Isolation, Structure, and Genetically Engineered Synthesis of Precorrin-5, the Pentamethylated Intermediate of Vitamin B_{12} Biosynthesis

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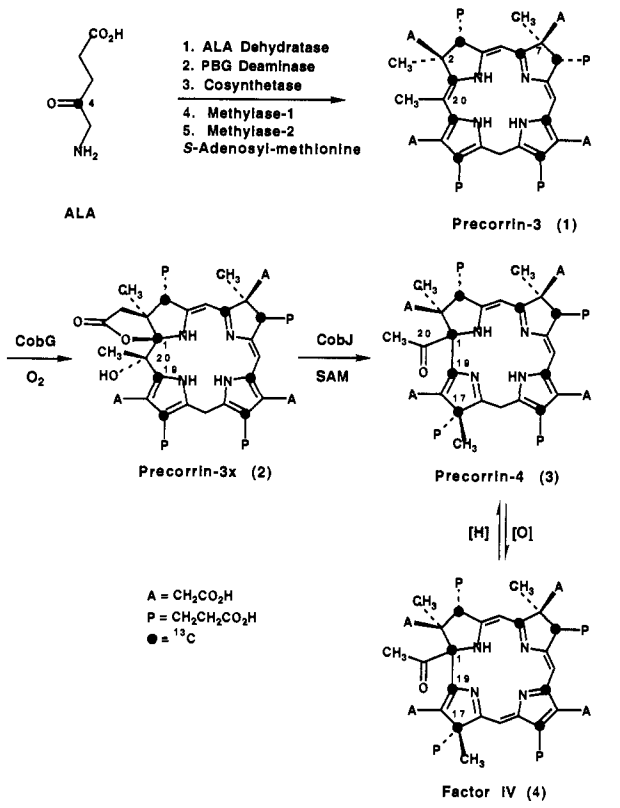
Recent work[1,2] on deciphering the functions of the biosynthetic genes of the vitamin B_{12} pathway in the aerobic bacterium *Pseudomonas denitrificans* has uncovered the mechanism of ring contraction connecting precorrin-3 (**1**) and -4 (**3**) (Scheme I), a remarkable process which features an O_2-dependent spring-loading step[1,3] controlled by CobG to arrive at precorrin-3x (**2**), followed by pinacol-like ring contraction and *S*-adenosylmethionine (SAM)-dependent C-methylation (at C-17 of **2**), both mediated by CobJ, to reach precorrin-4 (**3**).

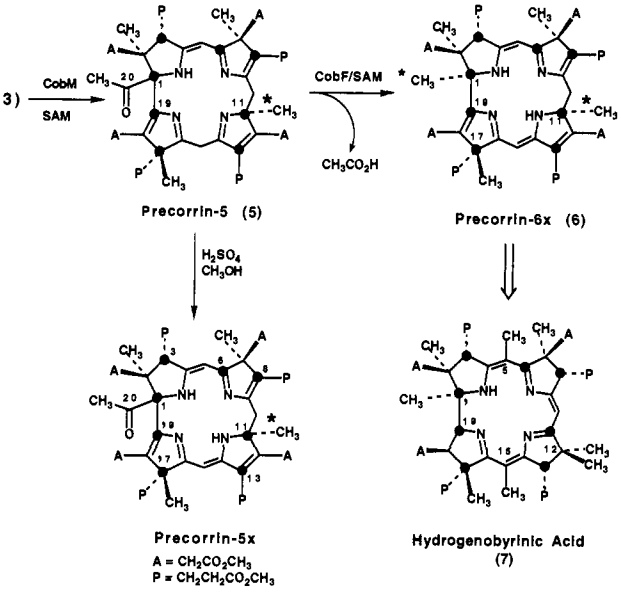
We are glad to report that the assignments of the functions of the next two genes in the sequence described below have uncovered the last of the precorrins, the pentamethylated precorrin-5 corresponding to C-methylation at position 11 of precorrin-4. With this finding, all of the intermediates between precorrin-3 and precorrin-6x (**6**) are now known.[4] Prior to the discovery of precorrin-4,[1] we had predicted[4,5] that CobM catalyzes insertion of a C-methyl group at C-11 on the basis of studies with model substrates. Using the true substrate precorrin-4[1] (**3**), biosynthetically labeled from [4-^{13}C]-5-aminolevulinic acid (ALA) as shown (●) (Scheme I), the ^{13}C NMR spectrum of the octacarboxylic acid isolated from the incubation mixture containing **3**, CobM,[4] and $^{13}CH_3$*–SAM revealed, in addition to C-1–C-19 coupling, a second coupled pair corresponding to a new CH_3* group at δ 23.7 (d, J = 37 Hz) and an sp^3 signal for C-11 (δ 76; d, J = 37 Hz). Proof that the C-1 acetyl function was still intact came from (a) the observation of a resonance for C-1 at δ 82 (d, J = 52 Hz) [also seen in precorrin-4[1] (**3**) and Factor IV[6] (**4**)] and (b) the retention of signals for the carbonyl at C-20 (δ 211) and the sp^3 center at C-15 (δ 27) when a second isotopomer of **4**, labeled with ^{13}C at C-15 and C-20 from [5-^{13}C]ALA, was used as substrate. The new isolate is thus precorrin-5, which isomerizes to precorrin-5x on esterification (Scheme II), as seen from the NMR data of its octamethyl ester[7] (Table I). When the gene product CobF[4] was included in the incubation, the signal for C-1 (δ 82) in the spectrum of (**5**) disappeared and was replaced by a resonance at δ 73 (dd) coupled to the signal for C-19 at δ 154 and to a new C-methyl group (*) at δ 29.5 (J = 36 Hz). Esterification of the free acid provided a sample with NMR and FAB-MS[7] data identical with those of precorrin-6x[8] [octamethyl ester] (**6**). This biosynthetic correlation confirms the structure and defines the absolute stereochemistry (except at C-1) of precorrin-5 as **5**.

Thus it appears that deacylation of precorrin-5 is catalyzed by the CobF enzyme to provide the short-lived intermediate precorrin-5y, which then undergoes C-1-methylation. The mechanistic rationale shown in Scheme III features the bis-imino chromophore in precorrin-5y extended from C-19 as an electron sink created by reaction with CobF. This structural motif, in which C-11 methylation insulates rings C and D electronically from rings

Scheme I



Scheme II



(1) Scott, A. I.; Roessner, C. A.; Stolowich, N. J.; Spencer, J. B.; Min, C.; Ozaki, S.-I. *FEBS Lett.* **1993,** *331,* 105.

(2) Debussche, L.; Thibaut, D.; Danzer, M.; Debu, F.; Fréchet, D.; Herman, F.; Blanche, F.; Vuilhorgne, M. *J. Chem. Soc., Chem. Commun.* **1993,** 1100.

(3) Spencer, J. B.; Stolowich, N. J.; Roessner, C. A.; Min, C.; Scott, A. I., manuscript in preparation.

(4) Roessner, C. A.; Warren, M. J.; Santander, P. J.; Atshaves, B. P.; Ozaki, S.-I.; Stolowich, N. J.; Iida, K.; Scott, A. I. *FEBS Lett.* **1992,** *301,* 73. The sequence shown in Scheme I depicts precorrin-4 (**3**) as the true intermediate which was isolated and characterized in our previous studies.[1] On the other hand, the Rhone Poulenc group has described the isolation and structure of Factor IV (**4**), the *oxidized* form of precorrin-4,[2] which is converted to precorrin-6x (**6**) in a cell-free system, presumably after a reduction back to **3**.

(5) Ozaki, S.-I.; Roessner, C. A.; Stolowich, N. J.; Atshaves, B. P.; Hertle, R.; Müller, G.; Scott, A. I. *J. Am. Chem. Soc.* **1993,** *115,* 7935.

(6) Thibaut, D.; Debussche, L.; Fréchet, D.; Herman, F.; Vuilhorgne, M.; Blanche, F. *J. Chem. Soc., Chem. Commun.* **1993,** 513.

(7) FAB-MS of the octamethyl ester of precorrin-5x (m-nitrobenzyl alcohol matrix): $^{12}C_{44}{}^{13}C_9H_{70}N_4O_{17}$ requires m/z 1043, found 1044 $(M + H)^+$. FAB-MS of the ^{13}C-enriched octamethyl ester of **6**: $^{12}C_{42}{}^{13}C_{10}H_{70}N_4O_{16}$ requires m/z 1016, found 1017 $(M + H)^+$.

(8) Thibaut, D.; Blanche, F.; Debussche, L.; Leeper, F. J.; Battersby, A. R. *Proc. Natl. Acad. Sci. U.S.A.* **1990,** *87,* 8800.

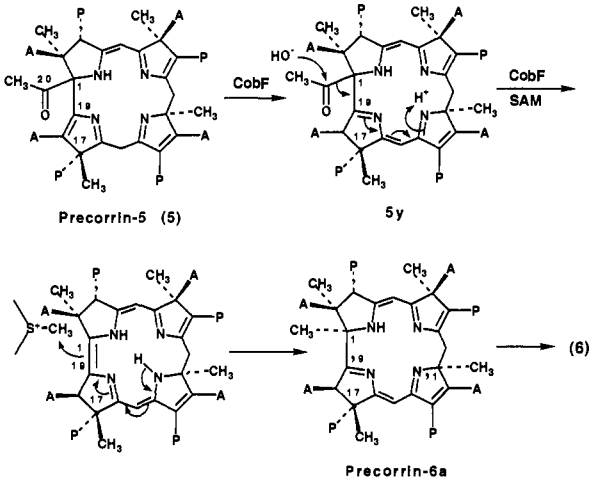
0002-7863/93/1515-10380$04.00/0

Table I. ^{13}C NMR Chemical Shifts (C_6D_6) for Precorrin-5x Methyl Ester Derived from (A) 4-^{13}C-ALA and (B) 5-^{13}C-ALA

A		B	
δ_C (J, Hz)	assignment	δ_C (J, Hz)	assignment
22.7 (d, 36)	C-11 methyl[a]	34.7 (d, 48)	C-10
54.2 (s)	C-3	78.0 (t, 69)	C-15
62.5 (s)	C-17	84.3 (d, 71)	C-5
70.8 (d, 36)	C-11	147.3 (d, 48)	C-9
79.5 (d, 49)	C-1	158.1 (d, 71)	C-4
131.3 (s)	C-8	160.6 (d, 69)	C-14
135.2 (s)	C-13	183.5 (d, 69)	C-16
146.6 (d, 49)	C-19	207.3 (s)	C-20
183.1 (s)	C-6		

[a] From $^{13}CH_3$–SAM; s, singlet, d, doublet, t, triplet.

Scheme III



A/B, not only facilitates deacetylation but also allows the return of electron density to C-1 followed by electrophilic C-methylation mediated by CobF/SAM. The process is completed by prototropic shift from C-18 in the kinetic product precorrin-6a (not isolated) to give precorrin-6x (**6**). That the facile removal of acetic acid from C-1 is also a *chemical* process was demonstrated with precorrin-5x, which loses acetic acid spontaneously (albeit slowly) at pH < 7.

When the entire repertoire of the eight enzymes[9,10] necessary for the nine-step synthesis of precorrin-5 was incubated with the substrates ALA and SAM, the genetically engineered synthesis of **5** was achieved in 30% overall yield based on ALA. This striking example of the *in vitro* reconstitution of a complex biosynthetic pathway serves to illustrate both the power and the generality of multienzyme synthesis,[11] featuring many different types of C–C, C–N, and C–O bond formations and rearrangements. Most importantly, the removal of the rigid, metabolic networking of the living cell, by using only those biosynthetic enzymes necessary to synthesize the target, ensures that each intermediate is dedicated to a predetermined pathway rather than being diverted into the main arteries of primary metabolism *in vivo*. We would like to suggest that with the demonstration of this nine-step, genetically engineered process, the multienzyme synthesis of complex natural products has come of age.[12]

Identification of the structures of precorrin-3x, precorrin-4, and now precorrin-5, completes our knowledge of the steps between precorrins-3 and -6x and consequently of all of the intermediates (and enzymes which interconnect them) between ALA and hydrogenobyrinic acid (**7**) in the aerobe *P. denitrificans*.[13-16] The details remain to be uncovered the details of the parallel but nonidentical pathway in anaerobic bacteria, e.g., *Salmonella typhimurium*[4,17] and *Propionibacterium shermanii*,[18] where, in the latter organism, the intermediates are known to be cobalt complexes beyond the stage of precorrin-3 and where the redox chemistry necessary for ring contraction must be independent of O_2.[19]

Acknowledgment. We thank the National Institutes of Health (NIDDK) and the Robert A. Welch Foundation for financial assistance and Marc Holderman for excellent technical assistance.

(9) In biosynthetic order these, are ALA dehydratase, PBG deaminase, urogen III synthase, urogen III methylase (CobA; 2 steps), precorrin-3 synthase (CobI or CbiL),[10] CobG,[1] CobJ,[1] CobM.[4]

(10) (a) Warren, M. J.; Roessner, C. A.; Ozaki, S.-I.; Stolowich, N. J.; Santander, P. J.; Scott, A. I. *Biochemistry* **1992**, *31*, 603. (b) The overall yield of 30% based on isolation of the pure ester precorrin-5x does not include optimization of the last two steps catalyzed by CobJ and -M. Earlier work[10a] had shown that, using the first five enzymes, precorrin-3 is synthesized from ALA in 79% overall yield (ca. 95% conversion for each step), while the conversion of precorrin-3 → -3x (CobG) is ~80%.[1,2] The CobJ, -M sequence is therefore estimated to be operating at ~70% efficiency.

(11) For recent reviews of genetically engineered syntheses of complex natural products, see: Scott, A. I. *Tetrahedron* **1992**, 48, 2559; *Pure Appl. Chem.* **1993**, *65*, 1299.

(12) Amato, I. *Science* **1992**, *257*, 1481; Pennisi, E. *Science News* **1992**, *142*, 148. Scott, A. I. *Chemistry Br.* **1993**, *29*, 687.

(13) The sequence between precorrin-6x (**6**) and hydrogenobyrinic acid (**7**) involves C-18,C-19 reduction to precorrin-6y,[14] bismethylation at C-5,C-15, and decarboxylation of the C-12 acetate side chain[15] (→ precorrin-8x), and finally [1,5]-sigmatropic shift of the C-11 methyl to C-12[16] to reach the cobalt free corrin **7**.

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(17) Roth, J. R.; Lawrence, J. G.; Rubenfield, M.; Kieffer-Higgins, S.; Church, G. M. *J. Bacteriol.* **1993**, *175*, 3303.

(18) Müller, G.; Zipfel, F.; Hlineny, K.; Savvidis, E.; Hertle, R.; Traub-Eberhard, U.; Scott, A. I.; Williams, H. J.; Stolowich, N. J.; Santander, P. J.; Warren, M. J.; Blanche, F.; Thibaut, D. *J. Am. Chem. Soc.* **1991**, *113*, 9893.

(19) Scott, A. I. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1223.