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Biosynthesis of vitamin B_{12}: the multi-enzyme synthesis of precorrin-4 and factor IV

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Background: In order to study the biosynthesis of vitamin B_{12}, it is necessary to produce various intermediates along the biosynthetic pathway by enzymic methods. Recently, information on the organisation of the biosynthetic pathway has permitted the selection of the set of enzymes needed to biosynthesise any specific identified intermediate. The aim of the present work was to use recombinant enzymes in reconstituted multi-enzyme systems to biosynthesise particular intermediates.

Results: The products of the *cobG* and *cobJ* genes from *Pseudomonas denitrificans* were expressed heterologously in *Escherichia coli* to afford good levels of activity of the corresponding enzymes, CobG and CobJ. Aerobic incubation of precorrin-3A with the CobG enzyme alone yielded precorrin-3B. When CobJ and *S*-adenosyl-L-methionine were included in the incubation, the product was precorrin-4. Both precorrin-3B and precorrin-4 are known precursors of vitamin B_{12} and their availability has allowed new mechanistic studies of enzymic transformations.

Conclusions: Our results show that the expression of the CobG and CobJ enzymes has been successful, thus facilitating the biosynthesis of two precursors of vitamin B_{12}. This lays the foundation for the structure determination of CobG and CobJ as well as future enzymic experiments focusing on later steps of vitamin B_{12} biosynthesis.

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Introduction

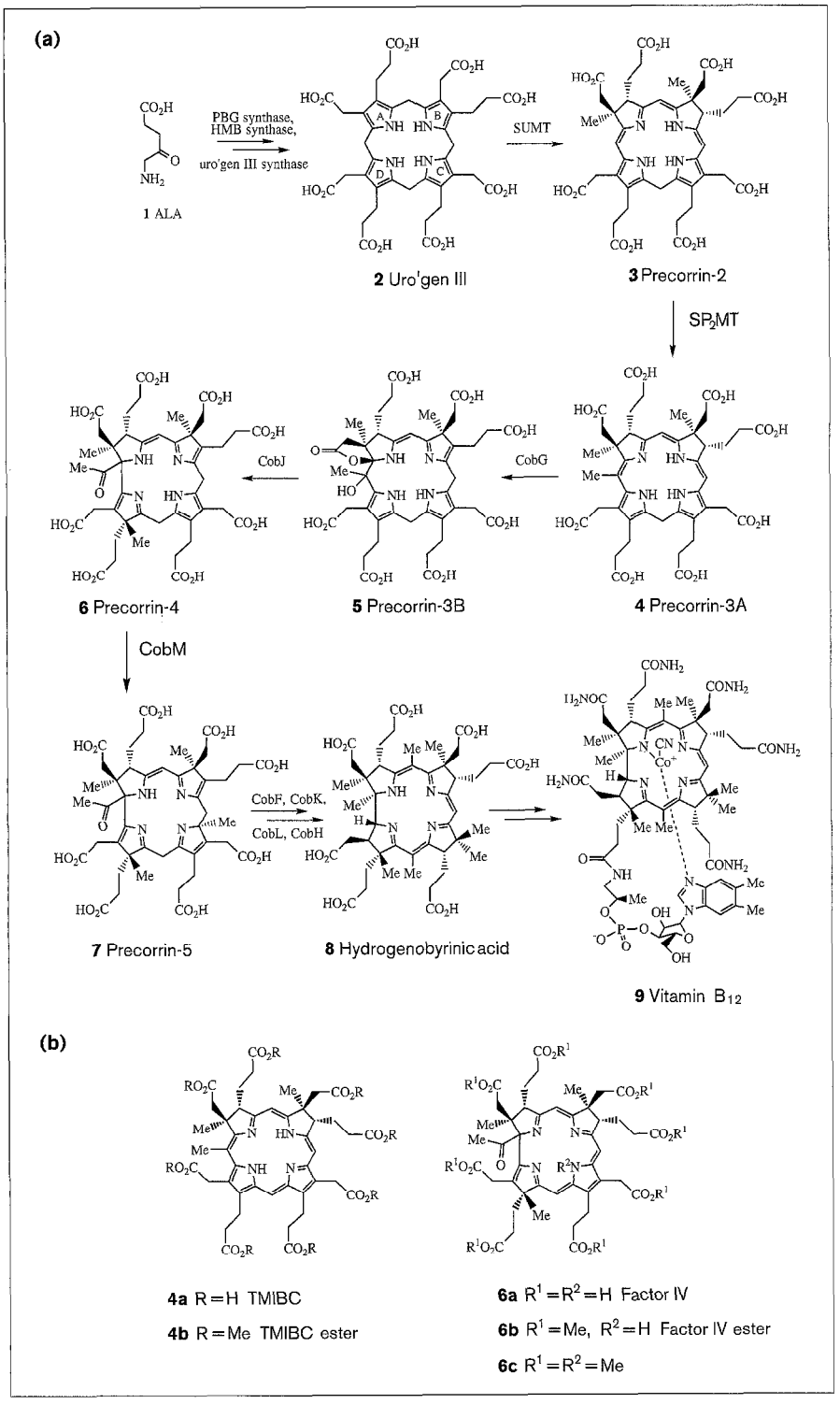
Enzymic synthesis of biosynthetic intermediates has been routinely employed in collaborative research on the biosynthesis of vitamin B_{12} (Figure 1a; **9**) carried out in Paris and Cambridge [1,2] and also in Texas [3]. The enzymes used for much of the European work were contained in cell-free systems prepared from strains of *Pseudomonas denitrificans* carrying all eight genes (or various subsets of these genes) necessary for the biosynthesis of hydrogenobyrinic acid (Figure 1a; **8**) from precorrin-2 (Figure 1a; **3**) [4]. In addition, many of the more recent experiments in our laboratory have been based on the use of recombinant enzymes in reconstituted multi-enzyme systems [5,6] and have led to further important discoveries about the biosynthetic pathway.

The work presented here depended on five recombinant enzymes produced by expression of their corresponding genes in an *Escherichia coli* host. Three enzymes encoded by the *hemB*, *hemC* and *hemD* genes were porphobilinogen synthase (PBG synthase; EC 4.2.1.24), hydroxymethylbilane synthase (HMB synthase; EC 4.3.1.8) and uroporphyrinogen III synthase (uro'gen III synthase; EC 4.2.1.75), respectively. The genes have been identified and characterised from several organisms, but those from *Bacillus subtilis* [7] were preferred for expression in *E. coli* [6]. The use of the *B. subtilis* genes had the great advantage that the uro'gen III synthase produced was stable, unlike the enzyme from other sources [8]. The remaining two enzymes encoded by the *cobA* and *cobI* genes were from *P. denitrificans* — *S*-adenosyl-L-methionine:uroporphyrinogen III methyltransferase (SUMT) [9] and *S*-adenosyl-L-methionine: precorrin-2 methyltransferase (SP_2MT) [10], respectively. Their expression in *E. coli* has been described [6].

These enzyme preparations from *B. subtilis* and *P. denitrificans* converted 5-aminolaevulinic acid (ALA; Figure 1a; **1**) preparatively and in high yield into three of the early intermediates involved in the biosynthesis of vitamin B_{12}: uro'gen III (Figure 1a; **2**), precorrin-2 (Figure 1a; **3**) and precorrin-3A (Figure 1a; **4**). Using this method, the enzymic synthesis of nonlabelled [11,12] and specifically labelled [13–17] forms of **2**, **3** and **4** has proved invaluable for investigating the biosynthetic pathway of vitamin B_{12}. Enzymic syntheses have also been used by other groups for various types of natural products including tetrapyrroles [18].

We wanted to use the same approach with recombinant enzymes to advance further along the biosynthetic pathway because this method has the advantage of allowing

Figure 1



Some early biosynthetic intermediates on the pathway to vitamin B_{12}. **(a)** The biosynthesis of precorrin-3B (**5**) and precorrin-4 (**6**) on the pathway to vitamin B_{12} (**9**). An intermediate is called a 'precorrin' if it precedes the formation of the corrin ring present in **8**. The number after 'precorrin' gives the number of methyl groups that have been introduced from *S*-adenosyl-L-methionine to form that substance during the steps going forward from uro'gen III (**2**). When this number is the same for two or more intermediates, they are distinguished by an added letter. Genes and gene products: *hemB*, PBG synthase (≡ALA dehydratase); *hemC*, HMB synthase (≡PBG deaminase); *hemD*, uro'gen III synthase (≡cosynthetase); *cobA*, *S*-adenosyl-L-methionine : uroporphyrinogen III methyltransferase (≡SUMT); *cobI*, *S*-adenosyl-L-methionine : precorrin-2 methyltransferase (≡SP2MT); *cobG*, CobG; *cobJ*, CobJ. **(b)** Derivatives of precorrin-3A (**4**) and precorrin-4 (**6**). TMIBC: trimethylisobacteriochlorin.

experiments on a specific enzymic transformation, or a selected series of such steps, without interference from competing enzymic reactions. Having a full knowledge of the biosynthetic organisation of the pathway to vitamin B_{12} [1], we focused on the two enzymes following SP_2MT encoded by the genes *cobG* and *cobJ*.

This paper describes the expression of the *cobG* and *cobJ* genes from *P. denitrificans* in an *E. coli* host and the use of their respective gene products in a coupled-enzyme synthesis of the tetramethylated intermediate for vitamin B_{12}, precorrin-4 (Figure 1a; **6**). Precorrin-4 was first detected by the group in Paris and was isolated as its didehydro

derivative, factor IV [19]. The structures shown for precorrin-4 and factor IV (Figure 1b; **6a**) were established using ^{13}C-labelling and nuclear magnetic resonance (NMR) spectroscopic analysis [14,19]. The factor IV enzymically synthesised in the present work was isolated chromatographically or as its octamethyl ester (Figure 1b; **6b**).

Results and discussion

Expression of *cobG* and *cobJ*

The strategy employed for the expression of the *cobG* gene from *P. denitrificans* in *E. coli* required site-directed mutagenesis to engineer an *Nde*I restriction endonuclease site over the ATG start codon of the gene (see the Materials and methods section, Construction of pSD37). Using this newly constructed *Nde*I restriction site, the mutated *cobG* gene was cloned downstream of the strong *E. coli* P*trp* promoter and the efficient ribosome-binding site from the λ *c*II gene to form a convenient cassette for further cloning. In contrast, expression of the *cobJ* gene of *P. denitrificans* in *E. coli* was achieved by simply cloning an appropriate *P. denitrificans* chromosomal fragment from pXL330 downstream of the tandem thermoinducible bacteriophage λ promoters in pCE30.

Expression of the *cobG* and *cobJ* genes in these heterologous systems led to the accumulation of proteins with M_r = 46,000 and M_r = 27,000, respectively, as measured by sodium dodecyl sulphate polyacrylamide gel electrophoresis analysis of cell-free extracts. The relative molecular masses of these accumulated proteins agree with the reported M_r for the CobG and CobJ enzymes purified from *P. denitrificans* [20] and with the predicted subunit M_r calculated from the amino acid sequence derived from the DNA sequence of the *cobG* and *cobJ* genes [4].

The CobG enzyme is known to catalyse the oxidative conversion of precorrin-3A into precorrin-3B (Figure 1a; **5**) [21], a step which requires oxygen [22]. In addition, CobJ has been shown to carry out the subsequent conversion of precorrin-3B into precorrin-4, a process which involves both ring contraction and methylation at C17 [19]. In order to assess the two enzyme preparations from the present work, we first tested the ability of the CobG enzyme to catalyse the conversion of precorrin-3A into precorrin-3B. Surprisingly, the initial preparations of CobG failed to do so. But, because CobG is an iron–sulphur protein [20], freshly prepared ferric chloride was added to the fermentations of the engineered *E. coli* strain. This change, together with a reduction in the aeration of the incubation, afforded cells yielding CobG enzyme which readily converted precorrin-3A into precorrin-3B. The latter is easily oxidised during handling to its didehydro derivative, factor IIIB [20] which was isolated by high performance liquid chromatography (HPLC) and identified by electrospray mass spectrometry. No further work was done on the synthesis of precorrin-3B because, having established that active CobG enzyme had been produced, our aim was to use the CobG and CobJ enzymes together to synthesise precorrin-4. The heterologous expression of the *P. denitrificans cobG* gene has also been briefly reported by the group in Texas [22].

Preparation of precorrin-4 and factor IV

In order to provide pure substrate, the precorrin-3A prepared by our coupled-enzyme system [6] was isolated and purified as the ester of its didehydro derivative, the trimethylisobacteriochlorin (TMIBC; Figure 1b; **4a**). Reduction was therefore necessary to return to precorrin-3A (the natural substrate for the CobG enzyme). Material produced by controlled catalytic hydrogenation of the ester **4b** (Figure 1b) followed by hydrolysis of the ester groups was successfully converted into precorrin-4 by the CobG and CobJ coupled-enzyme system in yields similar to those obtained in the method based on enzymic reduction (see below). This demonstrates unambiguously that both overproduced proteins are enzymically active. An experimentally simpler approach involved enzymic reduction of TMIBC, again prepared by hydrolysis of its ester, by the reductase present in cell-free extracts of *P. denitrificans* [23]. This simply involved addition of the cell-free extract and NADH to our engineered preparation of the CobG and CobJ enzymes containing TMIBC.

The amount of reductase to be added was studied with the aim of providing the optimum supply of precorrin-3A for the coupled-enzyme system. Interestingly, doubling the amount of reductase added either had little effect on the overall yield of precorrin-4 (measured as factor IV) or could even reduce it. It should be noted that the strain of *P. denitrificans* used as the source of the reductase does not express the *cobM* gene; this avoids conversion of the biosynthesised precorrin-4 into precorrin-5.

The optimum time for the incubation was 5–9 h (similar to that found by the group in Paris) [20]; the decreased yields observed after 9 h probably resulted from the decomposition of the labile precorrin-4, which outweighed its further production. By using an incubation period of 5 h, the maximum yield of precorrin-4 was obtained with maximal recovery of unused substrate.

In order to allow many small-scale yet accurate experiments on the production of factor IV, ^{14}C-labelling was used. The substrate, [2,7,20-*methyl*-^{14}C]precorrin-3A was prepared as reported [6], but using [*methyl*-^{14}C]*S*-adenosyl-L-methionine rather than unlabelled material. The molar specific activity of the resultant labelled TMIBC, and therefore of the [2,7,20-*methyl*-^{14}C]precorrin-3A prepared from it, was determined. The yield of factor IV could then be calculated from the total ^{14}C activity it carried. The best yields were 19–21% under the conditions outlined above, with ~15% of substrate recovered together with

11% of the corresponding monolactone. Oxidative lactonisation occurs readily when TMIBC is handled in the presence of oxygen [24]. This ^{14}C experiment also allowed the extinction coefficient for factor IV to be determined in water containing 0.1% trifluoroacetic acid: $\varepsilon = 3.8 \times 10^4$ at the λ_{max} of 370 nm.

Esterification of factor IV

The octamethyl ester derivative of factor IV was prepared using diazomethane. The molecular formula of the product was shown to be correct ($C_{52}H_{67}N_4O_{17}$) by accurate mass spectrometry measurement. A second product was also formed in this esterification step having a molecular formula $C_{53}H_{69}N_4O_{17}$ (by mass spectrometry measurement), corresponding to a nonamethyl derivative of factor IV. When the treatment with diazomethane was stopped after 15 s, only a small amount of $C_{53}H_{69}N_4O_{17}$ was formed so the required octamethyl ester ($C_{52}H_{67}N_4O_{17}$) was obtained in >75% yield. Longer times gave greater proportions of the nonamethyl product and for times >1 min, general decomposition set in. This unwanted nonamethyl material was not further examined but it was presumably the octamethyl ester of an *N*-methylated derivative, for example **6c** (Figure 1b); a mixture of *N*-methylated tautomeric forms of factor IV ester cannot, however, be excluded.

Significance

Recent developments have made it clear that the most powerful way to solve a complex biosynthetic problem is to identify the set of enzymes involved and then to use the strengths of analytical and organic chemistry to work out which step each enzyme catalyses and the structure of its product. The first of these stages is based on genetics and molecular biology. The elucidation of the complete biosynthetic pathway to vitamin B_{12} [1] is a dramatic example of how successful this approach can be. Knowledge of the functions of the various enzymes and of their sequence of action opens the way to heterologous expression of any of the component enzymes. With substantial quantities of protein thus available, there is the opportunity to biosynthesise the intermediate of interest in sufficient quantities, for example, for study by ^{13}C-nuclear magnetic resonance.

The work described here shows that two enzymes of the B_{12} biosynthetic pathway in *Pseudomonas denitrificans* which are particularly interesting have been successfully produced by heterologous expression of their genes in *Escherichia coli*. One is CobG, an iron–sulphur protein that carries out an oxidative transformation, and the other is CobJ, which catalyses a conversion involving both ring contraction and C-methylation. This lays the foundation for further advances, an important target being the crystallisation of these proteins for X-ray structure determination.

In addition, the CobG and CobJ enzymes have been applied in combination with those encoded by the *hemB*, *hemC*, *hemD*, *cobA* and *cobI* genes to allow the enzymic synthesis of precorrin-4 and factor IV from 5-aminolaevulinic acid and *S*-adenosyl-L-methionine. This provides a convenient source of precorrin-4 as a substrate for future enzymic experiments focusing on the later steps of vitamin B_{12} biosynthesis.

Materials and methods

General details

Electronic spectra were recorded on a Cecil CE 5501 ultra-violet (UV) spectrophotometer. Electrospray mass spectrometry was performed on a VG quadrupole mass spectrometer using VG Lab Base software (VG Analytical). ^1H-NMR spectra were recorded on a Bruker WH-400 instrument operating at 400 MHz and the solvent signal was used as internal reference. HPLC purifications were carried out using a Waters 600E multisolvent delivery system and pigments were detected by a Cecil CE 272 UV spectrophotometer equipped with a Venture RE 511.20 recorder. The reverse-phase column was a PhaseSep Nucleosil 5ODS and, before injection, samples were filtered through Acrodisc 3 (0.45 mm, Gelman Sciences). Radiochemical analyses were carried out on a United Technologies Packard 2000 CA Tricarb liquid scintillation analyser. Centrifugations were performed on an Europa 24M centrifuge and large scale fermentations, unless otherwise stated, were performed in a Microferm fermenter (New Brunswick Scientific) with constant stirring (200 rpm) and an air flow (4 l min^{-1}) using a Radiometer type TTT2 auto-titrator for pH control. All solvents were dried prior to use and water used throughout was distilled and further purified with a MilliQ system (Millipore). Solvents were evaporated on a Büchi Rotavapor at reduced pressure.

Bacterial strains and DNA

Bacterial strains and plasmid DNAs are listed in Table 1. *E. coli* AN1459 (*ilv thr supE recA srl::*Tn*10*) [25] and MC1060 (Δ(*lacIPOZYA*)*X74 galU galK strA2 hsdR*) [26] and *P. denitrificans* G3575 (Rif^r Δ(*cobF to cobM*)) [20] were used as hosts for all plasmids. *E. coli* TG1 (Δ(*lac-pro*) *thi supE hsd*D5/F′ *proAB lacI*^{q}ZΔ*M15*) [27] was used as the recipient host for all M13 derivatives. Bacteria were grown at 37°C in LB broth or on plates. For *E. coli* strain AN1459, cultures were grown at 30°C and media were supplemented with thymine (25 μg ml^{-1}). Antibiotic supplements where used were ampicillin (50 μg ml^{-1}) and chloramphenicol (20 μg ml^{-1}). Plasmids pCE30 [28], pXL1283 [6], pXL330 [29] and pXL229 [20] and bacteriophage M13tg131 [30] were as described. Oligonucleotides were synthesised on an Applied Biosystems oligonucleotide synthesiser and oligonucleotide-directed mutagenesis was performed using the system of Amersham. All other procedures used for DNA manipulations were essentially as described [31].

Reagents, chromatographic resins and enzymes

Enzymes used for DNA manipulations were obtained from Stratagene, Pharmacia or Boehringer-Mannheim and were used in buffers recommended by the suppliers. Reagents and buffer components, lysozyme, antibiotics and *S*-adenosyl-L-methionine were from Sigma and LiChroprep C-18 resin was from Merck. Radioactive [^{14}C-*methyl*]*S*-adenosyl-L-methionine (53 mCi mmol^{-1}) was from Amersham and culture media from Difco.

Plasmid constructions

Construction of pSD37. The *P. denitrificans cobG* gene was expressed in *E. coli* following the cloning of the *cobG* gene downstream of the *trp* promoter in a derivative of the pBR322-derived expression vector pXL694. Initially a 2.2 kb *Bgl*II / *Sph*I fragment from pXL330 [29], carrying the entire *cobG* gene, was subcloned into the *Bam*HI and *Sph*I sites of M13tg131 [30] leading to pXL1365. Oligonucleotide site-directed mutagenesis [32] was then performed on single-stranded pXL1365

Table 1

Bacterial strains and plasmid DNAs.

Bacterial strain or plasmid	Marker and replicon	Relevant properties	Reference or source
P. denitrificans			
G3575	Rif^r	Rif^r strain SC510 Δ(*cobF to cobM*), produces no cobalamin	[20]
E. coli			
AN1459		*ilv thr supE recA srl*::Tn*10*	[25]
MC1060		Δ(*lacIPOZYA*)*X74 galU galK strA2 hsdR*	[26]
TG1		Δ(*lac-pro*) *thi supE hsdD5*/F′ *proAB lacI*^{q}*Z*Δ*M15*	[27]
Plasmids			
M13tg131	f1	M13 bacteriophage derivative	[30]
pCE30	Amp^r ColE1	Carries bacteriophage λ promoters and cI*857* allele	[28]
pXL330	Amp^r ColE1	8.7 kb *Eco*RI (*cobF to cobM*) fragment from pXL151 cloned into pUC13	[29]
pXL1283	Amp^r ColE1	1.3 kb pXL1262 *Nde*I/*Sst*I (*cobA*) fragment cloned into pXL694	[6]
pXL229	Km^r RSF1010	3.3 kb *Eco*RI/*Bam*HI (*cobH to cobK*) fragment from pXL152	[20]
pXL1365	f1	2.2 kb pXL330 *Bgl*II/*Sph*I (*cobG*) fragment cloned into the *Bam*HI and *Sph*I sites of M13tg131	This study
pXL1404	f1	Site-directed mutant derivative of pXL1365 with an *Nde*I site insertion	This study
pSD37	Amp^r ColE1	1.9 kb pXL1404 *Nde*I/*Sph*I (*cobG*) equivalent fragment cloned into pXL1283	This study
pSD38	Amp^r ColE1	4.3 kb pXL330 *Sma*I (*cobH to cobK*) fragment cloned into pCE30	This study

using oligonucleotide 546 5′CATCAAATCCGT***CATATG***TTGAGGTT-CTCC3′) to construct the mutagenised plasmid pXL1404 which has a *Nde*I restriction endonuclease site (bold) over the translation initiation signal (italicised) in the *cobG* gene sequence 5′GGAGAACCTCAA-GTG*ATG*ACGGATTTGATG3′. A 1.9 kb *Nde*I/*Sph*I fragment containing the entire *cobG* gene equivalent to that in pXL1404 was then subcloned into pXL1283 [6] to give pSD37.

Construction of pSD38. The *P. denitrificans cobJ* gene was expressed in *E. coli* following the cloning of the *cobJ* gene downstream of the bacteriophage tandem λ P_R and P_L promoters in the vector pCE30 [28]. Plasmid pSD38 (8.3 kb) was constructed by ligation of the 4.3 kb *Sma*I fragment containing the *P. denitrificans cobJ* gene from pXL330 [29] into the *Sma*I site of pCE30.

Protein chemistry

Expression of the CobJ enzyme. E. coli AN1459/pSD38 was grown at 30°C in LB broth (1 l) supplemented with ampicillin (50 μg ml^{-1}) and thymine (25 μg ml^{-1}) to D_{595} ~0.6. After rapid temperature shift to 42°C, growth was continued at 42°C for 4 h. The culture was chilled and cells were harvested and lysed as described.

Expression of the CobG enzyme. E. coli MC1060/pSD37 was grown at 37°C in M9 medium (50 ml) supplemented with ampicillin (100 μg ml^{-1}), casamino acids (4% w/v final) and tryptophan (100 μg ml^{-1}) in a baffled flask for 16 h. This culture was then used to inoculate 1 l of the same medium with 10 μM $FeCl_3$ but without tryptophan supplement. Growth was continued at 37°C in a non-baffled conical flask for a further 18 h. The culture was chilled and cells were harvested and lysed as described.

Lysis of cells. Buffers for cell lysis were thoroughly deoxygenated under argon prior to use and all procedures during enzyme preparations were at 0–5°C. Chilled cell cultures were harvested (12,000×g, 15 min), resuspended to a density where D_{595} ~200 with 100 mM Tris HCl (pH 7.6), 10% (w/v) sucrose, poured into liquid N_2 and stored at −70°C. Cells were thawed and diluted to D_{595} ~100 to the final composition of lysis buffer (100 mM Tris HCl (pH 7.6), 10% (w/v) sucrose, 100 mM NaCl, 5 mM dithiothreitol, 20 mM spermidine HCl, 0.1% (v/v) Brij-58). Lysozyme (0.2 μg ml^{-1}) was added and the mixture was stirred for 2 h at 0°C. Cell-free extracts were then prepared by centrifugation (40,000×g, 30 min) and the supernatant obtained was dropped into liquid N_2 and stored at −70°C.

Preparation of TMIBC (4a) and precorrin-3A (4)

TMIBC was used as starting material for all enzymic experiments and was biosynthesised essentially as described [6]. This material was reduced enzymically to precorrin-3A in the incubation being run, as illustrated in the next section. Alternatively, catalytic hydrogenation of its octamethyl ester **4b** under conditions kindly provided by A. Eschenmoser (ETH, Zürich, Switzerland) afforded precorrin-3A octamethyl ester which was then hydrolysed before incubation. TMIBC octamethyl ester (200 μg; **4b**) in methyl acetate (1.5 ml) was stirred at room temperature under hydrogen for 0.5 h with platinum on activated carbon (5%; 2 mg) and the subsequent steps were carried out in a glove box (<5 ppm O_2). The catalyst was removed by filtration through a small Celite column and the filtrate evaporated. The yellow residue was dissolved in 2 M aqueous piperidine (200 μl) and stirred at room temperature for 48 h to afford precorrin-3A ready for enzymic incubations. To provide TMIBC for incubations in which it was enzymically reduced *in situ*, TMIBC octamethyl ester was first hydrolysed in 2 M aqueous piperidine as above.

Preparation of precorrin-4 (6) and factor IV (6a)

The final concentration of reagents in the buffer for enzymic incubations was 100 mM Tris Cl (pH 7.6), 5% (w/v) sucrose, 50 mM NaCl, 2.5 mM DTT, 0.6 mM *S*-adenosyl-L-methionine and 1.2 mM NADH. Buffers for incubations were thoroughly deoxygenated under argon prior to use and all the steps for preparation of incubation mixtures containing the enzymes were at 0–5°C. The volumes of enzyme solution used during the following incubations are related back to the volumes produced from known concentrations of suspended cells (see *Lysis of cells*). In this way, the directions hold good for any amount of cells used at the outset. The enzymic extracts obtained from *E. coli* AN1459/pSD38 (1 part) and *E. coli* MC1060/pSD37 (1 part) were thawed on ice and diluted twofold to the final concentration of incubation buffer containing precorrin-3A or TMIBC (5 μg ml^{-1}). When the substrate was TMIBC, a cell-free extract of G3575/pXL229 [20] was added to provide the reductase enzyme [23] which regenerates precorrin-3A. The mixture

was divided into volumes of 12 ml in conical screw cap tubes (17 mm × 120 mm) and incubated still and horizontally at 30°C in a constant temperature oven in the dark for 5 h. The tubes were not flushed with argon prior to sealing with the screw cap which ensured that air comprised ~20% of the total volume in each incubation vessel. The mixture was then cooled on ice, acidified with 2N HCl to pH 4.0, flushed with argon and the precipitated protein was removed by centrifugation (40,000 × g, 10 min, 4°C). The supernatant was passed through a LiChroprep column (2.0 × 0.5 cm) which had been previously washed with methanol and then water. The column was washed with water and pigments were eluted with 50% acetonitrile in water ready for separation by HPLC (PhaseSep Nucleosil 5ODS) using a gradient of 1% to 25% acetonitrile in aqueous KH_2PO_4/K_2HPO_4 buffer (100 mM, pH 7.0) at a flow rate of 1 ml min^{-1}. Fractions containing factor IV were detected by UV (370 nm) using an authentic sample [14] as standard; yield 20%. TMIBC (15%) was also recovered together with its monolactone (11%). The enzymically synthesised factor IV was shown to be identical with the standard sample by chromatography, by UV-visible absorption and also by mass spectrometry on its octamethyl ester.

*Esterification of factor IV (**6a**)*

The foregoing solution of factor IV was concentrated by adsorption onto a LiChroprep column and elution with the minimum volume of 50% aqueous acetonitrile as before. Methanol (1 ml) was added to the eluant (1 ml) and an ethereal solution of diazomethane was added dropwise but quickly to the stirred solution until a slight excess persisted. After 15 s, the organic solvents were evaporated and the aqueous solution was lyophilised to afford factor IV octamethyl ester as a blue amorphous residue. For unlabelled factor IV octamethyl ester **6b** by electrospray mass spectroscopy MH^+ 1019.4538; $C_{52}H_{67}N_4O_{17}$ requires M+H 1019.4501. When the treatment with diazomethane was extended beyond 15 s, a peak for a second product appeared in the mass spectrum at MH^+ 1033.4748. This indicates that this material is a nonamethyl derivative of factor IV ($C_{53}H_{69}N_4O_{17}$ requires M+H 1033.4657; see the Results and discussion section).

^{14}C-labelling experiments on factor IV

[2,7,20-*methyl*-^{14}C]TMIBC were prepared as described for unlabelled material [6] on a scale such that the volume of buffer solution containing all the enzymes was 100 ml. To this was added ALA (11 mg), then [^{14}C-*methyl*]*S*-adenosyl-L-methionine (200 µl at 25 µCi ml^{-1} in aqueous sulphuric acid). After a few minutes, unlabelled *S*-adenosyl-L-methionine (14 mg) was added and the incubation was continued and worked up as described [6] to yield [2,7,20-*methyl*-^{14}C]TMIBC octamethyl ester (777 µg), specific activity 4.25×10^6 dpm μmol^{-1}. This product was diluted with unlabelled TMIBC ester to give material of specific activity 4.36×10^5 dpm μmol^{-1} which was hydrolysed as usual and the resultant TMIBC was enzymically converted into factor IV as described above. The total activity of this product then allowed calculation of its yield. From the accurate quantity of factor IV so measured, its molar extinction coefficient at 370 nm, the strongest absorption in water containing 1% trifluoroacetic acid, was determined as $3.79 \times 10^4 M^{-1} cm^{-1}$.

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