Stylopeptide 2, a Proline-Rich Cyclodecapeptide from the Sponge *Stylotella* sp.[⊥,#]

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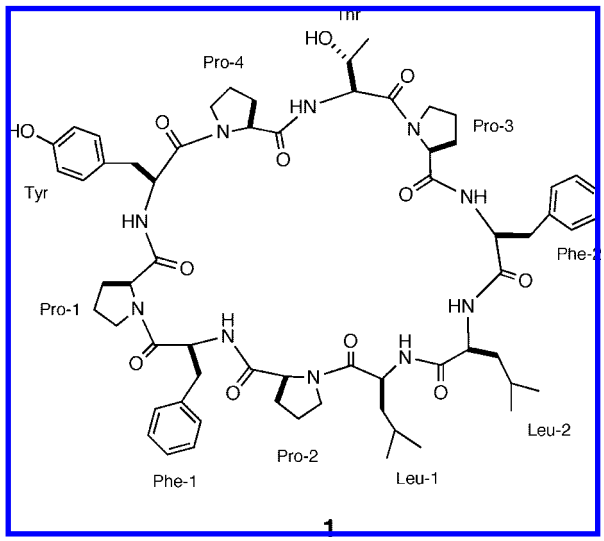
Received September 10, 2007

A new proline-rich cyclodecapeptide, designated stylopeptide 2, has been isolated from a cytotoxic extract of the Papua New Guinea marine sponge *Stylotella* sp. and found to correspond to structure **1**. The structural assignment was based on HRMS collision-induced dissociation tandem mass spectrometry (CID MS/MS), NMR spectroscopic data, and amino acid analysis, which led to assignment of the absolute configuration.

The number of nonribosomal-derived cyclic peptides isolated from marine sources continues to grow. Such peptides are relatively stable toward enzymatic degradation and, in being less polar, are more biologically available than the linear type. Proline-rich cyclopeptides constitute a relatively new class. Most members of this group are sponge-derived hepta- or octapeptides with nonpolar amino acid residues (usually with one or two aromatic representatives) interspersed among the proline units. Examples include the axinellins,[1] axinastatins,[2] hymenamides,[3] phakellistatins,[4] stylisins,[5] stylissamides,[6] stylopeptide 1,[7] wainunuamide,[8] and dominicin.[9] Phakellistatins 7–9 and 12 are examples of proline-rich cyclic decapeptides.[10] Herein we report the isolation and characterization of a new proline-type decapeptide, stylopeptide 2 (**1**), obtained from the same collection of the marine sponge *Stylotella* sp. that provided stylopeptide 1[7] and stylostatin 1.[11]



Dichloromethane−methanol extraction of the Papua New Guinea sponge *Stylotella* sp.,[7,11a] followed by solvent partitioning, provided a dichloromethane-soluble fraction that displayed activity against the P388 lymphocytic leukemia cell line (PS, ED_{50} 1.5 μg/mL). Successive Sephadex LH-20 partition chromatography followed by vacuum-liquid chromatography (VLC), preparative thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC) afforded a cyclodecapeptide designated stylopeptide 2 (**1**) as an amorphous solid. The peptide displayed the molecular formula $C_{63}H_{84}N_{10}O_{12}$ by high-resolution mass spectrometry (HRMS). Amino acid analysis identified its cation as a decapeptide composed of threonine, tyrosine, phenylalanine (2), leucine (2), and proline (4). Both its solubility in organic solvents and the 11 degrees of unsaturation suggested a cyclic structure.

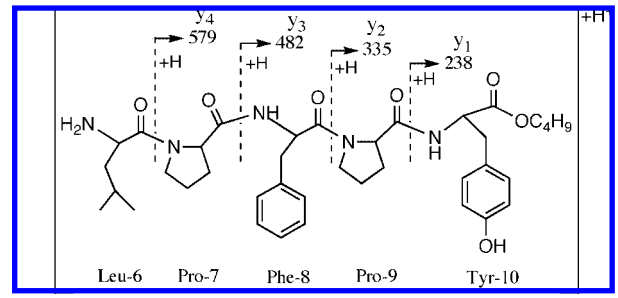


Figure 1. C-Terminal CID fragmentation of primary fragment ion *m*/*z* 692.

The ^{1}H NMR solution ($CDCl_3$) spectrum of peptide **1** clearly indicated that it exists as a mixture of conformers in solution, the ratio of which often varied somewhat. Because of the complexity of this spectrum, the amino acid sequence was initially investigated by collision-induced dissociation tandem mass spectrometry (CID-MS/MS)[12] using the parent peptide as well as its acyclic butyl ester derivative. The latter provided the most useful information. Thus, dissociation of the in-source C-terminal fragment ion at *m*/*z* 692 generated the product ions y_1 to y_5 depicted in Figure 1 and established the partial sequence Leu-Pro-Phe-Pro-Tyr, while N-terminal fragmentation of the protonated parent acyclic butyl ester (Figure 2) extended this sequence to two possibilities: Pro-Thr-Pro-Phe-Leu-Leu-Pro-Phe-Pro-Tyr (sequence 1) or Pro-Phe-Pro-Thr-Leu-Leu-Pro-Phe-Pro-Tyr (sequence 2). Evidence to support a Pro-Thr-Pro linkage was provided by the presence of the fragment ion at *m*/*z* 296, which can eliminate one of the proline residues to produce the ion at *m*/*z* 199 (Pro-Thr or Thr-Pro) (Figure 3a). Fragmentation of the ion observed at *m*/*z* 358, as depicted in Figure 3b, supported a Pro-1-Phe-2-Leu-3 assignment, but the same ion series could also have arisen from the adjacent Pro-Phe residues in the tripeptide Leu-6-Pro-7-Phe-8, after initial elimination of Leu-6. Further evidence supporting sequence 1 for stylopeptide 2 was provided by a comprehensive NMR analysis of the parent peptide.

[⊥] Dedicated to Dr. G. Robert Pettit of Arizona State University for his pioneering work on bioactive natural products.

[#] This paper is also dedicated to the memory of Mary R. Brennan SL (deceased January 25, 2007).

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10.1021/np0704856 CCC: $40.75 © 2008 American Chemical Society and American Society of Pharmacognosy
Published on Web 01/15/2008

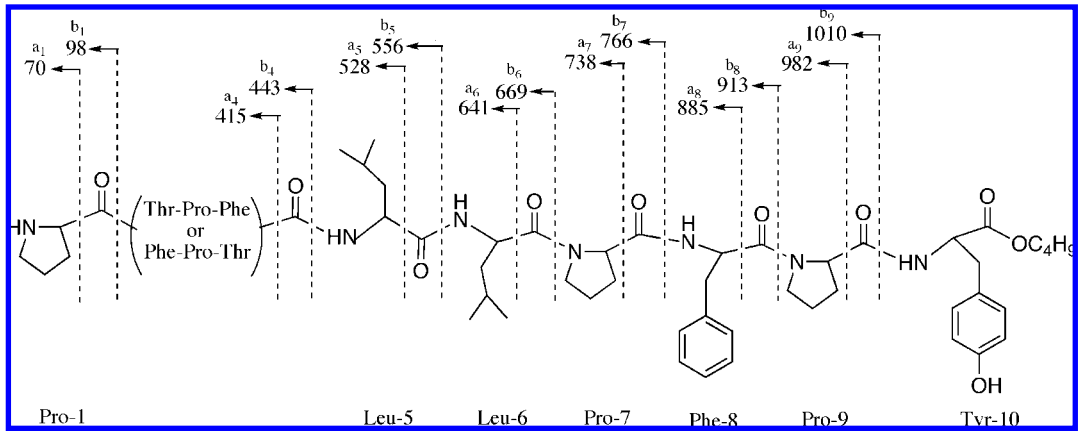

Figure 2. N-Terminal fragmentation of acyclic ester *m/z* 1247 $[M + H]^+$.

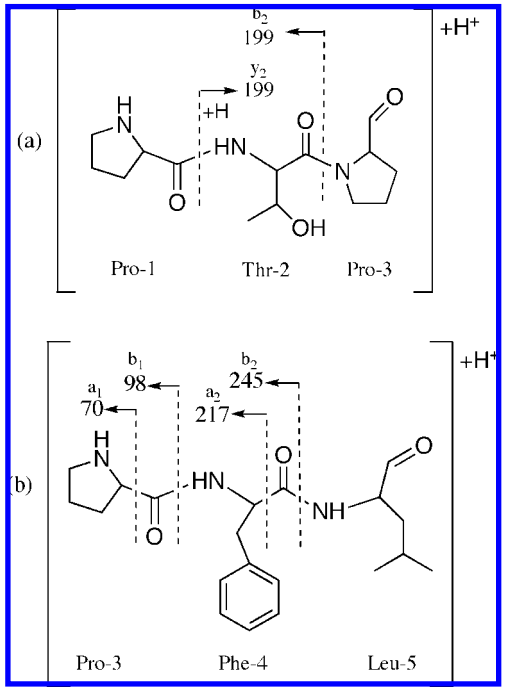

Figure 3. Two key tripeptide primary fragments and their CID fragmentation patterns: (a) *mz* 296.3; (b) *mz* 358.4.

The ^{1}H and ^{13}C NMR spectroscopic data for a major conformer of stylopeptide 2 (**1**) are presented in Table 1. Chemical shift values and COSY, TOCSY, and HMQC spectra verified the amino acid composition. The observed HMBC correlations that addressed sequencing are shown in Figure 4: Tyr NH to Pro-1 carbonyl carbon, Thr NH to Pro-4 carbonyl and α-carbons, and Leu-2 NH to Phe-2 carbonyl and α-carbon. The Leu NH correlations supported a Phe-Leu link, as given in sequence 1 above, and not the Thr-Leu link of sequence 2. Additional HMBC correlations are given in Table 1.

NOESY correlations across nine of the 10 peptide linkages provided further evidence for sequence 2 (Figure 4). The threonine unit is clearly between two prolines, as seen by correlations with its NH to the α-proton of Pro-4 and its α- and β-protons to the δa-proton of Pro-3. On the other hand, the NH of Leu-2 correlated with the α-proton of Leu-1 and the α- and β-protons of Phe-2, placing it between these two amino acids, as required by sequence 1. Additional confirmatory correlations are shown in Figure 4.

The solution ($CDCl_3$) conformation about the proline peptide linkages of **1** could be assigned from the chemical shift differences between the proline β- and γ-carbons.[13] Thus, Pro-1 and Pro-2 showed $\Delta_{\beta\gamma} = 9.15$ and 9.99, respectively, indicating that these proline peptide bonds are both *cis*. Conversely, $\Delta_{\beta\gamma} = 3.98$ and 3.18 for Pro-3 and Pro-4, respectively, allowed *trans*-geometry to be assigned to these peptide linkages. Confirmation for three of these assignments came from the NOESY spectrum.[14] Cross-peaks between Pro-2αH/Leu-1αH established *cis*-geometry for the Pro-2 peptide bond, while cross-peaks between Pro-3δH/Thr-αH and between Pro-4δH/Tyr-αH (Figure 4) confirmed *trans*-peptide links for these two prolines.

The absolute configuration of **1** was established by chiral HPLC analysis of the individual amino acids obtained upon hydrolysis with 6 N HCl. The retention times of the amino acids were compared with authentic samples of D- and L-proline, leucine, tyrosine, threonine, allo-threonine, and phenylalanine. All of the stylopeptide 2 amino acids were found to have the L-configuration, and the structure (**1**) of stylopeptide 2 was thereby completed.

In the National Cancer Institute's one-dose (10^{-5} M) 60-cell-line assay, stylopeptide 2 (**1**) inhibited two breast cancer cell lines: BT-549 displayed growth at 77% of the mean and HS 578T at 56% of the mean.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. IR spectra were recorded on a Perkin-Elmer 1330 IR spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were obtained on Bruker AC 200 (200 MHz) and Varian Unity 500 (500 MHz) spectrometers. ^{1}H NMR and ^{13}C NMR chemical shifts are referenced to 7.24 and 77.0 ppm, respectively, for $CDCl_3$. The liquid secondary ion mass spectrometry (LSIMS) and collision-induced decomposition tandem mass spectrometry (CID MS/MS) were carried out on a four-sector JEOL HX 110/110 instrument at the MIT MS Resource. Glycerol was used as the matrix. The energy of the Cs-ion beam was 15 keV. For the CID experiments, the monoisotopic precursor was selected with MS. Helium was used as the collision gas in the cell located between MS1 and MS2, and the collision energy was 7 keV; MS2 was scanned at constant B/E. The HRESIMS was obtained at the SUNY Buffalo mass spectrometry facility on a ThermoFinnigan MAT 95 XL with an ESI ion source in 60% methanol, 3 kV, heated capillary at 250 °C. The amino acid analysis was done at the MIT Biopolymers Laboratory. Sephadex LH-20 was used for gel filtration. TLC-grade Brinkmann silica gel 60H was used for VLC and preparative TLC. Preparative HPLC was performed with a RP C_{18} 5 μm column (190 mm × 10 mm) and chiral HPLC on a Chirex 3126 D-penicillamine column (150 mm × 2 mm). All solvents were reagent grade.

Isolation of Stylopeptide 2 (1). Approximately 500 kg of *Stylotella* sp. collected in Papua New Guinea was extracted with 1:1 dichloromethane−methanol, and the resulting extract was subjected to solvent partitioning as previously described.[7,11] The dichloromethane-soluble fraction (296 g) exhibited an ED_{50} of 1.5 μg/mL against the P388 leukemia cell line. A 150 g portion of this fraction was suspended in 3:2 dichloromethane−methanol, and the insoluble material was removed (filtration). The filtrate was subjected to partition chromatography on

Table 1. NMR Data (500 MHz, $CDCl_3$) for Stylopeptide 2 (**1**)

residue	position	δ_C mult.[a]	δ_H (*J*, in Hz)[b]	TOCSY	HMBC (H)
Pro-1	CO	170.79, qC			Pro-1 α, *β*b, Tyr NH
	α	61.17, CH	4.03 m	*β*, *γ*, *δ*	
	β	30.77, CH_2	1.89 m	α, *β*a, *γ*, *δ*	Pro-1 α
			2.44 m	α, *β*b, *γ*, *δ*	
	γ	21.62, CH_2	1.25 m	α, *β*, *γ*a, *δ*	Pro-1 α, *β*b
			1.74 m	α, *β*, *γ*b, *δ*	
	δ	46.86, CH_2	3.37 m	α, *β*, *γ*, *δ*a	Pro-1 α, *β*a
			3.47 m	α, *β*, *γ*, *δ*b	
Phe-1	CO	170.18, qC			Phe-1 α, *β*a,b
	α	51.87, CH	5.10 m	*β*, NH	Phe-1 *β*a,b
	β	37.20, CH_2	2.85 m	α, *β*a, NH	Phe-1 α, *ortho*
			3.03 m	α, *β*b, NH	
	γ	136.76, qC			Phe-1 α, *β*a,b, *ortho*
	ortho	129.58,[c] CH	7.29 m		
	meta and *para*	126.72, CH 127.90, CH 128.89, CH	7.22 m 7.21 m 7.23 m		
	NH		7.86 br s	α, *β*	
Pro-2	CO	169.30, q			Pro-2 α, *β*b
	α	60.55, CH	4.16 m	*β*, *γ*, *δ*	Pro-2 *β*a
	β	31.32, CH_2	1.96 m	α, *β*a, *γ*, *δ*	Pro-2 α
			2.07 m	α, *β*b, *γ*, *δ*	
	γ	21.34, CH_2	1.09 m	α, *β*, *γ*a, *δ*	Pro-2 α, *β*b
			1.64 m	α, *β*, *γ*b, *δ*	
	δ	46.86, CH_2	3.40 m	α, *β*, *γ*	Pro-2 α, *β*a
			3.41m	α, *β*, *γ*	
Leu-1	CO	171.47, qC			Leu-1 α
	α	51.34, CH	4.43 m	*β*, *γ*, *δ*, NH	Leu-1 *β*b
	β	40.83, CH_2	1.65 m	α, *β*a, *γ*, *δ*, NH	Leu-1 *γ*, *δ*
			1.68 m	α, *β*b, *γ*, *δ*, NH	
	γ	24.45, CH	1.60 m	α, *β*, *δ*, NH	Leu-1 *β*b
	δ	23.21, CH_3	0.88 d (6.0)	α, *β*, *γ*, NH	Leu-1 *β*a, *δ*
		23.39, CH_3	0.88 d (6.0)		Leu-1 *β*a, *δ*
	NH		7.68 br s	α, *β*, *γ*, *δ*	
Leu-2	CO	170.57, qC			Leu-2 α, *β*
	α	50.92, C	4.07 m	*β*, *γ*, *δ*, NH	Leu-2 NH
	β	40.83, CH_2	1.68 (2) m	α, *γ*, *δ*, NH	Leu-2 *δ*, NH
	γ	24.67, CH	1.59 m	α, *β*, *δ*, NH	Leu-2 *β*
	δ	20.88, CH_3	0.82 d (5.9)	α, *β*, *γ*, NH	Leu-2 *β*,*γ*
		21.03, CH_3	0.90 d (6.4)		Leu-2 *β*,*γ*
	NH		6.84 d (6.4)	α, *β*, *γ*, *δ*	Phe-2 α, CO
Phe-2	CO	171.01, qC			Phe-2 α, *β*a, Leu-2 NH
	α	54.60, CH	4.64 m	*β*, NH	Phe-2 *β*ab, Leu-2 NH
	β	36.10, CH_2	2.90 m	α, *β*a, NH	Phe-2 α, *ortho*
			3.24 m	α, *β*b NH	
	γ	138.37, qC			Phe-2 α, *β*a, *meta*
	ortho	126.03, C	7.16 m		Phe-2 *para*
	meta	129.13, CH	7.22 m		Phe-2 *ortho, para*
	para	128.32,[c] CH	7.28 m		
	NH		6.53 br s	α, *β*	
Pro-3	CO	170.79, qC			Pro-3 α
	α	61.57, CH	4.34 m	*β*, *γ*, *δ*	
	β	28.87, CH_2	1.75 m	α, *β*a, *γ*, *δ*	Pro-3 α
			1.85 m	α, *β*b, *γ*, *δ*	
	γ	24.89, CH_2	1.39 m	α, *β*, *γ*a, *δ*	Pro-3 *δ*a,b
			1.64 m	α, *β*, *γ*b, *δ*	
	δ	47.56, CH_2	3.36 m	α, *β*, *γ*, *δ*a	
			3.70 m	α, *β*, *γ*, *δ*b	
Thr	CO	169.30, qC			Thr α, *β*
	α	55.26, CH	4.78 d (9.2)	*β*, *γ*, NH	Thr *β*, *γ*, NH
	β	67.42, CH	4.18 m	α, *γ*, NH	Thr α, *γ*, NH
	γ	19.10, CH_3	1.21 d (6.9)	α, *β*, NH	Thr *β*
	OH		8.19 br s		
	NH		7.36 br s	α, *β*, *γ*	Pro-4 α, CO
Pro-4	CO	171.01, qC			Pro-4 α, *β*b, Thr NH
	α	58.68, CH	4.41 m	*β*, *γ*, *δ*	Pro-4 *β*b, *γ*a, *δ*b, Thr NH
	β	28.07, CH_2	2.04 m	α, *β*a, *γ*, *δ*	Pro-4 α, *γ*a
			3.44 m	α, *β*b, *γ*, *δ*	
	γ	24.89, CH_2	1.5 m	α, *β*, *γ*a, *δ*	Pro-4 α, *β*b, *δ*a
			1.92 m	α, *β*, *γ*b, *δ*	
	δ	47.56, CH_2	3.54 m	α, *β*, *γ*, *δ*a	Pro-4 *γ*a
			3.82 m	α, *β*, *γ*, *δ*b	
Tyr	CO	171.17, qC			Tyr α
	α	51.87, CH	4.92 m	*β*, NH	Tyr NH
	β	36.10, CH_2	2.82 m	α, *β*a, NH	Tyr α, *ortho*[d]
			2.97 m	α, *β*b, NH	

Table 1. Continued

residue	position	δ_C mult.[a]	δ_H (J, in Hz)[b]	TOCSY	HMBC (H)
	γ	127.58, qC			Tyr *meta*[d]
	ortho[d]	129.86, CH	6.84 d (8.4)	*meta*[d]	
	meta[d]	115.54, CH	6.76 d (8.4)	*ortho*[d]	Tyr *ortho*,[d] OH
	para[d]	155.49, qC			Tyr *ortho*,[d] *meta*[d]
	OH		8.90 br s		
	NH		7.46 br s	α, β	Pro-1 CO

[a] Determined from the DEPT spectrum. [b] With geminal protons, the lower field value is given the "a" designation, the higher field value is given the "b" designation. [c] Values may be interchanged. [d] Relative to the γ-carbon substituent.

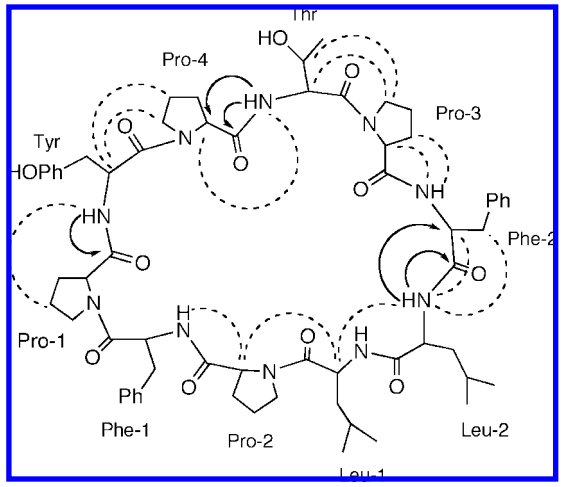

Figure 4. Key HMBC (→) and NOESY (---) correlations for stylopeptide 2 (**1**).

Sephadex LH-20, first with 3:2 dichloromethane−methanol, then with 3:2:2 hexane−toluene−methanol, to give four fractions. Fraction 4 (210 mg, ED_{50} 0.43) was subjected to VLC with 9:1 dichloromethane−2-propanol followed by preparative TLC (4:1 chloroform−2-propanol) to give 64 mg of a yellow powder. Preparative reversed-phase HPLC (4:1 methanol–water) afforded 20 mg of stylopeptide 2 (**1**) as a colorless, amorphous solid: $[\alpha]^{24}_D$ −131 (*c* 0.030, $CHCl_3$); IR ($CHCl_3$) λ_{max} 3680, 3400, 3300, 3050, 2960, 1650, 1530, 1450, 1270, 1100, 920, 700 cm^{-1}; ^1H NMR and ^{13}C NMR, see Table 1; HRESIMS *m/z* 1195.6158 $[M + Na]^+$ calcd for $C_{63}H_{84}O_{12}N_{10}Na$, *m/z* 1195.6162.

Butanolysis of Stylopeptide 2 (1). The peptide was heated with 3 N HCl in butanol for 3 h, and the resulting acyclic butyl ester derivative was purified by HPLC. LSIMS showed the $[M + H]^+$ molecular ion at *m/z* 1247.7, as compared to the original cyclic peptide, which had $[M + H]^+$ *m/z* 1173.6.

Absolute Configuration of the Amino Acids. A 1 mg sample of the peptide was heated with 6 N HCl at 110 °C for 18 h. The residual HCl was evaporated with N_2 at 90 °C, and the residue was suspended in 100 μL of water for chiral HPLC analysis on a Chirex 3126 D-penicillamine column with 2 mM $CuSO_4$ as solvent (flow rate 1 mL/min). Retention times (in min) of the authentic D- and L-amino acids were as follows: L-Thr 3.7, L-*allo*-Thr 3.8, D-*allo*-Thr 4.1, D-Thr 4.6; L-Pro 4.3, D-Pro 9.1; L-Leu 13.6, D-Leu 21.8; L-Tyr 17.5, D-Tyr 24.9; L-Phe 41.0, D-Phe 54.3. Retention times of the amino acids in the hydrolysate were as follows: Thr 3.7, Pro 4.4, Leu 14.0, Tyr 18.0, and Phe 39.9. Co-injection of the authentic samples with the hydrolysate confirmed the L-configuration for all of the amino acids.

. We are pleased to thank for financial assistance Outstanding Investigator Grant CA-44344-01-12 and Grants R01 CA90441-05 and 2R56 CA090441-06A1 from the Division of Cancer Treatment and Diagnosis, NCI, DHHS; the Fannie E. Rippel Foundation; the Arizona Disease Control Commission; and the Robert B. Dalton Endowment Fund (G.R.P.). We also thank NIH-NCRR for support of the Mass Spectrometry Resources, formerly at MIT (P41 RR00317, K. Biemann) and presently at BUSM (P41 RR10888, C.E.C.). The ThermoFinnigan MAT (SUNY at Buffalo) was purchased with a grant from NSF (CHE0091977). Other very helpful assistance was provided by Dr. D. L. Doubek.

Supporting Information Available: ^1H and ^{13}C NMR spectra of **1** are available free of charge via the Internet at http://pubs.acs.org.

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NP0704856