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

Marwa S. Donia, † Bin Wang, † Daniel C. Dunbar, † Prashant V. Desai, † Akshay Patny, ‡ Mitchell Avery, ‡ and Mark T. Hamann*,†

Department of Pharmacognosy, Medicinal Chemistry and National Center for Natural Products Research, School of Pharmacy, The University of Mississippi, University, Mississippi 38677

Received December 13, 2007

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. Didemnin B was the first marine natural product to enter phase I and II clinical trials as an anticancer agent. 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- or six-membered rings. 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 and water extracts were exhaustively extracted separately with dichloromethane and then ethanol. The dichloromethane and the ethanol extracts were combined on the basis of 3 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 C36H53N6O6S (calc 697.3747, ∆ = 3.4 mmu, mmu =
millimass unit), requiring 14 degrees of unsaturation. The peptidic nature of the compound was suggested by the IR, and \(^1H\) and \(^13C\) NMR spectra; four amide protons were observed at \(\delta 7.00, 7.61, 7.86,\) and \(9.14\) ppm, and six \(^13C\) NMR signals resonated between \(\delta 168\) 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 \(\alpha\)-proton of the isoprene group resonating at \(\delta 170.8\) ppm and to a quaternary aromatic carbon (\(\delta 78.0\) ppm).

The assignment of the thiazoline residue was accomplished using HMQC, HMBC, COSY, and HRESIMS spectrometric techniques. The methylene protons (\(\delta 4.03, 3.46\)) showed HMBC correlations to the carbonyl signal at \(\delta 174.5\), which was assigned as part of the valine 2 residue, and to the carbonyl signal at \(\delta 170.8\). The same protons also showed COSY correlations to a methine proton at \(\delta 5.13\), which provides a strong HMBC correlation to the valine 2 carbonyl (\(\delta 174.5\)). The \(cis/trans\) configuration of Pro was determined to be \(cis\) on the basis of the \(\Delta\delta_{\alpha\beta}\) (differential value of \(^13C\) chemical shifts of \(C\) and \(C\) 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 \(^1H–\(^13C\) correlation experiments (HMBC) and ROESY. The NH proton (\(\delta 9.10\)) and \(\alpha\)-proton (\(\delta 4.80\)) signals of threonine provide HMBC correlations to the carbonyl (\(\delta 171.1\)) signal of proline. The NH proton (\(\delta 7.80\)) and \(\alpha\)-proton (\(\delta 4.37\)) signals of valine 2 showed HMBC correlations to the threonine carbonyl resonance (\(\delta 168.9\)).

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 stereochimical 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 \(\alpha\)-carbon of the thiazoline moiety of mollamide B. Molecular modeling was utilized to provide a reasonable prediction of configuration at the \(\alpha\)-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 \(\alpha\)-carbon (D-Tzn (5): 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 (\(\Delta H_f\)) 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 \(\alpha\)-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]\(^+\) at 619.3259 (calc 619.3277, \(\Delta -1.8\) mmu) peak, indicating a molecular formula of \(C_{24}H_{20}N_2O_5S\), which accounts for 15 degrees of unsaturation and one more additional unsaturation unit than the known peptide keenamide A (3). The \(^1H\) NMR spectrum showed a comparable spectrum to keenamide A (3). Three \(^1H\) NMR resonances are lacking (\(\delta 5.0\) (d), 3.60, and 3.70 (m)) corresponding to the thiazoline unit, and instead an additional singlet peak at \(\delta 7.9\) ppm appears. Two additional aromatic carbon resonances (\(\delta 122.4, 149.2\) ppm) were observed, indicating the presence of an aromatic ring system. Analysis of HMBC data revealed that the aromatic singlet proton resonating at \(\delta 7.9\) ppm correlates to the leucine carbonyl carbon (\(\delta 170.7\) ppm) and to a quaternary aromatic carbon (\(\delta 149.2\) ppm). The same proton showed a strong correlation to the carbonyl

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

**Figure 2.** Energy-minimized conformations of MolB-R (1) and MolB-S (2).
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.\(\Delta\delta_{\text{H}}/\Delta\delta_{\text{C}}\) 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 (\(\Delta\delta_{\text{H}}/\Delta\delta_{\text{C}}\) 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 the thiazole-modified amino acid.

Mollamide B (1) and keenamide were examined for antimicrobial activity against MRSA, *Mycobacterium intracellulare*, *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-cancer activity against MRSA, *C. glabrata*, *C. krusei*, *C. albicans*, and *C. neoformans*, with IC\(_{50}\) and IC\(_{90}\) values of 18 and 35 \(\mu\text{g/mL}\), respectively. Mollamide B also exhibited significant percentage growth inhibition. 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 the relative sensitivity of solid tumors, normal cells, and leukemias.

Mollamide B (1) has been tested at 100 \(\mu\text{M}\) 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-
Elmer Lambda 3B UV/vis spectrometer. The 1H and 13C NMR spectra were recorded in CDC13 using Bruker spectrometers operating at 400 or 600 MHz for 1H and 100 or 150 MHz for 13C 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 (30–200 µm) were obtained from Natland International Corporation and Scientific Adsorbents Incorporation, respectively. TLC analyses were carried out using precoated silica gel G254 or aluminum oxide Alox-100 UV254 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 external color of the tunicate was 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* were collected in Manado Bay, Indonesia, in 2003. The 450 g dry wt of tunicate was extracted with H2O. The H2O extract was exhaustively partitioned in Manado Bay, Indonesia, in 2003. The 450 g dry wt of tunicate was extracted with EtOH, CH2Cl2, and 100% CH3CN. The mixture was further purified using a NH2 HPLC column and an isocratic elution system of 20% CH3Cl/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]D20 = −119.9 (c 0.25, CHCl3); UV (MeOH) λmax (log ε) 202 (4.53) nm; IR (KBr film) νmax 3356, 3256, 2966, 2931, 1683, 1530, 1447 cm−1; 1H NMR and 13C NMR, see Table 1; HRESIMS m/z 697 [M + H]+; HREIMS m/z [M + H]+ 697.3713 (calcd for C36H53N6O6S, 697.3747, ∆ 3.4 ppm).

**Mollamide C (2):** white solid; [α]D20 = 0 (c 0.1, CHCl3); UV (MeOH) λmax (log ε) 200 (5.15); IR (KBr film) νmax 3318, 2929, 2926, 2873, 1729, 1685, 1541, 1456, 1271, 1140, 1070 cm−1; 1H NMR and 13C NMR, see Table 2; HRESIMS m/z [M + H]+ 619.3259 (calcd for C30H46N6O6S, 619.3277, ∆ 1.8 ppm).

**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 H2O and derivatized with 20 % TFA and 20 % C5H5N. The mixture was heated at 40 °C for 15 h. The solutions were cooled to room temperature, neutralized with 5 µL of 2 N HCl, and evaporated to dryness. The solutions were cooled to room temperature, neutralized with 5 µL of 2 N HCl, and evaporated to dryness. The solutions were used for the determination of absolute configuration of the amino acids using a Waters NOVApak C18 (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 230 nm).

**Ozonolysis of Mollamide C (2).** A slow stream of O3 was bubbled into a 7 mL CH2Cl2 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 N₂, the residue was subjected to hydrolysis and derivatization as described above with molmollide B. The t/δ-configuration for serine, proline, and leucine were assigned using the C₁₃ column, and 1-isoleucine and l-allo-iso-leucine were assigned using the C₁₅ column as described above. Then the ion at m/z 384 was extracted for the four isoleucine isomers. The analysis of molmollide B (1) using the C₁₅ column established the presence of t-Pro (34.47) [t-Pro (36.13)], t-Ile (52.94) [t-Ile (52.63)], and L-Phe (52.41) [t-Phe (52.54)]. 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 molmollide C (2) using the C₁₅ column established the presence of t-Ser (21.56 min) [t-Ser (26.13)], t-I-Pro (34.07) [t-Pro (40.25)], and L-Leu (51.82) [L-Leu (60.85)]. Amino acid standards using LCTOF revealed retention times as follows: l-Ile (52.63) [l-allo-Ile (52.93), dl-Ile (70.42), d-allo-Ile (71.37)]. Due to the close retention time of l-Ile and l-allo-Ile, molmollide 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 molmollides 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 I were drawn using the BUILDERR module in Insight II (Accelrys Inc., San Diego, CA). All regular amino acids were drawn as S, while the α-carbons of the thiazoline moiety was drawn with both S and R configurations to provide two initial configurations of molecule I: 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 (ΔHf) 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 ΔHf 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 Mol-B-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 Mol-B-R and Mol-B-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 Mol-B-R appeared to match the experimental distances derived from the NMR data, while Mol-B-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.

Acknowledgment. We appreciate the assistance of F. T. Wiggers and A. G. Shilabin in acquiring NMR and LC-TOF data. We are grateful to the National Cancer Institute (NCI) and F. Valeriote at Wayne State for performing cytotoxicity assays; The National Center for Natural Products Research for performing the antimicrobial and antimalarial assays; R. F. Schinazi’s group at Emory for HIV-1; S. G. Franzblau’s group at UIC for Mtb; and A. M. S. Mayer for anti-inflammatory assays. Financial support for this work was provided by grant numbers R01AI36396 and P20 RR01292 from the NIAID and NCRR components of the National Institutes of Health (NIH), and the contents are solely the responsibility of the authors and do not necessarily represent the official view of the NIH. The Egyptian Government and the CDC are also very gratefully acknowledged for financial support. This investigation was conducted in a facility constructed with support from Research Facilities Improvements Program (C06 RR-14503-01) from the National Center for Research Resources, NIH.

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