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Multienzyme Synthesis and Structure of Factor S3

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Abstract: Factor S3, an unusual metabolite of *Propionibacterium shermanii*, is biosynthesized from porphobilinogen (PBG) by three enzymes, PBG deaminase, uro'gen III methyltransferase (M1), and the *Salmonella typhimurium cbiF* gene product, isolated from recombinant strains of *Escherichia coli*. PBG deaminase affords uro'gen I, and M1 performs the methylation of three β-pyrrolic carbons of uro'gen I to form 2,7,12-trimethylpyrrocorphin I. The *cbiF* gene product then methylates one α-pyrrolic carbon (C-16). Nonenzymatic insertion of zinc into the macrocycle forms the tetramethylated zinc corphinoid factor S3.

The tetramethylated zinc corphinoids factors S1 (**5**) and S3 (**6**), isolated by Müller[1,2] *et al.* from cell-free extracts of *Propionibacterium shermanii*, are derived from uroporphyrinogen I (uro'gen I) (**3**) by the addition of four methyl groups from (*S*)-adenosylmethionine (SAM) by methyltransferase enzymes presumably involved in vitamin B_{12} biosynthesis in this organism. NMR analysis of the octamethyl esters of ^{13}C-enriched factor S1 revealed methylation of four β-pyrrolic carbons (C-2, -7, -12, and -17), while factor S3 exhibited methylation at three β-pyrrolic (C-2, -7, and -12) and at one of four possible α-pyrrolic carbons (C-1, -6, -11, or -16). The precise assignment of the structure of factor S3 to one of the four isomers was not possible due to the extremely small sample sizes originally available from *P. shermanii*. Factors S1 and S3 are not intermediates in the biosynthesis of B_{12}, which is formed[3] from the nonsymmetrical isomer uro'gen III (**7**) via the di- and trimethylated derivatives, precorrin 2 (**8**) and precorrin 3 (**9**) (Scheme II). Uro'gen I and uro'gen III are produced by the action of PBG deaminase on porphobilinogen (PBG) (**1**) to give hydroxymethylbilane (HMB) (**2**), followed by nonenzymatic closure of the ring to yield uro'gen I or by the action of uro'gen III synthase to yield uro'gen III (Schemes I and II). The isolation of several B_{12} biosynthetic enzymes[4-7] from recombinant strains of *Escherichia coli* that express genes isolated from *E. coli*, *Salmonella typhimurium*, and *Pseudomonas denitrificans* has resulted in the characterization of three methyltransferases involved in B_{12} and/or siroheme biosynthesis. We report here that, in combination with PBG deaminase, the actions of two of these enzymes, *E. coli* uro'gen III methyltransferase and the *S. typhimurium cbiF* gene product, not only provide the means for the synthesis of milligram quantities of factor S3 from PBG but also allow an assignment to be made to one of the four possible isomers.

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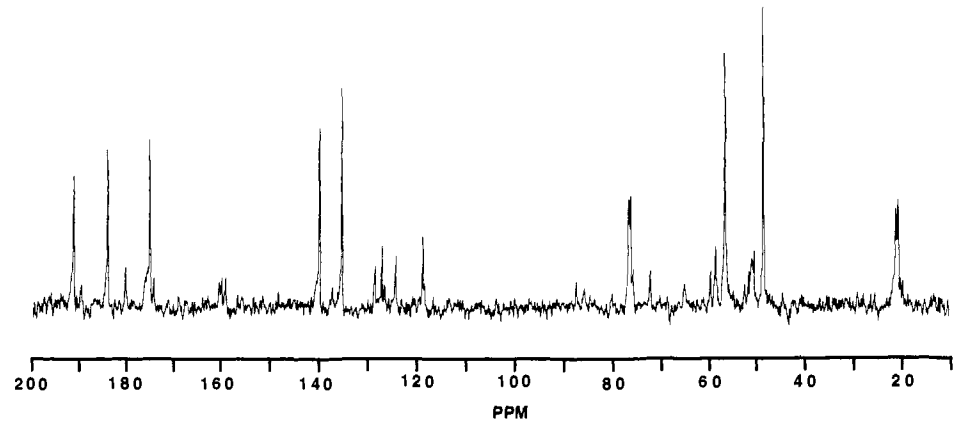
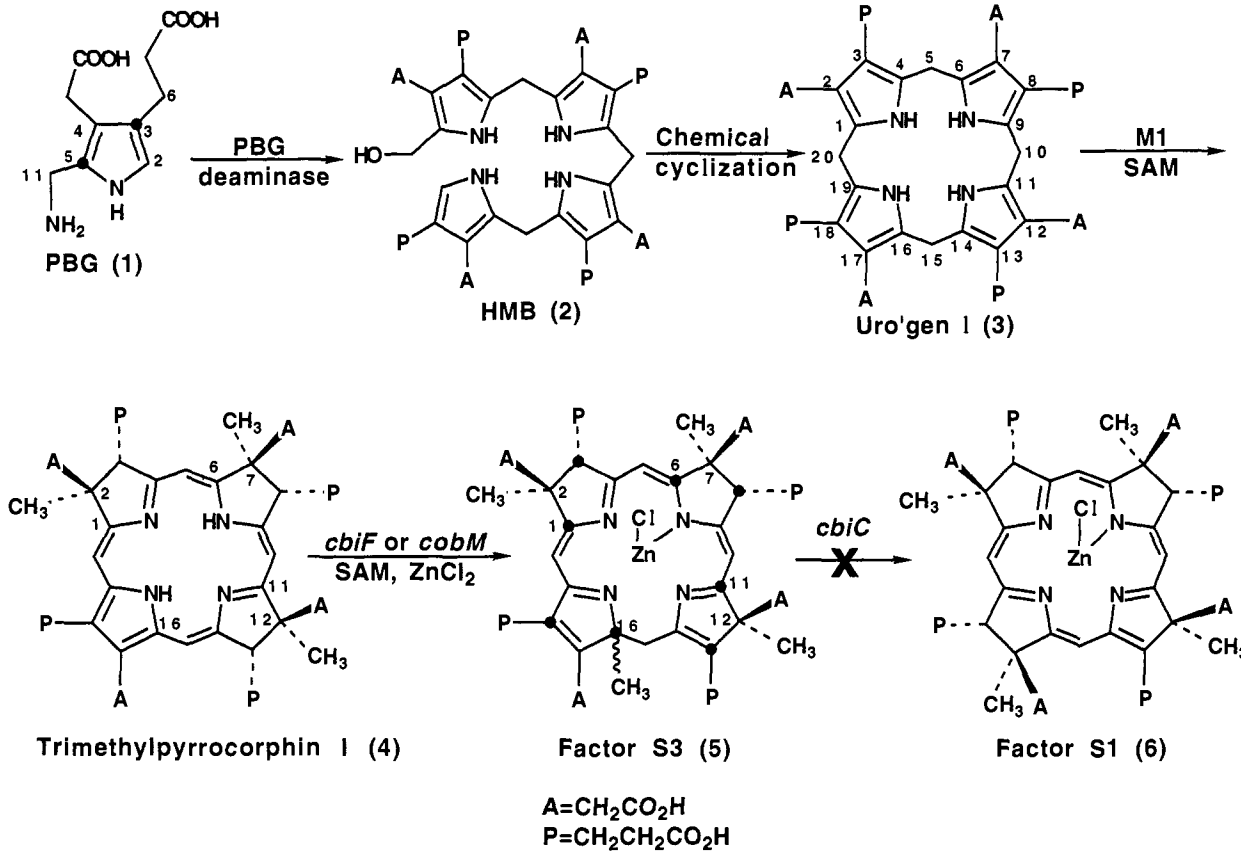


Figure 1. ^{13}C NMR spectrum of the product derived from incubation of trimethylpyrrocorphin I (4) with the *cbiF* gene product and [*methyl*-^{13}C]SAM. The trimethylpyrrocorphin I was generated from [3,5-$^{13}C_2$]PBG (1) and [*methyl*-^{12}C]SAM.

Scheme I



Results and Discussion

We have previously shown[5,6] that uro'gen III methyltransferase (M1), the *cysG* gene product used for siroheme biosynthesis in *E. coli*[5] and for both siroheme and vitamin B_{12} biosynthesis in *S. typhimurium*,[8] is normally responsible for the methylation of two β-pyrrolic carbons, C-2 and C-7, of uro'gen III to generate precorrin 2 (**8**). *E. coli* M1, however, is able to use uro'gen I (**3**), in addition to uro'gen III (**7**), as substrate and, in contrast to the corresponding enzyme isolated from *P. denitrificans*,[9] methylates yet a *third* β-pyrrolic carbon, C-12, to yield the corresponding trimethylpyrrocorphins I (**4**) and III (**11**).[10] The C-2, -7, and -12 methylation pattern and stereochemistry at these centers of trimethylpyrrocorphin I (**4**) are essential to the assignment of the structure of factor S3. We have also recently shown[4] that the *S. typhimurium cbiF* gene product methylates the α-pyrrolic C-11 position of precorrin 3 (**9**) to produce compound 4x[4] (**10**). Since the *cbiF*-encoded methylase has the specificity for an α-pyrrolic carbon, it seemed likely that the synthesis of factor S3 could be accomplished by the *cbiF*-directed methylation of an α-pyrrolic carbon of trimethylpyrrocorphin I (**4**). To test this hypothesis, trimethylpyrrocorphin I (**4**) was first generated from [3,5-$^{13}C_2$]PBG (**1**) and [*methyl*-^{12}C]SAM in the presence of PBG deaminase and M1 (see Experimental Section for details). It was then further incubated with the *cbiF* gene product and [*methyl*-^{13}C]SAM. The ^{13}C NMR spectrum (Figure 1) of the resulting product was strikingly similar to the spectrum of the octamethyl ester of factor S3, which had been isolated from extracts of *P. shermanii* incubated with similarly labeled substrates (see Table I for a comparison of chemical shifts). Critical to the

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Scheme II

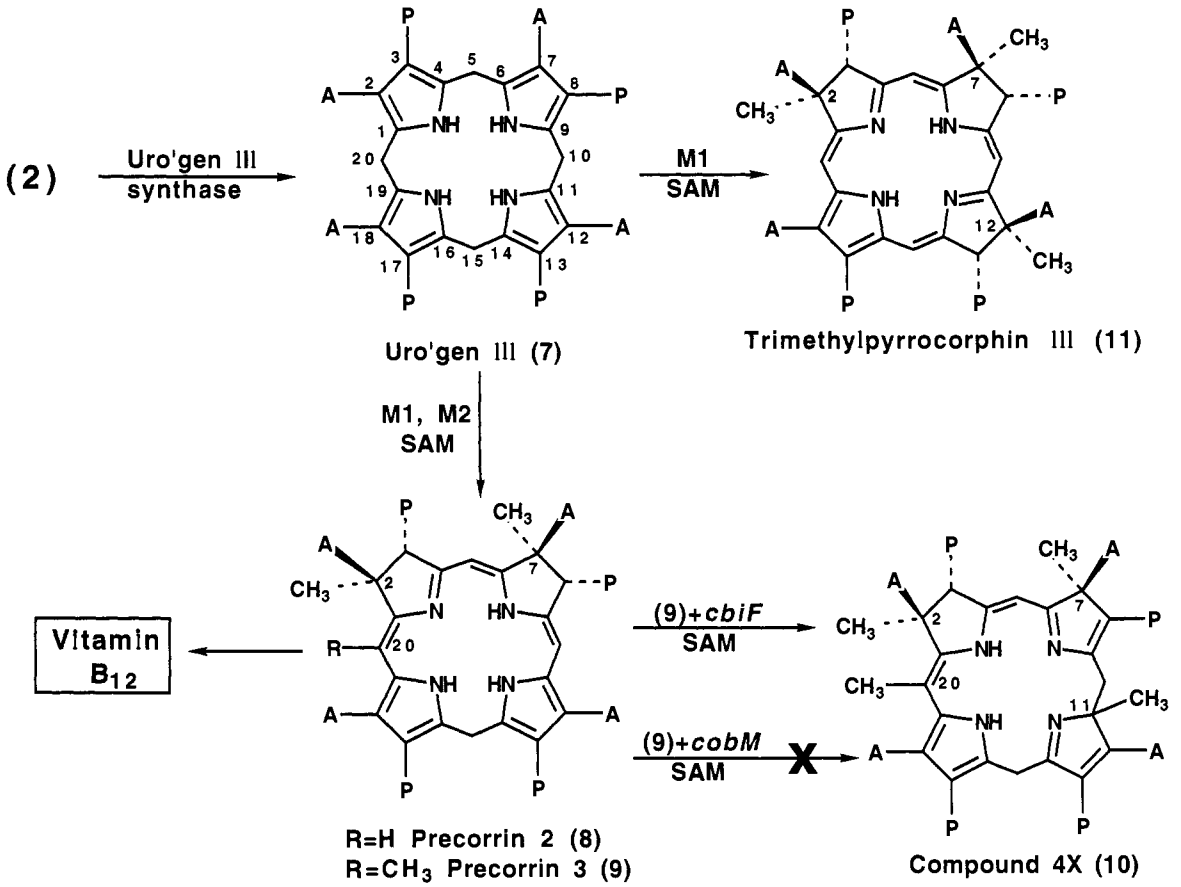

Table I. ^{13}C NMR Chemical Shifts (δ, J in Hz) of Metal-Free Factor S3, Factor S3, and Factor S3 Octamethyl Ester Derived from ^{13}C-Labeled PBG

	metal-free factor S3[a]	factor S3 (**5**)[a]	factor S3 octamethyl ester[b]
2-, 7-CH_3	21.0	20.1, 21.1	19.6, 17.3
12-CH_3	23.7	24.2	23.5
16-CH_3	21.1	22.5	20.9
C-1	175.9	177.7	175.5
C-2,7	49.8, 50.7	49.1, 51.3	46.5, 49.4
C-3,8	48.7, 56.7	51.1, 55.3	50.2, 52.0
C-4	155.4 (J = 81)	172.0 (J = 77)	168.6 (J = 76)
C-5	97.0 (J = 81)	96.2 (J = 77)	95.7 (J = 76)
C-6	184.6	187.4	182.8
C-9	169.5 (J = 60)	171.0 (J = 90)	167.9 (J = 65)
C-10	92.9 (J = 60)	92.2 (J = 90)	90.3 (J = 65)
C-11	191.5	184.7	182.3
C-12	60.9	60.3	57.8
C-13	136.0	137.6	133.9
C-14	145.5 (J = 53)	144.6 (J = 50)	145.9 (J = 52)
C-15	35.3 (J = 53)	34.4 (J = 50)	34.4 (J = 52)
C-16	76.5	80.2	79.7
C-17	164.9	163.3	ND
C-18	140.7	138.1	137.2
C-19	191.9 (J = 60)	183.2 (J = 60)	180.1 (J = 65)
C-20	92.9 (J = 60)	93.6 (J = 60)	90.6 (J = 65)

[a] The factors were synthesized with enzymes isolated from recombinant *E. coli* strains as described in this paper. The chemical shifts are referenced to internal dioxane (δ 66.5). [b] The factor was isolated from *P. shermanii*. The chemical shifts were derived from ^{13}C NMR spectra from which the structure of factor S3 was originally determined but not reported *in toto* in ref 1. The internal reference was $CDCl_3$ (δ 77.0).

assignment of the structure of the new compound was the observation of two doublets (J = 36.6 Hz) at δ 21.1 (CH_3) and δ 76.5 (C–N) consistent with the addition of a new ^{13}C-enriched methyl group on one of the four ^{13}C-labeled α-pyrrole carbons (C-1, -6, -11, or -16). Considering the conjugative array of trimethylpyrrocorphin I (**4**, Scheme I), C-16 is the only remaining nucleophilic center of these four carbons available for methylation by the CbiF protein. Additionally, trimethylpyrrocorphin III (**11**), synthesized from uro'gen III (**7**), was *not* a substrate for methylation by CbiF, a result which further reinforces the evidence that the fourth methylation occurs at C-16, since the only structural difference between trimethylpyrrocorphin I and trimethylpyrrocorphin III is inversion of the D ring in the latter structure where C-17 bears a propionate rather than an acetate side chain.

Also shown in Figure 1, five sp^2 and only *two* sp^3 carbons were observed using [3,5-$^{13}C_2$]PBG as substrate, indicating the concomitant migration of a double bond from the neighboring C-15 meso into the adjacent 13,14 endo position, a process paralleled in the conversion of precorrin 3 to compound 4x (**10**). The chemical shifts (Table I) of the resultant sp^3 meso carbon (C-15, δ 35.3) and of the remaining macrocycle carbons derived from [2,11-$^{13}C_2$]- or [4,6-$^{13}C_2$]PBG support the assignment of the new product as metal-free factor S3.

In the presence of 1 mM $ZnCl_2$, the chemical shifts of the ring carbons changed on complexation with Zn^{2+} to match even more closely those previously reported for factor S3 octamethyl ester (Table I), thus confirming the structure of factor S3 as (**5**) in which only the stereochemistry at C-16 remains unassigned.[11] Moreover, when the metal-free product was prepared with all four methyl groups ^{13}C-enriched, the methyl groups at C-2, -7, and -16 were unresolved and comprised a broad resonance at 21.0 ppm. The four methyl signals, however, became well resolved on addition of $ZnCl_2$ (Table I). These changes, induced on the addition of $ZnCl_2$, indicate that zinc had been spontaneously inserted into the macrocycle with a corresponding change of

electron distribution and ligand ruffling[12] of the periphery of the corphinate. Thus, metal-free factor S3 is converted to factor S3 in the presence of Zn^{2+}, suggesting that the macrocycle may coordinate the metal spontaneously when the electronic array is uniquely favorable, a process which may also play an early role in the anaerobic biosynthesis of vitamin B_{12} in *P. shermanii*, since recent evidence indicates that cobalt is inserted into precorrin 2 (**8**) in this organism.[13] In the aerobic *P. denitrificans*, however, cobalt is enzymatically inserted much later in the pathway into the cobalt-free hydrogenobyrinic acid diamide,[14] i.e., *after* formation of the complete corrin structure.

The octamethyl ester of the zinc complex, when analyzed by TLC, had an R_f value identical to that of the octamethyl ester of factor S3 isolated from *P. shermanii*, while FAB-MS of the esterified product derived from [2,11-$^{13}C_2$]PBG and [*methyl*-^{13}C]SAM revealed a molecular weight corresponding to $^{12}C_{42}{}^{13}C_{12}H_{67}O_{16}N_4$, consistent with the addition of a new methyl group to trimethylpyrrocorphin I with loss of ZnCl. From the above results, it is clear that the sequential enzymatic action of the enzymes PBG deaminase, M1, and the *cbiF* gene product on PBG, followed by zinc insertion, yields a product identical to factor S3. By combining these three enzymes with PBG and SAM, six steps in the synthesis of factor S3 (polymerization, cyclization, and four sequential methylations) can be accomplished in a two-stage incubation with >80% overall yield starting with 3 mg of PBG. The *P. denitrificans cobM* gene product, which shows homology with the *cbiF* gene product,[4] was also able to catalyze methylation of trimethylpyrrocorphin I (**4**) at C-16 to synthesize factor S3 but surprisingly, and in contrast to CbiF, was not able to perform the analogous C-11 methylation of precorrin 3 (**9**) to give compound 4x (**10**).

Recent studies[15] have shown that, in *P. denitrificans*, the *cobH* gene product catalyzes the migration of a methyl group from C-11 to C-12 by a mechanism involving [1,5]-suprafacial sigmatropic rearrangement to convert precorrin 8x to hydrogenobyrinic acid. A similar mechanism could in principle operate in the biosynthesis of factor S1[2] (**6**) from factor S3 (**5**). However, attempts to convert factor S3 to factor S1 with the homologous *S. typhimurium cbiC* gene product were unsuccessful, suggesting that another methyltransferase directly methylates trimethylpyrrocorphin I at C-17 to form factor S1. Indeed, recent preliminary results in our laboratory (Dr. Joe Spencer, unpublished) indicate that S1 is synthesized by methylation at C-17 of Zn-trimethylpyrrocorphin I by uro'gen III methyltransferase.

It is now clear that, although not on the vitamin B_{12} pathway, products such as factors S1 and S3 can provide valuable information about the function and specificity of the enzymes involved in corrin synthesis as well as spectroscopic models for the "missing" intermediates in the B_{12} pathway (e.g., tetra- and pentamethyl corphinoids in the type III series), whose detection and synthesis can now be approached using the repertoire of methyl transferases available from expression of *cysG* and the *cbi* and *cob* genes.[16]

Experimental Section

Chemicals. [5-^{13}C]-, [4-^{13}C]-, and [3-^{13}C]-5-aminolevulinic acid (ALA) isotopomers were prepared as previously described.[17,18] 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. (*S*)-adenosyl[$^{13}CH_3$]-L-methionine was prepared as previously described.[19] All other chemicals were purchased from Sigma Chemical Co. and were of the highest grade obtainable.

Enzyme Preparations. ALA dehydratase, PBG deaminase, and uroporphyrinogen III methyltransferase (M1) were purified from recombinant *E. coli* strains as previously described.[4,5] The *S. typhimurium cbiF* gene was expressed in a recombinant strain of *E. coli*, and a crude cell-free extract of the strain was used as the source of enzyme.

Trimethylpyrrocorphin I and Factor S3 Synthesis. Trimethylpyrrocorphin I was synthesized in 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, 15 mg of uro'gen III methyltransferase, and 10 mg of SAM incubated together under anaerobic conditions in an argon-purged glovebox for 15 h. The trimethylpyrrocorphin was isolated from the reaction mixture by binding it to a small column (1 × 1.2 cm) of DEAE Sephadex, followed by successive washes with H_2O and 0.2 M KCl, and eluting it with a minimal volume (2–3 mL) of 2 M KCl solution. For the synthesis of factor S3, trimethylpyrrocorphin I and SAM (5.0 mg) were added to a degassed cell-free extract derived from the *cbiF* expression strain. The mixture was incubated anaerobically for 15 h and the product isolated by adsorption to DEAE-Sephadex as described above. To convert metal-free factor S3 to factor S3, 1.0 mM $ZnCl_2$ was added directly to the sample in an NMR tube.

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. Samples in ~3 mL of 2 M KCl 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 glovebox. 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.

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(11) In a preliminary ^{1}H NMR experiment, the absence of intramethyl cross peaks in the 2D NOESY spectra of factor S3 octamethyl ester also supports the placement of each of the four methyl groups to separate pyrrole rings, thereby confirming the C-2, -7, -12, and -16 assignment. In contrast, had two methyl groups been located on adjacent carbons (such as C-1, -2, -7, and -12 as in the original structure), a strong NOE between the neighboring methyls would be expected.

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(16) In an alternate scenario, ring contraction could occur immediately after formation of precorrin 3 and prior to any further methylation, which could also explain why attempts to synthesize or isolate the theoretical tetra-, penta-, and hexamethylated corphinoids have so far proved difficult, in spite of the availability of both the substrate (precorrin 3) and the methyltransferases from both *S. typhimurium* and *P. denitrificans*.

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