two carbons.

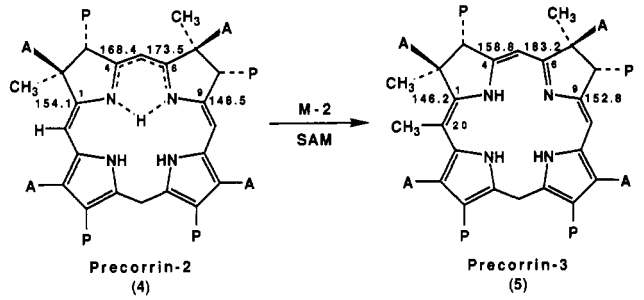
Our initial reports on the reaction catalyzed by the *cysG* gene product, M-1, showed that as the reaction catalyzed by M-1 proceeds, the UV/visible spectrum of the reaction changes from that of uro'gen III to a dipyrrocorphin (precorrin-2). The reaction catalyzed by M-2 in the same coupled enzyme system also led to the spectrum of a dipyrrocorphin, since methylation at C20 of precorrin-2 does not lead to a major change in the chromophore (Figure 1). The latter spectrum is the first reported UV/visible data for precorrin-3 and provides the necessary evidence that there has been no change in oxidation level or chromophore during the transformation of precorrin-2 to precorrin-3.

^{13}C NMR Studies on Precorrin-2 and Precorrin-3. The ^{13}C chemical shifts of the ring carbons of precorrin-2 and precorrin-3 derived from three isotopomers of PBG are summarized in Table I. Several important conclusions regarding the structures of precorrin-2 and precorrin-3 can be made via comparison of their chemical shifts. First and foremost in verifying the structure of precorrin-3 as **5** is the nearly 10 ppm downfield chemical shift difference observed for C20 upon methylation of **4**, analogous to that observed for the oxidized counterpart, factor III. The incorporation of a SAM-derived ^{13}C-labeled methyl group at C20, as evidenced by the C–C coupling of C20 in precorrin-3, provides direct evidence that M-2 catalyzes the SAM-dependent methylation of precorrin-2 at C-20.

Second, the observation of a triplet carbon at δ 21.9 ppm in both **4** and **5** derived from [2,11-^{13}C]PBG (Scheme I) indicates that both precorrins exist as 15-dihydropyrrocorphins,

[3] The spectrum of precorrin-2 derived from [4,6-$^{13}C_2$]PBG (Figure 4A) displays some differences from those published earlier (Warren et al., 1990b). Precorrin-2 is highly susceptible to oxidation, which can occur during prolonged data acquisition (16–20 h) while the sample is in the NMR tube. To overcome this problem, all spectra in this study were taken in 2-h blocks and initial data were compared to later data in order to observe any decomposition. Thus, the precorrin-2 spectra reported earlier, although correct in structural assignment, contain several spurious signals as a result of oxidation of precorrin-2 during overnight data acquisition.

Scheme II



in which the C15 resonance is coupled to those at C14 and C16. Moreover, the chemical shift values found for the ring C and D carbons, i.e. C11, 14 and C16, 19, both precorrins are dipyrrocorphins, thus confirming the proposed chromophores for precorrin-2 and -3 shown in Scheme I.

It is of further interest to note that the majority of the chemical shifts differences observed between precorrin-2 and precorrin-3 can be attributed to the minor changes in electronegativity and additional disruption of symmetry associated with methylation at C20. This is especially true for the southern half of the molecule, i.e., C10–C20, where, in precorrin-3, methylation at C20 has disrupted the chemical shift equivalence observed for the pyrrolic carbons of precorrin-2. The chemical shift difference between C1, -4, -6, and -9 in precorrin-2 and precorrin-3, however, seem to lie well outside of the range expected solely due to methylation at C20. In this case, the differences result instead from a different tautomeric arrangement of the C=C and C=N bonds in the northern half of the molecule.

The effect of tautomerization on chemical shift can be more easily assessed through assignment of C1, -4, -6, and -9 of precorrin-2 and precorrin-3. We can unambiguously assign both C4 and C9 in **4** and **5** due to direct C–C coupling to their adjacent meso carbons, C5 and C10, respectively. On the other hand, the assignment of the C1, C6 pair is less direct. But by comparison of chemical shifts of α-pyrrolic carbons in a number of reduced ring systems, and by symmetry to C9, C1 can be assigned with confidence in both precorrins to the upfield signal (δ 154.08 for **4**, δ 146.17 for **5**) of the C1, C6 pair. Accordingly, the extreme downfield shift of 183.17 ppm for C6 of **5** arises from the carbon of an imine bond, rather than a C=C meso double bond, suggesting the 4,5 double-bond arrangement shown in Scheme II. The chemical shifts for C4 and C6 of **4**, on the other hand, appear to reflect a more time-averaged structure, suggesting an equilibrium of both tautomers. Further support for this lies in closer inspection of the line widths of ^{13}C resonances of precorrin-2 and precorrin-3. In **4** the line widths of both C4 and C6 are substantially broadened (~35 Hz) in comparison to those of the remaining carbons of the molecule (<20 Hz), while in **5** the line widths of C4 and C6 are narrower (18–20 Hz), reflecting the presence of a single tautomeric species for **5**.

The above spectral data demonstrate that precorrin-3 is indeed synthesized at the dihydroisobacteriochlorin (≡ dipyrrocorphin) oxidation level and that the methyl group has been transferred to the C20 position of the macrocycle. Interestingly, insertion of the new methyl group at C20 gives rise to a single arrangement of the double bonds of the northern half of precorrin-3 (Scheme II). The structure of precorrin-3 is therefore different from that anticipated for this intermediate by simple analogy with precorrin-2. To investigate whether this transformation reflects a thermodynamically more stable conformation or simply corresponds to the kinetic product of enzyme catalysis, the NMR spectrum of reduced factor III octamethyl ester prepared by chemical reduction (H_2/Pt) was also determined. The spectrum of this specimen (not shown) gave signals with values very close to those obtained from the enzymatically produced precorrin-3, suggesting that the double-bond arrangement adopted by precorrin-3 is due to inherent subtle electronic effects induced by the methyl group positioned at C20. Such effects are known to be responsible for the site-specific C-methylations in the chemistry of dipyrrocorphins (Eschenmoser, 1988), and it is tempting to suggest that the same effects are responsible for the regio- and stereospecificities of the subsequent biological C-methylations[4] on the way to B_{12} via the putative precorrin-4 and precorrin-5, etc. Thus, while current ideas on corrin biosynthesis have relied on the chemical analogies proposed by Eschenmoser which suggest that ring contraction takes place *after* C-methylations in rings C and D and at C1, the order could be reversed in the biological system (although it lacks mechanistic and experimental precedent); i.e., precorrin-3 could suffer ring contraction *before* C-methylation in ring D. In addition, the recent disclosure of the structure of precorrin-6x (Thibaut et al., 1990b,c), which requires addition of NADPH to the medium to allow its transformation to *cobalt-free* corrin (hydrogenobyrinic acid) in a genetically engineered *P. denitrificans* system, may represent a second, oxygenative cobalt-free pathway to corrin. It is not yet clear if this intermediate is an oxidized, derailment product which cannot reenter the normal pathway by reduction or a normal intermediate in corrin synthesis in other B_{12}-producing bacteria, i.e., *P. shermanii*, *Clostridium tetranomorphum*, and *Salmonella typhimurium*, all of which are essentially anaerobic and most probably insert cobalt at an earlier stage (Müller, 1990; Müller et al., 1991) than *P. denitrificans*.

In light of the potential incongruence between the chemical models and the actual biological mechanism, the isolation and structure proof for precorrin-3 assumes major importance since it is the last known intermediate between uro'gen III and cobyrinic acid in anaerobic fermentations and, as such, becomes the requisite substrate for either the next SAM-dependent methyltransferase (M-3) or conceivably the ring contraction enzyme whose identification will be forthcoming as a result of the sequencing and expression of the genes of B_{12} biosynthesis in *S. typhimurium* and *P. denitrificans* (Jeter & Roth, 1987; Crouzet et al., 1990a,b).

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[4] The argument for modulation of both regio and stereoselectivity via electronic effects is based on the chemical models of Eschenmoser, which show that in the presence of a coordinating metal ion C-methylation occurs predominantly in ring C, while in metal-free systems, C-methylation of ring D is preferred (Leumann et al., 1983). Additionally, the ring D methyl group in corrinoids is unique in having β (≡upper face) stereochemistry, whereas those at C1, -2, -7, and -12 are delivered to the α (lower) face. It is suggested that this stereochemical control is governed by a lower face axial ligand (or binding site of the methyltransferase enzyme) on the central coordinated metal (Co^{3+} or Zn^{2+}). The isolation of several tetramethylated zinc complexes of uro'gen I from B_{12} producers together with recent evidence (Müller, 1990; Müller et al., 1991) that cobalt precorrin-3 is the true substrate for corrin biosynthesis also reinforces the idea that the electronic arrays of precorrin-2 and -3 (shown to be *different* in this work) and of subsequent precorrins represent electron-rich systems whose regio- and stereospecificities are finely tuned toward the remaining C-methylation reactions.

DNA Technologies Laboratory (TAMU) for synthesis of PCR primers.

Registry No. **1**, 106-60-5; **2**, 487-90-1; **3**, 1976-85-8; **4**, 82542-92-5; **5**, 114019-24-8; **6**, 14708-92-0; M-1, 73665-99-3; M-2, 131554-12-6; [5-^{13}C]-ALA, 79503-87-0; [4-^{13}C]-ALA, 114791-06-9; [3-^{13}C]-ALA, 123253-93-0; [2,11-$^{13}C_2$]-PBG, 58822-14-3; [3,5-$^{13}C_2$]-PBG, 108561-54-2; [4,6-$^{13}C_2$]-PBG, 123254-08-0; ALA dehydratase, 9036-37-7; vitamin B_{12}, 68-19-9; porphobilinogen deaminase, 9074-91-3; uro'gen III synthetase, 37340-55-9.

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