Tenuecyclamides A−D, Cyclic Hexapeptides from the Cyanobacterium *Nostoc spongiaeforme* var. *tenue*

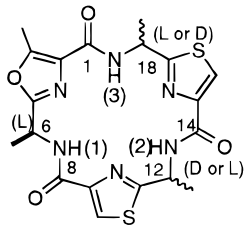
Ronny Banker and Shmuel Carmeli*

School of Chemistry, Raymond and Beverly Sackler Faculty of Exact Sciences, Tel Aviv University, Ramat Aviv, Tel Aviv 69978, Israel

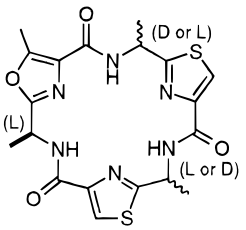
Received April 10, 1998

Four modified cyclic hexapeptides, tenuecyclamides A−D (**1**−**4**), were isolated along with the known antibiotic, borophycin (**5**), from the methanol extract of *Nostoc spongiaeforme* var. *tenue* (TAU strain IL-184-6). The planar structure of tenuecyclamides A−D (**1**−**4**) was determined by homonuclear and inverse-heteronuclear 2D-NMR techniques as well as by high-resolution mass spectrometry measurements. The absolute configuration of the asymmetric centers was studied by Marfey's method for HPLC. The stereochemistry of the asymmetric centers in tenuecyclamides A and B (**1** and **2**) could not be fully determined, while that of tenuecyclamides C and D (**3** and **4**) was unambiguously determined.

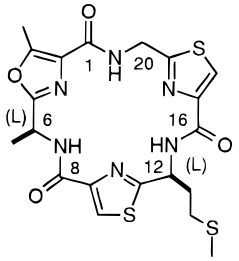
Cyanobacteria have become well-known as prolific producers of biologically active peptides with highly modified amino acid residues.[1,2] Several cyclic hexapeptides containing cyclically modified amino acids, such as westiellamide,[3] nostocyclamide,[4] dendroamides A−C,[5] and raocyclamides A and B,[6] have recently been isolated from cyanobacteria. Westiellamide is identical to trisoxazoline, isolated from the ascidian *Lissoclinum bistratum*.[7] It was suggested that westiellamide is produced by its obligate prokaryotic symbiont, *Prochloron* sp.[3] Five other modified cyclic hexapeptides are known from marine organisms, namely bistratamides A−D (from *L. bistratum*[8,9]) and dolastatin E (from the sea hare *Dolabella auricularia*[10]). As part of our continuing effort to isolate biologically active compounds from cultured cyanobacteria, we hereby report the isolation and structural elucidation of four novel cyclic hexapeptides, tenuecyclamides A−D (**1**−**4**), from the cultured *Nostoc spongiaeforme* var. *tenue* Rao (Nostocales, Nostocaceae).[11]



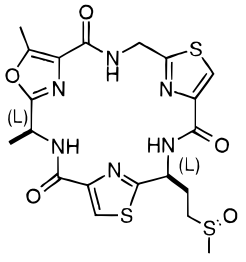
Tenuecyclamide A (**1**)



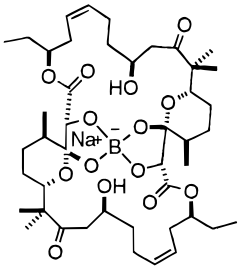
Tenuecyclamide B (**2**)



Tenuecyclamide C (**3**)



Tenuecyclamide D (**4**)



Borophycin (**5**)

Results and Discussion

N. spongiaeforme var. *tenue* was isolated from a litophytic sample collected in the Volcani Center, Bet Dagan, Israel. A pure clonal strain (TAU strain IL-184-6) was mass cultured in our laboratory. The MeOH extract of the freeze-dried cyanobacterium exhibited antimicrobial activity (against *Bacillus subtilis* and *Staphylococcus aureus*) and inhibited the division of embryos[6,12] of the Mediterranean sea urchin, *Paracentrotus lividus*. The crude extract was partitioned between CH_2Cl_2 and water, and the active dichloromethane layer was fractionated using Sephadex LH-20, silica, and C_{18}-HPLC columns to yield five pure compounds. The pure compounds, tenuecyclamides A−D (**1**−**4**), and the known boron-containing antibiotic borophycin (**5**) were isolated in yields of 0.05, 0.01, 0.02, 0.06, and 0.1%, respectively, on the basis of the dry weight of the bacteria. Tenuecyclamide A (**1**) was found to inhibit the division of sea urchin embryos with ED_{100} of 10.8 μM, tenuecyclamide C (**3**) with ED_{100} of 9.0 μM, and tenuecyclamide D (**4**) with ED_{100} of 19.1 μM. Tenuecyclamide B (**2**) was not tested.

Tenuecyclamide A (**1**) was isolated as a white amorphous solid. The molecular formula of **1**, $C_{19}H_{20}N_6O_4S_2$ (HREIMS), indicated 13 double-bond equivalents. Intense absorptions in the IR spectrum were observed at 3394 cm^{-1} (NH stretching vibration of secondary amide), 1667 cm^{-1} (amide-I band), and 1544 cm^{-1} (amide-II band), indicative of a peptide structure. The hexapeptide nature of **1** was deduced from the six nitrogen atoms in the formula and six sp^2 carbon signals in the amide region of the ^{13}C NMR spectrum. The presence of only three amide protons, two singlet aromatic protons, and one aromatic methyl in the ^1H NMR spectrum suggested three cyclically modified amino acids. Three standard amino acid residues (three alanyls) and three modified amino acids (two thiazole and one methyloxazole) were established on the basis of analy-

* To whom correspondence should be addressed. Tel.: 972-3-6408550. Fax: 972-3-6409293. E-mail: carmeli@ccsg.tau.ac.il.

10.1021/np980138j CCC: $15.00
Published on Web 08/15/1998

Table 1. NMR Data and Assignments for Compounds **1** and **2** in $CDCl_3$[a]

tenuecyclamide A (**1**)				tenuecyclamide B (**2**)			
position	δ_C, mult[b]	δ_H, mult, *J* (Hz)	LR H−C corrs[c]	position	δ_C, mult[b]	δ_H, mult, *J* (Hz)	LR H−C corrs[c]
1	160.70 s		4, 18, NH(3)	1	160.56 s		4, 18, NH(3)
2	128.65 s		4	2	128.43 s		4
3	153.63 s		4	3	153.99 s		4
4	11.60 q	2.70 s		4	11.65 q	2.69 s	
5	161.89 s		6, 7, NH(1)	5	161.65 s		6, 7, NH(1)
6	44.89 d	5.14 dq 6.2, 6.7	7, NH(1)	6	44.27 d	5.23 dq 6.7, 6.7	7
7	20.85 q	1.70 d 6.7	6	7	20.84 q	1.69 d 6.7	6
NH(1)		8.67 d 6.2		NH(1)		8.67 d 6.7	
8	160.05 s		6, 10, NH(1)	8	159.85 s		6, 10, NH(1)
9	148.81 s		10	9	148.94 s		10
10	123.91 d	8.16 s		10	123.66 d	8.14 s	
11	171.39 s		10, 12, 13, NH(2)	11	171.00 s		10, 12, 13, NH(2)
12	48.34 d	5.59 dq 6.4, 6.8	13, NH(2)	12	47.36 d	5.67 dq 7.8, 6.7	13
13	24.87 q	1.71 d 6.8	12	13	25.02 q	1.72 d 6.7	12
NH(2)		8.60 d 6.4		NH(2)		8.59 d 7.8	
14	159.98 s		12, 16, NH(2)	14	159.55 s		12, 16, NH(2)
15	148.66 s		16	15	148.50 s		16
16	124.97 d	8.24 s		16	124.41 d	8.18 s	
17	172.21 s		16, 18, 19, NH(3)	17	171.47 s		16, 18, 19, NH(3)
18	48.06 d	5.36 dq 6.0, 6.7	19	18	47.60 d	5.40 dq 6.6, 6.7	19
19	23.70 q	1.79 d 6.6	18	19	24.96 q	1.72 d 6.7	18
NH(3)		8.68 d 6.0		NH(3)		8.69 d 6.6	

[a] Carried out on an ARX-500 Bruker instrument. [b] Multiplicity and assignment from HMQC experiment. [c] Determined from HMBC experiment, $^nJ_{CH} = 8$ Hz, recycle time 1 s.

ses of ^1H, ^{13}C, COSY, HMQC, and HMBC spectra (Table 1). The two thiazole-alanine units could be established on the basis of the long-range H−C correlation between the aromatic protons (H-10 and H-16) and the neighboring carbons. The methyloxazole-alanyl unit was suggested on the basis of the long-range correlations of the aromatic methyl protons with C-1 to C-3 and of the proton and carbon NMR chemical shifts that are in good agreement with the values reported for the same units in dendroamides, A and B.[5] The three units could be sequenced to the total gross structure through the HMBC correlations of the alanyl CH and NH signals of one unit to the amide carbonyl of the neighboring unit (see Table 1).

Tenuecyclamide B (**2**) also had a molecular formula of $C_{19}H_{20}N_6O_4S_2$ on the basis of HREIMS data. The electron-impact mass spectrum exhibited some important fragment ions (*m*/*z* 138, 139) consistent with the presence of thiazole residues.[5] The IR amide absorptions of tenuecyclamide B were similar to those of tenuecyclamide A (1665 and 1541 cm^{-1}). The ^1H and ^{13}C NMR data for tenuecyclamide B, as well as the HMBC and HMQC correlations, are given in Table 1. The proton and carbon chemical shifts agree well with the values found for tenuecyclamide A, apart from the three double-quartet proton signals (H-6, H-12, and H-18), which are shifted slightly downfield. Therefore, we conclude that tenuecyclamide B contains the same three units as in tenuecyclamide A, differing in the stereochemistry of at least one of the alanine residues.

Tenuecyclamide C (**3**) had a molecular formula of $C_{20}H_{22}N_6O_4S_3$ on the basis of HREIMS data. The electron-impact mass spectrum showed some important fragment ions (*m*/*z* 136, 138) consistent with the presence of thiazole- and methyloxazole-containing residues.[5] In addition, the EIMS spectrum showed fragment ions at $[M - 48]^+$ (loss of MeSH) and $[M - 74]^+$ (loss of CH_2═CHSMe), indicative of a methionine residue. Three standard amino acid residues (glycine, alanine, and methionine) and three modified amino acids (two thiazole and one methyloxazole) were established on the basis of the analysis of ^1H, ^{13}C, COSY, HMQC, and HMBC spectra (Table 2). The thiazole-glycine and thiazole-methionine units were established on the basis of the long-range H−C correlation between the aromatic protons (H-10 and H-18, respectively) and the neighboring carbons. The chemical shifts of the thiazole-glycine unit coincide with values reported for the same unit in nostocyclamide.[4] The chemical shifts of the thiazole-methionine unit were found to be similar to those reported for dendroamide B.[5] The methyloxazole-alanyl unit is proposed on the basis of the long-range correlations of the aromatic methyl protons with C-1, C-2, and C-3 and the proton and carbon NMR chemical shifts that agree well with the values reported for the same units in the dendroamides[5] and nostocyclamide.[4] The $^1H-^{13}C$ HMBC correlations (see Table 2), especially NH(3) with C-1, NH-(1) with C-8, and NH(2) with C-16, assisted us in sequencing the three units into a cyclic hexapeptide structure.

Tenuecyclamide D (**4**) possesses a molecular formula of $C_{20}H_{22}N_6O_5S_3$ on the basis of HREIMS data. In addition to the amide bands, the IR spectrum of tenuecyclamide D displayed a strong absorption at 1046 cm^{-1} (S═O stretching vibration of sulfoxide). The presence of the sulfoxide group was supported by the electron-impact mass spectrum, which showed intense fragment ions, $[M - 64]^+$ (loss of CH_3SOH) and $[M - 90]^+$ (loss of CH_2═CHSOMe). The electron-impact mass spectrum also showed some important fragment ions (*m*/*z* 136, 138) consistent with the presence of thiazole- and methyloxazole-containing residues. The $^1H-^{13}C$ HMBC correlations strongly suggested the existence of methionine sulfoxide instead of a methionine amino acid unit in tenuecyclamide C. The changes in the chemical shifts of the relevant proton and carbon signals match those expected from oxidation of the methionine sulfur in **3** to sulfoxide in **4**. The proton and carbon signals assigned to positions 14 and 15 in **4** are downfield shifted relative to those of **3**, and carbon 13 is upfield shifted while the protons attached to it are downfield shifted. The ^1H and ^{13}C NMR data for tenuecyclamide D, including the HMBC and HMQC correlations (see Table 2), fully support the proposed structure **4**, which was also verified by comparison of the NMR chemical shifts of tenuecyclamide D with those of dendroamide C.[5] The spontaneous oxidation of tenuecyclamide C to tenuecyclamide D in solution ($CDCl_3$, overnight in the NMR tube) further support these findings.

Table 2. NMR Data and Assignments for Compounds **3** and **4** in $CDCl_3$[a]

tenuecyclamide C (**3**)				tenuecyclamide D (**4**)[d]			
position	δ_C, mult[b]	δ_H, mult, *J* (Hz)	LR H−C corrs[c]	position	δ_C, mult[b]	δ_H, mult, *J* (Hz)	LR H−C corrs[c]
1	160.86 s		4, 20, 20′, NH(3)	1	160.82 s		4, 20, 20′, NH(3)
2	128.29 s		4	2	128.28 s		4
3	153.83 s		4	3	153.68 s		4
4	11.54 q	2.69 s		4	11.51 q	2.68 s	
5	161.55 s		6, 7, NH(1)	5	161.55 s		6, 7, NH(1)
6	44.99 d	5.14 dq 6.0, 6.7	7, NH(1)	6	44.97 d	5.14 dq 5.1, 6.6	7, NH(1)
7	20.18 q	1.68 d 6.7	6	7	20.16 q	1.68 d 6.6	6
NH(1)		8.62 d 6.0		NH(1)		8.58 d	
8	159.99 s		6, 10, NH(1)	8	159.77 s		6, 10, NH(1)
9	149.05 s		10	9	149.17 s		10
10	124.20 d	8.18 s		10	124.69 d	8.215 s	
					124.61 d[e]	8.209 s[e]	
11	169.30 s		10, 12, 13, 13′, NH(2)	11	168.70 s		10, 12, 13, 13′, NH(2)
					168.62 s[e]		
12	51.00 d	5.79 ddd 7.3, 5.4, 4.9	13, 13′, 14, 14′, NH(2)	12	50.60 d	5.79 dt 7.3, 5.4, 4.9	13, 13′, 14, 14′, NH(2)
					50.81 d[e]		
13	37.33 t	2.19 ddt 14.1, 10.0, 4.9	12, 14, 14′	13	31.84 t	2.80 m	12, 14, 14′
						2.32 m	
		2.50 dddd 14.1, 10.1, 5.4, 5.0			31.01 t[e]	2.80 m[e]	
						2.31 m[e]	
14	28.96 t	2.33 ddd 13.0, 10.0, 4.9	12, 13, 13′, 15	14	49.77 t	2.83 m	12, 13, 13′, 15
						2.55 m	
		2.60 ddd 13.0, 10.1, 5.0			49.15 t[e]	2.83 m[e]	
						2.65 m[e]	
15	15.38 q	2.07 s	14, 14′	15	38.83 q	2.52 s	14, 14′
					38.48 q[e]	2.51 s[e]	
NH(2)		8.61 d 7.3		NH(2)		8.62 d 7.3	
						8.63 d 7.4[e]	
16	159.73 s		12, 18, NH(2)	16	160.10 s		12, 18, NH(2)
17	148.69 s		18	17	148.39 s		18
18	124.59 d	8.20 s		18	124.9 d	8.20 s	
19	165.30 s		18, 20, 20′, NH(3)	19	165.54 s		18, 20, 20′, NH(3)
20	41.00 t	4.81 dd 3.2, 17.8		20	40.96 t	4.79 dd 3.2, 17.8	18
		4.97 dd 4.7, 17.8				4.97 dd 4.7, 17.8	
NH(3)		8.51 dd 3.2, 4.7		NH(3)		8.47 dd 3.2, 4.7	

[a] Carried out on an ARX-500 Bruker instrument. [b] Multiplicity and assignment from HMQC experiment. [c] Determined from HMBC experiment, ${}^nJ_{CH} = 8$ Hz, recycle time 1 s. [d] A 5:1 mixture of *R* and *S* sulfoxides. [e] Signals of minor sulfoxide isomer.

To determine the absolute configuration of the alanine amino acids in tenuecyclamides A (**1**) and B (**2**), both compounds were subjected to acid hydrolysis (6 N HCl, 120 °C), and the acid hydrolysates were separated by HPLC and analyzed using Marfey's method.[13] The oxazole rings were cleaved by the hydrolytic conditions to generate the L-alanine in tenuecyclamides A (**1**) and B (**2**) while the thiazole rings remained intact. The configurations of the other two alanine units in both peptides remain unsolved even after ozonolysis, hydrolysis, and coupling with Marfey's reagent, since each compound gave a 1:2 mixture of D- and L-alanine. Therefore, both compounds contain one D- and two L-alanine units, but the stereochemistry of positions 12 and 18 could not be determined. Using the same method, the stereochemistry of the amino acids of tenuecyclamide C (**3**) were found to be L-alanine and L-methionine, and those of tenuecyclamide D (**4**), L-alanine and L-methionine sulfoxide. It is important to notice that under the ozonolysis conditions the methionine sulfoxide oxidized to methionine sulfone.

Experimental Section

Instrumentation. IR spectra were recorded on a Nicolet FTIR in $CHCl_3$ or neat. High-resolution MS were recorded on a Fisons VG AutoSpecQ M 250 instrument. UV spectra were recorded on a Kontron 931 plus spectrophotometer. NMR spectra were recorded on a Bruker ARX-500 spectrometer at 500.136 MHz for ^1H and 125.76 MHz for ^{13}C. ^1H, ^{13}C, DEPT, COSY-45, HMQC, and HMBC spectra were recorded using standard Bruker pulse sequences. HPLC separations were performed on an ISCO HPLC system (model 2350 pump and model 2360 gradient programmer) equipped with an Applied Biosystem Inc. diode-array detector.

Culture Conditions. An edaphic form of *N. spongiaeforme* var. *tenue* designated Tel Aviv University (TAU) strain no. IL-184-6 was isolated from a soil sample collected in Bet Dagan, Israel. A clonal strain was purified on BG-11 agar medium.[14] The cyanobacterium was cultured in 20 L glass bottles containing a BG-11 medium.[14] Cultures were illuminated continuously at an intensity of 100 μein/M^2/s from fluorescent tubes and aerated with 0.5% CO_2 in air (1 L/min) at an incubation temperature of 25 °C for 30−35 days. Yields of lyophilized cells typically ranged from 0.7 to 1.0 g/L of culture.

Isolation. Freeze-dried cyanobacterial cells (58 g) were extracted with MeOH (3 × 400 mL) overnight. The filtered extracts were combined and concentrated under reduced pressure. The crude extract was partitioned between CH_2Cl_2 and water. The lipophilic layer (2.3 g) was applied to a Sephadex LH-20 column (i.d. × h, 5 × 40 cm) eluted with 1:1 $CHCl_3$/MeOH (16 fractions of 15 mL). Fractions 8−14 were combined according to their antibacterial activity (*B. subtilis* and *S. aureus*) and cytotoxicity to fertilized sea urchin eggs. The combined fraction (0.8 g) was chromatographed over a silica 60H flash column (i.d. × l, 3 × 10 cm) employing a step gradient from hexane to EtOAc to MeOH (each fraction of 60 mL). The 12 fractions were separated qualitatively by TLC (Silica, 1:19 MeOH−$CHCl_3$). The combined fractions 5−7 (antimicrobial and cytotoxic) were further chromatographed isocratically (1:1 hexane/EtOAc) on a similar silica column to afford pure borophycin (**5**, 58.5 mg). Fractions 9−11 gave few deep violet spots under UV light but were not stained by vanillin. The combined fractions 9−11 (cytotoxic) were separated by reversed-phase HPLC (Allthec Econosil C_{18}, 10 μm, 250 × 22.5 mm) using 3:1 MeOH/H_2O (flow rate 5.0 mL/min) as eluent to yield four fractions. Fraction 4 was separated

again on a reversed-phase HPLC column (PhaseSep Spherisorb S5ODS1, 5 μm, 250 × 20 mm) using 7:3 CH_3CN/H_2O (flow rate 5.0 mL/min) as eluent. This separation yielded compounds **4** (34.8 mg, t_R 25 min), **3** (11.6 mg, t_R 26 min), and **1** (29.2 mg, t_R 30 min). Fraction 3 was further purified on a reversed-phase HPLC column (Allthec Econosil C_{18}, 10 μm, 250 × 22.5 mm) using 3:1 $MeOH/H_2O$ (flow rate 5.0 mL/min) as eluent to yield compound **2** (5.8 mg, t_R 28.8 min).

Tenuecyclamide A (1): amorphous solid; $[\alpha]^{25}_D$ −8.8° (*c* 2.5, MeOH); UV (MeOH) λ_{max} (ϵ) 243 (24 500) nm; IR ($CHCl_3$) ν_{max} 3394, 3612, 3020, 1667, 1639, 1593, 1544, 1518, 1501, 1213 cm^{-1}; for ^1H and ^{13}C NMR data, see Table 1; EIMS (70 eV) *m/z* (rel intensity) 460 (M^+, 100), 445 (15), 293 (10), 153 (10), 139 (10), 138 (20), 136 (8); HREIMS *m/z* 460.0989 (M^+, calcd for $C_{19}H_{20}N_6O_4S_2$, 460.0987).

Tenuecyclamide B (2): glassy solid; $[\alpha]^{25}_D$ −36.0° (*c* 0.5, MeOH); UV (MeOH) λ_{max} (ϵ) 244 (16 500) nm; IR (neat) ν_{max} 3361, 3020, 1665, 1637, 1541 cm^{-1}; for ^1H and ^{13}C NMR data, see Table 1; EIMS (70 eV) *m/z* (rel intensity) 460 (M^+, 100), 445 (10), 293 (7), 153 (8), 139 (12), 138 (17), 122 (6), 44 (16); HREIMS (70 eV) *m/z* 460.0985 (M^+, calcd for $C_{19}H_{20}N_6O_4S_2$, 460.0987).

Tenuecyclamide C (3): glassy solid; $[\alpha]^{25}_D$ +12.0° (*c* 0.5, MeOH); UV (MeOH) λ_{max} (ϵ) 244 (17 300) nm; IR ($CHCl_3$) 3397, 2964, 1667, 1597, 1543 cm^{-1}; for ^1H and ^{13}C NMR data, see Table 2; EIMS (70 eV) *m/z* (rel intensity) 506 (M^+, 100), 458 (100), 432 (75), 276 (25), 207 (17), 139 (25), 138 (25), 136 (17), 112 (20), 44 (19); HREIMS *m/z* 506.0863 (M^+, calcd for $C_{20}H_{22}N_6O_4S_3$, 506.0864), 458.0814 (calcd for $C_{19}H_{18}N_6O_4S_2$, 458.0830), 432.0663 (calcd for $C_{17}H_{16}N_6O_4S_2$, 432.0674).

Tenuecyclamide D (4): glassy solid; $[\alpha]^{25}_D$ +44.0° (*c* 0.5, MeOH); UV (MeOH) λ_{max} (ϵ) 247 (39 170) nm; IR ($CHCl_3$) ν_{max} 3396, 2977, 1669, 1641, 1542, 1046 cm^{-1}; for ^1H and ^{13}C NMR data, see Table 2; EIMS (70 eV) *m/z* (rel intensity) 522 (M^+, 30), 459 (85), 432 (100), 276 (43), 139 (22), 138 (15), 136 (17), 112 (12), 43 (19); HREIMS *m/z* 522.0817 (M^+, calcd for $C_{20}H_{22}N_6O_5S_3$, 522.0813).

Cytotoxicity Assay. Freshly collected specimens of the sea urchin *P. lividus* were used for this assay. To collect eggs and sperm, a 0.5 M KCl solution (1 mL) was injected into each specimen. The collected sperm and eggs were mixed prior to transferring them into the wells by pipet. Samples were dissolved in EtOH and added to seawater to give an adequate concentration at 1 mL of seawater in a 24-multiwell plate. The final concentration of EtOH was less than 1%. About 100 fertilized eggs were pipetted into each well, which contained sample solution, and the plate was kept at 24 °C. At appropriate periods, embryos were examined under a microscope. The dosage that inhibits 100% of the embryonic devision (ED_{100}) was determined. Crude extracts and fractions from chromatography were tested at a conncetration of 100 and 10 μg/mL. Pure compounds were tested from ca. 100 μg/mL and down by 2-fold dilutions.

Antibacterial Agar-Diffusion Assay.[15] Cultures of bacteria were obtained from stocks maintained at the Department of Plant Protection and Inspection Clinical Laboratory, Ministry of Agriculture, Bet-Dagan, Israel, as follows: *B. subtilis* (strain Bs1091-1) and *S. aureus* (strain Sau1091-3). The bacteria were maintained on agar slants, and subcultures were freshly prepared before use. Inocula were made in soybean-casein digest broth (5 mL) and grown for 24 h at 37 °C. The turbidity of the broth cultures was then adjusted with broth to obtain an optical density of 0.1 at 625 nm (using a spectrophotometer with a 1 cm light path). Freshly prepared and cooled Mueller-Hinton agar (34 g/L, pH 7.4) was poured into 90 mm Petri plates (approximately 20 mL). After the medium had been allowed to cool to room temperature, 100 μL aliqoutes of the various bacteria broth cultures were inoculated onto the plates and swapped evenly over the entire surface of the agar. The plates were then allowed to dry for 5 min before the disks were applied. The disks (6 mm) were impregnated with crude extracts or fractions from chromatography (at concentrations of 100, 10, and 1 μg per disk) and allowed to dry before they were applied to the surface of the inoculated plates (four disks per 90 mm plate) with sterile forceps. The plates were incubated for 24 h at 37 °C, and then zones of inhibition were measured.

Stereochemical Analysis. Portions (0.25 mg) of **1**−**4** in CH_2Cl_2 (1 mL) were ozonized by the addition of cold (−78 °C) ozone-saturated (blue) CH_2Cl_2 solution (1 mL). The reaction mixture was allowed to reach room temperature before the solvent was removed, and the residue was dissolved in 6 N HCl. It was then placed in a sealed glass bomb at 104 °C for 20 h. In another experiment, 0.25-mg portions of **1** and **2** were dissolved in 6 N HCl and placed in a sealed glass bomb at 104 °C for 20 h. After removal of HCl by repeated evaporation in vacuo, the hydrolysate was resuspended in water (200 μL) and derivatized with (1-fluoro-2,4-dinitrophenyl)-5-L-alanine amide (FDAA).[13] The derivatized amino acids (AA) from hydrolyzates were compared with similarly derivatized standard AA by HPLC analysis: Alltech C_{18} Econosphere, 5 μm, 4 × 250 mm, flow rate: 1 mL/min, UV detection at 340 nm, linear gradient elution from 9:1 50 mM triethylammonium phosphate (TEAP) buffer (pH 3)/CH_3CN to 1:1 TEAP/CH_3CN within 40 min. Retention times of the derivatized AA were L-Ala, 36.0 min; D-Ala, 39.4 min; L-Met, 35.8 min; D-Met, 42.2 min; L-Met-sulfone, 29.5 min, and D-Met-sulfone, 34.5 min. HPLC analysis of derivatized hydrolyzates of **1** and **2** established L-Ala, while that of derivatized hydrolyzates of ozonized **1** and **2** established 2 × L-Ala and D-Ala. HPLC analysis of derivatized hydrolyzates of ozonized **3** established L-Ala and L-Met and that of **4**, L-Ala and L-Met-sulfone.

Acknowledgment. We wish to thank Mrs. Lilach Eliass for running the antimicrobial assays and Mr. Shimon Hauftman for measuring the mass spectra.

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NP980138J