Mollamides B and C, Cyclic Hexapeptides from the Indonesian Tunicate *Didemnum molle*

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Two new cyclic hexapeptides, mollamides B (**1**) and C (**2**), were isolated from the Indonesian tunicate *Didemnum molle* along with the known peptide keenamide A (**3**). The structures were established using 1D and 2D NMR experiments. The relative configuration of mollamide B at the thiazoline moiety was determined using molecular modeling coupled with NMR-derived restraints. Their absolute configuration was determined using Marfey's method. The new peptides have been evaluated for their antimicrobial, antimalarial, anticancer, anti-HIV-1, anti-Mtb, and anti-inflammatory activities. Keenamide A and mollamide B show cytotoxicity against several cancer cell lines.

The ascidians have become one of the most important sources of marine natural products in the last decades, and a significant number of compounds with unusual structures and bioactivities have been isolated from various ascidians. The peptide metabolite didemnin B is an example of an important drug lead isolated from *Trididemnum solidum*, a Caribbean tunicate of the family Didemnidae.[1] Didemnin B was the first marine natural product to enter phase I and II clinical trials as an anticancer agent.[1] The genus *Didemnum* is an extremely productive source of bioactive cyclic peptides characterized by the presence of modified amino acids. Enzymatic heterocyclization of serine, threonine, and cysteine side chains with vicinal carbonyl groups results in the formation of five-ring heterocyclic oxazole and thiazole moieties.[2–4] Peptides with this functionality exhibit a diverse array of bioactivities. Thiazole-, oxazole-, and thiazoline-containing peptides have been isolated from the common Indo-Pacific ascidian *Didemnum molle* from many different geographic locations, and they share the peculiar reverse prenylated ethers of serine and threonine amino acids. They include the heptapeptides mollamide,[5] cyclodidemnamides,[6,7] and myotamides A and B[8] and the hexapeptides comoramides A and B[8] and didmolamides A and B.[9] As the chemical composition of this genus changes from one collection to another, Müller et al. proposed that these metabolites are actually biosynthesized by symbiotic prochlorophytes, and they isolated prokaryotic algal symbionts often associated with the didemnid family.[10] Furthermore, Schmidt et al. reported the full sequence and functional expression of the related peptides, patellamides, from the obligate symbiont *Prochloron didemni*.[11] Recently, Donia et al. showed that *Prochloron* spp. generate a diverse library of patellamides, and they used this information to engineer the production of a new cyclic peptide in *Escherichia coli*.[12]

Results and Discussion

An investigation of the tunicate *D. molle* collected from Manado Bay, Indonesia, yielded two new cyclic hexapeptides, mollamides B (**1**) and C (**2**), along with the known keenamide A (**3**), which was previously reported from the notaspidean mollusk *Pleurobranchus forskalii* collected from Manado, Indonesia. This mollusk was shown to feed on the ascidian *D. molle* on the basis of the analysis of the gut content.[13] All three peptides share the peculiar reverse prenylated ethers of serine and threonine amino acids.

The freeze-dried invertebrate was extracted with water to remove the foaming aqueous-soluble polysaccharides. The dried residue

1

2

3
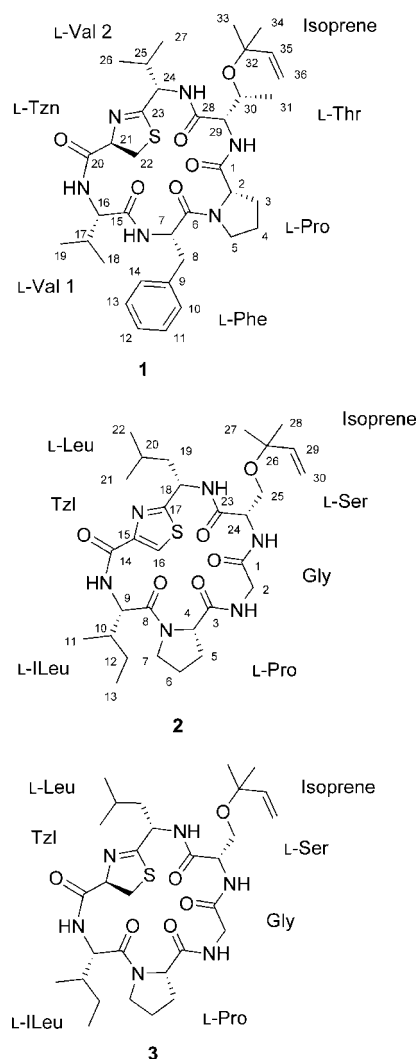
and water extracts were exhaustively extracted separately with dichloromethane and then ethanol. The dichloromethane and the ethanol extracts were combined on the basis of ^{1}H NMR analysis and were subjected to silica gel vacuum-liquid chromatography followed by reversed-phase and amino normal-phase HPLC to yield the two new cyclic hexapeptides mollamides B (**1**) and C (**2**) and the known peptide keenamide A (**3**).

Mollamide B (**1**) was obtained as a light yellow powder, and HRESIMS gave an [M + H]$^+$ peak at 697.3713 for a molecular formula of $C_{36}H_{53}N_6O_6S$ (calc 697.3747, Δ −3.4 mmu, mmu =

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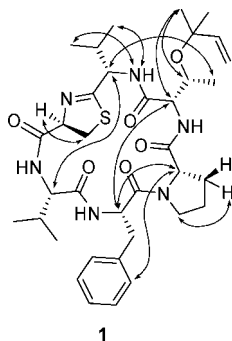† Department of Pharmacognosy.
‡ Department of Medicinal Chemistry.

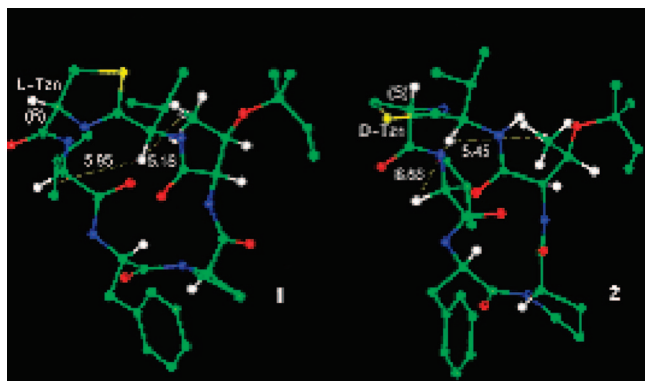**Figure 1.** Key ROESY correlations for mollamide B (**1**).

Figure 2. Energy-minimized conformations of MolB-*R* (**1**) and MolB-*S* (**2**).

millimass unit), requiring 14 degrees of unsaturation. The peptidic nature of the compound was suggested by the IR, and ^{1}H and ^{13}C NMR spectra; four amide protons were observed at δ 7.00, 7.61, 7.86, and 9.14 ppm, and six ^{13}C NMR signals resonated between δ 168 and 175 ppm, corresponding to amide carbonyls and indicating the presence of six amino acids. In addition, the IR spectrum showed bands at 3256, 3356, and 1683 cm^{-1}, confirming the presence of an amide functionality. The compound was negative to ninhydrin, suggesting a blocked N-terminus or a cyclic peptide.

The structure of mollamide B (**1**) was determined using both one- and two-dimensional NMR spectral measurements including COSY, HMQC, HMBC, and ROESY experiments. Six partial structures were constructed that were assigned as one phenylalanine, two valine units, one threonine carrying a dimethylallyl ether group, one proline unit, and one thiazoline unit. The ether linkage between the isoprene moiety and the threonine amino acid was assigned on the basis of the HMBC correlation between the β-proton of threonine resonating at δ 3.57 ppm and the quaternary carbon of the isoprene group resonating at δ 78.0 ppm.

The assignment of the thiazoline residue was accomplished using HMQC, HMBC, COSY, and HRESIMS spectrometric techniques. The methylene protons (δ 4.03, 3.46) showed HMBC correlations to the carbonyl signal at δ 174.5, which was assigned as part of the valine 2 residue, and to the carbonyl signal at δ 170.8. The same protons also showed COSY correlations to a methine proton at δ 5.13, which provides a strong HMBC correlation to the valine 2 carbonyl (δ 174.5). The *cis/trans* configuration of Pro was determined to be *cis* on the basis of the $\Delta\delta_{\beta\gamma}$ (differential value of ^{13}C chemical shifts of C_β and C_γ in Pro)[14] value of 8 ppm.

The amino acid sequence and the connection of amino acid residues of **1** were accomplished by long-range ^{1}H$-^{13}$C correlation experiments (HMBC) and ROESY. The NH proton (δ 9.10) and α-proton (δ 4.80) signals of threonine provide HMBC correlations to the carbonyl (δ 171.1) signal of proline. The NH proton (δ 7.80) and α-proton (δ 4.37) signals of valine 2 showed HMBC correlations to the threonine carbonyl resonance (δ 168.9). The NH

resonating at δ 7.00 and belonging to the valine 1 residue correlated with the thiazoline carbonyl carbon (δ 170.8) in the HMBC spectrum connecting both residues. The NH proton signal (δ 7.60) of the phenylalanine residue showed an HMBC correlation to the valine 1 carbonyl signal (δ 170.5). The connection between the proline and the phenylalanine amino acid residues was established by ROESY correlations between the α-proton of phenylalanine (δ 4.50) and the α-proton of proline (δ 3.44) and from the aromatic protons of phenylalanine to the α-proton of proline.

The absolute configuration of mollamide B (**1**) was determined by acid hydrolysis and derivatization with Marfey's reagent[15] followed by comparative HPLC analysis with derivatized standard D- and L-amino acids. The analysis established the L-configuration for each amino acid residue (L-valine, L-phenylalanine, L-proline, and L-threonine). A minor peak was also observed for D-valine, which resulted from partial racemization of the L-valine from the L-valinylthiazoline moiety. It has been reported that the thiazoline-based amino acid units in cyclic peptides tend to epimerize easily during hydrolysis.[16,17] The configuration at the thiazoline amino acid moiety using traditional degradation and derivatization techniques remains a challenge. During acid hydrolysis, the thiazoline moiety hydrolyzes to cysteine, which then incompletely derivatizes by FDAA (N-alkylation, S-arylation, formation of bis-derivative, and oxidation to cystine), leading to unpredictable results.[16] The synthesis of these compounds has historically been the only stereochemical solution to these moieties, but it is time-consuming and costly.

Due to the inherent limitations of experimental data to assign the absolute configuration of functional groups such as these, molecular modeling coupled with NMR-derived restraint information has exhibited significant potential for assigning relative and absolute configuration of many natural products and pharmaceutically relevant compounds.[18–20] A similar approach has been used to assign the unknown configuration at the α-carbon of the thiazoline moiety of mollamide B. Molecular modeling was utilized to provide a reasonable prediction of configuration at the α-carbon of the thiazoline moiety based on conformational changes to the molecule associated with D- or L-configuration at this center. Using the ROESY data as distance restraints (Figure 1), molecular mechanics calculations involving stepwise minimization followed by restrained simulated annealing were performed to generate an ensemble of conformations for the two possible configurations at the α-carbon (D-Tzn (*S*): MolB-*S*; L-Tzn (*R*): MolB-*R*). Additionally, since molecular mechanics energies are empirical in nature, more accurate semiempirical quantum mechanical calculations were performed to calculate the heats of formation (ΔH_f) for the lowest energy conformations. MolB-*R* appeared to be more stable than MolB-*S* by 8.5 kcal/mol. Also, MolB-*R* was found to be consistent with NMR-derived distance restraints, while MolB-*S* showed two significant violations (Figure 2). Thus, the configuration at the α-carbon of the thiazoline moiety was assigned to be *R*, corresponding to the L-amino acid configuration.

The minor component mollamide C (**2**) was obtained as a white solid. The HRESIMS showed an [M + H]$^+$ 619.3259 (calc 619.3277, Δ −1.8 mmu) peak, indicating a molecular formula of $C_{30}H_{46}N_6O_6S$, which accounts for 15 degrees of unsaturation and one more additional unsaturation unit than the known peptide keenamide A (**3**). The ^{1}H NMR spectrum showed a comparable spectrum to keenamide A (**3**). Three ^{1}H NMR resonances are lacking (δ 5.0 (d), 3.60, and 3.70 (m)) corresponding to the thiazoline unit, and instead an additional singlet peak at δ 7.9 ppm appears. Two additional aromatic carbon resonances (δ 122.4, 149.2 ppm) were observed, indicating the presence of an aromatic ring system. Analysis of HMBC data revealed that the aromatic singlet proton resonating at δ 7.9 ppm correlates to the leucine carbonyl carbon (δ 170.7 ppm) and to a quaternary aromatic carbon (δ 149.2 ppm). The same proton showed a strong correlation to the carbonyl

Table 1. NMR Spectroscopic Data for Mollamide B (**1**)[a,b]

amino acid	position	δ_C, mult.	δ_H (J in Hz)	HMBC[c]
proline	1	171.1, qC		
	2	61.2, CH	3.44, m	1, 3, 4, 5
	3	29.8, CH$_2$	2.1, 0.75, dd (6.0, 12.0)	1, 2, 4, 5
	4	21.8, CH$_2$	1.6, m	2, 3
	5	46.2, CH$_2$	3.3, 3.48, t (10.0)	3, 4
phenylalanine	6	170.1, qC		
	7	54.7, CH	4.5, t (7.2)	8
	8	37.6, CH$_2$	3.0, m	6, 7, 9, 10
	9	135.1, qC		
	10	129.5, CH	7.1, dd (1.2, 7.6)	8, 12, 14
	11	129.0, CH	7.2, m	9, 13
	12	127.6, CH	7.2, m	
	13	129.0, CH	7.2, m	9, 11
	14	129.5, CH	7.2, m	8, 10, 12
	NH		7.6, d	15
valine 1	15	170.5, qC		
	16	57.9, CH	4.18, t (7.6)	17, 18, 15
	17	32.4, CH	2.0, m	18, 19, 16, 15
	18	17.8, CH$_3$	0.97, m	16, 17
	19	18.7, CH$_3$	0.97, m	16, 17
	NH		7.0, d (7.6)	16, 20
thiazoline	20	170.8, qC		
	21	78.0, CH	5.13, m	20, 23
	22	34.0, CH$_2$	4.03, 3.46, dd (4.4, 11.2)	20, 21, 23
valine 2	23	174.5, qC		
	24	58.0, CH	4.37, dd (3.2, 6.0)	23, 25, 26, 27, 28,
	25	30.6, CH	2.27, m	26, 27
	26	16.1, CH$_3$	0.93, d (7.2)	24, 25, 27
	27	20.2, CH$_3$	1.0, d (6.8)	24, 25, 26
	NH		7.8, d (6.4)	24, 25, 28
threonine	28	168.9, qC		
	29	56.1, CH	4.8, dd (4.0, 9.6)	31, 30, 28
	30	70.1, CH	3.57, m	31, 29, 32, 28
	31	17.8, CH$_3$	1.43, d (6.8)	29, 30
	NH		9.1, d (9.6)	1
isoprene	32	78.0, qC		
	33	27.0, CH$_3$	1.32, s	34, 32, 35
	34	25.9, CH$_3$	1.37, s	33, 32, 35
	35	142.4, CH	5.8, dd (10.8, 17.6)	34, 33, 32
	36	115.3, CH$_2$	5.1, m	32, 35

[a] ^{1}H and ^{13}C NMR data were measured at 400 and 100 MHz, respectively. [b] All spectra were measured in CDCl$_3$ and referenced to the residual solvent signal at δ_H 7.26 and δ_C 77.4. [c] HMBC correlations are from the proton(s) stated to the indicated carbons.

carbon forming the amide bond with the isoleucine amino acid, supporting the presence of the thiazole-modified amino acid. Careful analysis of COSY, HMQC, and HMBC NMR data indicated that the rest of the molecule is identical to keenamide A. $\Delta\delta_{\beta\gamma}$ of the proline in mollamide C was calculated to be 2.8 ppm, showing that the proline amide bond is *trans*, which is the same as in keenamide A (**3**) ($\Delta\delta_{\beta\gamma}$ 3.1 ppm).

The absolute configuration of the thiazole-containing peptide mollamide C (**2**) was determined by the procedure described by McDonald and Ireland.[21] This procedure uses ozone to destroy the aromaticity of the thiazole in order to facilitate hydrolysis and prevent racemization. The analysis established the L-configuration of each amino acid residue (L-isoleucine, L-leucine, L-serine, and L-proline).

Mollamide B (**1**) and keenamide were examined for antimicrobial activity against MRSA, *Mycobacterium intracellulaire*, *Candida albicans*, *C. glabrata*, *C. krusei*, and *Cryptococcus neoformans* and exhibited no activity.

Mollamides B and C as well as keenamide have shown no anti-inflammatory activity in rat neonatal microglia, and neither of them inhibited cyclooxygenase enzyme (COX-2) activity in a cell-based assay.

Mollamide B (**1**) exhibited moderate antimalarial activity against *Plasmodium falciparum* (D6 clone and W2 clone), with IC$_{50}$ values of 2.0 and 2.1 μg/mL, respectively. Mollamide B also exhibited marginal activity against *Leishmania donovani*, with IC$_{50}$ and IC$_{90}$ values of 18 and 35 μg/mL, respectively, and against HIV-1 in human PBM cells with an EC$_{50}$ value of 48.7 μM *in vitro*.

Mollamide B (**1**) has been tested at 100 μM against four cancer cell lines: the non-small cell lung cancer cell line H460, the breast cancer cell line MCF7, and the CNS cancer cell line SF-268. Mollamide B showed significant percentage growth inhibition. The percentage growth was H460 (29), MCF7 (44), and SF-268 (42), which allowed the compound to be evaluated by the National Cancer Institute (NCI) in the 60-cell-line panel. No cell lines showed any sensitivity to mollamide B that exceeded the mean.

Mollamide C was tested in an *in vitro* disk diffusion assay that defines the differential cell killing among nine cell types examined. These are two leukemias (murine L1210 and human CCRF-CEM), five solid tumors (murine colon 38, human colon HCT-116, human lung H125, human breast MCF-7, and human prostate LNCaP), and a murine and human normal cell (hematopoietic progenitor cell, CFU-GM). The assay is designed to determine large differences in the relative sensitivity of solid tumors, normal cells, and leukemias. The greater the zone differential in the solid tumor colonies and only minor toxicity for both the leukemia and normal cells, the higher the selectivity of the compound to solid tumors. Mollamide C showed a unit zone differential value of 100 against L1210, human colon HCT-116, and human lung H125 and a value of 250 against murine colon 38 and was not considered to be solid tumor selective.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a JASCO DIP-310 digital polarimeter. IR and UV spectra were obtained using an AATI Mattson Genesis Series FTIR and a Perkin-

Table 2. NMR Spectroscopic Data for Mollamide C (**2**)[a,b]

amino acid	position	δ_C, mult.	δ_H (J in Hz)	HMBC[c]
glycine	1	169.0, qC		
	2	43.2, CH_2	3.5, d (3.6), 4.6, d (10.2)	1, 3
	NH		6.7, d (9.6)	
proline	3	170.8, qC		
	4	63.4, CH	4.0, dd (6.0,10.2)	5
	5	28.9, CH_2	2.1, dt (6.6), 2.0, m	3, 4, 6, 7
	6	26.1, CH_2	1.8, m	
	7	47.4, CH_2	3.6 (9.0, 11.4), 3.7 (9.6)	4, 5
isoleucine	8	161.8, qC		
	9	57.0, CH	4.5 (3.0, 10.2)	8, 10, 11, 13
	10	36.9, CH	1.9, m	11
	11	23.1, CH_2	1.3, m	
	12	11.9, CH_3	0.8, t (7.2)	10, 11
	13	16.7, CH_3	1.0, d (7.2)	9, 10, 11
	NH		8.9, d (8.4)	14
thiazole	14	171.6, qC		
	15	149.2, qC		
	16	122.4, CH	7.9, s	14, 15, 17
leucine	17	170.7, qC		
	18	50.6, CH	5.2, ddd, (4.2, 8.4, 15.0)	19, 23
	19	44.5, CH_2	1.8, dd (4.8, 9.0)	17, 18, 21
	20	26.4, CH	1.9, m	19, 21, 22
	21	21.4, CH_3	1.0, d	19, 20, 22
	22	23.6, CH_3	1.0, d (6.6)	19, 20
	NH		8.1, d (7.8)	18, 23
serine	23	171.7, qC		
	24	51.1, CH	4.9, ddd (3.6, 7.2, 9.3)	1, 23
	25	62.9, CH_2	3.4, 3.5 dd, (4.2, 9.0)	23, 26
	NH		8.5, d (9.6)	1
isoprene	26	77.4		
	27	25.6	1.2, s	28, 26, 29
	28	25.3	1.3, s	26, 27, 29
	29	142.4	5.7 dd, (10.8, 17.4)	26
	30	115.2	5.07 d, (10.8), 5.1 (17.4)	26, 29

[a] ^{1}H and ^{13}C NMR were recorded at 600 and 150 MHz, respectively. [b] All spectra were measured in $CDCl_3$ and referenced to the residual solvent signal at δ_H 7.26 and δ_C 77.4. [c] HMBC correlations are from the proton(s) stated to the indicated carbons.

Elmer Lambda 3B UV/vis spectrometer. The ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ using Bruker spectrometers operating at 400 or 600 MHz for ^{1}H and 100 or 150 MHz for ^{13}C NMR. Chemical shifts are reported in parts per million (ppm), and coupling constants (J) in Hz. The ESI-FTMS was acquired on a Bruker-Magnex BioAPEX 30es ion cyclotron Fourier transform mass spectrometer by direct injection into an electrospray interface. Silica gel (200−400 mesh) and alumina (63−200 μm) were obtained from Natland International Corporation and Scientific Adsorbents Incorporation, respectively. TLC analyses were carried out using precoated silica gel G_{254} or aluminum oxide Alox-100 UV_{254} 500 μM. HPLC were performed on a Waters 510 model system and Agilent 1100-Bruker microTOF system.

Animal Collection and Taxonomy. The tunicate was collected at 10−20 m depth from Manado Bay, Indonesia, on September 28, 2003, where it was extraordinarily abundant. The tunicate is massively encrusting with a tough and elastic texture. In life, the external color of the tunicate is grayish green. The animal was easily identified as *Didemnum molle* (Herdman, 1886), the most commonly encountered sea squirt in much of the Indo-Pacific.

Extraction and Isolation. Six kilograms of *D. molle* was collected in Manado Bay, Indonesia, in 2003. The 450 g dry wt of tunicate was extracted with H_2O. The H_2O extract was exhaustively partitioned between H_2O and CH_2Cl_2. The animal residue was extracted with EtOH. Both the EtOH and CH_2Cl_2 extracts were combined on the basis of TLC, yielding 19 g of dried extract. The extract was subjected to Si gel VLC using a step gradient elution system of hexanes/EtOAc/MeOH in 25% increments to yield 10 fractions. Fractions 6 and 7 eluted with EtOAc/MeOH (9:1) and EtOAc/MeOH (7.5:2.5), respectively, were enriched with peptides based on ^{1}H NMR analysis. Both fractions were purified separately with reversed-phase HPLC (Phenomenex Prodigy 10 μm, ODS 250 × 50 mm; λ 230 nm) eluting with a gradient system from 20% CH_3CN in H_2O to 100% CH_3CN. Mollamide B (**1**) (40 mg, 8.8 × 10^{-3} % dry wt) was eluted with 2% H_2O in CH_3CN. A mixture of keenamide A and a minor peptide was eluted with 14% H_2O in CH_3CN. The mixture was further purified using a NH_2 HPLC column (Phenomenex Luna 5 μm, 250 × 21.2 mm) and an isocratic elution

system of 20% CH_2Cl_2/hexanes, yielding mollamide C (**2**) (2.0 mg, 4.4 × 10^{-4} % dry wt) and keenamide A (**3**) (40 mg, 8.8 × 10^{-3} % dry wt).

Mollamide B (1): yellow solid; [α]^{25}$_D$ −119.9 (c 0.25, CHCl$_3$); UV (MeOH) λ_{max} (log ϵ) 202 (4.53) nm; IR (KBr film) ν_{max} 3356, 3256, 2966, 2931, 1683, 1650, 1530, 1447 cm^{-1}; ^{1}H NMR and ^{13}C NMR, see Table 1; LRESIMS m/z 697 [M + H]$^+$; HRESIMS m/z [M + H]$^+$ 697.3713 (calcd for $C_{36}H_{53}N_6O_6S$, 697.3747, Δ −3.4 mmu).

Mollamide C (2): white solid; [α]^{25}$_D$ 0 (c 0.1, CHCl$_3$); UV (MeOH) λ_{max} (log ϵ) 200 (5.15); IR (KBr film) ν_{max} 3318, 2959, 2926, 2873, 1729, 1685, 1628, 1541, 1456, 1271, 1140, 1070 cm^{-1}; ^{1}H NMR and ^{13}C NMR, see Table 2; HRESIMS m/z [M + H]$^+$ 619.3259 (calcd for $C_{30}H_{46}N_6O_6S$ 619.3277, Δ 1.8 mmu).

Determination of Absolute Configuration of the Amino Acids in Mollamide B (1). Hydrolysis of **1** (348 μg; 0.5 μmol) was achieved in 1 mL of 6 N HCl in a sealed vial at 100 °C for 20 h. Traces of HCl were removed under nitrogen. The resulting hydrolysate was resuspended in 100 μL of H_2O and derivatized with 20 μL of 10% Nα-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (FDAA) in acetone and 10 μL of 1 N NaHCO$_3$. The mixture was heated at 40 °C for 1 h.[15] The solutions were cooled to room temperature, neutralized with 5 μL of 2 N HCl, and evaporated to dryness.[13] HPLC analyses of the FDAA-derivatized hydrolysate of **1** and **2** and standard FDAA-derivatized amino acids were carried out using a Waters NOVAPAK C$_{18}$ (3.9 × 150 mm column) with a linear gradient of triethylammonium phosphate (50 mM, pH 3.0)/MeCN (90:10 to 60:40) in 60 min at 1 mL/min (UV detection at λ 340 nm). Because L-threonine and L-allo-threonine could not be resolved clearly using this method, they were separated using a Luna C$_5$ column (4.6 × 150 mm, 5 μm) with a gradient of 40 mM ammonium acetate, 70% CH$_3$CN, and 30% MeOH from 98:2 to 66:34 in 85 min at 0.8 mL/min, detected by microTOF, and then analyzed for the extracted ion at m/z 372 for the Marfey's derivative of threonine.

Ozonolysis of Mollamide C (2). A slow stream of O$_3$ was bubbled into a 7 mL CH$_2$Cl$_2$ solution of mollamide C (0.3 mg; 0.5 μmol) in a threaded bomb at 25 °C for approximately 8 min. After removal of the

solvent under a stream of N_2, the residue was subjected to hydrolysis and derivatization as described above with mollamide B. The D/L-configuration for serine, proline, and leucine were assigned using the C_{18} column, and L-isoleucine and L-allo-isoleucine were assigned using the C_5 column as described above. Then the ion at m/z 384 was extracted for the four isoleucine isomers.

The analysis of mollamide B (**1**) using the C_{18} column established the presence of L-Pro (34.47) [D-Pro (40.52)], L-Val (43.47), [D-Val (54.10)], and L-Phe (52.41) [D-Phe (59.86)]. Amino acid standards using LCTOF revealed retention times as follows: L-Thr (36.42 min) [L-allo-Thr (34.94), D-Thr (45.46), D-allo-Thr (41.37)].

The analysis of mollamide C (**2**) using the C_{18} column established the presence of L-Ser (21.56 min) [D-Ser (26.13)], L-Pro (34.07) [D-Pro (40.25)], and L-Leu (51.82) [D-Leu (60.85)]. Amino acid standards using LCTOF revealed retention times as follows: L-Ile (52.63) [L-allo-Ile (52.93), D-Ile (70.42), D-allo-Ile (71.37)]. Due to the close retention time of L-Ile and L-allo-Ile, mollamide C was co-injected with L-Ile and L-allo-Ile standards, which clearly revealed that Ile has the L-configuration. Thus, the analysis of mollamides B (**1**) and C (**2**) established the presence of all L-amino acids.

Molecular Modeling. In order to investigate the chirality at the α-carbon of the thiazoline moiety, two structures of molecule **1** were drawn using the BUILDER module in Insight II (Accelrys Inc., San Diego, CA). All regular amino acids were drawn as *S*, while the α-carbon of the thiazoline moiety was drawn with both *S* and *R* configurations to provide two initial configurations of molecule **1**: MolB-*R* and MolB-*S* (denoting the chirality at the α-carbon of the thiazoline moiety to be L-Tzn and D-Tzn, respectively).

The structures were first optimized using a series of concurrent minimizations employing the CVFF forcefield to a gradient of 0.001. The optimized structures were then subjected to simulated annealing using the DISCOVER (v 2.98) module of Insight II. All the simulations were performed using a distance-dependent dielectric. A forcing potential of 30 kcal/mol/rad² was applied to the omega dihedrals for all amino acids, except for the *cis*-proline, to maintain the *trans* geometry. Also, NMR-derived distance restraints based on ROESY correlations (Figure 1) were introduced during the MD simulations with a force constant of 20 kcal/mol/rad². The molecules were minimized beginning with 300 steps of steepest descents and 1000 steps of conjugate gradients to relieve any strain in the starting structures. The structures were then "heated" gradually from 100 to 1000 K in steps of 100 K with 1500 fs of simulation at each step. At 1000 K, the simulation was further continued for 20 000 fs, during which 20 conformations were sampled at an interval of 1000 fs. The conformations were then gradually "cooled" to 300 K in steps of 100 K with 2500 fs simulation at each step. The structures were finally minimized using 500 steps of steepest descents, 1500 steps of conjugate gradients, and 500 steps of VA09A. At this stage, the NMR-derived distance restraints were removed, and all 20 conformations were again subjected to the same minimization protocol as given above.

The structures were analyzed, and the lowest energy conformation of MolB-*R* obtained without any restraints appeared to maintain important interproton distances in accordance with the NMR data. The structures were then further optimized by semiempirical quantum mechanical calculations. The AM1 Hamiltonian was used to calculate the heat of formation (ΔH_f) using the MOPAC suite of programs (v 6.0). The semiempirical calculations were carried out without any restraints, and all degrees of freedom in the molecule were optimized. Minimization was run with the EF algorithm to a GNORM of 0.01. Since it is well known that most semiempirical methods underestimate the barrier to rotation in peptides, the key word MMOK was used to allow for a molecular mechanics correction. Stable points on the potential energy surface (PES) of the molecule were identified by running a FORCE calculation and were confirmed by the absence of any imaginary (negative) frequencies.

The ΔH_f values were calculated to be −175.2 kcal/mol for MolB-*R* and −166.7 kcal/mol for MolB-*S*. Thus, MolB-*R* appeared to be more stable by 8.5 kcal/mol. Also, significant violations were observed for

two important NMR-derived distance restraints in case of MolB-*S* (Figure 2). The distances between the α-proton of valine-1 and valine-2 were 5.95 and 6.86 Å, while those between the α-proton of valine-2 and the side chain methyl of threonine were 5.16 and 5.45 Å for MolB-*R* and MolB-*S*, respectively. The former restraint violation appears to be critical since the two atoms involved in the restraint flank the thiazoline ring, and thus their positions (coordinates) are expected to be affected by the chirality at the α-carbon of the ring. In other words, the optimized conformation of MolB-*R* appeared to match the experimental distances derived from the NMR data, while MolB-*S* showed two violations and was also energetically less stable than the former. On the basis of these observations, the chirality at the α-carbon of the thiazoline ring was assigned to be *R*, corresponding to the L-amino acid configuration for the thiazoline moiety.

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