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# Genetically Engineered Synthesis and Structural Characterization of Cobalt−Precorrin 5A and −5B, Two New Intermediates on the Anaerobic Pathway to Vitamin $B_{12}$: Definition of the Roles of the CbiF and CbiG Enzymes

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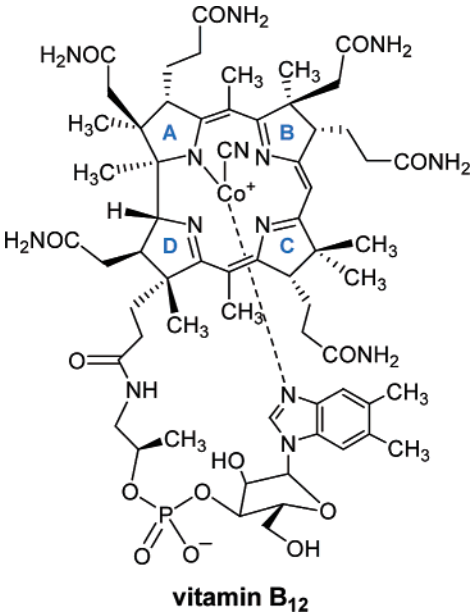
***Abstract:*** Two new cobalt corrinoid intermediates, cobalt−precorrin 5A and cobalt−precorrin 5B, have been synthesized with the aid of overexpressed enzymes of the vitamin $B_{12}$ pathway of *Salmonella enterica* serovar typhimurium. These compounds were made in several regioselectively $^{13}C$-labeled forms, and their structures have been established by multidimensional NMR spectroscopy. The addition of CbiF to the enzymes known to synthesize cobalt−precorrin 4 resulted in the formation of cobalt−precorrin 5A, and the inclusion of CbiG with CbiF produced cobalt−precorrin 5B, which has allowed us to define the role of these enzymes in the anaerobic biosynthetic pathway. CbiF is the C-11 methylase, and CbiG, an enzyme which shows homology with CobE of the aerobic pathway, is the gene product responsible for the opening of the ring A $\delta$-lactone and extrusion of the "$C_2$" unit. The discovery of these long-sought intermediates paves the way for defining the final stages of the anaerobic pathway. It is of considerable evolutionary interest that nature uses two distinct pathways to vitamin $B_{12}$, both conserved over several billion years and featuring completely different mechanisms for ring-contraction of the porphyrinoid to the corrinoid ring system. Thus the aerobic pathway utilizes molecular oxygen to trigger the events at C-20 leading to contraction and expulsion of the "$C_2$" unit as acetic acid from a metal-free intermediate, whereas the anaerobic route features internal delivery of oxygen from a carboxylic acid terminus to C-20 followed by extrusion of the "$C_2$" unit as acetaldehyde, using cobalt complexes as substrates.

## Introduction

Vitamin $B_{12}$, one of the most structurally complex low-molecular-weight natural products, has a distinctive corrinoid macrocycle (Figure 1).

Coenzyme $B_{12}$, which mediates a number of important biochemical transformations, features a 5-deoxyadenosyl moiety in place of the cyano group as the axial ligand on cobalt. Contraction of the macrocycle resulting in the direct connection between rings A and D and involving a "$C_2$" unit extrusion in its formation has attracted attention since the beginning of the biosynthetic studies on the cofactor.[1−3] Two pathways to make vitamin $B_{12}$ have been defined;[4,5] while aerobic organisms utilize molecular oxygen to achieve this ring contraction in a metal-free system and release the "$C_2$" unit as acetic acid, anaerobes resort to an early cobalt insertion in the macrocycle and a




**vitamin $B_{12}$**

***Figure 1.*** Structure of vitamin $B_{12}$ (cyanocobalamin).

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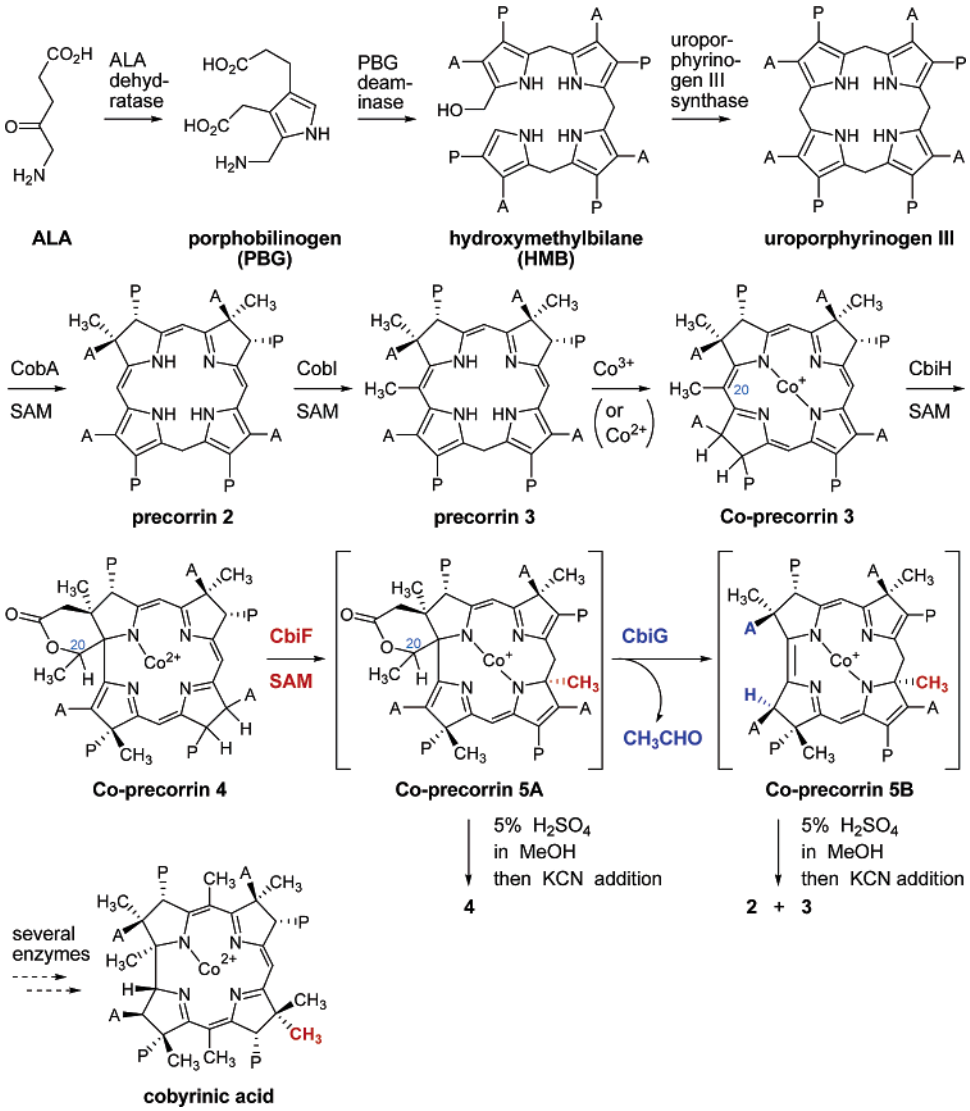
***Scheme 1*** [a,b]




[a] The proposed enzymatic synthesis of compounds **2**, **3**, and **4** and the biosynthetic intermediates and enzymes of the vitamin $B_{12}$ anaerobic pathway between ALA and cobyrinic acid are shown. Co−precorrin 4 has not been isolated, but its oxidized form Co−factor 4 has been isolated and characterized. [b] A = $CH_2CO_2H$; P = $CH_2CH_2CO_2H$; SAM = *S*-adenosyl-L-methionine.

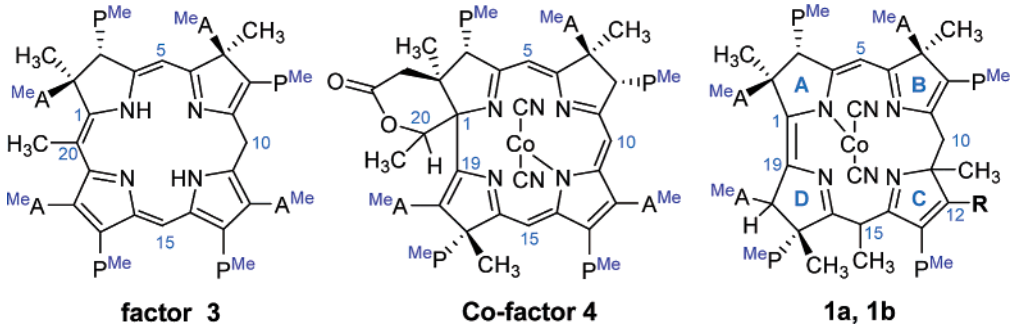


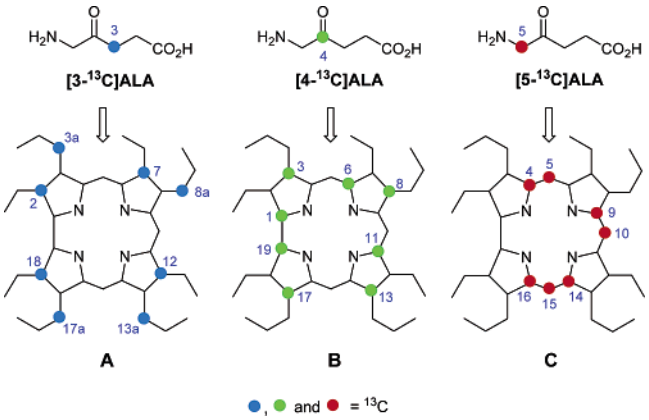

***Figure 2.*** Structures of compounds related to the biosynthetic intermediates of the vitamin $B_{12}$ aerobic and anaerobic pathways. **1a**, R = $CH_2CO_2CH_3$; **1b**, R = $CH_3$; $A^{Me}$ = $CH_2CO_2CH_3$; $P^{Me}$ = $CH_2CH_2CO_2CH_3$.

surprising ring A $\delta$-lactone formation followed by extrusion of acetaldehyde.[6,7] Most of the biosynthetic intermediates and enzymes of the aerobic pathway have been characterized, but despite intensive research on the anaerobic counterpart, the structures of the intermediates between cobalt−precorrin 4 and cobyrinic acid remain to be determined, and the roles of some of its enzymes are not known (Scheme 1).[5]

Previously, we reported the enzymatic synthesis of six unnatural corrinoids (**1a**, **1b** and four other related products) using the *Salmonella enterica* enzymes CbiH, CbiF, CbiG, and CbiT overexpressed in *Escherichia coli* (Figure 2).[8]

The unexpected "early" methylation at C-15 and decarboxylation at the ring C acetate terminus, attributed to the action of CbiT present in the enzyme lysates, prompted us to investigate

***Scheme 2*** [a]




[a] Isotopically enriched [3-$^{13}$C]-, [4-$^{13}$C]-, and [5-$^{13}$C]ALA are useful starting materials to enhance the $^{13}$C NMR signals in the product.

a similar approach in the absence of this enzyme, which should lead us to the long-sought cobalt-containing pentamethylated biosynthetic intermediate. For this purpose, a new construct was made by deleting the gene cbiT from the plasmid harbored in the strain used in our previous study; the new strain overexpresses only the enzymes CbiF, CbiG, and CbiH.

A powerful and widely used strategy for the structural elucidation of porphyrinoids has been the combination of $^{13}$C-labeled substrate incubations with high-field FT-NMR analysis. For vitamin $B_{12}$ in particular [3-$^{13}$C], [4-$^{13}$C], and [5-$^{13}$C] 5-aminolevulinic acid (ALA) isotopomers are especially useful in order to get information on the nature of each carbon of the macrocycle (Scheme 2).

In this work we report the synthesis and structural characterization of cobalt−precorrin 5A (**4**) and two isomeric forms of cobalt precorrin 5B (**2** and **3**) (Figure 3); these highly oxygen-sensitive products were isolated and analyzed under argon as the monocyano methylesters.

## Results and Discussion

In the first stage of the multienzyme synthesis, [4-$^{13}$C]ALA was incubated with *S*-adenosyl-L-methionine (SAM) and lysates of strains of *E. coli* that express the enzymes aminolevulinic acid dehydratase, porphobilinogen deaminase, uroporphyrinogen III synthase, CobA (precorrin-2 synthase), and CobI (precorrin-3 synthase) overnight under argon giving a yellow solution whose main component was precorrin 3 (Scheme 1). After addition of glycine cobalt(III) salt, SAM, and a lysate of a strain of *E. coli* that expresses the enzyme CbiH, the incubation was continued for 16 h to give cobalt−precorrin 4. In the final step the foregoing green solution was incubated for an additional 16 h with SAM and a lysate of a strain of *E. coli* that expresses the enzymes CbiH, CbiF, and CbiG which afforded a brown solution of the cobalt-containing products. Trapping of the porphyrinoids on DEAE Sephadex, esterification, and chromatographic purifications, all done in the absence of oxygen, yielded two yellow products, compounds **2b** and **3b** (**2b**/**3b** = 2:1) (Figure 3). These products were characterized by electrospray ionization mass spectrometry (ESI) and NMR spectroscopy. The accurate ESI mass measurements for compounds **2b** and **3b** indicated that for both products the $[M + Li]^+$ molecular formula matches $C_{44}{}^{13}C_8H_{66}CoLiN_5O_{16}$, which corresponds to a monocyano cobalt corrinoid with seven unsaturations. This unusual monocyano form was clearly indicated by the *odd* value corresponding to $M^+$ (note the previously observed correlation of *even* values for dicyano forms).[8] While pentacoordinate organocobalt complexes are rare, they have been previously reported.[9] The $^{13}$C NMR analysis of the isomeric products **2b** and **3b** showed that both have C-1 directly connected to C-19 as expected for any intermediate after cobalt−precorrin 4. The pairs of doublets associated with these carbons were located in the $sp^2$ region indicating the presence of a double bond between rings A and D. This fact suggested that the lactone function observed in cobalt−factor 4 had opened, and presumably, C-20 and its attached methyl group were extruded in the process. The COSY $^{13}$C−$^1$H NMR correlation experiment indicated that the only [4-$^{13}$C]ALA-derived carbon carrying a hydrogen is C-3; C-17 and C-11 are quaternary centers while C-6, C-8, and C-13 are $sp^2$ centers. The close similarity of **2b** and **3b** was also observed in their UV−vis spectra.

When the method described above was repeated using [3-$^{13}$C]-ALA as substrate the corresponding products **2a** and **3a** were obtained. The COSY $^{13}$C−$^1$H NMR correlation experiments of these labeled forms allowed us to confirm that C-18 carries a hydrogen, while C-12, at significant lower field when compared to that of C-2, C-7, and C-18, subtends a double bond to C-13.

A third labeled version made from [5-$^{13}$C]ALA afforded products **2c** and **3c**. The ESI mass analyses indicated the presence of only seven $^{13}$C-labeled carbons, confirming the presumed loss of C-20. The COSY $^{13}$C−$^1$H NMR correlation experiments showed that C-15 carries only one hydrogen, but unlike the products isolated in our previous study,[8] its chemical shift is in the aromatic region.

The complete $^{13}$C and $^1$H NMR data for isomers **2** and **3** are summarized in Tables 1 and 2, and the structures proposed are shown in Figure 3.

The absolute configuration at C-11 of compounds **2** and **3** has already been elucidated and shows that the C-11 methyl group is migrated to the α-methyl group of C-12 on the basis of the biosynthetic pathway of vitamin $B_{12}$ (Scheme 1).[10] The absolute configuration of the acetate side chain at C-18 of compound **2** was determined on the basis of the NOESY spectrum (Figure 4).

The NOE correlations were noted between H-18 and $H_2$-17a′ and between $H_3$-17a and $H_2$-18a in compound **2**. Although the NOESY NMR data do not allow assignment of the absolute configuration at C-18 for compound **3**, the stereochemistry shown in Figure 3 follows compound **2**, and the one observed in the later intermediate, cobyrinic acid (Scheme 1). The close NMR values observed for all carbons and hydrogens of **2** and **3** around ring D suggest that these compounds might have the same stereochemistry at that center (C-18). Correlation of all the data presented above with that of the six closely related

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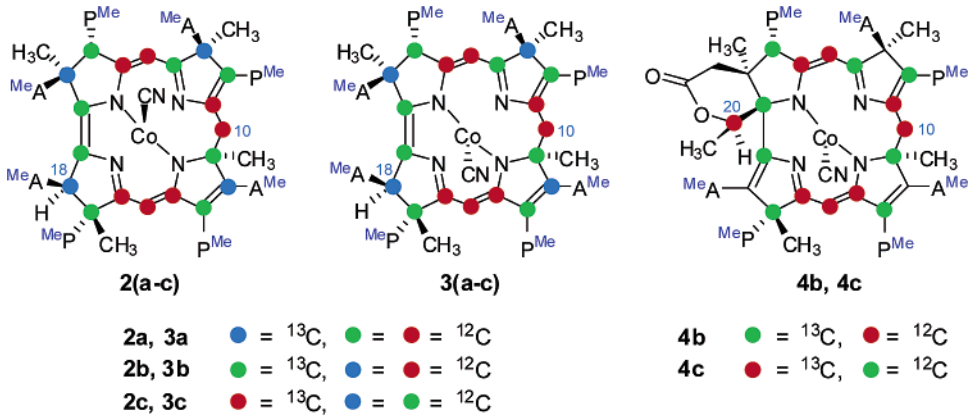



***Figure 3.*** Structures of CbiF and CbiG products (**2a**−**c**, **3a**−**c**) and CbiF product (**4b**, **4c**) from cobalt−precorrin 4. $A^{Me} = CH_2CO_2CH_3$; $P^{Me} = CH_2CH_2CO_2CH_3$.

***Table 1.*** $^{13}C$ NMR Chemical Shifts ($\delta$, ($J$, Hz)) of Cobalt−Precorrin 5B Octamethylester (**2a**−**c** and **3a**−**c**)

| | 2a | 3a |
|---|---|---|
| C-2 | 155.0 (s) | 156.4 (s) |
| C-7 | 57.0 (d, 2.7) | 57.0 (d, 2.7) |
| C-12 | 46.6 (s) | 46.3 (s) |
| C-18 | 44.5 (s) | 43.0 (s) |
| C-3a | 39.1 (s) | 39.7 (s) |
| C-8a | 27.3 (s) | 25.6 (s) |
| C-13a | 20.9 (s) | 20.7 (s) |
| C-17a | 20.3 (d, 2.7) | 20.0 (d, 2.7) |
| | **2b** | **3b** |
| C-6 | 177.0 (s) | 175.5 (s) |
| C-1 | 148.9 (d, 89.8) | 147.4 (d, 90.7) |
| C-19 | 143.9 (d, 89.8) | 143.7 (d, 90.7) |
| C-13 | 137.8 (d, 5.4) | 137.2 (d, 4.5) |
| C-8 | 132.8 (d, 2.7) | 132.1 (d, 2.7) |
| C-11 | 76.2 (d, 2.7, 5.4) | 78.1 (d, 2.7, 4.5) |
| C-3 | 55.5 (s) | 55.0 (s) |
| C-17 | 54.2 (s) | 54.0 (s) |
| | **2c** | **3c** |
| C-14 | 166.8 (d, 67.3) | 165.9 (d, 67.3) |
| C-16 | 165.9 (d, 72.7) | 166.7 (d, 72.7) |
| C-4 | 163.0 (d, 71.8) | 162.3 (d, 70.9) |
| C-9 | 146.9 (dd, 4.5, 50.3) | 144.5 (dd, 4.5, 50.3) |
| C-5 | 91.6 (dd, 4.5, 71.8) | 91.9 (d, 4.5, 70.9) |
| C-15 | 87.8 (dd, 67.3, 72.7) | 87.6 (dd, 67.3, 72.7) |
| C-10 | 33.6 (d, 50.3) | 33.8 (d, 50.3) |

products described in our recent study[8] indicates that the isomerism between compounds **2** and **3** is most likely due to the different facial attachment of the cyanide axial ligand. Quantum mechanical (B3LYP) calculations[11] performed on the model compounds **5a** and **5b**, replacing the $A^{Me}$ and $P^{Me}$ side chains with methyl, showed that an upper face cyanide coordination (the same as that depicted for vitamin $B_{12}$ in Figure 1) implies that $H_3$-11a (orange) is left in close proximity of the "vacant side" of this pentacoordinated system; a lower face cyanide coordination puts $H_{ax}$-10 (red; hydrogen at C-10 that is pointed above the plane of the macrocycle) in a similar situation (Figure 5).

This in turn translates into significantly different chemical shifts of 1.57 ppm observed in the experimental $^1H$ NMR value for $H_{ax}$-10 and 1.38 ppm for $H_3$-11a (see Table 2) as qualitatively

(11) See the Computational Methods section in the Supporting Information for a detailed description of the theoretical calculations.

***Table 2.*** $^1H$ NMR Chemical Shifts ($\delta$) of Cobalt−Precorrin 5B Octamethylester (**2** and **3**) and Cobalt−Precorrin 5A Octamethylester (**4**)

| | 2 | 3 | 4 |
|---|---|---|---|
| $H_2$-2a | 2.39, 2.43 | 2.68, 2.85 | 2.61, 2.64 |
| $H_3$-2a′ | 1.59 | 1.49 | 1.24 |
| H-3 | 4.07 | 4.27 | 3.52 |
| $H_2$-3a | 1.76, 2.14 | 1.89, 2.08 | 2.23, 2.81 |
| $H_2$-3b | 2.42, 2.42 | 2.37, 2.55 | 1.99, 2.34 |
| H-5 | 6.63 | 6.60 | 5.57 |
| $H_2$-7a | 2.58, 2.64 | 2.36, 2.47 | 2.33, 2.37 |
| $H_3$-7a′ | 1.17 | 1.20 | 0.99 |
| $H_2$-8a | 2.65, 2.77 | 2.38, 2.42 | 2.27, 2.59 |
| $H_2$-8b | 2.49, 2.63 | 2.30, 2.42 | |
| $H_2$-10 | 3.42, 3.59 | 1.85, 3.42 | 2.18, 3.31 |
| $H_3$-11a | 0.58 | 1.96 | 1.35 |
| $H_2$-12a | 3.36, 3.44 | 3.45, 3.45 | 3.28, 3.37 |
| $H_2$-13a | 2.82, 2.82 | 2.83, 2.94 | 2.76, 2.85 |
| $H_2$-13b | 2.60, 2.60 | 2.62, 2.62 | 2.57, 2.61 |
| H-15 | 6.46 | 6.49 | 5.91 |
| $H_3$-17a | 1.34 | 1.43 | 1.24 |
| $H_2$-17a′ | 2.35, 2.50 | 2.14, 2.22 | 1.97, 2.02 |
| $H_2$-17b′ | 2.40, 2.40 | 2.18, 2.18 | 2.10, 2.19 |
| H-18 | 4.21 | 4.14 | |
| $H_2$-18a | 2.72, 2.79 | 2.85, 2.98 | 3.01, 3.11 |
| H-20 | | | 4.50 |
| $H_3$-20a | | | 0.69 |

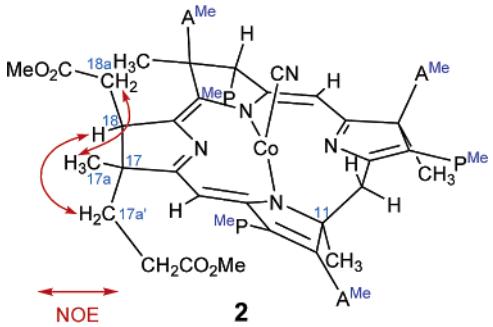



***Figure 4.*** $^1H-^1H$ correlations (NOESY) and the stereochemistry of compound **2**. $A^{Me} = CH_2CO_2CH_3$; $P^{Me} = CH_2CH_2CO_2CH_3$.

reproduced by the DFT calculations.[11] The calculated chemical shift can be found in Figure 9 of the Supporting Information. The *S* configurations at C-18 of compounds **2** and **3** (models **5c** and **5d**, respectively) were also explored computationally; however, in both models the configuration (*R* or *S*) at C-18 had virtually no effect on the calculated chemical shift for $H_{ax}$-10 and $H_3$-11a (see the Supporting Information).

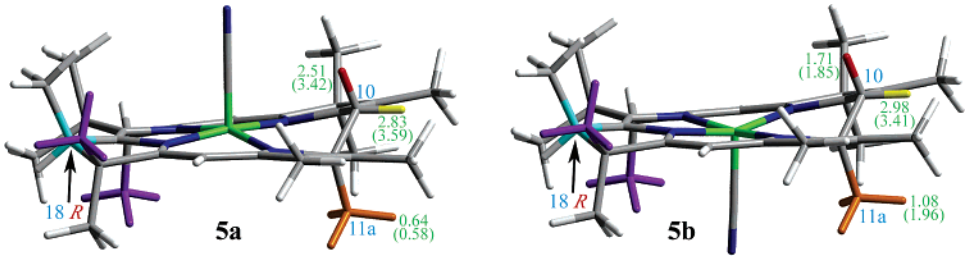



***Figure 5.*** 3D representation of the model compounds **5a** and **5b**. The theoretical (experimental) $^1$H NMR chemical shifts for $H_{ax}$-10, $H_{eq}$-10, and $H_3$-11a of **5a** (**2**) and **5b** (**3**) are shown in the green numbers.

***Table 3.*** $^{13}$C NMR Chemical Shifts ($\delta$, ($J$, Hz)) of Cobalt−Factor 5A Octamethylester (**4b** and **4c**)

| | 4b | | | 4c | |
|---|---|---|---|---|---|
| C-6 | 178.9 | (s) | C-14 or 16 | 173.0 | (d, 71.8) |
| C-19 | 153.3 | (dd, 3.6, 56.5) | C-4 | 171.6 | (d, 70.0) |
| C-13 | 136.8 | (d, 4.5) | C-16 or 14 | 165.5 | (d, 67.3) |
| C-8 | 130.4 | (d, 2.7) | C-9 | 143.2 | (dd, 50.3, 4.5) |
| C-1 | 84.7 | (d, 56.5) | C-5 | 87.4 | (dd, 4.5, 69.1) |
| C-11 | 77.3 | (dd, 2.7, 4.5) | C-15 | 84.5 | (dd, 68.2, 70.9) |
| C-17 | 60.2 | (d, 3.6) | C-20 | 82.3 | (s) |
| C-3 | 55.7 | (s) | C-10 | 32.8 | (d, 50.3) |

There was a large difference in the calculated chemical shift of C-3a and C-17a (purple) between **5a**/**5c** and **5b**/**5d** with the calculated chemical shifts in **5a** and **5b** being more consistent with the experimental results. The energy difference between all four models (**5a**−**d**) was less than 1 kcal mol$^{-1}$ with **5a** being lowest in energy and **5b** 0.5 kcal mol$^{-1}$ higher in energy consistent with the observed 2:1 ratio found experimentally for compounds **2** and **3**. The absolute configuration of compounds **2** and **3** thus determined is shown in Figure 3.

From a biosynthetic point of view, it is of interest to mention that compounds **2** and **3** can be obtained either via the reduced trimethylated intermediate precorrin 3 or from its oxidized counterpart, factor 3 (Figure 2). An explanation for this apparent puzzle could be the existence of endogenous nonspecific oxidoreductases in the *E. coli* lysates that would allow interconversion between precorrin 3 and factor 3; further investigation of this enigma is under way.

In the course of the preparation of the tetramethylated cobalt−corrinoid isomers **2b** and **3b** via precorrin 3 starting from [4-$^{13}$C]-ALA another distinctive brown product **4b** was isolated. Accurate ESI mass determination gave an $[M + Li]^+$ molecular formula corresponding to $C_{45}{}^{13}C_8H_{66}CoLiN_5O_{16}$; the difference of only one carbon unit compared with compounds **2b** and **3b**, the number of carbomethoxy groups (based on the integration of the region between 2.9 and 3.7 ppm in the $^1$H NMR), and the observation that one of the doublets is now an sp$^3$ center suggest the presence of a $\delta$-lactone in ring A as in the earlier isolate, cobalt−factor 4 (Figure 2). The complete $^{13}$C and $^1$H NMR data for compound **4** is summarized in Tables 2 and 3, and the structure proposed is shown in Figure 3.

The more important connectivities observed in the $^{13}$C−$^1$H correlation (COSY and HMBC) and the $^1$H−$^1$H correlation (NOESY) supporting this proposal are shown in Figure 6.

Quantum chemical calculations were also performed on model compounds (**6a**−**h**) for **4**, replacing the $A^{Me}$ and $P^{Me}$ side chains with methyl. There are four possible structures for the upper face cyanide coordination (**6a**−**d**) and another four for the lower face (**6e**−**h**) cyanide coordination shown in Figure 7 (theoretical

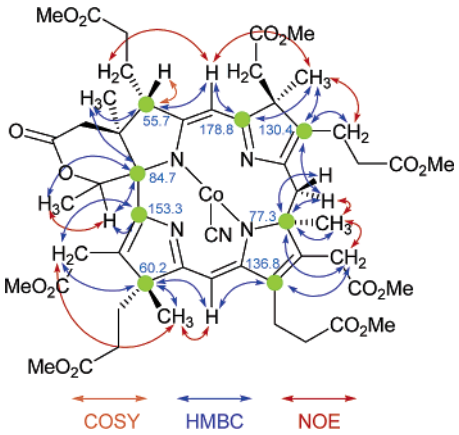



***Figure 6.*** Important $^{13}$C−$^1$H correlation (COSY and HMBC) and the $^1$H−$^1$H correlation (NOESY) of compound **4b**.

chemical shifts are reported in Figure 9 of the Supporting Information).

The energy for the models with an *R* configuration at C-1 (**6b**, **6d**, **6f**, and **6h**) are at least 30 kcal mol$^{-1}$ higher in energy than those with an *S* configuration and are therefore not discussed further. In contrast to the results for **5a** and **5b**, the calculated chemical shift for $H_{ax}$-10 and $H_3$-11a varied little with a change in the coordination face of the cyanide ($H_{ax}$-10 1.91−2.04 ppm and $H_3$-11a 0.42−0.89 ppm). In all structures of the upper face coordinated cyanide for **6**, the cyanide ligand is crowded by the lactone and is therefore pushed closer to $H_{ax}$-10 (Figure 8).

The lowest energy structure (**6e**) has a lower face coordinated cyanide and an *R* configuration at C-20. The calculated chemical shifts at C-1 and C-20 for **6e** are consistent with the experimental values. Model **6g** is only 1 kcal mol$^{-1}$ higher in energy than **6e** and has an *S* configuration at C-20, but the calculated chemical shifts for C-1 and C-20 are not consistent with the observed values. The only other model that has reasonable calculated chemical shifts for C-1 and C-20 and an *R* configuration at C-20 is model **6a**, but it is 1.4 kcal mol$^{-1}$ higher in energy than **6e** due to the crowding of the upper face by the lactone ring. The larger difference in energy between the upper and lower face coordination of the cyanide in **6** (1.4 kcal mol$^{-1}$) compared to that of **5** (0.5 kcal mol$^{-1}$) explains why only one isomer is observed for **4** and, on the basis of the energetic and calculated chemical shifts, the single isomer has a lower face cyanide coordination and an *S* configuration at C-1 and an *R* configuration at C-20 as shown in Figure 3.

The important realization that the free acid form of compound **4** is the precursor of the tetramethylated intermediate corresponding to compounds **2** and **3** prompted us to repeat a similar incubation experiment but this time intentionally leaving out

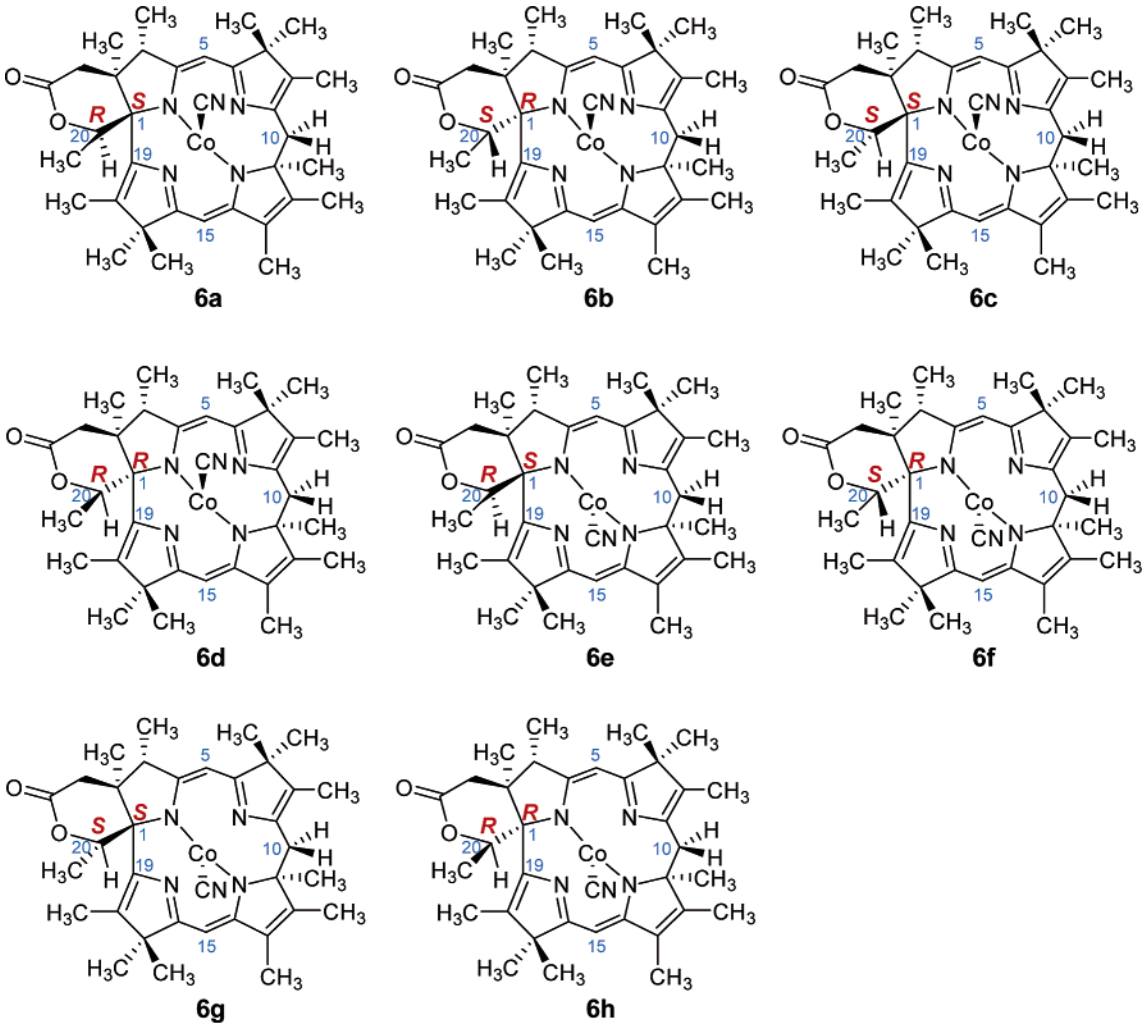



***Figure 7.*** Structures of the DFT model compounds (**6a**−**h**) for the experimental compound **4b**.

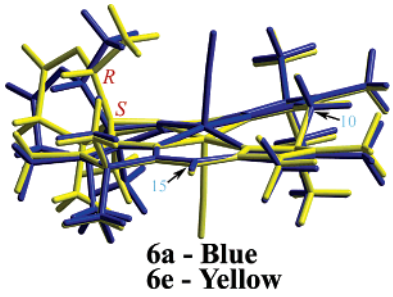



***Figure 8.*** 3D representation of the lowest energy upper and lower face model compounds **6a** and **6e**, respectively.

the enzyme CbiG, as this would define the role of this enzyme as the ring A lactone opening and "$C_2$" unit extrusion catalyst.

This time factor 3 derived from [4-$^{13}$C]ALA was used as the starting material, and after the addition of glycine cobalt(III) salt, lysates of *E. coli* strains expressing exclusively the enzymes CbiH and CbiF were used. After overnight incubation the corrinoid products were trapped on DEAE Sephadex, esterified, and purified as described before. On the basis of NMR analysis of the crude mixture, compound **4b** was determined to be present; however, compounds **2b** and **3b** were absent. This confirmed that the enzyme CbiG is the one responsible for the loss of C-20 and its attached methyl group (see Scheme 2).

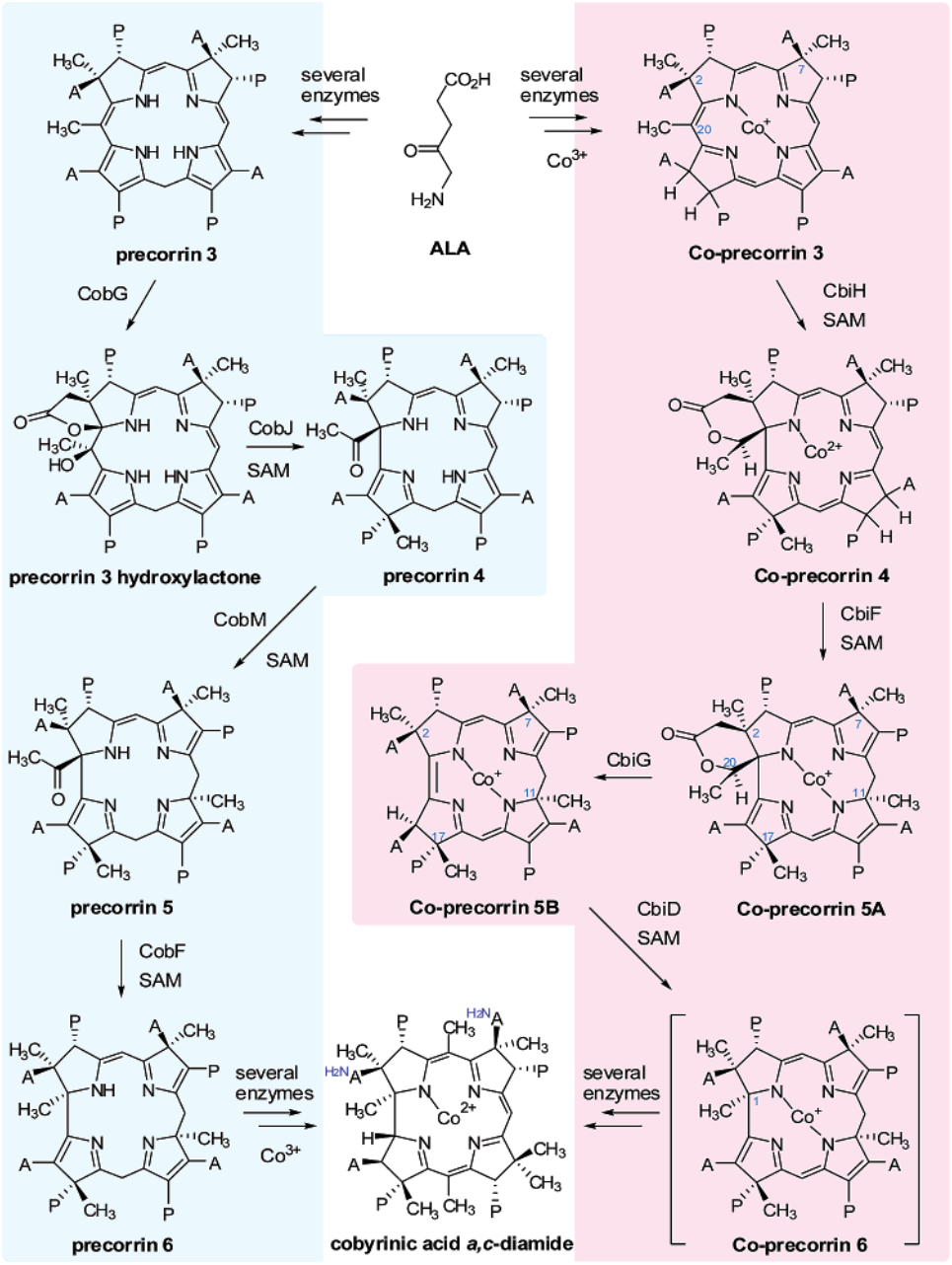
In one additional experiment starting from factor 3 derived from [5-$^{13}$C]ALA, compound **4c** was formed. NMR analysis confirmed the unique features present in this product, i.e., the singlet for C-20 is still present, its chemical shift is consistent with a lactone function in ring A, and C-10 appears in the characteristic region for a reduced meso carbon.

Following the nomenclature proposal for the vitamin $B_{12}$ precorrin intermediates of assigning figures to indicate the number of methyl groups derived from SAM, and letters to distinguish intermediates having the same number of introduced methyl groups, compound **4** can be properly named the heptamethylester of cobalt−precorrin 5A and compounds **2** and **3** isomeric forms of the octamethylester of cobalt−precorrin 5B, respectively.

## Conclusion

Unlike all the cobalt porphyrinoids that we have described in the past,[8] where only one nitrogen of the macrocycle is covalently bound to the metal ion and two cyanides appear as axial ligands attached to the trivalent cobalt, the products described in this study have two nitrogen atoms covalently coordinated to the metal and only one cyanide as axial ligand. Compound **4** (cobalt−precorrin 5A) corresponds to the long-awaited pentamethylated intermediate having five SAM-derived methyl groups at C-2, C-7, C-20, C-17, and C-11 and is the perfect anaerobic equivalent to the metal-free pentamethylated

***Scheme 3*** [a,b]




[a] The two pathways for the biosynthesis of vitamin $B_{12}$. Left scheme: the oxygen-dependent route from ALA to cobyrinic acid a,c-diamide in aerobic organisms (*P. denitrificans*). Right scheme: the proposed cobalt-dependent route from ALA to cobyrinic acid a,c-diamide in anaerobic organisms (e.g., *S. enterica* serovar typhimurium). [b] A = $CH_2CO_2H$; P = $CH_2CH_2CO_2H$; $A^{NH_2}$ = $CH_2CONH_2$; $P^{NH_2}$ = $CH_2CH_2CONH_2$; SAM = *S*-adenosyl-L-methionine.

product precorrin 5, that was made with the aid of enzymes of the aerobic organism *Pseudomonas denitrificans*,[12,13] showing the same hybridization for each of the carbons on the periphery of the macrocycle and retaining the $\delta$-lactone feature present in cobalt−precorrin 4 (Scheme 3).

Compounds **2** and **3** (cobalt−precorrin 5B) correspond to the deacylation product of the ring A lactone opening in which C-20 and its attached methyl group are lost. On the basis of mass spectra, detailed NMR analysis, and molecular modeling the most likely relationship between compounds **2** and **3** is that they are identical except for the face of attachment of the cyanide ligand.

Although the mechanistic proposals for this portion of the aerobic pathway involve the participation of a single enzyme (CobF) for the overall process of deacylation and subsequent C-1 methylation, a deacylated and not yet C-1-methylated intermediate **7** was postulated.[4]

Compounds **2** and **3** correspond, indeed, to the cobalt-containing version of this hitherto unisolated product **7**. We can venture to propose the existence of pentamethylated cobalt-free precorrin, the second one isolable from a SAM-free CobF incubation.

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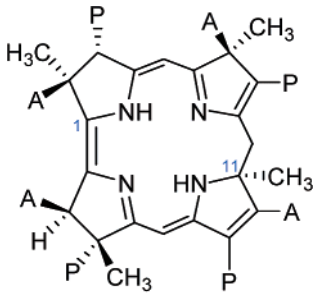
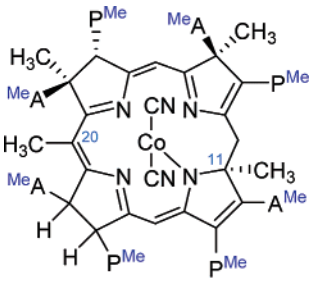


7

Since compound **4** was made with the aid of CbiF only, in addition to all the other enzymes necessary to make cobalt−precorrin 4, and compounds **2** and **3** were made with the sole addition of CbiG to the previous cocktail, it is now possible to confidently assign the specific function of each enzyme. CbiF, as expected from its homology to *P. denitrificans* CobM, is the SAM−cobalt−precorrin 4 methyl transferase which methylates the C-11 position; the product can be named cobalt−precorrin 5A. This enzyme has a high degree of nonspecificity and methylates several closely related porphyrinoids at this α-position. In fact, many of the attempts to obtain compound **4** in multienzyme incubations were hampered by the early action of CbiF on cobalt−precorrin 3 before CbiH had performed its function, leading to the derailment product cobalt−tetramethylcorphinoid **8**.[14,15]

CbiG is the cobalt−precorrin 5A hydrolase which hydrolyzes the ring A acetate δ-lactone with the loss of the C-20 carbon and its attached methyl group in the form of acetaldehyde;[7] so the product can be named cobalt−precorrin 5B.



8

The next enzymatic step on the anaerobic biosynthetic pathway, based on the established methylation sequence and homology to the aerobic counterpart, is methylation at C-1. The enzyme responsible for this alkylation appears to be CbiD (Scheme 3). However, the apparent requirements of the presence of CbiA and CbiP for its activity, in the reported engineered in vivo synthesis of cobyrinic acid diamide,[16] anticipates the challenge that will be encountered in deciphering its complex mode of action.

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**Supporting Information Available:** Experimental details and spectral data, as well as further computational details. This material is available free of charge via the Internet at http://pubs.acs.org.

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