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Structural characterization of novel cobalt corrinoids synthesized by enzymes of the vitamin B_{12} anaerobic pathway

Patricio J. Santander,* Yasuhiro Kajiwara, Howard J. Williams and A. Ian Scott*

Center for Biological NMR, Department of Chemistry, Texas, A&M University, PO Box 3255, College Station, TX 77843-3255, USA

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Abstract—Investigation on the use of the oxidized form (factor 3 (**3a**)) of the trimethylated intermediate (precorrin 3 (**2**)) as a substrate for the enzymes of the anaerobic pathway to vitamin B_{12} led to the synthesis of three pairs of novel cobalt corrinoids. The products were made with the aid of the *Salmonella typhimurium* enzymes CbiH, CbiF, CbiG, and CbiT, were synthesized in several ^{13}C labeled versions, and were isolated as methylesters after esterification. Structures were determined by detailed NMR and MS analyses. Each set of products was obtained in the decarboxylated (R=Me) and non-decarboxylated ($R=CH_2COOCH_3$) forms (at the C-12 position of the porphyrinoid).

1. Introduction

Two routes for the biosynthesis of vitamin B_{12} are known.[1,2] The path used by aerobes such as *Pseudomonas denitrificans* includes an oxygen-dependent step and a late cobalt insertion in the macrocycle[3] in contrast with anaerobes like *Salmonella typhimurium* which present a much earlier insertion of cobalt.[4]

Although in previous studies on the earlier enzymes of the anaerobic pathway[5,6] we have held to the general belief that the oxidation state of the di- and trimethylated intermediates is maintained at the same level (dihydroisobacteriochlorin), the fact that both in aerobes and anaerobes their oxidized counterparts have been successfully used as substrates when incubated with cell-free extracts[7,8] prompted us to investigate the possibility to proceed further in the path utilizing factor 3 (**3a**) (the purple oxidized form of the trimethylated intermediate) rather than precorrin 3 (**2**) (the yellow labile reduced form) as a substrate for CbiH and the next putative enzymes of the pathway.

As in previous studies,[6] we prepared precorrin 3 (**2**) from 5-aminolevulinic acid (**1**) by a multienzyme process, allowed its oxidation during the course of the esterification, and then isolated and purified factor 3 octamethylester (**3b**) by chromatography. The pure isobacteriochlorin methyl ester (**3b**) was hydrolyzed with piperidine and the octapiperidinium salt of factor 3 (**3c**) was used as a substrate for the subsequent enzymatic experiments (Scheme 1). The previous failure to obtain new isolates when utilizing only the CbiH and CbiF methylating enzymes and the suggestions that CbiG may play a role shortly after the macrocycle ring contraction process[9] led us to choose a strain of *Escherichia coli* that overexpresses four of the Cbi enzymes (CbiT,F,G,H) from *S. typhimurium*, which has been described in the past.[10]

2. Results and discussion

Precorrin 3 (**2**) was made by simultaneous incubation of 5-aminolevulinic acid (ALA) (**1**) with lysates of *E. coli* strains[10] overexpressing the enzymes aminolevulinic acid dehydratase, porphobilinogen deaminase, uroporphyrinogen III synthase, CobA and CobI, and *S*-adenosyl-L-methionine (SAM); trapping of the pigment, followed by esterification and TLC purification, yielded tens of milligrams of factor 3 octamethylester (**3b**).

The pure factor 3 octamethylester (**3b**) was hydrolyzed under argon in 2 M aqueous piperidine; after elimination of the excess of piperidine, the factor 3 octapiperidinium salt (**3c**) was incubated under argon with a lysate

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* Corresponding authors. Tel.: +1 979 845 4032; fax: +1 979 845 5992 (P.J.S.); e-mail: patricio@tamu.edu

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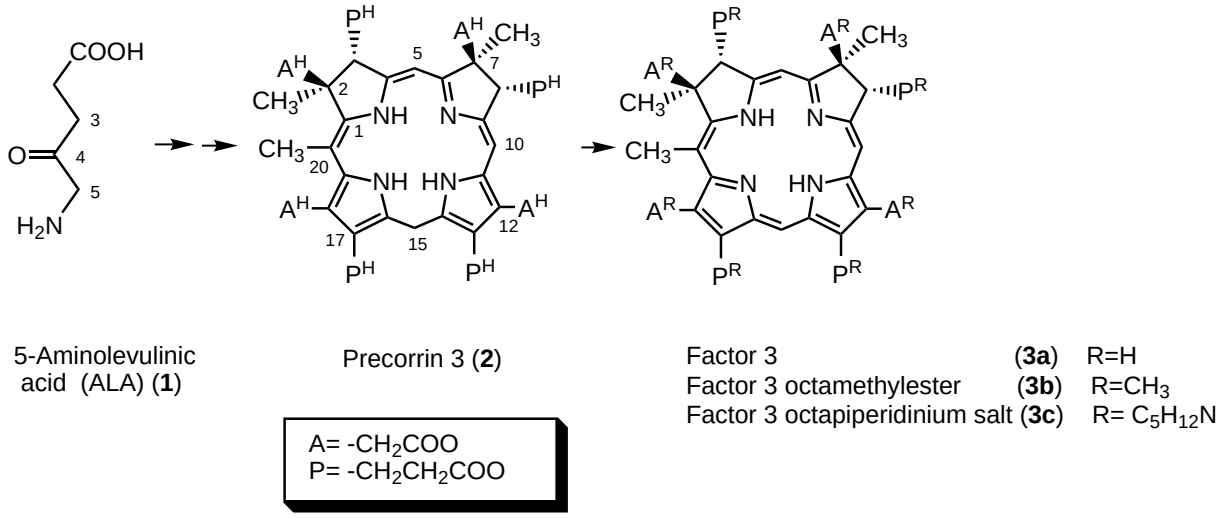


Scheme 1. Preparation of the substrate factor 3 octapiperidinium salt (**3c**).

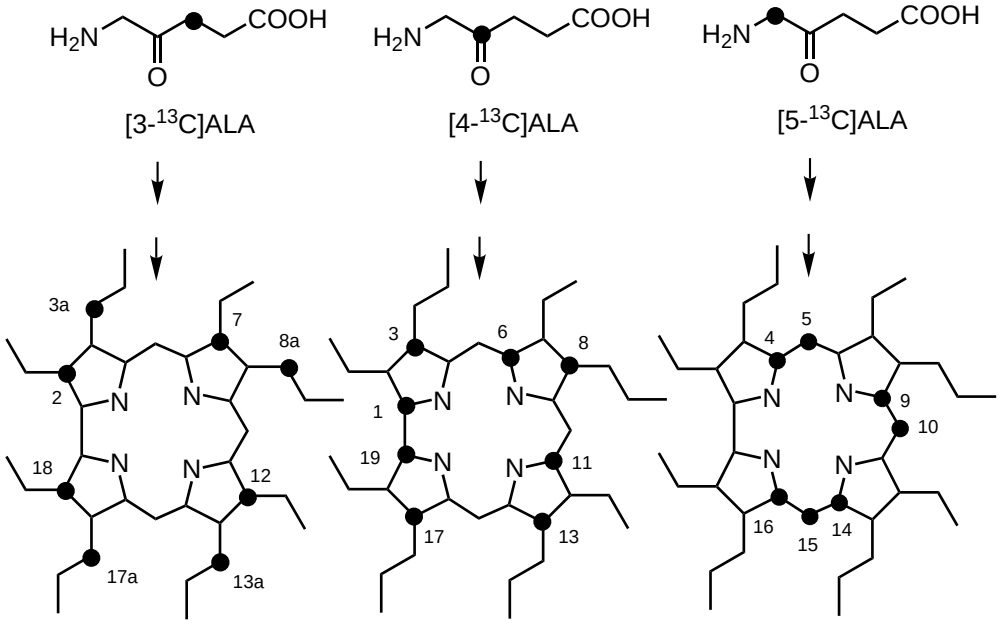
of *E. coli* containing the enzymes CbiH, CbiG, CbiF, and CbiT, SAM, and either cobalt (II) chloride or glycine cobalt (III) salt.

After overnight incubation, the corrinoid products were trapped and esterified with methanol/sulfuric acid. After exposure to air, TLC purification of the mixture of methylester products in solvent systems containing KCN afforded three distinctive colored bands (yellow, red and purple in the same order of elution). Each of these colored bands could be further resolved, by multiple elutions, into two products which were later shown to have the same arrangement of double bonds but to differ only in their molecular weights.

The ability to selectively label all the carbons on the periphery of the macrocyclic final products in different experiments, combined with the use of ^{13}C NMR techniques, gave us a very detailed knowledge of the fine differences among the products that were isolated.

The fate of each labeled carbon of the three alternative ^{13}C-isotopic versions of ALA used, into a generic corrinoid product, is shown in Scheme 2.

The ^{13}C NMR spectra of all six cyano cobalt porphyrinoids derived from [4-^{13}C]ALA showed two doublets in the region of 130–170 ppm of an unusually large coupling constant (89.7 Hz for the purple pair, 93.6 Hz for the red pair, and 82.7 Hz for the yellow pair) and their couplings were confirmed by INADEQUATE ^{13}C NMR experiments. This feature indicated a direct connection between carbons 1 and 19, showing that



Scheme 2. Fate of labeled carbons of alternative ^{13}C-isotopic versions of ALA into a generic corrinoid.

the macrocyclic ring contraction had taken place and that all the products are corrinoids.

The mass spectral data were determined by ESI in the positive mode, identifying the molecular ion (M^+) by the appearance of additional peaks corresponding to (M^++H), (M^++Li), (M^++Na), (M^++K), and (M^+−CN). As stated above, the mass spectral analysis showed that the pairs of products that were resolved by additional chromatographies differ by 58 mass units, suggesting that a monodecarboxylation of a side chain had occurred. The position of the decarboxylation was revealed by the change of chemical shift (^{1}H NMR, ^{13}C NMR) of the neighboring atoms.

The numbering system used is indicated in Figure 1, the complete sets of ^{13}C NMR and ^{1}H NMR data available are summarized in Tables 1 and 2, and the proposed structures (**4**–**9**) are shown in Figure 2.

From the outset of this study it became apparent that the six products were closely related. The first pair studied in detail were the purple products **6** and **7** which have λ_{max} (rel ε in CH_2Cl_2) as follows: 319 (0.25), 355 (0.44), 379 (0.28), 403 (0.31), 450 (0.19), 551 (1.00), 571 (0.97), and 586 (1.00) nm.

Initially the products were obtained as a mixture of approximately 2:1 ratio between the decarboxylated and non-decarboxylated forms. This made it easier to identify the sets of ^{13}C NMR signals for each species as well as the two families of peaks in the mass spectra.

The mass spectrum (ESI + mode) of compound **6** derived from [4-^{13}C]ALA shows *m/z* = 1145[(M+Na)$^+$, 100%], 1129[(M+Li)$^+$, 46%], 1123[(M+H)$^+$, 100%], and 1096[(M−CN)$^+$, 20%] consistent with the formula $C_{46}{}^{13}C_8H_{67}N_6O_{16}$.

A double labeled experiment, in which in addition of [4-^{13}C]ALA [$^{13}CH_3$]SAM was used, gave a product with three enriched methyl groups, two of them (at C-11 and C-17) showing a ^{13}C–^{13}C coupling.

Compound **7** has almost identical ^{13}C NMR data to **6**; however, the mass spectrum (ESI + mode) of the specimen derived from [4-^{13}C]ALA shows

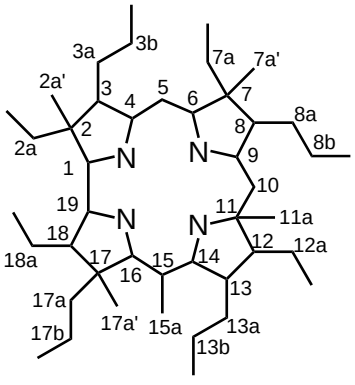


Figure 1. Numbering system of cobalt corrinoids **4**–**9**.

Table 1. ^{13}C NMR assignments for cobalt corrinoids **4**–**9** in C_6D_6

Compound	4	5	6	7	8	9
1	142.3	142.3	165.7	165.7	157.9	157.9
2			46.8	46.8	45.0	45.0
3	54.6	54.6	53.0	53.0	53.9	53.8
4	149.9	149.9	161.9	161.9	164.6	164.6
5	169.8	169.8	86.9	86.9	83.8	83.8
6	180.5	180.5	179.2	179.1	177.8	177.8
7			57.7	57.7	56.4	56.4
8	146.4	146.4	133.0	132.6	130.3	130.0
9	144.4	144.4	142.6	142.6	142.0	142.3
10	31.7	31.7	30.8	30.8	30.7	30.6
11	77.5	77.1	79.8	79.1	80.5	79.9
12			163.8	169.1	164.0	169.5
13	140.0	136.0	136.7	133.1	136.3	132.8
14	170.9	170.9	176.3	176.3	175.7	175.3
15	102.7	102.7	35.8	35.8	36.2	35.8
16	158.2	158.2	170.8	170.8	168.0	167.8
17	53.2	53.2	63.6	63.6	58.8	58.8
18			154.8	154.8	41.1	41.1
19	165.9	165.9	131.3	131.3	133.4	133.4
3a			23.7	23.7	25.1	25.1
8a			19.7	19.7	19.6	19.6
11a			23.4	23.9	23.9	24.4
13a			20.1	19.6	19.9	19.8
15a			17.0	17.0	17.0	17.0
17a′			21.5	21.6	19.1	18.9
17a			30.6	30.6	37.4	37.4

m/z = 1087[(M+Na)$^+$, 100%], 1071[(M+Li)$^+$, 44%], 1065[(M+H)$^+$, 12%], and 1038[(M−CN)$^+$, 29%] consistent with the formula $C_{44}{}^{13}C_8H_{65}N_6O_{14}$. The loss of one of the acetate carboxyl groups was also indicated by the absence of the signal for one of the carbomethoxy groups in the ^{1}H NMR spectrum.

A specimen of **7** derived from [4-^{13}C]ALA and esterified with CD_3OH shows *m/z* = 1124[(M+K)$^+$, 10%], 1108[(M+Na)$^+$, 24%], and 1086[(M+H)$^+$, 100%], 1059[(M−CN)$^+$, 88%] consistent with having seven methylesters.

The red products **8** and **9** have λ_{max} (rel ε in CH_2Cl_2) as follows: 308 (0.70), 322 (0.76), 333 (0.74 sh), 355 (0.46), 379 (0.47), 413 (0.36), and 505 (1.00) nm.

The mass spectrum (ESI + mode) of compound **8** derived from [3-^{13}C]ALA shows *m/z* = 1147[(M+Na)$^+$, 100%], 1125[(M+H)$^+$, 4%], 1098[(M−CN)$^+$, 21%] consistent with the formula $C_{46}{}^{13}C_8H_{69}N_6O_{16}$.

A specimen of **8** derived from [4-^{13}C]ALA and [$^{13}CH_3$]SAM shows fragments with *m/z* values 3 units heavier, consistent with having 3 methyl groups derived from SAM as indicated earlier for compound **6**.

The mass spectrum (ESI + mode) of compound **9** derived from [3-^{13}C]ALA shows *m/z* = 1105[(M+K)$^+$, 1%], 1085[(M+Na)$^+$, 100%], 1067[(M+H)$^+$, 2%], and 1040[(M−CN)$^+$, 34%] consistent with the formula $C_{44}{}^{13}C_8H_{67}N_6O_{14}$.

A specimen of **9** derived from [4-^{13}C]ALA shows *m/z* = 1105[(M+K)$^+$, 9%], 1089[(M+Na)$^+$, 14%],

Table 2. ^{1}H NMR assignments for cobalt corrinoids **4**–**9** in C_6D_6

Compound	4	5	6	7	8	9
H_3-2a′	1.55	1.55	1.33	1.33	1.39	1.39
H_2-2a	2.42, 2.54	2.42, 2.54	2.58, 2.74	2.58, 2.73	2.48, 2.52	2.48, 2.52
H-3	4.16	4.16	3.47	3.47	3.53	3.53
H_2-3a	2.31, 2.64	2.31, 2.64	2.00, 2.09	2.02, 2.14	2.13, 2.60	2.15, 2.69
H_2-3b			2.36, 2.48	2.36, 2.48	2.60, 2.75	2.60, 2.75
H-5			5.27	5.27	5.06	5.05
H_3-7a′	1.18	1.18	0.98	0.99	1.06	1.06
H_2-7a	2.11, 2.33	2.11, 2.33	2.32, 2.37	2.32, 2.36	2.34, 2.38	2.34, 2.38
H_2-8a	2.27, 2.39	2.27, 2.39	2.54, 2.64	2.49, 2.60	2.20, 2.62	2.24, 2.55
H_2-10	2.79, 3.19	2.67, 2.94	2.48, 3.14	2.31, 2.87	2.60, 3.09	2.47, 2.87
H_3-11a	1.29	1.16	1.50	1.38	1.50	1.39
H_2-12a	3.27, 3.45		3.18, 3.44		3.14, 3.35	
H_2-13a	2.43, 2.75	2.62, 2.69	2.55, 2.71	2.41, 2.62	2.19, 2.29	2.19, 2.29
H_2-13b		2.25, 2.27	2.33, 2.47	2.34, 2.45	2.24, 2.36	2.24, 2.36
H-15			4.63	4.59	4.47	4.44
H_3-15a	1.86	1.86	1.70	1.68	1.66	1.64
H_3-17a′	1.23	1.23	1.73	1.74	1.14	1.14
H_2-17a	2.09, 2.28	2.09, 2.28	3.56, 3.64	3.61, 3.64	2.04, 2.34	2.04, 2.34
H_2-17b			2.47, 2.47	2.47, 2.47	2.35, 2.60	2.35, 2.60
H-18	3.71	3.71			3.40	3.40
H_2-18a	2.76, 2.81	2.76, 2.81			2.54, 2.58	2.55, 2.64
H-18a			6.30	6.31		

1067[$(M+H)^+$, 24%], and 1040[$(M-CN)^+$, 100%] and after lithium addition 1073 [$(M+Li)^+$, 100%], 1040[$(M-CN)^+$, 47%].

Thus, the mass spectral data for the red pair (**8** and **9**) showed that these products are **2** mass units heavier than the purple products (**6** and **7** respectively), the ^{13}C NMR of the [3-^{13}C]ALA derived products indicated that the additional unsaturation involved C-18 with an exocyclic olefin as the only possible alternative.

HMBC NMR experiments provided unambiguous assignments given in Tables 1 and 2 as the [3-^{13}C]ALA derived version of products 6 and 7 showed both C-12 and C-18 in the olefinic region and H-18a (6.2 ppm).

The yellow products **4** and **5** have λ_{max} (rel ε in CH_2Cl_2) as follows: 307 (0.63), 332 (0.70), 344 (0.64), 388 (1.00), 429 (0.61 sh), 457 (0.50), 482 (0.54), 513 (0.37 sh), 630 (0.14), and 849 (0.43) nm.

The mass spectrum (ESI + mode) of compound **4** derived from [4-^{13}C]ALA shows $m/z = 1139$ [$(M+H)^+$, 100%], consistent with the formula $C_{46}{}^{13}C_8H_{67}N_6O_{17}$.

The mass spectrum (ESI + mode) of compound **4** derived from [5-^{13}C]ALA shows $m/z = 1138$ [$(M+H)^+$, 100%], consistent with the formula $C_{47}{}^{13}C_7H_{67}N_6O_{17}$.

The mass spectrum (ESI + mode) of compound **5** derived from [4-^{13}C]ALA shows $m/z = 1103$[$(M+Na)^+$, 11%], 1081 [$(M+H)^+$, 100%], and 1054[$(M-CN)^+$, 11%] consistent with the formula $C_{46}{}^{13}C_8H_{65}N_6O_{15}$.

The mass spectrum (ESI + mode) of compound **5** derived from [5-^{13}C]ALA shows $m/z = 1080$[$(M+H)^+$, 100%], 1053[$(M-CN)^+$, 6%] consistent with the formula $C_{47}{}^{13}C_7H_{65}N_6O_{15}$.

The distinct feature of the yellow series became more apparent in the analysis of the NMR spectra of the [5-^{13}C]ALA derived specimens. While the red and purple series showed the signals for the C-5 carbons in the traditional region of 95–100 ppm, for the yellow products these were dramatically shifted to 169.8 ppm suggesting a carbonylic form.

A comparison of the structural features of each pair with those of the known intermediates on the biosynthetic path indicates that the presence of exocyclic unsaturation in the purple pair (**6** and **7**) and the C-5 carbonyl form of the yellow pair (**4** and **5**) are not the product of enzymatic reactions but rather of purely chemical reactions. Knowing the generally poor stability of these compounds to oxygen exposure, we conducted experiments in which not only were the enzymatic incubations performed in a glove-box under argon (oxygen concentration under 20 ppm) but also the work up of the esterification, extraction, TLC purifications, and NMR sample preparations. The products obtained were exclusively the red pair indicating that the yellow and purple products are chemically derived from the red pair; thus compound **8** (red non-decarboxylated) would be the closest relative to the long sought, but elusive B_{12} intermediate cobalt-precorrin 5, which has been postulated but not yet isolated.

3. Conclusions

The methylation at C-15 for all products (4–9), a step that is expected to be preceded by methylation at C-l (among other changes), indicates that none of the isolates reported in this study correspond to an intermediate on the anaerobic pathway to B_{12}; however, several highly significant implications can be derived from the isolation of these three pairs of corrinoids. First, the

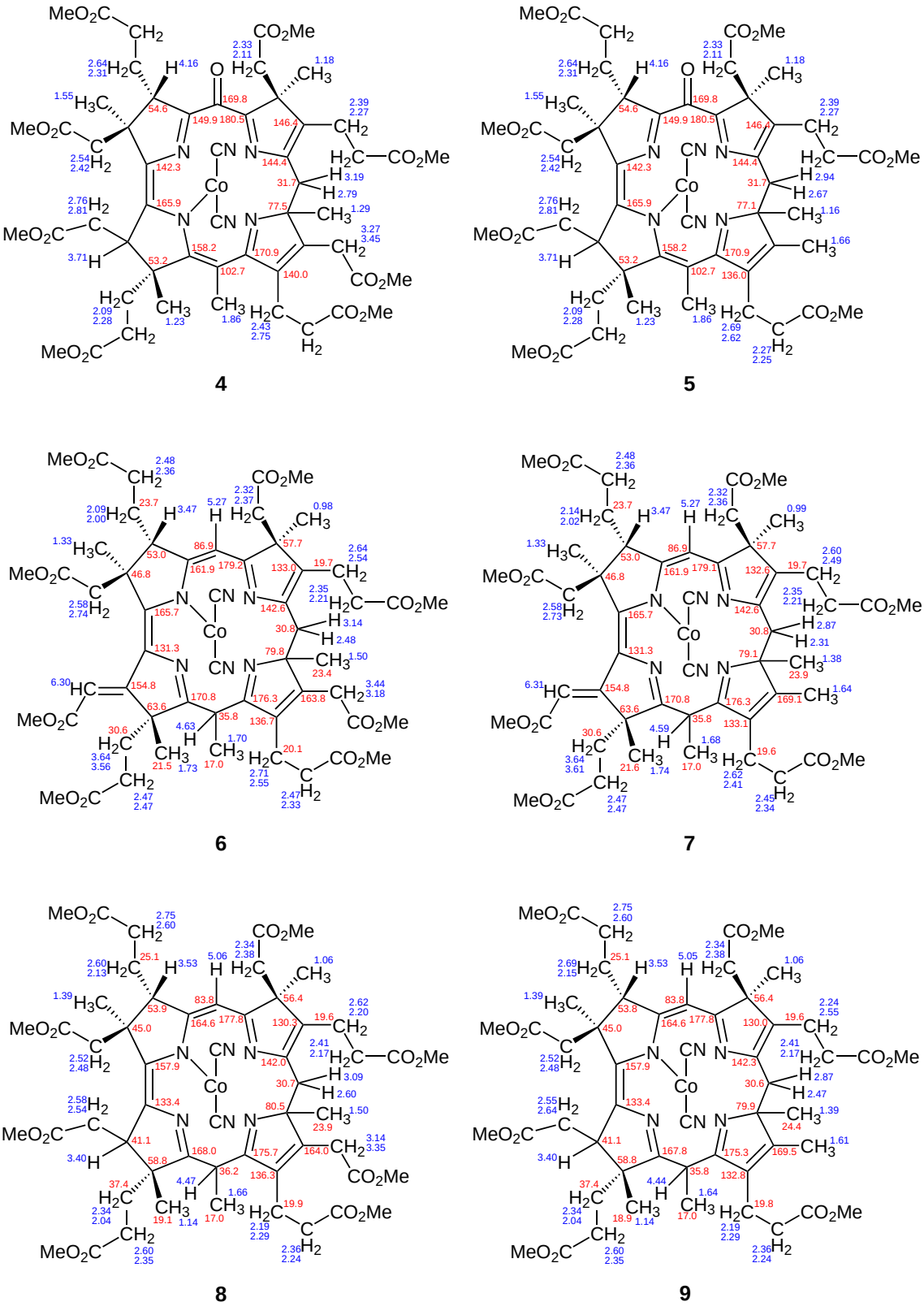


Figure 2. Structure and NMR assignments of cobalt corrinoids **4**–**9**.

unequivocal demonstration that the enzyme CbiH accepts factor 3, the oxidized form of the trimethylated intermediate, as a substrate and performs the C-17 methylation, macrocyclic ring contraction, and ring-A lactonization in the same fashion as it does it with precorrin 3 again raises the controversy on what is the true oxidation level of the real intermediates. Second, the structures of these new products provide reasonable

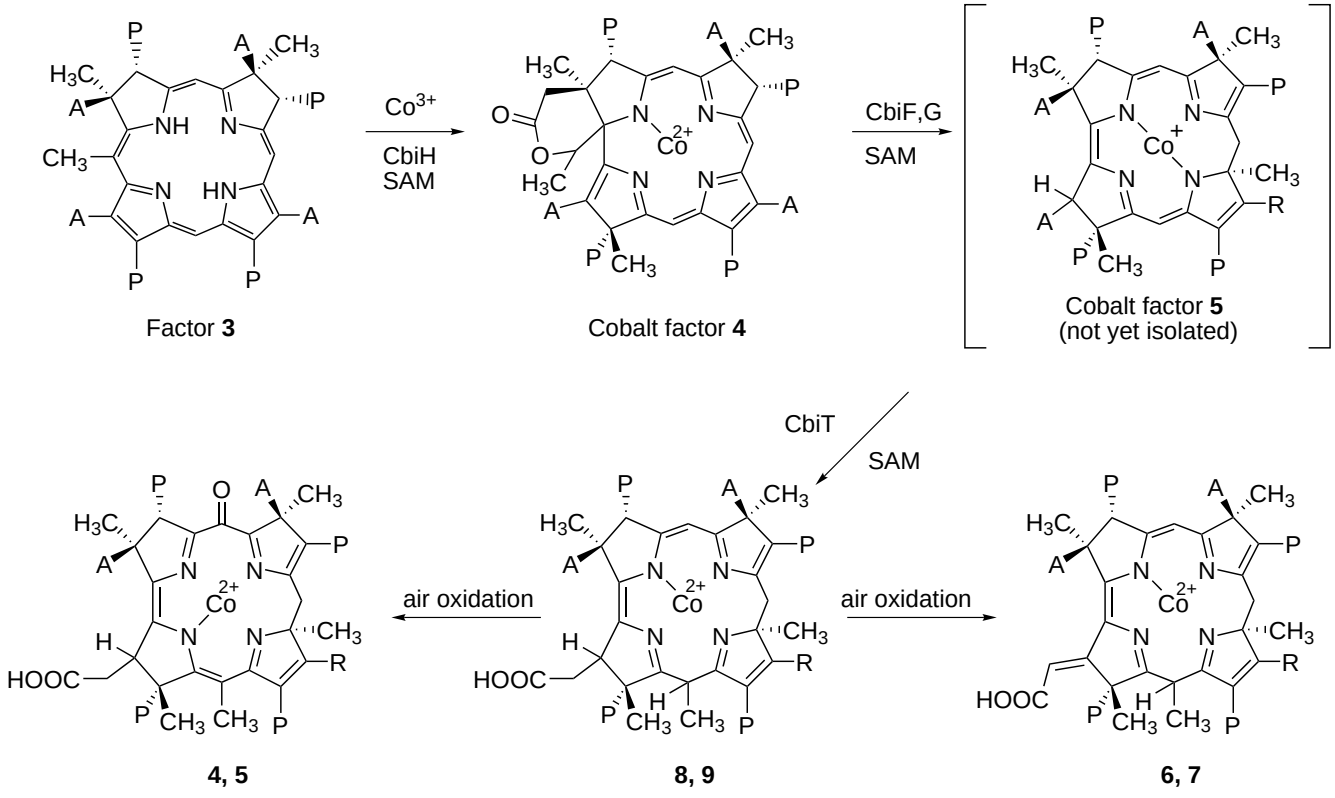
evidence that the next step after CbiH in the biosynthetic pathway is indeed the CbiF catalyzed methylation at C-11. Third, the surprising finding that CbiT possesses a second function, besides the previously assigned function,[10] based on the homology shown to a portion of the aerobic enzyme CobL;[11] it is now clear that apart from the C-12 acetate decarboxylating function, that was demonstrated by the production of all three pairs of pigments in the decarboxylated form, there is a methylating function that was shown by the methylation of C-15, in all six products. The methylating function has been previously hinted at by others[12] based on X-ray crystal structure studies which suggested the presence of a SAM binding site in the enzyme. The fact that aerobes have the methylating function at C-5 and C-15 and the decarboxylating function at C-12, all in the same enzyme (Cob L), had not allowed a determination of the timing between methylations at C-5 and C-15. It appears now, from our findings, that the methylation at C-5 would be the only function of CbiE and its timing could be addressed by appropriate experiments.

Lastly, the fact that all products have lost the C-2 unit (C-20 and its attached methyl group) suggests that the opening of the ring-A lactone may be catalyzed by CbiG, an enzyme that shows no homology with any of the gene products of the aerobic path. Based on all of the above results we suggest the pathway shown in Scheme 3 to explain the generation of the isolates and their connection with the intermediates postulated for the anaerobic B_{12} biosynthetic pathway. Investigations with a more restricted set of enzymes are currently under way to produce the true intermediates and determine the precise role of each enzyme.

4. Experimental

4.1. General procedures

^{1}H and ^{13}C NMR spectra were obtained in C_6D_6 on a Bruker WM-300 spectrometer operating at a carbon frequency of 75.47 MHz or a Bruker ARX500 NMR spectrometer operating at a carbon frequency of 125.76 MHz, and employing standard pulse sequences found in the XWIN-NMR software using either a 5- or 3-mm C/H probe. Peak positions are given in parts per million (δ) using the benzene signal (128 ppm for ^{13}C; 7.15 ppm for ^{1}H) as the internal standard. HSQC and HMBC experiments were performed in the ARX 500 instrument using 5 mm HCN or 3 mm HBB probes with *Z* gradient accessories. Supplied Bruker programs were modified for use on this spectrometer. UV–vis spectra were recorded on an Ocean Optics USB2000 Miniature Fiber Optic Spectrophotometer. Mass spectra were acquired on a PE SCIEX QSTAR quadrupole time-of-flight hybrid mass spectrometer using an electrospray ionization source. Purifications by thin layer chromatography (TLC) were carried out on EMD/Merck Silicagel 60 precoated plates. The *E. coli* strains overexpressing the enzymes utilized in this study have been described elsewhere.[10] Preparations of [5-^{13}C]ALA, [4-^{13}C]ALA, and [3-^{13}C]ALA were conducted as previously described.[13–15] Handling of oxygen sensitive mate-



Scheme 3. Proposed path for the generation of cobalt corrinoids **4**–**9**.

rial was done in a DRI-LAB glove-box DL-001-S-G Vacuum Atmospheres Company.

4.2. Preparation of factor 3 octamethylester (3b)

The [5-^{13}C], [4-^{13}C], and [3-^{13}C] derived versions were prepared as described below, starting from the corresponding isotopomer of 5-aminolevulinic acid (**1**). In 300 mL of 50 mM Tris–HCl buffer, 75 mM KCl, and 12 mM $MgCl_2$, pH 7.5, containing 100 mg *S*-adenosyl-L-methionine 1,4-butanedisulfonate, which had been degassed by several cycles of high vacuum and argon bubbling, lysates of *E. coli* strains overexpressing the enzymes ALA dehydratase, porphobilinogen deaminase, uroporphyrinogen III synthase, CobA, and CobI,[16] and 0.5 mL of Antifoam 204 (Sigma) were added. After several additional cycles of degassing, aminolevulinic acid (20 mg) dissolved in 4 mL of the same buffer was added followed by a few additional cycles of degassing. The flask was sealed and the incubation was continued for 16 h at 37 °C in the dark. The incubation mixture was centrifuged to remove any precipitated protein and the solution was passed slowly through 600 mg DEAE Sephadex A-25 to trap the precorrin 3 produced. After drying the resin with a few washes of argon saturated acetone and vacuum, the porphyrinoids were eluted and esterified for 16 h with 30 mL of 5% sulfuric acid in methanol. Exposure to air, which oxidizes precorrin 3 (**2**) to factor 3 (**3a**), dilution with dichloromethane (50 mL), neutralization with saturated $NaHCO_3$, and dilution with 100 mL water were followed by extraction of the purple solution with dichloromethane (3×50 mL). After evaporation of the solvent, the crude material was chromatographed on silica gel plates eluted with dichloromethane/methanol 97.3:2.7 which allowed the elimination of small amounts of coproporphyrin III tetramethylester and the unwanted epimeric forms of the main product factor 3 octamethylester (yield, approximately 60%).

4.3. Hydrolysis of factor 3 octamethylester

Factor 3 octamethylester was suspended in 3 mL of degassed 2 M piperidine in water and the hydrolysis was continued under argon in the dark for 48 h at room temperature. The solvent was evaporated at high vacuum and the residual piperidine was removed by several cycles of addition of 5 mL of degassed water followed by freeze-drying.

4.4. Enzymatic preparation of cobalt corrinoids

In 300 mL of 50 mM Tris–HCl buffer, 75 mM KCl, and 12 mM $MgCl_2$, pH 7.5, containing 100 mg *S*-adenosyl-L-methionine 1,4-butanedisulfonate which had been degassed by several cycles of high vacuum and argon bubbling, a lysate from 2 L of an *E. coli* strain overexpressing the *Salmonella typhimurium* enzymes CbiT, CbiF, CbiG, and CbiH, and 0.5 mL of Antifoam 204 (Sigma) were added. After several additional cycles of degassing, factor 3 octapiperidinium salt dissolved in 10 mL of the same buffer and 100 mg of cobalt (II) chloride (or 100 mg of glycine cobalt (III) salt) were added followed by a few additional cycles of degassing. The flask was sealed and the incubation was continued for 16 h at 37 °C in the dark.

4.5. Isolation and purification of the cobalt corrinoids (4–9)

The incubation mixture was centrifuged to remove any precipitated protein. KCN 100 mg was added and the solution was passed slowly through 1 g DEAE Sephadex A-25 to trap the porphyrinoids produced. After drying the resin with a few washes of argon saturated acetone and vacuum, the porphyrinoids were eluted and esterified for 16 h with 30 mL of 5% sulfuric acid in methanol. Dilution with dichloromethane (50 mL), neutralization with saturated $NaHCO_3$, and dilution with 100 mL of water were followed by addition of 100 mg KCN buffered with KH_2PO_4 (pH close to neutral) and extraction with dichloromethane (3×50 mL). After evaporation of the solvent, the crude material was chromatographed on silica gel plates eluted with dichloromethane/methanol 95:5 saturated with solid KCN. Workup exposed to air yielded three distinctive pairs of colored bands: yellow (**4** and **5**), purple (**6** and **7**), and red (**8** and **9**) in the same order of elution.

Production and isolation of only the red pair (**8** and **9**) was done by performing all operations, including extraction, evaporations, and chromatography in a glove-box.

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References and notes

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