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Review Article

Studies on the Biosynthesis of Antibiotics¹

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Chlorothricin; Granaticin; Naphthocyclinone.

Abstract

The biosynthesis of a number of antibiotics which have been studied in this laboratory is reviewed. These in-

clude indolmycin, pyrrolnitrin, dihydrophenylalanine, spectinomycin, chlorothricin, α -naphthocyclinone and granaticin. The results on the stereochemistry of some reactions involved in these biosyntheses are also discussed and these include the stereochemical course of the transfer of a methyl group from S-ade-

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nosylmethionine to the β -carbon of indolepyruvate and the stereochemistry of the reductive removal of the hydroxyl functions in the formations of 2,6-dideoxyhexoses.

Introduction

During the past decade our laboratory has engaged in studies on the biosynthesis of a number of microbial fermentation products with antibiotic activity. The compounds selected for investigation represent various structural types which were selected for various reasons. The choice of some of them reflects our interest in the metabolic conversions of tryptophan and related indolic precursors [16], and in the reactions of the shikimate pathway [14, 17] of aromatic amino acid biosynthesis. Some also reflect our interest in applying modern instrumental techniques, particularly NMR spectroscopy, to the study of biosynthetic processes [15]. The following article summarizes some of the results we have obtained in these studies.

Results

Indolmycin

This is the first antibiotic which we studied and its biosynthesis was investigated in collaboration with the group of Professor HORNEMANN of this department. The structure of indolmycin (Fig. 1) and its relative stereochemistry were established by SCHACH VON WITTENAU et al. [44] and by PREOBRAZHENS-KAYA et al. [40], and its absolute con-

Fig. 1. Structure of indolmycin.

figuration was determined by CHAN and HILL [6] as well as in our laboratory [24]. The compound is produced by *Streptomyces griseus* and has moderate antibiotic activity against resistant strains of *Staphylococcus* in mice. Early studies on the biosynthesis of this compound [24] showed that the indole ring and three carbons of the attached side chain are derived from the intact carbon chain of tryptophan and the carbon-bound methyl group of the side chain originates from methionine. The latter also provides the N-methyl group, the remaining carbon and two nitrogen atoms of the aminooxazolinone system coming from the amidino group of arginine (Fig. 2). The biosynthetic pathway (Fig. 3) involves transamination of tryptophan to indolepyruvate followed by methylation of the latter to give the R-isomer of 3-methylindolepyruvic acid. The two enzymes catalyzing these first two reactions have been isolated from *Streptomyces griseus* in our laboratory and the second one, a S-adenosylmethionine-dependent methyltransferase has been partially purified and characterized [53]. The subsequent steps in the biosynthetic sequence are reduction of the carbonyl group of methylindolepyruvate to give indolmycenic acid, followed by transfer of the guani-

BUILDING
BLOCKS OF
INDOLMYCIN

Fig. 2. Building blocks of indolmycin.

dino group of arginine to give the oxazolinone ring system of N-demethyl-indolmycin, which is then N-methylated in the last reaction step. Experiments using arginine labeled with ^{15}N in the amidino group have shown that the latter is transferred as an intact unit, i.e., without separating the two nitrogen atoms [61]. This conclusion follows from the finding that as indicated by mass spectral analysis of the precursor and the product, there is no change of the intramolecular ^{14}N - ^{15}N distribution during the transfer of the amidino group.

In a recent study we have determined the stereochemical course of the transfer of the methyl group from S-adenosylmethionine to the acceptor, indolepyruvate during this biosynthesis. Using tryptophan stereospecifically tritiated

at carbon atom 3 of the side chain which was available from earlier work [50], we were able to demonstrate that in the reaction the pro-R hydrogen at the methylene group of indolepyruvate is replaced by the methyl group with retention of configuration [61] (Fig. 4). The determination of the stereochemical course of the reaction at the methyl group required the synthesis of methionine carrying a chiral methyl group. This was accomplished starting from sodium acetate carrying a chiral methyl group by the reaction sequence outlined in Fig. 5 [32]. The key step is the alkylation of the homocysteine anion by the N,N-ditosylate of methylamine. This reaction is expected to proceed with inversion of configuration at the methyl group, the only alternative being racemization, in case the reaction pro-

Fig. 3. Pathway of indolmycin biosynthesis.

Fig. 4. Stereochemistry of the C-methylation in indolmycin biosynthesis at the acceptor carbon.

Fig. 5. Synthesis of methionine carrying a chiral methyl group.

ceeds by an $\text{S}_{\text{N}}1$ mechanism. Thus, if the methyl group in methionine is chiral, its absolute configuration follows from that of the starting acetate. Feeding of the methionine samples carrying a methyl group of R- or S-configuration followed by oxidation of the resulting indolmycin gave acetate from the C-methyl group of the antibiotic, which was analyzed for its chirality by the method of Arigoni et al. and CORNFORTH et al. [30, 10]. The results which are summarized in Table I, indicate

that the methylation of indolepyruvate proceeds with inversion of configuration at the methyl group. Since it is likely that any one transfer of the methyl group from a donor to an acceptor in the enzymatic process proceeds with inversion of configuration, this result suggests that the overall reaction involves a direct transfer of the methyl group from the sulphur of S-adenosylmethionine to the acceptor carbon without any intermediate alkylation of a functional group of the enzy-

Fig. 6. Summary of the stereochemical course of the C-methylation reaction involved in indolmycin biosynthesis.

Table I

Steric course of the S-adenosylmethionine: indolepyruvate methyl transferase reaction.

	Starting acetate	Methionine	Acetate from indolmycin	Indolmycin C-methyl group
Tritium retention in fumarase reaction	31 %	—	39 %	—
Methyl group configuration	S	R	S	S
Tritium retention in fumarase reaction	71 %	—	63 %	—
Methyl group configuration	R	S	R	R

me. The stereochemical results of this C-methylation reaction are summarized in Fig. 6.

Pyrrolnitrin

The antifungal antibiotic pyrrolnitrin (Fig. 7) is produced by various species of *Pseudomonas* and was discovered independently by ARIMA et al. [1] and by LIVELY et al. [29]. Its structure was elucidated by ARIMA's group [25] and has been confirmed by several syntheses [38, 56, 21] and by an X-ray analysis

PYRROLNITRIN

Fig. 7. Pyrrolnitrin

[35]. Pyrrolnitrin shows good fungistatic activity against a variety of dermatophytes, for example, *Trichophyton* species [39, 19]; it is suitable for topical application, for example, in athlete's

Fig. 8. Proposed pathway of pyrrolnitrin biosynthesis according to GORMAN and LIVELY (Ref. 20).

foot, etc. for which purpose it is marketed in Japan. Early studies on the biosynthesis of pyrrolnitrin carried out at Eli Lilly and Company [12] showed that of a number of compounds tested, only D-tryptophan- ^{14}C , and to a lesser extent the L-isomer, was incorporated into the antibiotic. Addition of tryptophan to the cultures also increased pyrrolnitrin production, but only the D-isomer was active in this respect. To account for the transformation of tryptophan into pyrrolnitrin GORMAN and LIVELY [20] proposed the pathway shown in Fig. 8. The first step was thought to be catalyzed by a chloroperoxidase. Investigations into the biosynthesis of pyrrolnitrin were continued in our laboratory in collaboration with the Lilly workers. Using specifically ^{15}N -labeled tryptophan samples it was shown (Table II) that both the indole nitrogen and the amino nitrogen of tryptophan are incorporated. Mass spectral analysis of the ^{15}N -labeled pyrrolnitrin samples indicated that, as predicted by the scheme in Figure 9, the indole nitrogen of tryptophan becomes the nitrogen of the nitro group and the

amino nitrogen of tryptophan gives rise to the pyrrole nitrogen of pyrrolnitrin (Fig. 9) [13]. The amino nitrogen of D,L-tryptophan is incorporated to a considerably lesser extent than the backbone of the amino acid and an experiment with D,L-tryptophan tritiated in the α -position of the side chain gave results as shown in Table II. This can be adequately explained by reversible transamination of the precursor. Rather surprisingly, D-tryptophan undergoes an even more extensive loss of the label from the amino group and also tritium from the α -position, than the racemate, a finding which does not fit in with the earlier assumption that D-tryptophan is the more immediate precursor of tryptophan than the L-isomer. A systematic comparison of the incorporation of tritium from the α -position of D-, D,L-, and L-tryptophan showed clearly (Table III) that the L-isomer is incorporated with very high retention of the tritium, whereas the D-isomer gives pyrrolnitrin with almost complete loss of the tritium. In a very recent experiment we synthesized and fed [47] tryptophan labeled intramolecularly with ^{13}C in the

Table II

Incorporation of ^{15}N -labeled tryptophan into pyrrolnitrin

Precursor	Incorporation of			Retention of	
	^{14}C	^3H	^{15}N	^3H	^{15}N
D,L-Tryptophan-(indole-2- ^{14}C -1- ^{15}N)	10.05 %	—	10.7 %	—	107 %
D,L-Tryptophan-(alanine-3- ^{14}C -2- ^{15}N)	15.6 %	—	2.9 %	—	19 %
D,L-Tryptophan-(alanine-3- ^{14}C -2- ^3H)	16.1 %	4.2 %	—	26 %	—
D-Tryptophan-(alanine-3- ^{14}C -2- ^3H -2- ^{15}N)	18.0 %	0.36 %	1.76 %	2 %	10 %

Fig. 9. Mass spectral analysis of ^{15}N -labeled pyrrolnitrins.

3-position of the chain and with ^{15}N in the amino group. Mass spectral analysis of the resulting pyrrolnitrin samples clearly showed a $M+2$ peak in excess of natural abundance in the sample derived from the L-isomer of the substrate, whereas no such peak was observed in the spectrum of the material derived from the D-isomer of tryptophan. All these data strongly indicate that L- and not D-tryptophan is the more immediate precursor of pyrrolnitrin, despite the fact that under any condition tested, ^{14}C -labeled D-tryptophan is incorporated into pyrrolnitrin more efficiently than the L-isomer.

Another surprising facet of pyrrolnitrin biosynthesis is the fact that the compound contains a chlorine atom in the 3-position of the pyrrole ring, although substitution of pyrrole should occur preferentially in the 2-position. As an explanation we considered the possibility that the introduction of this chlorine may not be a late step in the biosynthesis, but rather that this chlorine atom might be carried over from the initial stages in the biosynthesis. A biosynthetic process involving a 1,2-aryl shift as outlined in Fig. 10 could account for this. To probe this possibility we synthesized tryptophan labeled with

Table III

Incorporation of α -tritiated tryptophan into pyrrolnitrin

Precursor	Incorporation of ^{14}C	^3H	Tritium retention
D-Tryptophan- (alanine-3- ^{14}C -2- ^3H)	15.8 %	0.52 %	3.3 %
D,L-Tryptophan- (alanine-3- ^{14}C -2- ^3H)	13.5 %	4.07 %	30.2 %
L-Tryptophan- (alanine-3- ^{14}C -2- ^3H)	9.6 %	6.85 %	71.3 %

Fig. 10. Two alternative routes of pyrrolnitrin formation.

^{13}C in the 3-position of the side chain, fed this material to the producing culture of *P. aureofaciens* and analyzed the resulting pyrrolnitrin for the position of the ^{13}C by CMR spectroscopy. The results (Fig. 11) indicated that the ^{13}C was located in carbon atom 3 as would be predicted by the normal path-

way and not at C-4 as would be predicted by the route involving an aryl shift [33]. The CMR spectrum of pyrrolnitrin was assigned on the basis of chemical shift theory, single frequency decoupling experiments and comparison with model compounds and the crucial assignment of carbon 3 and 4 was specifi-

Fig. 11. CMR analysis of pyrrolnitrin.

cally confirmed by comparing the spectra of pyrrolnitrin and its amino analog. The location of the ^{13}C in position 3 was later also confirmed by detailed analysis of the proton NMR spectrum of pyrrolnitrin. As shown in Fig. 12, the presence of ^{13}C can be clearly detected by analysis of the long range

carbon-proton couplings of the hydrogens at positions 2 and 5. The larger coupling constant of the H-2 proton compared to the H-5 proton and the greater isotopic shift of H-2 clearly indicates that the ^{13}C is located in the 3-position [8].

In another experiment we examined

Fig. 12. Proton NMR analysis of ^{13}C -labeled pyrrolnitrin.

the possibility that the biosynthesis of pyrrolnitrin may proceed via oxindole rather than indolenine intermediates. Tryptophan deuterated in the 2-position of the indole ring was prepared and fed to the producing microorganism. The resulting pyrrolnitrin was found to be enriched with deuterium. To further probe the mechanism of the skeletal rearrangement in pyrrolnitrin biosynthesis we synthesized compound I, the hydroxy analog of the intermediate postulated by GORMAN and LIVELY (Fig. 13). The compound was obtained by oxidation of ^{14}C -labeled D,L-tryptophan as a mixture of the four possible stereoisomers with a *cis* ring junction [45]. The compound was clearly not incorporated into pyrrolnitrin, whereas tryptophan in a parallel culture gave very good incorporation of radioactivity [31].

The later stages of pyrrolnitrin biosynthesis were investigated by preparing and feeding a number of amino and deschloro analogs of the antibiotic. Aminopyrrolnitrin, compound II, (Fig. 13) had been shown earlier by the Lilly group to be efficiently incorporated into pyrrolnitrin [22]. We synthesized in tritium labeled form, the deschloro analogs of both pyrrolnitrin and aminopyrrolnitrin and found that the amino compound (III, Fig. 13) is efficiently incorporated into pyrrolnitrin whereas the corresponding nitro compound is not (IV, Fig. 13) [47]. The results indicated that there must be a compulsory reaction sequence from 3-(o-aminophenyl) pyrrole involving first the introduction of the two chlorine substituents and finally as the last step, oxidation of the amino to the nitro group.

Fig. 13. Incorporation of potential precursors into pyrrolnitrin.

The results obtained allow us to write a more defined reaction sequence for the biosynthesis of pyrrolnitrin. The fact that the hydrogen from the α -position of the tryptophan side chain is incorporated puts restraints on the position of the decarboxylation step in the overall reaction sequence since it excludes any intermediates in which a carbon derived from the α -position of tryptophan has lost the attached hydrogen. Although the non-incorporation of compound II does not exclude the intermediacy of the corresponding chloro analog, the known chemistry of such systems (tendency to undergo elimination to form the corresponding indole system) argues against a pathway involving such 3-substituted pyrrolo-[2, 3b]indoles. A plausible alternative is suggested by the recent isolation of an enzyme from *Pseudomonas* species which hydroxylates tryptophan derivatives in the 3-position of the side chain

[55, 41]. Studies by ROSENFELD's group [42] which were confirmed in our laboratory [58] indicate that the enzyme oxidizes these indoles to a methyleneindolenine derivative which then undergoes hydration. The analogous process involving the methyleneindolenine derivative of tryptophan which would then give ring closure to the corresponding amidine as shown in Figure 17, could be plausibly involved in the biosynthesis of pyrrolnitrin. Fragmentation of this amidine could lead directly to 3-(o-aminophenyl)pyrrole, the established intermediate in the biosynthesis. Thus, we propose the sequence shown in Fig. 14 as a plausible biosynthetic pathway for pyrrolnitrin formation.

Dihydrophenylalanine

L-2,5-Dihydrophenylalanine (DHPA) is a rather ubiquitous constituent of Actinomycetes which has been isolated from a variety of *Streptomyces* species

Fig. 14. Proposed pathway for pyrrolnitrin formation.

by several groups [60, 46, 18]. The compound exerts antibiotic activity by virtue of its action as an antagonist of phenylalanine. Initial experiments on its biosynthesis carried out by SCANNELL et al. [46] showed that radioactive shikimic acid is incorporated, but not phenylalanine (Fig. 15). Thus, the compound seems to be formed by the shikimate pathway, but not via the aromatic amino acids. We have confirmed this result and have shown that shikimic acid-[U- ^{14}C] labels only the six ring carbons of DHPA, but none of the side chain carbons (Fig. 16) [49]. Serine was

not incorporated, ruling out a pathway involving the condensation of the C_6 precursor with a C_3 unit providing the side chain. These results point to a pathway via chorismic acid as an intermediate. Two processes are known in nature for the attachment of the side chain of chorismic acid to the ring, one leading to the normal aromatic amino acids with attachment of the side chain at C-1 and a second leading to the m-carboxyphenyl amino acids with attachment of the side chain at the original C-3 of shikimate [28]. A distinction between these two possibilities was made

Fig. 15. 2,5-Dihydrophenylalanine and its biosynthetic precursors.

Fig. 16. Incorporation of various precursors into 2,5-dihydrophenylalanine.

in two ways. Shikimic acid deuterated in the 3-position was prepared and was found to be incorporated into DHPA with significant retention of deuterium. The process involving attachment of the side chain at C-3 should result in loss of the deuterium. Secondly, shikimic acid-[1,6-¹⁴C] was fed and the resulting DHPA was degraded by a route involving aromatization of the six membered ring. About half of the ¹⁴C was found to be located in the position adjacent to the site of attachment of the side chain, whereas, the para position was practically devoid of ¹⁴C.

This is as expected for the „normal“ rearrangement process, whereas the attachment of the side chain at C-3 of shikimate should have given a product containing half of the ¹⁴C in the para position and none ortho to the site of the attachment of the side chain (Fig. 16). These results then implicate chorismic acid and prephenic acid as intermediates in the biosynthesis of DHPA. We confirmed this conclusion by preparing both compounds in radioactively labeled form and demonstrating that both are efficiently incorporated into DHPA.

It will be noted that both in the pre-

cursor shikimate and in the product DHPA, the six membered ring is asymmetric, whereas the biosynthetic intermediate prephenate is symmetric. However, the two halves of the six membered ring in prephenate are enantiotopic and can be expected to be distinguished by an enzyme. It was therefore of interest to determine which half of the ring of prephenate becomes which side of the ring in DHPA. This question was answered by carrying out an asymmetric degradation of DHPA derived from shikimic acid-[1,6- ^{14}C]. The side chain of DHPA was degraded to give 2-(2,5-dihydroxyphenyl)ethanol which was then subjected to ozonolysis and reduction with sodium borohydride. Analysis of the two products, propane-1,3-diol and pentane-1,3,5-triol, showed that the latter contained all of the radioactivity. Thus, the labeling pattern of DHPA from shikimic acid-[1,6- ^{14}C] is as shown in Fig. 17. There are several ways in which the formation of the unconjugated diene system of DHPA from prephenate can be rationalized. One possibility would be initial reduction of

one of the ring double bonds of prephenate to give dihydroprephenate. This possibility was ruled out by preparing a mixture of all four stereoisomers of dihydroprephenate tritiated at C-4, and demonstrating that this mixture is not incorporated into DHPA. By process of elimination this finding would favor the pathway shown in Fig. 20, which involves an allylic rearrangement of prephenate, 1,4-reduction of the conjugated diene and finally a combined dehydration/decarboxylation [49]. Further experiments will be necessary to prove or disprove this proposed reaction sequence from prephenate to DHPA.

Spectinomycin

The aminocyclitol antibiotic spectinomycin was isolated independently by the groups at Upjohn Company and at Abbott Laboratories from *Streptomyces* species. It is a broad spectrum antibiotic, active against gram-positive and gram-negative organisms and is marketed in the U.S. for the treatment of gonorrhea. Its structure (Fig. 18) was determined by WILEY et al. [59] and

Fig. 17. Labeling pattern and proposed biosynthetic pathway of dihydrophenylalanine.

was confirmed by an X-ray analysis [9]. The spectinomycin molecule consists of two moieties, the aminocyclitol actinamine, which can be obtained from the antibiotic by acid hydrolysis, and the sugar actinospectose, a 4,6-dideoxy-2,3-diketo-hexose, which has only been isolated in the form of rearrangement products. Biosynthetic experiments on spectinomycin have been carried out by the groups of MITSCHER [34] and RINEHART [54]. MITSCHER demonstrated that both components of the spectinomycin molecule are derived from glucose and that the C-6 atom of glucose

becomes the methyl group of the deoxy sugar moiety. In addition, the MITSCHER group found that myo-inositol is efficiently incorporated into the actinamine moiety of spectinomycin. RINEHART's group confirmed MITSCHER's finding about the incorporation of glucose and in addition showed by ^{13}C labeling that C-6 of glucose gives rise to C-9 in the aminocyclitol portion of spectinomycin. This result defines the mode of incorporation of myo-inositol into spectinomycin and implies that the biosynthesis must involve an inversion of configuration at C-4 of glucose. We

Fig. 18. Structure and precursors of spectinomycin.

Table IV

Incorporation of double labeled glucose into spectinomycin

	Glucose-[6- ^{14}C , 6-T] T/ ^{14}C (T-retention)		Glucose-[6- ^{14}C , 4-T] T/ ^{14}C (T-retention)	
Glucose fed	2.34	(100 %)	2.34	(100 %)
Spectinomycin	1.78	(76 %)	0.57	(24 %)
Actinamine	1.20	(49 %)	0.02	(1 %)
Acetic acid	2.25	(96 %)	1.34	(57 %)

initiated studies on the biosynthesis of spectinomycin in collaboration with the group of Professor HURLEY at the University of Kentucky. In feeding experiments with glucose tritiated at C-4 and C-6, the results shown in Table IV were obtained. As expected, one half of the tritium from the 6-position of the glucose is lost during the conversion into the actinamine moiety of spectinomycin, but all the tritium from C-6 is retained during the incorporation into the deoxy sugar portion. In agreement with the implication of RINEHART's results, the tritium from the 4-position is completely lost during the incorporation of glucose into the actinamine moiety, but a substantial portion of the tritium is incorporated into the actinospectose moiety. Kuhn-Roth oxidation of spectinomycin from this experiment

gave acetic acid (from C-11 and C-22), which contained essentially all the tritium present in the antibiotic. This result indicates that the 4,6-dideoxyhexose moiety must be formed by a reaction involving a transfer of the hydrogen from the 4- to the 6-position of the hexose, most likely the dTDP-glucose oxidoreductase reaction. These preliminary experiments were carried out to set the stage for further studies on the stereochemical course of the formation of the aminocyclitol moiety and of the deoxy sugar moiety from glucose. These studies are now underway in our laboratory.

Chlorothricin

Chlorothricin (Fig. 19) represents a novel type of macrolide antibiotic. It was isolated from strain Tü 99 of *Strep-*

Structural formula of chlorothricin

Fig. 19. Structure of chlorothricin.

Table V

Incorporation of radioactive precursors into chlorothricin

Precursor fed:	Isolated chlorothricin:	
	Specific incorporation	Incorporation
Acetate-1- ¹⁴ C	2.6 — 49.5 %	—
Acetate-2- ¹⁴ C	18.4 — 40.5 %	—
Methionine-methyl- ¹⁴ C	28.7 %	—
Propionate-1- ¹⁴ C	26.4 %	—
Propionate-2- ¹⁴ C	2.9 — 59.0 %	—
Propionate-3- ¹⁴ C	17.3 %	—
Glucose-6- ¹⁴ C	—	1.4 — 1.9 %
Glucose-1- ¹⁴ C	—	0.36 — 0.72 %
Shikimate-U- ¹⁴ C	—	0.002 %
Leucine-U- ¹⁴ C	—	max. 1.64 %
Leucine-1- ¹⁴ C	—	max. 0.33 — 0.40 %

tomyces antibioticus [27] and its structure was determined by a combination of chemical methods and X-ray analysis of the aglycone [36, 4]. Studies on the biosynthesis of this compound were carried out in our laboratory and independently in the laboratory of Professor Pape, Münster. Feeding experiments with a variety of general precursors (Table V) showed good incorporation of acetate, methionine, propionate and

glucose [23]. Inspection of the structure of the aglycone suggests that it may be at least in part derived by the polyketide pathway. The methyl branches could arise either from propionate or methionine. To distinguish between these two possibilities, a chlorothricin sample, biosynthesized from methionine-¹⁴CH₃, was degraded as indicated in Fig. 20. The results clearly indicate that methionine labels only the methoxy

Degradation of chlorothricin from methionine-methyl-¹⁴C

Fig. 20. Degradation of chlorothricin from methionine-(methyl-¹⁴C).

group in the acyl moiety, but none of the carbon-bound methyl groups.

Further delineation of the incorporation of these precursors into chlorothri-

cin required determination of the isotopic labeling pattern in the antibiotic after feeding various labeled substrates. In view of the complexity of the anti-

Fig. 21. CMR spectrum of chlorothricin.

Fig. 22. Degradation of chlorothricin by methanolysis.

Fig. 23. CMR spectrum of methyl chlorothricolide.

biotic, extensive chemical degradation to isolate individual carbon atoms did not seem very practical and we therefore resorted to ^{13}C labeling and CMR analysis of the product as the most efficient way of determining the labeling pattern. The CMR spectrum of chlorothricin (Fig. 21) is rather complex. However, its interpretation is simplified by the fact that the antibiotic can be degraded as shown in Fig. 22 to give the methyl ester of the aglycone, an acylated sugar and methyl-2-deoxyrhamnose. The spectra of these three fragments can be recorded and interpreted separately. The spectrum of the aglycone as shown in Fig. 23 still shows closely spaced clusters of signals in the methylene region, and in the region of the olefinic carbons. However, using broad band and single frequency decoupling techniques, comparison with several derivatives and model com-

pounds, specific deuteration experiments and analysis of one bond carbon-carbon couplings of pairs of carbon atoms, an unequivocal assignment of

^{13}C -Distribution in the Acyl Moiety of Chlorothricin

Chemical shift, ppm rel. to TMS
($^{13}\text{COOH}/^{13}\text{CH}_3$, abundance rel. to nat. abundance = 100)

Fig. 24. ^{13}C -distribution in the acyl moiety of chlorothricin.

every signal in the spectrum could be made. Based on these assignments and those for the other two fragments, it was then possible to interpret the CMR spectrum of chlorothricin itself. This information was then used to analyze the spectra of chlorothricin and methyl chlorothricolide obtained in feeding experiments with acetate-[1- ^{13}C],

-[2- ^{13}C], and propionate-[1- ^{13}C] and -[3- ^{13}C]. The results for the acyl moiety are summarized in Fig. 24 and show that this portion of the antibiotic is derived from four acetate units by the polyketide pathway. The methoxy carbon as indicated earlier, is derived from the methyl group of methionine. Table VI shows the chemical shifts of chloro-

Table VI

CMR Chemical Shifts and ^{13}C -Enrichments of Chlorothricolide Methyl Ester
Relative signal intensity in sample from

Carbon No.	Chemical Shift (ppm from TMS)	Acetate-1- ^{13}C	Acetate-2- ^{13}C	Propionate-1- ^{13}C	Propionate-3- ^{13}C
2a	16.9 (q)	1.51	2.79		13.0
21a	20.7 (q)	1.63	3.47		
5	24.1 (t)	4.00	1.47		
4	26.6 (t)	1.20	2.20		
21	27.2 (d)	4.11	1.30		
13	28.0 (t)*	4.52	1.70		
14	28.4 (t)*	1.34	2.31		
15	32.3 (t)	4.64	1.44		
12	33.0 (t)	1.83	2.70		
22	35.0 (t)	1.98	1.70		
6	36.2 (t)	1.41	2.63		
3	37.6 (d)	4.40	1.47		
8	45.4 (d)	1.10	2.83		
18	45.9 (d)	1.10	1.82		
11	46.8 (d)	4.71	1.90		
2	47.7 (s)	1.63	1.83		
O-Me	51.6	1.10 (standard)	1.10 (standard)	1.10 (standard)	1.10 (standard)
7	73.3 (d)	5.01	1.89		
23	80.4 (s)	0.90	1.15		
25	115. (s)	1.1	1.83		
9	123.2 (d)	5.54	1.28		
17	124.4 (d)	5.43	1.10		
10	129.9 (d)	1.46	2.44		
20	134.0 (s)	1.10	2.08		
19	136.5 (d)	5.07	2.11	5.86	
16	138.0 (d)	2.82	3.04		
24	159.4 (s)	1.10	1.10		
26	164.9 (s)	2.75	2.30		
20a	166.5 (s)	1.00	2.00		2.29
1	177.5 (s)	1.20	2.20	4.25	

* These signal assignments may be interchanged.

thricolide methyl ester and the relative abundance values observed in this compound after feeding various precursors. It is evident that two molecules of propionate are incorporated into the aglycone, one giving rise to carbons 1, 2, and 2a and the other to carbons 19, 20 and 20a. It is noteworthy that the methyl group of this second propionate unit has been oxidized to a carboxyl group. In addition the results show incorporation of acetate in a pattern consistent with the polyketide pathway in the sense that a polyketide chain starts at carbon 21a and extends through the ring system to carbon atom 1 in the direction of decreasing number of the carbon atoms. This leaves five carbons unaccounted for which comprise the

tetronic acid moiety (carbons 23→26) and carbon 22.

Further experiments were carried out to define the origin of the two sugar moieties. A variety of feeding experiments with glucose (Table VII) showed specific incorporation of glucose into the sugar moieties and it was found that in the process of transformation into 2,6-dideoxyglucose, the hydrogens at C-2 and C-6 are completely retained, the hydrogens at C-1 and C-4 are partly retained, whereas the hydrogen at C-3 seems to be lost. Furthermore, degradation of chlorothricin obtained from glucose-[6-³H] and -[4-³H] (Table VIII) indicated that tritium from the 4-position of the sugar had been incorporated into one or more of the methyl groups,

Table VII

Incorporation of specifically labeled glucoses into chlorothricin and α -methyl-2-desoxy-D-rhamnoside

Position of label in glu- cose fed	T/ ¹⁴ C of glucose	¹⁴ C incorp.	T/ ¹⁴ C	T-ret	α -methyl-2-desoxy-D-rhamnoside T/ ¹⁴ C	T ret.
6- ¹⁴ C, 1-T	1.45	0.7%	0.21	14.4%	0.98	67.5%
6- ¹⁴ C, 2-T	1.93	0.5%	0.38	19.6%	1.93	100.0%
6- ¹⁴ C, 3-T	1.81	1.2%	0.13	7.1%	0.19	10.4%
6- ¹⁴ C, 4-T	2.50	1.9%	0.12	4.8%	0.90	36.0%
6- ¹⁴ C, 6-T	2.10	1.4%	0.54	25.7%	2.10	100.0%

Table VIII

Specific incorporation of glucose into chlorothricin

Glucose-6- ¹⁴ C-6-T	<i>S. antibioticus</i>	chlorothricin	Kuhn-Roth	acetic acid
T/ ¹⁴ C 2.1	→	0.63	oxidation	1.80 (86% T-retention)
Glucose-6- ¹⁴ C-4-T	<i>S. antibioticus</i>	chlorothricin	Kuhn-Roth	acetic acid
T/ ¹⁴ C 2.6	→	0.12	oxidation	0.60 (23% T-retention)

indicating that the formation of the sugar moiety may involve the dTDP-glucose oxidoreductase reaction.

The biosynthetic origin of chlorothricin can therefore be summarized as shown in Fig. 25. The acyl moiety is derived from four acetate units and a methyl group of methionine, the two sugar moieties are derived from intact molecules of glucose, and the aglycone is formed from propionate and acetate units via the polyketide pathway. Further studies are ongoing on the origin of the remaining five carbon atoms of the tetronic acid moiety. As indicated in Table VI, there seems to be some incor-

poration of acetate into carbons 25 and 26 of the tetronic acid moiety, but the results of these experiments were rather equivocal. However, an experiment with acetate-[1,2- $^{13}\text{C}_2$] clearly showed that both these carbons were labeled and that they must have arisen from the same acetate unit, since both carbons, in addition to the natural abundance singlet, showed doublets with large and equal coupling constants. This finding and the results of earlier studies on the biosynthesis of tetronic acids in other systems suggest that the remaining three carbons might be derived from a 4-carbon acid like oxalacetate. A plausible

Fig. 25. Biosynthetic origin of chlorothricin.

scheme explaining the formation of the tetronic acid moiety in chlorothricin and the presence of an oxygen function at C-25 is outlined in Fig. 26. Experiments are now underway to test this proposal.

α -Naphthocyclinone and Granaticin

These two compounds are members of the class of benzoisochromane quinone antibiotics which are produced by a variety of *Streptomyces*. α -Naphthocyclinone, as well as its congeners

Fig. 26. Proposed mechanism of formation of the tetronic acid moiety of chlorothricin.

Fig. 27. Structure of naphthocyclinones.

Granaticin

Fig. 28. Structure of granaticin.

β -naphthoclinone, γ -naphthocyclinone and β -naphthocyclinone epoxide (Fig. 27) are a series of dimeric pigments which were isolated from *Streptomyces arenae* strain Tü 495 and their structures were established by the

group of ZEECK [62, 63]. Granaticin, which was obtained from several *Streptomyces* strains [5, 7] is a monomer which has been further modified by attachment of a bicyclic ring system. The structure (Fig. 28) of granaticin was determined by a combination of chemical degradations and X-ray crystallography [26, 3]. The compound has moderate antitumor activity against P388 leukemia in mice [7]. Fig. 29 shows a number of other representatives of the large class of benzoisochromane-quinones. Of particular interest is the fact that both enantiomers of the modified benzoisochromane-quinone

Kalafungin

Actinorhodin

γ -Actinorhodin

R= OH, Nanaomycin A
R=NH₂, Nanaomycin C

Nanaomycin B

Nanaomycin D

Frenolicin

Griseusin A

Griseusin B

Fig. 29. Naturally occurring benzoisochromane quinones.

Fig. 30. Biosynthetic origin of α -naphthocyclinone.

system are found in nature, with nanaomycin D representing the stereochemical series corresponding to graticin and the naphthocyclinones, and kalafungin representing the enantiomeric series. The biosynthesis of α -naphthocyclinone was studied in feeding experiments with ^{13}C labeled acetate followed by CMR analysis of the labeled product [48]. A complete assignment of the CMR spectra of various naphthocyclinones had been made in ZEECK's laboratory and these were made available to us. The results which

are summarized in Fig. 30 show that the entire molecule of α -naphthocyclinone is made up from acetate units in the alternating labeling pattern characteristic of the polyketide pathway. The fact that carbons 10 and 10' are both derived from the methyl group of acetate lends support to a proposed biogenetic scheme advanced by ZEECK [63] which is summarized in Fig. 31. It envisions separate formation of the monomeric units, followed by dimerization via a dihydroquinone intermediate. In order to determine whether in α -naphthocyclinone both monomeric units had a backbone of eight acetate units, followed by loss of two carbons from the "left hand" portion at a late stage, or whether this compound was derived from the dimerization of two monomers of different numbers of carbon atoms, we attempted to locate the starter units in the two polyketide chains. Presumably one of the starter units would be C-16 and C-15. If the "left-hand" portion is derived from a C-16 precursor then the starter unit of

Fig. 31. Proposed biosynthesis of naphthocyclinones (ZEECK et al., Ref. 63).

this polyketide would have been lost in the elimination of the two carbon unit. If the precursor is a 14-C unit, then either C-6' and C-7' or C-14' and C-13' would have to be a starter unit. A feeding experiment with diethyl malonate- $[2-^{13}\text{C}]$ was carried out in the expectation that both the acetoxy group and the starter units would show no enrichment or less enrichment than the carbons representing the chain extension units. In agreement with expectations, the acetoxy group was not enriched. Both C-6' and C-14' showed normal enrichment, suggesting a 16-carbon precursor for the "left hand" portion. However, this conclusion is invalidated because of the surprising finding that C-16 also shows normal enrichment. It is at present not clear what the proper interpretation of this finding is. One possibility would be that malonyl CoA serves not only as a chain extension unit, but also as a starter unit, giving rise to a precursor carrying an extra carboxyl group attached to C-16 which is later eliminated. Further experiments will be necessary to clarify this question.

Studies on the biosynthesis of grana-

ticin were carried out using *Streptomyces violaceoruber* [52]. Again the approach of feeding ^{13}C -labeled acetate followed by CMR analysis of the product was used to establish that the benzoisochromane moiety of the antibiotic is derived from eight acetate units by the polyketide pathway as shown in Fig. 32. The CMR analysis was carried out on dihydro granaticin methyl ester, the CMR spectrum of which was interpreted on the basis of off-resonance and single frequency decoupling, N.O.E. measurements and comparison with model compounds. Based on observations made in the nanaomycin series [57] it was anticipated that formation of the five-membered lactone ring might be a late step in the biosynthesis. Formation of the five-membered lactone ring from the unhydroxylated acid occurs readily upon standing at room temperature in air and is thought to proceed by the mechanism outlined in Fig. 33. The notion that similar processes may operate in granaticin biosynthesis was confirmed by preparing dihydrogranaticin by hydrogenation of granaticin and demonstrating its transforma-

Fig. 32. Biosynthetic origin of granaticin.

Fig. 33. Mechanism of formation of the 5-membered lactone ring in the benzoisochromane quinone series.

tion into granaticin in cultures of *S. violaceoruber*. Furthermore, it was found that a cell-free extract of the organism was able to carry out the same transformation at rates far exceeding those of the nonenzymatic process. The reaction was shown to require atmospheric oxygen, but when carried out in an atmosphere of $^{18}\text{O}_2$, no ^{18}O was incorporated into the product. Indications are that the enzymatic process probably also in-

volves the direct cyclization as outlined in Fig. 33 rather than a hydroxylation/dehydration sequence.

The origin of the remaining six carbon atoms of granaticin was studied in feeding experiments with glucose labeled in various positions (Table IX). The results indicate that these remaining six carbons represent a 2, 6-dideoxy sugar moiety which is derived from the intact carbon skeleton of glucose. The results

Table IX

Incorporation of specifically labeled glucoses into granaticin

Positions of label in glucose fed	T/ ^{14}C of glucose	^{14}C -incorp.	Granaticin T/ ^{14}C	T-ret.	Acetic Acid % of T of grana- ticin	T/ ^{14}C	T-ret.
6- ^{14}C , 6-T	2.80	1.2 %	0.60	21%	82%	1.45	52%
6- ^{14}C , 4-T	5.96	0.76 %	1.25	21%		5.59	94%
3,4- ^{14}C , 4-T	7.3	0.33 %	3.17	43%	89%		
3,4- ^{14}C , 3-T	7.81	0.12 %	0.76	10%			
3,4- ^{14}C , 2-T	12.6	0.73 %	15.8	125%			
3,4- ^{14}C , 1-T	2.33	0.45 %	2.39	103%			

in Table IX indicate substantial incorporation of glucose into the acetate-derived portion of granaticin, which somewhat obscures the specific incorporation into the dideoxyhexose moiety. However, it is nevertheless clear that the transformation of glucose into the dideoxyhexose moiety proceeds with retention of the hydrogens at positions 1 and 2 and at least partial retention of the hydrogens at C-4 and C-6, whereas the hydrogen at C-3 is lost. Degrada-

tion of the granaticin by Kuhn-Roth oxidation to give acetic acid from the methyl groups again indicates that tritium from the 4-position of the glucose has been incorporated into the methyl group at C-6 of the dideoxyhexose. Operation of the dTDP-glucose oxidoreductase reaction is therefore again implicated in the formation of this dideoxyhexose.

To obtain more information on the stereochemistry and mechanism of the

TDP - glucose oxidoreductase reaction

Fig. 34. dTDP-glucose oxidoreductase reaction.

Fig. 35. Synthesis of (6R)- and (6S)-glucose-6-phosphate-[4-D-6-T].

dTDP-glucose oxidoreductase reaction (Fig. 34), we set out to determine the steric course of the transfer of the hydrogen from the 4- to the 6-position of the hexose [51]. To this end we prepared a sample of dTDP-glucose which was stereospecifically tritiated at C-6 and carried deuterium at C-4 in such a way that every tritiated molecule also contained deuterium. The enzymatic synthesis of this substrate started from 3R- and 3S-3-phosphoglycerate-3T, available from earlier work [14], and proceeded by the

sequence outlined in Fig. 35 and 36. The labeled dTDP-glucose was mixed with a 100-fold excess of unlabeled material and converted into dTDP-4-keto-6-deoxyglucose using the oxidoreductase purified from *E. coli*. The product was subjected to a Kuhn-Roth oxidation to give acetic acid from carbon atoms 6 and 5 which was analyzed for its chirality by the method of ARIGONI et al. and CORNFORTH et al. [30, 10]. The results which are summarized in Table X indicate that the methyl group is

Fig. 36. Preparation of dTDP-glucose from glucose-6-phosphate and conversion into dTDP-4-keto-6-deoxyglucose.

Table X

Chirality analysis of methyl group from dTDP-glucose oxidoreductase reaction

Chirality analysis of methyl group in dTDP-4-keto-6-deoxyglucose derived from (6R)-dTDP-glucose-(4-D, 6-T) (6S)-dTDP-glucose-(4-D, 6-T)		
Acetate, T/ ¹⁴ C	1.35	2.56
Malate, T/ ¹⁴ C	1.13	1.84
Fumarate, T/ ¹⁴ C	0.30	1.39
% Tritium retention in fumarase reaction	26 %	76 %
Configuration of methyl group	S	R

Table XI

Stereochemistry of 6-deoxyhexose formation in granaticin biosynthesis

	Precursor fed	
	(6R)-[4-D, 6-T]glucose	(6S)-[4-D, 6-T]glucose
Tritium retention-in chirality analysis of acetate from Kuhn-Roth oxidation of granaticin:	37%, 37%	78%
Configuration of methyl group:	S	R

Conclusion: Hydrogen transfer from C-4 to C-6 is intramolecular and stereospecific and occurs with inversion of configuration at C-6. Stereochemistry of reaction is the same as in *E. coli*.

placement of the hydroxyl group by hydrogen has occurred with retention of configuration. This finding and the observed loss of tritium from the 3-position of glucose are in agreement with a mechanism for the formation of the 2-deoxy function involving pyridoxamine phosphate as a co-factor, si-

milar to that observed by STROMINGER and co-workers for the formation of 3-deoxyhexoses [43].

In summary, the biosynthetic pathway of granaticin can be formulated as shown in Fig. 38. The organism initially synthesizes the 4-keto-2,6-dideoxyhexose nucleotide and the basic benzo-

Fig. 38. Biosynthetic pathway for granaticin.

isochromane-quinone moiety. These two units then combine to form a C-glycoside which in the form of its "boat" conformer undergoes a second carbon-carbon bond formation with the aromatic ring system to give dihydrograna-ticin. Enzymatic closure of the five membered lactone ring finally completes the biosynthetic sequence. Further work will be directed towards establishing the validity of this proposed scheme and towards elucidating its mechanistic details as well as questions relating to the substrate specificity and the stereospecificity of some of the enzymes involved.

Conclusion

The preceding summarizes the current status of our investigations into the biosynthesis of a number of antibiotics. While a considerable amount of information has been uncovered on the formation of these systems, much remains to be learned. The detailed chemistry of a number of the transformations which have been demonstrated to take place will have to be elucidated and the enzymology of some of the more interesting reaction steps should be studied. The utility of some of the microbial systems to carry out molecular modifications of some of the biologically active compounds should be explored, particularly in the benzoisochromane-quinone series. Finally, we are extending our studies to additional new compounds whose biosynthesis is not entirely apparent from inspection of their structures. In particular, we have initiated studies on the biosynthesis of boromy-

cin [11] and aplasmomycin [37]; the only two boron-containing antibiotics known to date.

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