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Bisebromoamide, a Potent Cytotoxic Peptide from the Marine Cyanobacterium *Lyngbya* sp.: Isolation, Stereostructure, and Biological Activity

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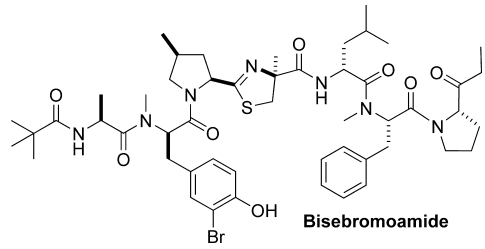
ABSTRACT



A novel cytotoxic peptide, termed bisebromoamide (1), has been isolated from the marine cyanobacterium *Lyngbya* sp. Its planar structure was determined by 1D and 2D NMR spectroscopy. The absolute stereostructure of 1 was determined by chemical degradation followed by chiral HPLC analysis. Bisebromoamide (1) exhibited potent protein kinase inhibition: the phosphorylation of ERK in NRK cells by PDGF-stimulation was selectively inhibited by treatment with 10−0.1 μM of 1.

Natural products, especially those from terrestrial plants and microbes, have long been a source of drug molecules, and pharmacologically active compounds from plants and microbes continue to play an important role in new investigational drugs.[1] However, considerable attention has recently been given to marine organisms due to their remarkable physiological activities.[2] In particular, cyanobacteria are prolific producers of bioactive secondary metabolites[3] and have been recognized as a source of potential pharmaceuticals.[4] For example, TZT-1027, a synthetic analogue of dolastatin 10, is currently being evaluated in phase I clinical trials in Japan, Europe, and the United States.[5] Dolastatin 10 was originally isolated from the sea hare *Dolabella auricularia*[6] and was recently obtained from a marine cyanobacterium.[7] Cryptophycin-309 and cryptophycin-249, which are synthetic analogues of the terrestrial cyanobacterial peptide cryptophycin-1, have undergone preclinical efficacy studies, and there is sufficient interest to consider entering them into a clinical trial.[8]

In our ongoing efforts toward finding novel marine cyanobacterial metabolites with antitumor activity,[9] we report here the structure determination and preliminary biological characterization of bisebromoamide (**1**), a marine cyanobac-

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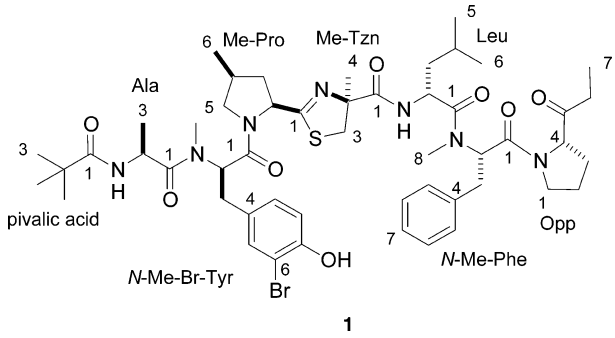
terial metabolite with antiproliferative activity at nanomolar levels from a cyanobacterium of the genus *Lyngbya* sp.



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The marine cyanobacterium *Lyngbya* sp. was directly harvested in Okinawa Prefecture. A crude organic extract of this material was subjected to bioassay-guided fractionation by solvent partition, ODS-HPLC, to yield bisebromoamide (**1**) as a colorless oil.

The HRESIMS spectrum of bisebromoamide (**1**) gave an [M + Na] pseudomolecular ion at *m*/*z* 1044.4212 that was consistent with the pseudomolecular formula $C_{51}H_{72}{}^{79}BrN_7O_8SNa$ (calcd for $C_{51}H_{72}{}^{79}BrN_7O_8SNa$, 1044.4244). The ^1H and ^{13}C NMR analysis showed that it was peptidic in nature; however, there were several resonances that were not attributable to the common ribosomally encoded amino acids, implying that **1** possessed a highly functionalized structure. The ^1H NMR data in $CDCl_3$ showed the presence of two amide NH groups (δ 7.47, 6.37) and two *N*-methylamide groups (δ 3.14, 3.06). The ^1H NMR spectrum showed a prominent intense singlet at δ 1.17 that was attributed to a *tert*-butyl group. A COSY analysis in $CDCl_3$ revealed that these exchangeable protons connected to alanine (Ala) and leucine (Leu) residues, respectively. Further two-dimensional NMR analysis in CD_3OD using COSY, HMQC, and HMBC data suggested the presence of *N*-methyl-3-bromotyrosine (*N*-Me-Br-Tyr), modified 4-methylproline (Me-Pro), *N*-methylphenylalanine (*N*-Me-Phe), and 2-(1-oxopropyl)pyrrolidine (Opp) residues (Table 1). Furthermore, HMBCs from a methyl singlet (H-4; Me-Tzn, δ 1.52) to carbonyl C-1 (Me-Tzn, δ 175.7), quaternary carbon C-2 (Me-Tzn, δ 84.9), and methylene carbon C-3 (Me-Tzn, δ 43.5), combined with H-3 (Me-Tzn, δ 3.23 and 3.37) to C-1 (Me-Pro, δ 180.3), suggested the presence of a 2-substituted thiazoline-4-methyl-4-carboxylic acid unit (Tzn = thiazoline). Finally, detailed HMBC experiments were used to determine the connectivity between six amino acids residues (Table 1). HMBC correlations H3 (pivalic acid)/C1 (pivalic acid) and H2 (Ala)/C1 (pivalic acid) suggested the connectivity of C1 (pivalic acid)−C2 (Ala) via an amide linkage and revealed that **1** possesses an *N*-pivalamide moiety. Although no additional connectivities were obtained from the NMR analysis, the Opp and C-terminus of the *N*-Me-Phe residue were unambiguously connected via an amide linkage, based on its molecular formula and degree of unsaturation. Thus, the planar structure of bisebromoamide (**1**) was determined (Figure 1).

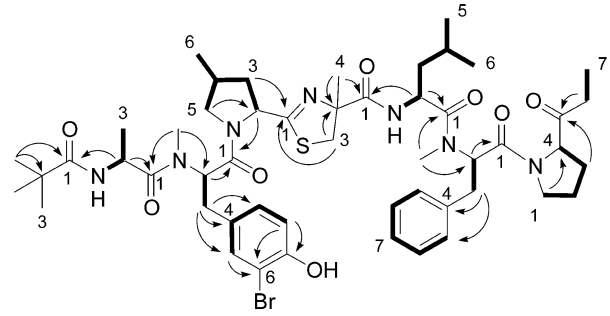

Figure 1. Gross structure of **1** determined by 2D-NMR spectroscopy (bold lines, $^1H-^1H$ COSY; arrows, HMBC correlations).

To assign the absolute configuration of the eight chiral centers, we sought to generate optically active fragments, for which some enantiomeric standards are commercially available (Ala, Leu), while others required laboratory synthesis by standard methods [*N*-Me-Tyr, Me-Pro, 2-methylcystine, *N*-Me-Phe, and Opp]. Acid hydrolysis of **1** generated Ala, *N*-Me-Tyr, Me-Pro, 2-methylcystine, Leu, *N*-Me-Phe, and Opp. The bromine atom of the *N*-Me-Br-Tyr moiety was lost during acid hydrolysis of **1**. The hydrolysate could be separated into single compounds except for a mixture of Ala and 2-methylcystine. Chiral HPLC established the stereochemistry of *N*-Me-Tyr, *N*-Me-Phe, and Leu to be D, L, and D, respectively. Treatment of Me-Pro, Opp, and the mixture of Ala and 2-methylcystine with Marfey's reagent,[10] followed by C18 HPLC, determined that the stereochemistries of Ala and 2-methylcystine were L and D, respectively. However, the Marfey derivatives of both Me-Pro and Opp from **1** completely epimerized during acid hydrolysis. On the other hand, the ozonolysis−acid hydrolysis sequence, which was developed to determine the stereochemistry of thiazole amino acids,[11] provided diastereomerically enriched 4(*S*)-Me-Pro [2*S*:2*R* = 7:3] (Scheme 1).

Scheme 1. Degradation Strategy To Liberate Chiral Subunits

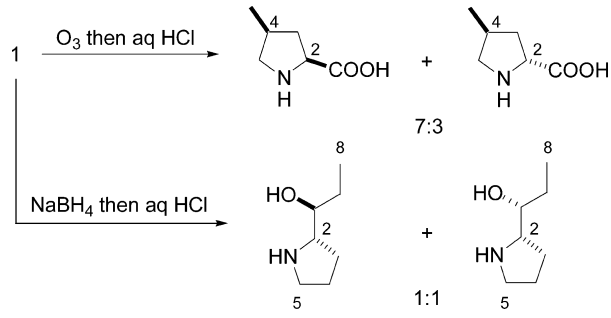

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Table 1. NMR Spectral Data of **1** in CD_3OD

position	$^1H^a$ (ppm)	$^{13}C^b$ (ppm)	HMBC (selected, H→C)
Opp			
1	3.54 m	48.7	4 (Opp)
2	1.88 m	25.7	
3	1.82 m, 2.20 m	29.1	5 (Opp)
4	4.56 dd (8.8, 4.4)	62.6	
5		211.6	
6	2.54 q (7.3)	33.6	5 (Opp)
7	1.03 t (7.3)	7.8	
N-Me-Phe			
1		170.4	
2	5.82 dd (10.3, 5.4)	56.5	1 (*N*-Me-Phe)
3	3.06 m	35.7	4, 5 (*N*-Me-Phe)
4	138.3		
5,9	7.27 dd (7.3, 2.4)	130.7	
6,8	7.20 dd (7.8, 7.3)	129.4	
7	7.09 dd (7.8, 2.4)	127.7	
8	3.07 s	31.1	2 (*N*-Me-Phe), 1 (Leu)
Leu			
1		174.3	
2	4.52 dd (10.8, 3.4)	50.2	1 (Leu), 1 (Me-Tzn)
3	0.67 m, 1.57 m	40.0	
4	1.52 m	25.7	
5	0.81 d (6.4)	21.7	
6	0.79 d (6.4)	23.7	
Me-Tzn			
1		175.7	
2		84.9	
3	3.23 d (11.2),	43.5	1 (Me-Pro), 2 (Me-Tzn)
		3.37 d (11.2)	
4	1.52 s	24.9	1, 2 (Me-Tzn)
Me-Pro			
1		180.3	
2	4.83 dd (10.7, 7.8)	66.5	1 (*N*-Me-Br-Tyr)
3	1.67 m, 2.37 m	39.6	1 (Me-Pro)
4	2.25 m	35.1	
5	2.94 m, 3.56 m	55.7	2 (Me-Pro)
6	1.04 d (6.8)	16.7	
N-Me-Br-Tyr			
1		171.4	
2	5.58 dd (9.3, 6.4)	57.8	1 (*N*-Me-Br-Tyr)
3	2.96 m	34.5	4, 5, 9 (*N*-Me-Br-Tyr)
4		131.2	
5	7.36 d (2.0)	135.1	
6		110.3	
7		154.1	
8	6.81 d (8.3)	117.1	
9	7.07 d (8.3, 2.0)	131.0	
10	3.12 s	32.3	2 (*N*-Me-Br-Tyr), 1 (Ala)
Ala			
1		175.2	
2	4.65 m	46.8	1 (Ala), 1 (pivalic acid)
3	0.95 d (7.3)	16.7	
pivalic acid			
1		180.7	
2		39.3	
3	1.14 s	27.7	1, 2 (pivalic acid)

[a] Recorded at 400 MHz. Coupling constants (Hz) are in parentheses. [b] Recorded at 100 MHz.

To prevent the racemization of Opp, reduction of the ketone with $NaBH_4$ followed by acid hydrolysis afforded 2(*S*)-(1-hydroxypropyl)piperidine [6*S*:6*R* = 1:1]. These analyses identified Me-Pro and Opp as (2*S*,4*S*) and (2*S*), respectively.

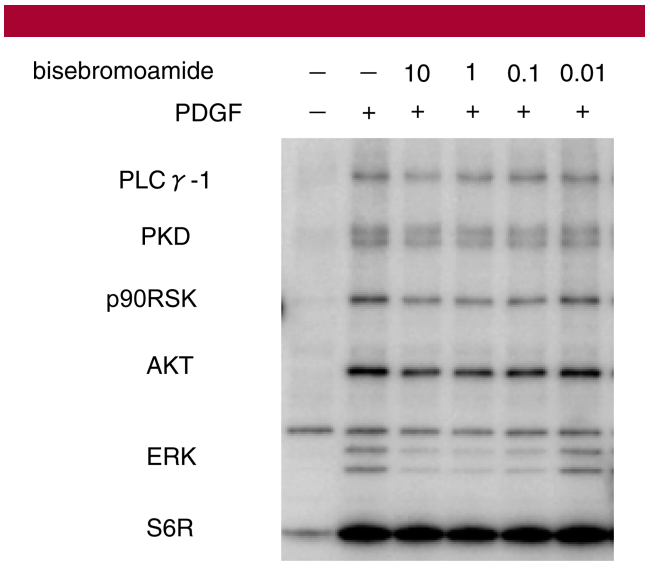

Figure 2. Effect of bisebromoamide (**1**) on PDGF-signaling. NRK cells were preincubated with the indicated concentrations of **1** for 3 h and then stimulated with PDGF for 10 min. The cells were lysed, and proteins were subjected to SDS-PAGE and analyzed by immunoblotting using a cocktail that contained antibodies against phosphorylated forms of the components. A detailed procedure of immunoblotting analysis is described in the Supporting Information.

Therefore, the absolute stereostructure of bisebromoamide (**1**) was determined to be as shown in formula **1**.

Bisebromoamide (**1**) contains a high degree of D-amino acids and *N*-methylated amino acids along with several other modified amino acid residues of nonribosomal origin. Furthermore, bisebromoamide (**1**) possesses a dense combination of unusual structural features, including a substituted Me-Tzn fused to a Me-Pro. Another unusual structural element is the Opp and *N*-Me-Br-Tyr. The Opp unit in **1** is unprecedented in natural products. In addition, **1** possesses an *N*-pivalamide moiety.

Bisebromoamide (**1**) exhibited cytotoxicity against HeLa S_3 cells with an IC_{50} value of 0.04 μg/mL. Bisebromoamide (**1**) was evaluated against a panel of 39 human cancer cell lines (termed JFCR39) at the Japanese Foundation for Cancer Research (see the Supporting Information). The average 50% growth inhibition (GI_{50}) value across all of the cell lines tested was 40 nM. In addition, bisebromoamide (**1**) exhibited potent protein kinase inhibition: the phosphorylation of ERK (extracellular signal regulated protein kinase) in NRK cells by PDGF (platelet-derived growth factor)-stimulation was selectively inhibited by treatment with 10 to 0.1 μM of **1** (Figure 2). Bisebromoamide (**1**) had no effect on the phosphorylation of AKT, PKD, PLCγ1, or S6 ribosomal protein at 10−0.1 μM. Some tubulin modulators have an effect on the phosphorylation of ERK. The pattern of differential cytotoxicity of **1** was evaluated by the Compare Program and was revealed not to be correlated with that shown by tubulin modulators. Immunoblotting analysis using an antiacetylated lysine antibody supported this result. Tubulin acetylation is one marker of microtubule stability and is affected after treatment with some tubulin modulators. The total levels of acetylated tubulin remained unchanged by treatment with **1**. Therefore, the ERK signaling pathways may be one of the intracellular targets of **1**. Aberrant activation of the Ras/Raf/MEK/ERK pathway is commonly observed in various cancers.[12] Thus, the therapeutic targeting of individual components of the Ras/Raf/MEK/ERK pathway has attracted much attention in the development of anticancer drugs. Recently, potent small-molecule inhibitors that target the components of the Ras/Raf/MEK/ERK pathway have been developed. Among them, RAF265, BAY 43-9006, and AZD6244 have reached the clinical-trial stage.[13] Bisebromoamide (**1**) is a promising lead agent in cancer drug discovery.

In summary, bisebromoamide (**1**), a marine cyanobacterial metabolite with antiproliferative activity at nanomolar levels, was isolated from a cyanobacterium of the genus *Lyngbya* sp. Further studies on the mode of action, cancer chemotherapeutic potential, and synthesis of bisebromoamide (**1**) are in progress.

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Supporting Information Available: Protein abbreviations, detailed experimental procedures, spectroscopic data, and HCC panel data for **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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