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Pembamide, a *N*-Methylated Linear Peptide from a Sponge *Cribrochalina* sp

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Essential title page information

Title

Pembamide, a *N*-Methylated Linear Peptide from a Sponge *Cribrochalina* sp.

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Abstract

A new highly *N*-methylated linear peptide, pembamide (**1**), has been isolated from the marine sponge *Cribrochalina* sp. (family Niphatidae) collected off the coast of Pemba (Tanzania). The planar structure of **1** was assigned on the basis of extensive 1D and 2D NMR spectroscopy and mass spectrometry. The absolute configuration of the amino acid residues in **1** was determined by application of the Advanced Marfey´s method. Compound **1** displayed significant cytotoxicity against three human tumor cell lines with GI_{50} values in the micromolar range.

Keywords: *Cribrochalina*; isolation; peptide; advanced Marfey's method; pembamide.

Numerous studies on *N*-methylamino acids (NMA) containing peptides[1] reveal that replacing natural amino acids with *N*-methylated analogs produce changes in the pharmacokinetic properties by blocking the proteolytic cleavage sites,[2,3] decreasing the number of hydrogen bonds, increasing lipophilicity and, in many cases, enhancing membrane permeability,[4,5,6] as well as altering the conformational characteristics or properties of the amide bonds.[7,8,9] Thus, cyclic *N*-methylamino acids such as vancomycin, cyclosporine and actinomycin D isolated from microorganisms have found clinical use in part due to the physical properties and chemical stability conferred by the NMA present in their structures.[10,11,12]

Highly *N*-methylated linear peptides have also been isolated from different marine sources. Thus, koshikamides[13] are peptides with four or more *N*-methylamino acids isolated from the marine sponge *Theonella* sp. Dictyonamide A,[14] was isolated from the fungus K063 separated from the red alga *Ceratodictyon spongiosum*, while the linear octapeptides RHM-1 and RHM-2[15] were obtained

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from a marine sponge-derived fungus *Acremonium* sp. Marine cyanobacteria are also sources of this type of compounds. For instance, apramides A-G,[16] dragonamides A-E,[17,18,19,20,21] almiramides A-C,[22] carmabin A,[23] dragomabin,[23] and kurahyne[24] have been isolated from the marine Cyanobacterium *Lyngbya majuscule* whilst micromide[25] was found from a species belonging to the genus *Symploca*.

On the other hand, marine sponges belonging to the genus *Cribrochalina* sp. have been found to contain a variety of structurally unique metabolites with interesting biological activities. These include the polyacetylenic alcohol duryne[26] that inhibits the growth of several human tumor cell lines including leukemia, colon, lung, gastric, and breast cancers, and the kapakahines[27], a family of cyclic peptides. Other studies have been related with marine alkaloids such as the manzadin family of bromopyrrole alkaloids,[28] pyrinadine A[29] which was the first pyridine alkaloid isolated from natural sources with an azoxy moiety and showed cytotoxic activity against L1210 murine leukemia and KB human epidermoid carcinoma cells in vitro. Also the tetrahydroisoquinoline cribrostatins 1-5, displaying some of them cytotoxic, antibacterial and antifungal activities,[30] were found in *Cribochalina* species.

2. Results and discussion.

As part of our ongoing efforts to find novel antitumor agents from marine organisms,[31] a detailed biological investigation of a specimen of the Tanzanian sponge *Cribrochalina* sp. was undertaken. Preliminary data from the extracts of this sponge displayed cytotoxic activity against the human tumor cell lines A-549 (lung), HT-29 (colon), and MDA-MB-231 (breast). Bioassay-guided fractionation of the active organic extract of this sample resulted in the isolation of a new highly *N*-methylated linear peptide which was named as pembamide (**1**). This compound shows significant cytotoxicity towards different human cancer cells.

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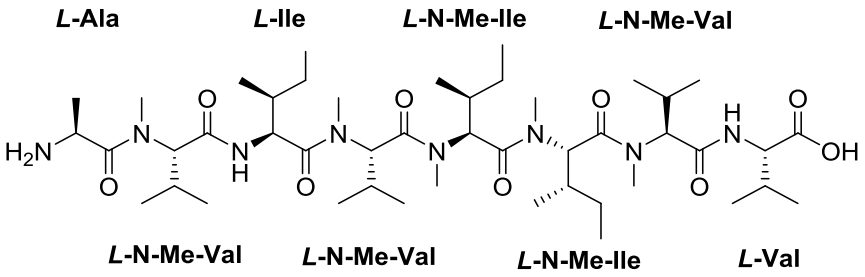

Pembamide (**1**)

A sample of the marine sponge *Cribrochalina* sp. collected by hand off the coast of Pemba Island, north of Tanzania, was extracted several times using CH_2Cl_2/MeOH (1:1). The crude extract was subsequently fractionated by vacuum flash chromatography (VFC) on Lichoprep RP-18 using a gradient mixture of H_2O, MeOH and CH_2Cl_2 with decreasing polarity. Bioguide fractionation against the former human tumor cell lines afforded an active fraction eluted with 100% MeOH that was subjected to reversed-phase HPLC to yield **1**.

Pembamide (**1**) was obtained as a colourless amorphous solid. The molecular formula of compound **1** was determined to be $C_{46}H_{86}N_8O_9$ (8 degrees of unsaturation) based on the $[M + H]^+$ ion peak at *m/z* 895.6596 observed in the (+)-HRESI-TOFMS and on NMR data. A peptidic structure was evident from the NMR data in CD_3OD (Table 1). The ^1H NMR spectrum of **1** revealed the presence of five tertiary *N*-methyl amide singlets at δ_H 3.16, 3.15, 3.09, 3.08 and 3.05, as well as a characteristic secondary amide NH doublet at δ_H 8.07 (deuterium exchangeable). Also, the presence of eight low-fielded signals (δ_H 5.32-4.30) assigned to the -protons of amino acids and 15 methyl groups between 1.45 and 0.77 ppm were representative of alkyl amino acids residues. Moreover, the ^{13}C NMR spectrum of **1** exhibits eight carbonyl carbons at δ_C 174.6, 174.3, 172.9, 172.2, 172.1, 172.0, 171.8 and 171.2 attributable to ester/amide functionalities and five *N*-methyl groups at δ_C 31.8, 31.4, 31.3, 31.2 and 31.0. Further examination of the ^{13}C NMR data

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showed the presence of eight carbons resonating in the δ_C 64.0-49.0 ppm range, and 25 signals in the high-field region, supporting the alkyl nature of the side chains of the amino acid residues. 2D NMR experiments of **1**, including COSY, TOCSY and edited-HSQC, established the presence of eight spin systems (Figure 1), three of which belong to the common amino acids alanine, valine and isoleucine. The identification of the remaining fragments was based on the long range HMBC correlations detected between *N*-methyl groups and the corresponding -protons of the amino acid residues. Thus, two *N*-methylated isoleucines were deduced by the presence of two HMBC cross peaks between the *N*-Me at δ_H 3.05 and C-2 at δ_C 58.0 ppm (*N*-MeIle-1) and between the *N*-Me at δ_H 3.09 and C-2 at δ_C 58.1 (*N*-MeIle-2). In the same way, long range correlations from three *N*-Me groups at 3.08, 3.16 and 3.15 ppm to the corresponding C-2 at δ_C 64.1, 59.7, 63.8 respectively, allowed us to identify the remaining amino acids as *N*-methyl valines (*N*-MeVal-1, *N*-MeVal-2 and *N*-MeVal-3), thereby accounting for all 8 degrees of unsaturation and signifying an overall linear arrangement.

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Table1. NMR Data of **1** in CD_3OD (500 MHz for ^1H and 125 MHz for ^{13}C)

Amino acid	Pos.	$_C$, mult.	$_H$, mult., *J* in Hz	Amino acid	Pos.	$_C$, mult.	$_H$, mult., *J* in Hz
Ala	NH_2	-	-	***N*-MeIle-1**	NMe	31.3, CH_3	3.05, s
	1	171.8, C	-		1	172.1, C	-
	2	49.0, CH	4.42, q, 7.0		2	58.0, CH	5.32, d, 11.5
	3	16.4, CH_3	1.45, d, 7.0		3	34.5, CH	2.09, m
					4	25.3, CH_2	1.23, m; 0.96 ,m
					5	19.6, CH_3	0.85[a]
					6	11.0, CH_3	0.85[a]
***N*-MeVal-1**	NMe	31.0, CH_3	3.08, s	***N*-MeIle-2**	NMe	31.4, CH_3	3.09, s
	1	171.2, C	-		1	172.9, C	-
	2	64.1, CH	4.65, d, 12.5		2	58.1, CH	5.30, d, 12.0
	3	27.4, CH	2.26, m		3	34.8, CH	2.13, m
	4	15.8, CH_3	0.89, d, 6.5		4	25.2, CH_2	1.23, m; 0.96, m
	5	18.9, CH_3	0.87, d, 6.5		5	19.0, CH_3	0.85[a]
					6	11.0, CH_3	0.86[a]
Ile	NH	-	-	***N*-MeVal-3**	NMe	31.8, CH_3	3.15, s
	1	174.6, C	-		1	174.3, C	-
	2	54.6, CH	4.70, d, 8.5		2	63.8, CH	4.70, d, 9.0
	3	37.8, CH	1.86, m		3	27.8, CH	2.24, m
	4	25.5, CH_2	1.52, m; 1.17, m		4	19.7, CH_3	0.96, d, 6.5
	5	18.2, CH_3	0.92, d, 7.0		5	19.0, CH_3	0.77, d, 6.5
	6	10.9, CH_3	0.89, t, 7.5				
***N*-MeVal-2**	NMe	31.2, CH_3	3.16, s	**Val**	NH	-	8.07, d, 7.0[b]
	1	172.2, C	-		1	172.0, C	-
	2	59.7, CH	5.22, d, 11.0		2	59.0, CH	4.30, d, 5.5
	3	28.6, CH	2.33, m		3	31.7, CH	2.16, m
	4	15.8, CH_3	0.88, d, 6.0		4	19.6, CH_3	0.92, d, 6.5
	5	18.6, CH_3	0.80, d, 6.5		5	19.8, CH_3	0.92, d, 6.5

[a] Signal overlapped. Multiplicity and coupling constant could not be determined. [b] Signal deuterium exchangeable.

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A combination of HMBC and ROESY data allowed us to determine the sequence of these units. Thus, long range correlations from -protons or *N*-Me groups to the carbonyl carbons of adjacent amino acids, plus ROESY correlations between the -protons and the *N*-Me protons allowed us to establish the sequence as Ala-*N*-MeVal-1-Ile-*N*-MeVal-2-*N*-MeIle-1-*N*-MeIle-2-*N*-MeVal-3-Val as shown as in Figure 1.

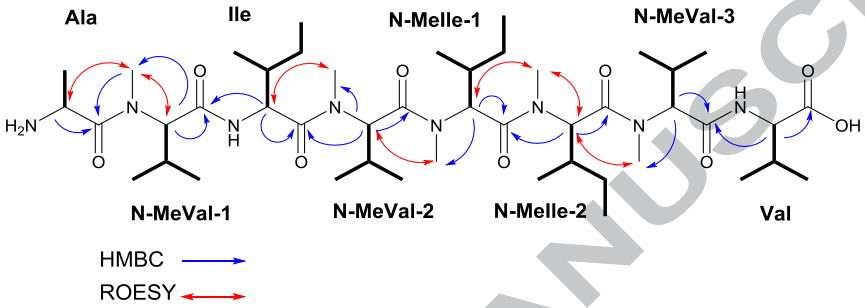

Figure 1. Selected ROESY (red) and HMBC (blue) and COSY (bold bonds) correlations for **1**.

The mass fragmentation pathways observed in ESIMS/MS of **1** confirmed the proposed sequence (Figure 2). Thus, the peaks at *m/z* 298 (Ala/*N*-MeVal-1/Ile), 411 (Ala/*N*-MeVal-1/Ile/*N*-MeVal-2), 538 (Ala/*N*-MeVal-1/Ile/*N*-MeVal-2/*N*-MeIle-1), 665 (Ala/*N*-MeVal-1/Ile/*N*-MeVal-2/*N*-MeIle-1/*N*-MeIle-2) and 778 (Ala/*N*-MeVal-1/Ile/*N*-MeVal-2/*N*-MeIle-1/*N*-MeIle-2/*N*-MeVal-3) displayed in the MS/MS experiment of **1** were used to confirm the former proposed sequence. The molecular formula of those fragments was confirmed by HRESIMS/TOF (Figure 2).

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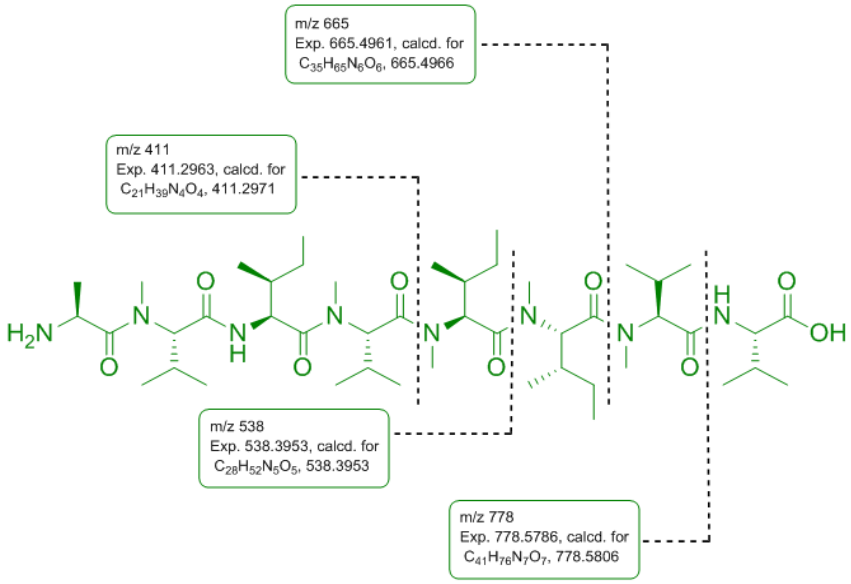
A



B

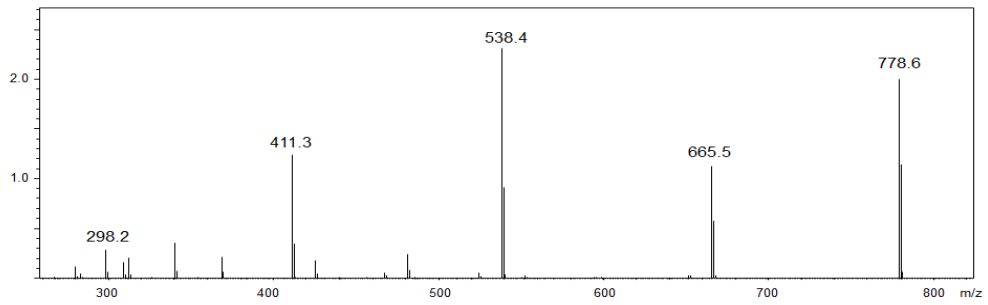


Figure 2. (A) Fragmentation pathway of pembamide (**1**) along with the (+)-HRESIMS/TOF of the fragments. (B) MS/MS ESI fragmentation at (*m/z*)= 778.6.

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The absolute configuration of all units in **1** was established as $_{L}$ using the Advanced Marfey Method.[32,33] Acid hydrolysis of **1** was subjected to Marfey´s derivatization with 1-fluoro-2-4-dinitrophenyl-5-$_{L}$-alanine amide ($_{L}$-FDAA) and racemic $_{LD}$-FDAA separately, and then analyzed by LC/MS. After comparing both chromatograms, we could assign the $_{L}$ configurations to the valine, alanine, and the three *N*-Me-valines. Since *N*-Me-$_{L}$-Val and $_{L}$-Ile have the same molecular weight, these two amino acids were distinguished by their UV spectrum. [34] Although Marfey's analysis clearly suggested the $_{L}$-configuration of the *N*-MeIle moieties, we were unable to distinguish *N*-Me-$_{L}$-Ile and *N*-Me-$_{L}$-*allo*-Ile by this method due to the lack of a standard of *N*-Me-$_{L}$-*allo*-Ile. In order to distinguish between the $_{D}$ and $_{L}$-*allo* configurations in **1**, racemization of the *N*-Me-$_{L}$-Ile standard was carried out, and the reaction mixture, containing both *N*-Me-$_{L}$-Ile and *N*-Me-$_{D}$-*allo*-Ile, was subjected to advanced Marfey´s analysis using LC-MS. The retention times of $_{L}$-Marfey derivatives of the *N*-Me-$_{L}$-Ile standard and the *N*-Me-$_{L}$-Ile units, in the hydrolysate of **1,** were identical. In the same way, we determined that the configuration for the remaining isoleucine was $_{L}$-Ile instead of $_{L}$-allo-Ile.

Cell proliferation assays against the human tumor cell lines A-549 (lung), HT-29 (colon), and MDA-MB-231 (breast) displayed that pembamide exhibited significant cytotoxic activity with a GI_{50} range at micromolar level (Table 2). As a positive standard antitumour compound, doxorubicin is usually included in our assays and it is tested in parallel to the compounds, following identical procedure. The results obtained with doxorubicin are included in the table 2

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Table 2. Cytotoxic Activity Data (M) of **1**.

Compound		Tumor cell lines		
		Breast, MDA-MB-231	Colon, HT-29	Lung, NSCLC A-549
Pembamide	GI_{50}	3.35	3.80	2.46
	TGI	8.15	3.91	> 11.2
	LC_{50}	> 11.2	4.13	> 11.2
Doxorubicin	GI_{50}	0.15	0.27	0.21
	TGI	0.50	0.86	0.85
	LC_{50}	2.41	>17.2	>17.2

GI_{50}, compound concentration that produces 50% inhibition on cell growth as compared to control cells, **TGI**, compound concentration that produces total growth inhibition as compared to control cells, and **LC_{50}**, compound concentration that produces 50% cell death as compared to control cells.

In summary, a new *N*-methylated linear peptide, pembamide (**1**), with a structure of Ala-*N*-MeVal-1-Ile-*N*-MeVal-2-*N*-MeIle-1-*N*-MeIle-2-*N*-MeVal-3-Val, was isolated from an extract of a sponge *Cribrochalina* sp., being the first time that this type of compounds is isolated from specimens belonging to this genus. Application of the advanced Marfey's method has allowed us to determine its absolute configuration. This compound showed significant cytotoxic activity against three human tumor cell lines. As in the case of other linear *N*-methylated peptides that have been isolated from marine sources, such as the octapeptides RHM1 and RHM2, the true origin of the compounds maybe from microorganisms living within the marine invertebrates.[35,36] As such, the actual source of pembamide remains to be determined.

5. Acknowledgments

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6. Supplementary data

Experimental procedure and ^{1}H, ^{13}C, HSQC, COSY, HMBC and ROESY NMR spectra of pembabide (**1**) along with its analysis by advance Marfey´s method. Supplementary data associated with this article can be found, in the online version, at xx.

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Highlights

- A new linear peptide, named Pembamide, was isolated from the marine sponge *Cribrochalina* sp.
- Structural elucidation was determined by a combination of NMR and MS-MS experiment.
- The absolute configuration of the amino acid residues was determined by application of the Advanced Marfey´s method.
- Pembamide has shown interesting cytotoxic properties against several tumour cell lines.

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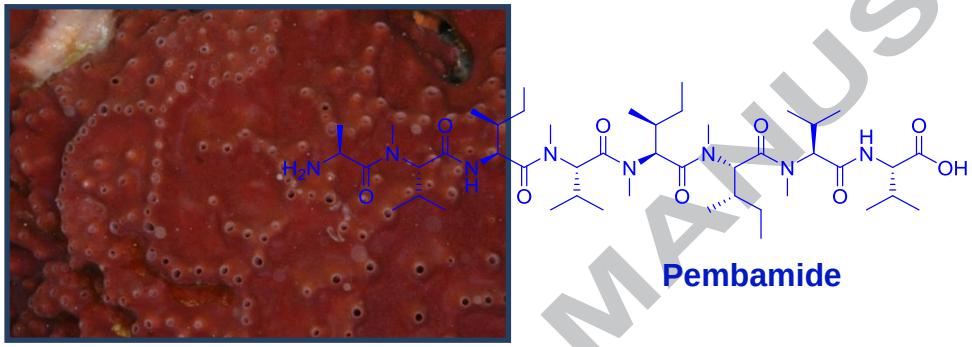
Graphical Abstract



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