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Antibiotic biosynthesis: from natural to unnatural compounds†

HG Floss

Department of Chemistry, Box 351700, University of Washington, Seattle, WA 98195-1700, USA

The evolution of the field of biosynthesis from the unravelling of the mode of formation of natural products to the use of such knowledge to create new compounds is reviewed using examples from the author's laboratory. The discussion focuses on the mode of operation of type II (spore pigment PKS) and type I (rifamycin PKS) polyketide synthases and their diversion to generate unnatural products, and on the genetics and biochemistry of deoxysugar formation in granaticin biosynthesis as a prerequisite to combinatorial enzymatic synthesis of unusual glycosides.
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The field of biosynthetic chemistry has evolved from the discovery of new natural products, their biosynthetic building blocks, the pathways by which they are assembled, the enzymes catalyzing this chemistry to the genes encoding the entire process. We are now coming full circle by using the knowledge and the tools we have developed to generate new, potentially bioactive molecules by combinatorial biosynthesis. The following review illustrates this evolution with some examples from the author's laboratory.

The story focuses on two classes of compounds. One is exemplified by granaticin [3,5,23], a benzoisochromane quinone antibiotic from *Streptomyces violaceoruber* Tü22 carrying a uniquely attached sugar moiety, and its L-rhodinoside, granaticin B (Figure 1). Granaticin contains the same polyketide backbone as actinorhodin, but the two compounds have opposite configurations at the two common stereocenters. It is worth remembering that granaticin provided one of the two examples, the other being medermycin, with which the feasibility of genetically engineering hybrid antibiotic structures was first demonstrated [17].

Early biosynthetic studies [37] established the biosynthetic building blocks of granaticin as acetate and glucose (Figure 1). As in all biosyntheses of antibiotic deoxysugar moieties, the transformations of the carbohydrate skeleton take place at the level of a sugar nucleotide. Two enzymes, dTDP-glucose synthase and dTDP-glucose 4,6-dehydratase, catalyzing the reactions shown in Figure 2, provide the general entry into this pathway [29,44]. The latter enzyme catalyzes the first deoxygenation reaction of the sugar backbone, at C-6, and it commits substrate irreversibly to the deoxysugar pathway. The enzyme catalyzes a unique hydride transfer from C-4 to C-6 of the substrate [12], and its involvement in the biosynthesis of granaticin has been demonstrated [37]. Based on a stereochemical analysis employing the chiral methyl group methodology [36], the detailed mechanism shown in Figure 2 has been proposed [37].

The second example involves the antitubercular antibiotics of the rifamycin family, exemplified by rifamycin B (Figure 3) [28]. The rifamycins are produced by *Amycolatopsis mediterranei* and they belong to the broader family of ansamycin antibiotics, which also includes geldanamycin, ansatrienin/mycotrienin and the antitumor agent, ansamitocin [11]. In contrast to the naphthalenic ansamycin, rifamycin, the latter compounds carry a benzenoid aromatic moiety in their macrocycle. All ansamycins are assembled by the polyketide pathway, and they share the feature that a unique aromatic acid, 3-amino-5-hydroxybenzoic acid (AHBA), serves as the polyketide starter unit [15,24]. Figure 3 summarizes the biosynthetic pathway to rifamycin B. AHBA is synthesized by a novel parallel branch of the shikimate pathway in which a nitrogen is introduced in the first step, followed by cyclization and dehydration to give an amino analog of dehydroshikimic acid. The latter is then aromatized by the enzyme AHBA synthase [25]. Polyketide chain extension of AHBA by two acetate and eight propionate units followed by lactam ring closure then leads to the presumed first macrocyclic product, proansamycin X, whereas premature chain termination after only three extension steps results in the formation of the compound P8/1-OG that is accumulated by some mutants of *A. mediterranei* [14]. Further downstream processing of proansamycin X then leads to the various rifamycins found in the fermentation including, ultimately, rifamycin B.

Following confirmation of the proposed pathway to AHBA by cell-free experiments with the proposed intermediates [25] attention turned to the pathway enzymes. The initial focus was on AHBA synthase as the unique enzyme of this new pathway, both because it could lead to a specific gene probe for the rifamycin biosynthetic gene cluster and because it catalyzes an unexpected reaction. Whereas AHBA synthase quantitatively converts aminoDHS to AHBA, the same substrate aromatizes non-enzymatically upon acid or base treatment to give quantitatively 3,4-dihydroxybenzoic acid (protocatechuic acid). Hence, AHBA synthase completely redirects the aromatization chemistry of its substrate. The enzyme from *A. mediterranei* was purified to homogeneity to give partial amino acid sequence data that were then used to clone the encoding gene by reverse genetics. Sequence analysis and expression of the gene then allowed elucidation of the reaction mechanism of AHBA synthase [26]. The enzyme uses PLP as a cofactor and catalyzes an α,β elimination of the elements of water from its substrate, as well as stereospecific proton elimination from C-6 as part of a 1,4-conjugate enolization

Correspondence: HG Floss, Department of Chemistry, Box 351700, University of Washington, Seattle, WA, 98195-1700, USA
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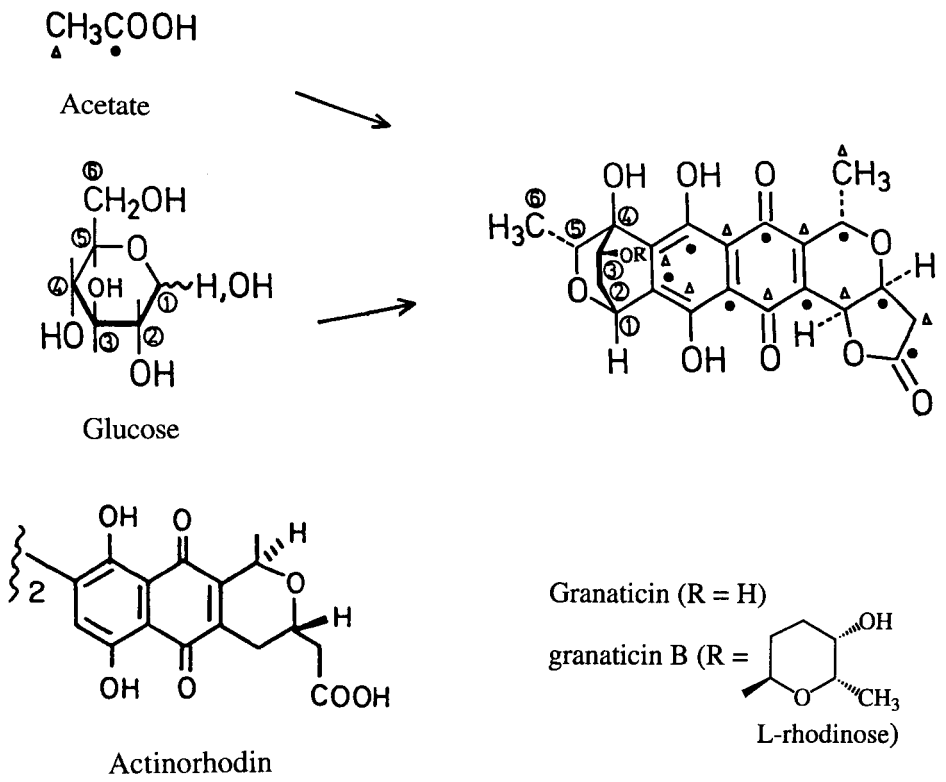


Figure 1 Structures of granaticin, granaticin B and actinorhodin, and the biosynthetic origin of granaticin.

(Figure 4). Since then, further improvements in the expression system have allowed, in collaboration with Dr Janina C. Eads of the University of Birmingham, the crystallization of the enzyme and solution of the crystal structure of the holoenzyme at 2.0 Å and of an enzyme–inhibitor complex at 2.2 Å [10]. Native AHBA synthase is a dimer with two active sites, each made up of amino acid residues from both subunits. Site specific mutagenesis experiments involving reconstitution of partially active chimeric enzyme from inactive mutants in which active site residues of opposite subunits had been replaced by inactive ones demonstrated that only the dimer is catalytically active (T.-W. Yu, J.C. Eads, H.G. Floss, unpublished results).

The two polyketides chosen as focus of this review are, of course, examples illustrating the two types of polyketide synthases (PKSs) operating in microbial systems. Rifamycin is assembled on a type I modular PKS of the kind first encountered in the biosynthesis of the polyketide backbone of erythromycin [7,8]. Granaticin, however, is synthesized on a type II PKS of the kind involved in the biosynthesis of compounds such as actinorhodin or tetracenomycin [18,20]. The granaticin PKS has been cloned from the *S. violaceoruber* Tü22 DNA [35] and shows a gene organization conforming to that of other type II aromatic PKSs, such as the *act*, *tcm* and *whiE* (spore pigment) PKSs (Figure 5). Extensive mix-and-match experiments with the components of these type II PKSs have been carried out and have led to the formulation of a series of programming rules for these enzymes [27,31], which assign specific functions to each of the component genes. A minimal PKS consisting of a keto synthase (KSα), a chain length factor (CLF or KSβ) and an acyl carrier protein (ACP) has been defined that is capable of assembling a complete polyketide. Recent studies on the *whiE* minimal PKS have shown that these enzymes exert very little control over the fate of this initially assembled polyketide. For example, the *whiE* minimal PKS produces a complex mixture of products, from which over 30 compounds of different shapes and sizes have been identified [34,45]. Some of the structures formed are quite unusual (Figure 6), such as the oxaadamantane TW93H. However, addition of just one more component, a cyclase, to the minimal PKS enzyme complex imposes extensive control over product formation, resulting in the synthesis of only one compound and its cellular metabolite [45]. Thus, it appears that the specificity of type II PKSs is controlled not so much by individual proteins but by the sum of all the protein components when they are assembled in the native multienzyme complex.

Returning to the rifamycin story, the AHBA synthase gene provided a means to isolate the rifamycin biosynthetic gene cluster, after a gene inactivation experiment had confirmed that this gene is indeed essential for rifamycin B biosynthesis [26]. Starting from cosmid clones carrying DNA surrounding the AHBA synthase gene the entire cluster was sequenced (Figure 7), the PKS region in the laboratory by Tang *et al.* [42] and independently also in that by Schupp *et al.* [33] and the non-PKS portion in our laboratory [2]. The cluster consists of four distinct regions. Region IV contains a series of genes encoding regulatory functions and resistance mechanisms and a substantial number of postsynthetic modification enzymes. This region has not yet been studied extensively. It is flanked on one side by region III that consists of the genes required for AHBA formation; however, one additional gene necessary for making AHBA, *rifJ*, is found separate from the AHBA cluster at the other end of region IV. Figure 8 compares the genes that we have demonstrated to be necessary and sufficient for AHBA formation [47] with the pathway of AHBA formation. There are clearly

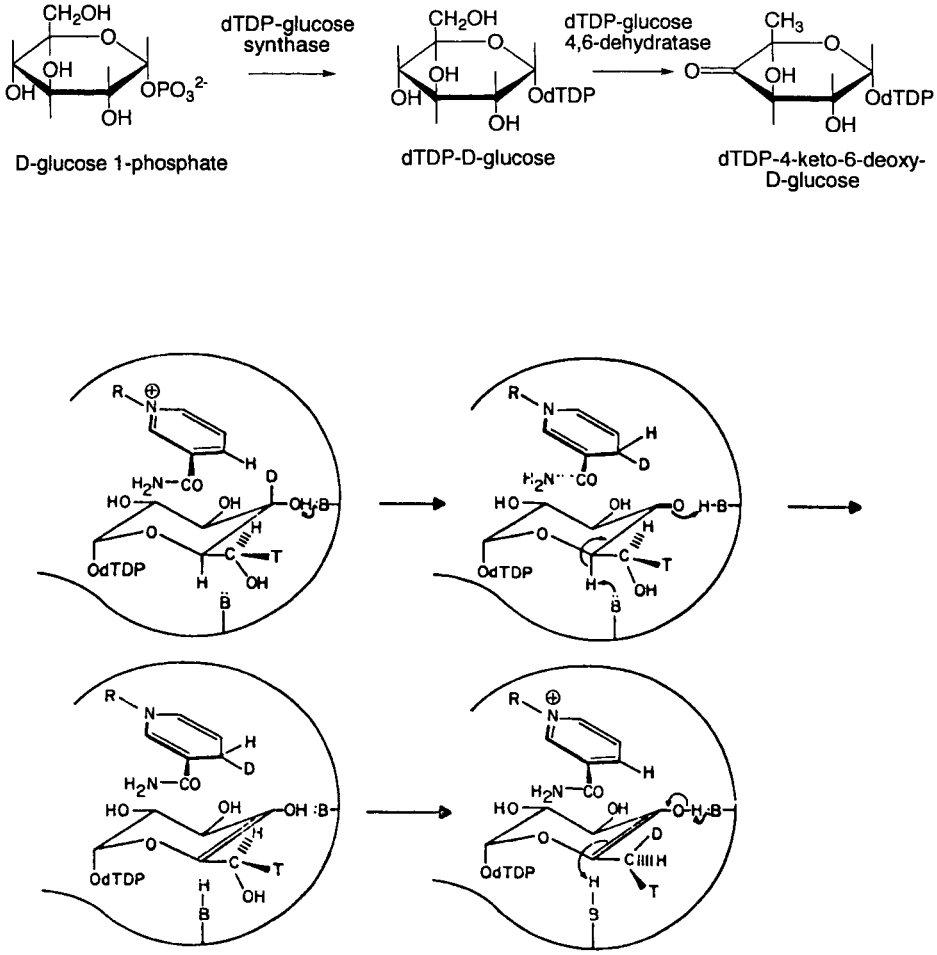


Figure 2 The dTDP-D-glucose synthase and dTDP-D-glucose 4,6-dehydratase reactions, and the mechanism of dTDP-D-glucose 4,6-dehydratase.

discrepancies between the two, suggesting that the pathway may be more complicated than initially thought. The original hypothesis for the origin and mode of introduction of the nitrogen is probably not correct, and alternative ways in which a nitrogen-containing version of the precursor, erythrose 4-phosphate, may be generated must be considered. All these genes have now been expressed in soluble form and their biochemical functions are under investigation.

Region II comprises the type I *rif* PKS that consists of five separate proteins containing the loading domain and 10 chain-extension modules, each with the appropriate modification domains, arranged colinearly with the biochemical assembly process (Figure 9). To probe the specificity of the loading domain, simple analogs of AHBA, 3-hydroxy- and 3,5-dihydroxybenzoic acid, were fed to a *rifK*(−) mutant of *A. mediterranei*. As expected the mutant produced no rifamycin, but surprisingly, accumulated copious amounts of analogs of the incompletely assembled polyketide P8/1-OG (Figure 3) lacking the amino group or carrying a hydroxy in place of the amino group [19]. This observation indicates that the loading domain of the *rif* PKS is promiscuous and polyketide synthesis can be initiated as effectively by the analogs as by AHBA. However, downstream domains of the PKS, most likely at the interface between module 3 of RifA and module 4 of RifB, recognize the structural difference in the starter region and reject the unnatural analogs of the normal assembly intermediates.

Unlike the macrolide type I PKSs, the *rif* PKS does not carry a type 1 thioesterase domain at the end of the last PKS module. Instead, *rifE* is immediately followed by a gene, *rifF*, with homology to mammalian acetyl CoA:arylamine *N*-acetyltransferases. Based on the chemical analogy it was assumed that this gene encodes the downloading enzyme that releases the completely assembled polyketide chain from the PKS by intramolecular nucleophilic displacement of the thioester bond by the nitrogen to give the macrolactam ring of proansamycin X. To test this notion *rifF* was inactivated by an in-frame deletion resulting, as expected, in loss of rifamycin B production. However, unexpectedly, the mutant did not accumulate the open-chain undecaketide, but rather an entire series of ketides of different chain lengths ranging from the tetra- through the decaketide (Figure 10) [46]. Similar results were reported by Stratmann *et al.* [41], who, working with a higher producing stain also isolated the expected undecaketide. This finding represents the clearest demonstration to date of the widely accepted processive nature of polyketide assembly on such type I PKSs, and it suggests that the *rif* PKS processes multiple polyketide chains simultaneously, as shown in Figure 9. Interestingly, small amounts of these incomplete polyketides were subsequently found in fermentations of the wild-type strain.

The accumulated ketides all carry the naphthoquinone structure characteristic of the rifamycins, although without the 8-hydroxy

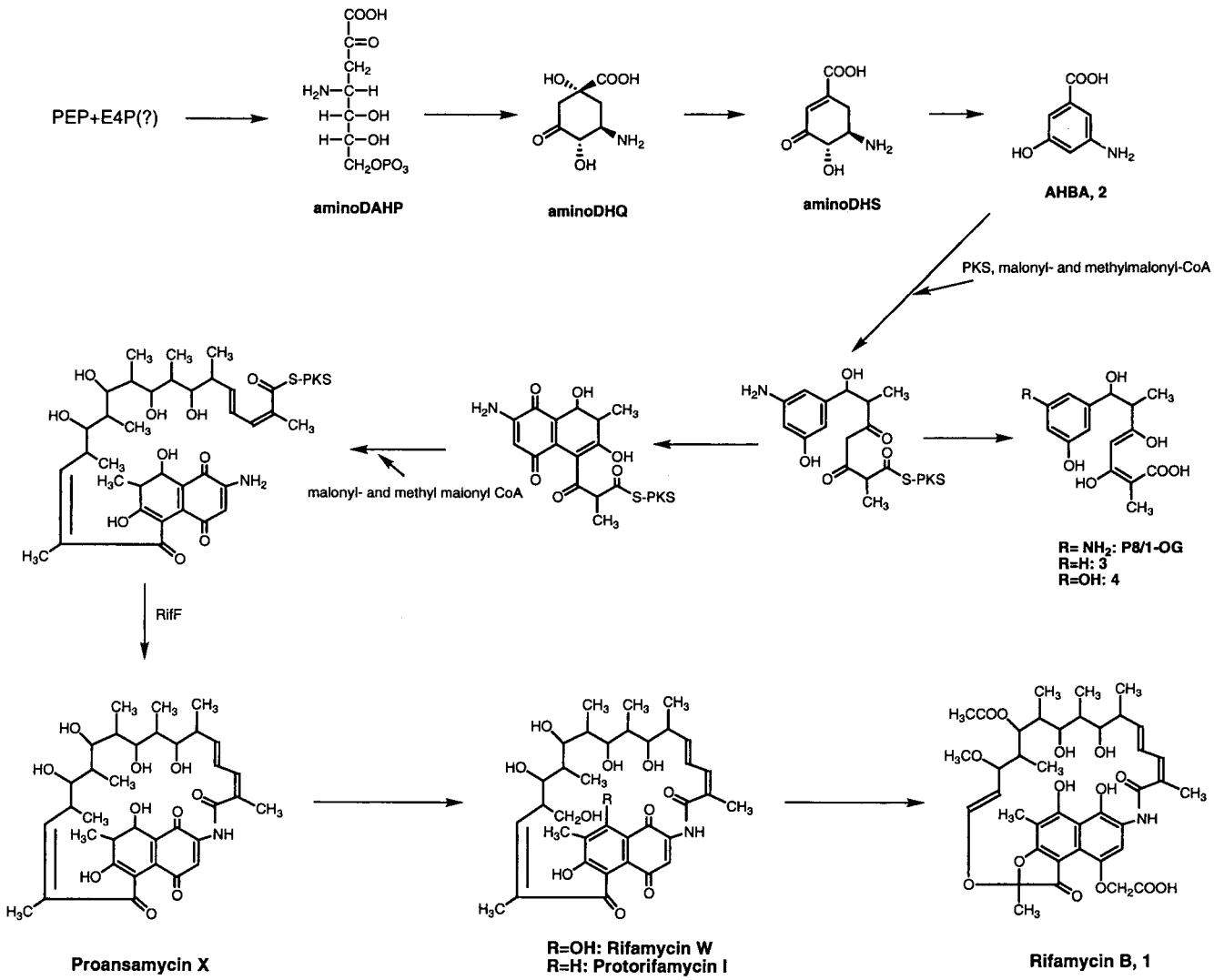


Figure 3 The biosynthetic pathway to rifamycin B in *A. mediterranei*.

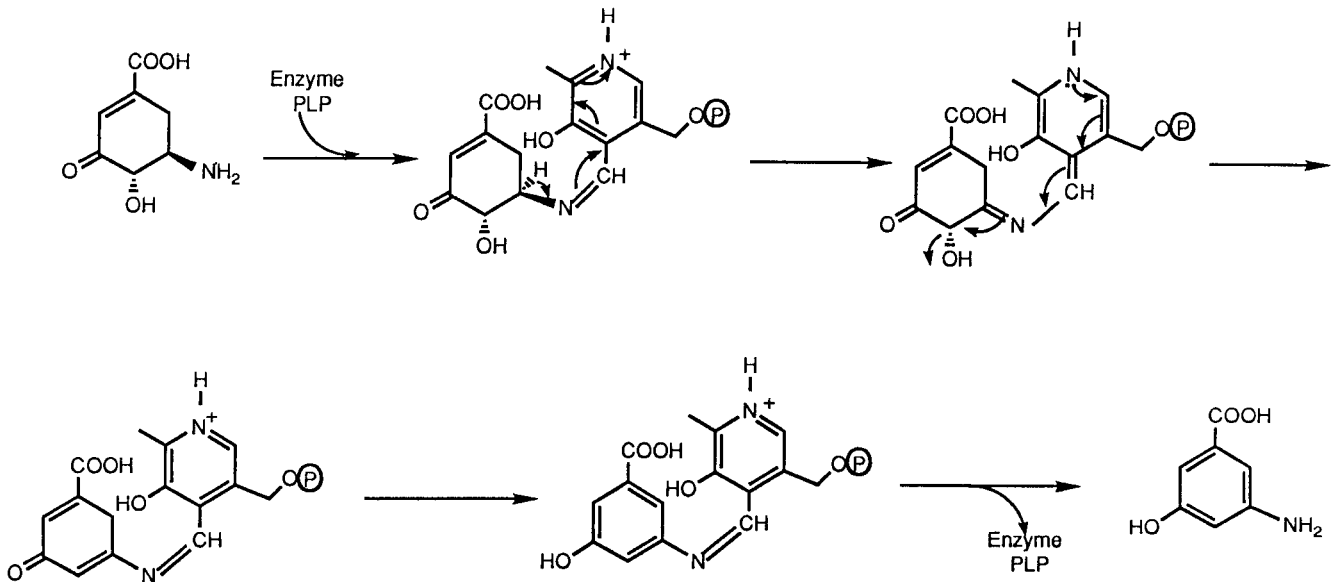


Figure 4 The reaction mechanism of AHBA synthase.

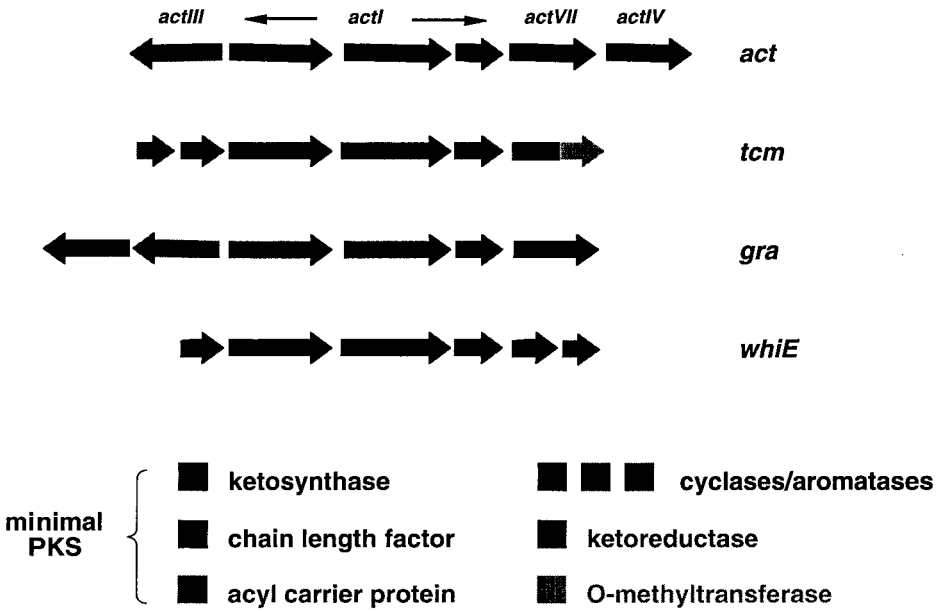


Figure 5 Organization of type II polyketide synthase (PKS) genes in Actinomycetes (*act* = actinorhodin, *tcm* = tetracenomycin, *gra* = granaticin, *whiE* = *S. coelicolor* spore pigment).

group, except the tetraketide, which still retains the original benzenoid structure of the starter unit. This strongly suggests that the naphthoquinone ring closure is not a postsynthetic modification but occurs during polyketide assembly, presumably between the

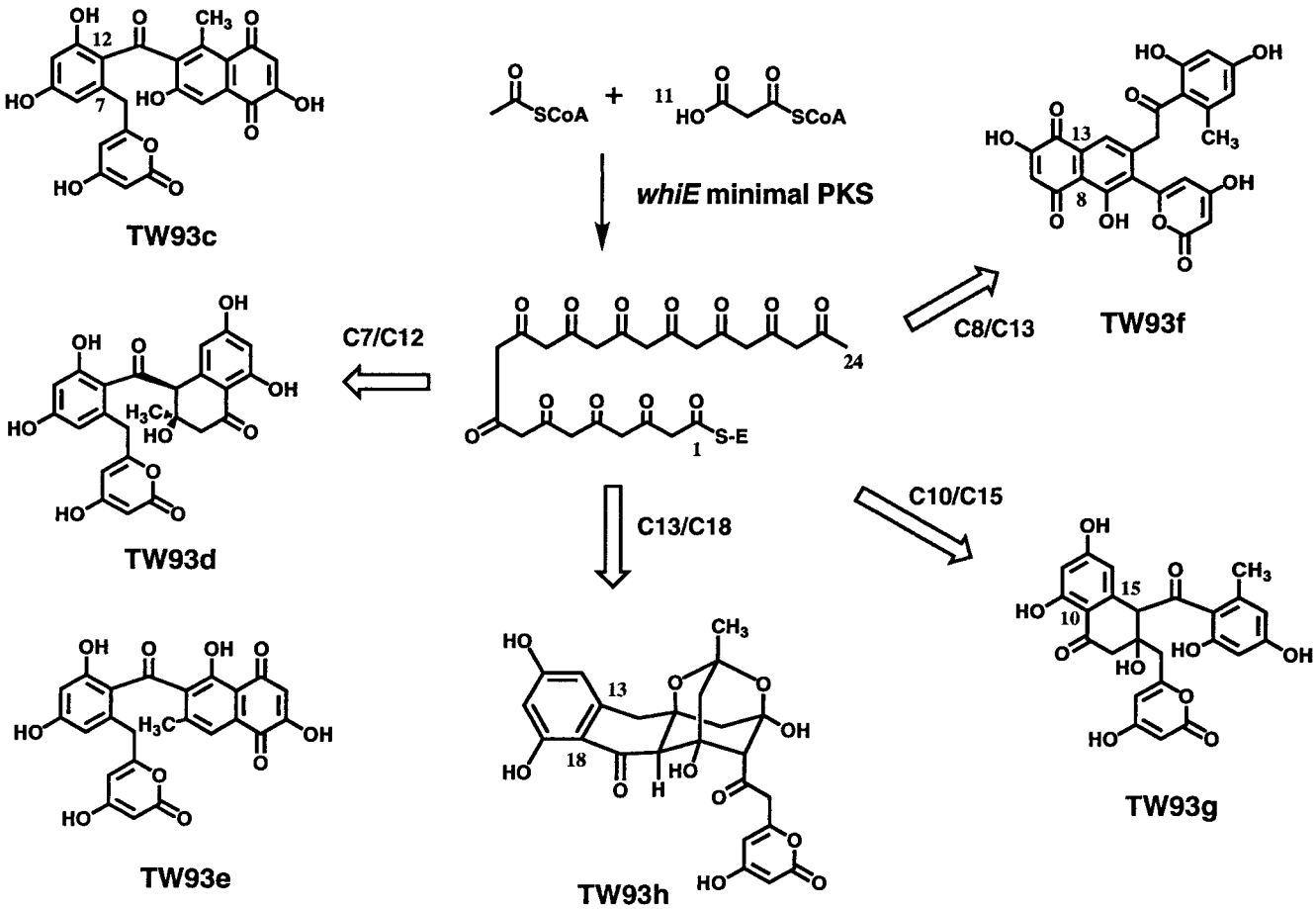
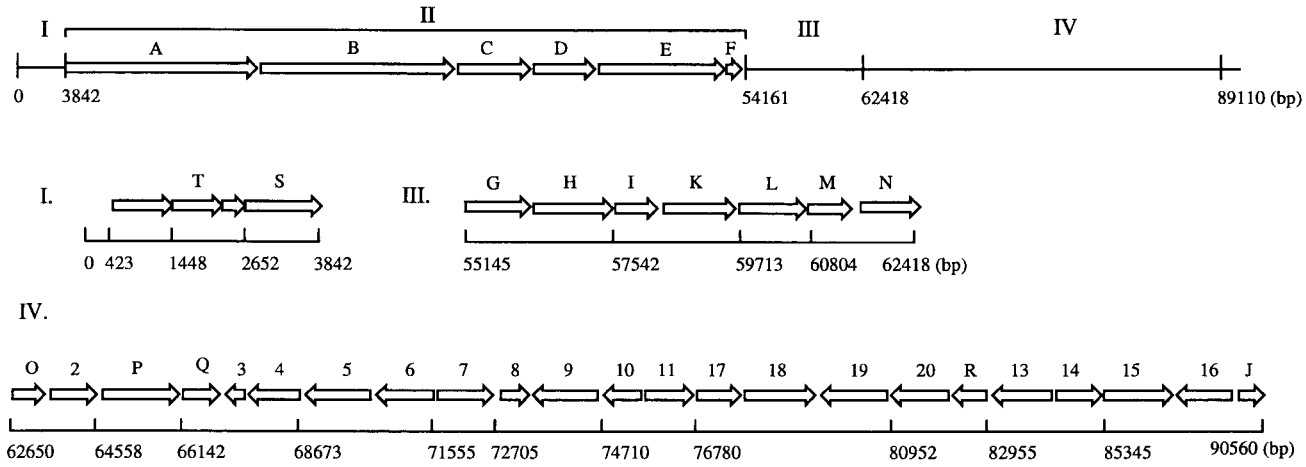
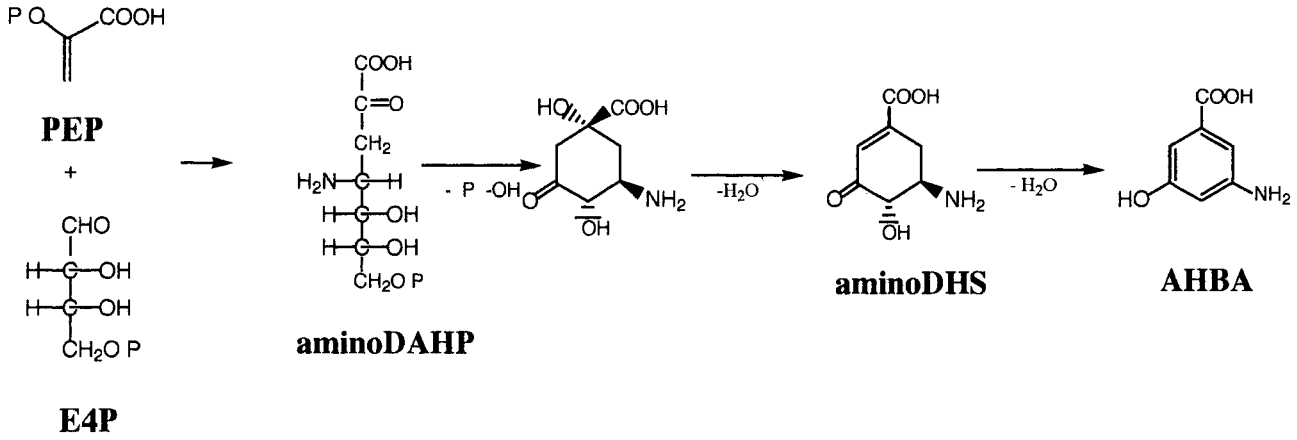


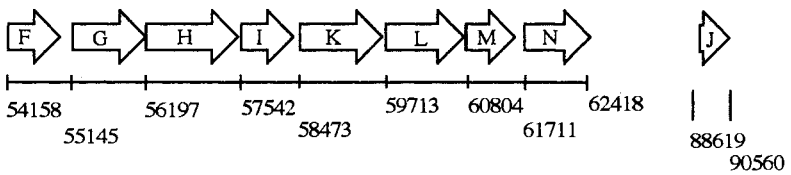
Figure 6 Structures of some minimal *whiE* PKS polyketide products and their proposed mode of formation.



RifA, RifB, RifC, RifD & RifE: polyketide synthase; RifF: amide synthase; RifG: aminoDHQ synthase; RifH: aminoDAHP synthase; RifI: Q/S dehydrogenase; RifK: AHBA synthase; RifL: oxidoreductase; RifM: phosphatase; RifN: kinase; RifO: putative regulator; RifP: efflux protein; RifQ: transcriptional repressor; RifR: thioesterase; RifS, Rif4, Rif5, Rif13 & Rif16: cytochrome P450 monooxygenase; RifT: unknown; Rif2: esterase; Rif3: unknown; Rif6: dNTP hexose dehydratase; Rif7: dNTP hexose glycosyl transferase; Rif8: dNTP hexose 3,5 epimerase; Rif9: aminotransferase; Rif10: oxidoreductase; Rif11: reductase; Rif14: methyl transferase; Rif15: transketolase; RifJ: aminoDHQ dehydratase

Figure 7 The rifamycin (*rif*) biosynthetic gene cluster from *A. mediterranei*.





RifG: AminoDHQ synthase (AroB *E. coli* K12)
RifH: AminoDAHP synthase (AroG *S. lycopersicum*)
RifI: Aminoquinate dehydrogenase (AroE *Synechocystis* sp.)
RifK: AHBA synthase (AHBAs *S. collinus*)
RifL: Oxidoreductase (Pur10 *S. ablboniger*)
RifM: Phosphatase (CbbzP *A. eutrophus*)
RifN: Kinase (XylR *Synechocystis* sp.)
RifJ: AminoDHQ dehydratase (AroD *A. pleuropneumoniae*)

Figure 8 Genes in the *rif* cluster involved in the biosynthesis of AHBA.

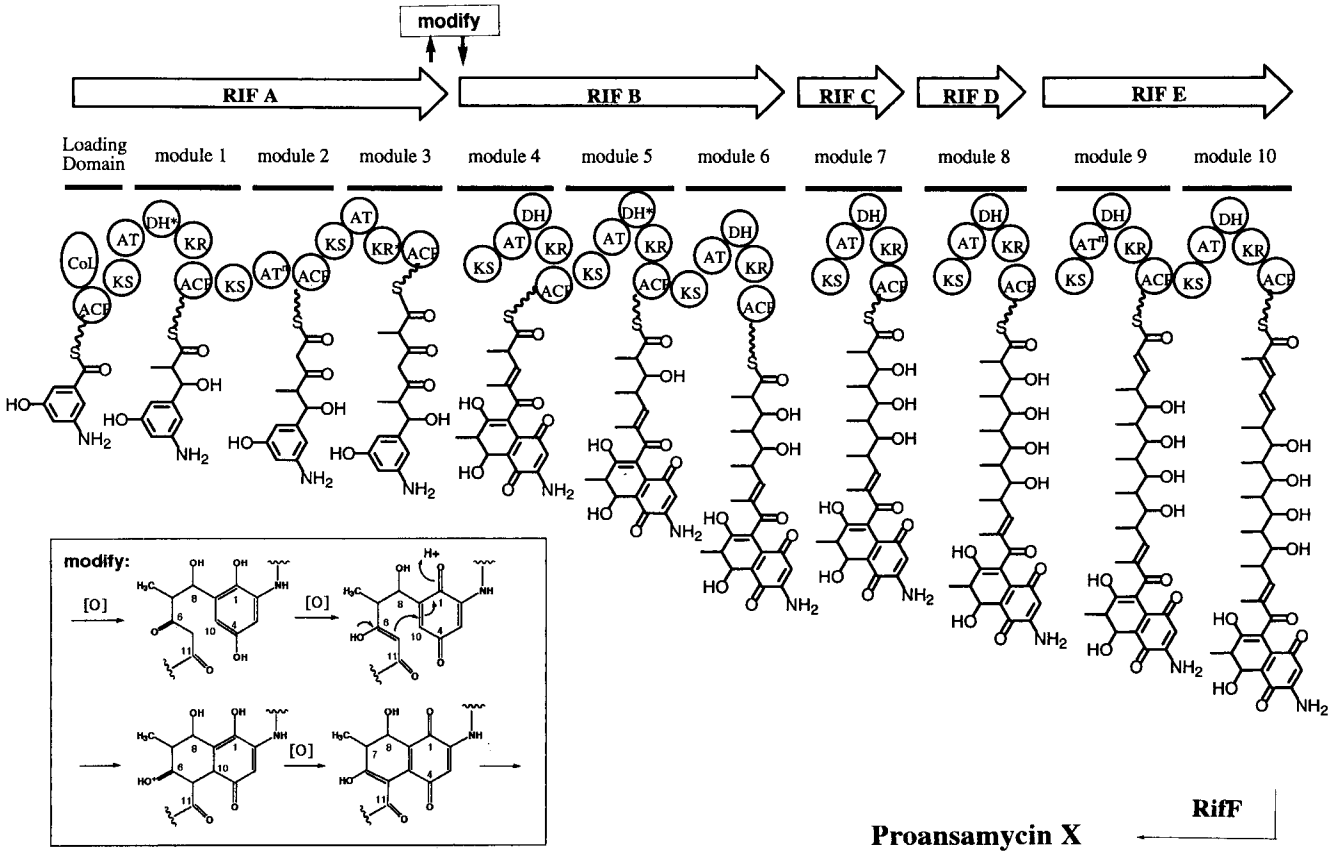


Figure 9 The deduced structure of the *rif* PKS and of the intermediates in the rifamycin polyketide assembly process, and a proposed mechanism for the naphthoquinone ring-closure reaction.

third and fourth chain extension step. A plausible mechanism for this reaction (Figure 9) involves hydroxylation of the benzene ring followed by oxidation to the *p*-benzoquinone, which may then undergo spontaneous cyclization by a Michael addition driven by reoxidation of the naphthohydroquinone to the quinone. Thus, the process may require no more than two enzymes, a hydroxylase and a quinone oxidoreductase, which may have to dock with the PKS multienzyme complex to modify the PKS-bound polyketide. The genes encoding the ring closure enzymes have not yet been identified.

Naphthoquinone ring closure appears to produce initially the 8-hydroxy-7,8-dihydronaphthoquinone structure as in proansamycin X, which is then converted to the 8-hydroxynaphthoquinone structure of the rifamycins. This may occur either by dehydration and hydroxylation, as suggested by the structure of the accumulated ketides from the *rifF*(−) mutant and by an

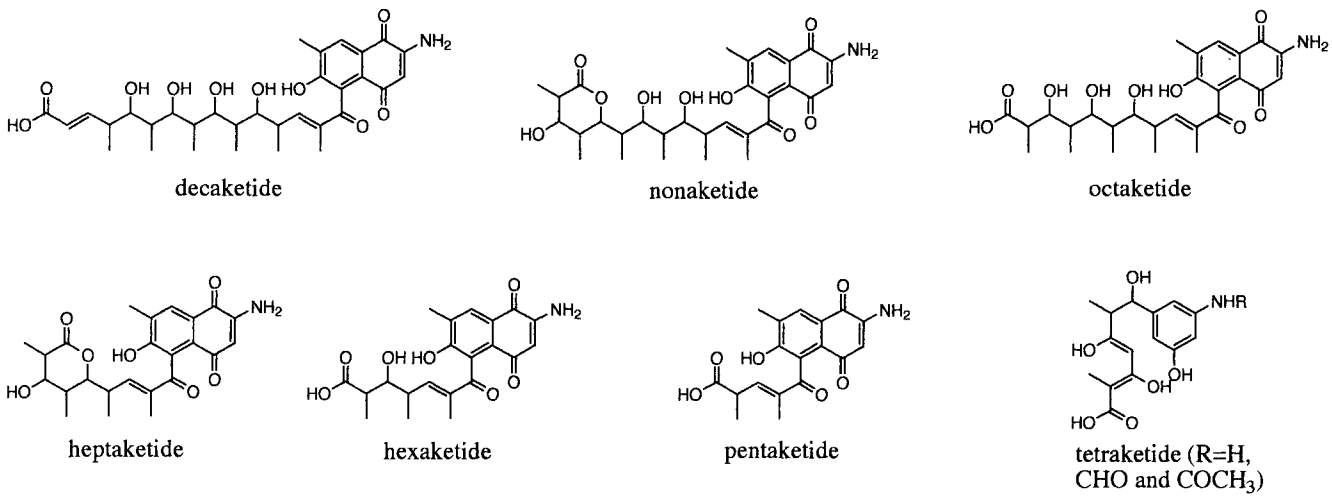


Figure 10 Ketides isolated from the *rifF*(−) mutant of *A. mediterranei*.

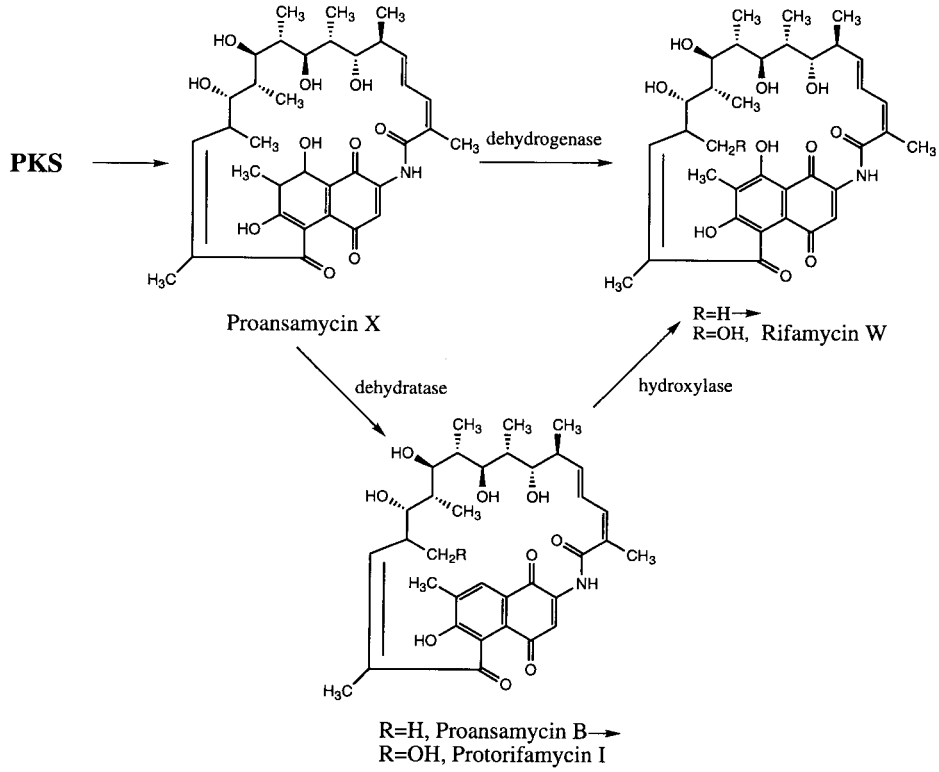
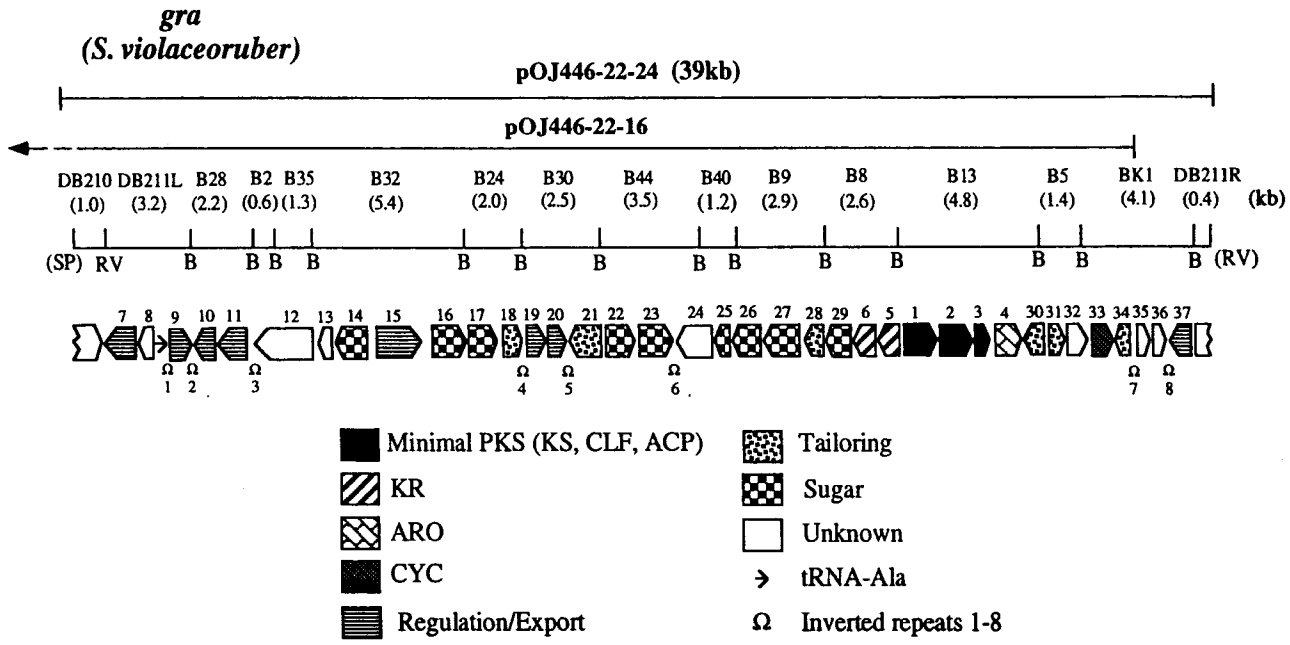


Figure 11 Two possible routes from the hypothetical intermediate proansamycin X to rifamycin W.

earlier report on the conversion of protorifamycin I into rifamycin W [13], or by a direct dehydrogenation of proansamycin X, as suggested by experiments of Rickards and coworkers [1], which showed retention of ^{18}O from the carboxyl group of AHBA in the 8-hydroxyl group of the rifamycins (Figure 11). In view of the contradiction between these two reports we repeated both experiments with appropriately blocked mutants and found no conversion of protorifamycin I into rifamycin B, but fully confirmed Rickards' ^{18}O experiments on the origin of the 8-hydroxy group of rifamycin from the carboxyl group of AHBA [46]. Thus, both protorifamycin I and the ketides accumulated by *A. mediterranei* are shunt metabolites, resulting from dehydration of the normal intermediates either on the enzyme or after their release during workup. The normal biosynthesis proceeds *via* 8-hydroxyl-7,8-dihydronaphthoquinone intermediates that are subsequently aromatized by direct dehydrogenation or, alternatively, by tautomerization to the fully aromatic 8-hydroxynaphthohydroquinone followed by reoxidation to the quinone. These proposals are fully supported by model reactions carried out by the Rickards group [32].

Using the AHBA synthase gene as a probe, biosynthetic gene clusters for several other ansamycin antibiotics have now been isolated. Two such clusters, one encoding formation of naphthomycin, a close relative of rifamycin with a larger macrocycle, and ansatrienin/mycotrienin, a benzenoid ansamycin, have been obtained from *Streptomyces collinus* and have been partially analyzed [6]. The naphthomycin cluster shows a similar arrangement of the AHBA synthesis genes relative to the PKS genes, whereas in the ansatrienin/mycotrienin cluster this arrangement is very different. In collaboration with the group of E. Leistner, Bonn, we have completed the sequence analysis of the ansamitocin biosynthetic gene cluster from *Actinosynnema pretiosum* (unpublished results), and the group of Sherman has reported the cloning and analysis of the mitomycin biosynthesis gene cluster from *S. lavendulae* [30].

Continuing with the granaticin story the focus now shifts to the sugar moieties of granaticin and granaticin B. A combined reverse genetics and consensus primer approach allowed the isolation from *S. violaceoruber* DNA of several cosmids carrying the dTDP-glucose 4,6-dehydratase gene, flanked by two other sugar biosynthesis genes encoding dTDP-glucose synthase and a glycosyltransferase [4]. Two of these cosmids carried all the genes necessary for the biosynthesis of granaticin and granaticin B, as was demonstrated by their transformation into *S. coelicolor* CH999 that resulted in transformants producing granaticin, granaticin B and their dihydro derivatives [21]. This then provided the impetus for sequencing the entire cosmid to reveal the complete granaticin biosynthesis gene cluster shown in Figure 12 [21]. While this sequencing was in progress another subclone of sugar genes from this cosmid was analyzed, which contained genes encoding a sugar nucleotide 3,5-epimerase, a homolog of *ascC* and *rfbH*, the sugar 3-deoxygenation genes of *Yersinia pseudotuberculosis* and *Salmonella typhimurium* [29,44], as well as a 4-ketoreductase and an unknown open reading frame. Our original assumption that these genes might be involved in the 2-deoxygenation reaction were not born out by gene inactivation experiments, carried out on the cosmid followed by transformation of the mutant cosmids into *S. coelicolor* CH999 [43]. All four of the mutant transformants still synthesized granaticin, but no longer made granaticin B, demonstrating that all four of these genes are involved in the biosynthesis of L-rhodinose. It seems likely that they act on the same precursor that must provide the sugar moiety of granaticin, dTDP-4-keto-2,6-dideoxy-D-glucose,



Deduced functions of the open reading frames.

ORF	Putative functional category	Gene	Homologue: Deduced role	Homologue: SM/ID (%) of product	Homologue: Origin	Nucleotide accession number
14	Sugar	*dnrS*	Glycosyl transferase	52/26	*S.peucetius*	L47164
15	Export	*actII-2*	Transmembrane protein	57/30	*S.coelicolor*	M64683
16	Sugar	*strD*	dTDP-1-glucose synthase	70/51	*S.griseus*	Y00459
17	Sugar	*strE*	dTDP-glucose-4,6-dehydratase	76/61	*S.griseus*	X62567
22	Sugar	*dnmV*	dTDP-4-keto-6-deoxyhexose reductase	51/44	*S.peucetius*	AF006633
23	Sugar	*rfbH (ascC)*	CDP-4-keto-6-deoxyglucose-3-dehydratase (E1)	71/53	*Y.pseudotuberculosis*	L01777
24	Unknown	--	--	--	--	
25	Sugar	*strM*	dTDP-4-keto-6-deoxyglucose-3,5-epimerase in streptomycin biosynthesis	52/46	*S.griseus*	X62567
26	Sugar	*rdmF*	Rhodomycin biosynthesis	65/47	*S.purpurescens*	U10405
27	Sugar	*dnmT*	dTDP-4-keto-6-deoxyglucose-2,3-dehydratase	53/46	*S.peucetius*	U77891
28	Tailoring?	*actVA-3*	Unknown	46/41	*S.coelicolor*	X58833
29	Sugar?	*lmbY*	FMN-dependent monooxygenase in lincomycin biosynthesis?	48/29	*S.lincolnensis*	X79146

Figure 12 The granaticin biosynthetic gene cluster from *S. violaceoruber* Tü22.

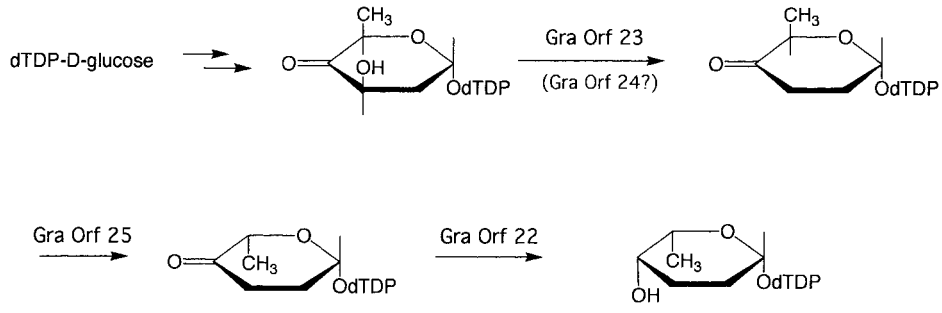


Figure 13 Genes/enzymes involved in the formation of L-rhodinose in *S. violaceoruber*.

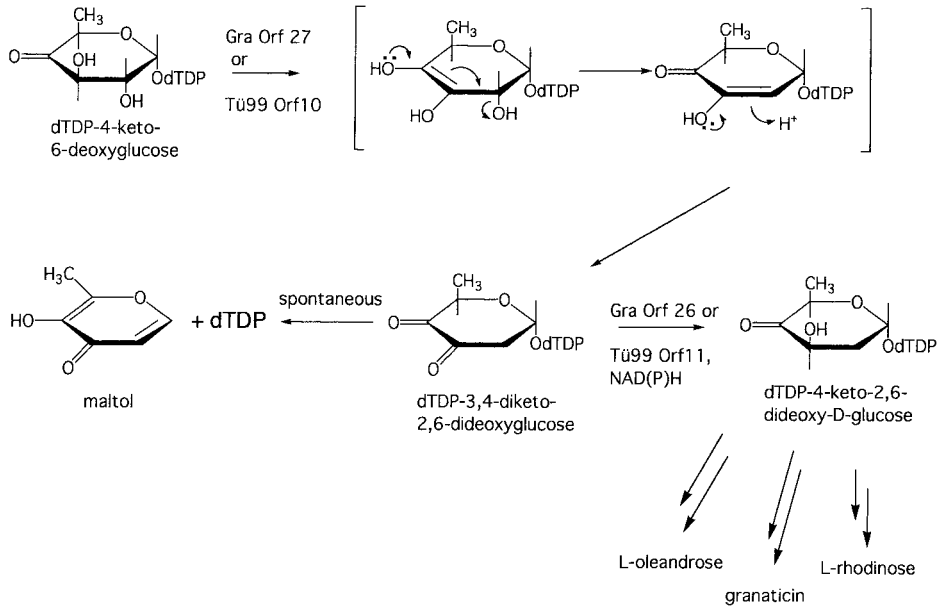


Figure 14 Action of the 2,3-dehydratase and 3-ketoreductase in the biosynthesis of dTDP-4-keto-2,6-dideoxy-D-glucose, and the mechanism of the 2-deoxygenation reaction.

presumably in the manner shown in Figure 13. Based on this finding it seems likely that the 3-deoxygenation in Streptomycetes occurs by the same mechanism as in *Yersinia* and *Salmonella*.

These findings left only two plausible candidates for the genes encoding the 2-deoxygenation reaction of the granaticin sugars, orfs 26 and 27. Both were expressed in soluble form in *E. coli* and their catalytic functions were studied. Incubation of dTDP-4-keto-6-deoxyglucose with *gra* orf 27 resulted in the isolation of two products, dTDP and maltol. When the reaction was followed in the NMR spectrometer, the intermediate formation of another compound, identified as dTDP-3,4-diketo-2,6-dideoxyglucose, was observed, which spontaneously decomposed to dTDP and maltol. However, addition of *gra* orf 26 and NAD(P)H to the incubation resulted in the isolation of a new compound identified as dTDP-4-keto-2,6-dideoxy-D-glucose (Figure 14) [9]. The same transformation was observed with two homologous genes expressed from the oleandomycin gene cluster of *S. antibioticus* Tü99. The product of the combined action of these two enzymes must then be the precursor of the sugar moiety of granaticin, of the L-rhodinose moiety of granaticin B as well as the L-oleandrose moiety of oleandomycin. A plausible mechanism for the 2-deoxygenation reaction catalyzed by *gra* orf 27 is shown in Figure 14. It is noteworthy that the three mechanisms for deoxygenation reactions of hexoses in antibiotic and bacterial cell wall formation, at C-2, C-3 and C-6, are different from each other, and they are all different from the mechanism of the most prominent sugar deoxygenation, that catalyzed by the enzyme ribonucleotide reductase [22,39,40].

The evolution of the field of biosynthesis has brought us to the point where we now have the knowledge and the tools to use biosynthetic chemistry, in conjunction with genetic engineering, to generate new molecules not (yet) encountered in Nature. Such applications of combinatorial biosynthesis should be viewed not as an alternative, but as a complement to combinatorial chemistry. Two major areas of application are clearly evident. One is the synthesis of new structural frameworks, for example as platforms for combinatorial chemistry. The most prominent examples in this area so far come from the polyketide field, and this work is clearly best done *in vivo*. The other involves "decorating" structural frameworks in new ways using postsynthetic biochemical modification systems. One of the more promising areas here involves glycosylation of various structural backbones with unusual sugars derived from antibiotic biosynthesis pathways. To realize the full potential of this approach, an *in vitro* strategy is considered more promising. This could involve (i) using recombinant enzymes expressed from antibiotic biosynthesis genes to prepare a collection of dTDP derivatives of unusual sugars as substrates, (ii) preparing a series of recombinant glycosyl transferases by overexpression of genes from antibiotic biosynthesis clusters, (iii) assembling a collection of structurally diverse antibiotic aglycones as sugar acceptors, (iv) combinatorializing reactions of these three partners and (v) broadening the substrate range of the glycosyltransferases by mutagenesis, e.g., by the DNA shuffling approach [16,38,48].

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