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Four New Cytotoxic Cyclic Hexa- and Heptapeptides from the Marine Ascidian *Didemnum molle*

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Abstract

Two novel cyclic hexapeptides, comoramides A and B and two new cyclic heptapeptides, mayotamides A and B were isolated, separately, from two collections of the compound ascidian *Didemnum molle* collected at two spots of the lagoon of Mayotte. The structure of all four peptides, including the absolute configuration of all amino acids, was elucidated by spectroscopic analysis, mainly NMR data, and degradation experiments. All four compounds exhibit mild cytotoxicity against several tumor cells. Obtaining four new cyclic peptides from two *D. molle* collections, which add to the two earlier reported cyclic peptides from an Australian and Philippine *D. molle*, supports the notion that these compounds originate from prokaryotic algal symbionts.

Keywords: Marine metabolites; peptides; NMR

Cyclic peptides have been isolated from a number of marine taxa, and many show remarkably high levels of cytotoxicity. [1] We have also reported recently the structure of a new cyclic peptide, oriamide, from the sponge *Theonella* sp. [2] Among the many interesting cyclic peptides from ascidians are mollamide [3] and cyclodidemnamide [4] isolated from the common Australian and Philippine Indo Pacific ascidian *Didemnum molle* (Herdman, 1886).

The present report describes the structure elucidation of four new cyclic peptides from two specimens of *D. molle* collected using SCUBA near Dzaoudzi Mayotte, and near San Peitro, in the Lagoon of Mayotte Comoro Islands, North-West of Madagascar. All four peptides differ from the two earlier reported peptides from this animal. It has been suggested that at least some

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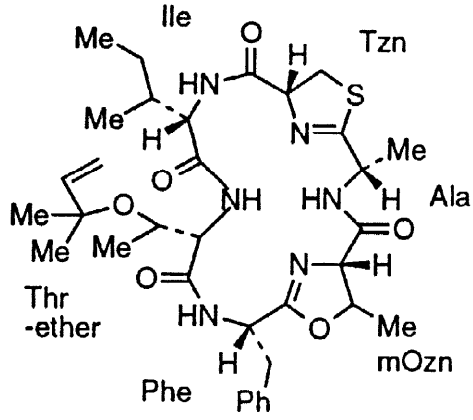
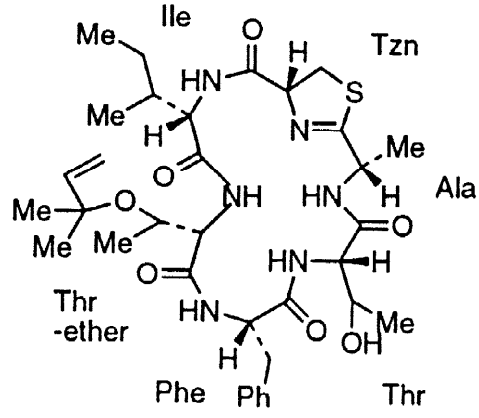
of the peptides are produced by symbiotic prochlorophytes, prokaryotic algal symbionts (*Prochloron* sp.) which are associated with many of the peptide-producing ascidians.[5] It is therefore not surprising to isolate different cyclic peptides from ascidians collected in distant or even close locations, as found in this work.

The chloroform-methanol (1:2) extract of freeze-dried *D. molle* from the two collection spots was rapidly chromatographed by VLC on silica gel with a solvent gradient from petrol ether to acetone to afford, from the San Peitro collection, two compounds designated comoramides A and B (**1** and **2**) and from the Dzaoudzi Mayotte site, a pair of others, mayotamides A and B, **3** and **4**, respectively.



1 comoramide A



2 comoramide B

Comoramide A (**1**) was isolated as an amorphous powder. The molecular formula, $C_{34}H_{48}N_6O_6S$, was determined by HRMS and ^{13}C NMR data. In the ^1H NMR spectrum, three NH signals at δ 7.05, 7.08 and 7.43 coupled to signals in the correct region for α-protons of amino acids suggested that the compound was a peptide. The ^1H, ^{13}C and HMBC NMR data indicated that there were six amino acids, including one carrying a dimethylallyl ether group and two amino acids which existed one as a thiazoline ring (Tzn) and the other as a 5-methyloxazoline (mOzn) heterocycle. COSY and TOCSY experiments were used to construct the major features of the constituent amino acids, and the resulting assignments were confirmed using the HMBC spectrum. From these data, isoleucine, threonine dimethylallyl ether, phenylalanine, 5-methyloxazoline (mOzn), alanine and a thiazoline amino acid (Tzn) were identified. After defining the six amino acid structures, HMBC (see Figure 1) and NOESY (see Figure 2) experiments were used to determine the connectivity between all six amino acids, thus constructing the cyclic hexapeptide structure of comoramide A (**1**).

Interestingly, a similarity exists between **1** and the Australian *D. molle* heptapeptide, mollamide, namely, two pairs of amino acids. Ile-Tzn and Phe-Thr dimethylallyl ether are the

Table 1.

500 MHz NMR Data for comoramides A (**1**) and B (**2**) in C_6D_6.

		1		**2**	
amino acid	position	^{1}H (#H, J [Hz])	^{13}C	^{1}H	^{13}C
	CO		170.6		170.6
Ile	α	4.99 dd (10.2, 30)	58.1	4.78 dd	58.2
	β	2.40 m	36.5	2.20 m	36.0
	γ	1.38 m (1H), 1.00 m (1H)	24.6	1.52 m, 1.05 m	26.0
	δ	0.84 t (6.5)(3H)	12.0	0.97 t	12.5
	β-CH_3	0.92 d (6.5)(3H)	16.2	0.85 d	17.0
	NH	7.05 d (2.7)		7.10 d	
	CO		170.7		170.7
Thr-ether	α	3.68 t (3.7)	57.1	3.72 t	58.1
	β	4.17 dd (11.3, 5.7)	66.7	4.40 m	65.0
	γ	1.12 d (6.5) (3H)	15.8	0.99 d	15.8
	1'		77.8		77.8
	2'	5.78 dd (17.5, 10.7)	142.7	5.67 dd	142.8
	3'	5.13 d (17.5), 5.03 d (10.7)	115.0	5.06 d, 4.96 d	115.0
	4'	1.28 s (3H)	24.4	1.40 s	25.0
	5'	1.19 s (3H)	27.7	1.26 s	28.0
	NH	7.43 d (3.5)		7.30 d	
	CO		168.5		168.6
Phe	α	4.91 ddd (10.4, 5.8, 3.9)	51.5	3.80 dd	53.3
	β	2.95 dd (14.1, 5.8) (1H)	37.2	3.10 dd	36.1
		2.79 dd (14.1, 10.4) (1H)		2.90 dd	
	1		135.2		135.2
	2,6	6.92 d (8.0)	129.7	6.92 d	129.7
	3,5	7.00 t (8.0)	128.3	7.00 t	128.2
	4	7.03 t (8.0)	126.9	7.03 t	126.9
	NH	7.08 d (3.9)		7.10 d	
	CO		171.1		171.0[a]
mOzr	α	4.35 d (4.0)	75.8	4.80 m	59.0
	β	4.59 dq (6.3, 4.0)	82.1	4.64 m	65.5
	γ	1.00 d (6.3) (3H)	21.3	0.70 d (6.5) (3H)	20.0
	CO		176.6		176.6
Ala	α	5.20 m	48.6	5.30 m	47.0
	β	1.67 d (6.4) (3H)	18.8	1.50 d	20.1
	NH	8.21 d (8.0)		8.69 d	
	CO		171.0		171.2
Tzn	α	4.85 ddd(10.6, 5.5, 2.1)	78.6	4.70 m	79.0
	β	4.16 dd (11.0, 5.7) (1H)	34.2	3.90 dd	35.0
		3.10 t (11.0) (1H)		2.90 t	

[a]Threonine

same in both. The absolute configuration of the amino acids in **1** was determined by hydrolysis with 6N HCl of **1**, or of **1** after ozonolysis and chiral TLC analysis[6] to be all of the L-series.

The solution conformation of **1** is best revealed by NOE's between the Ile-NH and the Thr ether α-Me, Thr-NH and the Ala-Me groups (See Figure 2).

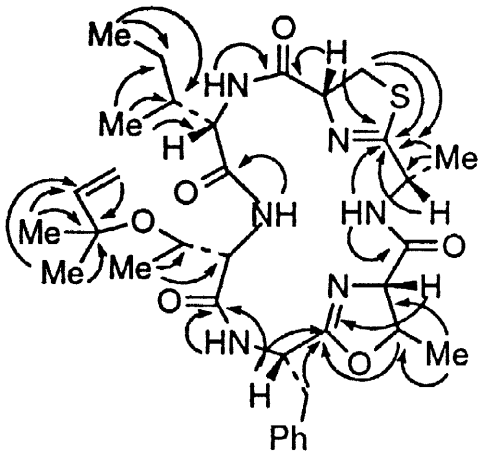


Figure 1. Key HMBC correlations used to determine the structure of comoramide A (**1**)

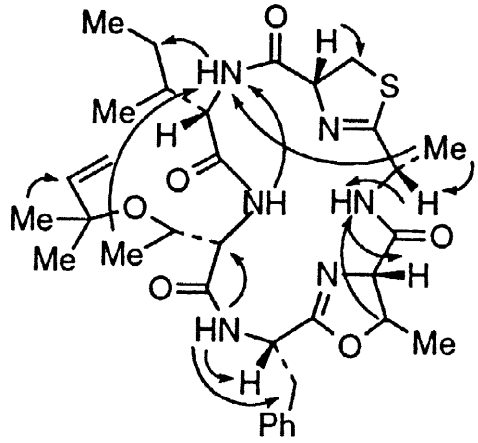


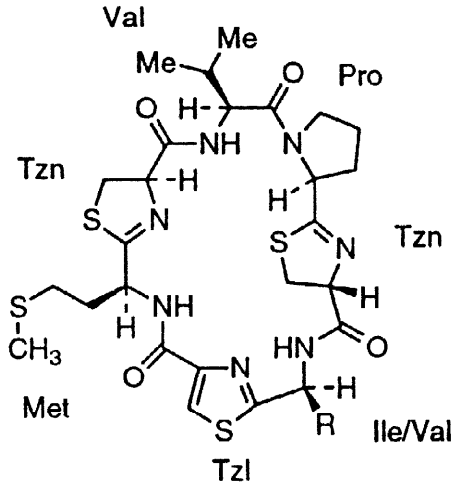
Figure 2. Key NOE's used to determine the structure of (**1**)

Comoramide B (**2**, $C_{34}H_{50}N_6O_7S$) was isolated as an amorphous powder. Full NMR data (see Table 1) and interpretation of the COSY, TOCSY, HMQC and HMBC data indicated that **2** was 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**. Micro acetylation (Ac_2O-pyridine) of **2** afforded the mono acetate (δ 1.57 s (3H), 6.00 m (1H) in C_6D_6) confirming the secondary OH group.

Mayotamide A (**3**), the first of two compounds from the second ascidian specimen investigated, was isolated as a highly air- and acid-sensitive white powder. The molecular formula, $C_{30}H_{43}N_7O_4S_4$, was determined by mass measurements and ^{13}C NMR data. A series of NH signals coupled to signals in the correct region for α-protons of amino acids, in the NMR spectrum, and CO resonances in the 170 ppm region suggested that compound **3** was also a peptide. The planar structure of **3** was assigned on the basis of 2D NMR data.

The combination of HMQC and HMBC experiments allowed all protons and carbons to be assigned (see Table 2, and Figure 3). Proton COSY and TOCSY together with HMBC experiments showed common correlation patterns for a valine, proline, isoleucine and methionine. Three additional unusual amino acids containing heteroatoms were assigned as one thiazole and two thiazoline ring containing acids (Tzl and Tzn), on the basis of ^{13}C data which paralleled literature data for similar structures.[4] Further evidence for the thiazole ring was the presence of one isolated proton at 7.60 on a carbon resonating at 124.5 ppm with a mutual coupling of 192 Hz. HMBC analysis supported the assignment of the four heterocycles of **3** and

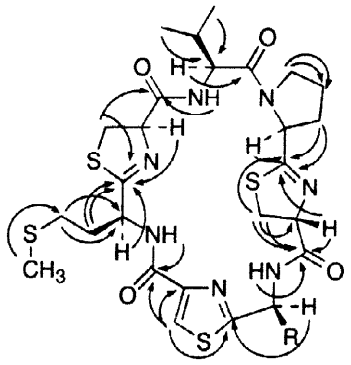
was also used together with a NOESY experiment to sequence the entire peptide (see Figures 3 and 4). Mayotamide A embodies the same Val-Pro-Tzn sequence as in cyclodidemnamide[4] and also contains an additional thiazoline (Tzn) ring. Replacement of one of the valines, the phenylalanine and the oxazoline of cyclodidemnamide by isoleucine, methionine and thiazoline in **3** is not expected to change much the conformation of its macrocycle.



3 R = CH(CH3)CH2CH3
4 R = $CH(CH_3)_2$

mayotamides A & B

Indeed, as in cyclodidemnamide[4] one of the methyl groups of the valine in the transannular position to the thiazole ring penetrates the thiazole shielding cone and is unusually upfield shifted to δ 0.15 in $CDCl_3$ (and 0.34 in C_6D_6, Table 2) supporting a similar conformation to that of cyclodidemnamide.[4] Further support for this conformation comes from a NOE seen between the above mentioned methyl and the methionine NH atom (see Figure 4) and also from the influence of the methionine sulfoxide group, in the case of the oxidised derivative, on the Val methyl group resonance, *vide infra.*



R = CH(CH3)CH2CH3

Figure 3. Key HMBC correlations used to determine the structure of comoramide A (**3**)

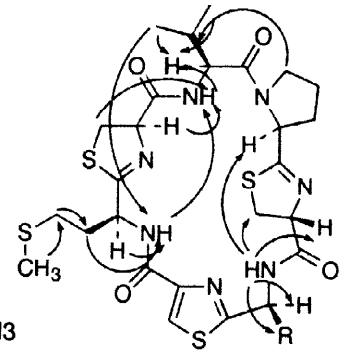


Figure 4. Key NOE's used to determine the structure of (**3**)

If no special care was taken during the isolation of the peptides, no mayotamides A and B (**3** and **4**) were obtained. Instead, each of compounds **3** and **4**, as understood eventually, gave during the isolation a non-separable 1:1 mixture of two compounds.[7] The same mixture was also obtained when pure **3** (or **4**) was left in the air. The major difference in the NMR spectrum of **3** and the transformed mixture was a change in the S-Me resonance, namely, the appearance

Table 2.

500 MHz NMR Data for mayotamides A (**3**) and B (**4**) in C_6D_6.

		1		**2**	
Amino acid	Position	^{1}H (#H, J [Hz])	^{13}C	^{1}H	^{13}C
	CO		170.7		171.2
Val	α	4.79 dd (9.9, 3.0)	54.5	4.85 dd	54.9
	β	1.50 m	31.3	1.50 m	31.7
	γ	0.78 d (6.5) (3H)	15.3	0.85 d (3H)	15.6
	γ'	0.34 d (6.5) (3H)	20.2	0.38 d (3H)	20.7
	NH	7.12 d (9.9)		7.10 d	
	CO		170.3		170.3
Tzn	α	4.74 dd (6.4, 4.5)	77.4	4.80 dd	77.8
	β	3.90 dd (11.2, 4.5) (1H), 3.35 m (1H)	36.6	3.95 dd 3.05 m	37.0
	CO		175.4		175.8
Met	α	5.40 m	51.2	5.50 m	51.6
	β	2.15 m (1H), 1.85 m (1H)	35.3	2.15 m, 1.80 m	35.9
	γ	2.35 t (7.0) (2H)	30.3	2.30 m	30.3
	S-Me	1.70 s (3H)	15.2	1.85 s (3H)	15.6
	NH	8.70 d (9.1)		8.90 d	
	CO		160.7		160.5
Tzl	α		148.7		148.6
	β	7.60 s	124.5	7.60 s	124.9
	CO		170.3		170.3[a]
Ile	α	5.48 t (8.5)	53.8	5.45 t (8.5)	56.3
	β	2.75 m	36.1	2.50 m	30.8
	γ	1.58 m (1H), 1.20 m (1H)	27.6		
	δ	0.90 t (6.5) (3H)	11.6	1.18 d (6.5)	20.0
	β CH_3	1.38 d (6.5)	16.1	1.42 d (6.5)	21.4
	NH	7.65 d (9.3)		7.75 d (9.2)	
	CO		170.5		170.5
Tzn	α	5.18 dd (10.2, 2.2)	78.8	5.15 dd	79.4
	β	3.15 m (1H), 2.80 m (1H)	36.9t		37.3
	CO		178.8		178.7
Pro	α	4.62 q (6.8)	60.2	4.70 q	60.6
	β	1.56 m (1H), 1.45 m (1H)	30.3	1.50 m, 1.40 m	30.2
	γ	1.20 m (1H), 0.80 m (1H)	24.9	1.17 m, 0.80 m	25.4
	δ	2.66 m (1H), 2.60 m (1H)	47.2	2.75 m, 2.70 m	47.6

[a]Valine

of two methyl groups resonating in $CDCl_3$ at δ_H 2.604 and 2.616 (instead of δ 1.70, in **3**) and δ_C 38.9 (instead of 15.2) ppm.[8] After the careful isolation of **3** and its transformation to the two oxidized derivatives ($C_{30}H_{43}N_7O_5S_4$, *m/z* 709, 30%) it became clear that the above methyl groups belong to two methyl sulfoxide groups which are obtained by air oxidation of the methionine methyl group (two diastereomers).[9]

Mayotamide B (**4**) was isolated as a white powder. The molecular formula, $C_{29}H_{41}N_7O_4S_4$, which was determined by HRMS and ^{13}C NMR data (see Table 2), suggested **4** to be a lower homolog of compound **3**. The ^1H, ^{13}C, HMQC, COSY, TOCSY and HMBC NMR data indicated clearly that mayotolide B is closely related to A (**3**), the only difference being replacement of the isoleucine of **3** by a valine in **4**. Mayotolide B therefore possesses the same five amino acid sequence (Val-Pro-Tzn-Val-Tzl) as in cyclodidemnamide.[4]

The chiral TLC analysis of compound **3**'s hydrolysate in comparison with authentic samples revealed that the amino acid residues bore L configuration. The various cyclic peptides were screened against several cultured tumor cell lines (A549, HT29 and MEL-28) and were shown to be mildly cytotoxic with IC_{50} values of 5-10 μg/ml.

Experimental Section

General Experimental Procedures. IR spectra were recorded on a Nicolet 205 FT-IR spectrometer. LRMS and HRMS were recorded on a Fisons, Autospec Q instrument. ^1H and ^{13}C NMR spectra were recorded on a Bruker ARX-500 spectrometer. All chemical shifts are reported with respect to residual $CHCl_3$ (δ 7.25 for ^1H and 77.0 ppm for ^{13}C). Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter using a 1cm microcell.

Biological material. The ascidian *Didemnum molle* was collected at two localities in the lagoon of Mayotte, at Dzaoudzi Mayotte (12°,52',03" south; 45°,16',25" east) and at San Peitro (12°,41',30" south; 45°,11' east), Comoro Islands, north-west of Madagascar, by SCUBA at a depth of 15 m. A voucher specimen is deposited at IUEM, La Reunion (AMT-15).

Extraction and Isolation. Each of the ascidians (50g) was homogenized and extracted with $CHCl_3$:MeOH (1:2) to give a brown gum (500 mg) after evaporation. This residue was rapidly chromotographed on silica gel (VLC) with a solvent gradient from light petrol ether to acetone. The fraction that eluted with petrol ether:acetone (1:1) was further chromotographed several times on silica gel with the same solvents to yield from the San Peitro sample comoramides A and B (**1** and **2**) (5 mg each), and from the Dzaoudzi Mayotte sample mayotamides A and B (**3** and **4**) (9 mg and 2 mg, respectively).

Comoramide A (1): amorphous powder; $[\alpha]_D$ + 0.5 (c 0.17, MeOH); υ_{max} ($CHCl_3$) 3690, 2930, 1650 cm^{-1}; ^1H and ^{13}C-NMR see Table 1; HREIMS observed *m/z* 668.3340 [M^+], $C_{34}H_{48}N_6O_6S$ requires *m/z* 668.3356; FABMS *m/z* 691 [M+Na^+, 10%], 669 [MH^+, 10%], 601

[M-68, 100%]; EIMS *m/z* 668 [M^+, 10%], 600 [M-68, 90%], 624 (85%), 599 (20%), 556 (100%), 527 (20%).

Comoramide B (2): amorphous powder; $[\alpha]_D$ -100 (c 0.05, MeOH); υ_{max} ($CHCl_3$) 3690, 2930, 1650, 1077 cm^{-1}; ^1H and ^{13}C-NMR see Table 1; HREIMS observed *m/z* 686.3450 [M^+], $C_{34}H_{50}N_6O_7S$ requires 686.3462; CIMS (CH_4) *m/z* 715 [MEt^+, 10], 687 [MH^+, 20%], 642 (20%), 619 (10%), 601 (20%), 575 (30%).

Mayotamide A (3): amorphous powder; $[\alpha]_D$ +77 (c 0.30, MeOH); υ_{max} ($CHCl_3$) 3680, 2920, 1648 cm^{-1}; ^1H and ^{13}C-NMR see Table 2; EIMS *m/z* 693 [M^+, 10%], 645 (50%), 629 (25%), 619 (100%), 605 (30%); EIMS of the sulfoxide *m/z* 709 [M^+, 30%], 694 (20%), 645 [M-CH_3SOH, 100%], 619 (85%), 605 (20%), 521 (20%), 462 (20%), HREIMS observed for the sulfoxide 645.2219 [M-CH_3SOH], $C_{29}H_{39}N_7O_4S_3$ requires 645.2225; observed 619.2082 [M-$C_3H_6O_6S$], $C_{27}H_{37}N_7O_4S_3$ requires 619.2069.

Mayotamide B (4): amorphous powder; $[\alpha]_D$ +130 (c 0.1, MeOH); υ_{max} ($CHCl_3$) 3680, 2920, 1648 cm^{-1}; ^1H and ^{13}C-NMR see Table 2; EIMS *m/z* 679 [M^+, 10%], 619 (45%), 605 (100%), 577 (10%); HREIMS observed *m/z* 679.2115, $C_{29}H_{41}N_7O_4S_4$ requires 679.2103.

Acknowledgement. We thank Dr. Monniot for the identification of the ascidian.

References and Notes

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[7] Comparison of the proton NMR spectra of the chromatography fractions during the isolation of **3** and **4** showed clearly their transformation to more stable derivatives. Secondly, obtaining two sulfoxides is consistent with the air oxidation of **3** and **4** during their isolation.

[8] When these signals were first observed during the isolation process, they were suspected to belong to N-Me groups.

[9] Interesting to note is the great easiness under which compound **3** (and **4**) undergoes air-oxidation, to a mixture of sulfoxides, in contrast to other methionine containing cyclic peptides. For example, dendroamide B[5] first isolated from a blue-green alga and also by us from an ascidian is stable to air and thus dendroamide C[5], the corresponding natural sulfoxide is a single diastereomer.