Genetically engineered synthesis of precorrin-6x and the complete corrinoid, hydrogenobyrinic acid, an advanced precursor of vitamin B_{12}

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Background: Genetically engineered synthesis, in which the gene products, cofactors, and substrates of a complete pathway are combined *in vitro* in a single flask to give the target, can be a viable alternative to conventional chemical construction of molecules of complex structure and stereochemistry. We chose to attempt to synthesize the metal-free corrinoid hydrogenobyrinic acid, an advanced precursor of vitamin B_{12}.

Results: Cloning and overexpression of the genes necessary for the *S*-adenosyl methionine dependent conversion of 5-aminolevulinic acid (ALA) to precorrin-3 and those required for the synthesis of hydrogenobyrinic acid from precorrin-3 completed the repertoire of the 12 biosynthetic enzymes involved in corrin synthesis. Using these enzymes and the necessary cofactors, the multi-enzyme synthesis of hydrogenobyrinic acid from ALA can be achieved in 20 % overall yield in a single reaction vessel, corresponding to an average of at least 90 % conversion for each of the 17 steps involved.

Conclusions: By replacing the cell wall with glass, and by mixing the soluble biosynthetic enzymes and necessary cofactors, the major segment of the physiological synthesis of vitamin B_{12} has been accomplished. Since only those enzymes necessary for the synthesis of hydrogenobyrinic acid from ALA are supplied, none of the intermediates is deflected from the direct pathway. This results in an efficiency which in fact surpasses that of nature.

Chemistry & Biology October 1994, **1**:119–124

Key words: Genetic engineering, hydrogenobyrinic acid, multi-enzyme synthesis, vitamin B_{12}

Introduction

As so many of the prime targets of contemporary synthetic efforts are the products of the biochemical machinery of plant, fungal, bacterial and even human metabolism, it is not without interest to contrast the rapid evolution of synthetic organic chemistry over the last 150 years (especially in the last two decades) [1] with that of nature's own strategy for natural product synthesis, which has taken some four billion years to evolve. We suggest that genetically engineered synthesis, in which the over-expressed gene products, cofactors, and substrates of a complete pathway [2] are recombined *in vitro* in a single flask to reach the target, not only expands the horizon of the application of enzymes in synthesis [3] but offers a viable alternative to conventional chemical construction of molecules of complex structure and stereochemistry. It is a fortunate consequence of the advances in the molecular biology of prokaryotic and eukaryotic organisms that the genes encoding the enzymes for natural product synthesis are now becoming accessible through the construction of genomic libraries for bacteria and cDNA banks for higher plants [4,5]. The catalytic machinery necessary for the total, enzymatic synthesis of nature's 100 000 known structures from methane to vitamin B_{12} (and probably a much greater number of as yet undiscovered compounds) is therefore becoming available, waiting for the bio-organic chemist to exploit the full power of this exquisitely designed synthetic chemistry.

In the aerobic organism *Pseudomonas denitrificans*, the biosynthetic pathway to vitamin B_{12} downstream from hydrogenobyrinic acid (HBA; Fig. 1), the first true corrinoid intermediate, has been completely elucidated at the enzyme level [6]. Cobalt is inserted into the macrocycle by a cobaltochelatase, which uses the *a,c*-diamide of hydrogenobyrinic acid as substrate [7], while the enzymes which catalyze the subsequent amidations (at *b*, *d*, *e* and *g*), addition of the 'nucleotide loop' (at *f*; see Fig. 1) and formation of the coenzyme form of vitamin B_{12} have been isolated from recombinant strains of *P. denitrificans* [6]. Also, much of the aerobic pathway upstream from hydrogenobyrinic acid has been uncovered [8–11] by expressing and identifying the functions of the eight biosynthetic gene products (enzymes 5–12; Fig. 1) required for the conversion of precorrin-2 to hydrogenobyrinic acid in *P. denitrificans*. Together with the first three enzymes of the siroheme pathway [8,12,13] from *Escherichia coli* (enzymes 1–3), these provide the entire repertoire of natural catalysts for the synthesis of the corrin structure from 5-aminolevulinic

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 ISSN 1074–5521

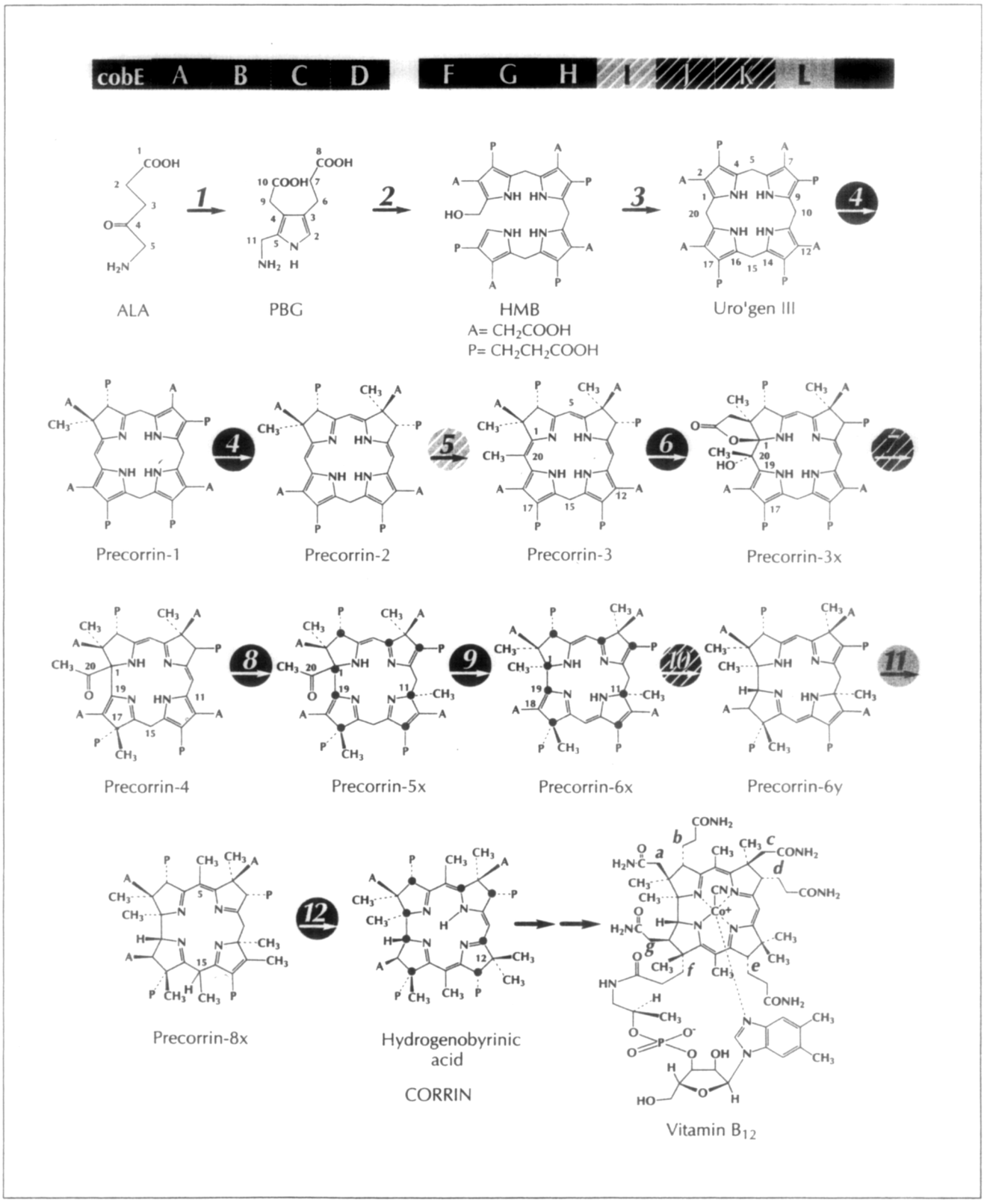

Fig. 1. The biosynthetic pathway from ALA to the corrins, hydrogenobyrinic acid (HBA) and vitamin B_{12}. **(a)** Organization of the *Pseudomonas* genes required for HBA synthesis. **(b)** The biosynthetic pathway for vitamin B_{12}, as reconstructed *in vitro* using the enzymes catalyzing reactions 1–12. The enzymes and the genes encoding them are: 1. ALA dehydratase (*hemB*), 2. PBG deaminase (*hemC*), 3. Uro'gen III synthase (*hemD*), 4. Precorrin-2 synthase (Uro'gen III methylase; *cobA*), 5. Precorrin-3 synthase (*cobI*), 6. Precorrin-3x synthase (*cobG*), 7. Precorrin-4 synthase (C-17 methylase/ring contractase; *cobJ*), 8. Precorrin-5 synthase (*cobM*), 9. Precorrin-6x synthase (deacylase/C-1 methylase; *cobF*), 10. Precorrin-6x reductase (*cobK*), 11. Precorrin-8x synthase (C-5,15 bismethylase and decarboxylase; *cobL*), 12. Precorrin-8x mutase (catalyzes the 1,5 sigmatropic shift of the methyl group from C-11 to C-12; *cobH*). The solid red circles show the labeling pattern derived from [4-^{13}C]-ALA for the three intermediates whose ^{13}C NMR spectra are shown in Figs 2 and 3.

acid (ALA). Particularly intriguing is the central section of this route from precorrin-3 to precorrin-6x, mediated by four enzymes (enzymes 6–9; Fig. 1) featuring O_2-dependent C-20 hydroxylation (CobG) [14] and three *S*-adenosyl methionine- (SAM-) dependent C-methylations (C-17, CobJ; C-11, CobM; C-1, CobF). The enzyme that performs the first of these methylations, CobJ, is also involved in contracting the ring of the macrocycle, while the remaining two modify the stereo-electronic array of the tetrapyrrolic template (via methyl group insertion) towards the final corrinoid structure necessary for coenzyme function.

Our earlier demonstration [15] of the multi-enzyme synthesis of precorrin-3 (enzymes 1–5, 6 steps) from ALA provided the basis for further reconstitution of the pathway, and the initial discovery of C-11 methylase activity [16] provided the first clue about the function of CobM (enzyme 8). This discovery was followed by the isolation of factor IV from *cobM* mutants of *P. denitrificans* [11,17] and the isolation and characterization of the actual precorrin-4 following its synthesis from precorrin-3 with the enzymes CobG and CobJ [9]. When CobM was included in the incubation [10], the product, precorrin-5x, could be isolated in quantitative yield based on ALA, reflecting complete conversion at each of the 11 steps of the 8-enzyme synthesis. With the genetic engineering of the remaining four enzymes completed [8], we now report on the successful conversion of ALA to precorrin-6x and, finally, to hydrogenobyrinic acid, using an ensemble of the 12 enzymes in a single flask.

Results and discussion

Synthesis of precorrin-6x

We have previously described [10] the synthesis, isolation, and NMR characterization of precorrin-5x methyl ester from an incubation containing ^{13}C-labeled precorrin-3, SAM, NADH, CobG, CobJ, and CobM. Addition of CobF to the complete precorrin-5 synthesizing system resulted in the appearance in the ^{13}C-NMR spectrum of not only the signals associated with precorrin-5x but also a new set of signals (Fig. 2) which correspond to those previously reported for precorrin-6x [18]. Attempts to convert isolated precorrin-5 to precorrin-6x in incubations containing only CobF were unsuccessful, however, suggesting that precorrin-5 is not the initial product of CobM, but rather an inactive form of precorrin-5 derived during the preparation of the sample for NMR analysis. As can be seen from the relative heights of the signals in Fig. 2, only about 50 % of the precorrin-5 was converted to precorrin-6x. We ascribe the appearance of two sets of closely spaced resonances in the spectrum of precorrin-5x and -6x to the presence of two species, epimeric at C-3, as observed in the cases of factor IV [17] and precorrin-8x [19], where

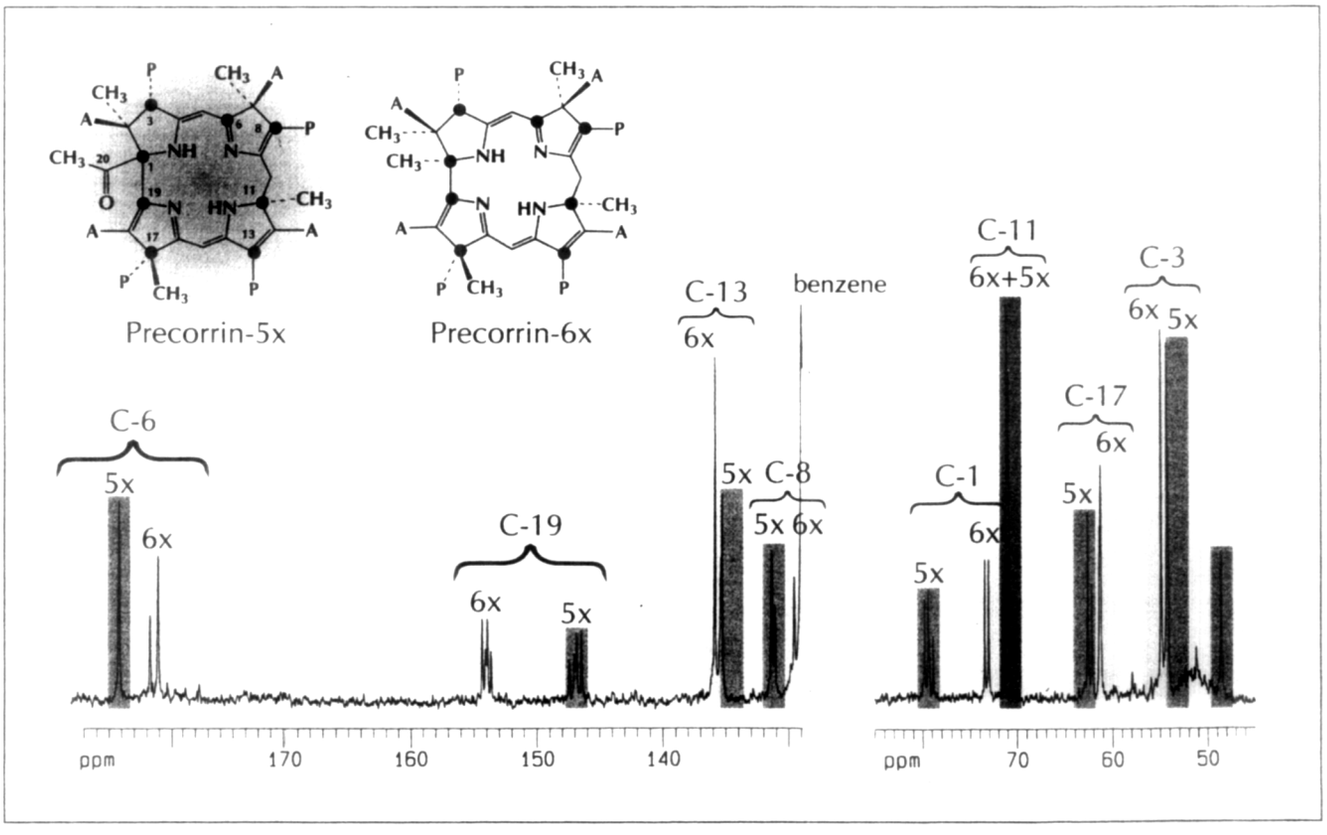

Fig. 2. Precorrin-6x can be generated *in vitro* by adding CobF to the enzymes that synthesize precorrin-5x. Top, structures of precorrin-5x and precorrin-6x methyl esters; atoms labeled with ^{13}C are marked in red. Bottom, the ^{13}C NMR spectrum of the products from an incubation of [4-^{13}C]-ALA-derived precorrin-3 with CobG, CobJ, CobM and CobF (enzymes 6–9 in Fig. 1) is shown. Both precorrin-5x and precorrin-6x are produced. NMR peaks are color-coded to the appropriate product.

multiple tautomeric and/or epimeric forms have been isolated. It is currently not known why the conversion of precorrin-5 to precorrin-6x is less efficient than the other reactions. The amount of CobF in the lysate is equal to or exceeds the amount used for each of the other methylases, but no studies on the relative stabilities of the enzymes have yet been done. It is not likely that there is a requirement for a small co-factor, other than the NADH or ATP provided in the reaction, as the synthesis of precorrin-6x can occur in a lysate derived from *P. denitrificans* that has been depleted of all small molecules by gel filtration and then supplemented with these two cofactors [20]. The low conversion rate of precorrin-5x to precorrin-6x may indeed be due to *in vitro* formation of a more stable but biochemically inert tautomer of precorrin-5, as in the case of precorrin-8x where only one of five known tautomers is a fully competent substrate for the next enzyme, CobH, the remaining tautomers showing low or no conversion.

Single-flask synthesis of hydrogenobyrinic acid

The nine enzymes required for the biosynthesis of precorrin-6x, and the three gene products responsible for the conversion of precorrin-6x to hydrogenobyrinic acid, CobK, CobL, and CobH ([21–23]; enzymes 10, 11 and 12, Fig. 1), were incubated aerobically for 15 h at 30 °C in a single flask containing ^{13}C-ALA, SAM, NADH and NADPH. The ^{13}C-NMR analysis revealed the presence of two products: precorrin-5x, which was found free in solution; and an enzyme-bound product, which was released by heat denaturation of the protein. The ^{1}H and ^{13}C-NMR data (D_2O) for the released product (derived from either [4-^{13}C] or [5-^{13}C] ALA) were fully consistent with literature values for hydrogenobyrinic acid [24,25]

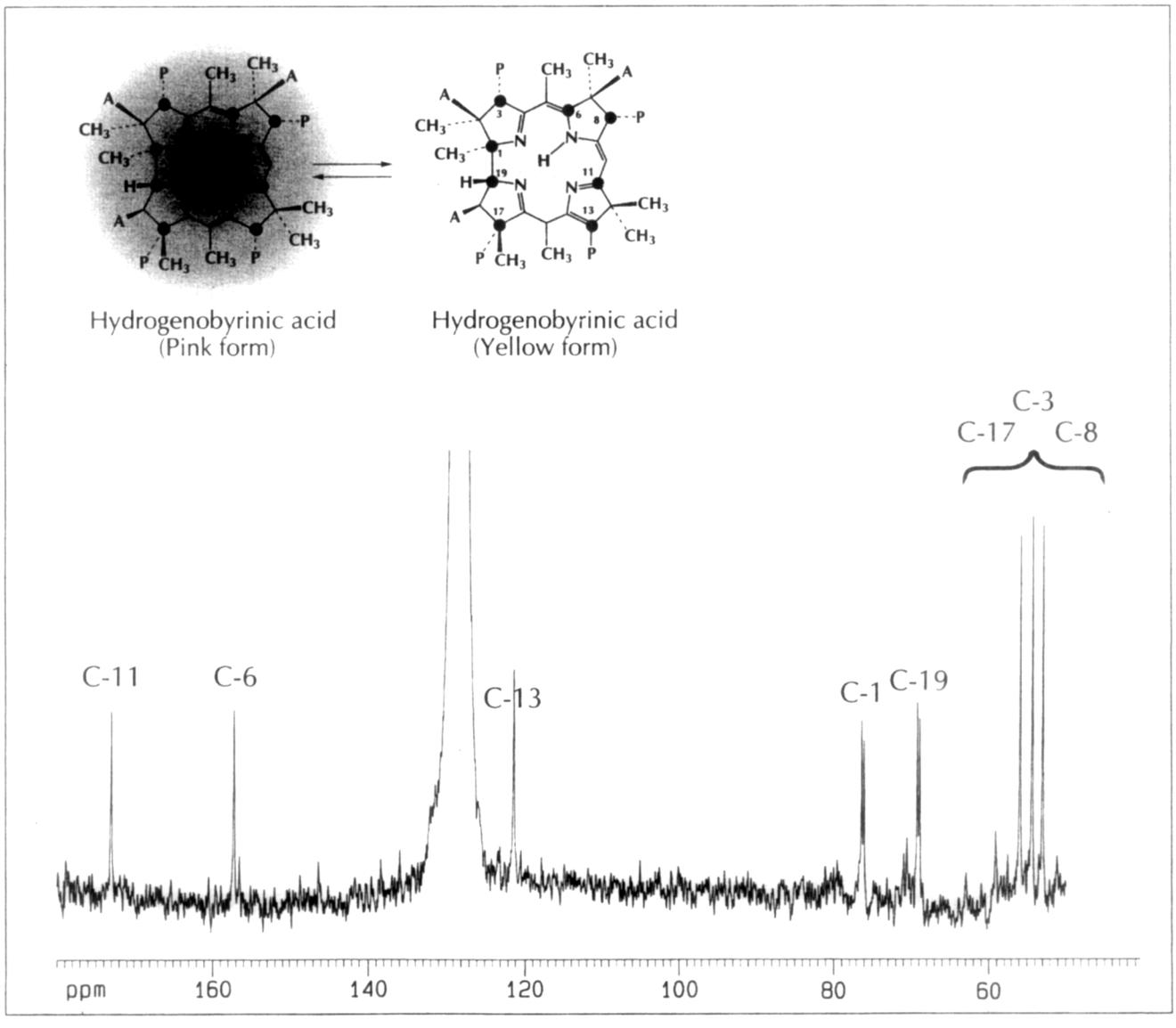

Fig. 3. Hydrogenobyrinic acid can be produced in a single flask using a total of 12 enzymes. Top, structures of two tautomers of hydrogenobyrinic acid, the one initially described [25] (pink shading) which can convert into a tautomer with a yellow chromophore (yellow shading). Atoms labeled with ^{13}C are marked in red. Bottom, the ^{13}C-NMR spectrum of the yellow form of the esterified hydrogenobyrinic acid isolated from a single flask incubation of [4-^{13}C]-ALA with all 12 enzymes shown in Fig. 1. This spectrum is fully consistent with the yellow form of hydrogenobyrinic acid being a tautomer of the pink form that was previously characterized [25].

a d revealed almost no other porphyrinoid impurities. T ie isolated hydrogenobyrinic acid tautomerized from tl e structure originally reported [25] (Fig. 3, pink form) t a compound with a yellow chromophore, the structure o which was established from the NMR spectrum of the c ompound derived from [4-^{13}C]-ALA (Fig. 3, bottom).

T he heptamethyl ester of a specimen derived by incorpo- r ition of seven ^{13}C-enriched carbons from 5 mg of [5-^{13}C]-ALA analyzed by fast atom bombardment–mass s pectrometry (FAB-MS) ($^{12}C_{45}{}^{13}C_7H_{74}N_4O_{14}$ 986.91 M+1); UV-Vis ($CHCl_3$): λ_{max} 270 (rel. int. 0.80), 319 sh ( ).82), 330 (1.00), 500 (0.48), 527 (0.52); CD ($CHCl_3$): λ 330 (rel. int. -1.00), 500 (-0.45), 527 (-0.47) nm, was i dentical in every respect to natural hydrogenobyrinic acid h eptamethyl ester [24] and corresponded to a yield of ~1 mg (~20 % yield based on the starting material, ALA).

As lysates of the *E. coli* cells harboring the overexpressed genes are used, rather than the purified enzymes, the conversion percentages are not yet optimized. The least efficient single step at present is the conversion of precorrin-5 to precorrin-6x (enzyme 9; 50 % yield), although this bottleneck is somewhat alleviated by coupling to the successive three enzymes.

Significance

We have described a procedure that combines three enzymes native to *E. coli* with nine enzymes from another organism (*P. denitrificans*) to allow the biosynthesis of a highly complex natural product from its simple five-carbon precursor. At first sight, it seems remarkable that as many as 12 biosynthetic enzymes can act in concert to follow the pathway in the same sequence as that found *in vivo*. It might have been predicted that product/substrate inhibition, binding to protein, or unfavorable kinetics would have militated against the (almost) perfect orchestration of 17 non-repetitive sequential reactions, involving regio- and stereo-selective C-methylations, oxidation, reduction, ring contraction, decarboxylation and finally [1,5]-sigmatropic rearrangement to reach the target corrinoid, but this is clearly not the case. Since each step can also be carried out as a single reaction catalyzed by the appropriate enzyme, it is clear that heterologous dimerization or other protein-protein interactions of the enzymes are not required for bioconversion.

The one-flask synthesis of the complete corrinoid structure, lacking only the peripheral amide functions of vitamin B_{12}, involves 12 enzymes in the creation of 9 stereocenters. The practical realization of this goal gives us confidence that the development of genetically engineered syntheses of equally challenging natural product structures will also be practical. The multi-enzyme synthesis of hydrogenobyrinic acid from ALA also completes the formal chemo-enzymatic synthesis of vitamin B_{12} itself, as hydrogenobyrinic acid can be converted to cyanocobalamin in a two-step process involving insertion of cobalt [24,25] and self assembly of the nucleotide loop specifically linked to the C-17 propionate side chain [26].

Materials and methods

Overexpression of the enzymes for the synthesis of hydrogenobyrinic acid from ALA

Overexpression of the 12 enzymes required to convert ALA to hydrogenobyrinic acid was performed by subcloning of the genes encoding the enzymes into plasmid expression vectors and transformation into strains of *E. coli* as previously described [8].

Synthesis of precorrin-3, precorrin-5x and precorrin-6x

Precorrin-3 was synthesized as described previously [8,15] in a reaction mixture containing SAM, ALA, ALA dehydratase, PBG deaminase, uro'gen III synthase, and cell lysates containing uro'gen III methyltransferase (CobA) and precorrin-2 methyltransferase (CobI). Precorrin-5x was synthesized in a reaction mixture containing SAM, NADH, precorrin-3, and cell lysates containing CobG, CobJ, and CobM as previously described [8,10]. For the synthesis of precorrin-6x, the reaction was also supplemented with a cell lysate containing CobF. The precorrin-6x was purified and analyzed as described previously [10].

Single-flask synthesis of hydrogenobyrinic acid

For synthesis of HBA, ALA (5 mg) was incubated aerobically in 200 ml Tris-HCl buffer, pH 8.0, containing SAM (80 mg), NADH (40 mg), NADPH (40 mg) for 15 h at 30 °C with a mixture of twelve enzymes: 1. ALA dehydratase (10 mg), 2. PBG deaminase (2 mg), 3. Uro'gen III synthase (2 mg), 4. Precorrin-2 synthase (Uro'gen III methylase; CobA), 5. Precorrin-3 synthase (CobI), 6. Precorrin-3x synthase (CobG), 7. Precorrin-4 synthase (C-17 methylase/ring contractase; CobJ), 8. Precorrin-5 synthase (CobM), 9. Precorrin-6x synthase (deacylase/C-1 methylase; CobF), 10. Precorrin-6x reductase (CobK), 11. Precorrin-8x synthase (C-5,15 bismethylase and decarboxylase; CobL), 12. Precorrin-8x mutase (catalyzes the 1,5 sigmatropic shift of the methyl group from C-11 to C-12; CobH). Enzymes 1–3 were purified from E. coli and enzymes 4–12 were obtained as lysates from 1–2 l (5–10 g of cells, wet weight) each of *E. coli* harboring plasmids bearing the appropriate *cob* gene [8].

The soluble products were adsorbed onto DEAE-Sephadex, then the enzyme-bound material released by heat denaturation of the protein (70 °C; 10 min), followed by centrifugation, adsorption of the products onto DEAE-Sephadex, elution with 10 % acetic acid and lyophilization. The resultant HBA was quantitated by spectroscopy [25] and convèrted to the heptamethyl ester (H_2SO_4-MeOH), which was purified by preparative reverse phase TLC (Rf = 0.6 (MeOH/H_2O, 9/1); Whatman KC18F).

Acknowledgements: This work was supported by grants from the National Institutes of Health and the Robert A. Welch Foundation. J.B.S. was a SERC Senior Fellow. We thank Professor G. Müller (Stuttgart) for a generous gift of hydrogenobyrinic acid and Sandra Estrada for technical support.

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Received: **26 Aug 1994**. Accepted: **6 September 1994**.