Biosynthesis of porphyrins and related macrocycles, part 43. Isolation and characterization of intermediates of coenzyme B_{12} biosynthesis, a cobyrinic acid triamide, the *a,c*-diamide and their *Co*-(5'-deoxy-5'-adenosyl) derivatives, from *Propionibacterium shermanii*

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Background: Vitamin B_{12} is synthesized by many different organisms, for example *Pseudomonas denitrificans* (aerobic) and *Propionibacterium shermanii* ('microaerophilic', or essentially anaerobic). The biosynthetic pathways in these two organisms show strong similarities but also some differences. There have been conflicting reports on where differences between these two organisms lie in the stages beyond the formation of the corrin macrocycle. Characterization of intermediates in the pathway will help resolve these conflicts.

Results: A single cobyrinic acid diamide and a single triamide have been isolated from *Pr. shermanii*. The diamide was shown to be the *a,c*-isomer. The triamide is not the *a,c,g*-isomer but it is indistinguishable from the single triamide isolated by other workers from *Ps. denitrificans*. The *Co*-(5'-deoxy-5'-adenosyl) derivative of the *a,c*-diamide was also isolated and fully characterized and the deoxyadenosyl derivative of the foregoing triamide has been shown to be present in the cells.

Conclusions: Our results support a unique pathway in *Pr. shermanii* proceeding from cobyrinic acid towards coenzyme B_{12}, at least as far as the adenosylated triamide intermediate. No evidence was found for multiple alternative pathways. The order of amidations of the carboxyl side-chains of cobyrinic acid up to the triamide stage is the same in *Pr. shermanii* and *Ps. denitrificans*.

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Introduction

Coenzyme B_{12} (compound **10**, Fig. 1) is a remarkable natural organo–cobalt complex in which the cobalt is held in a corrin macrocycle; it is also a molecule of considerable structural complexity. Vitamin B_{12} (compound **11**), the form in which the cobalto–corrin was originally isolated, has the adenosyl group on the cobalt atom replaced by a cyanide ligand introduced during the isolation procedure. The discovery of the natural synthetic pathway to B_{12} has been one of the greatest challenges to scientists working on biosynthesis. Recently an exciting period of research has led to the elucidation of all the steps for the construction of coenzyme B_{12} in the aerobic organism *Pseudomonas denitrificans* [1,2]. This organism builds the corrin macrocycle initially free of cobalt in the form of hydrogenobyrinic acid **4**. There have also been extensive studies [3,4] of another B_{12}-producing organism, *Propionibacterium shermanii*, which is grown under anaerobic or, at most, microaerophilic conditions. In contrast to the situation in *Ps. denitrificans*, cobyrinic acid **6**, the cobalt complex of hydrogenobyrinic acid **4**, is an intermediate en route to coenzyme B_{12} in *Pr. shermanii*.

Together, these studies [1,5,6] showed that several steps of the synthesis are identical in both of these organisms. First, the primary macrocyclic precursor uroporphyrinogen III (compound **1**) is converted into the dimethylated precursor precorrin-2 (compound **2**). Second, the order of introduction of the next six methyl groups after the second one is the same in the two organisms, with the third methyl placed at C-20. Third, C-20 and its attached methyl group, which are still present in precorrin-3A (compound **3**), are eliminated as acetic acid as a result of the ring-contraction process. This elimination generates the direct C-1 to C-19 bond characteristic of the corrin macrocycle.

Some differences between the biosynthetic routes used by the above aerobic and anaerobic (or microaerophilic) organisms are emerging, however. In particular, the cobalt ion is inserted early in the pathway in *Pr. shermanii*, at or close to the stage of precorrin-3A [7,8], whereas in *Ps. denitrificans* the biosynthesis continues without cobalt as far as hydrogenobyrinic acid *a,c*-diamide **5** (see Fig. 1) and only then is the cobalt ion introduced by a complex enzyme system [9]. It is also clear, largely from ^{18}O-labelling experiments [10–16], that some features of the mechanism of the oxidative ring-contraction process differ in the two organisms. On the other hand, there are indications that the biosynthetic pathways proceeding from the hexamethylated stage, at least, are closely related

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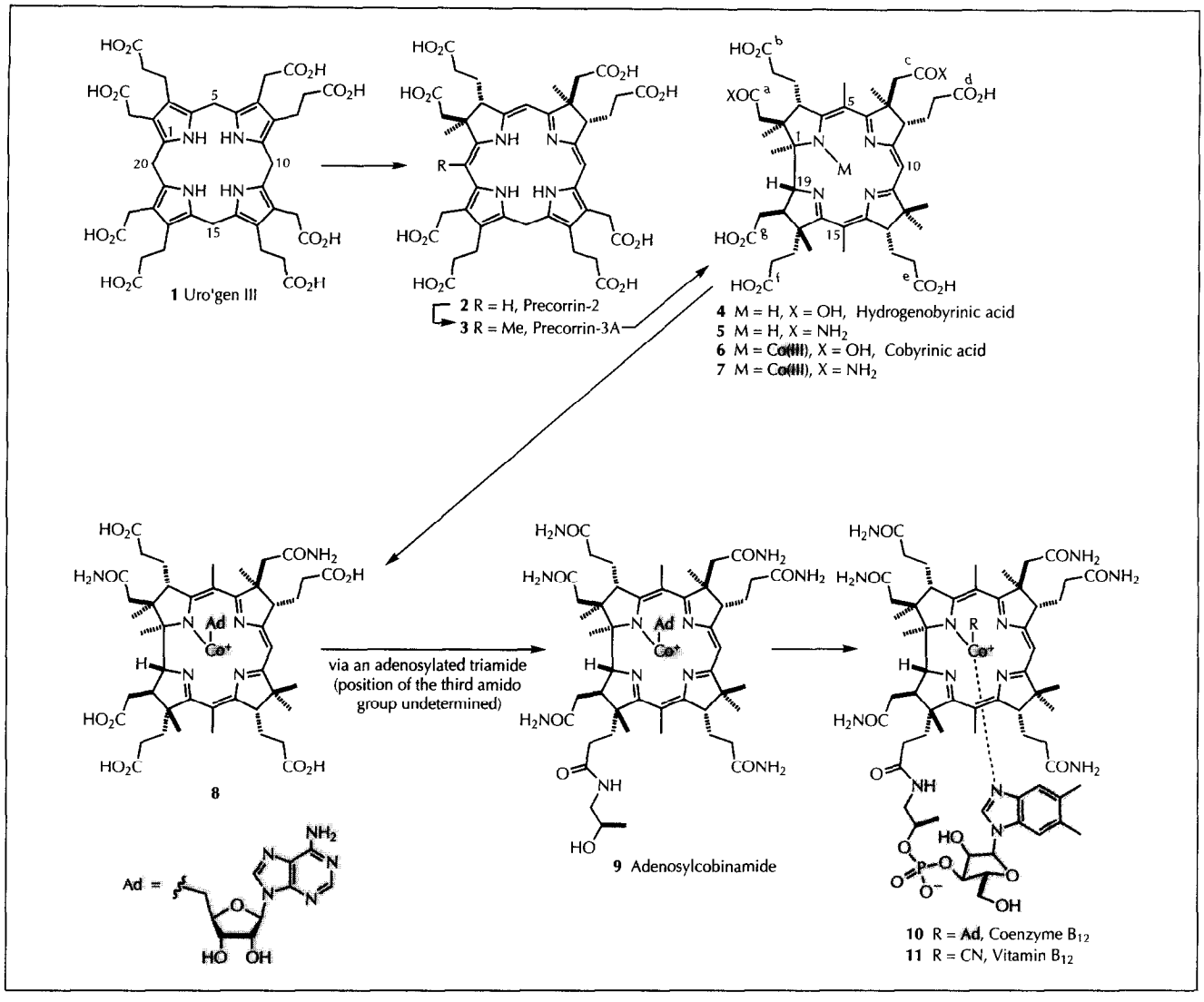

Fig. 1. Outline of the later stages of the biosynthetic pathway to coenzyme B_{12} (compound **10**) in *Pr. shermanii* and *Ps. denitrificans*. The synthetic pathways in the two organisms merge at the *a,c*-diamide **7**, and the order of amidation of the carboxyl side chains up to the triamide stage is the same in the two organisms. The cobalt ion is inserted at or close to the stage of precorrin-3A (compound **3**) in *Pr. shermanii*, whereas synthesis continues as far as *a,c*-diamide **5** in *Ps. denitrificans* before cobalt is introduced. Cobalt ion is highlighted in purple, adenosyl group in red and nucleotide loop in blue.

in the two organisms. It appears that over this part of the pathway, the various biosynthetic reactions firmly established for the cobalt-free macrocycles in *Ps. denitrificans* [1] may be similarly used but with their cobalt-containing analogues in *Pr. shermanii* [17].

Because of these various similarities and differences, there is considerable interest in further comparisons between the aerobic and anaerobic (or microaerophilic) organisms. We consider here events beyond the construction of cobyrinic acid **6**; the isolation of two amides of cobyrinic acid is reported, together with the isolation of their *Co*-(5'-deoxy-5'-adenosyl) derivatives.

Results and discussion

Paradoxically, this investigation grew out of the difficulties we were having in selecting *Pr. shermanii* cells that could make sufficient quantities of cobyrinic acid **6** for our experiments. To overcome this problem, it was necessary to test many different strains of cells, in several conditions of growth, for their ability to produce compound **6**. A rapid analytical method for compound **6** was needed and this involved trapping all the pigments on a reversed-phase adsorbant, followed by elution and high-pressure liquid chromatography (HPLC) analysis. Under standardized conditions, there was a linear relationship between the amount of compound **6** (over the range 0.1–0.75 μg) and the peak height on HPLC. The peak heights vary from one HPLC column to another and thus calibration is necessary, but then the analysis is widely applicable. While these studies were in progress, the separation of acidic corrins by HPLC using solvents different from the ones we had used was published [18], and we subsequently adopted these conditions for chromatographic comparison of amidated corrins (see below).

These chromatographic analyses showed that the batches of cells that produced considerable amounts of cobyrinic

acid **6** also contained other pigments. These additional pigments were especially rich in two large batches of cells, from which they were isolated by essentially the same procedure but using so-called 'fast protein liquid chromatography' (FPLC) in reversed-phase. This afforded two purple pigments and two yellow ones.

The two purple pigments were shown to be cobalt-containing corrins by their UV/visible spectra, and it is important to note that they were both single isomers, homogeneous by FPLC and HPLC. Their molecular weights, by fast atom bombardment mass spectrometry (FAB-MS), were two and three atomic mass units, respectively, less than that of cobyrinic acid **6**. These data indicated that the pigments were a diamide and a triamide of compound **6**.

It is clear by inspection that the biosynthesis of coenzyme B_{12} (compound **10**) from cobyrinic acid **6** involves amidation of all the carboxyl groups except for the one at site *f*, to which isopropanolamine becomes attached. These steps had been extensively studied in *Pr. shermanii* by three groups during the 1960s, led by Bernhauer, Friedrich and Pawelkiewicz. These were remarkable pioneering studies carried out without the help of HPLC, FPLC or NMR spectroscopy. Much valuable information was obtained but there were also serious disagreements, such as whether the seven carboxyl groups were modified in one specific sequence or whether modification can occur via several alternative pathways. Thus, some authors thought that two isomeric diamides (*a,c-* and *c,g-*) are biosynthesized in *Pr. shermanii*, leading to three triamides (*a,c,d-*, *a,c,g-* and *c,g,d-*). It was also thought that the isopropanolamine residue could be added at different stages in the amidation process, depending on the cultivation conditions. The work of others pointed to a unique pathway. The state of confusion is well described in the 1970 review by Friedmann and Cagan [19], which also gives the original references. The two amides isolated above were therefore of interest and were studied as follows.

The *a,c-*, *a,g-* and *c,g-* diamide pentamethyl esters of cobyrinic acid together with the *a,c,g-* triamide tetramethyl ester can be prepared by partial methanolysis of vitamin B_{12} (compound **11**) [20]. These amides are separable chromatographically and their structures had been rigorously determined by ^{13}C NMR analysis [20]. We prepared all four of these amido-esters exactly as previously described [20]. Our data precisely matched those of the earlier workers, leaving no doubt that we were handling the same substances. (All the cobalt-containing corrins discussed here were purified as their dicyano complexes; this has not been included in their names so as to avoid frequent repetition.) Hydrolysis of the *a,c-* and *c,g-*diamide esters gave the corresponding pentacarboxylic acids, which were compared by HPLC analysis using coinjection with the single diamide isomer isolated from *Pr. shermanii*. The natural diamide was indistinguishable from the *a,c*-isomer **7** but differed from the *c,g*-isomer. The *a,g*-isomer was not similarly compared because that isomer has a very different retention time from the *a,c*-isomer [18].

The natural triamide differed from all the amido-acids produced by hydrolysis of the fraction rich in the *a,c,g-*triamide ester prepared from vitamin B_{12}. The natural product is thus not the *a,c,g*-isomer, in contrast to the report that the *a,c,g*-triamide is generated in broken-cell enzyme preparations from *Pr. shermanii* [21]. However, a triamide was one of five partially amidated derivatives of cobyrinic acid (di- to hexa-amides) isolated from *Ps. denitrificans* [18]. The structures of the tri-, tetra- and penta-amides are unknown at present but there is strong evidence [18] that the amidations occur in a unique sequence starting from the *a,c*-diamide **7**. Since we only detected one triamide from *Pr. shermanii*, it was important to compare it with that from *Ps. denitrificans*. When the triamides from the two sources were co-injected onto the HPLC column, the result was a single peak. Bearing in mind the high resolving power of the HPLC conditions used, it is essentially certain that the triamides from the two organisms are identical.

The two yellow pigments from the *Pr. shermanii* cells were separated chromatographically. The pigment that ran slightly slower had a similar UV/visible spectrum to that of coenzyme B_{12} (compound **10**) and was converted by light into cobyrinic acid *a,c*-diamide **7**. Similar breakdown occurred under the conditions of fast-atom bombardment mass spectrometry (FAB-MS), since the recorded parent ion of the yellow pigment matched that from compound **7**. These results showed the yellow pigment to be a derivative of compound **7** which is alkylated on the cobalt atom; there are many precedents for photochemical cleavage of such cobalt–carbon bonds [22]. The ^1H NMR spectrum clearly indicated that the alkyl group is 5'-deoxy-5'-adenosyl; the spectrum showed eight low-frequency singlets (corresponding to the eight *C*-methyl groups of the macrocycle), a high-frequency singlet (10-H) and, importantly, two singlets at δ 8.32 and 8.11 (2-H and 8-H of adenosine) and a doublet at δ 5.12 (1-H of ribose). These latter signals are characteristic of an adenosyl group [23] and the sum of the evidence showed the yellow pigment to be *Co*-(5'-deoxy-5'-adenosyl)cobyrinic acid *a,c-* diamide **8**. The UV/visible spectrum of the faster-running yellow pigment was essentially identical to that of the above deoxyadenosyl derivative. On exposure to light it was cleaved to give a single corrin, which was the same triamide already characterized. It is clear that this second yellow product is *Co*-(5'-deoxy-5'-adenosyl)cobyrinic acid triamide, though final characterization by NMR spectroscopy eluded us due to lack of material.

Significance

One can see evolution in action as one looks at the chemistry of biosynthetic pathways used by different organisms. There is particular interest in

understanding how, during evolution, the early lack of oxygen, followed by its availability, affected the way anaerobic and aerobic organisms build the same final structure. The two micro-organisms we discuss here represent the aerobic and essentially anearobic worlds and both construct coenzyme B_{12} (compound 10).

Our work contributes to this understanding by clarifying the relationships between the later sections of the pathways leading to compound 10 in *Pr. shermanii* and *Ps. denitrificans*. Earlier reports came to seriously conflicting conclusions about the amidation steps by which cobyrinic acid is converted into adenosylcobinamide (compound 9). Although these pioneering studies (reviewed in [19]) supported early attachment of the adenosyl group to the cobalto–corrin system, the point (or points) at which this happened was not clear. Further, conclusions could not be drawn from enzymic experiments because broken-cell enzyme preparations from *Pr. shermanii* were able to adenosylate not only tri-, tetra- and penta-amides of cobyrinic acid [21] but also unnatural compounds such as a lactone and a lactam derivative of cobalamin [24].

We isolated four additional compounds from *Pr. shermanii* producing cobyrinic acid 6: a single diamide isomer of cobyrinic acid shown to be the *a,c*-isomer 7, a single triamide isomer and the deoxyadenosyl derivatives of these two compounds. The triamide was indistinguishable from the single triamide synthesized by *Ps. denitrificans*. The adenosylated diamide 8 has recently been established as an intermediate on the pathway to coenzyme B_{12} in *Ps. denitrificans* [25]. It seems likely that adenosylation occurs at the stage of the *a,c*-diamide 7 in *Pr. shermanii*, as shown in *Ps. denitrificans* [25]. The non-adenosylated triamide we isolated is probably produced by photochemical cleavage of the corresponding adenosylated material during handling in daylight. This would mean that the biosynthetic routes to coenzyme B_{12} in the two organisms, having differed in the earlier part of the pathway, merge at the *a,c*-diamide 7 and that the next intermediate, compound 8, is also the same. The order of the amidations of the carboxyl side-chains is the same in the two organisms up to the triamide stage. Further work is needed to determine the structures of the adenosylated tri-, tetra-, and penta-amides in *Pr. shermanii* and *Ps. denitrificans*.

Materials and Methods

Materials

Sep-Pak C_{18} cartridges (Waters) were washed with ethanol containing 1 % trifluoroacetic acid (10 ml) and then with 0.02 M hydrochloric acid (15 ml) before use. Samples for HPLC analysis or for FPLC were filtered through a membrane filter (0.2–0.45 μm, Gelman Sciences). HPLC analyses were carried out using a Waters 600E Multisolvent Delivery System with a Cecil Instruments CE 272 Ultraviolet detector set at 365 nm. For preparative scale reversed-phase column chromatography, a Pharmacia FPLC system was used consisting of two P-500 pumps, gradient programmer GP-250, monitor UV-M (365 nm) and column Prep RPC 15mm HR 10/10.

Buffer 1: degassed 50 mM aqueous potassium phosphate, pH 7.7, containing 0.2 mM dithiothreitol (DTT).

Buffer 2: 10 mM aqueous KCN containing pyridine (2.2 ml/l) and ethylene diamine tetra-acetic acid (EDTA) (230 mg/l), adjusted to pH 5.9 with acetic acid.

Buffer 3: 10 mM aqueous KCN containing pyridine (2.2 ml/l) adjusted to pH 4.1 with acetic acid.

Buffer 4: 0.1 M aqueous potassium phosphate, pH 5.0, containing 10 mM KCN.

Buffer 5: 0.1 M aqueous potassium phosphate, pH 6.5, containing 10 mM KCN [18].

Buffer 6: 50 % acetonitrile, 50 % 0.1 M aqueous potassium phosphate, pH 8.0, containing 10 mM KCN [18].

Estimation of cobyrinic acid **6** by HPLC

An aliquot of the broken-cell system (10 ml), prepared as in the next section, was diluted with buffer 3 (20 ml), acidified to pH 4 with 2M hydrochloric acid and centrifuged (1800 rpm, 10 min, 4 °C). The supernatant was passed through an acid-washed Sep-Pak C_{18} cartridge, then washed with 0.02 M hydrochloric acid (15 ml) and 5 % acetonitrile in buffer 3 (10 ml). The pigments were eluted with 20 % acetonitrile in buffer 3 (4 ml). The residue after freeze-drying the eluate was dissolved in buffer 4 (200 μl) and 10–20 μl was loaded onto a Spherisorb S5ODS1 HPLC column (4.6 x 250 mm) and eluted with a gradient of 0 to 20 % acetonitrile in buffer 4.

Standardization of the above procedure using added cobyrinic acid gave an average recovery of 72 %. A further 8 % could be recovered by redissolving the pellet from the centrifugation in buffer 1 (7.5 ml) and repeating the procedure.

Isolation of the corrin amides

Deep-frozen cells of *Pr. shermanii* (120 g) were thawed, suspended in buffer 1 (240 ml) and broken by three passes through a French pressure cell press (American Instruments Co., 10,000–16,000 psi). After centrifugation (27,000 x g, 45 min, 4 °C), the supernatant was diluted with buffer 3 (300 ml) and acidified to pH 4 with 2M hydrochloric acid. After further centrifugation (27,000 x g, 10 min), the supernatant was divided into six portions and each portion was passed through a Sep-Pak C_{18} cartridge. Each cartridge was washed with 0.02M hydrochloric acid (15 ml) and 5 % acetonitrile in buffer 3 (10 ml) and the pigments were eluted with 20 % acetonitrile in buffer 3 (5 ml). The residue after freeze-drying was dissolved in buffer 3 (10 ml), centrifuged (27,000 x g, 10 min), applied to an FPLC column and eluted with a gradient of 0 to 50 % acetonitrile in buffer 2. Fractions eluted with 2–12 % acetonitrile were combined, freeze-dried, reapplied to an FPLC column and eluted with a gradient of 0 to 25 % acetonitrile in buffer 2. Three fractions were obtained: (a) cobyrinic acid **6**, 3.6–5.6 % acetonitrile; (b) a purple/yellow fraction,

5.6–6.9 % acetonitrile; (c) an orange fraction, 6.9–8.9 % acetonitrile. Fraction (b) was further separated by FPLC, eluting with a gradient of 0 to 25 % acetonitrile in buffer 3, to give four compounds: (bi) flavin adenine dinucleotide, 5.4–5.65 % acetonitrile; (bii) flavin mononucleotide, 5.65–6.1 % acetonitrile; (biii) cobyrinic acid triamide, 8.0–8.75 % acetonitrile, 60.7 μg; (biv) cobyrinic acid *a,c*-diamide **7**, 8.75–9.4 % acetonitrile, 29.8 μg. Fraction (c) was also separated by FPLC, eluting with a gradient containing 0 to 25 % acetonitrile in buffer 3, to give two yellow fractions: (ci) *Co*-(5'-deoxy-5'-adenosyl)cobyrinic acid triamide, 7.5–8.0 % acetonitrile; (cii) *Co*-(5'-deoxy-5'-adenosyl)cobyrinic acid *a,c*-diamide **8**, 8.0–8.5 % acetonitrile.

The triamide gave the following spectroscopic data: UV/visible (H_2O+KCN): $\lambda_{max.}$ (nm (absorbance)): 576 (0.358), 537 (0.321), 365 (1.104); $\lambda_{min.}$: 555 (0.274), 326 (0.198). Mass spectrum (+ve FAB; glycerol–thioglycerol, 1:1): *m/z* found: 935.3780. $C_{45}H_{62}N_7O_{11}Co$ requires 935.3839.

The diamide **6** gave the following spectroscopic data: UV/visible (H_2O+KCN): $\lambda_{max.}$ (nm (absorbance)): 577 (0.128), 538 (0.117), 365 (0.399); $\lambda_{min.}$: 556 (0.101), 326 (0.098). Mass spectrum (+ve FAB; glycerol–thioglycerol, 1:1): *m/z* found: 936.3623. $C_{45}H_{61}N_6O_{12}Co$ requires 936.3679.

The adenosylated diamide **8** gave the following spectroscopic data: UV/visible (buffer 3): $\lambda_{max.}$ (nm (absorbance)): 457 (0.389), 367 (0.411), 303 (0.991); $\lambda_{min.}$: 414 (0.282), 361 (0.406). Mass spectrum (+ve FAB; glycerol–thioglycerol, 1:1): 936 (corresponding to Co–C cleavage). ^{1}H NMR (400 MHz, D_2O, well resolved signals only): δ 0.81 (3H, s), 0.92 (3H, s), 1.17 (3H, s), 1.40 (3H, s), 1.58 (3H, s), 1.77 (3H, s), 2.37 (3H, s), 2.42 (3H, s), 3.78 (1H, t, *J* 6.2 Hz), 4.24 (1H, br d, *J* 10 Hz), 4.34 (1H, dd, *J* 3.5 and 5.5 Hz), 5.62 (1H, d, *J* 3.1 Hz), 6.95 (1H, s), 8.11 (1H, s), 8.32 (1H, s).

The adenosylated triamide showed UV/visible (buffer 3): $\lambda_{max.}$ (nm (absorbance)): 458 (0.378), 366 (0.517), 303 (0.984); $\lambda_{min.}$: 415 (0.279), 356 (0.406).

Preparation of cobyrinic acid amide esters

Vitamin B_{12} (1 g) was partially methanolyzed as in [20] with a slightly modified work-up procedure involving column chromatography on silica gel (35 x 200 mm), eluting with 8 % MeOH in $CHCl_3$, to give the following fractions: (a) cobyrinic acid heptamethyl ester (57 mg); (b) cobyrinic acid *a*- and *c*-monoamide hexamethyl esters (150 mg); (c) cobyrinic acid *g*-monoamide hexamethyl ester (105 mg); (d) cobyrinic acid *c,g*- and *a,c*-diamide pentamethyl esters (85 mg); (e) cobyrinic acid *a,g*-diamide pentamethyl ester (202 mg); (f) cobyrinic acid *a,c,g*-triamide tetramethyl ester + others (61 mg). Fraction *d* (60 mg) was separated by preparative TLC, developing with chloroform–methanol (9:1) saturated with KCN, to give the pentamethyl esters of cobyrinic acid *a,c*-diamide **6** (28 mg) and cobyrinic acid *c,g*-diamide (16 mg). These amido esters were identified by comparison with literature data [20] using chromatography, NMR and mass spectrometry. Only values for materials relevant to the present work are quoted below.

Cobyrinic acid *a,c*-diamide pentamethyl ester: δ_H ($CDCl_3$+KCN): 1.19 (3H, s), 1.21 (3H, s), 1.35 (3H, s), 1.44 (3H, s), 1.47 (3H, s), 1.70 (3H, s), 2.14 (3H, s), 2.21 (3H, s), 2.27 (1H, m), 2.82 (1H, m), 3.03 (1H, t, *J* 5.5 Hz), 3.62 (3H, s), 3.67 (3H, s), 3.68 (3H, s), 3.69 (3H, s), 3.74 (3H, s), 5.52 (1H, s), 5.54 (1H, br s), 5.66 (1H, br s), 6.39 (1H, br s), 6.72 (1H, br s); *m/z* (+ve FAB, glycerol): 1058 (M^+), 1004 (M^+– 2HCN), 1003.

Cobyrinic acid *c,g*-diamide pentamethyl ester: δ_H ($CDCl_3$+KCN): 1.19 (3H, s), 1.22 (3H, s), 1.35 (6H, s), 1.50 (3H, s), 1.78 (3H, s), 2.16 (3H, s), 2.21 (3H, s), 2.97 (1H, m), 3.03 (1H, t, *J* 5.6 Hz), 3.62 (3H, s), 3.67 (3H, s), 3.68 (3H, s), 3.69 (6H, s), 5.35 (1H, br s), 5.52 (1H, s), 5.71 (1H, br s), 5.90 (1H, br s), 6.96 (1H, br s).

Cobyrinic acid *a,g*-diamide pentamethyl ester: δ_H ($CDCl_3$+KCN): 1.10 (3H, s), 1.15 (3H, s), 1.27 (3H, s), 1.35 (3H, s), 1.39 (3H, s), 1.49 (3H, s), 2.16 (3H, s), 2.19 (3H, s), 2.68 (1H, d, *J* 15.6 Hz), 2.80 (1H, m), 3.00 (1H, t, *J* 5.4 Hz), 3.35 (1H, dd, *J* 4.3 and 7.0 Hz), 3.40 (1H, s), 3.60 (3H, s), 3.65 (6H, s), 3.66 (3H, s), 3.68 (3H, s), 4.07 (1H, d, *J* 8.4 Hz), 5.51 (1H, s), 5.54 (1H, br s), 5.68 (1H, br s), 6.89 (1H, br s), 7.11 (1H, br s).

Comparison of the natural and partially synthetic di- and tri-amides by HPLC

The cobyrinic acid diamide pentamethyl esters were hydrolyzed with 2 M aqueous piperidine under N_2 in a glovebox (<10 ppm O_2) at ~10 °C for 48 h and then freeze-dried and analyzed on a μBondapack C_{18} HPLC column (10 μm; 3.9 x 300 mm) eluting at 1 ml min^{-1} with a gradient (31 min) from 95 % buffer 5 with 5 % buffer 6 to 80 % buffer 5 with 20 % buffer 6. The retention times were: cobyrinic acid *c,g*-diamide, 26.1 min; cobyrinic acid *a,c*-diamide, 26.8 min.

Photochemical cleavage of the adenosyl residue

A solution of the adenosylated diamide **8** in buffer 4 was exposed to sunlight for 1 h and the resultant solution was directly analyzed on a Spherisorb S5ODS1 HPLC column (4.6 x 250 mm) eluting at 1 ml min^{-1} with a gradient (45 min) of 0 to 20 % acetonitrile in buffer 4. The retention time of the product was identical with that of cobyrinic acid *a,c*-diamide **7**, 39.8 min (confirmed by coinjection), whereas the retention time of compound **8** was 43.2 min. In an exactly similar way, the adenosylated triamide was photochemically cleaved and the product was identified as the cobyrinic acid triamide.

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