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

David R. Houck, Li-Chun Chen, Paul J. Keller, John M. Beale, and Heinz G. Floss*

Contribution from the Department of Chemistry, The Ohio State University, Columbus, Ohio 43210. *Received January 4, 1988*

Abstract: The biosynthesis of the modified, sulfur-rich peptide antibiotic nosiheptide (**1**) was studied in feeding experiments with radioactive and ^{13}C-labeled precursors in *Streptomyces actuosus*. Following extensive chemical-shift assignments, ^{13}C-labeled samples of **1** were analyzed by ^{13}C NMR spectroscopy. In addition to supporting the expected origin of the thiazole ring and the L-cysteine moiety from cysteine, the L-threonine and butyrine moieties from threonine, and the hydroxyglutamate moiety from glutamate, the results demonstrated that the dehydroalanine moiety arises by dehydration of serine. The pyridine ring is formed uniquely by "tail-to-tail" condensation of two serine residues. As demonstrated by a double-labeling experiment, the indolic acid moiety arises by a novel intramolecular rearrangement of tryptophan in which the carboxyl group is connected to C2 of the indole and the α-carbon and the amino nitrogen are eliminated. Only the hydroxymethyl group at C4 of the indole ring is derived by a C-methylation, but not via 4-methyltryptophan. The results define the sequence of a hypothetical peptide precursor of **1**.

Nosiheptide (Figure 1), a metabolite of *Streptomyces actuosus*, is a highly modified peptide antibiotic produced commercially[1,2] as a growth promotant for poultry. Nosiheptide[2,3] and related compounds such as thiostrepton,[4] the thiopeptins,[5] and the micrococcins[6] form a class of sulfur-rich peptide antibiotics that as a group interfere with protein synthesis in Gram-positive bacteria. In general, their mode of action appears to be inhibition of the EF-G dependent activity of the 50S ribosomal subunit.[7] The complete structures of nosiheptide[8-10] and thiostrepton[11,12] have been determined by chemical degradation studies as well as X-ray crystallographic analysis. These cyclic peptides are each composed of two loops of highly modified amino acids. Structural moieties of thiostrepton and/or nosiheptide include thiazole rings, several dehydro amino acids, and unique ring systems apparently constructed from modified amino acids. Although some of the physicochemical properties are similar, nosiheptide has several structural features that are distinct from thiostrepton and the thiopeptins. First, nosiheptide has a 2,3,4-trisubstituted indole moiety in place of the quinaldic acid residue of thiostrepton. The piperidine ring of thiostrepton forms a junction for both peptide loops, whereas a pyridine ring completes a single loop in nosiheptide; a short loop containing the indole ring extends to the hydroxyglutamate residue of the larger loop, and an L-cysteine residue anchors the indolic acid via a thioester bond.

We are interested in the biosynthetic origins of these unusual structures and the biochemical machinery that assembles them. Until recently, the biosynthesis of this class of antibiotics had received little attention. In a preliminary paper, we have summarized our efforts to determine the biosynthetic precursors of the residues that constitute nosiheptide.[13] The study involved feeding ^{13}C-labeled amino acids to fermentations of *S. actuosus* and determining the ^{13}C enrichments in isolated nosiheptide by ^{13}C NMR spectroscopy. The results of these experiments are summarized in Figure 1. This paper presents the details of the biosynthetic studies and the NMR assignments. moreover, further biochemical information has been gleaned from the analysis of the incorporation of multiply ^{13}C-labeled precursors into nosiheptide.

Experimental Section

Materials. Authentic nosiheptide was obtained as a gift from Rhône-Poulenc Co. through the courtesy of Dr. J. Lunel. The $\alpha_2\beta_2$ complex of tryptophan synthase was the generous gift of Dr. Edith Wilson Miles of the National Institutes of Health, Bethesda, MD. Radioactive precursors were obtained from the following sources: L-[U-^{14}C]Alanine (150 mCi/mmol), L-[*methyl*-^{14}C]methionine (46 mCi/mmol), and L-[*U*-^{14}C]serine (160 mCi/mmol) from New England Nuclear; L-phenyl-[1-^{14}C]alanine (56 mCi/mmol), L-[*methylene*-^{14}C]tryptophan (58 mCi/mmol), and [^{14}C]formaldehyde (11 mCi/mmol) from Amersham; DL-[*ring*-7a-^{14}C]tryptophan (3.5 mCi/mmol) from ICN. The following companies supplied ^{13}C-enriched compounds (atom % ^{13}C): DL-[3-^{13}C]cysteine (97%) and [^{13}C]formic acid (98%), Cambridge Isotopes; L-[*methyl*-^{13}C]methionine (90%), ICN; L-[3-^{13}C]serine (90%) and DL-[1-^{13}C]serine (99%), Merck and Co. The [*U*-$^{13}C_3$]glycerol was synthesized from K^{13}CN and [1,2-$^{13}C_2$]acetic acid (both 99% ^{13}C, Los Alamos Stable Isotope Resource) via diethyl malonate and diethyl 2-acetoxymalonate;[14,15] 4-methyl-[*methylene*-^{14}C]tryptophan was prepared from 4-methylindole and [^{14}C]formaldehyde by published procedures.[16]

NMR Spectroscopy. ^{13}C and ^{1}H NMR spectra were acquired in DMSO-d_6 at 320 K at a field strength of 7.1 T on IBM AF-300 or Bruker WM-300 spectrometers. Assignments were based on chemical shift theory and data from 1D or 2D experiments. ^{13}C measurements were made under the following conditions: 30° pulse; repetition time, 1.1 s; spectral width, 16.13 kHz; 16K data sets; 2-Hz line broadening; and continuous broad-band ^{1}H decoupling. The 1D-INADEQUATE spectrum was acquired by using 16K data sets, an acquisition time of 0.5 s, and a total preparation period of 8 ms. 2D-INADEQUATE spectra were acquired over a 48-h period under the following conditions: repetition time of 1 s, preparation period of 8 ms, spectral width in F2 of ±5.8 kHz in 4K data sets, spectral width in F1 of ±11.6 kHz sampled in 256 increments, and zero-filled to 512 data points. Reverse INEPT experiments were performed with 16K data sets and a spectral width of 16.13 kHz. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS).

Organism and Fermentation. *S. actuosus* ATCC 25421 was obtained from the American Type Culture Collection, Rockville, MD. The strain

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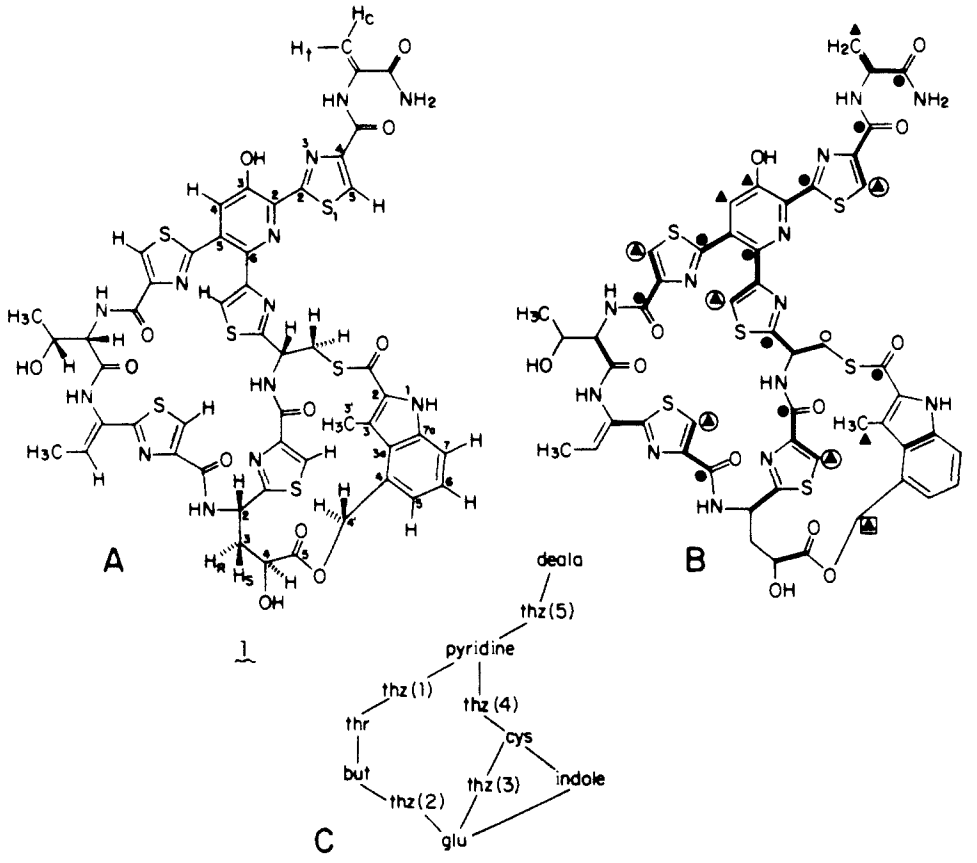

Figure 1. (A) Structure of nosiheptide. (B) The labeling sites in **1** from DL-[1-^{13}C]serine (●), L-[3-^{13}C]serine (▲), DL-[3-^{13}C]cysteine (○), L-[*methyl*-^{13}C]methionine (□), and [U-^{13}C]glycerol; the solid bars indicate intact transfer of ^{13}C–^{13}C bonds). Symbols are superimposed for carbon atoms labeled by two precursors in separate experiments. (C) The sequence of its residues.

was transferred from a lyophile tube and grown for 7 days (27 °C) on medium A. A strain selection and purification was carried out on this culture because it was heterogeneous with respect to nosiheptide production. Isolated colonies were picked and inoculated into medium B. This seed culture was incubated on a gyratory shaker (220 rpm with 5.1 cm, or 260 rpm with 2.5-cm throw) at 27 °C for 36–48 h. After growth was complete, aliquots (5 mL) were stored aseptically with glycerol (20%) as a cryoprotectant at −60 °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 all fermentations (medium B) for the feeding experiments. Medium C was inoculated with 2 mL of the seed culture (24–36 h) and incubated on a gyratory shaker for 84–96 h. The production of nosiheptide in medium C was very dependent on the rate of agitation (rpm) and throw (cm) of the gyratory shaker; at 220 rpm and 5.1-cm throw, antibiotic production began at 24 h and abruptly terminated at 84 h; yet, at a greater rate (260 rpm) and shorter throw (2.5 cm), nosiheptide production occurred between 36 and 96 h. Under ideal conditions the titer of the antibiotic was 175–250 mg/L.

The following media were used for this study: Medium A was used for solid culture and consisted of (grams/liter) yeast extract (0.15), casamino acids (0.3), malt extract (0.3), glycerol (10.0), sucrose (20.0), $CaCl_2$ (1.0), $MgCl_2$ (1.0), and agar (20.0). The pH was adjusted to 7.0 with NaOH before sterilization at 121 °C, 20 psig, 15 min. Medium B, the liquid seed medium, contained (grams/liter) glucose (30.0), $MgSO_4 \cdot 7H_2O$ (0.5), Promosoy (Central Soya, Ft. Wayne, IN) (20.0), corn steep liquor (20.0), $(NH_4)_2SO_4$ (3.0), and $CaCO_3$ (5.0). The pH was adjusted to 6.8 with NaOH prior to dispensing 45-mL volumes into each 250-mL flask (three baffles). Medium C was the production medium used for all the feeding studies and was composed of (grams/liter) L-glutamate (5.0), L-arginine (1.0), L-aspartate (1.0), $K_2HPO_4 \cdot 7H_2O$ (0.5), $MgSO_4 \cdot 7H_2O$ (1.0), Na_2SO_4 (2.0), $ZnSO_4 \cdot 7H_2O$ (0.01), $FeSO_4 \cdot 7H_2O$ (0.02), $CaCO_3$ (3.0), and glucose (40.0). The pH was adjusted to 7.25 before dispensing 45-mL volumes into each 250-mL flask (nonbaffled).

Isolation of Nosiheptide. The following procedure is an example of the routine isolation of nosiheptide from 500 mL of fermentation broth. This method was used for preparing nosiheptide samples for analysis by ^{13}C NMR spectroscopy.

The mycelia were separated from the whole broth by centrifugation (15 min at 3000 rpm) in two 250-mL polypropylene bottles. The supernatant was discarded; the pellet in each bottle was agitated with tetrahydrofuran (THF, 125 mL) plus 15 mL of a buffer composed of 1 M sodium citrate and 0.5 M sodium phosphate at pH 4.0. The mixture was agitated for 15 min, combined with hexane or petroleum ether (30 mL), and agitated for another 15 min. In some cases centrifugation was necessary for separation of the two phases. The organic phases were pooled and evaporated to dryness under vacuum at 35 °C in a rotary evaporator. Extraction of the aqueous phase was repeated, and the organic layers were combined and evaporated to dryness. The residue was dissolved in 100 mL of tetrahydrofuran (THF), and the resulting yellow or pink solution was filtered through Whatman No. 2 paper. The filtrate was combined with 200 mL of petroleum ether, stirred for 30 min, and placed in a cold room at 4 °C for 2–8 h. The resulting white precipitate was collected by centrifugation and then redissolved in 50 mL of CH_2Cl_2/CH_3CH_2OH, 4:1. Nosiheptide was precipitated from this solution by the addition of 100 mL of ether and collected by centrifugation. Precipitation was repeated from THF/hexane (1:2), and the pure nosiheptide was dried under vacuum for at least 12 h. The purity of the isolated nosiheptide was estimated by comparing HPLC integration (fluorescence as well as 254 nm detection) with the mass of the isolate and was usually >95%. In some of the stable isotope experiments 10 mg of authentic nosiheptide was combined with the sample prior to the final precipitation from THF/hexane.

Nosiheptide Assay and HPLC. A method was developed for rapid assay of nosiheptide in fermentation broths. The mycelial growth from a 2-mL aliquot of whole broth was collected by centrifugation (10 min at 3000 rpm); the supernatant was discarded. The pellet was mixed thoroughly by shaking for 10 min with 0.1 mL of buffer (1 M citrate and 0.5 phosphate, pH 4.0) and 1.7 mL of THF. The aqueous layer and the cell mass were separated from the THF by mixing the sample with 0.3 mL of hexane followed by centrifugation (10 min, 3000 rpm). An aliquot of the organic layer was combined with an equal volume of glacial acetic acid, and the resulting solution was directly injected (10 μL) onto a reversed-phase HPLC column.

The HPLC system was composed of a Rheodyne-7125 injector, a Waters M-45 solvent delivery system, a Hamilton PRP-1 column (10 μm, 4.6 × 250 mm), a Gilson-121 fluorometer, and a Hewlett Packard-3390 integrating recorder. The isocratic eluent system consisted of acetonitrile/*p*-dioxane/water/acetic acid (40:20:40:0.2) pumped at a flow rate of 1 mL/min. Nosiheptide was detected by fluorescence at emission wavelengths of 450–600 nm with excitation wavelength filtered at 390

nm. At the given detector sensitivity (one relative fluorescence unit = 1 RFU), the detector response was found to be a linear function of nosiheptide concentration within the range of 2–300 mg/L. For the nosiheptide peak, the capacity and asymmetry factors are $k' = 2.3$ and $\alpha = 0.7$, respectively.

Feeding Experiments. In general, isotopically labeled substrates were fed to cultures grown in the synthetic medium C. The labeled substrates were added when the nosiheptide titer reached 5–10 mg/L, usually at 32 h. Each radioactive precursor was diluted with an aqueous solution (pH 7.0) of unlabeled compound, sterilized by filtration, and then added to the 45-mL production culture. All radioactive experiments were conducted in duplicate or triplicate. A second addition of the labeled precursor (^{13}C or ^{14}C) was made 24 h after the initial feeding, and 24 h later the flasks were harvested by centrifugation. Nosiheptide was purified by the usual method, and radiochemical purity was confirmed by HPLC, with a radioactivity flow monitor (Packard Trace-II Radiochemical Detector) placed in series with the fluorescence or UV detectors.

A solution (13.1 mM) of DL-[3-^{13}C]cysteine was prepared in 75 mM MES (2-morpholinoethanesulfonic acid) buffer at pH 7.0. The labeled cysteine was gradually added to a production culture; 2 mL of the solution was added to each of 16 flasks (45 mL) at 40, 52, and 64 h (post-inoculation). After a total incubation time of 80 h, the cultures were combined in 250-mL centrifuge bottles. The nosiheptide was isolated as usual, but before the final precipitation from THF/hexane, 10 mg of authentic nosiheptide was added to the solution of labeled material. The dried precipitate (60 mg) was dissolved in 0.4 mL of DMSO-d_6 for NMR analysis.

L-[3-^{13}C]Serine was added as a 40 mM solution in water; 1 mL each was added to 15 flasks (40 mL) at 34 and 58 h. The cells were extracted 24 h later. This fermentation yielded 90 mg of isolated nosiheptide; 60 mg was dissolved in 0.4 mL of DMSO-d_6 for NMR spectroscopy. DL-[1-^{13}C]Serine (1 mL, 47.5 mM) was added to each of 15 flasks (45 mL) at 48 and 62 h. The fermentation was terminated after 96 h, and nosiheptide was isolated as usual except 10 mg of authentic material was added before the final precipitation. The total yield was 60 mg. L-[*methyl*-^{13}C]Methionine (1 mL, 27.4 mM) was added to each of 25 production flasks (at 41 and 65 h). The fermentation was harvested at 89 h and yielded 145 mg of nosiheptide. The L-[2,1′-$^{13}C_2$]tryptophan (1 mL, 16 mM) was added to each of 15 flasks (45 mL) at 32 and 56 h. The fermentation was terminated at 62 h. Nosiheptide was isolated as usual, and 10 mg of auuthentic material was added to bring the isolated yield to 45 mg. For the feeding experiment with [U-$^{13}C_3$]glycerol, an aqueous solution (800 mg/45 mL) was filter-sterilized and dispensed (1 mL each) into 15 flasks at 44 h. A second addition of 2 mL per flask was made at 48 h; cells were extracted 24 h later. Authentic nosiheptide (10 mg) was combined with the isolated material.

Preparation of [2-^{13}C]Indole. In a 125-mL Erlenmeyer flask, 3.5 g of *o*-toluidine (Aldrich, redistilled), 6.7 g of dicyclohexylcarbodiimide, and 40 mg of 4-(dimethylamino)pyridine were dissolved in 30 mL of dichloromethane. The mixture was stirred at room temperature for 10 min, and then a solution of 1 g of [^{13}C]formic acid (containing 5% H_2O), 1 mL of CH_3CN, and 5 mL of CH_2Cl_2 was added dropwise to the flask over a period of 15 min. This mixture was stirred for another 2 h under nitrogen and then filtered to remove the dicyclohexylurea. The filtrate was concentrated under reduced pressure and applied directly onto a silica gel column (Baker, 40 μm, 2 × 30 cm), which had been equilibrated with CH_2Cl_2/ether (95:5). The column was washed with three portions (250 mL) of the solvent in ratios of 95:5, 85:15, and 70:30 in succession. Fractions (50 mL) were monitored by TLC, and *N*-formyl-*o*-toluidine was found in fractions 12–14. Evaporation of the solvent gave an oil, and a slow stream of nitrogen blown over this material finally yielded 2 g of a pink, low-melting (50 °C) solid. The compound gave a single spot on TLC (CH_2Cl_2/ether, 1:1) and a single peak by GC–MS (m/z 136/135 = 9:1).

Labeled indole was prepared from *N*-[^{13}C]formyl-*o*-toluidine by a published procedure;[17] from 1 g of starting material, we obtained 600 mg of [2-^{13}C]indole (mp 50–51 °C; m/z 118/117 = 7.2:6.3). The product was isolated by column chromatography (silica gel) followed by crystallization (water).

Preparation of L-[2,1′-$^{13}C_2$]Tryptophan. The reaction mixture consisted of 5×10^{-5} M pyridoxal phosphate, 10 mM mercaptoethanol, 1.5 mM [2-^{13}C]indole (44 mg), 6.7 mM DL-[1-^{13}C]serine (14.9 mg), and 9 mg of $\alpha_2\beta_2$ complex of tryptophan synthase[18] in 250 mL of buffer (10 mM K_2HPO_4 and 100 mM Tris·HCl, pH 7.8). The entire mixture, less enzyme, was sterilized by filtration (0.22 μm) and placed in a sterile 500-mL Erlenmeyer flask. After addition of enzyme (9 mg, 0.5 mL), the mixture was incubated for 8 h at room temperature; 55 mg of [2-^{13}C]indole and 21 mg of DL-[1-^{13}C]serine were added, and the incubation was continued overnight. After addition of 10 mL of glacial acetic acid, the mixture was extracted with 2 volumes of ether. The aqueous solution was reduced to 15 mL, in vacuo (40 °C), and then adjusted to pH 5.9 with NaOH. Tryptophan was isolated from the mixture by reversed-phase chromatography on Dianion HP-20 (Mitsubishi Chemicals). The sample was applied to a 1.5 × 90 cm column (which had been equilibrated with water) and washed with 500 mL of water; the tryptophan was eluted with 20% aqueous ethanol. Evaporation of the solvent afforded white plates, which were recrystallized from water/ethanol (125 mg). Chemical shifts measured from the proton and carbon-13 NMR spectra were identical with those of authentic tryptophan; the ^{13}C NMR spectrum showed two intense signals: the carboxyl group at 177.3 ppm and indole C2 at 128.1 ppm. The ^{13}C enrichments at the carboxyl group (C1′) and ring carbon-2 (C2) of tryptophan were equal to those of the starting materials: DL-[1-^{13}C]serine (99 atom % ^{13}C) and [2-^{13}C]indole (50 atom % ^{13}C), respectively.

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Table I. ^1H NMR Assignments of Nosiheptide at 320 K

chemical shift, ppm	assignment	coupling constants, Hz
0.95 (d)	Thr-CH_3	6.4
1.72 (d)	but-CH_3	6.8
1.80 (ddd)	Glu H3*R*	2.5, 13.0, 12.0
2.44 (ddd)	Glu H3*S*	2.5, 12.9, 11.8
2.63 (s)	ind-CH_3	
3.56 (dd)	Cys H3	5.0, 13.9
3.86 (dd)	Cys H3	3.0, 13.8
4.00 (m)	Thr H3	obscured
4.09 (dd)	Glu H4	2.3, 11.9
4.57 (dd)	Thr H2	6.2, 8.4
5.00 (br)	deala NH_2	
5.40 (d)	ind H4′	11.7
5.59 (d)	ind H4′	11.8
5.63 (m)	Glu H2	obscured
5.76 (s)	deala H3c	
5.88 (ddd)	Cys H2	5.0, 9.6, 14.0
6.37 (s)	deala H3t	
6.46 (q)	but H3	6.9
7.12 (d)	ind H5	7.0
7.28 (dd)	ind H6	6.8, 8.2
7.58 (br)	Thr OH	
7.60 (d)	ind H7	8.3
7.64 (d)	Thr NH	8.3
7.72 (d)	Cys NH	9.6
7.82 (s)	pyr H4	
7.88 (s)	thz H5(4)	
8.00 (br)	Glu OH	
8.16 (s)	Thz H5(2)	
8.30 (s)	Thz H5(3)	
8.35 (d)	Glu NH	8.6
8.55 (s)	thz H5(5)	
8.65 (s)	thz H5(1)	
9.32 (s)	but NH	
10.04 (s)	deala NH	
11.19 (br)	ind NH	

Results

^1H NMR Assignments. The nosiheptide molecule contains 43 protons: 32 are bonded to carbon, 8 to nitrogen, and 3 to oxygen. The assignments determined from the ^1H NMR spectrum are listed in Table I. Most of the exchangeable protons were identifiable by adding a drop of D_2O to the DMSO-d_6 solution. Two rapidly exchanging protons were those of Glu-OH and Thr-OH. The rate of exchange of the peptide NH protons increased when the probe temperature was increased from 320 to 350 K. Three NH doublets were observed: Thr-NH, Cys-NH, and Glu-NH. The signal for the amino protons attached to deala-NH_2 was assigned to a very broad resonance at δ 5.00. For the most part, the ^1H signals were assignable from the homonuclear chemical shift correlated spectrum (COSY) of **1**. Cross peaks provided correlations for five separate amino acid spin systems and the indole system. Long-range ^1H–^1H couplings were also observed in the contour plots of COSY experiments. These could be enhanced by a delayed (long range) COSY experiment. Three of the thiazole C5 proton signals were assignable by long range J correlations. Notably, thiazole(4) H5 couples with both Cys-H2 (5 bond) and pyr-H4 (6 bond). The proton on pyr-C4 also couples

Table II. ^{13}C NMR Assignments and ^{13}C Enrichments in Nosiheptide Derived from ^{13}C-Labeled Precursors

^{13}C chemical shift, ppm	assignment	relative ^{13}C enrichments,[a] %, J_{cc} in **1** derived from: DL-[3-^{13}C]-cysteine	L-[CH_3-^{13}C]-methionine	L-[3-^{13}C]-serine	DL-[1-^{13}C]-serine
12.23	ind-CH_3(C3′)			2.8	
13.50	but-CH_3				
18.25	Thr-CH_3				
29.49	Cys-C3	7.2			
37.60	Glu-C3				
45.15	Glu-C2				
49.05	Cys-C2				
56.57	Thr-C2				
65.90	ind-CH_2O(C4′)		28.6	3.4	
66.40	Glu-C4				
66.50	Thr-C3				
103.60	deala-C3			6.7	
114.40	ind-C7				
118.35	ind-C3				
119.98	thz(4)-C5	9.5		2.2	
123.23	ind-C5				
124.45	thz(2)-C5	9.9		2.9	
124.70	ind-C3a				
124.91	ind-C6				
125.25	thz(3)-C5	9.9		2.8	
125.98	thz(1)-C5	8.1		2.4	
126.80	thz(5)-C5	8.3		2.8	
127.12	pyr-C4			3.3 (66 Hz)	
128.85	but-C3				
129.20	ind-C4				
129.27	but-C2				
129.90	pyr-C5				
130.40	ind-C2				
134.26	deala-C2				
135.00	pyr-C2				
137.60	ind-C7a				
142.52	pyr-C6				3.6
147.62	thz(2)-C4				
148.70	thz(3)-C4				
149.57	thz(5)-C4				
149.83	thz(1)-C4				
150.80	pyr-C3			3.1 (66 Hz)	
153.10	thz(4)-C4				
158.20	thz(5)-CO				3.0
159.45	thz(1)-CO				2.4
159.60	thz(2)-CO				4.0
159.80	thz(3)-CO				3.9
163.85	thz(1)-C2				6.3
165.0	deala-CO				5.4
166.32	thz(2)-C2				
167.10	tha(5)-C2				4.2
167.69	thr-CO				
168.98	thz(4)-C2				3.4
170.04	thz(3)-C2				
172.62	glu-CO				
181.80	ind-CO				6.2

[a] Natural abundance = 1.0. The figures are minimum values, because some of the samples were diluted with unlabeled carrier material.

with thz(5)-H5 (7 bond). These unusually long range couplings were confirmed by resolution enhancement of the ^1H spectrum and selective proton homodecoupling experiments. Other ^1H–^1H couplings through 7 bonds have previously been documented.[19] Signals for the thz(3)-H5 and thz(1)-H5 protons could not be distinguished by ^1H–^1H correlation, but all of the CH signals were assigned and confirmed by the ^1H–^{13}C correlations discussed in the next section.

^{13}C NMR Assignments. Depaire et al.[9,10] first studied the ^{13}C NMR spectrum of **1** before its complete structure was elucidated by X-ray analysis.[8] The assignments were based on chemical shift theory and spectral comparisons of **1** with the tri-*O*-acetate derivative as well as with molecular fragments obtained from acid and base hydrolysis. Since our biosynthetic conclusions would depend on unequivocal identification of ^{13}C enrichments in samples of **1**, we performed a series of NMR experiments that ultimately generated unambiguous assignments of virtually all the ^{13}C resonances (Table II). Initially, a DEPT experiment was used to distinguish the multiplicities of the protonated carbons. There are 51 lines in the spectrum corresponding to the 51 carbon atoms of **1**; the 22 carbon atoms bonded to protons include 15 methine, 4 methylene, and 3 methyl groups. In most of the nosiheptide samples obtained from our fermentations, we observed three signals (43, 171, and 174 ppm) of varying intensity that did not correspond to carbon signals of **1**. The methyl groups were the three most upfield signals. Between 120 and 129 ppm, nine lines could be assigned to sp^2-hybridized CH groups. With the ^1H NMR data in hand, firm assignments of all the proton-bearing carbons were obtained from ^1H–^{13}C homonuclear shift correlation experiments.

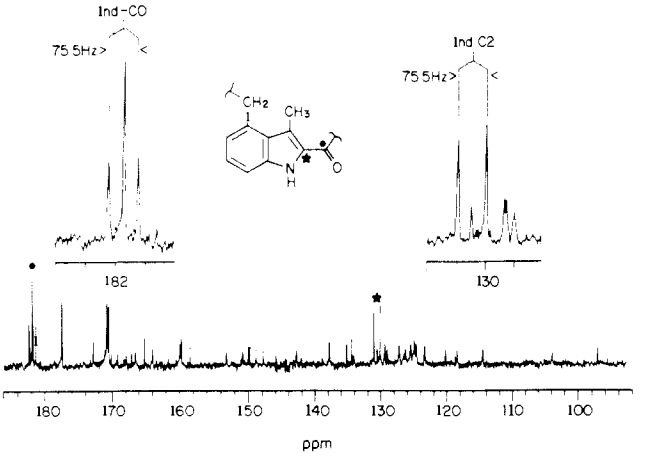

Figure 2. ^{13}C NMR spectrum of **1** derived from the feeding experiment with L-[2,1′-$^{13}C_2$]tryptophan.

Long-range ^1H–^{13}C shift correlation experiments (COLOC[20]) were used to identify most of the 29 quaternary carbons. Resonances of thiazole carbons 2 and 4 were identifiable by their respective two- and three-bond couplings with the thiazole C5 protons. Both indole C3 and C2 couple with the methyl protons of ind-C3′. Indole C7a couples with ind-H6; ind-C4 did not give a detectable signal in COLOC experiments. Although coupling of pyr-H4 with both pyr-C6 and pyr-C2 was observed, pyr-C3 and Pyr-C5 could not be assigned by this technique. Deala-C2 and the attached carbonyl carbon were assigned from their couplings with the vinylic hydrogen atoms.

The remaining quaternary signals were assigned from the ^{13}C–^{13}C coupling data that were obtained from ^{13}C-enriched samples of nosiheptide. The signal at δ 150.8 was assigned to pyridine C3 because coupling with pyr-C4 was observed in the sample derived from L-[3-^{13}C]serine; of course, both pyr-C4 and pyr-C2 had firm assignments prior to this conclusion. Since ind-C2 had an unambiguous assignment, similar logic was used to assign the signal of the attached thioester carbonyl (see Figure 2). The most complete ^{13}C–^{13}C coupling information was gained from the sample from the feeding experiment with [U-$^{13}C_3$]glycerol. Enrichments were high enough to permit the use of the 2D-INADEQUATE experiment[21,22] to determine connectivities in assemblies of carbon atoms derived from the same precursor molecule. The connectivities that were observed (Table III) completed the assignment of the ^{13}C NMR spectrum of nosiheptide (Table II). At this point only the signals for the four carbonyl groups that are attached to thiazole rings (δ 158.2–159.8) remained indistinguishable; this was not a problem in our work since these carbons are biochemically equivalent.[23]

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Table III. Data from 2D-Inadequate Experiment on Nosiheptide Derived from [*U*-$^{13}C_3$]Glycerol

carbons			δ_c, Hz, from transmitter[a]		double-quantum frequency, Hz	
A	B	J_{cc}(Hz)	A	B	predicted (A + B)	observed
Glu-CO	Glu-C4	62	5310	−2699	2611	2600
thz(3)-C2	Glu-C2	57	5114	−4304	810	800
Thr-CO	Thr-C2	57	4940	−3437	1503	1500
thz(2)-C2	but-C2	78	4835	2045	6880	6900
thz-CO	thz-C4	74	4284	3455	7739	7800
thz-C4	thz-C5	58	3455	1720	5175	5200
thz(4)-C2)	cys-C2	63	5031	−4010	1021	1000
Cys-C2	Cys-C3	56	−4010	−5480	−9490	−9500
deala-CO	deala-C2	65	4737	2422	7159	7200
deala-C2	deala-C3	75	2422	107	2529	2500
thz(5)-C2	pyr-C2	67	4895	2475	7370	7300
thz(1)-C2	pyr-C5	68	4654	2090	6744	6700
ind-C7a	ind-C3a	55	2671	1698	4368	4300

[a] Transmitter set at 102.181 ppm.

Table IV. Incorporation of ^{14}C-Labeled Amino Acids Into Nosiheptide

	amino acid		nosiheptide[a]	
precursor	μCi/mmol	mM	μCi/mmol	molar specific incorporation, %
L-[U-^{14}C]alanine	165	1.0	2	1.4
L-[*methyl*-^{14}C]methionine	130	0.9	24	18.5
L-[U-^{14}C]serine	38	1.5	14	37.0
L-phenyl-[1-^{14}C]alanine	1200	0.5	4	0.3
L-[2′-^{14}C]tryptophan	60	0.5	5	9.0
DL-[7a-^{14}C]tryptophan	50	0.5	5	9.0
DL-4-methyl-[*methylene*-^{14}C]tryptophan	53	1.0	NS	NS
L-[U-^{14}C]threonine	25	0.4	4	16.0
L-[U-^{14}C]cysteine	122	0.08	7	5.7

[a] NS indicates no significant radioactivity above background. The titer of nosiheptide was between 0.08 and 0.10 mM.

Biosynthesis. As an initial probe, incorporation of ^{14}C-labeled amino acids provided information to guide the experiments with stable isotopes; these data are given in Table IV. At levels approaching 1 mM, cysteine inhibited nosiheptide biosynthesis, but its incorporation into **1** was observed even at very low concentrations. Serine was anticipated to be an effective precursor since it is the progenitor of cysteine and also feeds one-carbon metabolism. Alanine, one of the possible precursors of dehydroalanine residues, did not label nosiheptide. Radioactivity from threonine was significantly incorporated into **1**. The substantial incorporation of L-[*methyl*-^{14}C]methionine suggested that both ind-C3′ and/or ind-C4′ might arise from methylations of, possibly, a cyclized phenylalanine residue. This hypothesis was, however, not borne out by the fact that L-phenyl-[1-^{14}C]alanine was an inferior precursor compared with ring and side chain labeled tryptophan.

Cultures of *S. actuosus* were then fed a number of ^{13}C-labeled amino acids to obtain positional information on the origin of the residues that make up nosiheptide; precursors fed included L-[3-^{13}C]serine, DL-[1-^{13}C]serine, L-[*methyl*-^{13}C]methionine, DL-[3-^{13}C]cysteine, and L-[2,1′-$^{13}C_2$]tryptophan. Relative ^{13}C abundances were calculated by normalizing the peak integrals within specific regions of the spectra of natural abundance and labeled samples. Table II reviews the specific enrichments that were observed from precursors carrying a single ^{13}C label.

Inspection of the structure of nosiheptide suggests that cysteine could potentially serve as the precursor to as many as seven residues, including the thiazoles, L-cysteine, and possibly dehydroalanine. Cysteine inhibited nosiheptide biosynthesis, so DL-[3-^{13}C]cysteine was gradually added to the fermentation over a period of 24 h. Despite this precaution, the titer dropped from a control of 195 mg/L to about 70 mg/L. Even so, the ^{13}C NMR spectrum of the product showed significant enrichments of Cys-C3 and each thz-C5. Deala-C3 was not labeled by cysteine, and this implicated serine as the precursor of the dehydroalanine moiety. Indeed, both L-[3-^{13}C]serine and DL-[1-^{13}C]serine specifically labeled dehydroalanine at C3 and C1, respectively. With one exception, the cysteine-derived residues were also labeled by serine; surprisingly, however, we were unable to detect incorporation of L-[3-^{13}C]serine into C3 of the L-cysteine residue.[24] Other enrichments from L-[3-^{13}C]serine were confirmed by use of a reverse INEPT experiment,[25] which selects for protons attached to ^{13}C-enriched sites [e.g. Thz-CH]. Therefore, both proton and carbon chemical shifts could be used to identify the sites of ^{13}C enrichment. Although Cys-C3 was not detectably enriched by L-[3-^{13}C]serine, Thz(4)-C2 (cysteine carboxyl) was labeled by DL-[1-^{13}C]serine.[26]

Rather significantly, pyr-C3 and -C4 were both labeled by L-[3-^{13}C]serine. In fact, these sites were simultaneously enriched to an extent that ^{13}C–^{13}C coupling was observable. The biochemical implications of that result were realized when DL-[1-^{13}C]serine was found to label C2 of thiazoles 1 and 5; the pyridine ring is thus believed to be constructed by "tail-to-tail" joining of two serine residues.

Although both indole C3′ and C4′ were labeled by L-[3-^{13}C]-serine, only the origin of the methylene group (C4′) could be traced to an initial methylation, because ind-C4′ was the only site enriched by L-[*methyl*-^{13}C]methionine. This result and the labeling of indole CO by DL-[1-^{13}C]serine immediately suggested that tryptophan was the progenitor of the indolic acid moiety. Knowing that serine provides the side chain of tryptophan,[18] we postulated that the carboxyl group of tryptophan may become connected to indole C2 during biosynthesis; the modification would require excision of the α-carbon and the attached amino group.

To test the above hypothesis, L-[2,1′-^{13}C]tryptophan was synthesized for a feeding experiment by a strategy involving condensation of [2-^{13}C]indole and [1-^{13}C]serine in a reaction catalyzed by tryptophan synthase. This route has the advantage of substrate and product specificity for the L isomers. The enzyme reaction proved to be a very efficient method for making labeled tryptophan.

(23) These carbonyl carbon signals have since been assigned, as shown in Table II, by analysis of a sample of **1** biosynthesized from L-[1,2-$^{13}C_2$]serine (Mocek, U.; Beale, J. M.; Unkefer, C.; Floss, H. G., unpublished result).

(24) Note that in our preliminary paper,[13] Cys-C2 was erroneously identified to have D rather than L configuration, and the result was interpreted accordingly.

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(26) We have since demonstrated unequivocal incorporation of L-serine into the cysteine residue in experiments with L-[1,2-$^{13}C_2$]- and L-[3-^{13}C,$^2H_2$]serine (Mocek, U.; Beale, J. M.; Unkefer, C.; Floss, H. G., unpublished results). The negative result in the initial feeding of L-[3-^{13}C]serine is almost certainly an experimental artefact. A tentative and, admittedly, speculative explanation is that the sample, which was subject to more manipulations prior to NMR analysis than later samples, had undergone partial hydrolysis of the thioester bond, spreading the ^{13}C of the labeled cysteine over two signals and thus creating the impression that the original Cys-C3 signal was not enriched.

Despite some technical difficulties (tryptophan inhibits nosiheptide biosynthesis), L-[2,1′-^{13}C]tryptophan was incorporated into **1** as we had postulated; the ^{13}C NMR spectrum clearly showed coupling of ind-CO and ind-C2 (Figure 2). This experiment proved that an unusual intramolecular rearrangement of tryptophan produces the indolic acid moiety of nosiheptide.

As demonstrated in the experiment with doubly labeled tryptophan, when two adjacent carbon atoms are simultaneously enriched with ^{13}C, the coupling of the NMR signals can serve as an assignment criterion and at the same time solve a biosynthetic problem (when the enrichment is the result of a biochemical process). For these reasons, [1,2-$^{13}C_2$]acetate,[27] [U-$^{13}C_3$]glycerol,[28] and [U-$^{13}C_6$]glucose[29] have become important tools in biosynthetic studies. We prefer [U-$^{13}C_3$]glycerol to [U-$^{13}C_6$]glucose, since this precursor typically yields higher ^{13}C enrichment and more readily interpretable coupling patterns. Thus, *S. actuosus* was fed [U-$^{13}C_3$]glycerol, with the objectives of completing the ^{13}C NMR assignments and defining all the precursor amino acids.

Not surprisingly, nosiheptide biosynthesized from [U-$^{13}C_3$]-glycerol produced a very complex ^{13}C NMR spectrum, due to extensive ^{13}C–^{13}C coupling. In fact, of the 51 carbons, only six carbons were clearly not coupled (all were part of the indole system). The overall enrichment was between 15 and 20%. Significant signal overlap obscured many of the resonances, but despite this complexity, a number of interpretations were made directly from the analysis of the proton decoupled ^{13}C NMR spectrum. First, signals for ind-CO and ind-C3′ were singlets; this agreed with the proposed rearrangement of tryptophan because the biosynthetic sequence would essentially excise the central atom of a ^{13}C–^{13}C–^{13}C unit (serine), eliminating ^{13}C coupling to the terminal carbon atoms. Secondly, the uniformly labeled glycerol did not enter the erythrose nor the ribose pool efficiently since ind-C5 and -C7 did not exhibit coupling to a neighboring ^{13}C atom (ind-C6), and ind-C2 and ind-C3 were not coupled to each other. Finally, the spectrum showed that all the residues derived from cysteine contained three-carbon doubly coupled species. A two-bond coupling of 7 Hz was clearly identifiable between the thz-CO and thz-C5 signals.

A contour plot of the 2D-INADEQUATE experiment uncovered the ^{13}C–^{13}C connectivities listed in Table III. The biochemical significance of the connectivities was then determined by comparing the double-quantum map with the percentage of coupled signal for each carbon. For instance, the most intense double-quantum signals, Glu-C4/C5, showed 66% coupling for each resonance. The satellites of thz(3)-C2 and Glu-C2 accounted for 34% of the total peak areas. Glu-C3 showed only 10% coupling and did not have a coupling partner in the INADEQUATE. Thus, this defined three biogenetically distinct parts of the hydroxyglutamate moiety. The data revealed that [U-$^{13}C_3$]glycerol was largely converted glycolytically to [1,2-$^{13}C_2$]acetate, which entered the TCA cycle. One turn of the tricarboxylic cycle labels carbons 4 and 5 of α-ketoglutarate, and a second turn labels carbons 1 and 2, providing the indicated hydroxyglutamate labeling and coupling pattern. The small coupling of the Glu-C3 signal is only statistical. By the same analysis, two separate acetate units should be apparent in the threonine residue (via oxaloacetate): Thr-C2 coupled to Thr-CO, and Thr-C3 to Thr-CH_3. Only a Thr-C2 to Thr-CO connectivity was observed in the double-quantum spectrum. The same labeling pattern was observed in the dehydrobutyrine residue [thz(2)-C2 to but-C2]. Although signals for both Thr-CH_3 and But-CH_3 were obscured by spectral overlap, ca. 10%, $^1J_{cc}$ = 37 Hz coupling could be clearly observed at the signals of their neighboring carbon atoms (Thr-C3 and but-C3).

Approximately 72% of the signals for the thz-C4, thz-C5, and thz-CO assemblies exhibited coupling. Moreover, the two-bond coupling between thz-C5 and thz-CO was observed in the double-quantum map. Considering the abundance of doubly coupled species in the thiazole rings, we anticipated observation of similar labeling patterns in other three-carbon units that are thought to arise from serine, namely dehydroalanine and the pyridine ring, particularly since these showed the highest enrichments in the serine feeding experiments. However, whereas 60% of deala-CO showed coupling, the integral for the satellites of the deala-C3 signal showed only 30%. Accurate integration of deala-C2 was precluded by signal overlap with pyr-C2. The same discrepancy was observed in the pyridine ring, because the single- and double-quantum spectra did *not* reveal connectivities of pyr-C2/C3 or pyr-C5/C4. Therefore, internally generated and externally fed serine may be utilized somewhat differently. The C2–C3 bond of a serine unit synthesized in the cells (from [U-$^{13}C_3$]glycerol) may be cleaved to some extent before transformation to dehydroalanine and the analogous pieces of the pyridine ring.

All of the carbon–carbon bonds that were transferred intact from [U-$^{13}C_3$]glycerol, as disclosed by this type of analysis, are shown in Figure 1 as heavy bars.

Discussion

The data obtained in this study demonstrate the origin of nosiheptide entirely from amino acids. For the precursors cysteine, serine, tryptophan, and the methyl group of methionine this was proven directly by feeding the ^{13}C-labeled compounds and demonstrating specific incorporation into the appropriate structural elements of **1**. Efficient incorporation of [^{14}C]threonine suggests that the threonine and probably also the dehydrobutyrine moiety arise from that amino acid. The origin of the dehydrobutyrine moiety from threonine is supported by the fact that the threonine and the dehydrobutyrine moiety of **1** biosynthesized from [U-$^{13}C_3$]glycerol showed the same labeling and coupling pattern. The coupling pattern of the γ-hydroxyglutamate moiety in the same sample conforms to that predicted for glutamic acid; however, direct verification by feeding [^{13}C]glutamate has not yet been done. The data are consistent with all precursor amino acids having the L configuration, although for the cysteine-derived moieties the evidence is not conclusive.

The origin of the dehydroalanine and the dehydrobutyrine moieties of **1** from the corresponding hydroxy amino acids has precedent in the biosynthesis of berninamycin.[30,31] Similarly, the formation of thiazole rings from cysteine has analogy in the generation of the thiazole ring of berninamycin from cysteine, the oxazole rings of the same compound from threonine,[30,31] and the oxazole ring of virginiamycin from serine.[32] Whether, as seems likely in the latter case,[33] the reaction proceeds via an aldehyde (in this case thioaldehyde) intermediate remains to be examined. Rather unusual is the formation of the pyridine ring by tail-to-tail linkage of two serine residues. The amino acid nature of all the building blocks is more evident in the corresponding structural element of other, related thiopeptide antibiotics, the tetrahydropyridine ring of thiostrepton[4] or micrococcin P.[34] This led Bycroft and Gowland to propose formation of this structure from two dehydroalanine residues in a single polypeptide chain.[34] Our results on **1** are consistent with this idea, although we can not yet rule out an alternative pathway in which one or both serine residues are first cleaved by serine hydroxymethyltransferase into glycine

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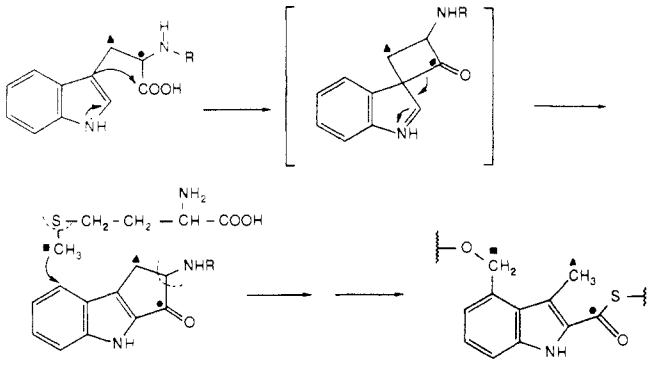

Figure 3. Formation of the indolic acid moiety of **1** from tryptophan.

and a C_1 fragment followed by assembly of the pyridine ring from these pieces.

The assembly of the indolic acid moiety from tryptophan represents a novel rearrangement of this amino acid, for which there seems to be no obvious biochemical or, for that matter, chemical precedent. The reaction presumably proceeds via a tricyclic intermediate (Figure 3), the formation of which can be easily rationalized (intramolecular acylation at indole C3 and rearrangement of the intermediate spiro indolenine by a 1,2-shift). The mechanism of ring opening of this tricyclic intermediate and of the extrusion of C2′ and the amino nitrogen is less obvious; future work will address this intriguing question. Methylation of the indole moiety of tryptophan is evidently not an early reaction step since labeled 4-methyltryptophan was not incorporated into **1**; the methyl group must therefore be introduced at some later stage in the biosynthesis. Finally, another interesting question is the mode of attachment of the tryptophan moiety to the rest of the molecule. All the other components of **1** can be arranged logically in a single polypeptide chain. However, the indolic acid moiety is not connected to this polypeptide by traditional amide bonds. Conceivably, the precursor tryptophan may be initially connected to this precursor polypeptide as an amide, and this bond is subsequently cleaved during the modification of the tryptophan to the indolic acid moiety. Again, this issue will be examined in future experiments.

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Registry No. 1, 56377-79-8; L-Cys, 52-90-4; L-Glu, 56-86-0; L-Thr, 72-19-5; L-serine, 56-45-1; L-tryptophan, 73-22-3.

A Convergent Synthesis of Triquinane Sesterterpenes. Enantioselective Synthesis of (−)-Retigeranic Acid A

Jonathan Wright, Gary J. Drtina, Richard A. Roberts,[1] and Leo A. Paquette*

Contribution from the Evans Chemical Laboratories, The Ohio State University, Columbus, Ohio 43210. *Received January 22, 1988*

Abstract: The total synthesis of enantiomerically pure (−)-retigeranic acid A has been achieved in a convergent manner from (*R*)-(+)-pulegone and (*S*)-(−)-limonene. In the pivotal coupling step, the Grignard reagent derived from optically pure bromide **46** was found to undergo exclusive 1,4-addition to α,β-unsaturated tricyclic ketone **10** (100% ee). The resulting two epimeric condensation products were separately carried forward through construction of the six-membered ring and introduction of the carboxyl functionality. Two diastereomers initially considered to be candidates for the structure of retigeranic acid B were thereby also prepared.

First isolated by Sheshadri in 1965 from the lichens of the *Lobaria retigera* group in the Western Himalayas,[2] retigeranic acid was initially believed to possess a tetracyclic skeleton.[3] Successful structural eludication was not completed, however, until 1972[4] when Shibata and co-workers established the correct skeletal arrangement and absolute configuration of this complex natural product by X-ray crystallography of the *p*-bromoanilide derivative.[5] At about this time, retigeranic acid was also isolated from *Lobaria isidiosa* and *Lobaria subretigera* in the Eastern Himalayas by Yoshimura.[6] Although the possible biological activity of **1** has not been determined, members of the genus *Lobaria* are considered to be important in perfumery and tanning and have been used as vegetable drugs for the cure of eczema and lung disorders.[2,6]

Retigeranic acid holds the distinction of being the first sesterterpene found to occur in lichens. This unusually structured pentacyclic carboxylic acid also bears no close correlation to any other existing sesterterpenes. Recently, Corey and his associates completed a total synthesis of racemic **1**[7] only to recognize that native retigeranic acid is composed of two isomeric substances of which **1** is the less prevalent constituent. This finding was subsequently confirmed in the Shibata laboratory. Evidently, recrystallization of the *p*-bromoanilides in preparation for the crystallographic analysis proceeded with fractionation in favor of the less soluble minor isomer. In light of these developments, the suggestion has been advanced to refer to **1** more accurately as retigeranic acid A.[8] In Corey's view, the major stereoisomer B was considered possibly to differ from **1** only at the methyl-bearing carbon in the E ring. This suggestion was later shown not to be tenable.[8]

Herein we describe the elaboration of carboxylic acids **1–3** in optically pure condition and show that retigeranic acid B is neither of the last two stereoisomers.[9] In planning this synthetic venture,

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