Achieving natural product synthesis and diversity via catalytic networking *ex vivo*

Charles A Roessner and A Ian Scott

Recent studies on *ex vivo* synthesis of natural products reveal that even complex multistep pathways can be successfully reconstructed. Genetic engineering of such reconstituted pathways has already been used to generate 'unnatural' natural products related to the original compound. In the future, it may be possible to use these approaches to make natural products that are currently inaccessible to conventional synthesis.

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

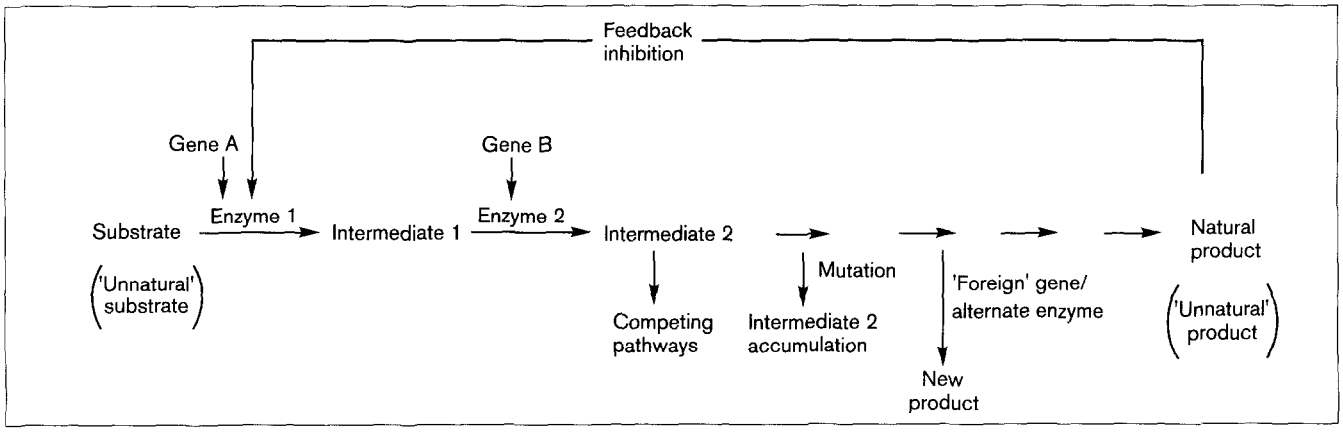
Chemistry & Biology May 1996, **3**:325–330

 ISSN 1074-5521

Natural product biosynthesis is a multi-step process often requiring as many as 15–20 gene products for the synthesis of a complex structure. Genetic engineering can function as a tool to increase production by removal of rigid branch points in the metabolic network, to overcome product inhibition, to identify intermediates by introduction of mutations or to synthesize new products by introduction of 'unnatural' substrates or 'foreign' genes from different organisms (Fig 1). There are many examples in the literature of whole-organism engineering, especially of prokaryotes. Some of the best examples come from the engineering of *Streptomyces* species to define the polyketide biosynthetic pathways and generate novel polyketides (reviewed in [1]). Whole-organism engineering can, however, be hampered by the difficulty of engineering higher eukaryotic organisms, and various properties of cells. An inability to take up some unnatural substrates can limit the substrates it is possible to use, and the cytoplasmic environment constrains reaction conditions (pH, temperature, etc.). Parts of the pathways may be shared by major metabolic pathways which, *in vivo*, compete for substrates but cannot be removed by mutation. Furthermore, using whole organisms does not reveal information about the mechanisms of the individual biosynthetic enzymes and requires no knowledge of the discrete intermediates bound or released from them. By contrast, *in vitro* multienzyme synthesis, while requiring the isolation and characterization of the full set of biosynthetic enzymes, is not beset by problems of metabolic trafficking or growth conditions and has the added benefit of allowing one to identify and manipulate the entire repertoire of biosynthetic intermediates and the enzymes that connect them to the natural product.

The strategy chosen for *in vitro* syntheses must depend on the nature of the organism from which the metabolic route to the natural product is derived. Prokaryotes often have lengthy operons encoding the enzymes for up to 20 steps of a pathway. Thus selection of mutants and complementation studies with a genomic library provides a logical approach, yielding a small number of clones that can be subcloned and

Figure 1



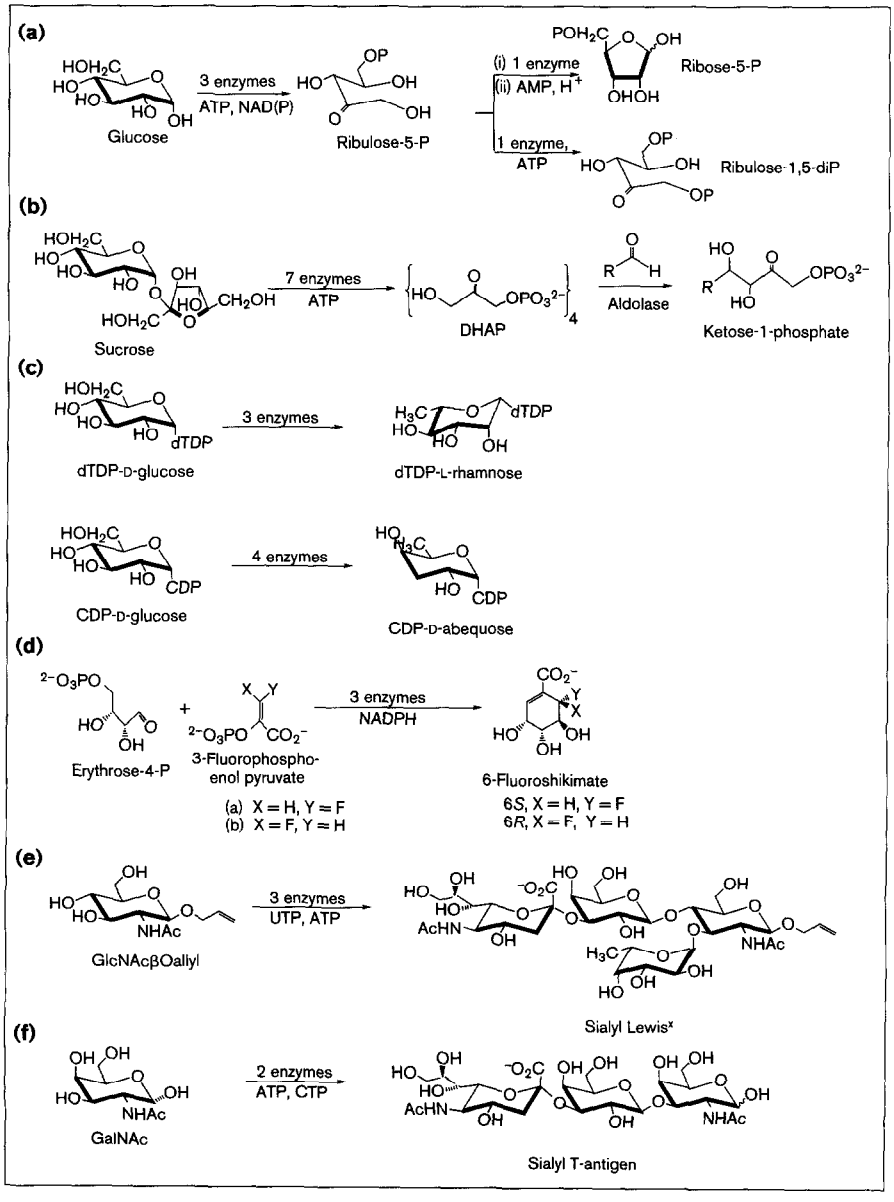
Natural product biosynthesis pathways can be engineered to enhance production or product diversity. Unnatural products may be obtained by supplying unnatural substrates. Limitations *in vivo* (such as cell toxicity, lack of substrate uptake, etc) may be overcome *in vitro*. Competing pathways can be overcome *in vitro* by using purified enzymes or a large excess of the desired enzymes. The accumulation of intermediates can be induced by inactivating the enzyme that normally uses the intermediate as a substrate, either by mutating it *in vivo* or omitting it *in vitro*. New products may be produced by introduction of genes from heterologous organisms *in vivo* or by addition of the corresponding enzymes *in vitro*, which may also reduce problems of expression, toxicity and unfavorable environmental conditions. Product inhibition may be overcome by replacing regulatory enzymes with isozymes, either naturally occurring or genetically altered, that do not exhibit feedback inhibition.

expressed to produce the complete repertoire of biosynthetic enzymes. In eukaryotes, however, the biosynthetic genes are seldom (if ever) clustered, so a cDNA library must be constructed and probed with oligonucleotides or antibodies corresponding to the proteins of interest. As these are lengthy procedures, this is an area that is ripe for development, and the advent of differential display or 'subtraction' libraries [2] is expected to have a profound impact. Whichever approach is used, the choice of expression vector is of paramount importance, and if the multienzyme synthesis requires expensive cofactors such as nucleoside triphosphates, NAD(P), S-adenosylmethionine (SAM) or 3'-phosphoadenosine-5'-phosphosulfate (PAPS), these can be regenerated [3] with a coupled enzyme system.

Multienzyme carbohydrate synthesis

The concepts of reconstituting a pathway or mixing enzymes from interlinked pathways in a single reactor was made a reality by the early work of Whitesides and Wong (reviewed in [3]) who used commercially available enzymes involved in the metabolism of monosaccharides to synthesize ribose-5-phosphate and ribulose-1,5-bisphosphate from glucose (Fig. 2a) [4]. A similar concept was more recently employed to convert sucrose to the versatile substrate dihydroxy acetone phosphate (DHAP), which was then further processed in the same reaction vessel by the addition of fructose-1,6-diphosphate aldolase in the presence of several aldehyde substrates to produce a variety of unusual branched-chain sugars (ketose-1-phosphates, Fig. 2b), a

Figure 2



Multienzyme synthesis of carbohydrates. A star indicates enzymes produced by genetic engineering. **(a)** Synthesis of ribose and ribulose-1,6-diphosphate with hexokinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphoribose isomerase and phosphoribulokinase. **(b)** The multienzyme one-flask synthesis of ketose-1-phosphates with invertase, xylose isomerase, hexokinase, glucose-6-phosphate isomerase, fructose-6-phosphate kinase*, fructose-1,6-biphosphate aldolase and triose-phosphate isomerase. **(c)** Synthesis of dTDP-L-rhamnose from dTDP-D-glucose with dTDP-D-glucose-4,6-dehydratase*, dTDP-4-keto-rhamnose-3,5-epimerase* and dTDP-6-deoxy-*L-lyxo*-hexulose-4-reductase*. Synthesis of abequose from CDP-D-glucose with CDP-glucose-4,5-dehydratase*, CDP-6-deoxy-*D-xylo*-4-hexulose-3-dehydrase*, CDP-6-deoxy-Δ3,4-glucoseen reductase* and CDP-3,6-dideoxy-*D-xylo*-4-hexulose-4-reductase*. **(d)** Synthesis of (6*R*)- and (6*S*)-fluoroshikimic acids from erythrose-4-phosphate and 3-fluorophosphoenol pyruvate with DHAP synthase*, dehydroquinate synthase, dehydroquinase and shikimate dehydrogenase. **(e)** Synthesis of sialyl-Lewis^x from GlcNAcβOallyl with β-1,4-galactosyltransferase, α-2,3-sialyltransferase* and α-1,3-fucosyltransferase*. **(f)** Synthesis of sialyl T-antigen from N-acetylgalactosamine with β-galactosidase and α-2,3-sialyltransferase*.

process which Fessner and Walter [5] termed 'artificial metabolism' as the routes evolved are not those of the normal glycolytic pathway. The yields were increased by replacing the rate-limiting enzyme with a recombinant isozyme with more favorable kinetics. Another synthesis (Fig. 2c) involves the monosaccharides found in the O-antigenic polysaccharide chains of *Salmonella enterica* (which may be used in vaccine production). Both rhamnose and abequose have been produced using cell-free extracts of *Escherichia coli* strains expressing the *S. enterica rbf* genes [6,7]. In another example, Duggan *et al.* [8] supplied erythrose-4-phosphate and the unnatural substrate 3-fluoro phosphoenolpyruvate to enzymes of the shikimate biosynthetic pathway to produce equal amounts of the 'unnatural' (*R*)- and (*S*)-6-fluoro analogs of shikimic acid (Fig. 2d), potentially useful as antibiotics.

Cell-surface oligosaccharides are highly diversified in structure and are important as antigenic determinants and for many cell functions such as receptor binding. Recent developments pioneered by C-H. Wong have resulted in the multienzyme synthesis of several of these complex oligosaccharides. Representative of Wong's work is the synthesis of the tetrasaccharide sialyl Lewis^x (Fig. 2e), a candidate for the treatment of reperfusion tissue injury, which can now be produced on the kilogram scale using recombinant enzymes and sugar nucleotide regeneration [9]. Along the same lines, Kren and Thiem [10] have described the single-pot preparation of the sialylated trisaccharide epitope of the T-antigen (Fig. 2f), which is expressed on the surface of several cell types, including epithelial cancer cells, and is thus important in immunological studies and for the synthesis of antitumor vaccines. In all of the examples above, important cofactors were regenerated with enzymatic systems at the steps (shown in Fig. 2) requiring cofactors.

Enzymatic synthesis of bacterial polyketides

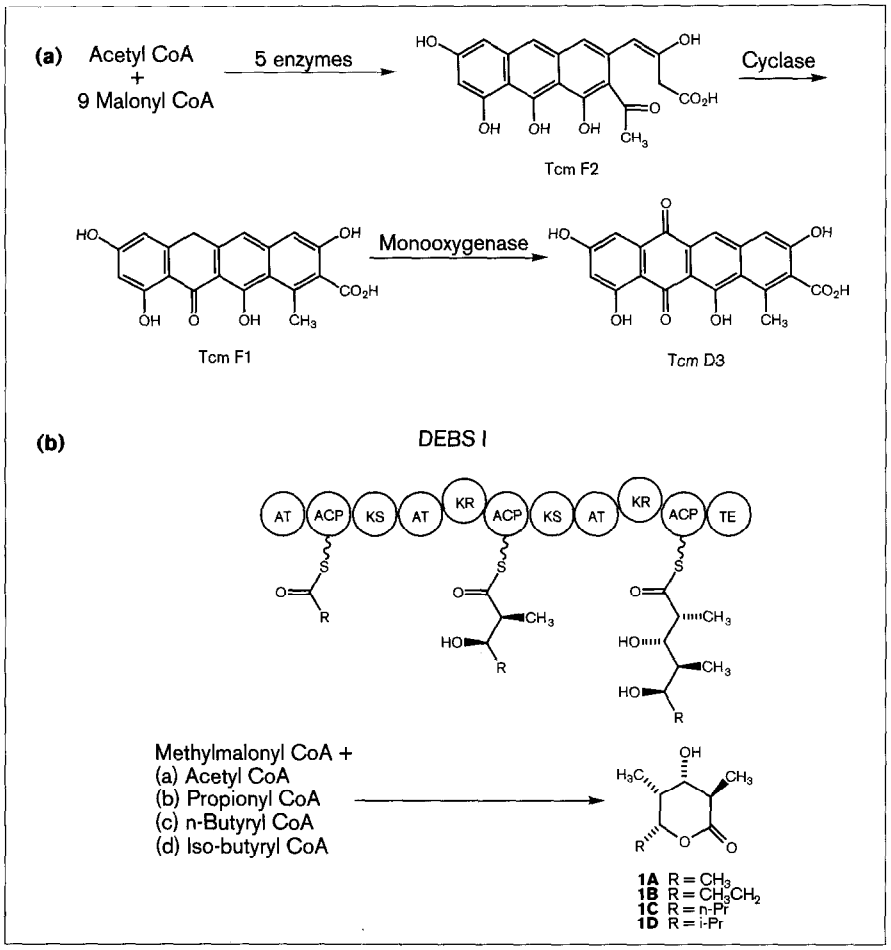
The polyketide family of natural products includes a number of important antibiotics, and their modular nature makes them particularly amenable to *in vitro* biosynthesis. Complex polyketides are assembled by a process akin to the synthesis of fatty acids, using acetyl-, propionyl- and butyryl-CoA as starter units, which are attached to large, multifunctional polyketide synthases (PKSs) by a thioester linkage. The elongation process involves a decarboxylative condensation usually with malonyl- or methylmalonyl-CoA. After each condensation, the growing polyketide chain can be tailored by reductive and subsequent dehydrative processes. Finally, the thioester is hydrolyzed and the released polyketide cyclized either by macro-lactone formation or by aldol/Claisen chemistry to yield an aromatic polyketide. These pathways have been analyzed by introducing genes for the PKSs and the subsequent reduction and cyclization steps into *Streptomyces* spp. There are two notable examples that represent a combination of whole-organism engineering and *in vitro* synthesis. In the first, Shen and Hutchinson [11] constructed a strain of *Streptomyces glaucescens* that overexpressed the tetracenomycin *TcmK,L,M* and *N* genes. A cell-free lysate derived from this strain produced tetracenomycin F2 (Tcm F2) from acetyl CoA and malonyl CoA (Fig. 3a). Addition of *TcmJ* to the other four genes greatly increased the yield of Tcm F2 *in vitro*. Tcm F1 was synthesized after addition of the purified Tcm F2 cyclase to the cell-free five-enzyme system, and addition of the necessary oxidase completed the system to yield Tcm D3 (Fig. 3a). This work represents the first example of *in vitro* multienzyme synthesis of aromatic polyketides.

In the second example [12], a strain of *Saccharopolyspora erythraea* was engineered to contain an altered 6-deoxyerythronolide B synthase (DEBS) gene resulting in the *in vitro* production of novel triketide lactones. DEBS, which synthesizes the aglycone core of erythromycin A, contains 30 active sites distributed among three large multienzymes, DEBS 1, DEBS 2 and DEBS 3 (molecular mass ~300 kDa each). The study of one of these domains *in vitro* was made possible by the relocation of the thioesterase/cyclase (TE, responsible for release and/or cyclization of the polyketide chain) from the end of the third multienzyme, DEBS 3, to the carboxy terminus of DEBS 1 (Fig. 3b). In a cell-free lysate derived from this strain, the lactone **1B** (Fig. 3b) was synthesized from propionyl CoA and methylmalonyl CoA. This represents the activity of the first part of the elongation process, which stops halfway because of the absence of DEBS 2 and 3. This elegant piece of engineering allowed the first cell-free synthesis of a lactonic triketide, a compound not normally found in cultures that produce erythromycin (a heptaketide). It was also found that there is a relaxed specificity for the starter unit; the system can tolerate acetyl-, propionyl-, and n-butyryl-CoA as substrates resulting in structures **1A**, **1C** and **1D**, respectively (Fig. 3).

Porphyrins and corrins

Another excellent example of how reconstitution of a multienzyme system has been exploited in the analysis of a complex pathway is illustrated by the system developed for the analysis of porphyrin and corrin synthesis (Fig. 4), in which of all of the *in vitro* variations outlined in Fig. 1 are represented. Genes from three different organisms were overexpressed; *hem* genes and *cysG* from *E. coli*, *cbi* genes from *Salmonella typhimurium* LT2, and *cob* genes from a strain of *Pseudomonas denitrificans* that is used for the commercial production of vitamin B_{12}. Overproduction of the enzymes allowed functional and mechanistic studies on some of them [13–19] and immobilization of the first three enzymes of the pathway (Fig. 4, steps 1–3) allowed the design of a reusable system for the production of uro'gen III from δ-aminolevulinic acid (ALA) on the 100 mg scale in 83 % yield [20]. By limiting the reaction to only those enzymes required for the synthesis of a given

Figure 3

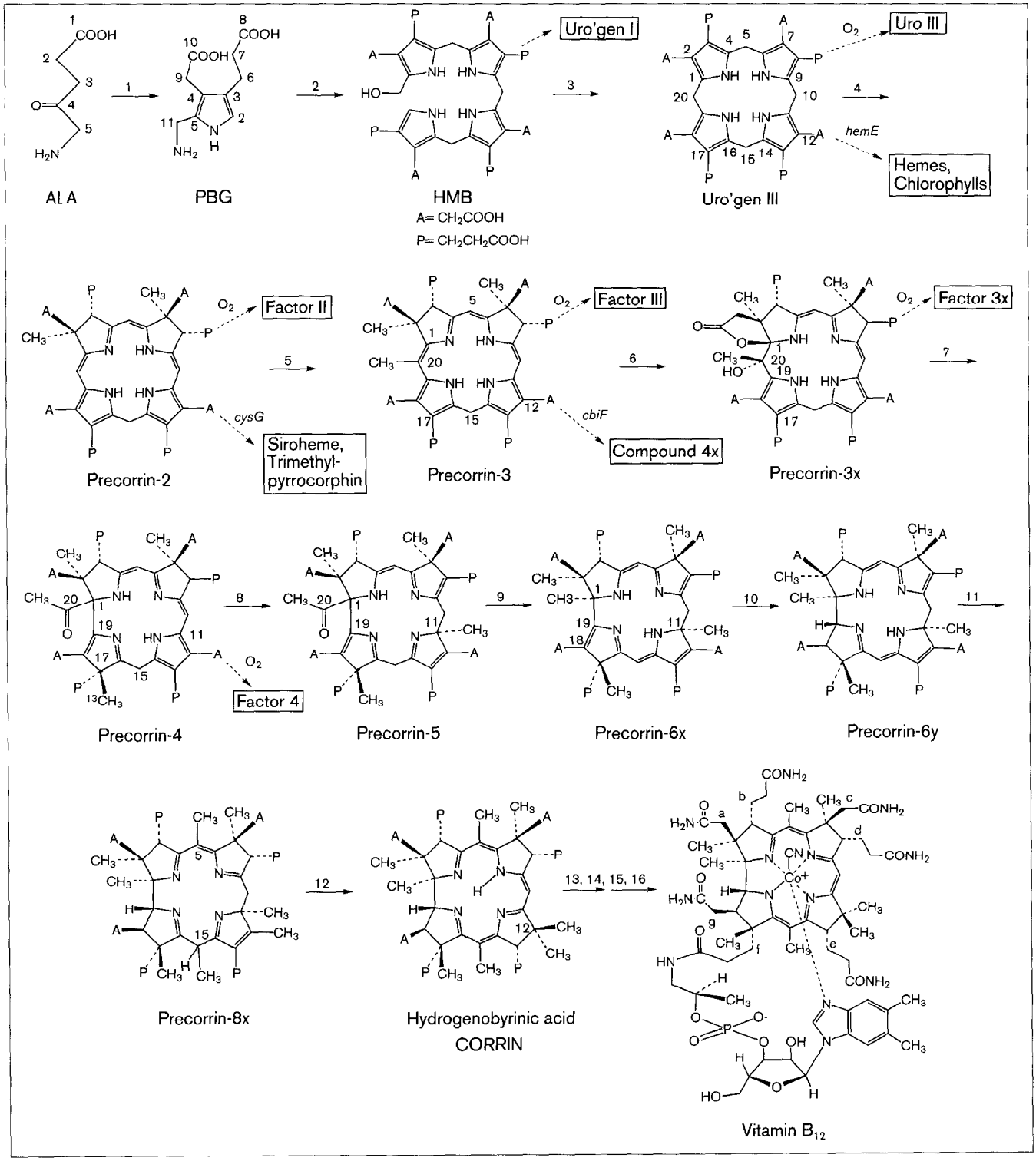


Polyketide biosynthesis. **(a)** The biosynthetic pathway from acetyl CoA and malonyl CoA to tetracenomycin in *Streptomyces glaucescens* showing steps catalyzed *in vitro* by TcmJKLMN, Tcm F2 cyclase and Tcm F1 monooxygenase to form Tcm D3. **(b)** Triketide lactones can be synthesized *in vitro* by an engineered triketide lactone synthase (DEBS1-TE). This includes an acyl carrier protein (ACP) domain (to which the growing polyketide is attached) and acyl transferase (AT), β-ketoacyl ACP synthase (KS) and β-ketoacyl reductases (KR) domains that synthesize the polyketide in a stepwise manner. The terminal thioesterase/cyclase (TE) domain releases the product from the synthase with cyclization. The different products, **1A–1D**, are produced by varying the starting substrate.

intermediate, precorrin-2, precorrin-3, precorrin-3x, precorrin-4 and precorrin-5 were synthesized and ^{13}C-labeled, and their structures fully characterized by NMR [21–24]. Several new 'unnatural' products were synthesized, for example, trimethylpyrrocorphin, the result of mismethylation (at C-12) of precorrin-2 by CysG [22], and compound 4x, resulting from mismethylation (at C-11) of precorrin 3 by the methylase encoded by *cbiF* [25]. These reactions were prevented in the synthesis of later intermediates by replacing CysG and CbiF with the homologous enzymes, CobA and CobM, which do not display the abnormal methylation properties. The synthesis of compound 4x led to the identification of the enzyme (encoded by *cbiF* or *cobM*) responsible for addition of the methyl group at C-11 of precorrin-4 to form precorrin-5 (step 8). The power of this multienzyme system was further demonstrated by the discovery that CobG (step 6) requires O_2 [26], an unexpected cofactor since many of the intermediates are sensitive to oxidation (Fig. 4).

The longest synthetic pathway successfully assembled in a single *ex vivo* reaction so far is our own reconstruction of the pathway yielding the corrin hydrogenobyrinic acid from the starting substrates ALA and SAM (reactions 1–12), using all 12 of the required enzymes that had been produced in *E. coli* by genetic engineering [27,28]. The overall yield (based on ALA) was 20 %, reflecting an average of at least 90 % conversion at each of 17 individual steps. Most crucial to a good yield was a high level of uro'gen III synthase activity to prevent the chemical formation of uro'gen I, and an excess of uro'gen III methyltransferase to prevent decarboxylation of uro'gen III (the first step of the heme pathway) by endogenous decarboxylase (encoded by the *hemE* gene). Thus a high yield of hydrogenobyrinic acid was made possible by supplying an excess of each desired enzyme obtained through genetic engineering to efficiently shunt each intermediate down the desired pathway and away from competing pathways. As the steps leading from hydrogenobyrinic acid to vitamin B_{12} (the chemical insertion of cobalt into hydrogenobyrinic acid to afford cobyrinic acid and the self assembly of the nucleotide loop (Fig. 4, steps 13–16)) have previously been demonstrated [29], the *in vitro* chemo-enzymatic synthesis of vitamin B_{12} is now complete.

Figure 4



The biosynthetic pathway from δ-aminolevulinic acid (ALA) to hydrogenobyrinic acid. The enzyme catalyzing each reaction and its corresponding gene are: (1) ALA dehydratase (*hemB*), (2) porphobilinogen (PBG) deaminase (*hemC*), (3) uroporphyringen III (uro'gen III) synthase (*hemD*), (4) uro'gen III methyltransferase (*cysG*, *cobA*), (5) precorrin 2 methyltransferase (*cobI*), (6) precorrin-3 oxidase (*cobG*), (7) precorrin-3 hydroxylactone methyltransferase (ring contraction; *cobJ*), (8) precorrin-4 methyltransferase (*cobM*, *cbiF*), (9) precorrin-5 methyltransferase (loss of acetic acid; *cobF*), (10) precorrin-6x reductase (*cobK*), (11) precorrin-6y decarboxylase and methyltransferase (*cobL*), (12) precorrin-8 methylmutase (*cobH*), (13) cobalt insertion, (14) esterification, (15) addition of nucleotide loop, (16) ammonolysis. Alternative pathways and observed derailment products are indicated in boxes.

In syntheses, such as the one described above, that require SAM as a methyl donor, enzymatic generation of SAM is desirable. Several problems have been encountered in attempts to synthesize SAM enzymatically, including product inhibition of the only enzyme available in large amounts, recombinant *E. coli* SAM synthetase. This problem limited accumulation of SAM to less than 1.0 mM, but has been overcome by production in *E. coli* of the homologous SAM synthetase (encoded by the *SAM2* gene) of *Saccharomyces cerevisiae*, which is not limited by product inhibition. The utility of this enzyme has been demonstrated by the successful supply of SAM *in situ* from ATP and methionine in the multienzyme synthesis of precorrin-2 (J. Park, J. Tai, C.A.R. and A.I.S., unpublished results).

Prognosis

It is now evident that the multienzyme synthesis of natural products has, within the last decade, passed from feasibility to practical reality [30,31]. The main problems still to be addressed include the development of rapid techniques for screening cDNA libraries to allow facile heterologous expression of plant enzymes, and the inclusion of a wider variety of enzyme activities (such as cytochrome P-450's and non-heme iron enzymes catalyzing hydroxylation) and reaction pathways involving coenzymes such as B_{12} and pyridoxal phosphate.

It now seems certain (although this could not have been foreseen) that there is virtually no limit to the number of enzymes that can be combined in a single reactor to produce a complex structure in good yield. What is truly remarkable is the lack of product/substrate inhibition, which is probably due to the irreversible nature of many of the later steps in a given sequence. It is our hope that the field will expand into the synthesis of natural products that are currently inaccessible by conventional chemical synthesis.

References

1. McDaniel, R., Ebert-Kosla, S., Hopwood, D.A. & Khosla, C. (1993). Engineered biosynthesis of novel polyketides. *Science* **262**, 1546–1550.
2. Sambrook, J., Fritsch, E.F. & Mariatis T. (1989). Preparation of radiolabeled DNA and RNA probes. In *Molecular Cloning: A Laboratory Manual* (2nd edn) (Nolan, C., ed.), pp. 10.38–10.50, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
3. Wong, C-H. & Whitesides, G.M. (1994). General Aspects. In *Enzymes in Synthetic Organic Chemistry.* (Baldwin, J.E. & Magnus, P.D., eds), pp. 19–22, Elsevier, New York.
4. Wong, C-H., McCurry, S.D. & Whitesides, G.M. (1980). Practical enzymatic syntheses of ribulose -1,5-bisphosphate and ribose 5-phosphate. *J. Am. Chem. Soc.* **102**, 7938–7939.
5. Fessner, W-D. & Walter, C. (1992). 'Artificial metabolisms' for the asymmetric one-pot synthesis of branched chain saccharides. *Angew. Chem. Int. Ed. Engl.* **31**, 614–616.
6. Marumo, K., Lindqvist, L., Verma, N., Weintraub, A., Reeves, P.R. & Lindberg, A. (1992). Enzymatic synthesis and isolation of thymidine diphosphate-6-deoxy-D-xylo-4-hexolose and thymidine diphosphate-L-rhamnose. *Eur. J. Biochem.* **204**, 539–545.
7. Lindqvist, L., Schweda, K.H., Reeves, P.R. & Lindberg A. (1994). *In vitro* synthesis of CDP-D-abequose using *Salmonella* enzymes of cloned *rfb* genes. *Eur. J. Biochem.* **225**, 863–872.
8. Duggan, P.J., Parker, E., Coggins, J. & Abell, C. (1995). Enzymatic synthesis of (6*R*)- and (6*S*)-fluoro shikimic acids. *Bioorg. Med. Chem. Lett.* **5**, 2347–2352.
9. Wong, C-H., Halcomb, R.L., Ichikawa, Y. & Kajimoto, T. (1995). Enzymes in organic synthesis: application to the problems of carbohydrate recognition (Part II) – Cloning and expression of glycosyl transferases and sugar nucleotide synthases. *Angew. Chem. Int. Ed. Engl.* **34**, 521–546.
10. Kren, V. & Thiem, J. (1995). A multienzyme system for a one-pot synthesis of sialyl T-antigen. *Angew. Chem. Int. Ed. Engl.* **34**, 893–895.
11. Shen, B. & Hutchinson, C.R. (1993). Enzymatic synthesis of a bacterial polyketide from acetyl and malonyl coenzyme A. *Science* **262**, 1535–1540.
12. Wiesmann, K.E.H., Cortés, J., Brown, M.J.B., Cutter, A.L., Staunton, J. & Leadlay, P.F. (1995). Polyketide synthesis *in vitro* on a modular polyketide synthase. *Chemistry & Biology* **2**, 583–589.
13. Jordan, P.M., *et al.*, & Scott, A.I. (1988). Identification of a cysteine residue as the binding site for the dipyrromethane cofactor at the active site of *Escherichia coli* porphobilinogen deaminase. *FEBS Lett.* **235**, 189–193.
14. Scott, A.I., *et al.*, & Roessner, C.A. (1989). Reconstitution of apoporphobilinogen deaminase: structural changes induced by cofactor binding. *FEBS Lett.* **242**, 319–324.
15. Scott, A.I., *et al.*, & Hoshino T. (1988). Site-directed mutagenesis and high-resolution NMR spectroscopy of the active site of porphobilinogen deaminase. *Biochemistry* **27**, 7984–7990.
16. Scott, A.I., *et al.*, & Pichon, C. (1988). Concerning the catalytic site of porphobilinogen deaminase. *J. Am. Chem. Soc.* **110**, 5998–5900.
17. Warren, M.J., Roessner, C.A., Santander, P.J. & Scott, A.I. (1990). The *Escherichia coli cysG* gene encodes S-adenosylmethionine-dependent uroporphyrinogen III methylase. *Biochem. J.* **265**, 725–729.
18. Spencer, J.B., Stolowich, N.J., Roessner, C.A. & Scott, A.I. (1993). The *Escherichia coli cysG* gene encodes the multifunctional protein, siroheme synthase. *FEBS Lett.* **335**, 57–60.
19. Warren, M.J., Bolt, E.L., Roessner, C.A., Scott, A.I., Spencer, J.B. & Woodcock, S.C. (1994). Gene dissection demonstrates that the *E. coli cysG* gene encodes a multifunctional enzyme. *Biochem. J.* **302**, 837–844.
20. Gonzalez, M.D. & Scott, A.I. (1995). Design of a reusable enzymatic system for the preparation of porphyrins of biological interest. *Bioorg. Med. Chem. Lett.* **4**, 743–746.
21. Warren, M.J., Stolowich, N.J., Santander, P.J., Roessner, C.A., Sowa, B.A. & Scott, A.I. (1990). Enzymatic synthesis of dihydrosirohydrochlorin (precorrin-2) and of a novel pyrrocorphin by uroporphyrinogen III methylase. *FEBS Lett.* **261**, 76–80.
22. Warren, M.J., Roessner, C.A., Ozaki, S-I., Stolowich, N.J., Santander, P.J. & Scott, A.I. (1992). Enzymatic synthesis and structure of precorrin-3, a trimethyl dipyrrocorphin intermediate in vitamin B_{12} biosynthesis. *Biochemistry* **31**, 603–609.
23. Scott, A.I., Roessner, C.A., Stolowich, N.J., Spencer, J.B., Min, C. & Ozaki, S-I. (1993) Biosynthesis of vitamin B_{12}: discovery of the enzymes for oxidative ring contraction and insertion of the fourth methyl group. *FEBS Lett.* **331**, 105–108.
24. Min, C., Atshaves, B.A., Roessner, C.A., Stolowich, N.J., Spencer, J.B. & Scott, A.I. (1993). Isolation, structure, and genetically engineered synthesis of precorrin 5, the pentamethylated intermediate of vitamin B_{12} biosynthesis. *J. Am. Chem. Soc.* **115**, 10380–10381.
25. Roessner, C.A., *et al.*, & Scott, A.I. (1992). Expression of nine *Salmonella typhimurium* enzymes for cobinamide biosynthesis: identification of the 11-methyl and 20-methyl transferases of corrin biosynthesis. *FEBS Lett.* **301**, 78–78.
26. Spencer, J.B., Stolowich, N.J., Roessner, C.A., Min, C. & Scott, A.I. (1993). Biosynthesis of vitamin B_{12}: ring contraction is preceded by incorporation of molecular oxygen into precorrin 3. *J. Am. Chem. Soc.* **115**, 11610–11611.
27. Roessner, C.A., *et al.*, & Scott, A.I. (1995). Overexpression in *Escherichia coli* of 12 vitamin B_{12} biosynthetic enzymes. *Prot. Expr. Purif.* **6**, 155–163.
28. Roessner, C.A., *et al.*, & Scott, A.I. (1994). Genetically engineered synthesis of precorrin 6-x and the complete corrinoid, hydrogenobyrinic acid, an advanced precursor of vitamin B_{12}. *Chemistry & Biology* **1**, 119–124.
29. Eschenmoser, A. (1988). Vitamin B_{12}: experiments concerning the origin of its molecular structure. *Angew. Chem, Int. Ed. Engl.* **27**, 5–29.
30. Scott, A.I. (1994). Towards the genetically engineered synthesis of natural products. *Chemistry & Biology* **1**, xxiv–xxv.
31. Scott, A.I. (1994). Genetically engineered synthesis of natural products. *J. Nat. Prod.* **57**, 557–573.