VOLUME 115, NUMBER 17
AUGUST 25, 1993

JOURNAL
OF THE
AMERICAN
CHEMICAL
SOCIETY

Biosynthesis of the Modified Peptide Antibiotic Nosiheptide in *Streptomyces actuosus*§

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Received March 5, 1993

Abstract: The biosynthesis of the highly modified thiopeptide antibiotic, nosiheptide (**1**), was studied by feeding radioactive and stable-isotope-labeled precursors to cultures of the producing organism, *Streptomyces actuosus*. The stable isotope enrichments and/or coupling patterns in the isolated **1** were analyzed by NMR spectroscopy using various 1D and 2D NMR techniques. The results complete the confirmation of the amino acid components of the antibiotic and shed light on a number of mechanistic and stereochemical aspects of its assembly from these basic building blocks. The dehydroalanine and butyrine moieties are formed by an *anti* elimination of water from serine and threonine, respectively, the thiazole rings from cysteine residues with loss of the *pro*-3*R* hydrogen in the oxidation step, and the hydroxypyridine moiety from two intact serine residues, situated nine amino acids apart in the peptide chain, and the carboxyl group of an adjacent cysteine. According to ^{15}N studies which included the complete assignment of the ^{15}N NMR resonances of **1**, the carboxy-terminal amide nitrogen of **1** originates from the amino group of serine, suggesting that the precursor peptide giving rise to **1** carries an additional carboxy-terminal serine residue which, except for its nitrogen, is removed during processing. The indolic acid moiety is elaborated separately by a direct rearrangement of tryptophan to 3-methylindole-2-carboxylic acid and methylation at C4; it seems to be attached to the peptide backbone prior to hydroxylation of the C4′ methyl group. Possible mechanisms of some of the key reactions and the mode of assembly of the modified peptide structure are discussed.

Introduction

Nosiheptide (**1**) (Figure 1), a metabolite of *Streptomyces actuosus*, belongs to a family of highly modified cyclic peptide antibiotics currently numbering about 20. The complete structure of **1** has been elucidated by chemical degradations[1,2] and X-ray crystallography.[3] The structural features of these compounds include a macrocycle containing four thiazole rings and a pyridine or tetrahydropyridine ring, and a side chain with one or two more thiazole rings and one or two dehydroalanines. The structure is completed by an extra loop which in the case of **1** simply consists of a unique indolic acid moiety but in the case of most other thiopeptides of a short peptide chain incorporating a quinaldic acid moiety.

Most of the thiopeptides inhibit protein synthesis in Gram-positive bacteria and share a common mode of action. They act on the 50S ribosomal subunit by tightly binding to the complex of 23S rRNA with ribosomal protein L-11 and inhibit the activities

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§ Dedicated to Prof. Wolfgang Steglich (Munich), on the occasion of his 60th birthday.
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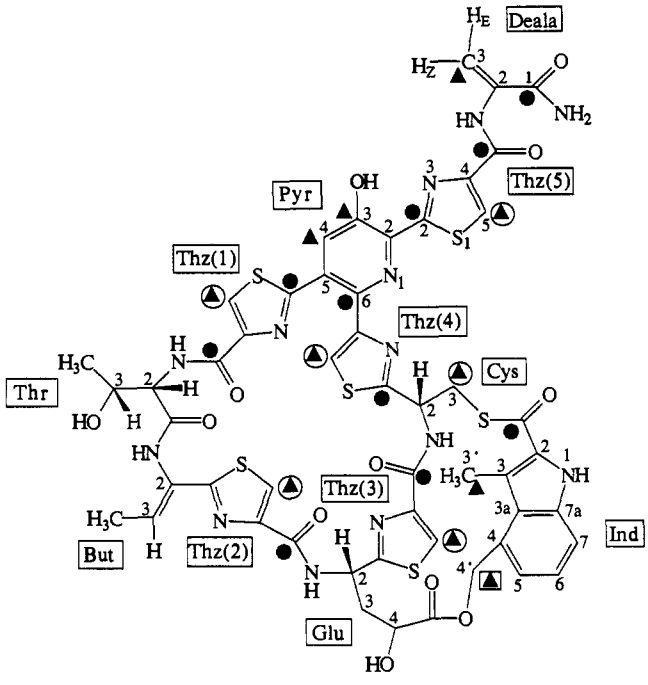


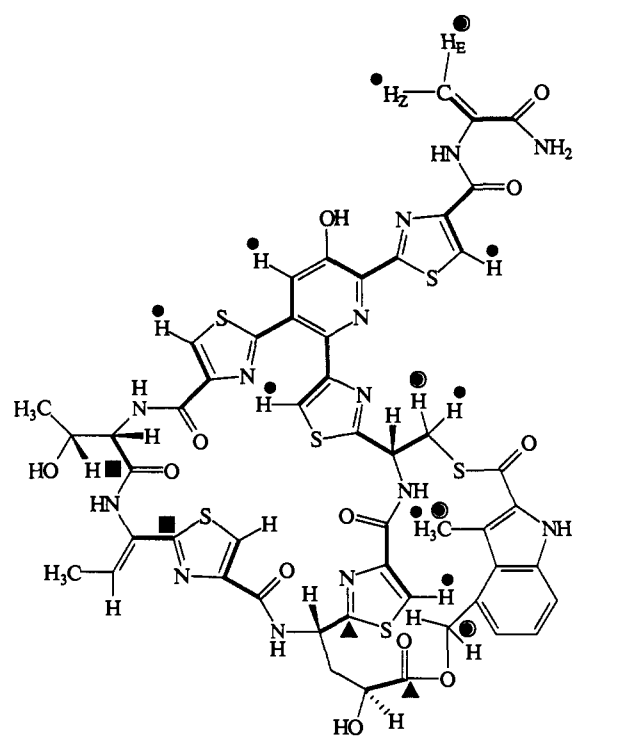
● DL-[1-^{13}C]serine ▲ L-[3-^{13}C]serine

○ DL-[3-^{13}C]cysteine □L-[*methyl*-^{13}C]methionine

Figure 1. Nosiheptide (**1**) and its labeling pattern from selected precursors.[10]

of elongation factors Tu and G.[4] Resistance to these antibiotics is due to methylation of O-2′ of an adenosine residue at a single site on the 23S rRNA, resulting in the inhibition of antibiotic binding.[4] In contrast, cyclothiazomycin,[5] which was recently reported by Seto *et al.*,[6] exhibits inhibitory activity against renin[6] as well as potent antifungal activity.[5a] Although nosiheptide is used commercially as a feed additive to increase weight gains in poultry and hogs,[7] none of the thiopeptide antibiotics have found applications in human medicine due to their low water solubility and poor resorption from the gastrointestinal tract.

Complete and unequivocal assignments of all signals in the ^{13}C and ^{1}H NMR spectra of **1** were established by applying a variety of 1D and 2D as well as multiple quantum techniques to unlabeled and biosynthetically multiple labeled samples.[8,9] In two earlier papers[9,10] we discussed feeding experiments with *S. actuosus* using various ^{14}C- and ^{13}C-labeled amino acids, which revealed the labeling pattern for **1** shown in Figure 1. The present paper details further biosynthetic studies which completely confirm the origins of the amino acid components of **1** and provide considerable additional insights into mechanistic and stereochemical aspects of the assembly of **1** from its basic building blocks.



— bond labels from S-[1,2-$^{13}C_2$]- or [2,3-$^{13}C_2$]serine

▲ S-[1,5-$^{13}C_2$]glutamic acid

■ R,S-[1-^{13}C]threonine

● deuterium from S-[3-^{13}C,$^{2}H_2$]serine

○ deuterium from (2S,3S)-[3-^{13}C,$^{2}H_1$]serine

Figure 2. Positional and bond-labeling patterns of **1** biosynthesized from ^{13}C and deuterium-labeled precursors.

Results

Precursor Amino Acids. In order to complete the confirmation of the amino acid precursors of nosiheptide, (*S*)-[1,5-$^{13}C_2$]glutamic acid and (*R,S*)-[1-^{13}C]threonine were fed to *S. actuosus* and the resulting nosiheptide samples analyzed by ^{13}C NMR. Nosiheptide biosynthesized from (*S*)-[1,5-$^{13}C_2$]glutamic acid was labeled in the carboxyl group of the hydroxyglutamic acid moiety (δ 172.62, 0.6% enriched) and C2 of thiazole 3 (δ 170.04, 0.65% enriched) (Figure 2). This result was as expected since it was known[9,10] that the thiazole rings derive from cysteine and the carboxyl group of the adjacent amino acid. As anticipated, (*R,S*)-[1-^{13}C]threonine labeled the carbonyl carbon of the threonine moiety (δ 167.69) and C2 of thiazole 2 (δ 166.32); the enrichments were 1.7% and 2.0%, respectively. This confirmed that the threonine and the dehydrobutyrine residue are derived from threonine (Figure 2).

Dehydroalanine. As shown earlier,[9,10] C1 and C3 of the dehydroalanine (Deala) moiety in **1** are labeled by C1 and C3, respectively, of serine. Intact incorporation of serine was demonstrated by observing the expected ^{13}C–^{13}C coupling patterns in the 2D INADEQUATE[11,12] spectra of the nosiheptide samples from feeding experiments with (*S*)-[1,2-$^{13}C_2$]- and (*S*)-[2,3-$^{13}C_2$]serine (Figure 2, Table I). Thus, the dehydroalanine unit clearly arises by elimination of water from a serine moiety. The steric course of this process was elucidated by two feeding

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Table I. NMR Spectral Data of **1** derived from (*S*)-[1,2-$^{13}C_2$]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 CO	165.00	64.1[a]	Deala 2				
Deala 2	134.26	64.2[b]	Deala CO	75.9[b]	Deala 3		
Deala 3	103.60			76.2[c]	Deala 2	0.56	0.25
Thz(5)CO	158.20	77.6[a]	Thz(5) 4				
Thz(5) 2	167.10	81.5[a]	Pyr 2				
Thz(5) 4	149.57	77.6[b]	Thz(5)CO	65.4[b]	Thz(5) 5		
Thz(5) 5	126.80			67.0[c]	Thz(5) 4	0.32	no inc[e]
Pyr 2	135.00	81.7[b]	Thz(5) 2	65.4[b]	Pyr 3		
Pyr 3	150.80			65.3[c]	Pyr 2		
Pyr 4	127.12			62.0[c]	Pyr 5	0.26	no inc
Pyr 5	129.90	71.6[b]	Thz(1) 2	58.0[b]	Pyr 4		
Pyr 6	142.52	78.7[a]	Thz(4) 4				
Thz(1) 2	163.85	71.1[a]	Pyr 5				
Thz(1) 4	149.83	76.3[b]	Thz(1)CO	65.8[b]	Thz(1) 5		
Thz(1) 5	125.98			67.0[c]	Thz(1) 4	0.23	no inc
Thz(1)CO	159.45	76.5[a]	Thz(1) 4				
Thr 2	56.57			52.0[c]	Thr CO		
Thr 3	66.50						
Thr CH_3	18.25						
Thr CO	167.69			51.0[b]	Thr 2		
But 2	129.27			49.7[c]	Thz(2) 2		
But 3	128.85						
But CH_3	13.50						
Thz(2) 2	166.32			45.0[b]	But 2		
Thz(2) 4	147.62	76.8[b]	Thz(2)CO	66.2[b]	Thz(2) 5		
Thz(2) 5	124.45			65.9[c]	Thz(2) 4	*d*	*d*
Thz(2)CO	159.60	76.7[a]	Thz(2) 4				
Glu 2	45.15			59.2[b]	Thz(3) 2		
Glu 3	37.60						
Glu 4	66.40			60.0[c]	Glu CO		
Glu CO	172.62			61.9[b]	Glu 4		
Ind 2	130.40						
Ind 3	118.35						
Ind 3a	124.70						
Ind 4	129.20						
Ind 4′	65.90			enr[f]		0.15	0.17
Ind 5	123.23						
Ind 6	124.91						
Ind 7	114.40						
Ind 7a	137.60						
Ind CO	181.80						
Ind CH_3	12.23	enr		enr		0.49	0.24
Cys 2	49.05	58.8[b]	Thz(4) 2	36.6[b]	Cys 3		
Cys 3	29.49			36.9[c]	Cys 2	0.44	0.24
Thz(4) 2	168.98	58.8[a]	Cys 2				
Thz(4) 4	153.10			66.7[b]	Thz(4) 5		
Thz(4) 5	119.98			66.6[c]	Thz(4) 4	0.23	no inc
Thz(3) CO	159.80	77.2[a]	Thz(3) 4				
Thz(3) 2	170.04			59.0[c]	Glu 2		
Thz(3) 4	148.70	77.1[b]	Thz(3)CO	65.9[b]	Thz(3) 5		
Thz(3) 5	125.25			65.5[c]	Thz(3) 4	0.19	no inc

[a] Carbon derived from C1 of (*S*)-[1,2-$^{13}C_2$]serine. [b] Carbon derived from C2 of (*S*)-[1,2-$^{13}C_2$]- and (*S*)-[2,3-$^{13}C_2$]serine. [c] Carbon derived from C3 of (*S*)-[2,3-$^{13}C_2$]serine. [d] No isotope-shifted signal detected. [e] No incorporation. [f] Enriched.

experiments with 3-deuterated serine samples, (*S*)-[3-^{13}C, $^{2}H_2$]serine and (2*S*,3*S*)-[3-^{13}C,$^{2}H_1$]serine. Nosiheptide biosynthesized from (*S*)-[3-^{13}C,$^{2}H_2$]serine retained both atoms of deuterium at C3 since the {^{1}H,^{2}H} ^{13}C NMR spectrum of this sample clearly showed the isotope-shifted peak 0.56 ppm upfield from the natural abundance signal at δ 103.60. The {^{1}H,^{2}H} ^{13}C NMR spectrum of the sample from the (2*S*,3*S*)-[3-^{13}C,$^{2}H_1$]serine feeding displayed a 0.25 ppm isotope shifted peak for the Deala C3 signal. Methylene groups that are stereospecifically labeled with deuterium can be analyzed by ^{2}H-decoupled 2D ^{1}H–^{13}C NMR shift correlation spectroscopy.[13] This experiment was carried out in the CH selective mode with the latter sample. The observed correlation between Deala C3 (δ 103.60) and its *Z* hydrogen (δ 6.37) indicated that the hydrogen was located in the *Z* and the deuterium in the *E* position. Additional one-dimensional single-frequency ^{2}H-decoupling {^{1}H} ^{13}C NMR experiments confirmed that the *pro-S* hydrogen from C3 of serine is stereospecifically incorporated into the *E* position at C3 of the dehydroalanine moiety. Assuming that the configuration of the serine moiety in the precursor peptide is still *S*, this result indicates an *anti* stereochemistry for the elimination of water to give dehydroalanine. The same steric course is evident from the configurations of precursor and product for the dehydration of threonine to the dehydrobutyrine moiety, again assuming that the configuration of the threonine moiety does not change upon incorporation into the peptide.

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Cysteine Moiety. The cysteine moiety (Cys) of nosiheptide has been shown to be derived from cysteine,[9,10] which in turn is formed from serine. The intact incorporation of serine into the cysteine moiety was verified by feeding (*S*)-[1,2-$^{13}C_2$]- and (*S*)-

[2,3-$^{13}C_2$]serine (Table I, Figure 2). Nosiheptide biosynthesized from (*S*)-[3-^{13}C,$^2H_2$]serine retained two atoms of deuterium at Cys C3 since the {^1H,^2H} ^{13}C NMR spectrum displayed a peak at δ 30.59, shifted 0.44 ppm upfield from the natural abundance signal of Cys C3. The feeding experiment with (2*S*,3*S*)-[3-^{13}C,$^2H_1$]serine revealed the retention of one atom of deuterium; a 0.24 ppm upfield shifted peak was observed. In the ^2H-decoupled CH selective 2D HETCOR spectrum a correlation between Cys C3 (δ 30.15) and Cys H3 at δ 3.86 proved that this proton is derived from the *pro-R* hydrogen of serine. No correlation was observed between Cys C3 and the other Cys H3 at δ 3.56. The conversion of serine to cysteine is thus a stereospecific process, but the steric course cannot be deduced from this experiment, because we lack individual assignments for these diastereotopic hydrogens in the ^1H NMR spectrum; extensive NOE experiments did not distinguish these signals. However, assuming that this conversion proceeds in *S. actuosus* with the same stereochemistry as in *Escherichia coli*, i.e., retention of configuration,[14] we can suggest assignments for these resonances as δ 3.86 for H_R and δ 3.56 for H_S at C3 of the cysteine moiety.

Thiazoles. The formation of the thiazole rings from one molecule of cysteine and the carboxy group of the adjacent amino acid was further confirmed by intact incorporation of (*S*)-[1,2-$^{13}C_2$]- and (*S*)-[2,3-$^{13}C_2$]serine; the nosiheptide samples clearly showed the expected ^{13}C–^{13}C coupling patterns in the 2D INADEQUATE spectra (Table I, Figure 2). In the feeding experiment with (*S*)-[3-^{13}C,$^2H_2$]serine, retention of one atom of deuterium was seen at C5 of all thiazole rings except thiazole 2; no deuterium was detectable at C5 of the latter. No incorporation of deuterium from (2*S*,3*S*)-[3-^{13}C,$^2H_1$]serine into the thiazole moieties was observed. Consequently, the hydrogens at C5 of thiazole 1, 3, 4, and 5 arise from the *pro*-3*R* hydrogen of serine, while the H5 of thiazole 2 may not originate from either of the methylene hydrogens of serine.

Pyridine Moiety. The pyridine moiety (Pyr) of nosiheptide originates from two molecules of serine and the carboxyl group of an adjacent cysteine. The two serine units are incorporated in an unusual manner; they are connected to each other through their methylene carbons.[9,10] Thus, the pyridine ring may be formed by a "tail-to-tail" condensation of two molecules derived intact from serine; alternatively, one or both of these serine molecules may first be metabolized to smaller carbon fragments, e.g., glycine and a C_1 unit, from which the pyridine ring is then assembled. The results of the feeding experiments with (*S*)-[1,2-$^{13}C_2$]- and (*S*)-[2,3-$^{13}C_2$]serine provided important information on this issue. The 2D INADEQUATE spectrum of nosiheptide biosynthesized in the (*S*)-[2,3-$^{13}C_2$]serine feeding experiment exhibited connectivities between Pyr C3 and Pyr C2 as well as Pyr C4 and Pyr C5. Nosiheptide biosynthesized from (*S*)-[1,2-$^{13}C_2$]serine showed the connectivities Pyr C2 to Thz(4) C2 and Pyr C5 to Thz(1) C2 (Table I, Figure 2). These results indicate that the three-carbon segments Pyr C3–Pyr C2–Thz(4) C2 and Pyr C4–Pyr C5–Thz(1) C2 do indeed each arise intact from serine. This finding dismisses the possibility that the hydroxypyridine residue arises from serine less directly, e.g., via glycine and methylenetetrahydrofolate or some other metabolic breakdown products of serine. Feeding (*S*)-[3-^{13}C,$^2H_2$]serine revealed the retention of one atom of deuterium at C4 of the hydroxypyridine moiety; the resulting **1** showed a peak shifted 0.26 ppm upfield from the nondeuterated signal at δ 126.73. Deuterium from (2*S*,3*S*)-[3-^{13}C,$^2H_1$]serine was not incorporated; therefore the *pro*-3*S* hydrogen of serine must be the one that is eliminated in the aromatization of the pyridine moiety (Table I, Figure 2).

Indolic Acid Moiety. Initial biosynthetic experiments[9,10] had indicated that the indolic acid moiety (Ind) is derived from (*S*)-tryptophan. More detailed investigations with a double-labeled sample of tryptophan further showed that formation of the indolic acid proceeds by an intramolecular migration of the tryptophan carboxyl group to C2 of the indole ring.[10] In this process the α-carbon and the amino group of the tryptophan side chain are lost, and the methylene carbon is transformed into the methyl group of the indolic acid. The hydroxymethyl group at C4 was shown to be derived from the methyl group of (*S*)-adenosylmethionine.[9,10] Although the origins of the carbon skeleton of the indolic acid moiety were thus known, the exact sequence of reactions of this transformation of tryptophan and their mechanisms were not at all evident.

To probe whether the methylene group of the side chain passes through a higher oxidation state, e.g., the aldehyde, during the conversion of tryptophan into the indolic acid moiety of **1**, we synthesized (*R*,*S*)-[3-^{13}C,$^2H_2$]tryptophan by the method of Weygand and Linden[15] and fed it to *S. actuosus*. The {^1H,^2H} ^{13}C NMR spectrum of the resulting **1** showed a ^{13}C resonance for the indolic acid methyl group which was deuterium-shifted upfield by 0.49 ppm, indicating the retention of both deuterium atoms. Consistent with this, **1** biosynthesized from (*S*)-[3-^{13}C,$^2H_2$]serine also retained two atoms of deuterium, whereas **1** from the (2*S*,3*S*)-[3-^{13}C,$^2H_1$]serine feeding experiment retained one atom of deuterium at this position (Table I). Hence, mechanisms involving intermediates of a higher oxidation level at that carbon atom can be ruled out.

An earlier experiment, which showed no incorporation of radiolabeled 4-methyltryptophan, had already suggested that methylation in the 4-position of indole is not the first step in the conversion sequence.[10] This tentative conclusion was confirmed in another feeding experiment with (*R*,*S*)-4-methyl[3′-^{13}C]tryptophan, which also was not incorporated at all. This finding is in notable contrast to the situation in the biosynthesis of thiostrepton,[16] where the formation of the quinaldic acid moiety is initiated by methylation of tryptophan at C2 of the indole ring.

One plausible late precursor of the indolic acid moiety is 4-(hydroxymethyl)-3-methylindole-2-carboxylic acid (**2**), which can be obtained as the major product of alkaline hydrolysis of nosiheptide.[1] A tritium label was introduced into the hydroxymethyl group of this compound as outlined in Figure 3 to yield 4-[4′-^3H]-**2**, 522.8 μCi of which as then fed to *S. actuosus*. An aliquot of the isolated **1** from this experiment was diluted with unlabeled material and recrystallized to constant specific radioactivity. The incorporation was found to be only 1.2%. This contrasts with the result obtained when a sample of 3,4-[4′-^3H]dimethylindole-2-carboxylic acid ([4′-^3H]-**3**), easily obtained by the route shown in Figure 3, was fed to *S. actuosus*. The resulting nosiheptide showed an incorporation of 6.8% after similar purification to constant specific radioactivity. This sample of **1** was then degraded in order to determine whether the tritiated **3** had indeed been incorporated specifically into the indolic acid moiety. Base hydrolysis followed by esterification afforded 4-(hydroxymethyl)-3-methylindole-2-carboxylic acid methyl ester which had 95% of the specific molar radioactivity of **1**. These results indicate that **3** is probably indeed one of the free intermediates in the biosynthetic conversion of L-tryptophan to the indolic acid moiety of **1**, whereas the substantially lower incorporation of **2** argues against its role as a free intermediate.

The most likely next earlier precursor, 3-methylindole-2-carboxylic acid (**4**), was synthesized carrying a ^{13}C label in the carboxyl group following a procedure reported by C. D. Jones.[17] The ^{13}C NMR spectrum of **1** biosynthesized from this material showed a greatly enhanced signal at δ 181.8 corresponding to an enrichment of 65% in the carboxyl group of the indolic acid moiety.

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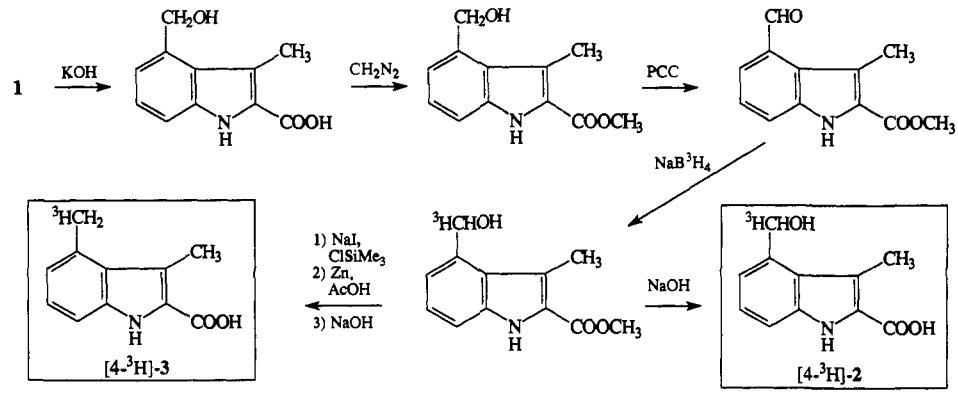

Figure 3. Preparation of [4-^{3}H]-**2** and [4-^{3}H]-**3**.

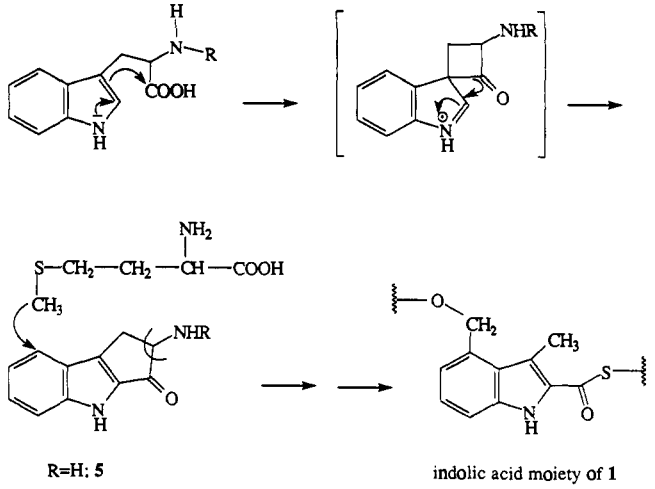

Figure 4. Originally proposed mechanism for the conversion of tryptophan into the indolic acid moiety of **1**.

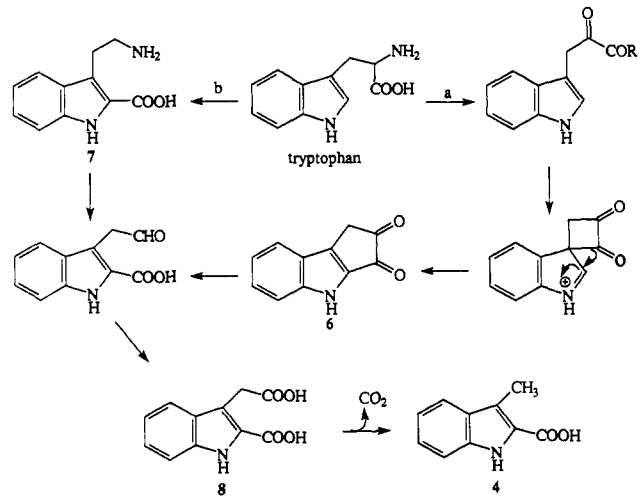

Figure 5. Alternative pathways for the formation of the indolic acid moiety of **1**.

Given this extremely efficient incorporation, **4** must be a rather immediate precursor of the indolic acid moiety.

A possible mechanism for the formation of the indolic acid moiety we considered[10,18] is shown in Figure 4. Acylation of the indole ring of tryptophan by the side chain carboxyl group, possibly via a spiro intermediate which then undergoes a 1,2-shift, produces the tricyclic amine **5**. This would then undergo fragmentation to yield **4**, ammonia, and formaldehyde. This pathway was tested by a feeding experiment with *rac*-2-amino-3-oxo-[1-^{13}C]cyclopent[*b*]indole hydrochloride ([1-^{13}C]-**5**), synthesized by a published procedure[19] from (*R,S*)-[3′-^{13}C]tryptophan, which in turn was prepared from $^{13}CO_2$ gas by the standard procedure of Weygand and Linden.[15] Surprisingly, absolutely no incorporation into nosiheptide was observed. In a variant of this mechanism, tryptophan may first undergo transamination to the α-keto acid before the intramolecular acylation of the indole ring to give, in this case, a tricyclic diketone intermediate, 2,3-dioxocyclopent-[*b*]indole (**6**). The latter could be converted into **4** by a mechanism analogous to the one shown in Figure 4, or alternatively, hydrolytic opening of the C ring might give 2-carboxyindole-3-acetaldehyde, which could then be oxidized to 2-carboxyindole-3-acetic acid (**8**) and decarboxylated to **4** (Figure 5, path a). However, a feeding experiment with the tricyclic diketone [1-^{13}C]-**6**, prepared by deamination of [1-^{13}C]-**5**, again gave no incorporation of the label into **1**.

As another hypothetical mechanism (Figure 5, path b), we considered a pyridoxal phosphate-mediated decarboxylation of tryptophan in which the enzyme-bound CO_2 generated from the carboxyl group is immediately reattached intramolecularly to C2 of the indole ring. The resulting tryptamine-2-carboxylic acid (**7**) would then be metabolized, possibly again via 2-carboxyindole-3-acetaldehyde and **8** to give 3-methylindole-2-carboxylic acid (**4**). This idea was probed in a feeding experiment with [*indole*-^{15}N]-**7**.[20] No incorporation of ^{15}N into the resulting nosiheptide was detected. As a further test of this and the previous mechanism we also synthesized [*methylene*-^{13}C]-**8**, which potentially may be an intermediate on either pathway, and fed it to *S. actuosus*. Again, absolutely no incorporation into **1** was detected. The negative outcome of the experiments with all these potential intermediates between tryptophan and **4** leaves the question of the detailed mechanism of this transformation open. It in fact suggests the possibility that the entire process may take place in a concerted fashion on the same enzyme without any free intermediates.

Another issue we examined is the formation of the C4′ methylene group of the indolic acid moiety. Nosiheptide biosynthesized from (*S*)-[3-^{13}C,$^2H_2$]- and (2*S*,3*S*)-[3-^{13}C,-$^2H_1$]serine each retained one atom of deuterium at C4′ of the indolic acid; the {^{1}H,^{2}H} ^{13}C NMR spectra displayed deuterium-shifted resonances 0.31 ppm upfield from the natural abundance signal at δ 65.90. One hydrogen from C3 of serine was therefore eliminated during the generation of this methylene group; if that process is stereospecific,[21] this would have to be the *pro-R* hydrogen. Two explanations can be considered for this obser-

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(21) Due to differences in conditions the two experiments do not reveal if equal amounts of deuterium are retained from both precursors. Therefore we cannot rule out the possibility, although unlikely, that one hydrogen is removed from C3 of serine nonstereospecifically, resulting in incorporation of one atom of deuterium per ^{13}C from the dideuterated precursor and between 0.5 and 1 atom from the stereospecifically deuterated one.

Table II. ^{15}N NMR Signal Assignments for Nosiheptide and Incorporation of ^{15}N-Labeled Precursors into 1 by *S. actuosus*

							$^{15}NH_4Cl$ feeding expt		[^{15}N]Gly feeding expt		[^{15}N]Gly + unlabeled serine feeding expt	
nitrogen position	$\delta_{^{15}N}$ (ppm)	correlated proton	$\delta_{^1H}$ (ppm)	correlated proton	$\delta_{^1H}$ (ppm)	nat abund pk ht (cm)	pk ht (cm)	rel abund[a]	pk ht (cm)	rel abund[a]	pk ht (cm)	rel abund[a]
Thz(4)N	325.3	Thz(4)H5	7.88[b]	Cys H2	5.88[b]	4.05	4.50	1.12	5.85	1.60	5.20	1.16
Thz(1)N	322.8	Thz(1)H5	8.65[b]			4.95	4.30	0.88	6.05	1.35	5.10	0.94
Pyr N	317.8					2.70	3.30	1.23	8.30	3.41	6.25	2.10
Thz(3)N	313.3	Thz(3)H5	8.30[b]	Glu H2	5.63[b]	3.45	3.65	1.07	4.75	1.53	4.10	1.08
Thz(2)N	310.5	Thz(2)H5	8.16[b]			3.10	3.15	1.03	4.35	1.55	4.10	1.20
Thz(5)N	303.9	Thz(5)H5	8.55[b]	Deala NH	10.04[d]	3.06	4.00	1.32	3.55	1.29	2.00	0.60
Ind NH	137.3	Ind NH	11.19[c]			3.30	2.20	0.70	3.45	1.17	2.80	0.85
Deala NH	130.7	Deala NH	10.04[c]	Deala $H3_E$	5.76[e]	3.90	3.55	0.91	10.25	2.90	10.45	2.44
But NH	127.9	But NH	9.32[c]			3.20	2.90		2.45		3.05	
Glu NH	126.6	Glu NH	8.35[c]	Glu H2	5.63[e]	4.10	4.45	1.10	4.85	1.30	4.20	0.93
Cys NH	123.6	Cys NH	7.72[c]	Cys H2	5.88[e]	3.25	3.35	1.04	5.35	1.83	3.20	0.90
Thr NH	115.9	Thr NH	7.64[c]	Thr H2	4.57[e]	2.30	2.45		2.35		2.75	
Deala NH_2	106.6					3.00	2.75	0.93	8.00	2.97	6.80	2.06

[a] Normalized peak height relative to average normalized peak height = 1.00 for Thr NH and But NH. [b] ($^3J_{NH}$). [c] ($^1J_{NH}$). [d] ($^4J_{NH}$). [e] Relayed.

vation: (i) the elimination of the *pro-R* hydrogen occurs during the metabolic conversion of serine to methionine or (ii) two hydrogens are removed from the methyl group after the methylation, e.g., by oxidation of C4′ to the aldehyde during the biosynthesis. A feeding experiment with [*methyl*-^{13}C,$^2H_3$]-methionine yielded nosiheptide which in the ^{13}C NMR showed the deuterated resonance shifted 0.61 ppm. Thus, two atoms of deuterium from the methionine methyl group were retained at Ind C4′, ruling out the possibility of an additional hydrogen loss after the methylation step. Consequently, one hydrogen from C3 of serine must be eliminated during the metabolic conversion of this carbon into the methyl group of methionine. This conversion presumably proceeds via 5,10-methylenetetrahydrofolate; a plausible explanation for a stereospecific loss of the *pro*-3*R* hydrogen is that the 5,10-methylenetetrahydrofolate is in rapid equilibrium with 5,10-methenyltetrahydrofolate. This redox process involves removal of the methylene hydrogen in 5,10-methylenetetrahydrofolate, which originates from the *pro*-3*R* hydrogen of serine.[22]

^{15}N NMR Assignments and Origin of Nitrogens. The carboxy terminus of the peptide chain in most of the thiopeptide antibiotics, including **1**, is blocked as a carboxamide function. The mode of formation of this carboxamide group and the origin of its nitrogen are not obvious. Early on we hypothesized that this nitrogen atom may originate from the amino nitrogen of the tryptophan, which is rearranged to the indolic acid moiety; as descibed above, the α-carbon and this nitrogen are lost in the process. This would imply that the tryptophan giving rise to the indolic acid moiety is initially part of the original peptide chain. This notion was tested by a feeding experiment with [3′-^{13}C, *amino*-^{15}N]tryptophan. The resulting **1** showed the expected incorporation of ^{13}C into the methyl group of the indolic acid moiety (8.0% enrichment), but no detectable incorporation of the ^{15}N label into the terminal amide function. Although one can make the argument that the ^{15}N may have been washed out of the precursor by reversible transamination prior to its incorporation into the antibiotic, it seems unlikely that this process would be so fast as to result in no ^{15}N incorporation at all. Thus, the outcome of this experiment casts serious doubt on this hypothesis.

Alternatively, the carboxamide group may arise by an amidation of the carboxy group; the nitrogen should then be derived either from the ammonium ion pool or from the amide function of glutamine. As another possibility the amide nitrogen may arise from the amino group of an amino acid that is no longer present in the final antibiotic. Such an amino acid could be part of a larger propeptide, for example as a component of a signal peptide or leader sequence, that would be cleaved at some point in the further processing of the peptide. In order to leave the amino acid nitrogen behind in the amide bond, the peptide linkage obviously must be cleaved by a mechanism other than simple hydrolysis. Precedent can be found in the formation of the analogous terminal carboxamide function of the mammalian pituitary hormone releasing factors, like TRH, by hydroxylation of a carboxy-terminal glycine and subsequent hydrolysis of the resulting carbinolamine.[23] While this precedent might suggest glycine as the source of the amide nitrogen, a structural comparison with the related thiopeptide antibiotic, thiostrepton,[24] containing two serine-derived carboxy-terminal dehydroalanine moieties, would point to serine as another candidate.

To determine the source of the terminal amide nitrogen, we carried out a series of feeding experiments with $^{15}NH_4Cl$ and [^{15}N]glycine. Interpretation of the data required unequivocal assignments of all the ^{15}N resonances of **1**. These were established using a biosynthetically enriched sample from a $^{15}NH_4Cl$ feeding experiment as well as a natural abundance sample. Multiplicities of the ^{15}N signals were determined by a ^{15}N DEPT experiment which clearly distinguished the terminal amide nitrogen from other resonances. The assignments of the ^1H resonances[8] then served as a basis for assigning the ^{15}N signals by inverse proton detected $^{15}N/^1H$ correlation spectroscopy. This technique, a valuable tool for the assignment of ^{15}N resonances, was found to be very sensitive and rapid with our biosynthetically enriched sample. Three separate experiments were employed: (i) 2D inverse heteronuclear multiple quantum correlation with BIRD pulse[25]; (ii) 2D inverse heteronuclear multiple bond correlation[26]; and (iii) 2D inverse heteronuclear multiple quantum relay correlation with BIRD pulse.[27] The BIRD pulse was employed to allow detection of only protons coupled to ^{15}N, eliminating solvent resonances and noncoupled species. The complete set of ^{15}N assignments and observed correlations are listed in Table II.

Once the assignments were in hand, the biosynthesized samples could be analyzed in a straightforward manner by inverse-gated decoupling techniques. Relative ^{15}N enrichments in **1** were calculated by normalizing the peak intensities of the spectra of natural abundance and labeled samples with Thr NH and But NH as the reference peaks (Table II). The sample from the $^{15}NH_4Cl$ feeding experiment gave relative spectral line intensities that were quite similar to those of the natural abundance sample. There was, however, significant general enrichment in the $^{15}NH_4Cl$-derived sample as judged by the rapidity of data accumulation relative to the nonlabeled material, although in the

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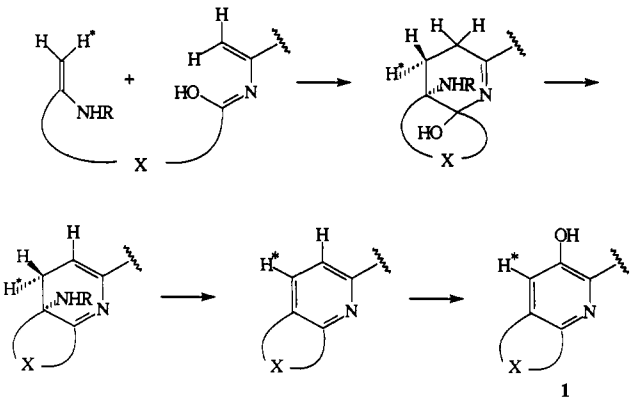
absence of a standard the absolute enrichment could not be quantitated. In contrast, the [^{15}N]glycine feeding experiment resulted in approximately 3-fold higher enrichments at Deala NH_2 (the terminal amide) and the directly serine-derived nitrogens, Deala NH and Pyr N, than at the threonine-derived reference nitrogens. The cysteine-derived nitrogens, Cys NH and Thz(1)–(5) Ns, showed relative enrichments of about 1.5-fold (Table II). These data point to either glycine or serine, which is biosynthesized from glycine, as the immediate source of the terminal amide nitrogen. To determine whether glycine or serine is the more proximate precursor, [^{15}N]glycine was fed again along with a 3-fold excess of nonlabeled serine. If serine were the proximate source of the carboxamide nitrogen, one would expect that the intensities of the signals for Deala NH_2, Deala NH, and Pyr N would remain the same relative to each other, but would show a decrease in magnitude relative to the reference signals. If the terminal amide nitrogen were furnished by glycine, one would expect that only the Deala NH and Pyr N signals but not Deala NH_2 would decrease relative to the reference signals. The NMR analysis of the sample of **1** from this experiment showed no significant difference in the relative signal intensity pattern of the three most heavily labeled nitrogens, Deala NH_2, Deala NH, and Pyr N, compared to that from the previous [^{15}N]glycine experiment (Table II). The ^{15}N experiments thus strongly suggest that serine is the source of the terminal amide nitrogen, although because of the possibility of a very rapid equilibration between serine and glycine, glycine cannot be ruled out completely.

Discussion

The results reported here complete the confirmation of the amino acid origin of all the structural components of nosiheptide. They also shed light on the mode of assembly of the basic building blocks into the final antibiotic structure and on the nature and mechanisms of some of the amino acid modifications taking place during this biosynthesis.

The data confirm the origin of the α,β-dehydroamino acid moieties, Deala and But, by dehydration of the corresponding β-hydroxyamino acids, serine and threonine, and they also establish the steric course of these dehydrations. The observed stereochemistry conforms to the axiom, pointed out by Knowles,[28] that enzyme-catalyzed 1,2-dehydrations which involve elimination of a proton α to a carboxyl group,[29,30] as exemplified by the reactions catalyzed by fumarase or enolase, proceed in an *anti* fashion. A number of enzymes catalyze 1,2-dehydrations with *syn* geometry, but most of these, e.g., the type I dehydroquinate dehydratase,[31] operate by a very different mechanism.

The formation of the thiazole rings of **1** has analogy in the generation of the thiazole rings in berninamycin,[32] in myxothiazol,[33] and in bleomycin[34] from cysteine and of the oxazole ring in virginiamycin[35] from serine. In the latter case, the stereochemistry of the process has been studied previously.[36] The incorporation of (*S*)-serine proceeds with loss of the *pro-S* hydrogen and retention of the *pro-R* hydrogen from C3 of the amino acid. Our results on the stereochemistry of thiazole ring formation in **1** agree with this observation, assuming that the conversion of serine into cysteine in *S. actuosus* proceeds with the same stereochemistry as that catalyzed by the *E. coli* enzyme, i.e., with retention of configuration at C3.[14] This assumption is quite plausible given the overwhelming tendency of pyridoxal phosphate-catalyzed β-replacement reactions to proceed with retention of configuration at Cβ.[37] An attempt to verify this assumption experimentally by determining the configuration at C3 of the cysteine moiety of **1** derived from (2*S*,3*S*)-[3-^{13}C,^{2}H$_1$]serine was unsuccessful; we were not able to derive independent NMR assignments for the two diastereotopic methylene hydrogens at that position.



1

Figure 6. Proposed mode of formation of the hydroxypyridine moiety of **1**.

The stereochemical result on the formation of the oxazole ring of virginiamycin was interpreted[36] to suggest the intermediacy of an aldehyde at the carbon derived from C3 of serine. For reasons which will be discussed in detail in conjunction with the biosynthesis of thiostrepton,[40] analogous thioaldehyde intermediates in thiazole ring formation are rather unlikely in the biosynthesis of thiostrepton or, by analogy, of **1**. A curious and at present unexplained observation is the failure to detect any deuterium at C5 of thiazole 2 in the feeding experiment with 3-dideuterated serine. A complete removal of all deuterium from this position in one thiazole, but not in the others, seems somewhat implausible, and this finding may only be a spectroscopic artifact. However, it should also be remembered that thiostrepton carries a dihydrothiazole rather than a thiazole ring in the corresponding position in the macrocycle,[24] and the metabolic fate or mode of formation of thiazole 2 in **1** may differ from that of the other thiazole rings. Apart from this anomaly, the stereochemical data are entirely consistent with a uniform mechanism for the formation of the thiazole rings.

The pyridine moiety of **1** is generated in a rather unique way by connecting C3 of a serine and the carboxyl carbon of an adjacent cysteine to C3 and C2 of another serine eight amino acids removed in the peptide chain. This crucial reaction also establishes the essential macrocyclic framework of **1** and related thiopeptide antibiotics. Following an earlier proposal by Bycroft and Gowland[38] we assume that both serine residues are first dehydrated to dehydroalanine. The formation of the pyridine ring can then be written, in a formal sense, as a cycloaddition reaction (Figure 6), although we do not necessarily mean to imply that the process has to be concerted. Loss of water from the resulting vinylogous carbinolamine, aromatization by elimination of the nitrogen and an adjacent proton of the ene component, and hydroxylation then

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complete the hydroxypyridine structure. The nitrogen may be eliminated as ammonia or if, as in thiostrepton and other thiopeptides, the original precursor peptide has additional amino-terminal residues, as the carboxy-terminal amido group of a cleavage peptide. As the feeding experiments with (*S*)-[3-^{13}C,$^{2}H_2$]serine and (2*S*,3*S*)-[3-^{13}C,$^{2}H_1$]serine have shown, the hydrogen retained at C4 of the pyridine moiety must originate from the *pro*-3*R* position of serine. This defines the stereochemistry of the final 1,2-elimination reaction aromatizing the pyridine ring. Assuming that the two dehydroalanine residues undergoing the cyclization are formed with the same stereochemistry as the Deala moiety of **1**, i.e., that they carry the deuterium from the *pro*-3*R* position of serine in the 3*Z* position, and that the addition to the ene component is a suprafacial process, the aromatization reaction must involve an *anti* elimination of the elements of ammonia (Figure 6).[39] Analogous studies on the stereochemistry of formation of the tetrahydropyridine moiety of thiostrepton[40] support the above assumptions and thus the deduced steric course of the elimination step.

Our earlier work[10] had shown that the indolic acid moiety of **1** is formed by a unique rearrangement of tryptophan, probably not via 4-methyltryptophan. The present results confirm that methylation at the 4-position of indole is not the first step in this transformation and demonstrate that both 3-methylindole-2-carboxylic acid (**4**) and 3,4-dimethylindole-2-carboxylic acid (**3**) are efficient and specific precursors. 4-(Hydroxymethyl)-3-methylindole-2-carboxylic acid (**2**), on the other hand, was not incorporated nearly as efficiently, suggesting that the indolic acid precursor is attached to the peptide backbone prior to hydroxylation of the methyl group at C4. Enzymatic studies demonstrating that **3** and **4**, but not **2**, can be converted to the adenylate derivative by an activating enzyme from *S. actuosus*[41] support this conclusion. Whether in the normal pathway attachment of the indolecarboxylic acid moiety to the peptide backbone occurs before or after methylation at C4 cannot be decided from the available data. This leaves the question of how tryptophan is converted into **4**. The results of the experiments reported here demonstrate that none of the plausible intermediates implied in the various hypothetical pathways envisioned (Figures 4 and 5), including the rather attractive cyclopent[*b*]indoles **5** and **6**, are incorporated into **1**. Although, as in all negative feeding experiments, the possibility of nonincorporation due to cell impermeability to the precursors cannot be ruled out, the very efficient incorporation of **4** argues against such permeability barriers. The most plausible alternative is that the conversion of tryptophan to **4** occurs in a concerted fashion on a single enzyme or enzyme complex without any free or diffusible intermediates. A possible mechanism, a modification of the one considered most plausible earlier (Figure 4), is depicted in Figure 7. It is proposed that the carbocation resulting from the initial acylation of C2 of the indole by the side chain carboxyl group does not stabilize by proton loss to **5**, but that the positive charge immediately initiates the fragmentation of the system, leading to **4**. Further mechanistic insight into this intriguing rearrangement reaction will probably come only from detailed studies at the enzymatic level.

In conjunction with the antibiotic structure the results of the biosynthetic experiments define the minimal sequence of a precursor peptide for nosiheptide: *N*-Ser(1)-Cys(2)-L-Thr(3)-Thr(4)-Cys(5)-L-Glu(6)-Cys(7)-L-Cys(8)-Cys(9)-Ser(10)-Cys(11)-Ser(12)-(Ser(13))-C. Residues 1–12 are retained, mostly in modified form, in the antibiotic, but only the amino nitrogen of Ser(13) is incorporated into the final structure. It is entirely possible that the precursor peptide carries additional amino acids at the amino- and/or the carboxy-terminal end, which are removed during the peptide processing. As a further complication, one or more of the amino acids in this precursor peptide may have D rather than L configuration; the cysteine residue corresponding to Cys(5) in the related antibiotic thiostrepton has D configuration. The tryptophan giving rise to the indolic acid moiety is apparently not part of the original peptide; this moiety is probably attached to the peptide backbone at a later stage. The thiopeptide antibiotics as a family share the same overall architecture; their assembly involves some highly conserved and some variable building blocks and some variation in the modifications to the peptide precursor. This suggests similarities in the biosynthetic machinery assembling them and a fairly high degree of conservation in the blueprint specifying amino acid sequence and the nature and position of certain postsynthetic modifications. The antibiotic thiostrepton by analogy to the findings about **1** would be generated from a peptide at least 18 amino acids long.

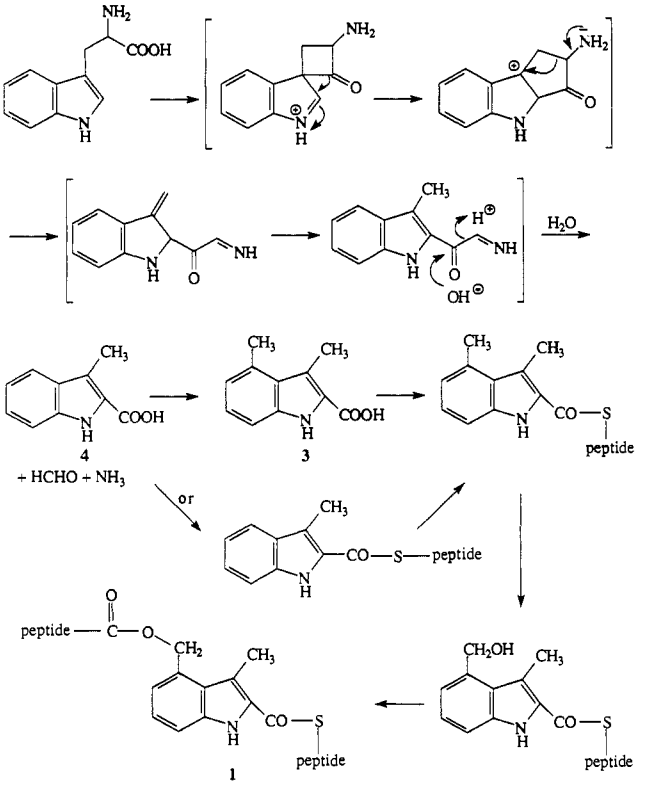

Figure 7. Proposed mechanism of the rearrangement of tryptophan into **4** and its further conversion into the indolic acid moiety of **1**.

With chain lengths of 13–18 amino acids in a nonrepetitive sequence, the thiopeptides fall between the simple peptide antibiotics,[42] e.g. gramicidin S,[43] bacitracin,[44] tyrocidine,[45] or cyclosporin,[46] which are all synthesized on enzyme templates, and the larger lanthionine-containing antibiotics, such as nisin,[47] epidermin,[48] or subtilin,[49] which are modified from ribosomally assembled propeptides. The available evidence points to an enzyme-mediated rather than a ribosomal process for the assembly of the amino acid building blocks into the peptide sequence of **1**. This follows from the fact that chloramphenicol at concentrations which shut down protein synthesis completely does not imme-

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diately block the formation of **1**.[50] More importantly, Southern hybridization experiments with oligonucleotide probes against partial sequences of both the nosiheptide and thiostrepton precursor peptides, designed to reflect the codon usage of streptomycetes, failed to detect any homologous sequences in the DNA from the respective producing organisms.[50a,51]

A peptide synthase may first assemble a linear peptide of the sequence inherent in **1**, which is subsequently processed by separate enzymes into the final antibiotic structure. Such an initial precursor peptide might be isolable from blocked mutants of the organism which serve as secretor mutants in cosynthesis of antibiotic. A number of **1**-nonproducing mutants of *S. actuosus* have been generated, but all of these proved to be very unstable, and they could not be characterized cleanly by cosynthesis assay.[52,53] Another approach would be to synthesize the linear peptide precursor and to test it for specific conversion into **1** in the producing organism. There are obvious difficulties with this rather direct approach. One is that, as discussed above, we cannot predict the exact structure of this precursor with certainty. Another is that such a peptide, when administered exogenously, may be hydrolyzed to its components much more rapidly than it is incorporated into **1**. We have synthesized H_2N-[U-^{14}C]Ser-Cys-Thr-Thr-Cys-Ser-Cys-Cys-Cys-Ser-Cys-Ser-$CONH_2$, the structure we considered most plausible as a linear precursor peptide for **1**; however, a preliminary experiment did not detect any incorporation of radioactivity from this compound into **1** in whole cells of *S. actuosus*.[54]

Far more likely, in analogy to the apparent mode of operation of the template-type peptide synthases producing other simple peptide antibiotics,[55] **1** may be assembled by a process in which the addition of amino acids to the growing peptide chain and their structural modification are interspersed in the biosynthetic sequence and may occur on the same enzyme complex. Such a process would also have analogy in the mode of assembly of macrocyclic polyketide antibiotics, such as erythromycin.[56] However, segments of the biosynthetic process, for instance the generation of the indolic acid moiety, probably do occur on separate enzymes independent of the peptide assembly. Further insight into the mode of assembly and modification of the building blocks into the peptide structure of **1** is as likely to come from a genetic analysis of the system as from further biochemical experiments. In collaboration with the laboratory of W. R. Strohl, a modest beginning has been made in this direction.[51,57]

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. FAB/mass spectra were acquired on a VG 70 SEQ tandem hybrid instrument at the University of Washington Mass Spectrometry Facility. Routine GC/MS identifications of synthetic intermediates were performed on a Hewlett-Packard 5790A gas chromatograph with an HP 5970 mass selective detector. A New Brunswick G25 rotary shaker (2.5-cm throw) was used for the fermentations.

Materials. All chemicals and solvents were reagent grade and were used as received with the following exceptions. Tetrahydrofuran (THF) and diethyl ether were distilled from sodium benzophenone ketyl. Dichloromethane, chloroform, and acetonitrile were distilled from phosphorus pentoxide. Dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) were distilled from calcium hydride at reduced pressure. *S. actuosus* ATCC 25421 was obtained from the American Type Culture Collection, Rockville, MD. Authentic nosiheptide was a gift from Rhône-Poulenc Co. through the courtesy of Dr. J. Lunel. Ingredients for fermentations were purchased from Difco and Sigma, except Promosoy 100, which was provided by Central Soya, Ft. Wayne, IN. DL-[1-^{13}C]Threonine (purity 80% by ^{1}H NMR, containing 20% allothreonine) was prepared from K^{13}CN and 2-(benzyloxy)propanal via the Strecker synthesis.[58] *S*-[1,2-$^{13}C_2$]Serine (99% ^{13}C), *S*-[2,3-$^{13}C_2$]serine (99% ^{13}C), *S*-[3-^{13}C,$^2H_2$]serine (99% ^{13}C, 99% ^{2}H), and (2*S*,3*S*)-[3-^{13}C,$^2H_1$]serine (99% ^{13}C, 93% ^{2}H)[60] as well as *S*-[1,5-$^{13}C_2$]glutamic acid[61] were gifts from the Los Alamos Stable Isotope Resource. The following companies supplied stable-isotope-labeled compounds: [^{13}C,$^2H_3$]iodomethane (99% ^{13}C and 98% ^{2}H) and sodium [^{15}N]nitrite (99%), MSD Isotope; $^{13}CO_2$ gas (99%) and L-[*methyl*-^{13}C]methionine (90%), ISOTEC Inc.; ethyl [1-^{13}C]bromoacetate (99%) and [^{15}N]aniline (99%), Cambridge Isotope Laboratories; $^{15}NH_4Cl$ and [^{15}N]glycine (both 99%), Aldrich. [^{3}H]$NaBH_4$ (600 mCi/mmol) was purchased from New England Nuclear.

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 *p*-anisaldehyde, phosphomolybdic acid, ninhydrin, or aqueous potassium permanganate solution followed by heating. Preparative TLC was carried out 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. 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 a Fisher Recordall recorder.

NMR Spectroscopy. NMR spectra of nosiheptide samples were acquired in DMSO-d_6 at 320 K on an IBM AF-300 spectrometer operating at a field strength of 7.1 T (300-MHz proton frequency). ^{1}H chemical shifts are reported in parts per million 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), and integration. ^{13}C NMR spectra were recorded at a frequency of 75.4 MHz. A continuous wave deuterium decoupler was employed for analysis of ^{13}C–^{2}H bond labeled samples. 2D INADEQUATE spectra were acquired over 48 h under the following conditions: repetition time of 1 s, spectral width in F2 either ±12.8 or ±13.8 kHz in 4K data sets, spectral width in F1 either ±12.8 or ±13.8 kHz in 1K data sets sampled either in 128 increments and zero-filled to 453 data points, or in 256 increments and zero-filled to 512 data points. ^{15}N NMR spectra were recorded at 30.4 MHz with an inverse probe and inverse accessory. ^{15}N chemical shifts were referenced externally to $^{15}NH_4Cl$. 2D experiments were conducted with the decoupler as the observe channel (90° ^{1}H pulse, 10.5 μs) and the broadband transmitter as the X-nucleus channel (90° ^{15}N pulse, 18.0 μs). To avoid signal modulation by spin rate fluctuations, samples were measured in a nonspinning mode and shimmed accordingly.

Organism and Fermentation, Isolation of Nosiheptide, Nosiheptide Assay, and HPLC. Detailed procedures were reported in an earlier paper.[10]

Feeding Experiments. In general, isotopically labeled substrates were fed to cultures grown in a synthetic medium.[10] Each precursor was dissolved in water unless otherwise noted, sterilized by filtration, and then added to the production culture. The labeled substrates were fed

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Table III. Conditions of Feeding Labeled Precursors to **1**-Producing *S. actuosus*

expt	precursor	amount	no. of flasks	additions at	harvest at	amount of **1** isolated
1	(*R,S*)-[1-^{13}C]threonine	100 mg in 18 mL	9	42 and 66 h	99 h	37 mg
2	(*S*)-[1,5-$^{13}C_2$]glutamic acid	100 mg in 40 mL	20	48 and 72 h	101 h	130 mg
3	(*S*)-[1,2-$^{13}C_2$]serine	100 mg in 20 mL	20	48 and 72 h	101 h	95 mg
4	(*S*)-[2,3-$^{13}C_2$]serine	100 mg in 20 mL	20	48 and 72 h	101 h	106 mg
5	(*S*)-[3-^{13}C,$^{2}H_2$]serine	100 mg in 20 mL	20	48 and 72 h	101 h	100 mg
6	(2*S*,3*S*)-[3-^{13}C,$^{2}H_1$]serine	150 mg in 20 mL	20	42 and 66 h	97 h	42 mg
7	(*R,S*)-4-methyl[3′-^{13}C]tryptophan	100 mg in 40 mL	20	48 and 72 h	98 h	100 mg
8	(*S*)-[*methyl*-^{13}C,$^{2}H_3$]methionine	100 mg in 14 mL	7	41 and 66 h	100 h	36.7 mg
9	(*R,S*)-[3′-^{13}C,$^{2}H_2$]tryptophan	100 mg in 40 mL	20	48 and 72 h	99 h	44 mg
10	(*R,S*)-[3-^{13}C,*amino*-^{15}N]tryptophan	80 mg in 40 mL	20	48 and 72 h	99 h	88 mg
11	3-methyl[*carboxy*-^{13}C]indole-2-carboxylic acid (**4**)	180 mg in 10 mL[a]	10	41 and 65 h	96 h	71 mg
12	2-amino-3-oxo[1-^{13}C]cyclopent[*b*]indole·HCl (**5**)	40 mg in 12 mL	4	42 and 66 h	98 h	27 mg
13	2,3-dioxo[1-^{13}C]cyclopent[*b*]indole (**6**)	47 mg in 3 mL[b]	15	49 and 73 h	144 h	30 mg
14	2-carboxy[*indole*-^{15}N]tryptophan (**7**)	200 mg in 60 mL	20	42 and 66 h	100 h	131 mg
15	2-carboxy[*methylene*-^{13}C]indole-3-acetic acid (**8**)	85 mg in 30 mL	15	55 and 79 h	148 h	40 mg
16	$^{15}NH_4Cl$	100 mg in 10 mL	5	41 and 65 h	97 h	18.5 mg
17	[^{15}N]glycine	100 mg in 10 mL	5	41 and 65 h	97 h	27 mg
18	[^{15}N]glycine + unlabeled serine	100 mg + 30 mg in 36 mL	18	41 and 65 h	100 h	98 mg
19	4-[4′-^{3}H](hydroxymethyl)-3-methyl-2-carboxylic acid (**2**)	1.5 mg, 522 μCi in 8 mL[c]	4	43 and 66 h	98 h	12.1 μCi[d]
20	3,4-[4′-^{3}H]dimethylindole-2-carboxylic acid (**3**)	0.65 mg, 425 μCi in 4 mL[c]	2	41 and 65 h	97 h	17 mg, 31.1 μCi[d]

[a] 5% Na_2CO_3 solution. [b] Absolute EtOH. [c] 25% Na_2CO_3 solution. [d] Crude product.

in two equal portions. The first addition was made when the nosiheptide titer reached 5–10 mg/L and the second approximately 24 h later. At the times indicated the cultures were harvested by centrifugation and **1** was purified as described.[10] 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 follows. An aliquot (1.487 μCi) of **1** from [4′-^{3}H]-**2** (Table III, experiment 19) was diluted with authentic **1** (43.2 mg, theoretical specific radioactivity 0.0344 μCi/mg) and precipitated repeatedly using the following two methods: (A) to the solution of **1** in a minimum amount of CH_2Cl_2–EtOH (4:1) was added 2 vol of Et_2O; (B) to the solution of **1** in a minimum amount of THF was added 2.5 vol of petroleum ether. These procedures afforded 18 mg of **1** with a constant specific radioactivity of 0.0183 μCi/mg. On this basis, the incorporation of **2** into **1** was calculated to be 1.2%. An aliquot (3.66 μCi) of **1** from [4′-^{3}H]-**3** (Table III, experiment 20) was diluted with nonlabeled **1** to a total weight of 13.6 mg (theoretical specific radioactivity 0.197 μCi/mg). After purification to constant specific activity as described above, 8.8 mg (0.163 μCi/mg) of **1** was obtained corresponding to an incorporation of 6.8%. The remainder of the original sample (27.4 μCi, 15.7 mg) was purified by method A to give 23.1 μCi of nosiheptide (15 mg). An aliquot of 2.31 μCi was diluted with authentic **1** to a total weight of 20 mg (theoretical specific radioactivity 0.116 μCi/mg) and precipitated as described above to constant specific activity (0.114 μCi/mg). On this basis, the incorporation of **3** into **1** was calculated to be 6.2%. A sample of the purified **1** (100 mg, 19.6 μCi/mmol) was hydrolyzed with base as described below to give 8.8 mg (55%) of tritiated **2**, which was esterified with diazomethane to the methyl ester (5.9 mg, 9.1 μCi/mmol).

Synthesis of Labeled Precursors. DL-4-Methyl-[3′-^{13}C]tryptophan. Labeled 4-methyltryptophan was prepared from 4-methylindole and $^{13}CO_2$ via 4-methyl[*methylene*-^{13}C]gramine in analogy to a published procedure.[15] The product was isolated by reverse-phase chromatography on a 1.5 × 30 cm Diaion HP-20 column (Mitsubishi Chemicals) preequilibrated with water. Following application of the sample the column was washed with 500 mL of water and then eluted with 20% ethanol to afford 203 mg of 4-methyl[3′-^{13}C]tryptophan (0.93 mmol, 46.8% from 4-methylindole): ^{1}H NMR (300 MHz, DMSO-d_6/D_2O, 100:1) δ 7.34 (d, *J* = 8.0, 1H), 7.28 (s, br, 1H), 7.12 (t, *J* = 7.8, 1H), 6.91 (d, *J* = 7.9, 1H), 3.84 (m, 1H), 3.74 (ddd, *J* = 134.2, 14.1, 3.5, 1H), 3.19 (ddd, *J* = 134.2, 14.0, 3.5, 1H), 2.74 (s, 3H); ^{13}C NMR (75.4 MHz, DMSO-d_6) δ 29.77 (enriched).

Methyl 4-(Hydroxymethyl)-3-methylindole-2-carboxylate. A solution of sodium hydroxide (44 mL, 12.5 N) was added to a suspension of **1** (2.2 g, 1.7 mmol) in THF (110 mL). After being heated at reflux for 17 h, the mixture was allowed to cool to room temperature. Water (60 mL) was added and the mixture washed with EtOAc (2 × 50 mL). The aqueous layer was acidified (pH 2) with concentrated HCl and then extracted with Et_2O (5 × 50 mL). The combined organic extracts were dried ($MgSO_4$) and concentrated to give 408 mg of crude **2**. This material (370 mg) in 20 mL of THF was treated with excess diazomethane in ether. After being stirred for 2 h at room temperature, the reaction mixture was quenched with 5 mL of glacial acetic acid, and 100 mL of Et_2O was added. This mixture was washed with saturated aqueous $NaHCO_3$ (3 × 20 mL) and a small amount of water. The combined organic layers were dried ($MgSO_4$) and concentrated. The yellow residue was purified by silica gel chromatography (hexane–EtOAc, step gradient 4:1 to 1:1) to give the methyl ester as a colorless oil (217.6 mg, 1 mmol, 58.4%): R_f 0.4 (hexane–EtOAc, 4:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.73 (s, br, 1H), 7.27 (m, 2H), 7.09 (d, *J* = 8.1, 1H), 5.07 (s, 2H), 3.93 (s, 3H), 2.84 (s, 3H); GC/MS (*m/z*, rel int) 219 (M^+, 70).

Methyl 4-Formyl-3-methylindole-2-carboxylate. Pyridinium chlorochromate on aluminum oxide (516 mg, 1 mmol/g) was added to a solution of methyl 4-(hydroxymethyl)-3-methylindole-2-carboxylate (122 mg, 0.56 mmol). After being stirred for 2 h at room temperature, the mixture was filtered and the remaining solid washed with THF (50 mL). The filtrate was evaporated *in vacuo* to give a yellow residue, which was purified on a preparative TLC plate (2 mm) using EtOAc–hexane (1:1) to yield methyl 4-formyl-3-methylindole-2-carboxylate (118 mg, 0.54 mmol, 97%): mp 195–197.5 °C; R_f 0.75 ($CHCl_3$–Et_2O, 7:3); ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.63 (s, 1H), 9.04 (s, br, 1H), 7.76 (dd, *J* = 7.8, 0.8, 1H), 7.61 (dd, *J* = 7.8, 0.8, 1H), 7.40 (t, *J* = 7.7, 1H), 3.96 (s, 3H), 2.89 (s, 3H); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 191.4, 162.6, 136.8, 132.5, 124.8, 124.7, 120.3, 117.9, 52.0, 13.8.

4-([4′-^{3}H]Hydroxymethyl)-3-methylindole-2-carboxylic Acid ([4′-^{3}H]-2). A mixture of methyl 4-formyl-3-methylindole-2-carboxylate (6.5 mg, 29 μmol) and [^{3}H]$NaBH_4$ (5 mCi, 600 mCi/mmol) in methanol (0.7 mL) was stirred for 2 h at room temperature. The mixture was applied directly to an aluminum-backed TLC plate (0.2 mm, 20 × 17 cm) which was developed with $CHCl_3$–Et_2O (6:4). The band at R_f 0.6 was cut out and eluted with THF to yield methyl 4-([4′-^{3}H]hydroxymethyl)-3-methylindole-2-carboxylate as a colorless solid (2.8 mCi, 4.5 mg, 21 μmol, 87%). This material was dissolved in 1 mL of THF, and 0.3 mL of 1 N sodium hydroxide solution was added. The mixture was stirred overnight at room temperature and then for 3 h at 60 °C. After removal of THF *in vacuo* and addition of water (1 mL), the basic solution was washed with EtOAc (3 × 1 mL). The aqueous layer was acidified to pH 2 with 1 N HCl and the resulting solution extracted with EtOAc (5 × 1 mL). The combined organic layers were washed with water (1 mL), dried (Na_2SO_4), and concentrated to yield [4′-^{3}H]-**2** (1.14 mCi, 3.5 mg, 17 μmol, 77%) as a colorless solid: ^{1}H NMR (300 MHz, THF-d_8) δ 10.48 (s, br, 1H), 7.25 (d, *J* = 8.2, 1H), 7.11 (t, *J* = 7.7, 1H), 7.02 (d, *J* = 7.0, 1H), 4.96 (s, 2H), 2.83 (s, 3H).

Methyl 4-([4′-^{3}H]Hydroxymethyl)-3-methylindole-2-carboxylate. This material (3.78 mCi, 5 mg, 22.8 μmol, 81%) was obtained from methyl 4-formyl-3-methylindole-2-carboxylate (6.0 mg, 28 μmol) and tritiated with $NaBH_4$ (5 mCi, 600 mCi/mmol) essentially as described above.

Methyl 3,4-[4′-^{3}H]Dimethylindole-2-carboxylate. Chlorotrimethylsilane (5.9 mL, 46 mmol) was slowly added with stirring to a mixture of the previously prepared methyl ester of [4′-^{3}H]-**2** (5 mg, 3.78 mCi, 22.8 μmol) and anhydrous sodium iodide (17.1 mg, 114 μmol) in dry acetonitrile (0.5 mL). During the addition, the reaction vessel was warmed

to 45 °C. After 45 min the mixture was diluted with acetonitrile (0.5 mL) and acetic acid (15 mL). Zinc dust (11 mg, 7 equiv) was added in portions at room temperature. After 15 min the dark yellow reaction mixture was heated to 80 °C and stirred for 5 h at this temperature. It was then allowed to cool to room temperature, filtered through a pad of Celite, and washed with Et_2O (3 × 5 mL). The filtrate was washed with 5 mL of saturated aqueous $NaHCO_3$, dried ($MgSO_4$), and concentrated *in vacuo* to yield an orange solid. The crude product was purified by preparative TLC (hexane–EtOAc, 9:1). The band of R_f 0.29 was cut out and eluted with EtOAc (10 mL) and the solvent removed *in vacuo* to yield methyl 3,4-[4′-^{3}H]dimethylindole-2-carboxylate (1.91 mCi, 2.8 mg, 14 μmol, 50.5% radiochemical yield). A nonlabeled sample synthesized in the same way on an 8-times-larger scale and purified by column chromatography on silica gel (hexane–EtOAc, 9:1) (yield 72.2%) was characterized by ^{1}H NMR and GC/MS: mp 143–144 °C; R_f 0.29 (hexane–EtOAc, 9:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.62 (s, br, 1H), 7.15 (m, 2H), 6.81 (m, 1H), 3.93 (s, 3H), 2.82 (s, 3H), 2.72 (s, 3H); GC/MS (*m/z*, rel int) 203 (M^+, 51). Chromatographic comparison showed that the radioactive and nonlabeled samples were identical.

3,4-[4′-^{3}H]Dimethylindole-2-carboxylic Acid ([4′-^{3}H]-3). Aqueous NaOH (0.5 mL, 1 N) was added to a solution of methyl 3,4-[4′-^{3}H]dimethylindole-2-carboxylate (1.91 mCi, 2.8 mg, 13.8 μmol) in THF (0.5 mL). After being stirred at 90 °C for 12 h, the mixture was allowed to cool to room temperature, and the THF was removed *in vacuo*. The residue was diluted with water (1 mL) and washed with Et_2O (2 × 1 mL). The aqueous layer was acidified with 1 N HCl and the resulting solution extracted with EtOAc (3 × 2 mL). The combined organic layers were concentrated *in vacuo* and afforded [4′-^{3}H]-3 (1.5 mg, 0.981 mCi, 7.9 μmol, 51.4% radiochemical yield) as an orange solid: R_f 0.2 ($CHCl_3$–MeOH, 9:1); ^{1}H NMR (300 MHz, THF-d_8) δ 10.42 (s, br, 1H), 7.12 (d, *J* = 8.0, 1H), 6.99 (dd, *J* = 8.0, 7.1, 1H), 6.68 (d, *J* = 7.1, 1H), 2.85 (s, 3H), 2.70 (s, 3H). The spectroscopic data of this compound were identical to those of an authentic nonlabeled sample.

3-Methyl[*carboxy*-^{13}C]indole-2-carboxylic Acid ([*carboxy*-^{13}C]-4). This compound was prepared from *N*-(*p*-toluylsulfonyl)-*o*-aminoacetophenone and ethyl [1-^{13}C]bromoacetate by the procedure of Jones[17] in 38.8% overall yield based on ethyl bromoacetate. It was obtained as a yellow oil: ^{1}H NMR (300 MHz, THF-d_8) δ 10.45 (s, 1H), 7.58 (d, *J* = 8.2, 1H), 7.33 (d, *J* = 7.1, 1H), 7.18 (m, 1H), 7.00 (m, 1H), 2.57 (s, 3H); ^{13}C NMR (75.4 MHz, THF-d_8) δ 163.83 (enriched).

***rac*-2-Amino-3-oxo-[1-^{13}C]cyclopent[*b*]indole Hydrochloride ([1-^{13}C]-5).** This compound was prepared from DL-[3′-^{13}C]tryptophan, synthesized from $^{13}CO_2$ using a literature procedure,[15] by the method of Ohki and Nagasawa.[19] It was obtained as colorless crystals from ethanol–water: mp 290–300 °C; ^{1}H NMR (300 MHz, D_2O) δ 7.79 (d, *J* = 8.2, 1H), 7.49 (d, *J* = 8.3, 1H), 7.41 (t, *J* = 7.6, 1H), 7.17 (t, *J* = 7.6, 1H), 4.43 (m, 1H), 3.54 (ddd, *J* = 150.0, 16.6, 6.8, 1H), 3.04 (ddd, *J* = 150.0, 16.7, 2.6, 1H); ^{13}C NMR (75.4 MHz, D_2O) δ 26.17 (enriched).

2,3-Dioxo-[1-^{13}C]cyclopent[*b*]indole ([1-^{13}C]-6). *rac*-[1-^{13}C]-5 (70 mg, 0.32 mmol) was suspended in dry acetonitrile (6 mL) under an atmosphere of dry argon at 0 °C. After being stirred at 0 °C for 10 min, triethylamine (dry, 50 μL, 0.37 mmol) was added. The reaction mixture gradually formed a solution over 10 min. *N*-Bromosuccinimide (67 mg, 0.37 mmol) was added and the reaction mixture stirred for 10 min. Triethylamine (50 μL, 0.37 mmol) was added and stirring continued for an additional 25 min. The reaction was quenched by addition of sodium phosphate buffer (6 mL, pH 3.5, 50 mM) and the mixture stirred for 20 min at 0 °C. EtOAc was then added, and the solution was washed with water. The organic layer was dried ($MgSO_4$) and the solvent removed *in vacuo*. The crude product was purified by flash chromatography (Et_2O) to give [1-^{13}C]-6 (47 mg, 0.25 mmol, 78%) as a yellow solid: mp 245–247 °C dec; ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.27 (s, 1H), 7.80 (d, *J* = 8.0, 1H), 7.50 (d, *J* = 4.0, 2H), 7.20 (m, 1H), 3.62 (d, *J* = 137.0, 2H); ^{13}C NMR (75.4 MHz, DMSO-d_6) δ 32.43 (enriched).

2-Carboxy-[*indole*-^{15}N]tryptamine ([*indole*-^{15}N]-7). This compound was prepared from [^{15}N]aniline via 1,2,3,4-tetrahydro-1-oxo-β-[*indole*-^{15}N]carboline by the procedures of Shapiro and Abramovitch[20] in 22.2% overall yield: mp 229–230 °C dec; ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.79 (d, *J* = 98.4, 1H), 7.53 (d, *J* = 8.0, 1H), 7.34 (d, *J* = 8.0, 1H), 7.08 (t, *J* = 7.6, 1H), 6.96 (t, *J* = 7.0, 1H), 3.22 (t, *J* = 6.2, 2H), 3.03 (t, *J* = 6.2, 2H); ^{13}C NMR (75.4 MHz, DMSO-d_6) δ 165.53, 134.79, 134.59, 127.58 (d, *J* = 3.5), 122.07, 118.57, 118.34, 111.82, 110.32 (d, *J* = 3.6), 39.90, 22.28; ^{15}N NMR (30.4 MHz, DMSO-d_6) δ 143.01 (enriched).

2-Carbethoxy-[*methylene*-^{13}C]gramine. Anhydrous THF (22 mL) was placed in a flame-dried, three-neck 250-mL flask under an atmosphere of dry argon. Ten milliliters of a 1 M solution of lithium aluminum hydride in THF was then added via a syringe. The resulting clear solution was frozen in a liquid nitrogen bath and the flask evacuated. $^{13}CO_2$ gas (250 mL, 10 mmol) was then introduced via a gas syringe. The flask was warmed to 0 °C in an ice–water bath and stirred at 0 °C for 20 min. THF was then removed *in vacuo* at 0 °C (ice bath). The light gray residue was cooled in liquid nitrogen, and methanol (6 mL) was added carefully with a syringe. This was followed by the addition of a solution of dimethylamine in acetic acid (prepared by the careful addition of 10 mL of glacial acetic acid to 8 mL of 40% aqueous dimethylamine at 5 °C). The flask was warmed to 0 °C and stirred for 5 min. The reaction mixture was allowed to warm to room temperature, and then ethyl indole-2-carboxylate (1.15 g, 6 mmol) was added. The mixture was heated until reflux of the methanol was observed. The resulting solution was stirred at room temperature for 18 h. It was finally heated to 70 °C for 30 min and then allowed to cool to room temperature. The solution was acidified (pH 2) with dilute HCl and extracted with CH_2Cl_2 (2 × 100 mL). The organic layers were discarded, and the aqueous layer was taken to pH 8 by careful addition of saturated sodium bicarbonate solution. The resulting solution was extracted with CH_2Cl_2 (3 × 200 mL), the combined organic extracts were dried ($MgSO_4$), and solvent was removed *in vacuo* to leave 473 mg (1.92 mmol, 32%) of a white solid: mp 82–84 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.15 (s, 1H), 7.83 (d, *J* = 8.0, 1H), 7.28 (m, 2H), 7.12 (m, 1H), 4.40 (q, *J* = 7.0, 2H), 2.32 (d, *J* = 5.0, 6H), 2.32 (s, trace unlabeled), 1.42 (t, *J* = 7.0, 3H); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 52.69 (enriched).

2-Carbethoxyindole-3-[*methylene*-^{13}C]acetonitrile. 2-Carbethoxy-[*methylene*-^{13}C]gramine (450 mg, 1.83 mmol) was dissolved in anhydrous dimethyl sulfoxide (12 mL). Potassium cyanide (260 mg, 3.66 mmol) was added and the mixture stirred at room temperature for 10 min. Dimethyl sulfate (0.5 mL) was added dropwise over 15 min and the solution stirred at room temperature under an atmosphere of dry argon for 15 h. Water (30 mL) was added to the reaction mixture, and the resulting suspension was acidified to pH 1 with dilute hydrochloric acid and extracted with Et_2O (2 × 50 mL). The organic extracts were combined and dried ($MgSO_4$), and the solvent was removed *in vacuo*. The crude product was purified by flash chromatography (ether–hexane, 1:1), yielding 2-carbethoxyindole-3-[*methylene*-^{13}C]acetonitrile (225 mg, 0.99 mmol, 54%): mp 108–110 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.40 (s, 1H), 7.73 (d, *J* = 8.0, 1H), 7.36 (m, 2H), 7.19 (t, *J* = 8, 1H), 4.43 (q, *J* = 7.0, 2H), 4.22 (d, *J* = 136.0, 2H), 1.43 (t, *J* = 7.0, 3H); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 30.65 (enriched).

2-Carboxyindole-3-[*methylene*-^{13}C]acetic Acid ([*methylene*-^{13}C]-8). 2-Carbethoxyindole-3-[*methylene*-^{13}C]acetonitrile (225 mg, 0.99 mmol) was dissolved in THF (10 mL) and 1 N sodium hydroxide solution (10 mL) added. The solution was heated with rapid stirring at 90 °C for 2 days. The reaction mixture was allowed to cool to room temperature and the THF partially removed *in vacuo*. The solution was extracted with Et_2O (2 × 25 mL) and the aqueous layer acidified with dilute 1 N HCl. The solution was extracted with EtOAc (3 × 50 mL), the combined organic layers were dried ($MgSO_4$), and the solvent was removed *in vacuo* to leave 199 mg (0.9 mmol, 92%) of a creamy-white solid: mp 191–194 °C; ^{1}H NMR (300 MHz, acetone-d_6) δ 7.71 (d, *J* = 8.0, 1H), 7.51 (d, *J* = 8.0, 1H), 7.28 (m, 1H), 7.10 (dt, *J* = 7.5, 1.0, 1H), 4.22 (d, *J* = 129.3, 2H); ^{13}C NMR (75.4 MHz, acetone-d_6) δ 30.65 (enriched).

(*S*)-[*methyl*-^{13}C,^{2}H$_3$]Methionine. This was prepared by a modification[62] of the procedure of Dolphin and Endo.[63] (*S*)-Homocystine (300 mg, 1.12 mmol) was placed in a three-neck flask equipped with a glass-coated magnetic stirring bar, a rubber stopper, a gas outlet tube to a bubbler, and a dry ice cold finger with a gas inlet tube at the top of the finger. The whole apparatus was flushed with nitrogen and the flask cooled to −78 °C. (*S*)-Homocystine was dissolved in liquid ammonia (36 mL), and sodium was added in small pieces to the solution until the blue color persisted for 15 min. The blue color was then discharged with a minimum amount of ammonium chloride. [^{13}C,^{2}H$_3$]Iodomethane (0.158 mL, 2.46 mmol) was added and the resulting mixture stirred at −78 °C for 4 h. The cold finger was kept at −78 °C for another hour. The ammonia was then allowed to evaporate under a stream of nitrogen. The resulting solid was dissolved in water (3 mL) and acidified to pH 6 with 1 N HCl. The crude product was purified by ion exchange chromatography (Dowex 50 WX8, NH_4^+ form, 100–200 mesh, 80 mL) by eluting first with 80 mL of water followed by 0.5 N NH_4OH solution. The ninhydrin-positive fractions were collected and freeze-dried to yield (*S*)-[*methyl*-^{13}C,-^{2}H$_3$]methionine (325 mg, 2.12 mmol, 90%) as a colorless solid: R_f 0.46

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(acetonitrile–water, 3:1); ^{1}H NMR (300 MHz, D_2O) δ 3.86 (t, J = 6.6, 1H), 2.66 (td, J = 7.6, 4.2, 2H), 2.15 (m, 2H); ^{13}C NMR (75.4 MHz, D_2O) δ 16.11 (septet, J = 21.8 Hz) (enriched).

(*R,S*)-[3′-^{13}C,$^2H_2$]Tryptophan. (*R,S*)-[3′-^{13}C,$^2H_2$]Tryptophan was prepared from $^{13}CO_2$ and lithium aluminum deuteride via [^{13}C,$^2H_2$]-formaldehyde and indole using a published procedure.[15] From 234 mg (2 mmol) of indole we obtained 110 mg of [3′-^{13}C,$^2H_2$]tryptophan (0.53 mmol, 26.6%) after purification as described above: ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.60 (d, J = 7.78, 1H), 7.33 (d, J = 8.08, 1H), 7.16 (d, J = 1.35, 1H), 7.06 (ddd, J = 7.83, 6.92, 1.22, 1H), 6.97 (ddd, J = 8.07, 6.89, 1.06, 1H), 3.58 (d, J = 4.2, 1H); ^{13}C NMR (75.4 MHz, DMSO-d_6) δ 26.82 (s, br, 0.58 ppm shifted upfield compared to nonlabeled material; sharp singlet upon deuterium decoupling).

(*R,S*)-[3′-^{13}C,*amino*-^{15}N]Tryptophan. [*methylene*-^{13}C]Gramine was prepared from $^{13}CO_2$ gas and indole.[15] Diethyl [^{15}N]formamidomalonate was synthesized from sodium [^{15}N]nitrite.[64] The two compounds were then converted to (*R,S*)-[3′-^{13}C,*amino*-^{15}N]tryptophan by the method of Weygand and Linden.[15] [*methylene*-^{13}C]Gramine (155 mg, 0.89 mmol) and diethyl [^{15}N]formamidomalonate (272 mg, 1.25 mmol) yielded 82 mg of (*R,S*)-[3′-^{13}C,*amino*-^{15}N]tryptophan (0.4 mmol, 44.8%): ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.64 (d, J = 7.8, 1H), 7.27 (d, J = 8.0, 1H), 7.30 (s, 1H), 7.09 (t, J = 7.9, 1H), 7.01 (t, J = 7.9, 1H), 3.71 (m, 1H), 3.21 (dm, J = 138.7, 1H), 3.02 (dm, J = 138.8, 1H); ^{13}C NMR (75.4 MHz, DMSO-d_6) δ 109.39 (d, J = 49.7, C3), 55.3 (dd, J = 34.4, 6.16, C-2′), 27.4 (s, enriched, C3′); ^{15}N NMR (30.4 MHz, DMSO-d_6) δ 134 (s, indole nitrogen), 42.4 (s, enriched, amino nitrogen).

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Acknowledgment. We are greatly indebted to the Los Alamos Stable Isotope Resource, supported by NIH (Grant RR 02231) and USDOE/OHER, and particularly Dr. Clifford J. Unkefer for providing numerous stable-isotope-labeled compounds and to Dr. J. Lunel, Rhône-Poulenc, for generous supplies of nosiheptide. Financial support of this work by the National Institutes of Health (Research Grant AI 20264) is also gratefully acknowledged.