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Revised structure and structure–activity relationship of bisebromoamide and structure of norbisebromoamide from the marine cyanobacterium *Lyngbya* sp.

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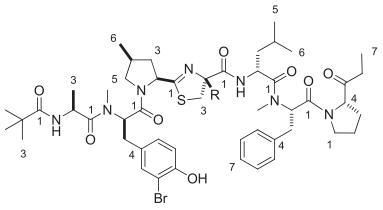
ABSTRACT

Novel potent cytotoxic peptides bisebromoamide (**1**) and norbisebromoamide (**2**) have been isolated from the marine cyanobacterium *Lyngbya* sp. The planar structure of these peptides was elucidated through the extensive application of 1D and 2D NMR techniques. The absolute stereostructure of **1** was determined by chemical degradation followed by chiral HPLC analysis. Recently, Tao and co-workers achieved synthesis of bisebromoamide, and the configuration of thiazoline moiety was revised. We re-investigated the stereochemistry of thiazoline moiety of **1**. The structure–activity relationships of bisebromoamide (**1**) were investigated with the use of natural and synthetic analogs. Furthermore, bisebromoamide (**1**) potently inhibited protein kinase: the phosphorylation of ERK in NRK cells by PDGF-stimulation was selectively inhibited by treatment with 10–0.1 μM of **1**.

1. Introduction

About half of the drugs that are currently in clinical use are of natural origin,[1] which indicates that natural products play a highly significant role in the drug discovery and development process.[2] Natural products, especially those from terrestrial plants and microbes, have long been a traditional source of drug molecules. Indeed, pharmacologically active compounds from plants and microbes play important roles in new investigational drugs.[1] However, much attention has recently been given to compounds from marine organisms due to their remarkable physiological activities.[3] In particular, cyanobacteria are prolific producers of bioactive secondary metabolites[4] and are considered to be a source of potential pharmaceuticals.[5] For example, TZT-1027, a synthetic analog of dolastatin 10, is currently being evaluated in phase I clinical trials in Japan, Europe and the United States.[6] Dolastatin 10 was originally isolated from the sea hare *Dolabella auricularia*[7] and recently from a marine cyanobacterium.[8] Cryptophycin-309 and cryptophycin-249, synthetic analogs of terrestrial cyanobacterial peptide cryptophycin-1, have undergone preclinical efficacy studies, and there is sufficient interest to consider entering them into a clinical trial.[9]

In our ongoing efforts to identify novel marine cyanobacterial metabolites with antitumor activity,[10] we isolated bisebromoamide (**1**),[11] which shows antiproliferative activity at nanomolar levels, from a cyanobacterium of the genus *Lyngbya*. Recently, Tao and co-workers achieved synthesis of bisebromoamide, and the stereochemistry of thiazoline moiety was revised.[12] We report here the full details of a structure analysis of **1** including re-investigation of the stereochemistry of thiazoline moiety of **1** and the isolation and structure of norbisebromoamide (**2**). We also report the structure–activity relationships of natural bisebromoamides and related analogs.



Bisebromoamide (1) : R = Me
Norbisebromoamide (2) : R = H

2. Results and discussion

The marine filamentous cyanobacterium *Lyngbya* sp. was collected at Okinawa Prefecture and extracted with MeOH. The MeOH extract of this material was partitioned between EtOAc and H_2O. The EtOAc-soluble material, which exhibited cytotoxicity against

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HeLa S_3 cells, was further partitioned between 90% aqueous MeOH and hexane. The material obtained from the aqueous MeOH portion was subjected to bioassay-guided fractionation by using ODS silica gel and ODS-HPLC to give bisebromoamide (**1**) and norbisebromoamide (**2**) as a colorless oils.

In its ESIMS spectrum, bisebromoamide (**1**) showed 1:1 doublet ion peals at *m*/*z* 1044 and 1046, indicative of the presence of one bromine atom. The HRESIMS spectrum of bisebromoamide (**1**) gave an [M+Na]+pseudomolecular ion at *m*/*z* 1044.4212 that was consistent with the composition $C_{51}H_{72}{}^{79}BrN_7O_8SNa$ (calcd for $C_{51}H_{72}{}^{79}BrN_7O_8SNa$, 1044.4244). The IR spectrum showed bands at 3522, 3436, 3328, and 1640 cm^{-1} that were assigned to hydroxyl and amide groups. ^1H and ^{13}C NMR analysis showed that it was peptidic in nature. However, there were several resonances, such as a signal at δ_C 211.6 assigned to a ketone group, that were not attributable to the common ribosomally encoded amino acids, implying that **1** possessed a highly functionalized structure. ^1H NMR data in $CDCl_3$ showed the presence of two amide NH groups (δ_H 7.47, 6.37) and two *N*-methylamide groups (δ_H 3.14, 3.06). A COSY analysis in $CDCl_3$ revealed that these exchangeable protons connected to alanine (Ala) and leucine (Leu) residues, respectively. Most prominent in the ^1H NMR spectrum was an intense singlet at δ_H 1.17 that was attributed to a *tert*-butyl group, which is uncommon for natural products. An analysis of the coupling constants of aromatic protons revealed a 1,2,4-trisubstituted benzene ring. The presence of *N*-methyl-3-bromotyrosine was revealed by comparing the ^1H and ^{13}C NMR data with those for geodiamolide B[13] and miuraenamide A.[14] Further two-dimensional NMR analysis in CD_3OD using COSY, HMQC, and HMBC data suggested the presence of modified 4-methylproline (4-Me-Pro), *N*-methylphenylalanine (*N*-Me-Phe) and 2-(1-oxo-propyl)pyrrolidine (Opp) residues. Furthermore, HMBCs from a methyl singlet (H-4; MeTzn, δ_H 1.52) to carbonyl C-1 (MeTzn, δ_C 175.7), quaternary carbon C-2 (MeTzn, δ_C 84.9), and methylene carbon C-3 (MeTzn, δ_C 43.5), combined with that from H-3 (MeTzn, δ_H 3.23, and 3.37) to C-1 (Me-Pro, δ_C 180.3) suggested the presence of a 2-substituted thiazoline-4-methyl-4-carboxylic acid unit. These NMR data were similar to those of tantazoles,[15] mirabazoles,[16] and largazole,[17] which possess the same unit. Finally, detailed HMBC experiments (see Supplementary data) were used to determine the connectivity between six amino acids residues.

HMBC correlations between H3 (pivalic acid)/C1 (pivalic acid) and H2 (Ala)/C3 (pivalic acid) suggested the connectivity of C3 (pivalic acid)-C2 (Ala) and revealed that **1** possesses an *N*-pivalamide moiety (see Supplementary data). Although no additional connectivities were obtained from the NMR analysis, the Opp and C-terminus of *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 to be as shown in **1**.

To assign the absolute configuration of the eight chiral centers, our strategy was to generate optically active fragments by acid hydrolysis, for which some enantiomeric standards are commercially available (Ala, Leu); others required laboratory synthesis by standard methods [*N*-Me-Tyr, 4-Me-Pro, 2-methylcystine, *N*-Me-Phe, and Opp]. Acid hydrolysis of **1** generated Ala, *N*-Me-Tyr, 4-Me-Pro, 2-methylcystine, Leu, *N*-Me-Phe, and 2-Opp. Since the bromine atom was lost during acid hydrolysis, *N*-Me-Tyr was obtained from **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 4-Me-Pro, 2-Opp, and the mixture of Ala and 2-methylcystine with Marfey's reagent,[18] followed by C_{18} HPLC determined that the stereochemistry of Ala and 2-methylcystine were L and D, respectively.[11] Although we reported that the absolute stereochemistry of 2-Me-Cys of **1** was D, Ye and co-workers achieved synthesis of bisebromoamide, and the stereochemistry of thiazoline moiety was revised.[12] So we re-investigated the stereochemistry of thiazoline moiety of **1**. Because HPLC analysis of Marfey derivative of 2-methylcystine from **1** gave complicated result due to partial oxidation of SH group, reduction of 2-methylcystine to 2-Me-Cys with ethyl mercaptan followed by the treatment of Marfey's reagent gave Marfey derivatives of Ala and 2-Me-Cys, respectively. HPLC analysis of Marfey derivatives of Ala and 2-Me-Cys surely established the stereochemistry of 2-Me-Cys to be L. Marfey derivatives of both 4-Me-Pro and 2-Opp from **1** were 1:1 mixtures: these units were completely epimerized during acid hydrolysis. Whereas the ozonolysis-acid hydrolysis sequence, which was developed to determine the stereochemistry of thiazole amino acids,[19] provided diastereomerically enriched 4(*S*)-Me-Pro [2*S*:2*R*=7:3]. To prevent racemization of 2-Opp, reduction of ketone with $NaBH_4$ followed by acid hydrolysis gave 2(*S*)-(1-hydroxypropyl)-piperidine [6*S*:6*R*=1:1]. These analyses identified 4-Me-Pro and 2-Opp as (2*S*, 4*S*) and (2*S*), respectively. Therefore, the absolute stereostructure of bisebromoamide (**1**) was determined to be as shown in formula 1.

The molecular formula of the minor congener norbisebromoamide (**2**) was determined to be $C_{50}H_{70}BrN_7O_8S$ by HRESIMS. Norbisebromoamide (**2**) showed the same NMR spectral features as **1** (Table 1), except that its ^1H NMR spectrum lacked the singlet methyl at δ_H 1.52 for MeTzn H-4 and instead showed a signal of methine at δ 5.05. Interpretation of these NMR data coupled with the molecular formula indicated that **2** was a norderivative of **1**.

The stereochemistries of *N*-Me-Phe, *N*-Me-Tyr, and Ala in norbisebromoamide (**2**) were determined to be L, D, and L, respectively, based on similar experiments. The stereochemistries of Leu, 4-Me-Pro, Opp, and Cys could not be determined due to their epimerization and/or racemization, but the similarity of its NMR spectra to those of **1** suggests that norbisebromoamide (**2**) possess the same stereochemistries as **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 non-ribosomal origin. Furthermore, bisebromoamide (**1**) possesses a dense combination of unusual structural features, including a substituted 4-Me-Tzn fused to a 4-Me-Pro. Another unusual structural elements are the 2-Opp and *N*-methyl-3-bromotyrosine. The 2-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 disease-oriented panel composed of 39 human cancer cell lines (HCC panel) at the Japanese Foundation for Cancer Research (see Supplementary data). The average GI_{50} value across all of the cell lines tested was 40 nM. Although **1** exhibited potent cytotoxicity against 39 human cancer cell lines, the lines were uniformly sensitive to **1**.

In addition, bisebromoamide (**1**) potently inhibited protein kinase: 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**. Bisebromoamide (**1**) had no effect on the phosphorylation of AKT, PKD, PLCγ1, or S6 ribosomal protein at 10 to 0.1 μM. Therefore, the ERK signaling pathways may be intracellular targets of **1**. Aberrant activation of the Ras/Raf/MEK/ERK pathway is commonly observed in various cancers.[20] Thus, **1** was considered to be a potential anticancer drug, which acts against aberrant activated cells of the ERK-MAP pathway, with few side-effects. 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.[21] Bisebromoamide (**1**) may lead to anticancer drugs.

Finally, we investigated the structure–activity relationships of bisebromoamide (**1**) through the use of natural bisebromoamides

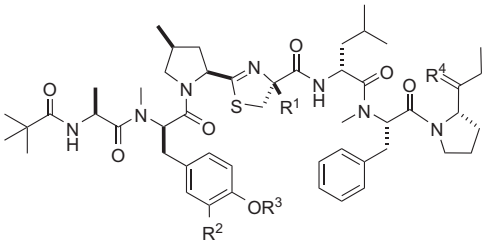
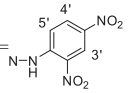
Table 1
NMR spectral data of **2** in CD_3OD

Norbisebromoamide (**2**)		
Position	^1H (ppm)[a]	^{13}C (ppm)[b]
Opp		
1	3.56 m	47.3
2	1.90 m	24.8
3	2.18 m, 1.82 m	28.1
4	4.57 dd (8.9, 5.0)	65.5
5		210.7
6	2.55 q (7.3)	32.5
7	1.04 t (7.3)	6.6
N-Me-Phe		
1		169.4
2	5.80 dd (10.9, 5.7)	55.5
3	3.06 m	34.6
4		137.5
5,9	7.26 dd (7.0, 2.4)	129.7
6,8	7.15 dd (7.9, 7.0)	128.4
7	7.08 dd (7.9, 2.4)	129.9
10	3.07 s	30.0
Leu		
1		173.2
2	4.55 dd (10.8, 3.4)	49.0
3	0.66 m, 1.56 m	38.5
4	1.55 m	24.7
5	0.81 d (7.2)	22.6
6	0.79 d (7.2)	20.6
Tzn		
1		172.2
2	5.05 dd (9.2, 4.0)	78.1
3	3.31 m, 3.64 m	36.6
4		
Me-Pro		
1		181.8
2	4.89 m	61.7
3	1.67 m, 2.37 m	38.6
4	2.25 m	35.0
5	2.94 m, 3.61 m	54.4
6	1.05 d (6.8)	15.7
N-Me-Br-Tyr		
1		170.4
2	5.59 dd (9.0, 6.5)	56.7
3	2.95 m	33.6
4		130.2
5	7.35 d (1.8)	134.1
6		109.5
7		153.1
8	6.81 d (8.5)	116.1
9	7.07 dd (8.5, 1.8)	130.8
10	3.11 s	31.0
Ala		
1		174.2
2	4.64 q (7.2)	45.8
3	0.94 d (7.2)	15.7
Pivalic acid		
1		179.7
2		38.4
3	1.12 s	26.8

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

and synthetic analogs. Alcohol **3**, hydrazone **4**, methylether **5**, and debromobisebromoamide (**6**) were prepared from **1** and evaluated with regard to their cytotoxicities against HeLa S_3 cells. The data shown in Table 2 indicate that norbisebromoamide (**2**) and all of the prepared analogs exhibit potent cytotoxicity, like bisebromoamide (**1**). Therefore, the ketone, bromine atom, and phenolic hydroxyl group of **1** are not important for its cytotoxicity. These functional groups can be used to prepare probe molecules for studying the mode of action and target biomolecules. In particular, we intend to prepare photoreactive group- or biotin-modified probe molecules to identify the target biomolecules.

Table 2
Cytotoxicities of natural bisebromoamides and synthetic analogs

Sample	IC_{50} (ng/mL)[a]
	
1: R^1=Me, R^2=Br, R^3=H, R^4=O (bisebromoamide)	40
2: R^1=H, R^2=Br, R^3=H, R^4=O (norbisebromoamide)	45
3: R^1=Me, R^2=Br, R^3=H, R^4=H, OH	77
4: R^1=Me, R^2=Br, R^3=H, R^4= 	91
5: R^1=Me, R^2=Br, R^3=Me, R^4=O	72
6: R^1=Me, R^2=H, R^3=H, R^4=O	82

[a] Cytotoxicities against HeLa S_3 cells.

3. Conclusion

In summary, bisebromoamide (**1**) and norbisebromoamide (**2**), marine cyanobacterial metabolites with antiproliferative activity at nanomolar levels, were isolated from a cyanobacterium of the genus *Lyngbya* sp. Further studies on the mode of action, cancer chemotherapeutic potential and chemical synthesis of bisebromoamide (**1**), and norbisebromoamide (**2**) are in progress.

4. Experimental section

4.1. Material

The marine cyanobacterium *Lyngbya* sp. was collected at the reef of Bise, Okinawa, Japan (26°40′N, 127°52′E), in April 2007.

4.2. Extraction and isolation

Approximately 1300 g (wet weight) of cyanobacterium was extracted with MeOH (3 L) for 1 week. The extract was filtered, and the filtrate was concentrated. The residue was partitioned between ethyl acetate (3×0.3 L) and H_2O (0.3 L). The material obtained from the organic layer was partitioned between 90% aqueous MeOH and hexane. The aqueous MeOH fraction (0.36 g) was first separated by column chromatography on ODS (6 g) using 40% aqueous MeOH, 60% aqueous MeOH, 80% aqueous MeOH, and 100% MeOH. The fraction (41 mg) eluted with 80% MeOH was subjected to HPLC [Cosmosil $5C_{18}$-AR-II (φ 20×250 mm); flow rate 5 mL/min; detection, UV 215 nm; solvent 65% MeCN] to give bisebromoamide (**1**) (9.6 mg, t_R=36.5 min) and norbisebromoamide (**2**) (0.8 mg, t_R=27.1 min).

*4.2.1. Bisebromoamide (**1**).* Colorless oil; $[\alpha]_D^{22}$ +17.8 (*c* 1.00, $CHCl_3$); IR ($CHCl_3$) 3522, 3436, 3328, 3009, 1640 cm^{-1}; ^1H NMR, ^{13}C NMR, and HMBC data see Supplementary data; ESIMS (rel int) *m*/*z* 1046 $[M+Na+2]^+$ (100), 1044 $[M+Na]^+$ (100); HRESIMS *m*/*z* $[M+Na]^+$ 1044.4212 (calcd for $C_{51}H_{72}{}^{79}BrN_7O_8SNa$, 1044.4244).

*4.2.2. Norbisebromoamide (**2**).* Colorless oil; $[\alpha]_D^{25}$ +11.5 (*c* 0.82, $CHCl_3$); IR ($CHCl_3$) 3522, 3328, 3001, 1640 cm^{-1}; ^1H NMR, ^{13}C NMR

data see Table 1; ESIMS (rel int) m/z 1032 $[M+Na+2]^+$ (100), 1030 $[M+Na]^+$ (100); HRESIMS m/z $[M+Na]^+$ 1030.4097 (calcd for $C_{50}H_{70}{}^{79}BrN_7O_8SNa$, 1030.4088).

4.3. Acid hydrolysis of 1

Bisebromoamide **1** (500 μg, 0.49 μmol) and 9 M HCl (0.1 mL) were added to a reaction tube, which was then sealed under reduced pressure. The mixture was heated at 110 °C for 72 h, diluted with H_2O (1 mL), and evaporated. The acid hydrolysate could be separated into the individual components except for a mixture of Ala and 2-methylcystine. [Conditions for HPLC separation: column, Cosmosil $5C_{18}$-PAQ (4.6×250 mm); solvent, MeOH/H_2O=5/95; flow rate, 1.0 mL/min; detection at 254 nm. Retention times (min) of components: Ala and 2-methylcystine (3.6), Leu (3.9), *N*-Me-Tyr (7.0), *N*-Me-Phe(10.8)]. The absolute stereochemistries of Leu, *N*-Me-Tyr, and *N*-Me-Phe were determined by chiral HPLC analysis. Leu: [column, CHIRALPAK(MA+) (4.6×50 mm); solvent, 2 mM $CuSO_4$; flow rate, 1.0 mL/min; detection at 254 nm] t_R (min): D-Leu (7.5), L-Leu (13.2).

N-Me-Tyr: [column, CHIRALPAK(MA+) (4.6×50 mm); solvent, MeOH/2 mM $CuSO_4$=95/5; flow rate, 0.5 mL/min; detection at 254 nm] t_R (min): *N*-Me-D-Tyr (11.3), *N*-Me-L-Tyr (14.1).

N-Me-Phe: [column, CHIRALPAK(MA+) (4.6×50 mm); solvent, MeCN/2 mM $CuSO_4$=90/10; flow rate, 0.5 mL/min; detection at 254 nm] t_R (min): *N*-Me-D-Phe (11.3), *N*-Me-L-Phe (12.5).

An aqueous solution of a mixture of Ala and 2-methylcystin was treated with ethyl mercaptan at room temperature for 18 h. The absolute stereochemistry of obtained Ala and 2-methylcysteine was determined by HPLC analysis of their Marfey derivatives. Ala: [column, Cosmosil $5C_{18}$-AR-II (4.6×250 mm); solvent, MeOH/0.02 M AcONa=65/35; flow rate, 1.0 mL/min; detection at 340 nm] t_R (min): D-Ala (9.2), L-Ala (5.0).

2-Methylcysteine: [column, Cosmosil $5C_{18}$-MS-II (4.6×250 mm); solvent, MeOH/0.02 M AcONa=60/40; flow rate, 1.0 mL/min; detection at 340 nm] t_R (min): D-2-methylcysteine (8.9), L-2-Methylcysteine (8.2).

4.4. Ozonolysis-acid hydrolysis of 1

Ozone gas was bubbled through a solution of bisebromoamide **1** (4.0 mg, 3.9 μmol) in MeOH (4 mL) at −78 °C for 30 min. Excess ozone was evacuated by bubbling nitrogen, and dimethyl sulfide (0.1 mL) was added. The mixture was warmed to room temperature and concentrated to give a colorless oil. This oil and 9 M HCl (0.1 mL) were added to a reaction tube and the tube was sealed under reduced pressure. The mixture was heated at 110 °C for 48 h, diluted with H_2O (1 mL), and evaporated. The residue was purified by reversed phase HPLC [column: Cosmosil $5C_{18}$-PAQ (20×250 mm); solvent: MeOH/H_2O/TFA=1/99/0.1; flow rate: 5.0 mL/min; detection at 215 nm] to afford Me-Pro (t_R=21.8 min, 2*S*:2*R*=7:3). Based on the ^1H NMR data, the relative stereochemistries of the two stereoisomers were established. The major isomer was assigned as *cis*-4-methylproline and the minor isomer was assigned as *trans*-4-methylproline. The absolute stereochemistry of 4-Me-Pro was determined by HPLC analysis of its Marfey derivative. [Column, Cosmosil $5C_{18}$-MS-II (4.6×250 mm); solvent, MeOH/0.02 M AcONa=70/30; flow rate, 1.0 mL/min; detection at 340 nm] t_R (min): (2*S*,4*S*)-4-Me-Pro (4.5), (2*R*,4*R*)-4-Me-Pro (8.0).

4.5. Reduction-acid hydrolysis of 1

To a stirred solution of **1** (3.5 mg, 3.4 μmol) in MeOH (0.5 mL) at room temperature was added sodium borohydride (30 mg, 0.79 mmol). After being stirred at room temperature for 2 h, the mixture was diluted with AcOEt (5 mL) and H_2O (5 mL). The organic layer was separated, and the aqueous layer was extracted with AcOEt (2×10 mL). The organic layer and the extracts were combined, washed with saturated aqueous NaCl (15 mL), dried (Na_2SO_4), and concentrated. The residual oil (3.3 mg, 3.2 μmol) and 9 M HCl (0.1 mL) were added to a reaction tube, and the tube was sealed under reduced pressure. The mixture was heated at 110 °C for 48 h, diluted with H_2O (1 mL), and evaporated. The residue was purified by reversed phase HPLC [column: Cosmosil $5C_{18}$-PAQ (20×250 mm); solvent: MeOH/H_2O/TFA=1/99/0.1; flow rate: 5.0 mL/min; detection at 215 nm] to afford 2(*S*)-(1-hydroxypropyl)-piperidine [t_R=33.9 min, 6*S*:6*R*=1:1]. The absolute stereochemistry of C2 was determined by HPLC analysis of its Marfey derivative.

Condition 1: [column, Cosmosil $5C_{18}$-MS-II (4.6×250 mm); solvent, MeOH/0.02 M AcONa=70/30; flow rate, 1.0 mL/min; detection at 340 nm] t_R (min): 2(*R*)-(1-hydroxypropyl)-piperidine [6*S*:6*R*=1:1] (9.7, 3.3), 2(*S*)-(1-hydroxypropyl)-piperidine [6*S*:6*R*=1:1] (15.3, 3.3).

Although the retention time of one diastereomer of Marfey derivatives of authentic samples was identical to that from natural **1** (retention time: 15.3 min), two diastereomers of Marfey derivatives of authentic samples could not be separated under the above condition (retention time: 3.3 min). However, these diastereomers could be separated under the following conditions.

Condition 2: [column, Cosmosil $5C_{18}$-MS-II (4.6×250 mm); solvent, MeCN/0.02 M AcONa=40/60; flow rate, 1.0 mL/min; detection at 340 nm] t_R (min): 2(*R*)-(1-hydroxypropyl)-piperidine [6*S*:6*R*=1:1] (33.4), 2(*S*)-(1-hydroxypropyl)-piperidine [6*S*:6*R*=1:1] (30.4).

*4.5.1. Alcohol **3**.* To a stirred solution of **1** (2.5 mg, 2.44 μmol) in MeOH (0.50 mL) was added $NaBH_4$ (30.0 mg, 0.79 mmol). The mixture was stirred at room temperature for 2 h, diluted with H_2O (5 mL) and AcOEt (5 mL), and then extracted with EtOAc (3×10 mL). The combined extracts were washed with brine (15 mL), dried (Na_2SO_4), and concentrated. The residual oil was purified by column chromatography on silical gel (5 g, hexane/EtOAc 1:15) to give alcohol **3** (2.1 mg, 84%) as a colorless oil: TLC, R_f 0.10 (hexane/EtOAc 1:15); $[\alpha]_D^{25}$ +2.5 (*c* 0.17, $CHCl_3$); IR ($CHCl_3$) 3431 (br), 3022, 1641 cm^{-1}; ^1H NMR see Supplementary data; ESIMS (rel int) m/z 1048 $[M+Na+2]^+$ (100), 1046 $[M+Na]^+$ (100); HRESIMS m/z $[M+Na]^+$ 1046.4413 (calcd for $C_{51}H_{74}{}^{79}BrN_7O_8SNa$, 1046.4401).

*4.5.2. Hydrazone **4**.* To a solution of 2,4-dinitrophenyl hydradine (125 mg, 0.63 mmol) in MeOH (5.0 mL) was added concd H_2SO_4 (0.3 mL) at room temperature. This solution (3 mL) was added dropwise to a stirred solution of bisebromoamide (**1**) (3.3 mg, 3.22 μmol) in MeOH (2.0 mL) at room temperature. The mixture was stirred for 3 h, diluted with H_2O (5 mL) and AcOEt (5 mL), and then extracted with EtOAc (3×15 mL). The combined extracts were washed with brine (15 mL), dried (Na_2SO_4), and concentrated. The residual oil was purified by PLC (hexane/EtOAc 1:15) to give hydrazone **4** (3.6 mg, 92%) as a yellow oil: TLC, R_f 0.58 (hexane/EtOAc 1:15); $[\alpha]_D^{25}$ +9.1 (*c* 0.46, $CHCl_3$); IR ($CHCl_3$) 3522, 3004, 1642, 1519 cm^{-1}; ^1H NMR see Supplementary data; ESIMS (rel int) m/z 1204 $[M+H+2]^+$ (100), 1202 $[M+H]^+$ (100); HRESIMS m/z $[M+H]^+$ 1202.4689 (calcd for $C_{57}H_{77}{}^{79}BrN_{11}O_{11}S$, 1202.4708).

*4.5.3. Methylether **5**.* To a stirred solution of **1** (3.0 mg, 2.94 μmol) in DMF (0.2 mL) were added K_2CO_3 (5.2 mg, 0.037 mmol), Bu_4NI (2.7 mg, 0.0073 mmol), and MeI (0.05 mL, 0.80 mmol). The mixture was stirred at room temperature for 4 h, and the reaction mixture was diluted with saturated NH_4Cl aqueous (1 mL) and AcOEt (5 mL) and then extracted with EtOAc (3×10 mL). The combined extracts were washed with brine (15 mL), dried (Na_2SO_4), and concentrated. The residual oil was purified by PLC (hexane/EtOAc 1:20) to give methylether **5** (3.0 mg, 99%) as a colorless oil: TLC, R_f 0.80 (hexane/EtOAc 1:20); $[\alpha]_D^{25}$ +5.2 (*c* 0.30, $CHCl_3$); ^1H NMR see Supplementary data; ESIMS (rel int) m/z 1038 $[M+H+2]^+$ (100), 1036 $[M+H]^+$

(100); HRESIMS m/z $[M+H]^+$ 1036.4611 (calcd for $C_{52}H_{75}{}^{79}BrN_7O_8S$, 1036.4581).

*4.5.4. Debromobisebromoamide (**6**).* A mixture of bisebromoamide (**1**) (1.0 mg, 0.98 μmol) and 5% Pd/C (4.6 mg) in EtOH (0.5 mL) was stirred under a hydrogen atmosphere at room temperature for 5 h. The mixture was filtered through a pad of Celite, and the residue was washed with MeOH. The filtrate and the washings were combined, concentrated, and purified by PLC ($CHCl_3$/MeOH 10:1) to give a debromobisebromoamide (0.9 mg, 92%) as a colorless oil: TLC, R_f 0.25 ($CHCl_3$/MeOH 10:1). $[\alpha]_D^{25}$ +1.6 (*c* 0.07, $CHCl_3$); ^1H NMR see Supplementary data; HRESIMS m/z $[M+Na]^+$ 966.5140 (calcd for $C_{51}H_{73}N_7O_8SNa$, 966.5139).

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Supplementary data

Detailed experimental procedures, spectroscopic data, and HCC panel data for **1**. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2010.11.106. These data include MOL files and InChiKeys of the most important compounds described in this article.

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