Tetrahedron Vol. 47, No. 31, pp. 6045-6058, 1991
Printed in Great Britain

0040-4020/91 $3.00+.00

GENETIC ENGINEERING OF HYBRID ANTIBIOTICS - A PROGRESS REPORT

BY

HEINZ G. FLOSS[a] AND WILLIAM R. STROHL[b]

Department of Chemistry, University of Washington, Seattle, WA 98195[a]

and

Department of Microbiology, The Ohio State University, Columbus, OH 43210[b]

(*Received in USA* 10 *January* 1991)

Interspecies cloning of antibiotic biosynthesis genes in streptomycetes can be used to generate new hybrid natural product structures; the application of this approach to polyketide and its extension to thiopeptide antibiotics are summarized.

1. Introduction

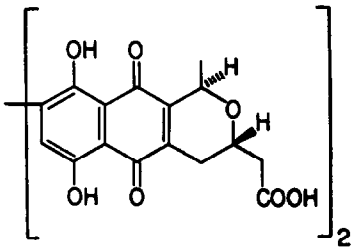
In the course of the development of new antibiotics and other medicinally or agriculturally useful microbial metabolites, the need frequently arises to modify the structure of the naturally occurring compound in order to improve the clinical utility, minimize undesirable side effects and in general explore the relationship between chemical structure and biological activity of a particular pharmacophor. Not infrequently, the material most abundantly produced by the particular organism does not have the ideal combination of properties required for a compound to reach market. Hence, even the eventual commercial production may have to be redirected towards a modified structure. Such structural modifications can be achieved either by chemical means, total synthesis or chemical alteration of the natural product, or by biological means, i.e., diverting the normal biosynthetic machinery into producing analogs. While the chemical approach is usually most expeditious when dealing with relatively simple molecules, the biological approach becomes increasingly attractive as the complexity of the structures increases.

A variety of ways are available to alter natural product structures by biological means. Microbial transformation is one principal approach which is widely practiced. In this case one utilizes the synthetic capabilities of microorganisms which do not produce the particular metabolite under study to carry out chemical reactions on a molecule foreign to them. The industrially most important examples of this approach are, of course, the various microbial transformations on the steroid ring system (1) which are a key to the production of various steroid-based medicinal agents. A large body of knowledge exists by now about the various kinds of type reactions carried out by particular microorganisms (2), and this allows one to choose a panel of microorganisms which are likely to carry out a predictable range of reactions on a given molecule.

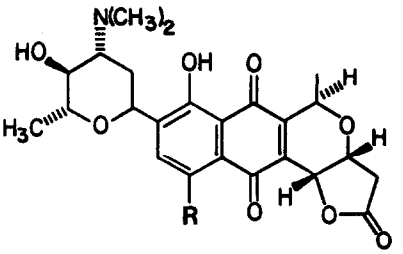
The major alternative is to alter the outcome of the normal biosynthesis of the compound under study by the producing organism; this is based on the fact that many enzymes in secondary metabolism show much less stringent substrate specificity than enzymes in primary metabolism and can therefore handle a variety of analogs of their normal substrates. The oldest of these approaches is false precursor feeding. In this case, an analog of one of the normal precursors is added to the fermentation and results in the formation of an altered metabolite incorporating this altered precursor. The commercially most important example is the production of modified penicillins, for example penicillin G by addition of sodium phenylacetate or penicillin V by addition of sodium phenoxyacetate to the penicillin fermentation (3). In this case the false precursor added in large concentration must in essence dilute out the endogenous normal precursor in order to lead to predominant production of the modified metabolite. This feature is avoided in refinements of this approach developed by Rinehart and his coworkers, called mutasynthesis (4), and by Ōmura and colleagues, called hybrid biosynthesis (5). These approaches apply particularly to multibranched, convergent biosynthetic pathways in which a final product is assembled from several components, each of which is made separately by the producing organism. The biosynthesis of one of these components is blocked, either by a mutation (mutasynthesis) or by an inhibitor (hybrid biosynthesis), and the missing piece is then replaced by externally added analogs. This can result in the production of a natural product analog to the complete exclusion of the normal compound. Mutasynthesis has been applied most extensively in the field of aminoglycoside antibiotics and hybrid biosynthesis mainly to polyketide-derived metabolites. Ultimately, however, the most desirable option would be to construct new, modified biosynthetic pathways by rearranging the chemical capabilities of organisms, i.e., by incorporating genes recruited from other, related organisms which code for enzymes that can function in place of or in addition to those enzymes already present in the parent. Such genetically engineered organisms should then be capable of producing new structures not normally encountered as natural products without the need to add any artificial precursors.

2. Genetic Engineering of Hybrid Natural Products

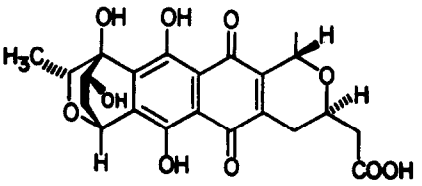
The feasibility of genetically engineering such organisms which carry and express biosynthetic genes originating from different parents and which produce new "hybrid" natural products was demonstrated by David Hopwood and his colleagues in collaboration with the laboratory of Ōmura and that of one of the authors (6). The class of compounds serving as the subject of that study were the benzoisochromane quinone antibiotics, of which actinorhodin (I) is the oldest known representative. Following the cloning of the entire actinorhodin biosynthetic pathway gene set on a 22kbp DNA fragment and their expression in a heterologous host (7), Hopwood and coworkers cloned various subsets of these genes into *Streptomyces sp.* AM-7161, the producer of the antibiotic medermycin (II). Transformants carrying the *actV* gene, thought to code for a terminal hydroxylase in actinorhodin biosynthesis, produced a new antibiotic, mederrhodin A (III), which differed from the parent by the presence of an additional hydroxyl group at C-6. When the entire actinorhodin biosynthetic gene cluster



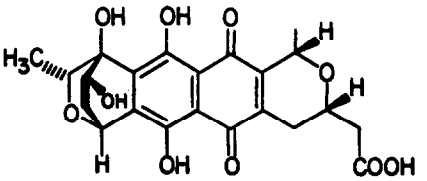
I: actinorhodin



II: R=H, medermycin
III: R=OH, mederrhodin A



IV: dihydrogranaticin



V: dihydrogranatirhodin

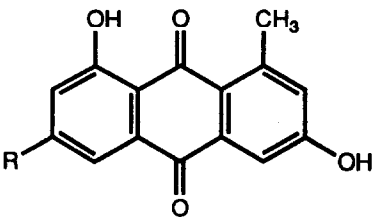
was introduced into *S. violaceoruber* Tü22, the producer of granaticin and dihydrogranaticin (IV), the transformant produced a new compound, called dihydrogranatirhodin (V). Dihydrogranaticin has the opposite stereochemistry as actinorhodin in the pyran ring, but dihydrogranatirhodin was found to have actinorhodin stereochemistry at one chiral center and granaticin stereochemistry at the other. Interestingly, the mode of interaction between the two gene sets seems to be different in these two examples. Transformants of *Streptomyces* AM-7161 carrying a plasmid with the entire actinorhodin biosynthesis gene set produced both actinorhodin and medermycin, but no new hybrid compounds. Conversely, transformants of *S. violaceoruber* Tü22 wild-type or a mutant blocked in granaticin synthesis carrying only parts of the actinorhodin biosynthesis genes produced granaticin/dihydrogranaticin but no new compounds. This finding points to the complexities of the interactions arising when foreign genes are introduced into a particular genetic environment.

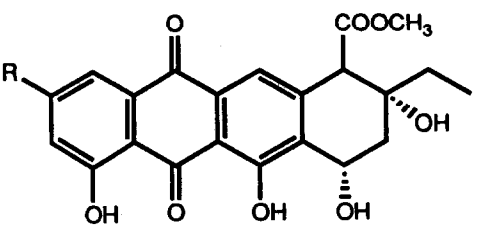
Following the report of these initial examples, which established the principal feasibility of this approach, a number of other cases of generation of hybrid antibiotics by interspecies cloning of antibiotic biosynthesis genes have been published. For example, an antibiotic non-producing mutant of *Saccharopolyspora erythraea*, the producer of erythromycin, was transformed with a gene library from *Streptomyces antibioticus*, the producer of the related macrolide antibiotic, oleandomycin. The non-producing mutant was deficient in the synthesis of the early pathway intermediate, 6-deoxyerythronolide B. Antibiotic producing transformants were found to synthesize a mixture of 2-norerythromycins which lack the methyl group at carbon 2 of the macrolide ring of the erythromycins (8). In another case, the *carE* gene from the biosynthetic pathway to carbomycin, a 16-membered macrolide antibiotic, was cloned from *S. thermotolerans* into *S. ambofaciens*, which produces the

related 16-membered macrolide spiramycin (9). The *carE* gene is thought to encode an isovaleroyl transferase, and the resulting transformant of *S. ambofaciens* produced a new compound, 4″-isovaleroyl spiramycin. Other examples of the production of new compounds in interspecific transformants of streptomycetes have been noted, but the compounds have not yet been identified.

3. Hybrid Structures by Cloning Genes between Actinorhodin and Anthracycline Producers

Further efforts to delineate the scope and limitations of the interspecies cloning approach to the generation of new hybrid natural product structures are being made as a collaborative venture between the laboratories of the two authors. One direction of this work involves the cloning of biosynthetic genes from the actinorhodin pathway into producers of anthracycline antibiotics. When an 8.8 kbp *Pst*I DNA fragment from *S. coelicolor* carrying the *actI*, *actIII*, *actIV* and *actVII* loci (10), which encode early steps in actinorhodin biosynthesis (11), was cloned into *S. galilaeus* ATCC 31133, the producer of aclacinomycin A, formation of a new compound, 1,7-dihydroxy-9-methylanthraquinone (aloesaponarin II) (**VI**) was observed (12). It was demonstrated that aloesaponarin II was biosynthesized by the assembly of 8 acetate units, indicating that it is formed by the polyketide synthase from the actinorhodin pathway (coded for by *actI*) rather than by the polyketide synthase from the anthracyclinone pathway. Aloesaponarin II-formation was also observed in class VI mutants of *S. coelicolor*, and the same DNA fragment also conferred aloesaponarin II biosynthesis upon *S. peucetius*, *S. parvulus*, *S. azureus* and *Streptomyces C5*. Further subcloning of the 8.8 kbp *Pst*I fragment to a 2.8 kbp *Xho*I fragment carrying only the *actI* locus revealed that only this locus was required for aloesaponarin II biosynthesis by *S. galilaeus* ATCC 31133. However, *actI* did not support the synthesis of aloesaponarin II in *S. azureus*, suggesting that *S. galileus* 31133 provided the *actIII*, *actVII* and *actIV* activities. Thus, under those circumstances aloesaponarin II formation in *S. galilaeus* is the result of cooperation between genes from the donor and from the host.





VI: R=H, aloesaponarin II

VII: R=OH, desoxyerythrolaccin

VIII: R=H, aklavinone

IX: R=OH, 2-hydroxyaklavinone

When the DNA fragment encoding only the *actI* locus was cloned into *S. galilaeus* ATCC 31671, a mutant producing 2-hydroxyaklavinone (**IX**) instead of aklavinone (**VIII**), the formation of a different anthraquinone

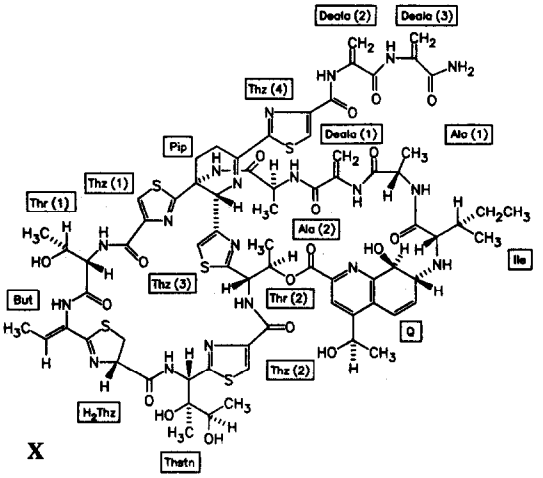
was observed. This compound, desoxyerythrolaccin (**VII**), carries an additional hydroxy group. Based on the proposal by Hallam *et al.* (13) that the *actIII* gene encodes a reductase that reduces a β-keto group in the polyketide chain to the secondary alcohol, resulting in loss of the oxygen function at that position, it was surmised that *S. galilaeus* 31671 lacked the gene equivalent to *actIII*. Hence, cloning of the *actI* locus into that background should indeed result in an anthraquinone carrying an extra hydroxy group.

The correctness of this hypothesis was confirmed by cloning the *actIII* gene into *S. galilaeus* 31671. As expected, the resulting transformant produced aklavinone instead of 2-hydroxyaklavinone, indicating that the *actIII* gene can function in place of its missing homolog from the anthracyclinone pathway. One of the intriguing prospects opened up by this finding is the possibility of converting any anthracycline producing strain into a producer of the corresponding 2-hydroxyanthracyclines by specific inactivation of the *actIII*-homologous gene.

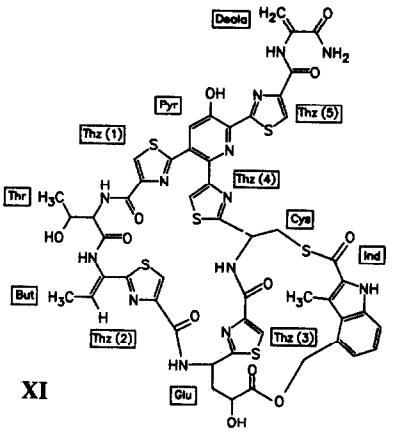
These observations clearly demonstrate the potential for using interspecies cloning of antibiotic biosynthesis genes in streptomycetes in a rational and predictive way to generate new hybrid natural product structures. What is needed to implement this approach is a thorough understanding of the relationships between genes and their functions in specific antibiotic biosynthetic pathways. In addition, implementation of such an approach requires an understanding of the regulatory mechanisms which govern the expression of antibiotic biosynthesis genes in streptomycetes (14). This is exemplified, for example, by the fact that cloning of the *actI*, *actIII*, *actVII* and *actIV* genes into *S. azureus* (not known to produce any polyketide antibiotics) resulted in production of aloesaponarin II whereas cloning of the same genes into *S. actuosus* (also not a producer of polyketide antibiotics) did not result in formation of the same metabolite. Expression of the *act* genes is controlled by a regulatory gene, *actII* (13,14); apparently, *S. azureus* carries a gene functionally homologous to *actII* whereas *S. actuosus* does not.

4. Thiopeptide Antibiotics

As a second major area in which to explore the potential of the genetic engineering approach to hybrid antibiotic production, we embarked on studies on a family of antibiotics called the thiopeptides. This family, as exemplified by its parent, thiostrepton (**X**)(15), is characterized by a high sulfur content and by a highly modified peptide structure incorporating a macrocyclic ring containing several thiazole units. In addition to thiostrepton and a series of closely related structures, this family includes nosiheptide (**XI**)(16), a slightly simpler molecule of similar architecture, which exhibits the same mode of action and has found use in agriculture as a feed additive to promote weight gains in animals (17). The thiopeptide antibiotics all inhibit protein synthesis in gram-positive bacteria by binding to the 23 S rRNA and one of the ribosomal proteins; resistance to these antibiotics is conferred by a resistance gene which codes for a methyltransferase that specifically methylates the 2´-position of a particular adenosine residue in the 23 S rRNA (18). The initial discovery of the thiopeptide antibiotics caused considerable excitement because of their broad and potent antibiotic spectrum; however, this excitement quickly faded when it was found that the compounds are not very active *in vivo*. Their water



X



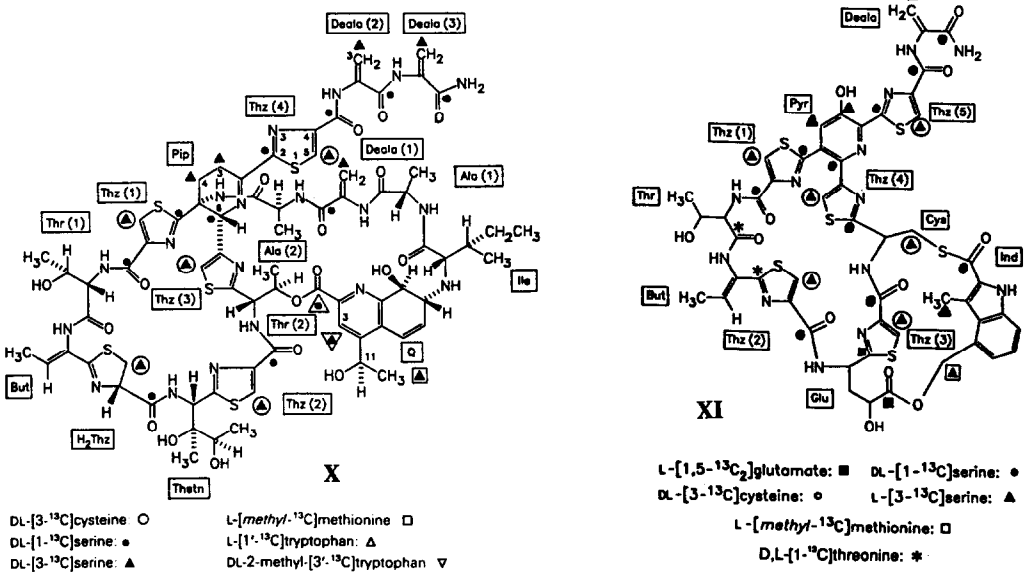
XI

Structures of the thiopeptide antibiotics, thiostrepton (**X**) and nosiheptide (**XI**)

insolubility prevents parenteral use and their resorption from the gastrointestinal tract when given orally is very slow, resulting in slower build-up of serum levels of the antibiotic than required to kill pathogens before they acquire resistance. Obviously, this makes these compounds targets for structure modification in order to improve their solubility or pharmacokinetic properties. The complexity of their structures, however, makes chemical structure modification all but impossible, pointing to biological structure modification as the most logical alternative. Thus, we embarked on a broad program aimed at developing methodology for the biological structure modification of thiopeptide antibiotics. The components of this program are (i) analysis of the biosynthetic pathway to thiopeptide antibiotics, particularly identification of the basic precursors and intermediates in the pathway, and isolation of enzymes catalyzing individual reaction steps, (ii) development of the molecular genetics of the producing organisms, *S. azureus* and *S. laurentii* (thiostrepton) and *S. actuosus* (nosiheptide) and cloning of the genes coding for the biosynthetic enzymes, (iii) establishment of the essential structural elements required for antibiotic activity and, by inference, of those parts of the molecules which can be altered without loss of activity, (iv) interspecies cloning of thiopeptide biosynthesis genes between producers of different compounds with the aim of generating transformants which produce new hybrid structures. So far, we have been working on objectives (i) and (ii), and the following gives a progress report on those studies.

Identification of the basic building blocks of these antibiotics and delineation of many aspects of their biosynthetic pathways involves the feeding of stable isotope-labeled precursors followed by NMR analysis of the labeled products. This requires, first of all, a complete and unambiguous assignment of all the relevant signals in the NMR spectra of the target compounds, **X** and **XI**. This task, which used to be very formidable, is now accomplished fairly readily using modern 2-D and inverse detection NMR methods (19). With these NMR assignments in hand, feeding of various ^{13}C-labeled amino acids resulted in the labeling patterns shown in Figure 1 (20,21). It was thus established that the dehydroalanine moieties of both compounds arise by

Figure 1. Labeling patterns of thiostrepton (X) and nosiheptide (XI) after feeding ^{13}C-labeled precursors.



dehydration of serine. Threonine gives rise to the threonine moieties as well as the dehydrobutyrine moieties in both compounds. The isoleucine and dihydroxyisoleucine moieties in **X** as well as the hydroxyglutamic acid moiety in **XI** arise from the corresponding parent amino acids. The thiazole moieties in both antibiotics arise from an intact molecule of cysteine plus the carboxyl group of the next amino acid in the peptide, the latter giving rise to C-2 of the thiazole ring.

Nosiheptide carries in an extra loop attached to the macrocyclic ring an unique trisubstituted indole moiety. This structure has not been encountered in nature before, and its biosynthetic origin was not obvious from inspection of the structure. Feeding experiments with labeled precursors (20) established that this moiety arises by a novel rearrangement of a molecule of tryptophan in which the carboxyl group of the side chain is attached to C-2 of the indole ring, followed by expulsion of the α-carbon and the attached nitrogen and reduction of the β-carbon to a methyl group. In addition, C-4 of the indole moiety undergoes C-methylation, presumably followed by hydroxylation. A number of potential intermediates in the transformation of tryptophan into this indolic acid moiety were synthesized and tested for their incorporation (22). The results indicated that methylation in the 4-position of the indole is not the first step in the transformation, but that both 3-methylindole-2-carboxylic acid and 3,4-dimethylindole-2-carboxylic acid are specific precursors of the indolic acid moiety. However, 4-hydroxymethyl-3-methylindole-2-carboxylic acid was not incorporated very efficiently, suggesting that the indolic acid precursor is attached to the peptide backbone at the stage of the 3,4-dimethyl compound. A hypothetical pathway was envisioned (Figure 2) involving acylation of the 2 position of the indole by the side chain carboxyl group to give a tricyclic intermediate (**XII**) which then undergoes fragmentation to 3-methylindole-

2-carboxylic acid. However, the tricyclic compound **XII**, when synthesized with a ^{13}C-label in the molecule, was not at all incorporated into the indolic acid moiety of nosiheptide. Another possible mechanism would

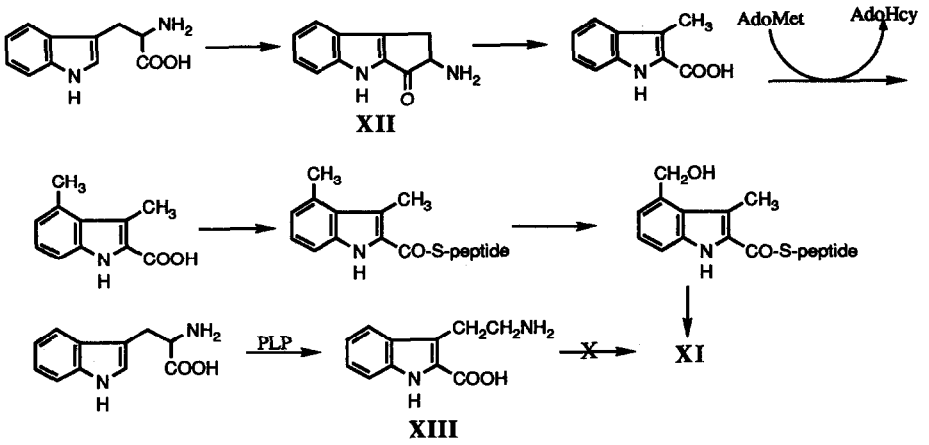


Figure 2. Hypothetical pathway to the indolic acid moiety of nosiheptide.

involve a pyridoxal phosphate-catalyzed decarboxylation and transfer of the carboxyl group to C-2 of the indole. The resulting compound, tryptamine-2-carboxylic acid (**XIII**), was also synthesized, carrying an ^{15}N label in the indole nitrogen, but was again not incorporated into the indolic acid moiety of nosiheptide. Although these results cast doubt on either of the two mechanisms, neither one is ruled out conclusively, because the hypothetical intermediates may exist only in an enzyme bound state, and the entire transformation may be carried out in a concerted fashion on a single enzyme. Obviously, further studies at the enzymatic level will be necessary to clarify this issue. Such studies have become possible by the identification of key intermediates in the transformation and are now in process.

Thiostrepton also carries a structurally unique moiety in the extra loop attached to the peptide backbone. This moiety is a substituted quinolinic acid carrying a hydroxyethyl side chain. The origin of this moiety, again, was traced to tryptophan as the precursor amino acid (21). Tryptophan undergoes a ring expansion from the 5-membered indole to the 6-membered quinoline system by cleavage of the bond between the indole nitrogen and C-2, and reconnection of the indole nitrogen to the α-carbon of the side chain. In this fashion, tryptophan accounts for all the carbon atoms of the quinaldic acid moiety of **X** except the methyl group of the hydroxyethyl side chain which originates from the methyl group of methionine. 4-Hydroxyethylquinolinic acid was found to be a specific precursor of the quinaldic acid moiety (23), indicating that the transformations of tryptophan up to this point occur prior to attachment to the peptide backbone. In contrast to the biosynthesis of the indolic acid moiety of **XI**, it was found that in this case methylation of tryptophan at the 2-position of the indole ring is the first step in the reaction sequence (21). This methylation reaction has been observed in a cell-free system and some of its characteristics have been examined (24).

Both **XI** and **X** contain a pyridine or tetrahydropyridine moiety which are located at the critical juncture of the macrocyclic ring system. The feeding experiments (20,21) revealed that this moiety arises from two molecules of serine, which are carbon-carbon linked through their carbon atoms 3, and the carboxyl group of an adjacent cysteine moiety. Intact incorporation of the two serine moieties was confirmed by experiments with double-labeled precursors followed by ^{13}C-^{13}C coupling analysis (21-23). A possible mechanism for the assembly of the pyridine/tetrahydropyridine ring from these precursors, based on an earlier proposal by Bycroft and Gowland (25), might involve the conversion of both serine moieties into dehydroalanine followed by an intramolecular hetero-Diels-Alder reaction or a similar process.

To obtain more information on the mode of assembly of this pyridine/tetrahydropyridine ring as well as the mechanism of some of the other reactions involved in the assembly of these two antibiotics, we traced the fate of the two hydrogens from C-3 of serine in these two biosyntheses. This involved the feeding of [3-^{13}C,$^{2}H_2$]serine and 3*S*[3-^{13}C,$^{2}H_1$]serine followed by product analysis by ^{13}C-NMR with broad band ^{1}H and either broad band or single frequency ^{2}H-decoupling and by ^{1}H-^{13}C correlation spectroscopy with deuterium decoupling (22,23). The results of this analysis revealed that the dehydration of serine to dehydroalanine proceeds by an *anti* elimination of water, that the formation of the thiazole rings and of the quinaldic acid moiety of thiostrepton proceeds with loss of the pro-*S* and retention of the pro-*R* hydrogen and that the dihydrothiazole ring in thiostrepton still contains both deuterium atoms from C-3 of serine. The latter finding indicates that the cysteine residue in the peptide chain giving rise to this dihydrothiazole must have D configuration. Finally, these experiments established the hydrogen stereochemistry at the two carbons of the tetrahydropyridine ring derived from C-3 of serine as shown in Figure 3, establishing specific boundary conditions for any mechanism invoked for the assembly of this ring system.

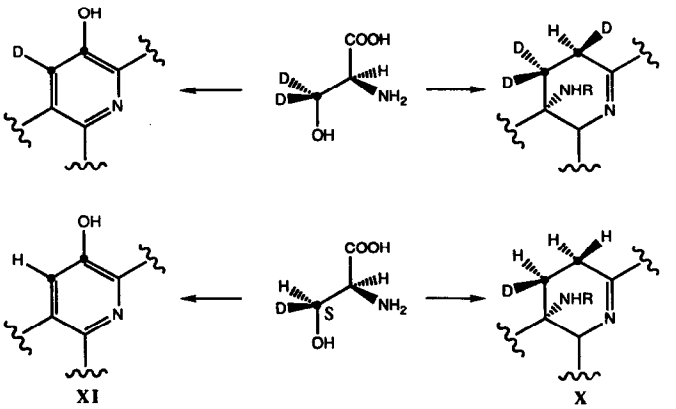


Figure 3.
Stereochemistry of deuterium labeling of the pyridine/tetrahydropyridine rings of **X** and **XI** from 3-deuterated serine samples.

From the results of these studies minimal sequences of peptide precursors for nosiheptide and thiostrepton can be written. In addition to the amino acids retained as such or in modified form in the final antibiotics, the original precursor peptide very likely carries at least one additional carboxy-terminal serine moiety, and possibly additional amino acids. This follows from the finding by ^{15}N-labeling experiments followed by ^{15}N-NMR

spectroscopy that the terminal amide nitrogen of **XI** arises specifically from the amino acid serine (22). In analogy to the formation of the terminal amide function of some of the pituitary peptide hormones in animals (26), it is assumed that this serine residue is attached to the carboxy terminus of the peptide and is then removed by an oxidative process leaving behind its nitrogen. The precursor peptide for **XI** thus must be at least 13 amino acids long and, by analogy, that for **X** 18 amino acids. The available evidence points to an enzyme-mediated rather than a ribosomal process for the assembly of these precursor peptides. This follows from the fact that chloramphenicol at concentrations which shut down protein synthesis does not immediately block the formation of either **XI** or **X**. More importantly, oligonucleotide probes against partial sequences of the nosiheptide or thiostrepton precursor peptides, selected to reflect the codon useage bias of streptomycetes, failed to hybridize to any fragments in partial digests of the DNA from the corresponding producing organism (27).

Based on the information available to date, one can estimate that the biosynthesis of nosiheptide and thiostrepton requires on the order of about 20 enzymes each, one of which, the peptide synthase, may have a rather high molecular weight (28). Together with regulatory genes, between 20 and 30 kbp of DNA are thus probably required to code for each of these biosyntheses. We have now embarked on the task of cloning these genes and identifying their individual functions. The strategy employed is based on the assumption that, in analogy to all the *Streptomyces* antibiotic biosynthesis pathways examined so far, the genes coding for nosiheptide and thiostrepton biosynthesis, respectively, are clustered on the chromosome of the producing organism, and that this cluster also contains the gene conferring upon the organism resistance to the antibiotic it produces (29). Hence, we started this effort by cloning from the nosiheptide producer, *S. actuosus*, an 8.5 kbp *Bam*HI DNA fragment which conferred nosiheptide resistance upon the sensitive host, *S. lividans* (30). Further subcloning of the nosiheptide resistance gene on a 2.4 kbp *Pst*I-*Bam*HI fragment which was subsequently sequenced, revealed the presence of two open reading frames located close to each other and reading in the same direction (31). One of these, *nshR*, showed a very high degree of homology to the previously cloned (32) and sequenced (33) thiostrepton resistance gene. The second open reading frame contained 699 nucleotides and was separated from *nshR* by a short stem loop region. Comparison of the deduced amino acid sequence of the protein coded by *orf699* with protein sequence data bases revealed the presence of an α-helix-β-turn-α-helix motif which is typical of many DNA binding proteins (31). This suggested that *orf699* may code for a regulatory protein. Evidence for a regulatory function of *orf699* (now termed *nshA*) is provided by the finding that *nshA* mutants generated from the *S. actuosus* wild-type by integrating a mutated form of *nshA* in a pIJ101-derivative plasmid into the genome by protoplast-curing of the plasmid showed an altered phenotype (28). These mutants initially overproduced **XI** and upon complementation with wild-type *nshA* regained normal levels of **XI** production. However, the mutants were unstable, reverting after about 5 transfers to low or no nosiheptide production, very likely by a second suppressor mutation. Further evidence for a regulatory role of *nshA* in nosiheptide biosynthesis comes from the unusual relationship between it and the *nshR* gene. Transcription of *nshR* occurs not only from its own promoter, but also from two promoters upstream of *nshA* in a polycistronic message (31). Since a hairpin

loop separates *nshR* from *nshA* this suggests the possible involvement of an antiterminator function to allow read-through of this structure. One of the promoters upstream of *nshA* is active only between 44 h and 140 h (with maximal activity at 96 h), indicating temporal regulation of this promoter coincident with the time frame of nosiheptide production (28).

Sequencing of the additional 1.1 kbp *Pst*I-*Pvu*II DNA fragment downstream of *nshR* revealed the presence of two additional potential open reading frames, *orfB* and *orfC*, which read in the opposite direction from *nshR*. It seems likely that at least one of these, *orfC*, may code for one of the biosynthesis genes of the nosiheptide pathway. This follows from hybridization experiments with three probes representing three different regions of the sequenced *S. actuosus* DNA against the DNA from a variety of other streptomycetes (Figure 4). Probe

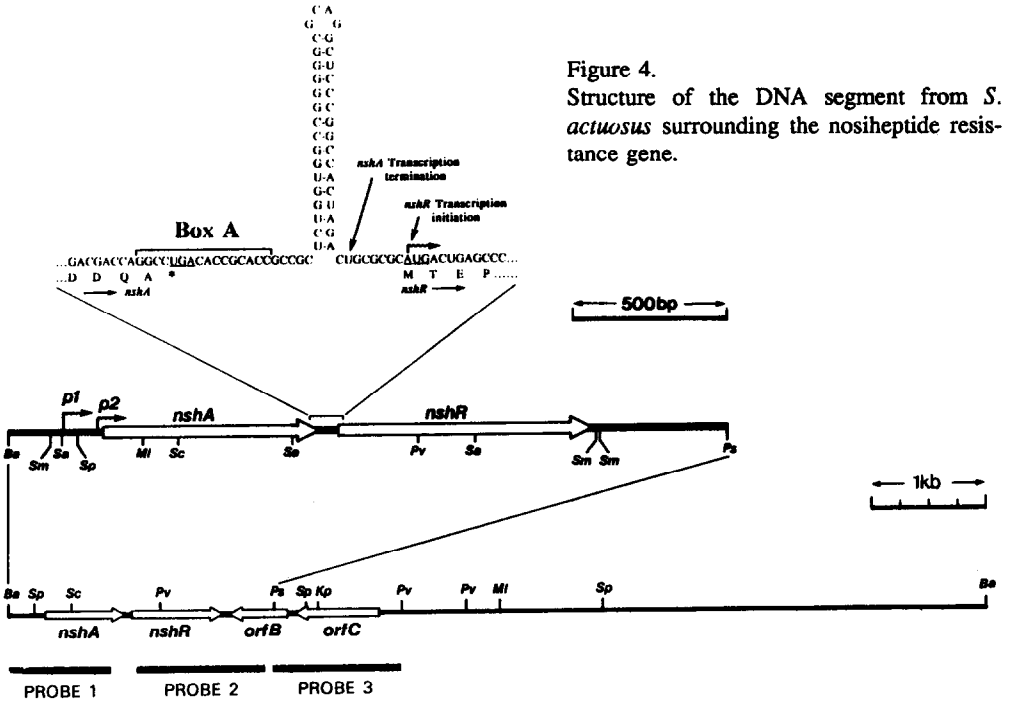


Figure 4.
Structure of the DNA segment from *S. actuosus* surrounding the nosiheptide resistance gene.

1 (from the *Bam*HI site at nt1 to nt844 of *nshA*; see ref. 31), representing *nshA*, hybridized against all thiopeptide antibiotic producers and to a majority of other streptomycetes which are not producers of thiopeptide antibiotics. Thus, regulatory genes homologous to *nshA* are ubiquitous in streptomycetes. Probe 2 representing *nshR* and part of *orfB* (from nt1066 to the *Pst*I site at nt2326) hybridized against all producers of **X**, **XI** and other thiopeptides, indicating that homologous resistance genes are present in all thiopeptide producers. Probe 3 representing *orfB* and *orfC* (1.1 kpb *Pst*I to *Pvu*II fragment; see Fig. 4), however, hybridized only against the three species of *Streptomyces* known to produce **XI**, not against other thiopeptide producers or streptomycetes not producing thiopeptides. Hence, it seems very likely that *orfC* may be very specifically involved in nosiheptide

biosynthesis (28). Attempts are now underway to define its function in the biosynthetic pathway by inactivating the corresponding gene in wild-type *S. actuosus*. If this should result in loss of **XI** production (and possibly accumulation of an intermediate) this would confirm our assumption that the biosynthesis genes are clustered around the resistance gene and would open the way for the cloning of the additional pathway genes by chromosome walking.

Efforts have also been initiated to clone by a similar strategy the genes coding for the biosynthesis of **X**. Once the genetic work on both these pathways is sufficiently advanced, the stage will be set for attempts to realign the chemical capabilities of the two organisms resulting hopefully in the production of novel hybrid antibiotic structures.

5. Outlook

The genetic approach to the generation of new hybrid natural product structures is still very much in its infancy. A far more detailed understanding of gene structure, gene function and gene regulation in streptomycetes will have to be developed in order to use this approach in a rational and predictive fashion. However, the potential clearly exists to expand greatly on the existing range of structures produced in streptomycetes by constructing altered biosynthetic pathways through interspecific cloning of antibiotic biosynthesis genes. Given the rapid advances in *Streptomyces* genetics in recent years, it hopefully will not be very long until the full potential of this approach can be realized.

6. Experimental Procedures

Feeding experiments were carried out with shake cultures of *S. actuosus* ATCC 25421 (nosiheptide) and *S. laurentii* ATCC 31225 (thiostrepton) as previously described (20). The purified samples of the labeled antibiotics were analyzed by NMR spectroscopy (19) on an IBM AF-300 spectrometer. The labeled precursors were obtained commercially, synthesized chemically (22,23) or prepared microbiologically by C. Unkefer, Los Alamos Stable Isotope Resource (21).

The cloning of the nosiheptide resistance gene and the sequence analysis of it and *nshA* have been described (31). The same sequencing techniques (34,35) were used to determine the nucleotide sequence of the 1.1 kbp *Pst*I-*Pvu*II DNA segment downstream of *nshR*, which contains the C-terminus of *orfB* and all of *orfC*. Hybridization experiments with DNA probes 1, 2 and 3 (Figure 4) were carried out at a stringency allowing for ca. 80% homology (two washes with 0.5 x SSC and 0.2% SDS for 30 min each at 68° C). Time-dependent transcriptional analyses were carried out by high-resolution S1 mapping using the Na•TCA method of Murray (36), and are described in greater detail elsewhere (28).

Acknowledgements

The authors thank a large and enthusiastic group of co-workers who are listed in the appropriate references and deserve all the credit for the experimental work reported here. Financial support of this work by the NIH (AI 20264) and NSF (DMB 8607619 and DMB 8904133) as well as the services of the Los Alamos Stable Isotope Resource (supported by NIH grant RR02231) are gratefully acknowledged.

References and Notes

1) W. Charney, H.L. Herzog "Microbial Transformations of Steroids", Academic Press, New York, 1967.

2) K. Kieslich "Microbial Transformations of Non-Steroidal Cyclic Compounds", John Wiley and Sons/Georg Thieme Publishers, Stuttgart, 1976.

3) O.K. Behrens, J. Corse, J.P. Edwards, L. Garrison, R.G. Jones, Q.F. Soper, F.R. van Abeele and C.W. Whitehead *J. Biol. Chem.* **1948**, *175*, 793; W.J. Halliday and H.R.V. Arnstein *Biochem. J.* **1956**, *64* 380.

4) K.L. Rinehart, Jr. *Pure and Appl. Chem.* **1977**, *49*, 1361.

5) S. Ōmura, H. Ikeda, H. Matsubara and N. Sadakane *J. Antibiot.* **1980**, *33*, 1570.

6) D.A. Hopwood, F. Malpartida, H.M. Kieser, H. Ikeda, J. Duncan, I. Fujii, B.A.M. Rudd, H.G. Floss and S. Ōmura *Nature (London)* **1985**, *314*, 642; S. Ōmura, H. Ikeda, F. Malpartida, H.M. Kieser and D.A. Hopwood *Antimicrob. Agents Chemother.* **1986**, *29*, 13.

7) F. Malpartida and D.A. Hopwood *Nature (London)* **1984**, *309*, 462.

8) J.B. McAlpine, J.S. Tuan, D.P. Brown, K.D. Grebner, D.N. Whittern, A. Buko and L. Katz *J. Antibiot.* **1987**, *40*, 1115.

9) J.K. Epp, M.L. Huber, J.R. Turner and B.E. Schoner in "Genetics and Molecular Biology of Industrial Microorganisms", C.L. Hershberger, S.W. Queener and G. Hegeman, Eds., Amer. Soc. Microbiol., Washington, D.C., 1989, pp. 35-39.

10) B.A.M. Rudd and D.A. Hopwood *J. Gen. Microbiol.* **1979**, *114*, 35.

11) H.G. Floss, S.P. Cole, X.-G. He, B.A.M. Rudd, J. Duncan, I. Fujii, C.-j. Chang and P.J. Keller in "Regulation of Secondary Metabolite Formation", H. Kleinkauf, H.v. Döhren, H. Dornauer and G. Nesemann, Eds., VCH Verlag, Weinheim, 1985, pp. 283-304; S.P. Cole, B.A.M. Rudd, D.A. Hopwood, C.-j. Chang and H.G. Floss *J. Antibiot.* **1987**, *40*, 340.

12) P.L. Bartel, C.-B. Zhu, J.S. Lampel, C.D. Dosch, N.C. Connors, W.R. Strohl, J.M. Beale and H.G. Floss *J. Bacteriol.* **1990**, *172*, 4816; W.R. Strohl, P.L. Bartel, N.C. Connors, C.-B. Zhu, D.C. Dosch, J.M. Beale, H.G. Floss, K. Stutzmann-Engwall, S.L. Otten and C.R. Hutchinson in "Genetics and Molecular Biology of Industrial Microorganisms", C.L. Hershberger, S.W. Queener, G. Hegeman, Eds., Amer. Soc. Microbiol., Washington, D.C., 1989, pp. 68-84.

13) S.E. Hallam, F. Malpartida and D.A. Hopwood *Gene* **1988**, *74*, 305.

14) W.R. Strohl, P.L. Bartel, Y. Li, N.C. Connors and R. Woodman *J. Industrial Microbiol.*, in press.

15) J.F. Pagano, M.J. Weinstein, M.A. Stout and R. Donovick, *Antibiot. Ann.* **1956**, 554; B. Anderson, D. Crowfoot Hodgkin and M.A. Viswamitra *Nature (London)* **1970**, *225*, 233.

16) T. Prange, A. Ducruix, C. Pascard and J. Lunel, *Nature (London)* **1977**, *265*, 285; T. Endo and H. Yonehara *J. Antibiot.* **1978**, *31*, 623; C. Pascard, A. Ducruix, J. Lunel and T. Prange *J. Amer. Chem. Soc.* **1977**, *99*, 6418.

17) F. Benazet, M. Cartier, J. Florent, C. Godard, G. Jung, J. Lunel, D. Manet, C. Pascal, J. Renaut, P. Tarridec, J. Theilleux, R. Tissier, M. Dubost and L. Ninet, *Experientia* **1980**, *36*, 414; L. Keppens and G. DeGrote *Rev. Agric. (Brussels)* **1979**, *32*, 159.

18) E. Cundliffe and J. Thompson *J. Gen. Microbiol.* **1981**, *126*, 185.

19) U. Mocek, L.-C. Chen, P.J. Keller, D.R. Houck, J.M. Beale and H.G. Floss *J. Antibiot.* **1989**, *42*, 1643; U. Mocek, J.M. Beale and H.G. Floss *J. Antibiot.* **1989**, *42*, 1649.

20) D.R. Houck, L.-C. Chen, P.J. Keller, J.M. Beale and H.G. Floss *J. Amer. Chem. Soc.* **1988**, *110*, 5800.

21) P. Zhou, D. O'Hagan, U. Mocek, Z. Zheng, L.D. Yuen, T. Frenzel, C. Unkefer, J.M. Beale and H.G. Floss *J. Amer. Chem. Soc.* **1989**, *111*, 7274.

22) R. Tsuchiya, Ph.D. dissertation, University of Washington, Seattle, 1991.

23) Z. Zeng, Ph.D. dissertation, Ohio State University, Columbus, 1990.

24) T. Frenzel, P. Zhou and H.G. Floss *Arch. Biochem. Biophys.* **1990**, *278*, 35.

25) B.W. Bycroft and M.S. Gowland *J.C.S., Chem. Comm.* **1978**, 256.

26) C.C. Glembotski, B.A. Eipper and R.E. Mains *J. Biol. Chem.* **1984**, *259*, 6385.

27) R.H. Woodman, Ph.D. dissertation, Ohio State University, Columbus, 1991.

28) Y. Li, D.C. Dosch, R.H. Woodman, H.G. Floss and W.R. Strohl *J. Industrial Microbiol.*, in press.

29) J.F. Martin and P. Liras *Annu. Rev. Microbiol.* **1989**, *43*, 173.

30) D.C. Dosch, W.R. Strohl and H.G. Floss *Biochem. Biophys. Res. Comm.* **1988**, *156*, 517.

31) Y. Li, D.C. Dosch, W.R. Strohl and H.G. Floss *Gene* **1990**, *91*, 9.

32) C.J. Thompson, T. Kieser, J.M. Ward and D.A. Hopwood *Gene* **1982**, *20*, 51.

33) M.J. Bibb, M.J. Bibb, J.M. Ward and S.N. Cohen *Mol. Gen. Genet.* **1985**, *199*, 26.

34) R. Kraft, J. Tardiff, K.S. Krauter and L.A. Leinwand *Biotechniques* **1988**, *6*, 544.

35) H. Zhang, R. Scholl, J. Browse and C. Somerville *Nucleic Acids Res.* **1988**, *16*, 1220.

36) M.G. Murray *Anal. Biochem.* **1986**, *158*, 165.