Absolute Stereochemistry of Precorrin-3x and Its Relevance to the Dichotomy of Ring Contraction Mechanism in Vitamin B_{12} Biosynthesis

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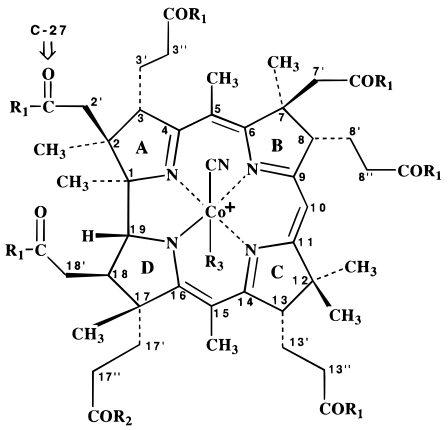
Abstract: In Nature, two pathways of vitamin B_{12} biosynthesis have been discovered. In the aerobe *Pseudomonas denitrificans* molecular oxygen is utilized in the formation of the novel hydroxy-γ-lactone intermediate precorrin-3x. In the subsequent conversion of precorrin-3x to hydrogenobyrinic acid catalyzed by the *Ps. denitrificans* Cob enzymes, we demonstrate that no oxygen exchange of the peripheral carboxylic acid functions with the medium takes place. This result is in sharp contrast to the anaerobic pathway in *Propionibacterium shermanii*, where a unique carboxylate function (ring A acetate) undergoes extensive oxygen exchange. The stereochemistry of precorrin-3x at the C-20 center was determined using NMR and it is proposed that the C-20 hydroxyl is *cis* to the oxygen terminus of the γ-lactone at C-1, a result that has a distinct bearing on the ring contraction mechanism of corrin biosynthesis in *Ps. denitrificans.* The implications of these findings for both the aerobic and anaerobic pathways are discussed and two discrete mechanisms for ring contraction and the subsequent loss of acetic acid are presented.

During the anaerobic biosynthesis of vitamin B_{12} **(1)** in *Propionibacterium shermanii* it has been observed[1,2] that a unique and substantial exchange of the carbonyl oxygens derived from the original carboxyl oxygens of the precusor 5-amino-levulinic acid (ALA) with the aqueous medium has taken place at the ring A acetamido group (C-27)[3] of **1**. This oxygen exchange was later confirmed[4] and shown to occur prior to cobyrinic acid (**2**) biosynthesis in the same organism. A



Vitamin B_{12} (1) R_1=NH_2; R_2,R_3=$NHCH_2CH(CH_3)O{\cdot}PO_2^-O$

Cobyrinic Acid (2) R_1,R_2=OH; R_3=CN

mechanism has been proposed[5] invoking formation of a mixed anhydride (**5b**) in the detachment of the methyl ketone function at C-1 from precorrin 5 (**5a**), followed by regiospecific hydrolysis (at **a** rather than **b**) as shown in Scheme 1. The evidence for this postulate rests on the observed transfer of ^{18}O from the ring A acetate carboxyl to the acetic acid isolated after the ring contraction step.[5] This experiment was performed with *Pr. shermanii* extracts where no intermediates beyond the cobalt complex of precorrin-3 (Scheme 2) have been found,[6] and where the mechanism of ring contraction remains unknown.

In sharp contrast in the aerobic pathway to B_{12}, discovered in *Pseudomonas denitrificans*,[7,8] *both* ^{18}O-labeled ring A acetate carboxyl oxygens have been shown[9] to be completely *retained* as late in the pathway as precorrin-5 (**5a**). It therefore became crucial to determine if any ^{18}O exchange occurs in *Ps. denitrificans* between precorrin-5 and HBA (Scheme 1).

In previous studies[9,10] we demonstrated that the insertion of a hydroxyl group at C-20 from molecular oxygen and lacton-

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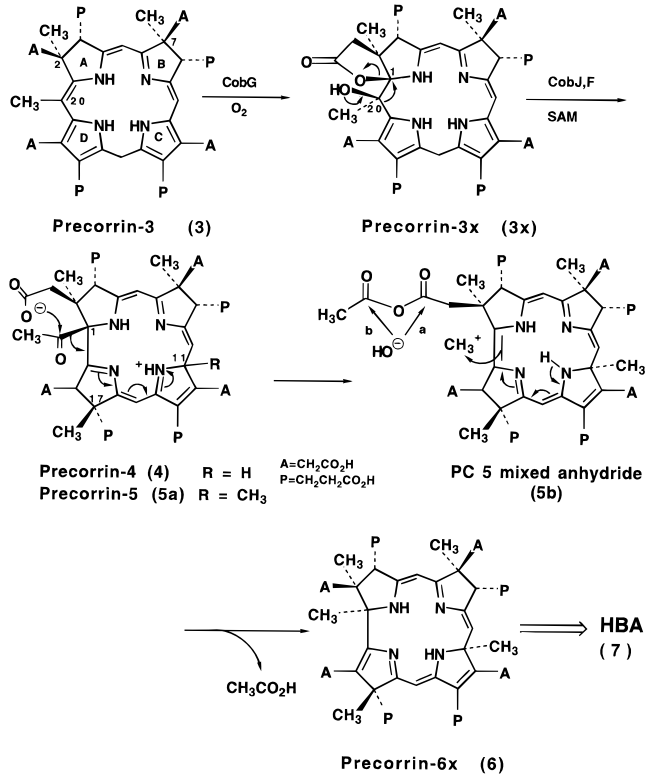
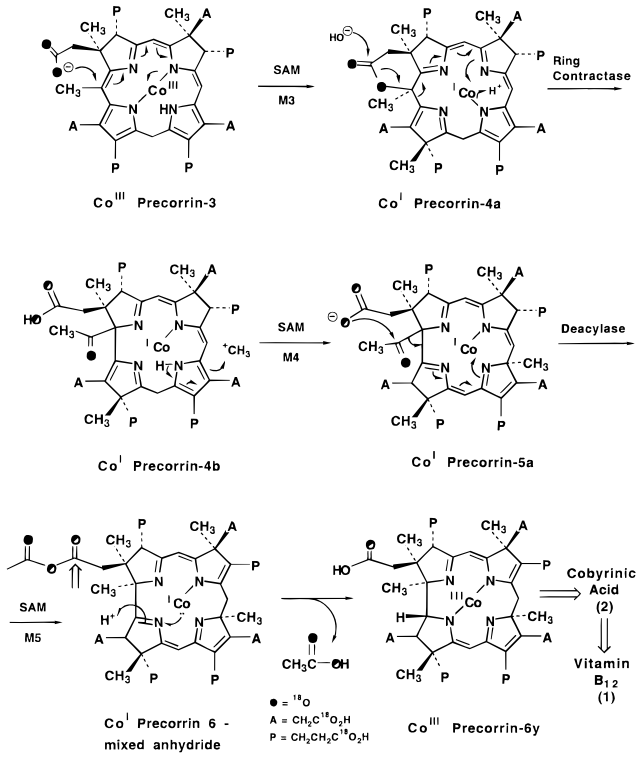
Scheme 1



Scheme 2



ization of the ring A acetate onto C-1 occur during aerobic synthesis of precorrin-3x (also referred to as precorrin-3B[11]) from precorrin-3 (**3**) without exchange with the medium. The absolute stereochemistries of the newly created oxygenated centers at C-20 and C-1, which have implications for both the mechanism of precorrin-3x formation and the subsequent ring contraction and deacetylation steps, have now been determined. We also describe the conversion of ^{18}O enriched ALA to the complete corrin HBA (**7**) and the analysis of the ^{13}C-NMR spectrum of the free acid, using the isotope induced chemical shift[12] to analyze the extent of exchange of ^{16}O for ^{18}O in all seven carboxylic acid functions of HBA.

Experimental Section

Materials. Unlabeled 5-aminolevulinic acid (ALA) and its [5-^{13}C]- and [4-^{13}C]-labeled isotopomers were prepared as previously described.[13,14] The sample of [1-^{13}C; 1,1,4-$^{18}O_3$]-ALA was a generous gift from M. Kajiwara (Tokyo). (*S*)-Adenosyl-L-methionine (SAM), ATP, NADH, NADPH, and DEAE Sephadex-A25 were purchased from Sigma Chemical Co. Isopropyl-β-D-thiogalactoside (IPTG) was purchased from Ambion Inc. Precorrin-3 was synthesized anaerobically from ALA and SAM using a minor modification of our recent method that utilizes CobA lysates in place of purified *E. coli* Uro-gen III methyltransferase (CysG) preventing over methylation by CysG.[8c] In this case however, an *E. coli* strain which harbors a pUC18 derived plasmid containing both *CobA* and *CobB* genes (J. Park, unpublished) was used, which has the advantage of supplying similar CobA activities to our previous strain[8c] without requiring IPTG induction. Enzymatically synthesized precorrin-3 was isolated on DEAE sephadex and checked for purity by NMR spectroscopy.[15]

Bacterial Strains and Enzymes. *Escherichia coli* ALA dehydratase, PBG deaminase, and Uro'gen III cosynthase were prepared and purified as described previously.[8,15] Lysates of bacterial strains harboring the *Ps. denitrificans Cob*I, G, J, M, F, K, L, and H gene products required for the production of HBA were prepared as described.[8b,c]

Preparation of Precorrin-3x and Hydrogenobyrinic Acid (HBA). Precorrin-3 (10 mg), NADH (20 mg), and 40 mL of CobG lysates (from 2-L cells) were combined and diluted with 0.1 M Tris buffer (pH 8) to a final volume of 100 mL and incubated aerobically with gentle swirling for 4 h. The resulting precorrin-3x was isolated by absorption onto DEAE Sephadex, washing successively with 0.3 M KCl and water, and then eluting with 20% acetic acid. The eluant was lyophilized and the residue treated with diazomethane. The resulting heptamethyl ester of precorrin-3x was separated from residual octamethyl esters of precorrin-3/Factor III via HPLC on a C18 column (Alltech Econosil 10 × 250 mm) eluting with CH_3OH/H_2O (85/15).

The synthesis of HBA was accomplished in similar fashion combining in a single vessel 10 mg each of precorrin-3, SAM, ATP, NADH, NADPH and lysates containing the requisite gene products (CobG, J, M, F, K, L, H). The overnight (30 °C) incubation was however preceded by a short (2−3 h) preincubation at 18 °C which increases the conversion of precorrin-5 to precorrin-6x (J. Wang, unpublished results). The incubation mixture containing the protein-bound HBA was treated with DEAE Sephadex to remove incomplete corrinoids (mainly precorrin-5), and the HBA was released from the protein by thermal denaturization (70 °C, 15 min), absorbed to DEAE, and isolated as described above.

NMR Spectroscopy and Molecular Modeling. ^1H and ^{13}C NMR spectra were obtained at 500.13 and 125.77 MHz, respectively, on a

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Bruker ARX-500 spectrometer. Spectra were recorded at 22 °C employing standard one-pulse sequences for 1D spectra and were referenced to the residual C_6D_6 signal at 7.14 ppm (^{1}H) or 128.0 ppm (^{13}C). Two homonuclear 2D experiments, DQ-COSY,[16] and ROESY,[17] were acquired in the TPPI phase sensitive mode[18] using a SW of 4500 Hz. For the ROESY experiment a spin lock of 5 kHz field strength was applied at the carrier frequency (4.0 ppm) for 300 ms. For both experiments, 400 t_1 increments were acquired with 128 scans per increment. The t_1 domains were further extended to 1024 points using linear prediction, and the resulting 1024 × 1024 matrices were processed using a shifted sine bell filter in both dimensions before each transformation.

2D proton detected heteronuclear (^{1}H−^{13}C) correlated spectroscopy was performed on selectively ^{13}C-enriched samples using standard HMQC and HMBC sequences.[19] The single-bond HMQC experiment utilized a 3.5-ms delay and GARP ^{13}C-decoupling during acquisition. The mulitiple-bond HMBC experiment was optimized for long-range couplings (70 ms), employing a low pass filter to eliminate one-bond couplings and was acquired without decoupling. Typically, 256−400 t_1 increments of 128 scans were acquired and extended to 512 points via linear prediction. The final 512 × 1024 data point spectra were processed in both directions using a Gaussian function, and a magnitude calculation was performed on the F_1 dimension. All data acquisition and processing routines were accomplished using UXNMR operating on the Bruker X32 workstation.

Molecular modeling and energy minimization routines were accomplished using SYBYL.[20] Models of precorrin-3x with both possible stereochemistries at C-20 were constructed using standard bond lengths, bond angles, and proper hybridization before an initial series of minimizations. The resulting structures were then subjected to quenched molecular dynamics (QMD) by heating to 500 K over 10 ps. Dynamic runs were then performed in a NTV ensemble for a total time of 500 ps with sampling every 5 ps. Each of the 100 structures were thoroughly minimized using 1000 steps of steepest descent algorithm. The resulting lowest energy structures were compared and the interproton distances for each stereoisomer analyzed.

Results

Synthesis of Hydrogenobyrinic Acid (HBA, 7). ALA was converted in 20% yield to HBA using the 12-enzyme system described previously,[8c] which effectively reconstitutes the aerobic pathway of *Ps. denitrificans.* During the enzymatic formation of HBA, it had been observed that almost all of the product was absorbed on the enzyme (CobH), which catalyzes the conversion of precorrin-8x to **7**, thereby imparting a yellow color to the protein. HBA could be freed from CobH by thermal denaturization and isolated on DEAE. Elution of the product resulted in conversion to the pink form as it migrated through the column. The resulting pink tautomer was found to be identical in UV−vis, ^{1}H, and ^{13}C NMR spectral characteristics with HBA (free acid) previously isolated from an engineeered strain of *Ps. denitrificans*.[21]

Fate of Carboxylate Oxygens Derived from ^{18}O-ALA during Aerobic Biosynthesis of HBA. A sample of [1-^{13}C; 1,1,4-$^{18}O_3$]-ALA (10 mg) was converted to HBA using the 12-enzyme system and analyzed by ^{13}C NMR spectroscopy. To eliminate potential oxygen exchange upon esterification, HBA was analyzed in the free acid form after acetic acid elution and removal of the solvent via lyophilization. The resulting ^{13}C NMR spectrum (Figure 1) of the carboxylic acid region of the heptaacid clearly demonstrates the equivalence of all seven sets of signals, each consisting of three resonances, the upfield, highest intensity signal of each set being attributable[12] to carboxylates containing two ^{18}O atoms. Thus the results indicate that no exchange of any of the acetate carboxyls had occurred during the conversion of ALA to HBA using the Cob enzymes of *Ps. denitrificans*. Had a single exchange event occurred, a decrease or complete loss of an upfield signal from one of the seven sets of signals would have been observed with a concomitantly large increase in the adjacent center signal corresponding to a species containing single ^{18}O and ^{16}O atoms, along with a smaller increase in the downfield resonance from the original "triplet". Multiple or complete exchange with solvent, as in the case of *Pr. shermanii*, would result in a single resonance at the downfield position corresponding to the native chemical shift (i.e., two ^{16}O atoms), with little or no upfield components of the original "triplet" observed.

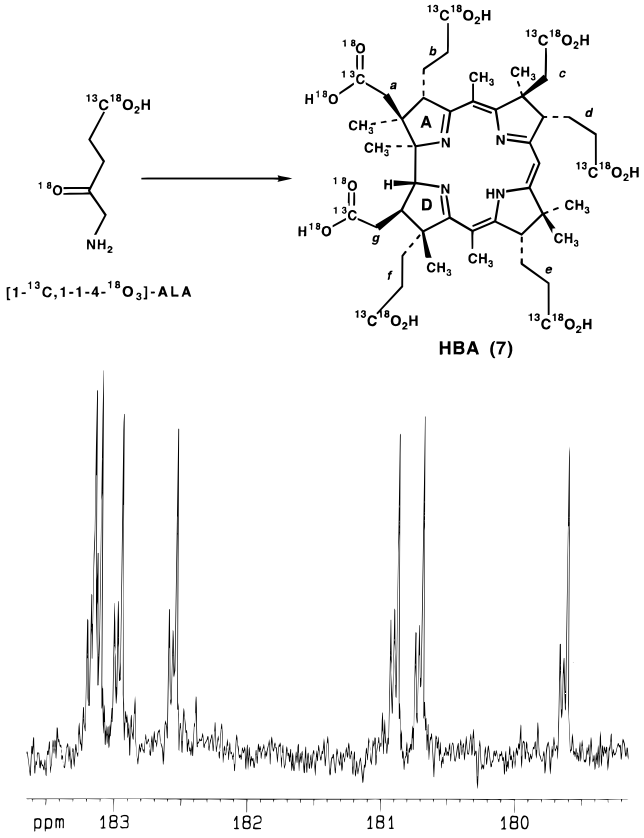

Figure 1. 125-MHz ^{13}C-NMR spectrum (D_2O) of hydrogenobyrinic acid (HBA, **7**) derived from [1-^{13}C; 1,1,4-$^{18}O_3$]-ALA (99 atom % ^{13}C, 90 atom % ^{18}O) using the multienzyme system described in the Experimental Section. The upfield, highest intensity resonance of each group originates from the two-^{18}O-atom perturbation (6.8 Hz, 0.054 ppm) on the ^{13}C carbonyl and clearly demonstrates that ^{18}O is fully retained in all seven carboxylates. The two lower intensity resonances correspond to ^{13}C carbonyls containing either one or zero ^{18}O atoms, resulting from the ∼10% ^{16}O originally present in the ALA.

Stereochemical Assignment of the C20 Center of Precorrin-3x. Due to a full complement of eight acetate and propionate side chains, the resulting overlap from these resonances in the aliphatic region (Figure 2A) prevents the direct, unambiguous assignment of the proton spectrum necessary for the elucidation of the stereochemistry of precorrin-3x solely by ^{1}H NMR. However, precorrin-3x can be conveniently prepared from various ^{13}C-enriched precusors, and following complete

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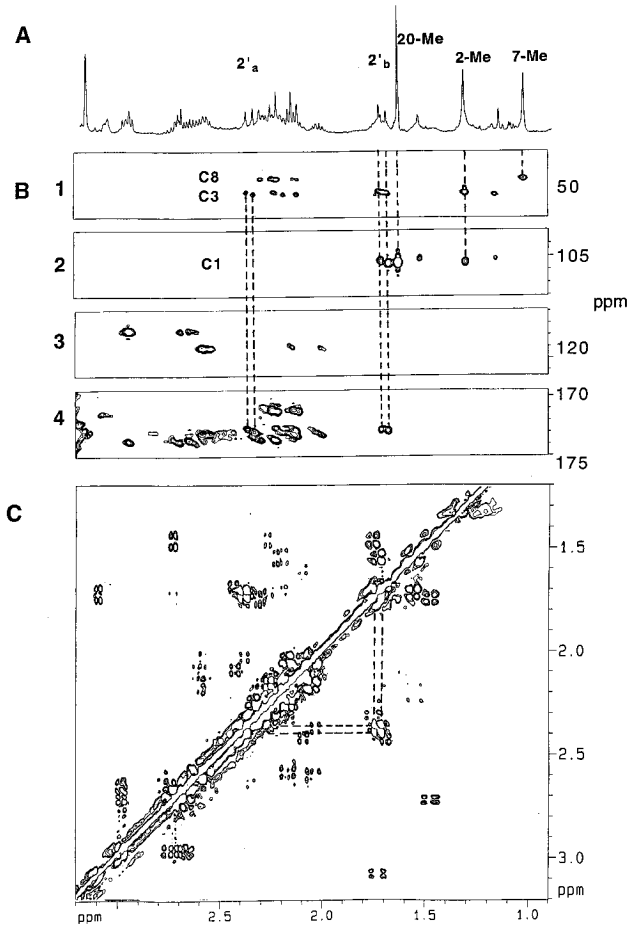

Figure 2. 500-MHz ^{1}H-NMR spectra of precorrin-3x heptamethyl ester in benzene-d_6. (A) Upfield aliphatic region from the 1D proton spectrum of **3x** prepared from unlabeled ALA; (B) sections from the ^{1}H−^{13}C HMBC 2D spectrum of **3x** prepared from roughly equal amounts of [1-^{13}C]- and [4-^{13}C]-ALA. Each panel corresponds to the equivalent proton frequecies (F_2 dimension) as in spectrum (A), taken at four different F_1 (carbon) ranges. Lines illustrate shared correlations between neighboring carbons to a single proton resonance (see text for complete details). (C) Portion of the phase sensitive 2D DQ-COSY of unlabeled **3x** corresponding to the same regions as in (A) and (B). The AB coupling pattern of 2′-acetate protons is highlighted.

^{13}C-NMR assignment, heteronuclear correlated spectroscopy can be employed to aid in the assignments of these protons.

First, the ^{13}C chemical shifts of the macrocyclic carbons derived from 4-^{13}C and 5-^{13}C ALA were assigned as summarized in Table 1. With the exception of C-3 and C-8, all of the carbons of precorrin-3x derived from these two labels could be initially assigned on the basis of chemical shift and C−C coupling information. The unambiguous assignments of C-3 and C-8 (as well as further confirmation of the remaining 14 carbons) and their correlations to neighboring protons can be deduced by analysis of the HMBC/HMQC network (Figure 2B). Of primary importance for the stereochemistry at C-20, the assignment of ring A of precorrin-3x proceeded as follows. Unambiguously assigned carbons C-20 and C-1 both show strong correlations to the C-20 methyl protons (δ 1.65), while C-1 displays additional correlations to C-2 methyl and acetate protons (panel 2, Figure 2B). Both of these protons also correlate to C-3 (δ 51, panel 1, Figure 2B), thereby confirming the assignment of the latter. Finally, C-3 correlates to the C-5 meso proton (not shown), which also correlates to C-6, thus leading into the sequential assignments of rings B, C, and D summarized in Table 1.

Table 1. Carbon-13 NMR Assignments and Proton Correlations of Precorrin-3x Heptamethyl Ester in Benzene-d_6

	δ ^{13}C (J_{CC})	HMBC (HMQC) correlations
C1	105.17	1.30, 1.72
C3	51.05	1.34, 1.74, 2.25, 2.36, 4.85
C4	159.18 (75.1 Hz)	1.72, 3.08
C5	83.75 (75.4, 7.6 Hz)	(4.85)
C6	178.66	1.03, 1.15, 2.13, 2.24, 4.85
C8	49.43	1.05, 2.13, 2.24, 2.33, 5.89
C9	150.75 (82.6, 7.6 Hz)	1.75, 2.70
C10	102.89 (82.2 Hz)	(5.89)
C11	126.95	3.43, 5.89
C13	118.82	2.23, 2.36, 2.77, 2.81, 3.43
C14	125.55 (50.2 Hz)	2.55, 2.60
C15	22.95 (50, 50 Hz)	(2.95, 3.96)
C16	127.25 (50.1 Hz)	2.95
C17	117.37	2.87, 2.91, 3.16, 3.40
C19	131.60	1.66, 2.87, 2.91, 3.16, 3.40
C20	78.65	1.66
C2-Me	20.4	1.7, 2.4
C7-Me	18.3	2.2, 2.3
C20-Me	25.9	1.5, 1.8
carbonyls		
C7-acetate	170.9	2.16, 2.25
C12-acetate	172.0	3.43
C2-acetate	172.5	1.72, 2.38
C3-propionate	172.6	2.14, 2.22
C17-propionate	172.8	2.04, 2.17, 2.58
C8-propionate	173.3	2.26, 2.34
C13-propionate	173.4	2.65, 2.75, 2.97
C18-acetate	176.6	3.40

A 2D double quantum filtered (DQ) COSY experiment was next performed to assign the remaining protons, particularly those which were "silent" in the HMBC/QC experiments. For example, the 2′-acetate methylene protons display a well-separated AB coupling pattern (δ 1.72, 2.38) in the DQ-COSY spectrum (Figure 2C) presumably due to their distinct environments fixed by the γ-lactone ring. However, only one of these protons (δ 1.72) was found to correlate to C1 in the HMBC experiment above (panel 2, Figure 2B). Comparison of cross peaks of identical proton chemical shift between the HMBC (Figure 2B) and DQ-COSY (Figure 2C) spectra leads to the assignment of all the remaining acetate and propionate spin systems summarized in Table 2.

Finally, proton−proton through-space connectivities were established via the 2D spin-locked NOE or ROESY experiment. At 500 MHz, the molecular weight of precorrin-3x, hence its rotational correlation time, would result in weak or absent cross peaks in the regular 2D-NOESY experiment. To solve this problem a lower field strength can often be used sucessfully,[22] however the losses in sensitivity and spectral dispersion upon going to 300 MHz were unacceptable in the case of precorrin-3x.

Figure 3 shows a portion of the 2D ROESY spectrum of precorrin-3x containing the NOE correlations for the 3 methyl groups obtained with a spin lock mixing period of 300 ms. It can be seen that a strong cross peak is observed between the C-2 methyl protons (1.3 ppm) and C-20 methyl protons at 1.65 ppm. A strong cross peak is also apparent between the C-2 methyl group and one of the 2′-acetate protons at 1.72 ppm together with a weaker interaction to one of the 3′-propionate pairs. No other correlations are indicated between the C-20 methyl group to neighboring 2′- or 18′-acetate protons, although the single 2′-acetate proton (δ 1.72), which correlates with the C-2 methyl, lies too close in chemical shift to the C-20 methyl

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Table 2. Proton Assignments and Correlations of Precorrin-3x Heptamethyl Ester in Benzene-d_6

assignment	δ (ppm)	ROESY correlations
2-Me (s)	1.33	3′, 20-Me, 3, 2′b
2′a (d) $J = 17$ Hz	1.72	2′b
2′b (d) $J = 17$ Hz	2.38	2′a
3 (d,br)	3.07	2-Me, 3′, 3″
3′ (m)	1.55, 1.72	2-Me, 3
3″ (m)	2.14, 2.22	3
5 (s)	4.95	7′a, 8′, 7-Me, 3, 3′
7-Me (s)	1.04	8′, 5, 7′ab
7′a (d) $J = 14.5$ Hz	2.16	7-Me, 8
7′b (d) $J = 15.1$ Hz	2.26	7-Me, 8
8 (d,br)	2.72	7′ab, 8′
8′ (m)	1.47,1.75	8, 7-Me
8″ (m)	2.26, 2.38	
10 (s)	5.89	8, 12′, 8′, 8″
12′ (dd)	3.43, 3.57	10, 13′
13′ (dd)	2.96	12′
13″ (m)	2.65, 2.75	
15 (br)	2.98, 3.95	
17′ (dd)	2.58	
17″ (m)	2.04, 2.17	
18′ (dd)	3.40, 3.48	
20-Me (s)	1.6	2-Me, 3′
7 × CO_2Me	3.17, 3.25, 3.26, 3.30, 3.32, 3.35, 3.41	

to resolve a potential cross peak. This evidence therefore suggests that the methyl group attached to C-20 must be facing *down*, that is on the same face as the C-2 methyl group.

This conclusion is borne out in the molecular model of precorrin 3x. Of the four stereoisomers possible about the C-1, C-20 centers, the two containing the γ-lactone formed by attack of the acetate carbonyl on the lower face of C-1 can be ruled out due to the excessive strain of the resultant *trans* fusion of the two five membered rings. In the two stereoisomers about C-20 in which the lactone is on the upper face of C-1, the C-20−C-2 methyl−methyl interproton distances can be solved by molecular modeling. These were found to be 2.34 Å for the C-20 methyl on the lower face and 5.0 Å for the methyl group in the opposite configuration, the latter distance being at the upper limit for an observable NOE. A model of precorrin-3x depicting the NMR-favored orientation with the C-20 methyl group down, viewed edgewise and looking toward C-20, is shown in Figure 4. Further inspection of the model offers an explanation for the absence of the usually favorable H−H interaction between meso (C-20 Me) and the β-pyrrole substituent (C-18 acetate) as is the case between the H-5 proton (δ 4.95) and the C-7 CH_3 as demonstrated in Figure 2C. Due to the sp^3 centers at C-15 and C-20, ring D of precorrin-3x is puckered upward, out of the usually planar geometry observed with fully conjugated porphyrins. This has the effect of increasing the C-18−C-20 interproton distance to 3.6 Å when the C-20 methyl group is below the plane. This distance is decreased to 1.3 Å when the C-20 methyl group is modeled above the plane. The absence of any observable interactions between C-20 C**H**$_3$ and C-2′ C**H**$_2$ as well as C-18′ C**H**$_2$ thus provides strong evidence that the C-20 methyl is in the α- (below plane) configuration leading to the definition of the stereochemistry as shown.

Discussion

The ^{13}C-NMR spectrum of HBA (**7**) (Figure 1) derived from [1-^{13}C, 1,1,4-$^{18}O_3$]-ALA clearly shows that all seven carboxylates have retained the initial $^{18}O/^{16}O$ ratio, in confirmation of a recent observation.[23] This lack of exchange not only reinforces

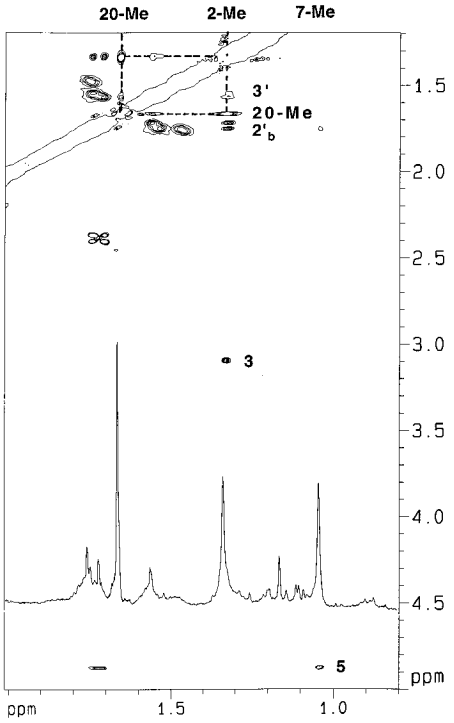


Figure 3. Region of the 500-MHz 2D ROESY spectrum of **3x** displaying the methyl group connectivity, obtained with a 300-ms spin-lock period. All cross peaks are opposite in phase relative to the diagonal. The connectivity between the C-2 and C-20 methyl protons is indicated.

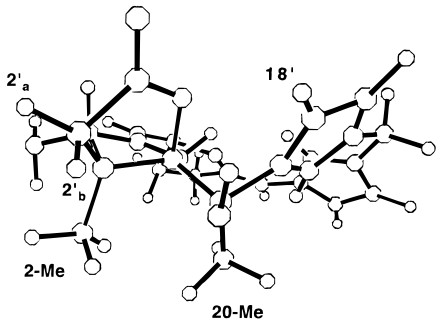
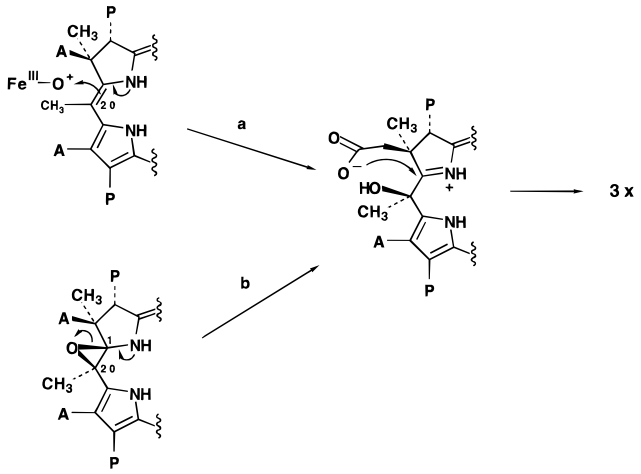


Figure 4. Molecular model of Precorrin-3x illustrating the close proximity between the C-2 and C-20 methyl groups in the stereoisomer formed via *cis*-lactonization and oxygen insertion from the upper face. The molecule is pictured edgewise, looking toward the ring A lactone (lefthand side) and ring D (right). With the exception of the C-2 acetate, the remaining acetate and propionate side chains have been replaced in this figure only with protons for clarity.

the earlier findings and conclusion[6] that the *Pr. shermanii* and *Ps. denitrificans* pathways differ in the stage at which cobalt is inserted—into precorrin-2 in the former and after HBA formation in the latter organism—but also indicates a dichotomy of mechanism for *both* the ring contraction and deacetylation steps. Thus, in *Ps. denitrificans*, molecular oxygen is the source of the C-20 hydroxyl in precorrin-3x[9,10] **(3x)** which, on ring contraction, leads to the methyl ketone function at C-1 in precorrins 4 (**4**) and 5 (**5a**) (Scheme 1). Pivotal to the stereoelectronic control of the ring contraction process is the absolute stereochemistry of precorrin-3x, which we have determined by examination of the ROESY spectrum as discussed above. A strong NOE is observed between the C-20 CH_3 and the ring A C2-methyl group (Figure 3), an assignment confirmed by lack of NOE from the CH_3 at C-20 to any other proton.

(23) Li, Y.; Alanine, A. I. D.; Vishwakanna, R. A.; Balachandran, S.; Leeper, F. J.; Battersby, A. R. *J. Chem. Soc., Chem. Commun.* **1994**, 2507.

Scheme 3



Such an interaction is possible, as confirmed by molecular modeling, only when the orientation of the lactone is on the upper face, and the C-20 methyl on the lower face. Thus the configuration of the C-20 hydroxyl is *cis* to the oxygen terminus of the γ-lactone at C-1. This finding has important implications for the mechanisms of precorrin-3x formation and for the subsequent ring contraction process. First, formation of precorrin-3x must involve direct insertion of oxygen from the upper face of precorrin-3 (Scheme 3). The enzyme resposible for oxygen insertion, CobG, is known to be an iron−sulfur protein and may involve a $Fe^{III}-O^{+}$ species ($\equiv OH^{+}$), which finds a chemical analogy in the oxidation of the C-5 position of corrins with Udenfriend's reagent[24] (Fe^{II}, ascorbate, O_2), leading to the insertion of hydroxyl followed by γ-lactone formation from the upper face of the imine, as in mechanism (a). On the other hand, mechanism (b) requires initial formation of the 1,20 epoxide, again on the upper face, and for steric reasons requires opening of the epoxide through participation of the nitrogen lone pair, rather than by direct attack by the carboxylate of the C-2 acetate side chain (Scheme 3). A second implication of the *cis* hydroxy-lactone array dictates that, during the subsequent rearrangement catalyzed by the SAM-requiring enzyme, CobJ (Scheme 1, **3x** → **4**), migration of ring D takes place below the plane of the ring leading to a β-oriented methyl ketone function at C-1 in precorrin-4, after elimination of the lactonic function *without oxygen exchange* of the ring A carboxylate.

Having determined the stereochemistry of precorrin-3x at C-1 and C-20 (and hence precorrins 4 **(4)** and 5 **(5)** at these centers), we can now comment further on the dichotomy of mechanism associated with the two pathways. Thus, in *Ps. denitrificans*, acetic acid must be lost by hydrolysis of precorrin-5 involving external water or other nucleophile, associated with the conversion of precorrin-5 → precorrin-6x (**6**) (Scheme 1), a process catalyzed by the methyl transferase enzyme CobF,[25] without requiring participation of the ring A carboxylate in mixed anhydride formation.[26] However, in *Pr. shermanii* which does not use O_2, participation of the ring A acetate could result in the formation of a δ-lactone, mediated by a two-electron valency change of cobalt as previously suggested,[27] thereby installing ^{18}O from the ring A carboxylate at C-20 (Scheme 2). A hydrolytic step[5] would then set up the pinacol-like ring contraction and also exchange ^{18}O in the ring A carboxylate. Internal deacetylation[27] forms the mixed anhydride, whose regiospecific hydrolysis [(⟹); Scheme 2] accounts for the nearly complete loss of label from the original ring A carboxylate.[1,2,4]

There is no doubt that there are at least two pathways to vitamin B_{12} in nature. The first is the ancient, anaerobic route used by archaebacteria (and by *Pr. shermanii*), which features early insertion of cobalt and exchange of oxygen at the ring A acetate as a necessary consequence of functionalization at C-20 with the internal oxygen derived from the acetate carboxyl. We suggest that the functionalization of C-20 and the resultant ring contraction and deacetylation mechanisms are driven by the versatility of the valency changes of the coordinating cobalt , which, serving as the primodial equivalent of O_2, carries out the equivalent functions. Thus, in the second, aerobic pathway, O_2 is used to set up the pinacolic ring contraction and cobalt is inserted *after* corrin formation, a sequence which requires neither participation (and hence isotopic exchange) of the unique ring-A acetate carboxylate nor the presence of cobalt in both the ring contraction and deacetylation processes. It is fascinating that Nature should have evolved and conserved both pathways to such a complex and essential cofactor.

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(25) Min, C.; Atshaves, B. P.; Roessner, C. A.; Stolowich, N. J.; Spencer, J. B.; Scott, A. I. *J. Am. Chem. Soc.* **1993**, *115*, 10380.

(26) Alternatively, the mixed anhydride (**5b**) could also be formed in the aerobic pathway and then suffer regiospecific hydrolysis at the carbonyl group b to leave the original $^{18}O/^{16}O$ ratio unchanged. In view of the opposite regiospecifity of this hydrolysis in the anaerobic pathway we consider such a scenario as extremely unlikely.

(27) Scott, A. I. *Heterocycles* **1994**, *39*, 471.