Didmolamide A and B, Two New Cyclic Hexapeptides from the Marine Ascidian *Didemnum molle*

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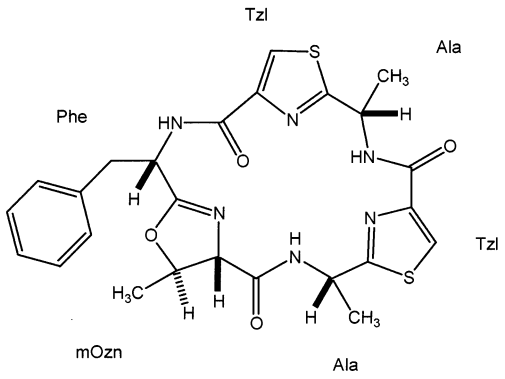
Two novel cyclic hexapeptides, didmolamides A and B, were isolated from the compound ascidian *Didemnum molle* collected in Madagascar. The structure of the two peptides was elucidated by interpretation of MS, COSY, HMQC, and HMBC data. The absolute configuration of all amino acids was determined to be L using Marfey's method for HPLC.

Ascidians have been shown to be a rich source of cyclic peptides.[1] Among the many interesting cyclic peptides from ascidians are the lissoclinamides[2,3] and prepatellamid A[4] isolated from *Lissoclinum patella* and the tamandarins A and B, which were isolated from an unidentified didemnid ascidian from Brazil.[5] We also recently reported the structure of four cyclic hexapeptides, comoramides A and B and mayotamides A and B, from the ascidian *Didemnum molle* that was collected in the lagoon of Mayotte.[6] Yet other cyclic peptides, mollamide and cyclodidemnamide, were isolated from the same species collected in other places around the world.[7,8]

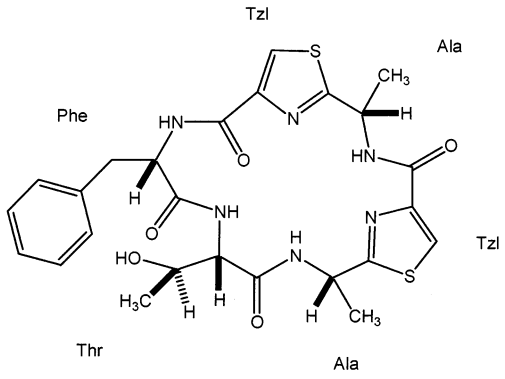
The present report describes the isolation and structure elucidation of two new cyclohexapeptides, didmolamides A and B from the ascidian *Didemnum molle*, collected in Madagascar by scuba at a depth of 5 m. The chloroform−methanol (1:2) extract of the ascidian was subjected to partition by the method of Kupchan et al.[9] The chloroform fraction was repeatedly subjected to Sephadex LH-20 columns to afford didmolamide A (**1**, 15.5 mg) and didmolamide B (**2**, 8.3 mg).

The EI mass spectrum of **1** exhibited a molecular ion $[M]^+$ at *m/z* 538. The molecular formula, $C_{25}H_{26}N_6O_4S_2$, was determined by HRMS and ^{13}C NMR data. In the ^1H NMR spectrum, three NH signals at δ 8.56, 8.24, and 7.73 ppm coupled to signals in the correct region for α-protons of amino acids (Table 1) suggested that the compound was a peptide. The ^1H, ^{13}C, and HMBC NMR data indicated that there were six amino acids in **1**, of which two existed as thiazole rings (Tzl) and one as a 5-methyl oxazoline (mOzn) heterocycle. The phenylalanine and two alanine residues were suggested on the basis of COSY, TOCSY, and HMBC experiments. HMBC data (Figure 1) also provided information on the amino acid sequence, thus constructing the cyclic hexapeptide structure of didmolamide A (**1**).

Acid hydrolysis of **1** and derivatization with Marfey's reagent,[10] followed by HPLC analysis, revealed that Phe and mOzn (which hydrolyzed to Thr) residues in **1** were of the L-form. Furthermore, an observed NOE between the methyl group of mOzn and the α proton of the oxazoline determined the 5*R*-configuration, in agreement with the 2*S*,3*R*-configuration of threonine in **2**, vide infra. Ozonolysis of **1** led to degradation of the alanylthiazole moiety to yield



1 didmolamide A



2 didmolamide B

Ala, which was also determined by Marfey's method to be of the L-form.

The EI mass spectrum of **2** exhibited a molecular ion $[M]^+$ at *m/z* 556. The molecular formula, $C_{25}H_{28}N_6O_5S_2$, was determined by HRMS and ^{13}C NMR data. The NMR data of **2** (Table 1 and Figure 2) indicated it to be identical to **1** except for the 5-methyloxazoline ring, which in **2** is replaced by threonine, the most likely biogenetic precursor of the methyloxazoline ring of **1**.

Degradation and derivatization of **2**, under the same conditions as described for compound **1**, determined the L-configuration of all six amino acids (the 2*S*,3*R*-configuration of the Thr is suggested on the basis of the measured NOE between the α proton and the γ methyl of the mOzn

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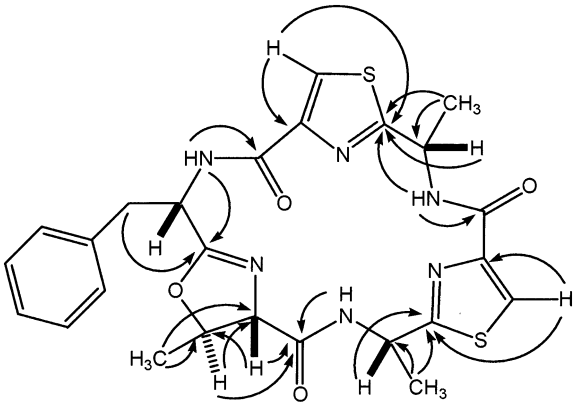

Figure 1. HMBC correlations in didmolamide A (**1**).

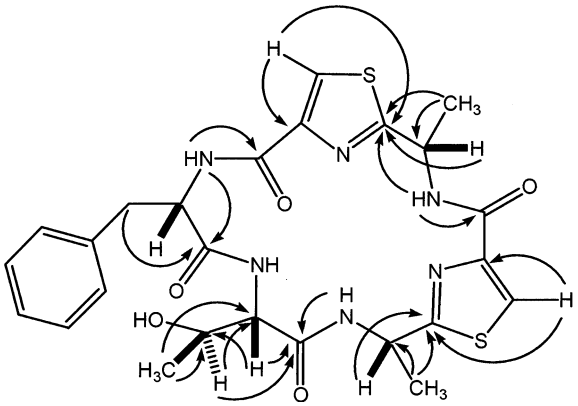

Figure 2. HMBC correlations in didmolamide B (**2**).

Table 1. 500 MHz NMR Data for Didmolamide A (**1**) in C_6D_6 and B (**2**) in $CDCl_3$−MeOH

amino acid	position	**1** H (#H, *J* in Hz)	**1** ^{13}C	**2** ^{1}H	**2** ^{13}C
Ala	CO		171.4		171.6
	α	5.14 m	48.1	5.30 m	46.1
	β	1.2 d (6.5) (3H)	24.9	1.48 d (6.5) (3H)	22.0
Tzl	CO		159.2		160.0
	α		149.0		149.0
	β	7.76 s	123.7	7.95 s	123.9
Phe	CO		167.5		171.5
	α	5.28 t (4.0)	48.5	5.00 t (4.0)	54.5
	β	3.02 dd (14.0, 4.0)	37.5	3.20 dd (14.0, 4.0)	37.2
		3.31 dd (14.0, 4.0)		3.10 dd (14.0, 4.0)	
	1		135.2		135.1
	2, 6	6.91 d (8.0)	129.8	6.93 d (8.0)	129.8
	3, 5	7.01 t (8.0)	128.3	7.10 t (8.0)	128.4
	4	7.03 t (8.0)	126.8	7.05 t (8.0)	126.9
	NH	8.24 d (7.5)			
mOzn	CO		169.0		169.8
	α	3.85 d (9.5)	74.1	4.01	60.1
	β	4.72 m	81.9	4.20 m	66.3
	γ	1.30 d (6.5) (3H)	22.9	0.80 d (6.5) (3H)	18.9
Ala	CO		170.8		170.4
	α	5.14 m	47.0	5.38 m	47.9
	β	1.35 d (3H)	24.5	1.55 d (6.5) (3H)	23.2
	NH	7.73 d (7.0)			
Tzl	CO		159.3		160.5
	α		149.8		147.8
	β	7.67 s	123.2	7.90 s	123.0

in **1**). Both didmolamides were screened against several cultured tumor cell lines (A549, HT29, and MEL28) and were shown to be mildly cytotoxic, with IC_{50} values of 10−20 μg/mL.

D. molle represents another example of a marine organism in which the chemical composition changes from one collection to the other; whether this is due to changes in associated microorganisms or other factors (changes in locality, the period of the year, reef-streams, temperature, etc.) is unknown. As similar cyclic hexapeptides such as nostocyclamide,[11] raocyclamides,[12] and tenuecyclamides[13] have been isolated from cyanobacteria, it can be suggested that the cyclic hexapeptides in *D. molle* also originate from a cyanobacterium.

Experimental Section

General Experimental Procedures. Optical rotations were obtained with a Jasco P-1010 polarimeter. IR spectra were obtained with a Bruker FTIR Vector 22 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on Bruker ARX-500 and Avance-400 spectrometers. ^{1}H, ^{13}C, COSY, HMQC, and HMBC were recorded using standard Bruker pulse sequences. EIMS and HR-EIMS were recorded on a Fisons, Autospec Q instrument.

Biological Material. The tunicate *Didemnum molle* was collected at two localities in Madagascar, at the lagoon of Tulear (aquarium) in December 1999 (Tu 504) and at Andavadoc, 100 km from Tulear (21°58′348″ south; 43°12′241″ east) (Tu 648) in May 2000, by scuba at a depth of 5 m. A voucher specimen is deposited at IUFM, La Reunion (voucher numbers AMT-504, 648).

Extraction and Isolation. The ascidian (380 g) was homogenized and extracted with $CHCl_3$−MeOH (2:1) to give a brown gum (2.5 g). This gum was subjected to partitioning by the method of Kupchan et al.[9] The chloroform fraction (1.04 g) was repeatedly chromatographed on a Sephadex LH-20 column, eluting with heptane−$CHCl_3$−MeOH (2:1:1) to afford didmolamide A (15.5 mg, 0.004%) and didmolamide B (8.3 mg, 0.0022%).

Didmolamide A (1): an oil; $[\alpha]^{25}_{D}$ −35.7° (*c* 0.59, MeOH); IR ($CHCl_3$) ν_{max} 3680, 2925, 1647 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; EIMS *m*/*z* 538 [M^{+}] (85), 512 (35), 469 (20), 447 (75), 295 (45), 239 (60), 174 (50), 138 (60), 120 (70), 104 (85), 91 (100); HREIMS *m*/*z* 538.1461 (calcd for $C_{25}H_{26}O_4N_6S_2$, 538.1457).

Didmolamide B (2): an oil; $[\alpha]^{25}_{D}$ −216° (*c* 0.11, MeOH); IR ($CHCl_3$) ν_{max} 3680, 2925, 1648 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; EIMS *m*/*z* 556 [M^{+}] (5), 538 (100), 512 (15), 447 (85), 138 (55), 91 (35); HREIMS *m*/*z* 556.1578 (calcd for $C_{25}H_{28}O_5N_6S_2$, 556.1562)

Determination of the Stereochemistry of the Amino Acids Phe and Thr. A 0.5 mg portion of **1** was dissolved in 6 N HCl (ca. 0.5 mL) and was heated at 110 °C for 20 h. The HCl was then removed under vacuo. The hydrolysate was resuspended in water and derivatized with (1-fluoro-2,4-dinitrophenyl)-5-L-alanine amide (FDAA).[10] The *N*-[(dinitrophenyl)-5-L-alanine amide]amino acid derivatives were compared with similarly derivatized standard amino acids by HPLC analysis: Merck puropherstar column, 5 μm, 4.6 × 12.5 mm, flow rate 1 mL/min, UV detector at 340 nm, linear gradient elution from 9:1 50 mM triethylammonium phosphate (TEAP) buffer (pH 3)/acetonitrile to 1:1 TEAP/acetonitrile within 60 min. The determination of the absolute configuration of each amino acid was confirmed by spiking the derivatized hydrolysates with the derived authentic amino acids. The HPLC analysis established L-Phe and L-Thr.

Determination of the Stereochemistry of Ala. A fine stream of O_3 was bubbled into a solution of compound **1** [0.5 mg of compound **1** in MeOH (0.5 mL)−CH_2Cl_2 (5 mL)] for 20 s. Dimethyl sulfide (2 drops) was then added, and the reaction mixture was left overnight at room temperature. After removal of the CH_2Cl_2 and DMSO, the residue was dissolved in 6 N HCl (0.5 mL) and was subjected to acid hydrolysis as described above. Derivatization with FDAA, as described above, established the L-form for both residues of Ala.

Determination of the Stereochemistry of the Amino Acids of Didmolamide B (2). Degradation and derivatization of compound **2**, in the same manner as described for compound **1**, determined the L-form of all six acids, as in **1**.

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