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Chemoenzymatic Synthesis of an Unnatural Tetramethyl Cobalt Corphinoid†

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Abstract—The chemoenzymatic synthesis and structural characterization by ^{13}C NMR of a tetramethyl cobalt–corphinoid produced by methylation of cobalt-precorrin-3 using CbiF are described.

Introduction

Two distinct pathways for the microbial biosynthesis of vitamin B_{12} have been established.[12] Both pathways feature the introduction of 8 methyl groups (derived from *S*-adenosylmethionine (SAM)) into the porphyrinoid template, with the subsequent loss of one of these (at C-20) as part of a two carbon unit, a process intimately connected with the ring contraction of the porphyrinoid to the corrinoid structure.

Although all the organisms examined seem to share the same methylation sequence,[3–6] aerobes such as *Pseudomonas denitrificans*, follow an oxygen dependent ring contraction and a late cobalt insertion,[7] while anaerobes, like *Propionibacterium shermanii*, follow an oxygen-free early insertion of cobalt.[8,9] Recent progress in the characterization of the enzymes of the anaerobic pathway (derived from the *cbi* genes) to the vitamin[10] led us to investigate the action of CbiF, the putative fourth *Salmonella typhimurium* SAM-dependent methyltransferase, on cobalt-precorrin-4 (**1**) (Fig. 1) which was expected to lead to methylation at C-11.

In a previous study,[10] we prepared cobalt-precorrin-4 (**1**) from 5-aminolevulinic acid (**2**) by a stepwise multienzymatic synthesis of precorrin-3 (**4**) followed by nonenzymatic insertion of cobalt in the macrocycle and subsequent methylation at C-17 with CbiH (a *S. typhimurium* methyltransferase). Since in this multienzyme process the yields are not quantitative and the intermediates are not isolated before they are made available to the next enzyme, the final product of the incubation consisted of a mixture of cobalt-precorrin-4 (**1**) and unreacted cobalt-precorrin-3 (**5**). Initial attempts to methylate these products with CbiF indicated that, after esterification and air exposure, the major isolable products were neither methylated at C-17 nor ring contracted, hinting that they were derived from a nonspecific methylation of cobalt-precorrin-3. Like other methyltransferases of the B_{12} pathway (e.g. CysG) CbiF has already been shown to methylate other metal-free porphyrinoids non-specifically.[11,12] In this study we describe the preparation and structural characterization by ^{13}C NMR of the tetramethyl cobalt-corphinoid (TCC) (**6**) produced by the methylation of cobalt-precorrin-3 (**5**) using this enzyme.

Results and Discussion

Cobalt-precorrin-3 (**5**) was made by sequential generation of precorrin-3 (**4**) from aminolevulinic acid (**2**) using lysates[13] of *E. coli* strains overexpressing the enzymes aminolevulinic acid dehydratase, porphobilinogen deaminase, uroporphyrinogen III synthase, CobA and CobI; followed by a non enzymatic complexation with cobalt chloride. This product was methylated with *S*-adenosylmethionine using a lysate of a strain of *E. coli* that overexpresses CbiF. Trapping of the porphyrinoids, followed by esterification and chromatographic purification, yielded a major product that was characterized by UV–vis spectroscopy, mass spectrometry and ^{13}C NMR spectroscopy.

The tetramethyl cobalt–corphinoid dicyano octamethylester (**6**) has λ_{max} (rel ε) as follows: 300 (0.56 sh), 350 (0.36), 401 (1.00), 490 (0.08), 590 (0.22) and 640 (0.17 sh).

^{13}C NMR analysis of the spectrum of **6** made from [5-^{13}C]-aminolevulinic acid (Fig. 2) shows a doublet of doublets centered at 93.9 ppm ($J_{CC} = 76$ Hz, and

† Dedicated to the memory of Sir Derek Barton.

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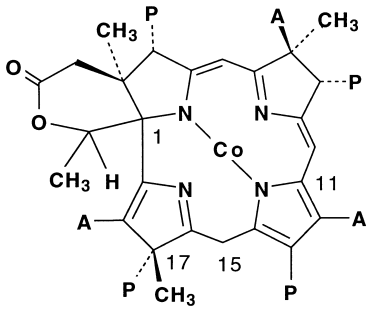


1 (Co-precorrin 4)

Figure 1.

J_{CC} = 64 Hz) and a singlet at 100.6 ppm, which are characteristic of species derived from the uroporphyrinogen type III. Signals for the other meso positions are at 99.2 ppm (J_{CC} = 75 Hz) (C-5) and an unusually high field doublet for C-10 centered at 30.6 ppm (J_{CC} = 49 Hz) indicative of a reduced meso position which is coupled to C-9 appearing at 145.2 ppm (J_{CC} = 48 ppm).

More critical to the structural assignment of the new compound was the spectrum of the specimen derived from [4-^{13}C]-aminolevulinic acid (Fig. 3). Noticeably absent are the C-1/C-19 pair of doublets commonly associated with ring contraction, instead only a lone singlet observed at 78.8 ppm, whose chemical shift is consistent with alkylation at an α-pyrrolic position observed in other CbiF catalyzed methylations at C-11.[11,12] Two signals between 40 and 60 ppm are observed indicating that out of the four propionate termini carbons (C-3, C-8, C-13 and C-17) only two are sp^3 hybridized, the other two showing sp^2 signals in the region between 135 and 137 ppm. The remaining three carbon signals are located in the region between 173 and 179 ppm, characteristic of carbons involved in an olefinic system and vicinal to nitrogen. The last very characteristic signal at 78.8 ppm corresponds to an alkylation at an α pyrrolic position and is assigned to C-11. The assignments are shown on Table 1.

The FAB-mass spectrum of **6** shows prominent peaks at 1114 and 1088 corresponding to the parent compound and the loss of one cyanide ligand, respectively, while the MALDI-mass spectrum shows as major peaks 1088 and 1062 corresponding to the respective losses of one and two cyanide ligands.

Of the three structures uniquely compatible with both the NMR and MS data (Fig. 4), only structure III contains the C-8/C-9 double bond reminiscent of other CbiF methylation products. The proof of structure III was obtained via additional 2-DNMR experiments (including HMQC, HMBC and proton inverse detection) on both isotopically labeled samples, in particular, the 2-D HMBC spectrum of [4-^{13}C]-ALA derived material shown in Figure 5. Starting from the unique carbon signal at 78.8 ppm (assumed to be C-11), complete correlation pathways can be traced in either direction, thus confirming that the correct structure corresponds to III in which the olefinic propionate terminus is at C-8 and the 'reduced' ring is the southern ring D having both acetate and propionate termini carrying hydrogens (see Fig. 5 for complete details).

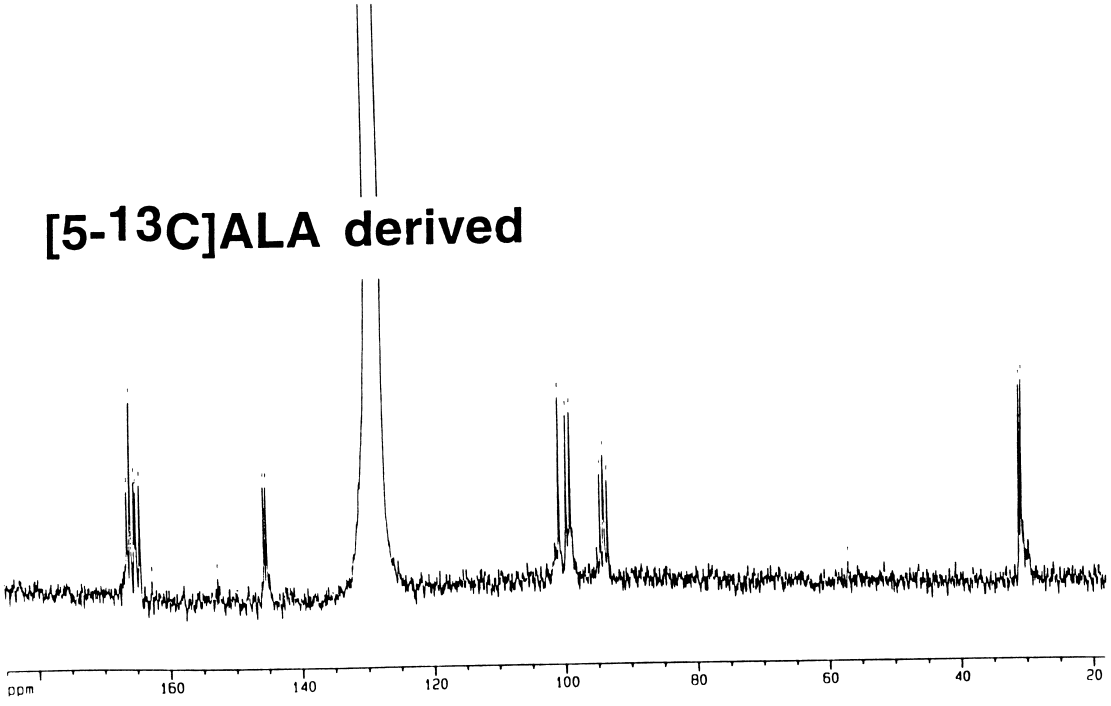


Figure 2.

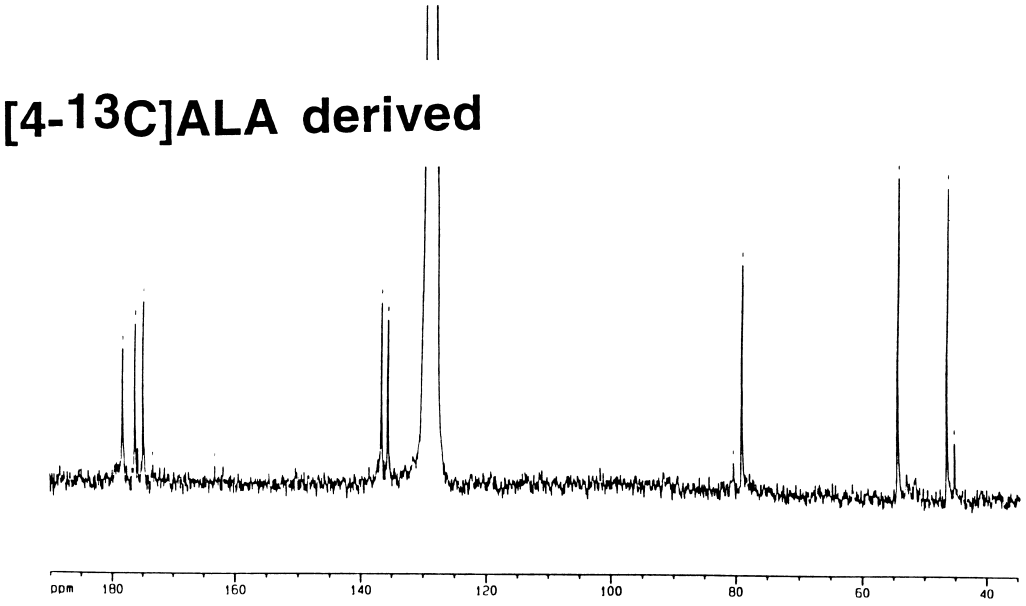


Figure 3.

Table 1. ^{13}C NMR chemical shifts (C_6D_6) for tetramethyl cobalt-corphinoid dicyano octamethylester (**6**) derived from (A) [4-^{13}C]ALA and (B) [5-^{13}C]ALA

A		B	
δ (J,Hz)	assignment	δ (J,Hz)	assignment
46.3	C-17	30.6 (d, 49)	C-10
54.2	C-3	93.9 (dd, 76, 64)	C-15
78.8	C-11	99.2 (d, 75)	C-5
135.4	C-8	100.6 (s)	C-20
136.4	C-13	145.2 (d, 48)	C-9
174.8	C-19	165.9, 165.4 and 164.6	C-14, C-16 and C-4
176.1	C-1		
178.1	C-6		

A remarkable aspect of this unusual chromophore system is the fact that it corresponds perfectly to that of Factor S3 (**7**), a metabolite previously shown to accumulate as a derailment product derived from uroporphyrinogen I (**8**) (Fig. 6) in whole cells and cell-free incubations of *P. shermanii*, the only difference being the pattern of methylation: 2, 7, 11 and 17 for Factor S3 and 2, 7, 11 and 20 for the corphinoid described in this study.

Conclusion

The scope of the non-specificity of CbiF has been further widened by demonstrating that in addition to the metal free precorrin-3 (**4**) and the 2,7,12-trimethylpyrrocorphin (**9**) derived from uroporphyrinogen I (**8**), a cobalt complex of precorrin-3 (**5**), presumably in the form of a pyrrocorphynate (as shown in Scheme 1), can also serve as a good substrate for this unusual methyltransferase.

Experimental

^{13}C NMR spectra were obtained in C_6D_6 on a Bruker ARX500 NMR spectrometer operating at 125.76 MHz for ^{13}C, and employing standard pulse sequences found in the XWIN-NMR software. 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. UV–vis spectra were recorded on a Varian DMS 90 spectrophotometer. FAB mass spectra were acquired on a VG Analytical 70S high resolution, double focusing, magnetic sector mass spectrometer in a nitrobenzylalcohol

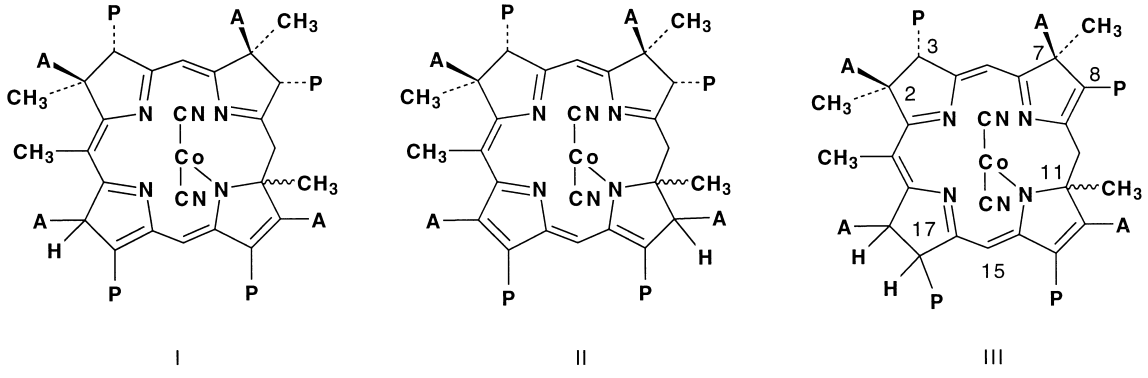


Figure 4.

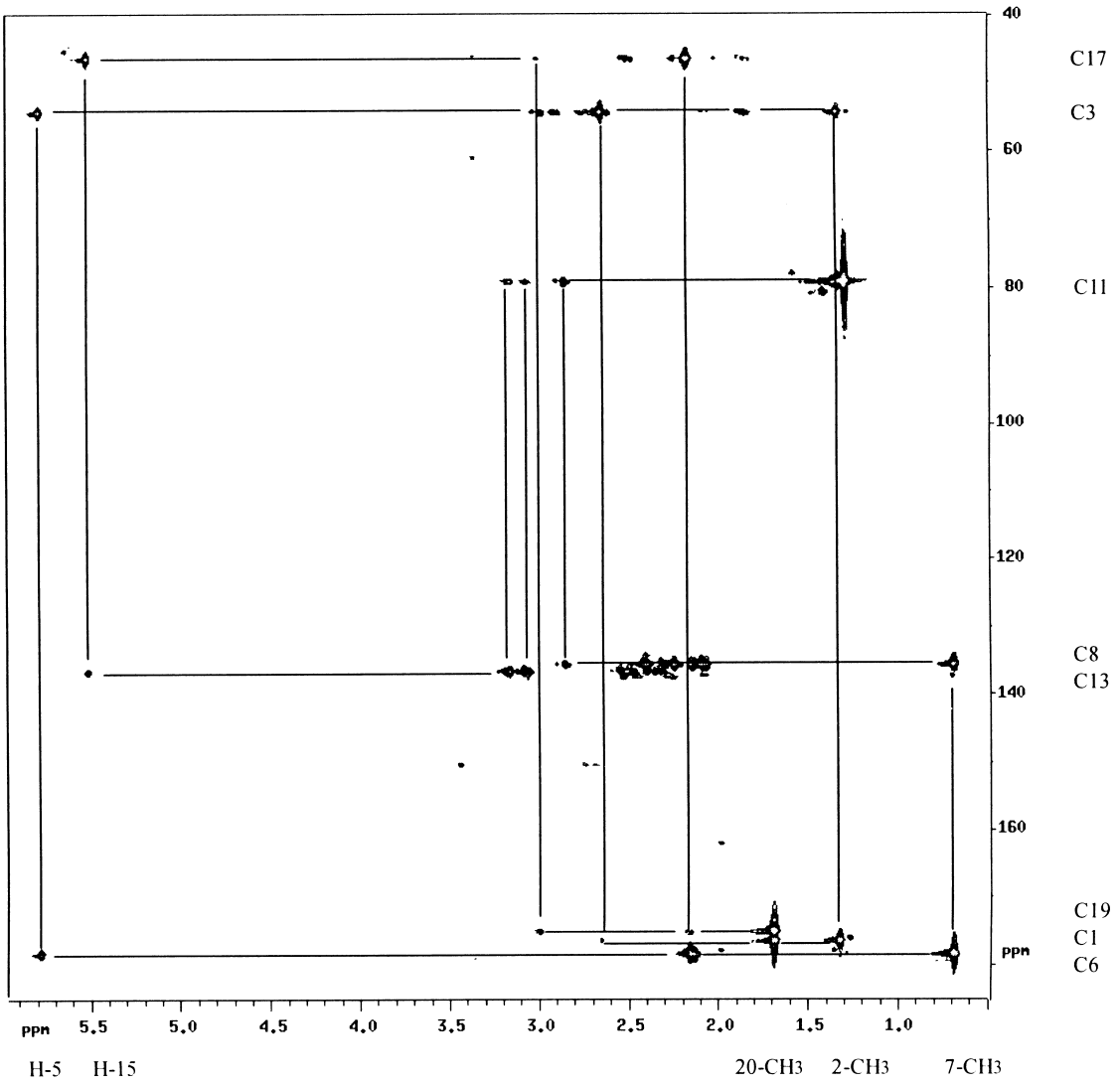


Figure 5. 2-D HMBC spectrum of the tetramethyl cobalt-corphinoid (**6**) derived from [4-^{13}C]-ALA. The two correlation pathways in opposite directions originating from C-11 (78.8 ppm) are given by solid lines, and the key proton correlations are denoted. The counter clockwise correlation pathway goes from C-11 through the vinylic C-8, H-5 meso, and the sp^3 C-3, while the clockwise correlation pathway goes through the H-15 meso on to the other sp^3 center, C-17, thus confirming positioning of the chromophore.

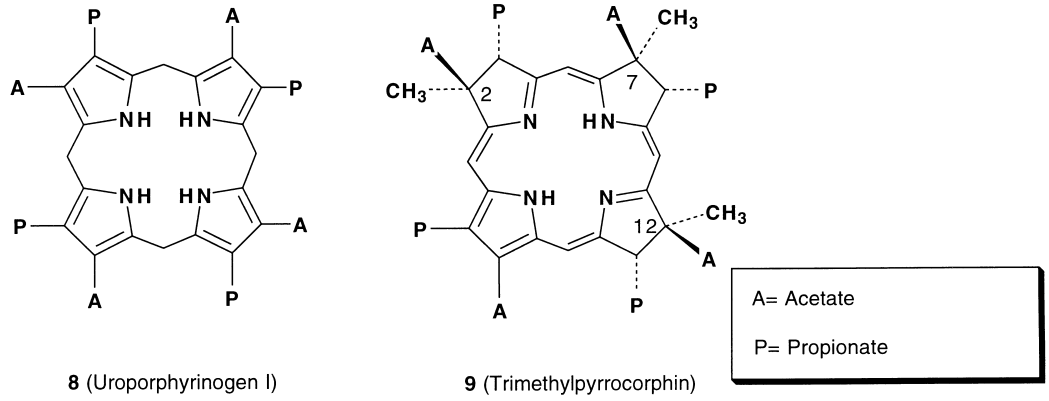
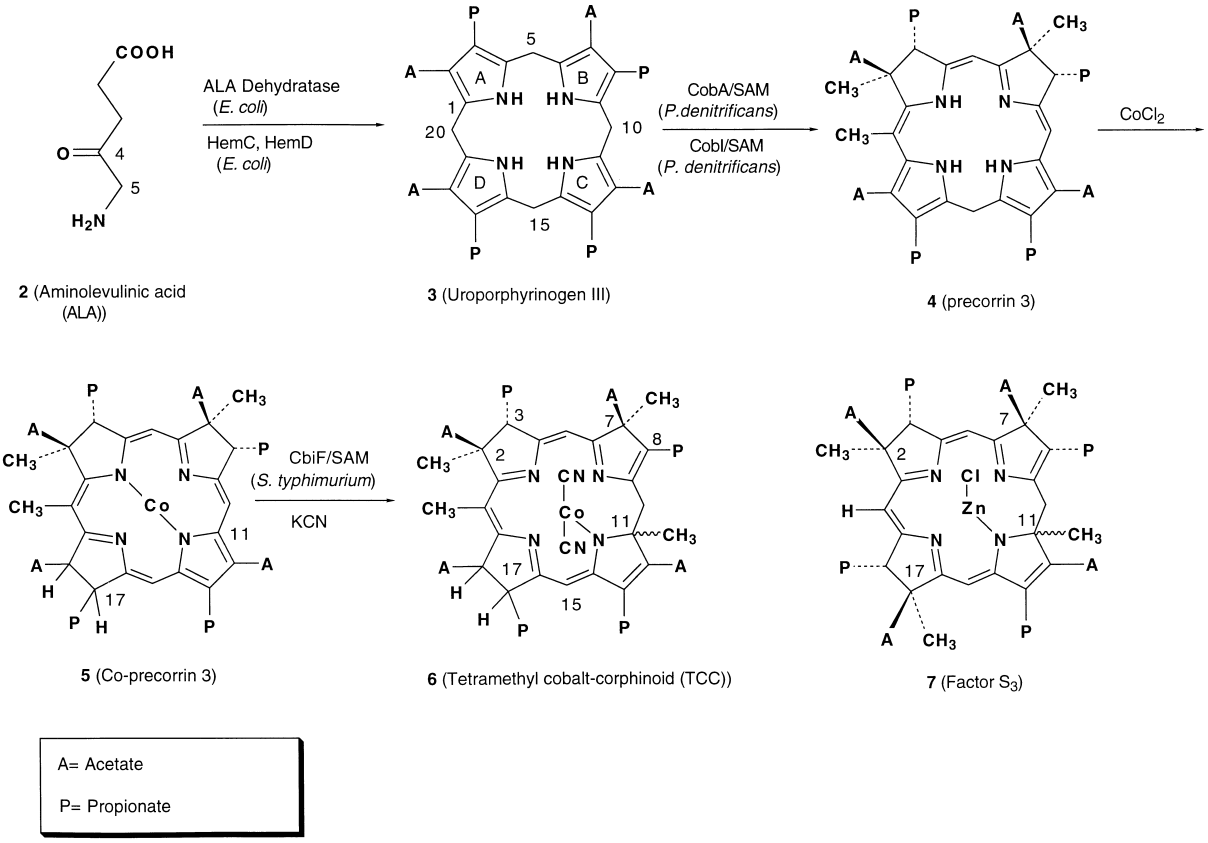


Figure 6.

matrix. MALDI-mass spectrometry analyses were performed on a PerSeptive Biosystems Voyager Elite XL spectrometer. Purification by thin layer chromatography (TLC) were carried out on Altech plates. The *Escherichia coli* strains overexpressing the enzymes utilized in this study have been described elsewhere.[13] Preparations of [5-^{13}C]ALA and [4-^{13}C]ALA were conducted as previously described.[14,15]



Scheme 1.

Preparation of cobalt-precorrin-3

The [5-^{13}C]- and [4-^{13}C]-ALA derived versions were prepared as described below, starting from the corresponding isotopomer of aminolevulinic acid (**2**). In 75 mL of 50 mM Tris:HCl buffer, 75 mM KCl, 12 mM $MgCl_2$, pH 7.5 which had been degassed by three cycles of high vacuum and argon bubbling, aminolevulinic acid (10 mg) was dissolved and after addition of 10 mL of a lysate (10 mg of protein/mL) of an *E. coli* strain that overexpresses ALA dehydratase the incubation was continued at 37 °C for 2 h. Lysates from strains overexpressing porphobilinogen deaminase, uroporphyrinogen III synthase, CobA and CobI were added, followed by 100 mg of *S*-adenosylmethionine dissolved in 10 mL of the same buffer and the incubation (in a total volume of 300 mL) was continued for an additional 16 h. The porphyrinoids were adsorbed on 600 mg of DEAE Sephadex A-25 previously washed with degassed, argon saturated buffer. After washing the resin with 0.2 M KCl, the yellow porphyrinoids, consisting mainly of precorrin-3 (**4**) were eluted with 15 mL of 2.5 M KCl; $CoCl_2.6H_2O$ (50 mg) dissolved in 10 mL of degassed water was added and the preincubation continued for six hours to achieve cobalt complexation during which time a dark green color developed.

CbiF methylation of cobalt-precorrin-3

A lysate from 2 L of culture of an *E. coli* strain overexpressing CbiF was prepared in 200 mL of 50 mM Tris:HCl buffer, 75 mM KCl, 12 mM $MgCl_2$, pH 7.5 which had been degassed by three cycles of high vacuum and argon bubbling. After addition of 100 mg of *S*-adenosylmethionine dissolved in 10 mL degassed water, the foregoing cobalt-precorrin-3 solution was added and the incubation continued at 37 °C for 16 h.

Isolation and purification of the tetramethyl cobalt-corphinoid (TCC)

After dilution with 500 mL of degassed water, the porphyrinoids were adsorbed onto 1 g of DEAE Sephadex A-25, followed by freeze drying and esterification under argon in 50 mL of 5% sulfuric acid in methanol. After neutralization with saturated $NaHCO_3$ and dilution with 200 mL of water containing 50 mg of buffered KCN, the green solution of porphyrinoids was extracted with 3×100 mL portions of dichloromethane. Chromatographic purification on silica gel plates eluted with 4% methanol in dichloromethane afforded several sharp bands at R_f values between 0.4 to 0.5.

Acknowledgements

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