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Urumamide, a novel chymotrypsin inhibitor with a β-amino acid from a marine cyanobacterium *Okeania* sp.

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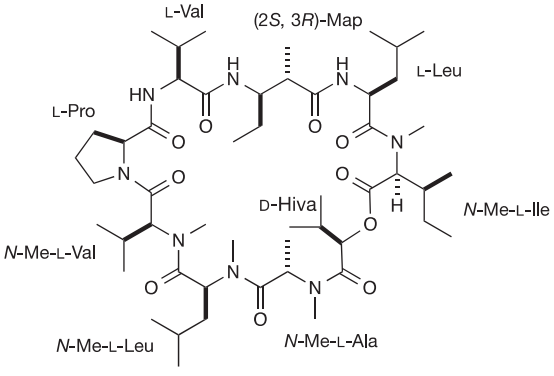
ABSTRACT

Urumamide, a novel cyclic depsipeptide that contains a β-amino acid, was isolated from a marine cyanobacterium *Okeania* sp. Its gross structure was determined by spectroscopic analyses, and the absolute configuration was established based on Marfey's analyses and chiral HPLC analyses of hydrolysis products. Biologically, urumamide inhibited the growth of human cancer cells. In addition, urumamide inhibited chymotrypsin.

Marine cyanobacteria produce many interesting peptides that are biosynthesized by nonribosomal peptide synthetases (NRPS).[1] These peptides are noteworthy not only because of their remarkable structures but also because of their biological activities, such as their ability to inhibit the growth of human cancer cells and/or serine proteases. In our continuing search for new compounds from marine cyanobacteria, we have isolated related peptides, including bisebromoamide,[2] kurahyne,[3] kurahamide,[4] and maedamide.[5]

Here, we report the discovery of urumamide (**1**), a cyclic depsipeptide composed of seven α-amino acids, one α-hydroxy acid, and one β-amino acid, 2-methyl-3-aminopentanoic acid (Map), from a marine cyanobacterium *Okeania* sp. In addition, **1** inhibited the growth of human cancer cells and inhibited chymotrypsin, but not elastase or trypsin. To the best of our knowledge, this is the first report to show that a Map-containing peptide exhibits chymotrypsin–inhibitory activity. Several related peptides containing a Map moiety, such as lyngbyastatin 1,[6] majusculamide C,[7] and guineamide A,[8] have been discovered in marine cyanobacteria. In addition, some types of cyclic depsipeptides have recently been isolated from cyanobacteria, including medusamide A,[9] companeramide A,[10] and precarriebowmide.[11]

The marine cyanobacterial samples (800 g, wet weight) were collected at Ikei Island, Okinawa, and extracted with methanol. The extract was filtered, concentrated, and partitioned between EtOAc and H_2O. The EtOAc–soluble material was further partitioned between 90% aqueous MeOH and hexane. The material obtained from the aqueous MeOH portion was subjected to fractionation with reversed-phase column chromatography (ODS silica gel, MeOH–H_2O) and repeated reversed-phase HPLC to give urumamide (**1**) (11.8 mg) as a colorless oil.



urumamide (**1**)

The molecular formula of **1** was found to be $C_{51}H_{90}N_8O_{10}$ by HRESIMS (*m*/*z* 975.6838, calcd for $C_{51}H_{91}N_8O_{10}$ $[M+H]^+$ 975.6858). The NMR data for **1** are summarized in Table 1. The ^1H NMR spectrum revealed the presence of four singlets corresponding to *N*-methyl amide substituents (δ 3.12, 3.11, 3.01,

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Table 1
NMR data for urumamide (**1**) in CD_3OD[a]

Unit	Position	δ_C[b]	δ_H[c] (*J* in Hz)	COSY	HMBC (H→C)	Selected NOESY
Val	1	174.3				
	2	62.9	3.86, dd (4.8, 8.1)	3, NH	1, 3, 5	
	3	30.6	2.02, m	2, 4, 5	1, 2, 4, 5	
	4	20.9	1.16, d (6.5)	3	2, 3, 5	
	5	20.0	1.08, m	3	2, 3, 4	
	NH		8.36, d (4.8)	2	2, 3, 1 (Pro)	2 (Pro)
Map	1	176.0				
	2	45.7	2.46,m	3, 6	1, 3, 4, 6	
	3	55.9	3.77, m	2, 4a, 4b, NH	1, 2	
	4a	21.5	0.93, m	3		
	4b		1.61, m	3, 5	3, 5	
	5	11.9	0.85, t (7.2)	4b	3, 4	
	6	13.5	1.09, m	2	1, 2, 3	
	NH		8.62, d (9.7)	3	3, 1 (Val)	
Leu	1	176.8				
	2	51.5	4.79, m	3a, 3b, NH		
	3a	39.7	1.38, m	2, 3b, 4	4	
	3b		1.98, m	2, 3a	2, 4, 6	
	4	26.5	1.87, m	3a, 5, 6		
	5	24.9	0.90, m	4	3, 4, 6	
	6	20.7	0.98, m	4	3, 4, 5	
	NH		7.94, d (8.1)	2	2, 3, 1 (Map)	
N-Me-Ile	1	173.3				
	2	61.9	5.39, d (6.3)	3	1, 3, 4, 6, *N*-Me, 1 (Leu)	
	3	36.8	2.24, m	2, 4a, 4b, 6	4, 5	
	4a	28.8	1.39, m	3, 4b, 5	2, 3, 5, 6	
	4b		1.57, m	3, 4a, 5	2, 3, 5, 6	
	5	12.4	0.95, m	4a, 4b	3, 4	
	6	16.9	1.09, m	3	2, 3, 4	
	N-Me	32.9	3.12, s		2, 1 (Leu)	2 (Leu)
Hiva	1	171.2				
	2	76.4	5.29, d (9.0)	3	3, 5, 1 (*N*-Me-Ile)	
	3	31.8	2.24, m	2, 4, 5	1, 2, 4	
	4	18.0	0.99, m	3	2, 3, 5	
	5	19.2	1.11, m	3	2, 3, 4	
N-Me-Ala	1	172.5				
	2	51.9	5.55, m	3	1, 3, *N*-Me, 1 (Hiva)	
	3	14.7	1.23, d (6.7)	2	1, 2	
	N-Me	30.7	3.01, s		2, 1 (Hiva)	
N-Me-Leu	1	173.0				
	2	53.9	5.57, m	3a, 3b	1, 3, 4, *N*-Me	*N*-Me (*N*-Me-Val)
	3a	38.0	1.33, m	2, 3b, 4	4, 5	
	3b		1.75, m	2, 3a	2, 4, 5	
	4	26.6	1.50, m	3a,5, 6	3	
	5	20.9	0.99, m	4	3, 4, 6	
	6	24.3	1.06, d (6.7)	4	3, 4, 5	
	N-Me	33.3	2.69, s		2. 1 (*N*-Me-Ala)	
N-Me-Val	1	172.4				
	2	60.1	5.19, d (11.2)	3	1, 3, 4, 5, *N*-Me	2 (Pro)
	3	28.8	2.24, m	2, 4, 5	2	
	4	19.5	0.91, m	3	2, 3, 5	
	5	18.7	0.98, m	3	2, 3, 4	
	N-Me	31.2	3.11, s		2, 1 (*N*-Me-Leu)	2 (*N*-Me-Leu)
Pro	1	174.0				
	2	60.9	4.93, m	3	3, 4, 5	NH (Val), 2 (*N*-Me-Val)
	3	32.7	2.09, m	2, 4a, 4b	4, 5	
	4a	23.2	1.90, m	3, 4b	3	
	4b		2.09, m	3, 4a	3, 5	
	5a	48.0	3.47, m	4a, 4b, 5b	3, 4	
	5b		3.51, m	4a, 4b, 5a		

[a] ^1H–^{13}C connectivities were determined by the HMQC method.
[b] Measured at 100 MHz.
[c] Measured at 400 MHz.

2.69). In addition, three broad signals (δ 8.62, 8.36, 7.94) of amide protons were observed. In the ^{13}C NMR spectrum, nine carbonyl signals (δ 176.8, 176.0, 174.3, 174.0, 173.3, 173.0, 172.5, 172.4, 171.2) were observed. Based on further analyses of the ^1H NMR, ^{13}C NMR, COSY, HMQC, HMBC, DEPT 135, and DEPT 90 spectra, urumamide was confirmed to contain seven α-amino acids: valine (Val), leucine (Leu), *N*-Me-isoleucine (*N*-Me-Ile), *N*-Me-alanine (*N*-Me-Ala), *N*-Me-leucine (*N*-Me-Leu), *N*-Me-valine (*N*-Me-Val), and proline (Pro). In addition, the presence of two unusual residues, one α-hydroxy acid and one β-amino acid, was also clarified as

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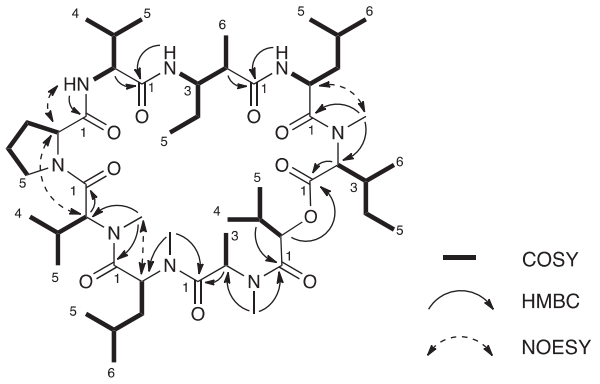

Figure 1. Gross structure of urumamide (**1**) based on 2D NMR correlations.

Table 2
IC_{50} values of urumamide (**1**) for serine protease activities in vitro

Serine proteases	IC_{50} (μM)		
	Chymotrypsin	Elastase	Trypsin
Urumamide (**1**)	33 ± 9	>100	>100
PMSF	480 ± 230	1300	6900

follows. The presence of the α-hydroxy acid residue, a 2-hydroxyisovaleric acid residue (Hiva), was determined on the basis of the low-field shifted signals of the α-position (δ_H 5.29, δ_C 76.4). Meanwhile, the presence of the β-amino acid, Map, was established based on the chemical shifts at the α-position (δ_H 2.46, δ_C 45.7) and the β-position (δ_H 3.77, δ_C 55.9).

The sequences of these partial structures were determined based on HMBC and NOESY data (Table 1 and Fig. 1). HMBC correlations, NH of Val/C-1 of Pro, NH of Map/C-1 of Val, and NH of Leu/C-1 of Map, connected the three residues: Val-Map-Leu. Moreover, three HMBC correlations, H-2 of Hiva/C-1 of *N*-Me-Ile, *N*-Me of *N*-Me-Ala/C-1 of Hiva, and *N*-Me of *N*-Me-Leu/C-1 of *N*-Me-Ala, revealed the sequence as follows: *N*-Me-Ile—Hiva—*N*-Me-Ala-*N*-Me-Leu. Finally, the NOESY correlations, H-2 of Leu/*N*-Me of *N*-Me-Ile, H-2 of *N*-Me-Leu/*N*-Me of *N*-Me-Val, H-2 of *N*-Me-Val/H-2 of Pro, and H-2 of Pro/NH of Val, allowed us to determine the gross structure of **1** as shown in Figure 1.

The absolute configuration of **1** was determined as follows. The stereochemistry of all α-amino acid residues was assigned to be L-form based on the results of Marfey's analyses.[12] Meanwhile, the absolute stereochemistry of the α-hydroxy acid, Hiva, was determined to be D-form on the basis of chiral HPLC analyses.

With regard to Map, (2*R*,3*R*)- and (2*S*,3*R*)-Map were synthesized as described elsewhere.[13] Subsequently, the two Maps were derivatized with D/L-FDLA to afford four authentic standards: D-FDLA-(2*R*,3*R*)-Map, L-FDLA-(2*R*,3*R*)-Map, D-FDLA-(2*S*,3*R*)-Map, and L-FDLA-(2*S*,3*R*)-Map. Meanwhile, we also prepared the L-FDLA derivative of the Map derived from **1**. The HPLC retention time of the L-FDLA derivative of the natural Map matched that of the authentic standard, L-FDLA-(2*S*,3*R*)-Map. Therefore, the stereochemistry of the Map unit was determined to be 2*S*,3*R*.

The growth-inhibitory activities of urumamide against HeLa and HL60 cells were evaluated by the MTT assay. The cells were placed in 96-well plates and treated with various concentrations of compounds (5–50 μM for HeLa cells, 5–50 μM for HL60 cells) for 72 h. As a result, **1** showed weak growth–inhibitory activity against HeLa and HL60 cells with IC_{50} values of 18 ± 0.5 μM and 13 ± 0.5 μM, respectively (n = 3).

In addition, **1** was evaluated for its ability to inhibit serine proteases. The activities of chymotrypsin, elastase and trypsin were tested in vitro. As shown in Table 2, **1** showed inhibitory activity against chymotrypsin with an IC_{50} value of 33 ± 9 μM (n = 3). The inhibitory activity of **1** was much stronger than that of phenylmethylsulfonyl fluoride (PMSF) which was used as a positive control. Moreover, there was no apparent inhibition of elastase or trypsin at 100 μM. So far, a series of dolastatin 13[14] analogs, such as symplocamide A,[15] lyngbyastatins 4–10,[16] and molassamide,[17] has been reported as a strong serine protease inhibitor (IC_{50} 2.5 nM–10 μM). These compounds share the cyclic structure composed of six amino acid residues including 3-amino-6-hydroxy-2-piperidone, and the previous research revealed the detailed structure activity relationships of dolastatin 13 type compounds based on the X-ray structural analyses.[18] Although the serine protease inhibitory-activity of urumamide (**1**) is weaker than them, the structure of urumamide (**1**) is completely different from dolastatin 13 analogs. Therefore, **1** can be expected to be a novel lead for protease inhibitors.

In conclusion, urumamide (**1**), a novel cyclic depsipeptide was isolated from a marine cyanobacterium, *Okeania* sp. The structure of **1** was established by spectroscopic analyses, Marfey's method and chiral HPLC analyses of acid hydrolysates. The structure of urumamide (**1**) contains seven L-α-amino acids, one hydroxy acid, D-Hiva, and one β-amino acid, (2*S*,3*R*)-Map. The stereochemistry of Map is the same as that of previously reported peptides bearing Map. Urumamide exhibited weak growth–inhibitory activity against human cancer cells and inhibited chymotrypsin. To the best of our knowledge, this is the first report to show that a Map-containing peptide exhibits chymotrypsin–inhibitory activity. Other Map-containing peptides may also exhibit the same activity.

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Supplementary data

Supplementary data (^{1}H, ^{13}C, COSY, NOESY, HMQC and HMBC NMR spectra in CD_3OD for urumamide (**1**). HPLC chromatograms for determination of the absolute configurations. A phylogenic tree of the urumamide-producing cyanobacterium. Detailed experimental procedures) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2016.08.012.

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