Biosynthesis of the Modified Peptide Antibiotic Thiostrepton in *Streptomyces azureus* and *Streptomyces laurentii*

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Abstract: The biosynthesis of the thiopeptide antibiotic thiostrepton (**1**) has been investigated by administration of isotopically labeled precursors to cultures of *Streptomyces azureus* and *Streptomyces laurentii*. The amino acid origin of all the components of the antibiotic was demonstrated. Experiments with (*S*)-[1,2-$^{13}C_2$]- and (*S*)-[2,3-$^{13}C_2$]serine showed intact incorporation of serine into the thiazoline and thiazole rings as well as the dehydroalanine, alanine, and tetrahydropyridine moieties. (*S*)-[3-^{13}C,$^{2}H_2$]Serine and (2*S*,3*S*)-[3-^{13}C,$^{2}H_1$]serine were used to elucidate the stereochemistry of the various transformations of serine. The quinaldic acid moiety arises from (*S*)-tryptophan and the methyl group of methionine; using methionine carrying a chiral methyl group, it was shown that this methylation proceeds with retention of configuration of the methyl group. Efficient incorporation of (*R*,*S*)-2-methyl-[3′-^{13}C]tryptophan proved that the methylation is the first step in the sequence, followed by an intramolecular ring expansion reaction in which the bond between N1 and C2 of the indole ring is cleaved and a new bond is formed between this nitrogen and the side chain α carbon. This quinaldic acid moiety is elaborated separately and then attached to the peptide backbone. Possible mechanisms of some of the key reactions and the mode of assembly of the modified peptide structure are discussed.

Introduction

The antibiotic thiostrepton (**1**, Figure 1) was first isolated from fermentation broths of *S. azureus*[1] and later from *S. laurentii*.[2] It proved to be identical with bryamycin produced by cultures of *S. hawaiiensis*.[3] The complete structure of **1** has been determined by chemical degradations[4] and X-ray crystallographic analysis.[5] Thiostrepton belongs to a small family of highly modified, sulfur-rich peptides, called the thiopeptide antibiotics, which also includes siomycins,[6] thiopeptins,[7] micrococcins,[8,9] sporangiomycin,[10] thiocillins,[11] nosiheptide,[12] and A10255.[13] These compounds all display strong similarities in their structural features, sharing the structural elements of a macrocycle containing several thiazoles or dihydrothiazoles and anchored through a pyridine or reduced pyridine ring, a side arm of usually one thiazole and one or more dehydroalanines, and an additional loop of variable size. Consistent with that common architecture, a common mechanism

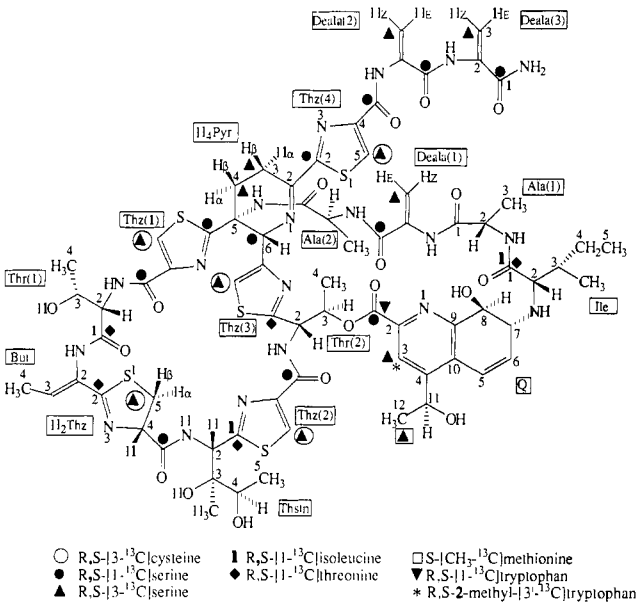

Figure 1. Structure of **1** and its labeling pattern from precursor amino acids carrying single ^{13}C labels.

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Table I. Incorporation of ^{13}C-Labeled Precursors into **1**

carbon	(*R,S*)-[1-^{13}C]-serine	(*R,S*)-[3-^{13}C]-serine	(*R,S*)-[3-^{13}C]-cysteine	(*R,S*)-[1-^{13}C]-tryptophan	(*S*)-[CH_3-^{13}C]-methionine	(*R,S*)-[1-^{13}C]-threonine	(*R,S*)-[1-^{13}C]-isoleucine	(*R,S*)-2-methyl-[3′-^{13}C]-tryptophan
Ile CO						2.5	4.1	
Thz(2) 2						2.3	6.4	
Thr(1) CO						1.8		
Thz(3) 2						1.7		
H_2Thz 2						1.2		
Q CH_3		1.8			22.9			
H_4Pyr 3		2.0						
H_4Pyr 4		*a*						
H_2Thz 5		1.4	3.5					
H_4Pyr 6	1.6							
Deala(1) 3		2.6						
Deala(2) 3		2.5						
Deala(3) 3		2.5						
Thz(3) 5		1.8	3.1					
Q 3		1.6						40
Thz(1) 5		1.8	3.1					
Thz(2) 5		1.6	2.9					
Thz(4) 5		1.6	2.9					
Thz(4) CO	1.6							
Q CO	1.4			8.5				
Thz(1) CO	1.5							
Deala(2) CO	2.4							
Thz(2) CO	1.5							
Deala(1) CO	3.0							
Deala(3) CO	2.4							
Thz(4) 2	1.7							
Thz(1) 2	1.8							
H_2Thz CO	1.5							

[a] The signal is probably enriched, but due to overlap, the degree of incorporation could not be determined.

of action appears to be responsible for their biological activity, inhibition of protein synthesis in Gram-positive bacteria.[14] They bind to the complex of 23S ribosomal RNA and ribosomal protein L11, inhibiting the action of the GTP-dependent elongation factors EF and Tu.[15,16] The producing organisms protect themselves from the action of their own antibiotics through a resistance gene[17,18] which codes for a methylase[19] that specifically methylates the 2′ position of adenosine 1067 in the 23S rRNA,[16,20] blocking antibiotic binding.

Earlier work[8,21] had provided assignments for the all signals in the ^1H and ^{13}C NMR spectra of **1**; they were partly revised in a later study.[22] With these data in hand, the ground was laid for an investigation of the biosynthesis of **1**. We now report the results of these studies. Some of the data have been communicated in preliminary form.[23]

Results

Precursor Amino Acids. Feeding experiments with labeled precursors were initially carried out with shake cultures of *S. azureus* ATCC 14921 and later with a strain of *S. laurentii* ATCC 31255 which gave better yields of **1**.[24] Following establishment of suitable fermentation conditions and optimal timing for precursor additions, a series of feeding experiments were carried out which demonstrated incorporation of ^{14}C-labeled serine, tryptophan, methionine, and threonine into **1** (data not shown). These were followed up by experiments with some of the same amino acids carrying ^{13}C labels in specific positions. While most of these were commercially available, two of them, isoleucine and threonine, had to be synthesized. (*R,S*)-[1-^{13}C]Isoleucine was obtained from K^{13}CN and racemic 2-methylbutanal by Strecker synthesis as a 26:74 mixture with (*R,S*)-[1-^{13}C]alloisoleucine.[25] Similarly, (*R,S*)-[1-^{13}C]threonine in a 84:16 mixture with (*R,S*)-[1-^{13}C]allothreonine was prepared by Strecker synthesis from K^{13}CN and 2-(benzyloxy)propanal;[25] the protecting group was removed hydrolytically during the reaction. The requisite aldehyde was obtained by reduction of 1,1-dimethoxypropanone, benzylation, and cleavage of the acetal with TFA.[26]

The labeling patterns deduced by ^{13}C NMR analysis of the samples of **1** resulting from these feeding experiments (Table I) are summarized in Figure 1. They confirm the rather predictable origin of the two threonine moieties from threonine and of the isoleucine moiety from isoleucine. Threonine also gives rise to the butyrine moiety, which must thus arise by a dehydration reaction. Isoleucine is also the precursor of the thiostreptine moiety, which represents a dihydroxylated isoleucine whose carboxyl group has been incorporated into a thiazole ring. Interestingly, substantial incorporation of (*R,S*)-[1-^{13}C]threonine into the two carbon atoms derived from the carboxyl group of isoleucine is seen, Thz(2) C2 δ 166.38 (2.3%) and Ile CO δ 173.71 (2.5%). These findings reflect the role of threonine as a precursor in isoleucine biosynthesis.

We did not feed alanine carrying a single ^{13}C label because we felt that it would be incorporated with too much scrambling of the isotope to give useful information. However, we did observe the appropriate labeling and ^{13}C–^{13}C coupling patterns in the alanine moieties, albeit less intense than in the more directly

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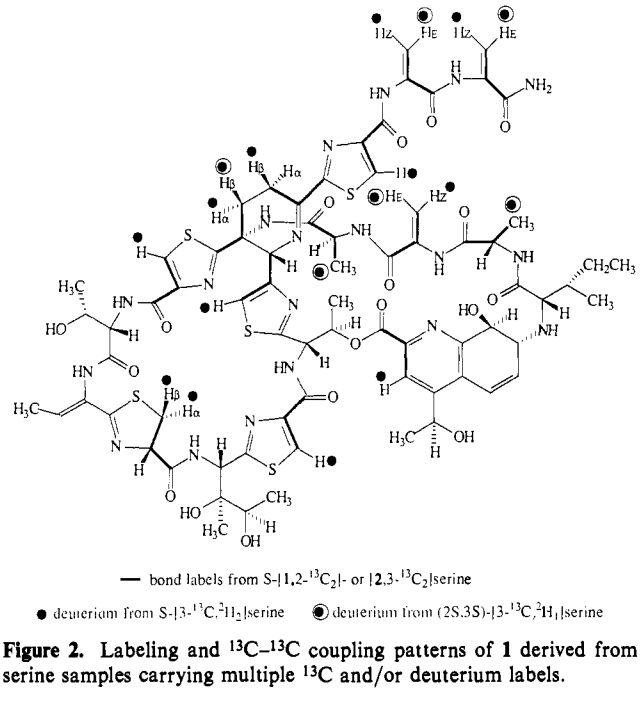

Figure 2. Labeling and ^{13}C–^{13}C coupling patterns of **1** derived from serine samples carrying multiple ^{13}C and/or deuterium labels.

serine-derived moieties, in two feeding experiments with (*S*)-[1,2-$^{13}C_2$]- and (*S*)-[2,3-$^{13}C_2$]serine (Figure 2 and Table II), consistent with a metabolic pathway by which serine undergoes α,β-elimination to pyruvate, which is then transaminated to alanine. As expected for such a pathway, two deuterium atoms and one deuterium were incorporated into each alanine moiety respectively from (*S*)-[3-^{13}C,$^2H_2$]- and (2*S*,3*S*)-[3-^{13}C,$^2H_1$]serine (Figure 2 and Table II).

Dehydroalanine. Carbon atoms 1 and 3 of each of the three dehydroalanine moieties are derived specifically from C1 and C3, respectively, of serine (Table I). The ^{13}C–^{13}C coupling pattern seen after feeding (*S*)-[1,2-$^{13}C_2$]- and (*S*)-[2,3-$^{13}C_2$]serine (Table II) confirmed the intact incorporation of (*S*)-serine into these moieties. Thus, as in the case of nosiheptide,[27,28] the dehydroalanine moieties are formed by dehydration of serine residues in the peptide chain. Two deuterium atoms were retained in each of the dehydroalanine residues from (*S*)-[3-^{13}C,$^2H_2$]serine since the {^1H,^2H}^{13}C NMR spectrum showed the three Deala C3 signals shifted 0.5 ppm upfield from the corresponding natural abundance signals. One deuterium atom per dehydroalanine unit was retained from (2*S*,3*S*)-[3-^{13}C,$^2H_1$]serine. The 2D selective ^1H,^{13}C heteronuclear shift correlation NMR spectrum with ^2H decoupling[29] of the latter thiostrepton sample displayed a correlation between the Deala C3 peaks and the Deala H_z hydrogens which are therefore derived from the *pro-R* hydrogen of C3 of serine (Table II). Assuming that the configuration of the serine moieties in the peptide chain is still *S*, this result establishes an *anti* geometry for the dehydration of serine to dehydroalanine. The same stereochemistry is evident from the configurations of the precursor and product for the dehydration of threonine to the butyrine moiety of **1**, assuming again that the configuration of the precursor threonine in the peptide chain is still *S*.

Thiazoles. The four thiazole rings as well as the dihydrothiazole ring and their attached carboxy groups (or, in the case of thiazole(3) the attached H_4Pyr C6) each arise from a molecule of cysteine and the carboxyl group of the adjacent amino acid, which provides carbon 2. This follows from the specific incorporation into the appropriate positions of ^{13}C not only from (*R*,*S*)-[1-^{13}C]- and (*R*,*S*)-[3-^{13}C]serine but also from (*R*,*S*)-[3-^{13}C]cysteine (Table I). In addition, each thiazole C2 was labeled by C1 of the appropriate amino acid, i.e., threonine in the case of the dihydrothiazole ring and thiazole(3), isoleucine for thiazole(2), and serine for thiazoles(1) and -(3). The intact incorporation of the precursor carbon skeleton was confirmed by observation of the expected ^{13}C–^{13}C couplings in the **1** samples from the (*S*)-[1,2-$^{13}C_2$]- and (*S*)-[2,3-$^{13}C_2$]serine feeding experiments (Table II). One atom of deuterium was retained at C5 of each thiazole ring after feeding (*S*)-[3-^{13}C,$^2H_2$]serine, whereas no deuterium was incorporated from (2*S*,3*S*)-[3-^{13}C,$^2H_1$]serine. Therefore, the hydrogens at C5 of the thiazole rings are derived from the *pro-R* hydrogen of C3 of serine. Incorporation of two atoms of deuterium from (*S*)-[3-^{13}C,$^2H_2$]serine at C5 of the dihydrothiazole ring was clearly evident from the appearance of a signal shifted 0.51 ppm upfield relative to the corresponding resonance at δ 33.90 for the unlabeled material (Table II). This observation is important because it demonstrates that this carbon atom could not have passed through a higher oxidation state in the course of the metabolic transformations leading to the thiazoline ring. Surprisingly, however, no corresponding isotope-shifted signal was detectable for this resonance in the spectrum of **1** derived from (2*S*,3*S*)-[3-^{13}C,$^2H_1$]serine. Taken at face value, this observation would suggest no deuterium incorporation at this position, a totally implausible result in view of the clear incorporation of two deuterium atoms from (*S*)-[3-^{13}C,$^2H_2$]serine. However, the enrichment of **1** from the feeding experiment with the stereospecifically monodeuterated precursor was substantially lower than that from the experiment with the dideuterated one, and the failure to detect the expected signal was most likely due to the poorer signal to noise of the spectrum.

Piperidine Moiety. The formation of the tetrahydropyridine ring seems to follow the same pattern as that of the hydroxypyridine ring in nosiheptide,[27,28] i.e., a tail-to-tail connection of two serine units which provide the ring nitrogen and carbon atoms 2,3,4,5, and whereas carbon 6 is contributed by an adjacent cysteine. Consistent with this mode of assembly, C3 of serine labels H_4Pyr C3 and H_4Pyr C4, and C1 of serine labels Thz(1) C2, Thz(4) C2, and H_4Pyr C6 (Table I). (*S*)-[1,2-$^{13}C_2$]Serine and (*S*)-[2,3-$^{13}C_2$]serine were used to probe for intact incorporation of the carbon skeleton of serine. The 2D-INADEQUATE[30] spectra of **1** obtained from the (*S*)-[1,2-$^{13}C_2$]- and (*S*)-[2,3-$^{13}C_2$]serine feeding experiments showed connectivities H_4Pyr C2–Thz(4) C2 and H_4Pyr C5–Thz(1) C2 as well as H_4Pyr C3–H_4Pyr C2 and H_4Pyr C4–H_4Pyr C5, respectively (Table II). These results clearly demonstrate that the segments H_4Pyr C3–H_4Pyr C2–Thz(4) C2 and H_4Pyr C4–H_4Pyr C5–Thz(1) C2 are each derived intact from serine.

Boundary conditions for the intruiging mechanism of connecting the hydroxymethyl carbons of the two serine units were established by the feeding experiments with (*S*)-[3-^{13}C,$^2H_2$]- and (2*S*,3*S*)-[3-^{13}C,$^2H_1$]serine which determined the fate of the hydrogens at C3 of the precursor (Table II). The ^{13}C NMR spectrum of **1** biosynthesized from (*S*)-[3-^{13}C,$^2H_2$]serine showed a signal for H_4Pyr C3 at δ 24.58 shifted 0.25 ppm upfield, indicating the incorporation of one atom of deuterium. No clear result was obtained for H_4Pyr C4 due to severe overlap with other peaks. The same problem was encountered with **1** from the (2*S*,3*S*)-[3-^{13}C,$^2H_1$]serine feeding experiment. To resolve the overlapping resonances, 2D selective ^1H,^{13}C heteronuclear shift correlation NMR with ^2H decoupling[29] was employed since it only displays carbons bearing a single proton. The spectrum of **1** from (*S*)-[3-^{13}C,$^2H_2$]serine showed one correlation between H_4Pyr C3 (δ 24.57) and H_4Pyr H3α (δ 2.76), which demonstrates

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Table II. NMR Spectral Data of **1** Derived from (*S*)-[1,2-^{13}C]Serine (A), (*S*)-[2,3-$^{13}C_2$]Serine (B), (*S*)-[3-^{13}C,$^{2}H_2$]Serine (C), and (2*S*,3*S*)-[3-^{13}C,$^{2}H_1$]Serine (D)

		incorporation from					
		A		B		C	D
carbon	δ (ppm)	J_{C-C} (Hz)	coupling partner	J_{C-C} (Hz)	coupling partner	upfield isotope shift	upfield isotope shift
Deala(3) CO	165.99	65[a]	Deala(3) 2				
Deala(3) 2	133.02	65[b]	Deala(3) CO	77[b]	Deala(3) 3		
Deala(3) 3	104.27			77[c]	Deala(3) 2	0.53	inc. of one ^{2}H[d] (H_B)
Deala(2) CO	162.00	68[a]	Deala(2) 2				
Deala(2) 2	134.20	68[b]	Deala(2) CO	76[b]	Deala(2) 3		
Deala(2) 3	103.28			77[c]	Deala(2) 2	0.52	inc. of one ^{2}H[d] (H_B)
Thz(4) CO	159.56	78[a]	Thz(4) 4				
Thz(4) 2	168.35	45[a]	H_4Pyr 2				
Thz(4) 4	149.92	78[b]	Thz(4) CO	65[b]	Thz(4) 5		
Thz(4) 5	127.55			65[c]	Thz(4) 4	0.18	no inc.[d]
H_4Pyr 2	161.94	49[b]	Thz(4) 2	49[b]	H_4Pyr 3		
H_4Pyr 3	24.57			47[c]	H_4Pyr 2	0.25 (H_R)	no inc.[d]
H_4Pyr 4	29.16			35[c]	H_4Pyr 5	inc. of two ^{2}H[e]	inc. of one ^{2}H[d] (H_S)
H_4Pyr 5	57.57	59[b]	Thz(1) 2	34[b]	H_4Pyr 4		
H_4Pyr 6	64.21	59[a]	Thz(3) 4				
Thz(1) CO	161.67	77[a]	Thz(1) 4				
Thz(1) 2	169.73	61[a]	H_4Pyr 5				
Thz(1) 4	146.30	78[b]	Thz(1) CO	66[b]	Thz(1) 5		
Thz(1) 5	124.88			68[c]	Thz(1) 4	0.20	no inc.[d]
Thr(1) CO	165.47						
Thr(1) 2	55.63						
Thr(1) 3	66.46						
Thr(1) CH_3	18.92						
But 2	128.45						
But 3	132.53						
But CH_3	15.20						
H_2Thz CO	171.97	56[a]	H_2Thz 4				
H_2Thz 2	170.16						
H_2Thz 4	78.98	57[b]	H_2Thz CO	31[b]	H_2Thz 5		
H_2Thz 5	34.78			31[c]	H_2Thz 4	0.50	*f*
Thstn 2	53.06						
Thstn 3	77.19						
Thstn 3 CH_3	18.34						
Thstn 4	67.73						
Thstn 5	15.88						
Thz(2) CO	162.10	78[a]	Thz(2) 4				
Thz(2) 2	166.38						
Thz(2) 4	150.09	78[b]	Thz(2) CO	66[b]	Thz(2) 5		
Thz(2) 5	125.36			67[c]	Thz(2) 4	0.19	no inc.[d]
Thr(2) 2	55.83						
Thr(2) 3	72.00						
Thr(2) CH_3	18.68						
Q CO	160.80	85[a]	Q 2				
Q 2	143.56	85[b]	Q CO	63[b]	Q 3		
Q 3	122.26			63[c]	Q 2	0.26	no inc.[d]
Q 4	153.41						
Q 5	123.15						
Q 6	129.99						
Q 7	59.02						
Q 8	67.34						
Q 9	154.55						
Q 10	127.20						
Q 11	64.35						
Q CH_3	22.53						
Ile CO	173.71						
Ile 2	65.60						
Ile 3	38.49						
Ile 3 CH_3	15.52						
Ile 4	24.57						
Ile 5	11.25						
Ala(1) CO	168.84	53[a]	Ala(1) 2				
Ala(1) 2	49.35	53[b]	Ala(1) CO	34[b]	Ala(1) CH_3		
Ala(1) CH_3	18.88			34[c]	Ala(1) 2	0.48	0.20
Deala(1) CO	162.92	67[a]	Deala(1) 2				
Deala(1) 2	132.28	67[b]	Deala(1) CO	77[b]	Deala(1) 3		
Deala(1) 3	102.79			80[c]	Deala(1) 2	0.54	inc. of one ^{2}H[d] (H_B)
Ala(2) CO	173.31	53[a]	Ala(2) 2				
Ala(2) 2	51.95	53[b]	Ala(2) CO	36[b]	Ala(2) CH_3		
Ala(2) CH_3	18.81			37[c]	Ala(2) 2	0.53	0.27
Thz(3) 2	169.97						
Thz(3) 4	157.17	59[b]	H_4Pyr 6	67[b]	Thz(3) 5		
Thz(3) 5	118.16			67[c]	Thz(3) 4	0.21	no inc.[d]

[a] Carbon derived from C1 of (*S*)-[1,2-^{13}C]serine. [b] Carbon derived from C2 of (*S*)-[1,2-^{13}C]- and (*S*)-[2,3-$^{13}C_2$]serine. [c] Carbon derived from C3 of (*S*)-[2,3-$^{13}C_2$]serine. [d] Due to overlap with other peaks, no isotope-shifted signals were detected. The incorporation was determined by 2D selective $^{1}H,^{13}C$ heteronuclear shift correlation NMR with ^{2}H decoupling.[29] [e] Severe overlap in this region of the NMR spectrum did not give a clear result for this carbon signal. No incorporation was observed in the 2D selective $^{1}H,^{13}C$ heteronuclear shift correlation NMR spectrum with ^{2}H decoupling. Since the incorporation of one deuterium was proven in the experiment with (2*S*,3*S*)-[3-^{13}C,$^{2}H_1$]serine, one has to assume the presence of two atoms of deuterium in **1** derived from (*S*)-[3-^{13}C,$^{2}H_2$]serine. [f] No isotope-shifted signal was detected.

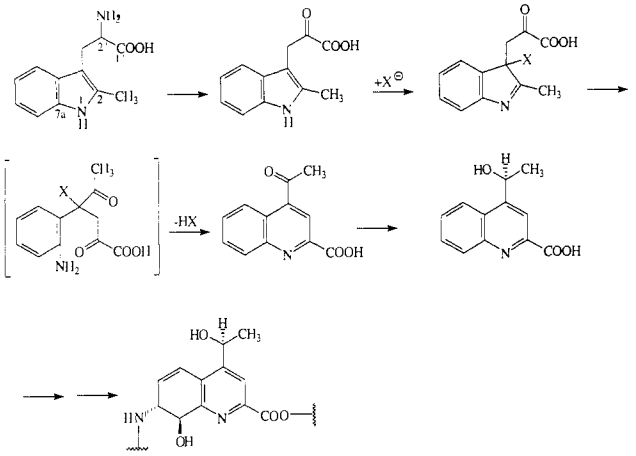

Figure 3. Proposed mechanism for the ring expansion of 2-methyltryptophan to the quinaldic acid moiety of **1**.

that the deuterium incorporated at C3 is located in the β (*pro-R*) position. No correlation was observed for H_4Pyr C4; thus it could be either labeled with two atoms of deuterium or none. However, in the spectrum of **1** from the (2*S*,3*S*)-[3-^{13}C,$^{2}H_1$]serine feeding experiment, a cross peak for H_4Pyr C4 (δ 29.16) and H_4Pyr H4α (δ 3.92) was evident, which indicates that one atom of deuterium is incorporated at H_4Pyr H4β (*pro-S* position). This also implies that there must be two deuterium atoms present at H_4Pyr C4 of the **1** derived from (*S*)-[3-^{13}C,$^{2}H_2$]serine. No deuterium is present at H_4Pyr C3 of **1** derived from (2*S*,3*S*)-[3-^{13}C,$^{2}H_1$]serine; consequently, the deuterium retained at C3 of the tetrahydropyridine moiety from the dideuterated precursor is derived stereospecifically from the *pro-R* position at C3 of serine (Figure 2).

Quinaldic Acid Moiety. Initial experiments showing incorporation of both radiolabeled tryptophan and [*methyl*-^{14}C]methionine into **1** (data not shown) suggested that the quinaldic acid moiety may arise from these two precursors. This was confirmed in subsequent work with ^{13}C-labeled precursors. Inspection of the structure of the quinaldic acid moiety indicates that L-tryptophan can account for the origin of all its carbon atoms except C12, the methyl group, which may then be derived from the methyl group of methionine. Consistent with this hypothesis, (*R*,*S*)-[1′-^{13}C]tryptophan labeled the quinaldic acid carboxyl group at δ 161.6 (8.5% enrichment) and (*S*)-[*methyl*-^{13}C]methionine significantly enhanced the signal of Q C12 at δ 23.63 (22.9% enrichment) (Table I). Thus, the formation of the quinaldic acid moiety must involve a ring expansion of the indole to the quinoline system. This process could proceed by either (a) cleavage of the N1/C7a bond and connection of the side chain nitrogen to C7a or (b) cleavage of the N1/C2 bond and connection of the side chain C2′ to N1. In order to distinguish between these two possibilities, a sample of (*S*)-[1′,2′-^{13}C,*indole*-^{15}N]tryptophan was prepared enzymatically from [^{15}N]indole[31] and (*S*)-[1,2-$^{13}C_2$]serine with tryptophan synthase by adapting a published procedure.[27] The **1** biosynthesized from (*S*)-[1′,2′-$^{13}C_2$,*indole*-^{15}N]tryptophan showed ^{13}C enrichment in the quinaldic acid carboxyl group (δ 160.80) and Q C2 (δ 143.56) and one-bond coupling of these two signals to each other (J_{C-C} = 85 Hz). Additional couplings to ^{15}N were displayed by both signals: a one-bond coupling of 3.02 Hz by Q C2 and a two-bond coupling of 8.08 Hz by Q CO.[32] This leads to the conclusion that alternative b must be followed in the expansion of the indole ring of tryptophan to the quinaldic acid system (Figure 3) and that this rearrangement process is strictly intramolecular. Serine is the precursor of the tryptophan side chain, and the coupling patterns displayed by the quinaldic acid moiety of **1** after feeding (*S*)-[1,2-$^{13}C_2$]- and (*S*)-[2,3-$^{13}C_2$]serine (Table II) are entirely consistent with the proposed rearrangement. This process involves the elimination of one of the methylene hydrogens of the tryptophan side chain, and hence ultimately of C3 of serine. The experiments with the two deuterated serine samples showed incorporation of deuterium from (*S*)-[3-^{13}C,$^{2}H_2$]serine but not from (2*S*,3*S*)-[3-^{13}C,$^{2}H_1$]serine (Table II), suggesting that this hydrogen loss is stereospecific. Assuming that the steric course of the conversion of serine into tryptophan is the same in *S. laurentii* as in *Neurospora crassa* and *Escherichia coli*, retention of configuration at C3,[33] the hydrogen retained at Q C3 must originate from the *pro*-3*S* position of the tryptophan side chain. No mechanistic significance can be attached to this finding at the present time.

The question at what stage in the conversion sequence of tryptophan into the quinaldic acid moiety the methyl group is introduced was investigated. In the biosynthesis of nosiheptide, the methylation was found to be a late step, occurring only after rearrangement of tryptophan to an indole-2-carboxylic acid.[28] To investigate this issue, we synthesized a sample of (*R*,*S*)-2-methyl-[3′-^{13}C]tryptophan by following literature procedures.[34] When this material was fed to *S. laurentii*, ^{13}C NMR analysis of the resulting **1** revealed a 40% enrichment at Q C3 (δ 122.26), indicating excellent utilization of this precursor in the formation of the quinaldic acid moiety. Trapping experiments in which (*R*,*S*)-2-methyl-[3′-^{13}C]tryptophan (200 mg/L) was added to cultures of *S. laurentii* showed 5–10% dilution of ^{13}C in the reisolated material. Furthermore, butanol extraction of the mycelia, followed by derivatization and GC–MS analysis, revealed the presence of 2-methyltryptophan in the cultures. Its concentration was highest just prior to the appearance of **1** in the fermentation. The demonstration that *S. laurentii* is able both to synthesize 2-methyltryptophan and to incorporate it efficiently into **1** leaves little doubt that this amino acid is an intermediate in the biosynthesis of the quinaldic acid moiety. Thus, rather surprisingly, in contrast to the formation of nosiheptide, in the biosynthesis of thiostrepton the methylation of tryptophan is the first step in the reaction sequence. This conclusion was further confirmed by enzymatic studies demonstrating the formation of 2-methyltryptophan from tryptophan and (*S*)-adenosylmethionine (AdoMet) in cell-free extracts of *S. laurentii*.[35]

The steric course of this methylation was examined using methionine carrying a chiral methyl group.[36] (*methyl-R*)-[*methyl*-$^{2}H_1$,^{3}H]Methionine and (*methyl-S*)-[*methyl*-$^{2}H_1$,^{3}H]methionine[37] containing methyl groups of 80% and 75% enantiomeric excess, respectively, were fed to cultures of *S. azureus*; the resulting samples of **1** were degraded by Kuhn–Roth oxidation[38] to give acetic acid from the C-methyl groups and their attached carbons. Since the feeding of [*methyl*-^{13}C]methionine had shown incorporation of ^{13}C only into Q C12, the dilution of the acetic acid from this position with acetic acid derived from other C-methyl groups in the molecule was of no consequence for the stereochemical analysis. The configurations of the acetic acid samples were determined by the method of Cornforth *et al.*[39] and Arigoni and co-workers[40] using a procedure

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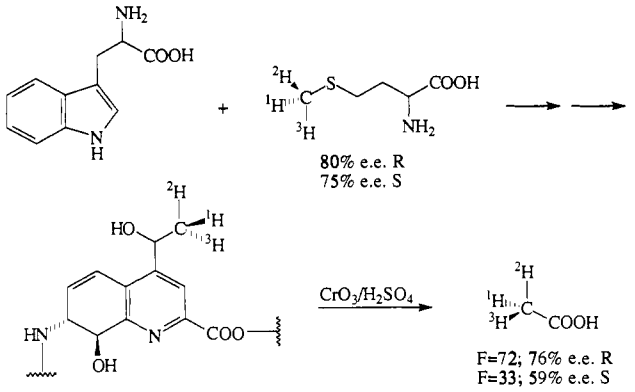

Figure 4. Steric course of the methylation reaction in the biosynthesis of the quinaldic acid moiety of **1**.

routinely employed in our laboratory.[41] Surprisingly, the analyses revealed that the methyl groups in the acetic acid had the same configuration as in the respective methionine samples from which they were derived (76% ee *R* and 59% *S*, respectively), i.e., that the methyl transfer must proceed with retention of configuration (Figure 4). This is only the second example of a methionine-dependent methylation that proceeds with net retention of configuration, the only previously observed case being a methylation in the biosynthesis of the antibiotic thienamycin.[42] The vast majority of methyltransferases that use AdoMet as the substrate transfer the methyl group with inversion of configuration, presumably via a single S_N2 transition state (cf. refs 36 and 43). In a follow-up experiment, it was ascertained that the methyl group is nevertheless transferred intact, i.e., with all three hydrogens. Feeding of (*S*)-[*methyl*-^{13}C,$^{2}H_3$]methionine gave **1** in which the {^{1}H,^{2}H}^{13}C NMR signal for Q C12 (δ 23.38) was shifted upfield by 0.85 ppm, characteristic of three atoms of deuterium attached to a ^{13}C.

A logical advanced intermediate in the formation of the quinaldic acid moiety from 2-methyltryptophan is 4-(1-hydroxyethyl)quinaldic acid (HEQ). This compound was prepared in labeled form from quinaldic acid methyl ester by acetylation at C4[44] and reduction of the resulting methyl 4-acetylquinaldate with sodium [^{3}H]borohydride. After hydrolysis, racemic 4-(1-hydroxy-[1-^{3}H]ethyl)quinaldic acid was obtained. The expected position of the tritium label was confirmed by ^{3}H NMR, which showed a single peak at δ 5.90. Thiostrepton biosynthesized from this precursor was isolated, diluted with an unlabeled carrier material, and purified to constant specific radioactivity by HPLC and precipitation from different solvents. The final values indicated a low but significant incorporation (3.6%) of the tritiated HEQ into **1**. This finding lends credence to the notion that HEQ is indeed an intermediate in the formation of the quinaldic acid moiety of **1** from tryptophan.[45]

Discussion

The results presented here establish the basic building blocks from which the structure of **1** is assembled, and they provide some information on the nature and mechanisms of the various amino acid modifications that take place during this biosynthesis. Many of the findings parallel what we have learned in studies on the biosynthesis of the related but simpler antibiotic, nosiheptide.[27,28] The overall architecture of these two molecules is quite similar, apart from the fact that nosiheptide does not contain the large second peptide loop with the quinaldic acid moiety, but instead carries a single indolic acid moiety which bridges the macrocycle. However, in the conserved part of the structure, there are a few amino acid replacements, namely Thr(2) in **1** by cysteine in nosiheptide and the isoleucine-derived Thstn moiety in **1** by a hydroxylated glutamic acid. Our findings are in perfect agreement with those recently reported for the thiopeptide antibiotic A10255.[47]

As in the case of nosiheptide,[28] the dehydroalanine and butyrine moieties of **1** are derived from serine and threonine moieties, respectively, by *anti* 1,2-eliminations of water. Inherent in the stereochemical conclusion is the assumption that these precursor amino acid residues in the peptide chain still have *S* configuration. Although the steric course of this reaction conforms to the pattern, as pointed out by Knowles,[48] that 1,2-eliminations in which the abstracted proton is α to a strong acidic group proceed in an *anti* fashion, this coincidence by no means implies that the enzymatic process has to be concerted. The isoleucine residue giving rise to the Thstn moiety is modified by the introduction of two hydroxy groups at adjacent carbons. This could either involve two independent hydroxylations or a dehydrogenation to a 3,4-dehydroisoleucine residue followed by epoxidation and hydrolytic opening of the epoxide. The hydroxylations are suggested by the analogy to nosiheptide where the glutamic acid residue in the equivalent position must undergo a hydroxylation; the configuration at C3 of Thstn is consistent with replacement of H by OH in a retention mode, the common steric course of hydroxylations at saturated carbons.[49] On the other hand, the epoxidation route has a parallel in the modification of HEQ to the quinaldic acid moiety of **1**, which must proceed via an epoxide. An attempt was made to differentiate between these two mechanisms by determining whether one or both of the Thstn oxygens come from atmospheric O_2. However, several fermentations run under an $^{18}O_2$-containing atmosphere gave low yields and levels of ^{18}O incorporation too low to analyze. Hence, this issue remains unresolved.

Thiazole ring formation in **1** from cysteine and the carboxyl group of the next amino acid in the peptide chain also follows the pattern previously encountered in nosiheptide[27] as well as in bleomycin,[50] berninamycin,[51] and myxothiazole[52] and in the analogous formation of the oxazole rings in berninamycin[51] and virginiamycin.[53] The parallel with nosiheptide and virginiamycin extends to the stereochemistry of the reaction; as in the present case, both thiazole ring formation in nosiheptide[28] and oxazole ring formation in virginiamycin[54] were found to proceed with stereospecific loss of the *pro-S* and retention of the *pro-R* hydrogen from C3 of serine. Making the reasonable assumption[55] that the conversion of serine into cysteine in *Streptomyces* takes the same steric course as in *E. coli*,[56] retention of configuration at C3,[57]

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(57) We had hoped to verify the correctness of this assumption experimentally by determining the configuration at H_2Thz C5 of **1** biosynthesized from (2*S*,3*S*)-[3-^{13}C,$^{2}H_1$]serine, but failed because we did not observe the expected deuterium-shifted signal for H_2Thz C5 in the {^{1}H,^{2}H}^{13}C NMR spectrum.

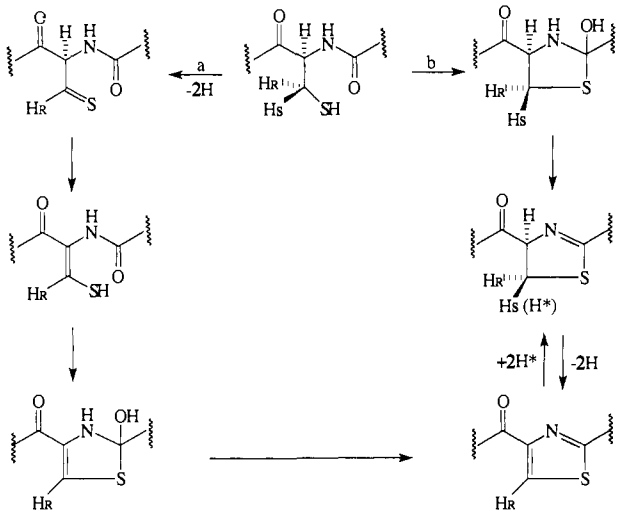
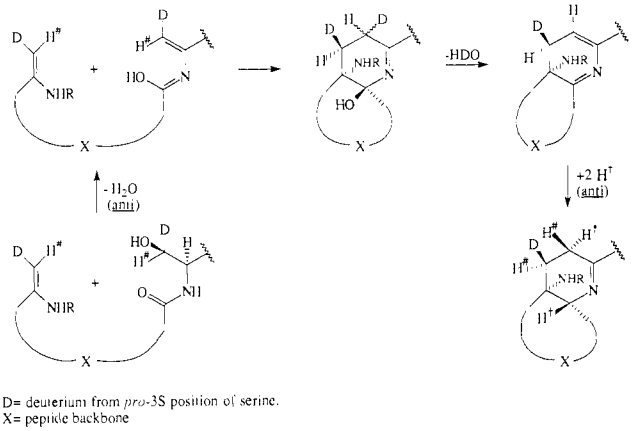


Figure 5. Two possible mechanisms for thiazole ring formation in the biosynthesis of **1**.

the hydrogen lost would also be the *pro-S* hydrogen in cysteine. Assuming further that the precursor cysteine moieties in the peptide chain have *S* configuration, this would indicate an *anti* stereochemistry for the 2,3-dehydrogenation of the cysteine moieties to give the carbon–carbon double bonds in the thiazole rings, the same steric course as deduced for oxazole ring formation in virginiamycin.[54] Since most biological dehydrogenations α,β to a carbonyl function proceed with *syn* geometry,[58,59] it was argued in the case of oxazole formation[54] that cyclization to a dihydrothiazole followed by dehydrogenation was an unlikely mechanism. It was proposed instead that the transformation is initiated by dehydrogenation of C3 to an aldehyde which in its enol form then cyclizes by nucleophilic attack on the amide carbonyl group. However, the majority of the alcohol dehydrogenases[60] remove the *pro-R*, not the *pro-S*, hydrogen from the primary alcohol carbon of their substrates. Thus, stereochemistry does not offer very compelling arguments for either of these mechanisms. A stronger case can be made for an α,β-dehydrogenation and against an aldehyde mechanism for thiazole ring formation in **1** on the basis of the results of the present study. The two options are outlined in Figure 5. The crucial observation that two atoms of deuterium from C3 of serine are incorporated at C5 of the H_2Thz moiety of **1** reveals that this ring cannot arise by reduction of a thiazole and that it cannot be formed via a thioaldehyde intermediate, because either event would necessitate the removal of one of the original deuterium atoms and its replacement by a nonlabeled hydrogen. Thus, the dihydrothiazole is most likely an intermediate stage in the formation of the thiazole rings, i.e., path b of Figure 5 is operative. Of course it is possible, although not very likely, that the dihydrothiazole and the thiazole rings are formed by entirely different mechanisms. The finding that both hydrogens at H_2Thz C5 originate from C3 of serine has another important implication. The configuration at C4 of H_2Thz corresponds to that of an *R* amino acid. Since the above result establishes that the dihydrothiazole function cannot have arisen by reduction of an achiral thiazole, this must represent the configuration of the original precursor amino acid in the peptide chain. Therefore the cysteine in this position of the peptide must



D= deuterium from *pro*-3S position of serine.
X= peptide backbone

Figure 6. Proposed mechanism and stereochemistry of formation of the H_4Pyr moiety of **1**.

have D (*S*) configuration,[61] although it may still originate from free (*R*)-cysteine. Situations like this have been encountered, for example, in the biosynthesis of gramicidin S, where an (*R*)-phenylalanine residue in the final antibiotic arises from free (*S*)-phenylalanine which is epimerized during the biosynthesis by a racemase associated with the peptide synthase,[62] and the formation of the (*R*)-valine moiety of the ACV tripeptide from (*S*)-valine.[63] It is tempting to speculate that this configurational inversion may block the action of a dehydrogenase which oxidizes the dihydrothiazole to the thiazole ring, providing a reason for the presence of the dihydrothiazole moiety in this particular position. However, there is at present no evidence for or against this hypothesis.

One of the more unusual features of thiopeptide antibiotic biosynthesis is the formation of the tetrahydropyridine ring, and in the process also of the macrocycle, by a connection of a serine and the carboxyl group of an adjacent cysteine to another serine 8 amino acid residues removed in the peptide chain. Following an earlier proposal by Bycroft and Gowland,[9] it is assumed that both serines first undergo dehydration to dehydroalanine, presumably with the same *anti* stereochemistry by which the other Deala moieties are formed (Figure 6). The mechanism of the subsequent cyclization is unclear, and there is no indication at this point as to whether the reaction is stepwise or concerted. Absent any redox changes, it should lead to a hydroxytetrahydropyridine. A subsequent 1,4-dehydration and 1,4-reduction then complete the structure. Although the stereochemistry of this process cannot yet be fully delineated, because the configuration of the postulated intermediate cyclization product is unknown, the experiments with the two deuterated serine samples establish some boundary conditions. Since deuterium from (2*S*,3*S*)-[3-^{13}C,^{2}H$_1$]serine occupies the *pro-S* position at C4 of H_4Pyr, the addition to the double bond of the more amino-terminal dehydroalanine, as one would expect, must be suprafacial. The final reduction of the 1,4-diene involves *anti* addition of two hydrogens to the *Si* face at C3 and the *Re* face at C6. The configuration of the initial cyclization product is a matter of conjecture. The suggested configuration for C6 (Figure 6) is based on the consideration that *R* configuration at C6 would make the geometry of the system most productlike, necessitating a minimum of motion in the subsequent two steps.

The formation of the quinaldic acid moiety by a ring expansion of the indole system of 2-methyltryptophan to the quinoline system has biochemical precedent in the formation of the cinchona alkaloids, like quinine, from their indole alkaloid precursors, as

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demonstrated by Leete and Wemple.[64] As in other rearrangements of the indole ring system of tryptophan,[65] the reaction is presumably initiated by attack of an X^+ species on C3 of the indole (Figure 3), followed in this case by hydrolytic opening of the resulting indolenine. The alternative imine formed between the indole nitrogen and the side chain carbonyl group, the latter generated by transamination, can then rearomatize by loss of HX. The chemical feasibility of this transformation has been demonstrated long ago in the hypochlorite-catalyzed conversion of 2-methyltryptophan into 4-acetylquinaldic acid.[66] Enzymatic reduction of the 4-acetyl group will produce HEQ, the next established precursor along the pathway. It should be noted that the rearrangement reaction as formulated includes an oxidation, since X is added as X^+ and eliminated as X^-; alternative mechanisms can be formulated in which the oxidation state of X does not change and which lead directly to HEQ. The further conversion of HEQ into the quinaldic acid moiety of **1** presumably proceeds via a 7,8-arene oxide which is then opened by nucleophilic attack of the isoleucine amino group. Recent work[67] strongly suggests that the latter steps take place only after HEQ has been attached to the peptide backbone by ester linkage to the hydroxyl group of Thr(2). The correct[68] enantiomer of HEQ was shown[67] to be the only substrate for an enzyme from *S. laurentii* activating the carboxyl group to the adenylate in preparation for ester formation.

The methylation involved in the formation of the quinaldic acid moiety of **1** presents some unusual features. Although it is quite plausible that methylation is an early step in the transformation of tryptophan into HEQ (it would be mechanistically difficult at subsequent stages in the process), it is rather surprising that the corresponding C4 methylation of tryptophan in the formation of the indolic acid moiety of nosiheptide occurs late in the transformation sequence after the skeletal rearrangement.[28] Even more surprising is the unusual retention of stereochemistry observed for the transfer of the methyl group from AdoMet to C2 of the indole ring, for which no mechanistic imperative is obvious. Since all three methyl hydrogens of methionine are retained in the product, this stereochemistry is not the fortuitous outcome of a more complex process, like the methylation of sterol side chains,[43] in which the methyl group passes through the stage of an intermediate methylene group. Other known instances of methyl transfer with retention involve methylation at a non-nucleophilic carbon[42] or transfers of methyl groups from unactivated positions,[70] i.e., reactions which presumably require mechanistic features beyond a simple single S_N2 transfer. No such requirements are obvious for the methylation at C2 of a 3-substituted indole, although normal indole chemistry would suggest the possibility of a mechanism involving initial methylation at C3 followed by a 1,2 migration of the methyl group. However, such a mechanism would not explain the overall retention of stereochemistry since, following initial transfer with inversion, the 1,2 migration should proceed through a bridged carbocation, and hence with retention, to give overall inversion of configuration. Additional enzymatic studies[35] have ruled out other possibilities as well, e.g., initial methylation at N1 followed by migration to C2, but have not yet pinpointed a plausible alternative. Thus, the mechanism of this stereochemically unusual methylation reaction remains for the time being obscure.

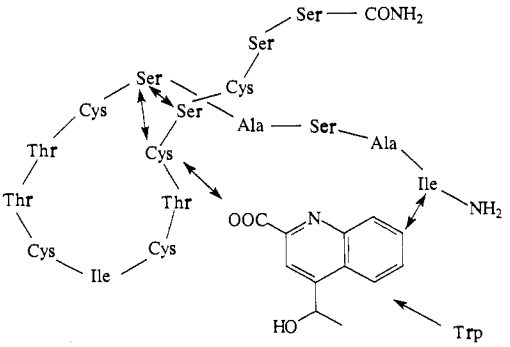

Figure 7. Formal mode of assembly of **1** from a linear peptide precursor.

The data reveal that **1** results from extensive modification of a peptide of the linear sequence N-*S*-Ile(1)-*S*-Ala(2)-Ser(3)-*S*-Ala(4)-Ser(5)-Cys(6)-*S*-Thr(7)-Thr(8)-*S*-Cys(9)[61]-*S*-Ile(10)-Cys(11)-*S*-Thr(12)-Cys(13)-Ser(14)-Cys(15)-Ser(16)-Ser(17)-(Ser(18))-C. In analogy to the findings for the nosiheptide[28] biosynthesis, it is assumed that the carboxy-terminal amide nitrogen of **1** also arises from an additional carboxy-terminal serine (Ser(18)) which is removed by an oxidative process[71] leaving its nitrogen attached to the peptide chain. Several attempts to generate evidence supporting or refuting this assumption by tracing the biosynthetic origin of this nitrogen, as was done successfully in the nosiheptide fermentation, gave completely ambiguous results because in *S. laurentii* all nitrogen sources were utilized indiscriminately to label all nitrogens randomly. Cys(9) in the peptide chain must have *S* configuration, presumably due to a postsynthetic modification, and it is possible that one or more of the other amino acids of unspecified stereochemistry have the unnatural configuration. As part of the postsynthetic modifications, the peptide chain must fold back on itself, bringing Ser(14) and Cys(13) close to Ser(5), to allow the cycloaddition between these moieties which generates both the H_4Pyr moiety and the large macrocycle (Figure 7). The quinaldic acid moiety is evidently not part of the original precursor peptide but must be attached at a later stage. The second macrocycle is generated by attack of the amino-terminal nitrogen of the peptide chain on the arene oxide function of the epoxidized quinaldic acid attached to Thr(7).

The assembly of such a large peptide of nonrepetitive sequence from its amino acid precursors may either occur by a ribosomal process, as in the biosynthesis of the lanthiobiotics,[72] or by a template-directed non-ribosomal enzymatic process. The latter mechanism operates in the biosynthesis of a substantial number of mostly smaller peptide antibiotics,[73] like gramicidin,[74] tyrocidine,[75] bacitracin,[76] cyclosporin,[77] and aminoadipoyl–cysteinyl–valine (ACV),[78] the precursor of the penicillins and cephalosporins. The available evidence points to a template-directed process for

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the assembly of the thiopeptide antibiotics nosiheptide and **1**. Attempts to demonstrate the presence of nucleotide sequences in the DNA of the producing organisms which could code for the amino acid sequences of nosiheptide or **1** by Southern hybridization with appropriate labeled oligodeoxynucleotides failed to reveal any homologous DNA in *S. actuosus* or *S. laurentii*, respectively.[79] Additionally, in the case of nosiheptide, it was shown that chloramphenicol at concentrations which completely shut off protein synthesis does not immediately inhibit antibiotic formation.[79a,80] The peptide synthase may assemble a linear peptide of the sequence indicated above, which then is further modified by individual separate enzymes. Alternatively, and more likely in view of the mode of operation of other peptide synthases,[81] the assembly as well as the modifications may occur on the same multi-enzyme complex and may be interspersed in their order such that amino acids are modified after their attachment to the growing peptide chain before the further extension of the chain. Such a multi-enzyme complex would contain functional units catalyzing activation and amide bond formation for each individual amino acid as well as the appropriate modification reactions. Whether all the enzyme functions required to assemble and modify the entire backbone of **1** are located on one multi-enzyme complex or whether the molecule is assembled in segments on several complexes remains to be established. These and other questions about the biosynthesis of **1** are now being examined at both the biochemical and the genetic level.

Experimental Section

General Procedures. All reactions were carried out under an atmosphere of dry nitrogen at room temperature in oven-dried glassware unless otherwise noted. Melting points are uncorrected. Radioactive samples were counted in a Beckman LS 1801 liquid scintillation counter using Aquasol-2 (New England Nuclear) scintillation cocktail. Routine GC–MS identifications of synthetic intermediates were performed on a Hewlett-Packard 5790A gas chromatograph with a 5970 mass selective detector. A New Brunswick G25 rotary shaker was used for fermentations.

Materials. All chemicals and solvents were reagent grade and were used as received, with the exception that diethyl ether was distilled from sodium benzophenone ketyl. Authentic thiostrepton was obtained as a gift from Squibb Corp. or purchased from Calbiochem, San Diego, CA. Ingredients for fermentations were purchased from Difco and Sigma. The $\alpha_2\beta_2$ complex of tryptophan synthase was a gift from Dr. Edith Miles of the National Institutes of Health, Bethesda, MD. (*S*)-[1,2-$^{13}C_2$]-, (*S*)-[2,3-$^{13}C_2$]- (both 99% ^{13}C), (*S*)-[3-^{13}C,$^2H_2$]- (99% ^{13}C, 99% ^2H), and (2*S*,3*S*)-[3-^{13}C,$^2H_1$]serine (99% ^{13}C, 93% ^2H) were provided by the Los Alamos Stable Isotope Resource. (*methyl-R*)-[*methyl*-$^2H_1$,^3H]Methionine and (*methyl-S*)-[*methyl*-$^2H_1$,^3H]methionine were prepared earlier in this laboratory by following a published procedure.[37] The biosynthesized thiostrepton samples were degraded to acetic acid by Kuhn–Roth oxidation as described earlier,[38] and the configurational analysis of these acetic acid samples was carried out by following the method of Cornforth *et al.*[39] and Arigoni and co-workers[40] using a standard protocol of our laboratory.[41] (*S*)-[$^{13}C^2H_3$]Methionine was synthesized from [^{13}C,$^2H_3$]iodomethane and (*S*)-homocysteine.[28] The following companies supplied stable isotope-labeled compounds: [^{15}N]ammonium chloride (99.5%), potassium[^{13}C] cyanide (99%), $^{13}CO_2$ gas (99%), ISOTEC Inc.; (*R,S*)-[3-^{13}C]cysteine (97%), Cambridge Isotope Laboratories; (*R,S*)-[1-^{13}C]serine (99%), (*R,S*)-[3-^{13}C]serine (90%), (*S*)-[*methyl*-^{13}C]methionine (98%), (*R,S*)-[1-^{13}C]tryptophan (99%), [^{13}C,$^2H_3$]iodomethane (99% ^{13}C and 98% ^2H), MSD Isotopes. Sodium[^3H] borohydride (1.4 Ci/mmol) was purchased from Amersham.

Chromatography. Reactions were monitored by thin-layer chromatography (TLC) using E. Merck silica gel 60F-254 glass plates (0.25 mm). Compounds were visualized by illumination with UV light and/or spraying with ethanolic phosphomolybdic acid, ninhydrin, or aqueous potassium permanganate solution, followed by heating. Preparative TLC was conducted on E. Merck silica gel 60F-254 glass plates (1 or 2 mm); in the case of radioactive materials, silica gel 60F-254 aluminum sheets (0.2 mm) were used. Column chromatography was performed on E. Merck silica gel 60 (230–400 mesh). Solvents for chromatography were HPLC grade.

NMR Spectroscopy. Spectra of thiostrepton samples were acquired in chloroform-*d*/methanol-d_4 (4:1) on an IBM AF-300 spectrometer operating at 7.1 T. ^1H NMR spectra were recorded at 300 MHz. Chemical shifts are reported in ppm downfield from tetramethylsilane with the solvent resonance as an internal standard. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broadened, m = multiplet), coupling constants (Hz), integration. ^{13}C NMR spectra were recorded at 75.4 MHz. A continuous-wave deuterium decoupler was employed for analysis of samples containing ^{13}C–^2H bonds. 2D-INADEQUATE spectra were acquired over 48 h under the following conditions: repetition time of 1 s, spectral width in F2 either +14.8 or +15.1 kHz in 4K data sets, spectral width in F1 either +14.8 or +15.1 kHz in 1K data sets sampled either in 128 increments and zero-filled to 512 data points or in 256 increments and zero-filled to 380 data points. NMR spectroscopy of tritiated samples was carried out at 320.13 MHz on an IBM AF-300 spectrometer equipped with a tritium channel and a dual ^1H–^3H 5-mm probe. Tritium spectra were referenced to the proton resonance of TMS with a conversion factor of 1.066 639 74 for obtaining the equivalent tritium frequency.

Organism and Fermentation. *S. azureus* ATCC 14921 and *S. laurentii* ATCC 31255 were obtained from the American Type Culture Collection, Rockville, MD. The strains were transferred from a lyophile tube and incubated on yeast malt agar (YME agar) for 3 days at 28 °C. Isolated colonies were picked from the slants and inoculated into medium A. This seed culture was incubated on a gyrotary shaker at 250 rpm for 2 days. After growth was complete, aliquots (2 mL) were stored aseptically with glycerol (15%) as a cryoprotectant at −78 °C (frozen vegetative mycelia or FVM). The FVMs could be stored for at least 1 year with no significant loss of production capability and were used to start a new generation on YME agar. These slants were then used to start all fermentations (medium A) for the feeding experiments. A 5-mL sample of a seed culture was used to inoculate 50 or 100 mL of production medium (medium B) in 250- or 500-mL Erlenmeyer flasks. These incubations were carried out at 26–27 °C for 72–80 h on a gyrotary shaker at 250 rpm. Under ideal conditions, the titer of the antibiotic was 70–80 mg/L.

The following media were used for this study: Medium A, the liquid seed medium, contained (g/L) soybean flour (15), dextrose (30), $CaCO_3$ (2.5), and NaCl (1). Medium B was the production medium used for all the feeding experiments and was composed of (g/L) glucose (100), soybean flour (20), yeast extract (1), corn steep liquor (35), KH_2PO_4 (1), Na_2HPO_4 (1.4), $(NH_4)_2SO_4$ (1), $CaSO_4$ (2), $MgSO_4{\cdot}7H_2O$ (0.2), $FeSO_4{\cdot}7H_2O$ (0.2), $MnSO_4{\cdot}2H_2O$ (0.5), $Na_2MoO_4{\cdot}2H_2O$ (0.005), H_3BO_3 (0.005), $CuSO_4{\cdot}2H_2O$ (0.01), $ZnSO_4{\cdot}7H_2O$ (0.01), and $CoCl_2{\cdot}6H_2O$ (0.05). The pH value of the production medium was adjusted to 7.0 with NaOH before sterilization for 15 min at 121 °C.

Isolation of Thiostrepton. The following procedure is an example of the routine isolation of thiostrepton from 250 mL of fermentation broth. This method was used for preparing thiostrepton samples for analysis by ^{13}C NMR spectroscopy.

Following the completion of the fermentation, an equal volume of chloroform was added to the culture and the mixture homogenized in a blender. The aqueous layer with the broken mycelia was then separated from the chloroform layer by centrifugation at 7000 rpm for 10 min. Extraction of the aqueous phase was repeated, and the organic layers were combined and evaporated to dryness under vacuum below 35 °C in a rotary evaporator. The residue was dissolved in a minimum amount of chloroform, and thiostrepton was precipitated by addition of *n*-hexane. The precipitate was collected by centrifugation. This procedure was repeated twice. The precipitates were combined and then redissolved in a minimum amount of methylene chloride/ethanol (4:1). Thiostrepton was precipitated by the addition of diethyl ether and collected by centrifugation. The purity of the isolated thiostrepton was estimated by comparing HPLC integration (254-nm detection) with the mass of the isolate; it was usually >90%.

Thiostrepton Assay and HPLC. Rapid assays of thiostrepton in the fermentation broth were carried out by HPLC. A 1-mL aliquot of the whole broth was agitated with the same amount of chloroform and collected by centrifugation (10 min at 3000 rpm); an equal amount of glacial acetic acid was added to the organic layer. An aliquot of this solution (15 μL) was injected directly into the HPLC column.

The HPLC system consisted of a Beckman 116A pump, a Beckman 210A injector, a Hamilton PRP-1 C_{18} column (10 mm, 250 × 4.1 mm), a Waters UV absorbance detector (equipped with a 254-nm filter), and

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Table III. Conditions of Feeding Labeled Precursors to **1**-Producing *S. azureus* and *S. laurentii*

expt no.	precursor	amount	no. of flasks	time of addition (h)	time of harvest (h)	amount of **1** isolated
1	(*R,S*)-[1-^{13}C]serine	250 mg[a]	20	34	72	24 mg
2	(*R,S*)-[3-^{13}C]serine	250 mg[a]	16	11, 27, 36	71	23 mg
3	(*R,S*)-[3-^{13}C]cysteine	50 mg[a]	20	12, 27, 37	72	21 mg
4	(*R,S*)-[1′-^{13}C]tryptophan	30 mg[a]	10	8, 32	72	11.5 mg
5	(*S*)-[*methyl*-^{13}C]methionine	150 mg[a]	10[b]	32, 52	72	26.5 mg
6	(*S*)-[*methyl*-^{13}C,^{2}H$_3$]methionine	200 mg[a]	10[b]	32, 52	72	42 mg
7	(*S*)-[1,2-^{13}C$_2$]serine	100 mg[c]	14	32, 52	76	71 mg
8	(*S*)-[2,3-^{13}C$_2$]serine	100 mg[c]	14	32, 52	76	90 mg
9	(*S*)-[3-^{13}C,^{2}H$_2$]serine	100 mg[c]	18	32, 52	76	43 mg
10	(2*S*,3*S*)-[3-^{13}C,^{2}H$_1$]serine	100 mg[c]	17	32, 52	80	45 mg
11	(*R,S*)-2-methyl-[3′-^{13}C]tryptophan	100 mg[c]	20	32, 46	72	100 mg
12	(*S*)-[1′,2′-^{13}C$_2$,*indole*-^{15}N]tryptophan	70 mg[c]	10	32, 52	72	10 mg
13	(*R,S*)-[1-^{13}C]isoleucine	100 mg[c]	16	32, 52	76	80 mg
14	(*R,S*)-[1-^{13}C]threonine	100 mg[c]	15	32, 52	72	47 mg
15	4-(1-hydroxy[1-^{3}H]ethyl)quinaldic acid	400 μCi[c]	5	40	78	4 mg, 40 μCi
16	(*methyl-R*)-[*methyl*-^{2}H$_1$,^{3}H]methionine	10 μCi[a]	1	32, 44	72	*d*
17	(*methyl-S*)-[*methyl*-^{2}H$_1$,^{3}H]methionine	10 μCi[a]	1	32, 44	72	*d*

[a] *S. azureus*. [b] In this case, the flask size was 500 mL with 100 mL of production medium. [c] *S. laurentii*. [d] Not determined, carrier added prior to isolation.

a Fisher Recordall recorder. The isocratic solvent system consisted of 60% acetonitrile in water at a flow rate of 0.5 mL/min. The retention time of thiostrepton under these conditions was approximately 9 min.

This method was used to establish the production curve and monitor fermentations. The calibration curve was obtained using a set of standard thiostrepton solutions of different concentrations. At a given detector sensitivity, the detector response was found to be a linear function of thiostrepton concentration within the range 2–150 mg/L.

Feeding Experiments. In general, the isotopically labeled substrates were added to the production cultures shortly after initiation of antibiotic synthesis. Each precursor was dissolved in deionized water and sterilized by filtration through a sterile 0.2-μm Millipore filter (Gelman). The labeled precursors were administered to the cultures in two or three portions as indicated (Table III). The cultures were harvested by centrifugation and **1** was purified as described above. The conditions of the individual experiments are listed in Table III.

In the experiments with tritiated precursors, the crude **1** obtained was further purified to constant specific radioactivity, as in the following example. Thiostrepton (4 mg, 40 μCi) obtained from feeding 4-(1-hydroxy-[1-^{3}H]ethyl)quinaldic acid was diluted with unlabeled thiostrepton (100 mg), and the mixture was dissolved in a minimum amount of chloroform and precipitated with *n*-hexane. This procedure was repeated twice and afforded 54.2 mg of **1** with a constant specific activity of 0.144 μCi/mg. On the basis of this, the incorporation of 4-(1-hydroxy-[1-^{3}H]ethyl)quinaldic acid was calculated to be 3.6%.

Synthesis of Labeled Precursors. (*R,S*)-[1-^{13}C]Isoleucine. This compound was prepared from potassium [^{13}C]cyanide (1.36 g, 20.6 mmol), ammonium chloride (1.26 g, 23.5 mmol), and 2-methylbutanal (2.2 mL, 21 mmol) by Strecker synthesis.[25] It was obtained as a white solid which proved to be a mixture of (*R,S*)-[1-^{13}C]isoleucine and (*R,S*)-[1-^{13}C]alloisoleucine. ^{13}C NMR (75.4 MHz, D_2O): δ 172.7, and 172.3 ppm (enriched, carboxyl groups). The mixture of the two amino acids was converted into the *N*-pentafluoropropionyl isopropyl ester derivatives as described in the literature.[82] The product mixture was taken up in methylene chloride and analyzed by GC–MS using a chiral column (Chiralsil Val III). Four peaks with almost identical fragmentation patterns were observed. The ratio of peak 1:peak 2:peak 3:peak 4 = 3.2:1:3.2:1.2; therefore the ratio of (*R,S*)-[1-^{13}C]isoleucine and (*R,S*)-[1-^{13}C]alloisoleucine was either 74%:26% or 26%:74%. (*S*)-Isoleucine was derivatized in the same fashion, and coinjection of both samples established that the minor component is (*R,S*)-[1-^{13}C]isoleucine (26%) and the major one is (*R,S*)-[1-^{13}C]alloisoleucine (74%).

(*R,S*)-[1-^{13}C]Threonine. This compound was synthesized from 2-(benzyloxy)propanal (1.9 g, 11.5 mmol), prepared by a published procedure,[26] potassium[^{13}C] cyanide (0.5 g, 7.57 mmol), and ammonium chloride (0.5 g, 9.3 mmol) by Strecker synthesis.[25] A white solid (550 mg, 4.5 mmol, 60%) was obtained which consisted of a mixture of (*R,S*)-[1-^{13}C]threonine (74%) and (*R,S*)-[1-^{13}C]allothreonine (26%). The ratio was determined by integration of the signals for the methyl groups of the two compounds in the ^{1}H NMR spectrum. ^{13}C NMR (75.4 MHz, D_2O): δ 170.95, 169.99 ppm (enriched, carboxyl groups).

(*S*)-[1′,2′-^{13}C$_2$,*indole*-^{15}N]Tryptophan. This compound was prepared from [^{15}N]indole, synthesized from [^{15}N]ammonium chloride and *o*-toluyl chloride via *o*-[^{15}N]toluidine[31] and (*S*)-[1,2-^{13}C$_2$]serine by a published procedure.[27] It was obtained as a white solid which was further purified by recrystallization from water/ethanol (149 mg, 0.72 mmol, 51.8%). The ^{13}C NMR spectrum showed two enriched signals: δ 171.64 (d, $^{1}J_{C-C}$ = 51.9, C1), 55.16 (d, $^{1}J_{C-C}$ = 51.9, C2). The following signals displayed couplings: δ 136.76 (d, $^{1}J_{C-N}$ = 15.0, C2), 127.60 (d, $^{2}J_{C-N}$ = 4.0, C3a), 124.60 (d, $^{1}J_{C-N}$ = 13.2, C7a), 27.30 (d, $^{1}J_{C-C}$ = 34.7, C3′).

(*R,S*)-2-Methyl-[3′-^{13}C]tryptophan. 2-Methyl-[*methylene*-^{13}C]gramine was prepared from $^{13}CO_2$ gas and 2-methylindole[34b] and then converted into (*R,S*)-2-methyl-[3′-^{13}C]tryptophan using the method of Weygand and Linden.[34a] 2-Methyl-[*methylene*-^{13}C]gramine (168 mg, 0.96 mmol) and diethyl formamidomalonate (272 mg, 1.25 mmol) yielded (*R,S*)-2-methyl-[3′-^{13}C]tryptophan (90 mg, 0.41 mmol, 42.9%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.51 (d, *J* = 7.13, 1H), 7.22 (d, *J* = 7.32, 1H), 6.94 (m, 2H), 3.50 (m, 1H), 3.28 (dm, *J* = 121.6, 1H), 2.84 (dm, *J* = 121.6, 1H), 2.28 (s, 3H). ^{13}C NMR (75.4 MHz, DMSO-d_6): δ 26.69 (enriched).

Methyl 4-(1-hydroxy-[1-^{3}H]ethyl)quinaldate. Methyl 4-acetylquinaldate (0.6 mg, 2.62 μmol), synthesized by a literature procedure,[44] was dissolved in methanol (0.5 mL), and the solution was added to sodium [^{3}H]borohydride (5 mCi, 1.4 Ci/mmol). The mixture was kept at room temperature while the reaction was followed by TLC (hexane/ethyl acetate (1:1), R_f = 0.2). After 30 min, the reaction was complete and the solution was acidified with 0.01 N HCl. The mixture was stirred overnight at room temperature. Water (0.2 mL) was then added and stirring continued for additional 3 h. The product was purified by preparative TLC on silica gel (hexane/ethyl acetate). The radioactive band at R_f = 0.2 was cut out and eluted with methanol. The product (total radioactivity 0.4 mCi) was not further purified. The eluate was concentrated to dryness and the residue dissolved in water (5 mL) and 0.01 N NaOH solution (0.2 mL). The mixture was stirred overnight at room temperature, then neutralized with 0.01 N HCl, and the solvent was evaporated *in vacuo*. The total radioactivity was 0.39 mCi (98%). ^{3}H NMR (320.13 MHz, D_2O): δ 5.90.

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