

ORIGINAL ARTICLE

Fradiamine A, a new siderophore from the deep-sea actinomycete *Streptomyces fradiae* MM456M-mF7

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New bioactive substances were identified from several marine actinomycetes strains by LC-HRESI-MS based non-targeted metabolomics. A new siderophore and its derivative, named fradiamines A and B, were isolated from the extract of the deep-sea actinomycetes *Streptomyces fradiae* MM456M-mF7 by Diaion CHP-20P, Sephadex LH-20 column chromatography and HPLC. Fradiamine A was a new compound, but fradiamine B was previously patented as a sweetness enhancer. Their structures were determined by NMR and LC-HRESI-MS/MS analysis. Fradiamines A and B contained two alkyl amines asymmetrically bonded to citrate, a type of structure derived from actinomycetes and other bacteria and rarely observed in siderophores. Fradiamines A and B showed moderate antibiotic activity against *Clostridium difficile* with IC₅₀ values of 32 and 8 µg ml⁻¹, respectively.

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INTRODUCTION

The secondary metabolites of terrestrial actinomycetes are major sources of novel bioactive compounds.^{1,2} Recently, discovering efficiency of the medically useful antibiotics with novel structures has been decreased.^{3,4} The deep-sea provides unique environmental factors different from those of the terrestrial environment, such as temperature, aeration and nutrition availability. These factors all affect the expression of secondary metabolite genes.^{5,6} Numerous novel bioactive compounds were isolated from marine organisms.⁷ Marine actinomycetes also produced novel useful compounds, including the anticancer candidates salinosporamide A isolated from *Salinispora tropica*⁸ and thiocoraline isolated from *Micromonospora marina*.⁹ Novel bioactive compounds are identified using biological activity-based screening methods. However, many bioactive compounds escape detection because suitable tests for measuring their biological activity are still missing. Physicochemical screening methods can also be used to detect novel bioactive compounds and avoid the limitations of bioactive assays. For example, the antimicrobial dienomycin isolated from *Streptomyces* sp.,¹⁰ and the antifungal and protein kinase inhibitory substance staurosporine isolated from *Streptomyces staurosporeus*¹¹ were discovered from the secondary metabolites of actinomycetes by color reaction-based physicochemical screening. In recent years, LC-HRESI-MS-based non-targeted metabolomics have been used in many laboratories. Several novel bioactive compounds were discovered using this method including photo-oxidative hemolysis inhibitors, trehangelines isolated from *Polymorphospora rubra*,¹² and the antitrypanosomal substance mangromycin isolated from *Lechevalieria aerocolonigenes*.¹³ Therefore, we expected that novel

compounds will be isolated from marine actinomycetes by physicochemical screening.

In this study, we screened secondary metabolites of various actinomycetes isolated from deep-sea sediment for novel bioactive compounds by LC-HRESI-MS analysis-based non-targeted metabolomics, a type of physicochemical screening.^{14,15} We identified two components not listed in the database from the extract of actinomycete strain MM456M-mF7 isolated from deep-sea sediment collected from the coast of Sagami Bay, Japan. The components were named fradiamines A (1) and B (2). Herein, we describe the isolation, structure determination and biological activities of fradiamines A and B.

RESULTS AND DISCUSSION

Screening

Screening was performed based on LC-HRESI-MS analysis of the solvent extracts of fermentation materials, and extracted peaks from the MS data were identified using our in-house accurate masses database. The MS data analyses were performed using MS component extraction software to obtain accurate masses and the retention time index. These values were collated to our in-house accurate masses library (12 019 compounds). In the course of the screening, we found two components not listed in our library from actinomycete strain MM456M-mF7. The components were identified as protonated molecules of *m/z* 435.2089 (1) and 449.2243 (2).

Taxonomy of the strain MM456M-mF7

The strain MM456M-mF7 was isolated from sediment from the inside of *Calyptogena* community, Sagami Bay, Japan (35°0.940'N

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The authors dedicate this work to Professor Satoshi Omura, a distinguished Nobel Prize awardee in Physiology or Medicine 2015.

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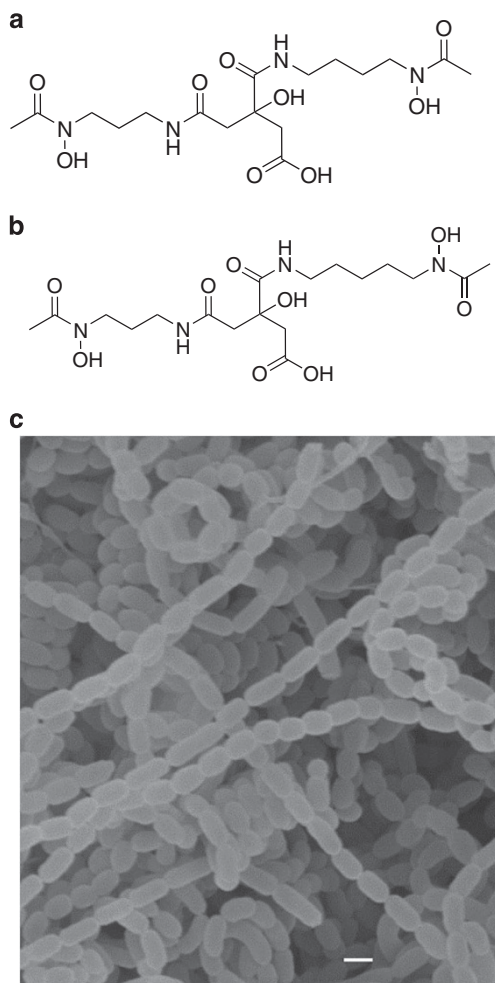


Figure 1 Structures of **1** (a) and **2** (b). (c) Scanning electron micrograph of strain MM456M-mF7 after growing on inorganic salt-starch agar (ISP medium no. 4) for 7 days at 30 °C. Scale bar, 1 μm .

139°13.222'E, depth: 806 m). Strain MM456M-mF7 formed well-branched substrate mycelia. This strain formed straight to flexuous aerial mycelia with imperfect or regular spirals of three to five turns (Figure 1c). Mature spore chains consisted of 11 to 30 or more spores. The spore was oval with a smooth surface and $0.6 \times 0.6\text{--}1.4 \times 0.5 \mu\text{m}$ in size. The substrate mycelia were pale yellowish brown. The aerial mycelia were pinkish white to pink. These characteristics were observed on inorganic salt-starch agar (ISP medium no. 4). The type of diaminopimelic acid isomer in whole-cell hydrolysates of the strain MM456M-mF7 was the LL-form. The partial 16S ribosomal RNA gene sequence (1426 bp) was determined. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of MM456M-mF7 is LC120624. The strain showed high similarity with *Streptomyces fradiae* (NBRC 12773^T, 1426/1426 bp, T: Type strain, 100%). These phenotypic and genotypic data suggested that strain MM456M-mF7 belongs to *S. fradiae*. Therefore, the strain was designated as *S. fradiae* MM456M-mF7.

Fermentation and isolation

Fradiamines A (**1**) and B (**2**) were produced by solid-state culture. A total of 7 ml of seed culture was transferred into a 500-ml K-1 flask containing 40 g of a producing medium. The fermentation was carried out at 30 °C for 14 days in the dark.

Table 1 Physicochemical properties of compounds **1** and **2**

	1	2
Appearance	Colorless syrup	Colorless syrup
Optical rotation $[\alpha]_D^{25}$	+14.1° (c 0.39, MeOH)	+15.8° (c 0.85, MeOH)
UV $\lambda_{\text{max}}^{\text{H}_2\text{O}}$	End absorption	End absorption
IR (KBr) $\nu_{\text{max}} \text{ cm}^{-1}$	3397, 2938, 2874, 1715, 1637, 1543, 1420, 1227	3400, 2938, 2867, 1716, 1633, 1542, 1419, 1227
Molecular formula	$\text{C}_{17}\text{H}_{30}\text{N}_4\text{O}_9$	$\text{C}_{18}\text{H}_{32}\text{N}_4\text{O}_9$
HRESI-MS (m/z)		
Calculated	435.2086 ($\text{C}_{17}\text{H}_{30}\text{N}_4\text{O}_9+\text{H}$) ⁺	449.2242 ($\text{C}_{18}\text{H}_{32}\text{N}_4\text{O}_9+\text{H}$) ⁺
Found	435.2089 (M+H) ⁺	449.2243 (M+H) ⁺
Color reaction		
Positive	I ₂ , Rydon–Smith, iron(III) chloride	I ₂ , Rydon–Smith, iron(III) chloride
Negative	Ninhydrin	Ninhydrin

A total of 600 g of the fermentation material was extracted with EtOH and was filtered. The filtrate containing the fradiamines was purified by column chromatography with CHP-20P eluted with 50% MeOH, and rechromatographed on the same column eluted with 0.01 M NaOH. Compounds **1** and **2** were separated during this process.

The fractions containing **1** were chromatographed on a CHP-20P column by stepwise-gradient elution from 0 to 60% MeOH. The fractions were further purified with reverse-phase octadecylsilane and HILIC (hydrophilic interaction liquid chromatography) HPLC to give 10.5 mg of **1** as a colorless syrup.

The fractions containing **2** were further purified by CHP-20P column chromatography, eluted with 50% MeOH, loaded on a LH20 column and eluted with 50% MeOH to give 187.3 mg of **2** as a colorless syrup.

Structure determination

The physicochemical properties of the fradiamines are summarized in Table 1. The UV spectra of **1** and **2** showed end absorption. Under the presence of molar equivalent of iron(III) chloride, characteristic absorbance was observed in visible range (Supplementary Figures S3 and S4). The IR spectra of **1** and **2** showed specific absorption derived from the amide bond (ν_{max} of **1** was 1637 and 1543 cm^{-1} and that of **2** was 1633 and 1542 cm^{-1}). The color reaction of **1** and **2** on silica gel TLC were positive to the Rydon–Smith reaction and iron (III) chloride, and negative to ninhydrin. These data suggested that **1** and **2** were peptidic compounds. The molecular formulas of **1** and **2** were determined as $\text{C}_{17}\text{H}_{30}\text{N}_4\text{O}_9$ and $\text{C}_{18}\text{H}_{32}\text{N}_4\text{O}_9$, respectively, by LC-HRESI-MS. The ¹H, ¹³C NMR and HMQC spectral data of the compounds are summarized in Table 2.

The ¹³C NMR and DEPT135 spectra of **1** revealed the presence of two methyls, nine *sp*³ methylenes, five carbonyls and an oxygenated *sp*³ quaternary carbon. The presence of two amide protons in the ¹H NMR and four exchangeable protons were suggested by the molecular formula. The correlations from ¹H-¹H COSY and HMBC are summarized in Figure 2a. The ¹H-¹H COSY spectrum revealed the connectivities from δ 8.02 (1'-NH) to δ 3.47 (4'-H) and from δ 7.76 (1''-NH) to δ 3.42 and 3.49 (5''-H). These data suggested that **1** contained C3 and C4 alkyl diamine units. The HMBC spectrum revealed that **1** contains a citric acid core and two acetylated alkyl

Table 2 ^1H and ^{13}C NMR data of **1** and **2** in $\text{DMSO-}d_6$

Position	1				2			
	δ_{C} (p.p.m.)	Mult ^a	δ_{H} (p.p.m.)	Mult ^a	δ_{C} (p.p.m.)	Mult ^a	δ_{H} (p.p.m.)	Mult ^a
1	171.6	s			171.3	s		
2	43.1	t	2.41, 2.60	d 14.6, d 14.7	43.0	t	2.44, 2.63	d 14.8, d 14.8
3	74.3	s			74.3	s		
4	42.4	t	2.44, 2.50	m, m	42.2	t	2.46, 2.51	d 14.3, d 14.3
5	170.5	s			170.6	s		
6	173.5	s			173.3	s		
1'			8.02	br			8.06	brt 5.3
2'	36.2	t	3.02	m	36.2	t	3.02	m
3'	26.5	t	1.62	m	26.5	t	1.62	m
4'	45.1	t	3.47	m	45.1	t	3.47	m
5'			9.73	brs			9.73	brs
6'	170.3	s			170.3	s		
7'	20.3	q	1.96	s	20.3	q	1.95	s
1''			7.76	t 5.3			7.74	t 5.8
2''	38.2	t	2.99, 3.07	m	38.5	t	3.02	m
3''	26.3	t	1.34	m	28.7	t	1.38	m
4''	23.6	t	1.47	m	23.4	t	1.19	m
5''	46.6	t	3.42, 3.49	m, m	26.1	t	1.47	m
6''			9.73	brs	46.8	t	3.44	m
7''	170.1	s					9.73	brs
8''	20.4	q	1.96	s	170.2	s		
9''					20.4	q	1.95	s

^aMultiplicity and coupling constant in Hz.

amines. The long-range couplings observed in the HMBC experiments can be summarized as follows. The cross-peaks from δ 2.41 and 2.60 (2-H) to δ 171.6 (C-1), δ 74.3 (C-3), δ 42.4 (C-4) and δ 173.5 (C-6), and from δ 2.44 and 2.50 (4-H) to δ 43.1 (C-2), δ 74.3 (C-3), δ 170.5 (C-5) and δ 173.5 (C-6) supported the presence of citric acid. The cross-peak from δ 1.96 (7'-H) to δ 170.3 (C-6') and δ 1.96 (8''-H) to δ 170.1 (C-7'') supported the presence of two acetyl groups. The nitrogen-bearing methylene at δ 3.47 (4'-H) showed a correlation with the carbonyl carbon at δ 170.3 (C-6'), thus establishing the connectivity of the C3 alkyl diamine to the *N*-acetyl group. The alkyl proton at δ 3.42 and 3.49 (5''-H) showed a correlation with the carbonyl carbon at δ 170.1 (C-7''), establishing the connectivity of the C4 alkyl diamine to another *N*-acetyl group in the same manner. The amide proton at δ 8.02 (1'-H) showed a correlation with the carbonyl carbon at δ 170.5 (C-5); thus, the C3 alkyl amine and the carbonyl carbon (C-5) formed a peptide bond. The amide proton at δ 7.76 (1''-H) showed a correlation with the carbonyl carbon at δ 173.5 (C-6), establishing the connectivity of the C4 alkyl amine to the C-6 carbonyl carbon via a peptide bond. The remaining atoms were estimated to be hydroxyl groups connected with C-1, C-3, N-5' and N-6'' to form a carboxylic acid OH, a tertiary OH and two *N*-hydroxy groups, respectively, from the molecular formula of **1**. The MS/MS fragments (m/z 147.1128 and m/z 133.0970) of **1** and downfield shifts of the *N*-hydroxy groups bearing a methylene at C-4' and C-5'' compared with the amide NH bearing a methylene at C-2' and C-2'' and positive color reaction with iron(III) chloride on TLC supported the position of the *N*-hydroxy groups (Figure 2c). The planar structure of **1** was determined as shown in Figure 1a.

The NMR data of **2** were very similar to that of **1**. The presence of a longer methylene chain was suggested by the ^1H - ^1H COSY and the HMBC revealed a longer methylene chain connected from δ 7.74 (1''-NH) to δ 3.44 (6''-H) (Figure 2b). Moreover, the observed

fragment ion at m/z 161.1285 instead of m/z 147.1128 from MS/MS analysis (Figure 2d) also supported the presence of the longer methylene chain. Based on these results and the molecular formula, the planar structure of **2** was determined as shown in Figure 1b.

The compounds contained a carboxylic acid and two *N*-hydroxyl groups, features of iron chelators. Thus, we determined that both compounds were siderophores. Compound **1** was new and **2** was patented as a sweetness enhancer.¹⁶ These compounds had two alkyl diamines asymmetrically bonded to the citric acid core. A related structure, aerobactin, known as a bacterial siderophore, was isolated from *Aerobacter aerogenes*,¹⁷ rhizobactin 1021 was isolated from *Rhizobium meliloti*¹⁸ and schizokinen was isolated from *Bacillus megaterium*.¹⁹ However, almost no similar structures were identified from actinomycetes. Moreover, structures containing asymmetrically bonded alkyl diamines with a citric acid core are very rare in siderophores including bacterial siderophores. LC-HRESI-MS analysis is a suitable method for screening secondary metabolites of actinomycetes for new compounds with unique structures.

Antimicrobial activity

It is known that some siderophores have moderate antibiotic activities against some bacteria, such as nocardamine isolated from *Nocardia* sp.²⁰ and madurastatins isolated from *Actinomadura madurae*.²¹ Thus, the antimicrobial activity of **1** and **2** was evaluated along with other synthetic or natural siderophores against Gram-positive and Gram-negative bacteria including anaerobic bacteria. The antimicrobial activities of the compounds are shown in Table 3. Compounds **1** and **2** exhibited moderate antimicrobial activities against *Clostridium difficile* BAA-1382 at 32 and 8 $\mu\text{g ml}^{-1}$, respectively. The antimicrobial activities of the compounds were, however, cancelled dose-dependently under the presence of iron(III) chloride (Supplementary Table S1). The spectra of antimicrobial activities of

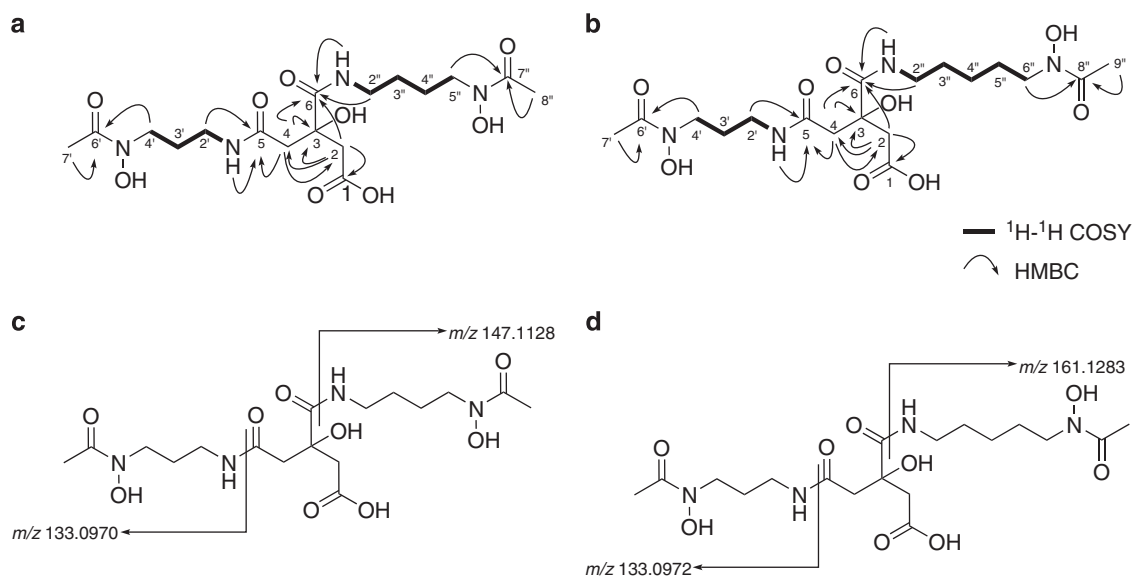


Figure 2 Key COSY (bold lines) and HMBC (single-headed arrows) correlations for compounds **1** (a) and **2** (b). MS/MS analyses of **1** (c) and **2** (d).

Table 3 Antimicrobial activities of **1** and **2**

Test microorganisms		MIC ($\mu\text{g ml}^{-1}$)				
		1	2	Tiron	Nocardamine	Mycobactin J
<i>Clostridium bifermentans</i>	JCM 1386T	> 128	> 128	> 128	16	4
<i>C. butyricum</i>	JCM 1391T	64	128	32	16	16
<i>C. difficile</i>	BAA-1382	32	8	16	8	32
<i>C. indolis</i>	JCM 1380T	> 128	16	32	16	> 64
<i>C. innocuum</i>	JCM 1292T	> 128	32	64	8	> 64
<i>C. limosum</i>	JCM 1427T	> 128	128	128	16	2
<i>C. perfringens</i>	B-0866	> 128	> 128	> 128	32	> 128
<i>C. ramosum</i>	JCM 1298T	> 128	64	64	32	> 64

the compounds were similar to those of other siderophores (Supplementary Tables S2 and S3).

METHODS

General

The optical rotations of the purified compounds were measured using a P-1030 polarimeter (JASCO, Tokyo, Japan). The UV spectra were recorded with a U-2800 UV-Vis spectrophotometer (Hitachi High-Technologies, Tokyo, Japan). The IR spectra were recorded with an FT/IR-4100 Fourier transform infrared spectrometer (JASCO). The ¹H and ¹³C NMR spectra were measured with an AVANCE III 500 spectrometer (Bruker, Billerica, MA, USA) and an ECZ600R spectrometer (JEOL RESONANCE, Tokyo, Japan) at 25 °C using tetramethylsilane as an internal reference. The mass spectra were recorded using a Q Exactive or LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA).

Screening

A total of 239 deep-sea actinomycetes were extracted with EtOH. The 10-fold diluted EtOH extract was analyzed with LC-HRESI-MS. HPLC was performed using a SPELCO Ascentis Express C₁₈ column (2.1 mm i.d. × 50 mm; Sigma-Aldrich, St Louis, MO, USA) at 40 °C. The mobile phase consisted of 0.1% HCOOH aq. (A) and 0.1% HCOOH-CH₃CN (B), with a gradient of 95% A to 0% A (0–5 min), 0% A (5–8.5 min) and 95% A (8.5–12 min). The flow rate was 0.4 ml min⁻¹. Compound **1** and **2** was eluted at 1.32 and

1.60 min, respectively. MS data acquisition was performed sequentially in the order of positive full MS, negative full MS and positive MS/MS using a heated ESI probe with a spray voltage of 4 kV, a capillary temperature of 350 °C and an S lens RF level of 50. The MS range was scanned from *m/z* 133.5 to 2000 (full MS resolution 35 000 and MS/MS resolution 17 500). The MS data analyses were performed by Xcalibur 2.2 (Thermo Fisher Scientific) and the component extraction software SIEVE 2.2 was used (Thermo Fisher Scientific) to get accurate masses and the retention time index.

Taxonomic studies of the strain MM456M-mF7

Morphological properties were observed following incubation at 30 °C for 21 days on yeast extract-malt agar (ISP medium no. 2), oatmeal agar (ISP medium no. 3) and inorganic salt-starch agar (ISP medium no. 4). Detailed observation of mycelial morphology was performed using a scanning electron microscope (SU-1510; Hitachi High-Technologies) after the strain MM456M-mF7 was incubated on ISP medium no. 4 at 30 °C for 7 days. The type of diaminopimelic acid isomers in the whole-cell hydrolysates was determined by the method of Stanek and Roberts.²² The total DNA of MM456M-mF7 was prepared using a Genomic DNA Extraction Kit Mini (RBC Bioscience, New Taipei, Taiwan) according to the manufacturer's instructions. 16S rRNA (positions 56–1495, *Escherichia coli* numbering system²³) was amplified by PCR and sequenced. A search for the most closely related sequences was performed using the BLAST algorithm at the DNA Data Bank of Japan.

Fermentation

A slant culture of MM456M-mF7 was inoculated into a 500 ml baffled Erlenmeyer flask containing 110 ml of seed medium consisting of 2% (w v⁻¹) galactose, 2% (w v⁻¹) dextrin, 1% (w v⁻¹) Bacto-soytone (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), 0.5% (w v⁻¹) corn steep liquor (Kogo Starch, Chiba, Japan), 1% (w v⁻¹) glycerol, 0.2% (w v⁻¹) (NH₄)₂SO₄, 0.2% (w v⁻¹) CaCO₃ and 1.8% (w v⁻¹) artificial seawater (Nihon Pharmaceutical, Tokyo, Japan) in deionized water (pH 7.4 before sterilization). The seed culture was incubated in a rotary shaker (180 r.p.m.) at 27 °C for 2 days. The producing culture was performed by solid-state fermentation. A total of 7 ml of seed culture was transferred into a 500 ml K-1 flask containing 40 g of a producing medium consisting of pressed barley (15 g) and deionized water (25 g). The fermentation was carried out at 30 °C for 14 days in the dark.

Isolation

Purification was guided by obtaining accurate masses using LC-HRESI-MS analysis. The whole fermentation material (600 g) was extracted two times with an equal amount of EtOH and the extract was passed through filter paper. The filtrate was diluted 10-fold with deionized water and adjusted to pH 2.0 with HCl. The diluted filtrate was applied to a Diaion CHP-20P column (500 ml; Mitsubishi Chemical, Tokyo, Japan). After washing with water, the compounds were eluted with 50% MeOH. The eluate was concentrated and dissolved in MeOH to remove insoluble contaminants. The MeOH supernatant was concentrated *in vacuo* to give 4.4 g of crude material. The material was adjusted to pH 2.0 and chromatographed on a CHP-20P column (200 ml), eluted with 0.01 M NaOH, and the fractions of interest were neutralized with HCl. At this stage, **1** and **2** were almost separated. The NaOH fractions primarily containing **1** (496.3 mg) were adjusted to pH 2.0 and then loaded on a CHP-20P column (110 ml), and eluted using a stepwise gradient of 0–60% MeOH (10% increment). Compound **1** was eluted from 20 to 40% MeOH (77.1 mg). The fractions of interest were further purified using a preparative HPLC column (Capcell Pak C₁₈ MG, 20 mm i.d. × 250 mm; Shiseido, Tokyo, Japan), eluted with 8% aqueous acetonitrile at a flow rate of 10 ml min⁻¹. Compound **1** was eluted from 23 to 27 min. Finally, the combined fractions were purified using a preparative HILIC HPLC column (PC HILIC, 20 mm i.d. × 250 mm; Shiseido), with linear-gradient elution from 95 to 50% aqueous acetonitrile over 45 min at a flow rate of 10 ml min⁻¹. Purified **1** was eluted from 22 to 24 min to give a colorless syrup (10.5 mg). The NaOH fractions primarily containing **2** (687.2 mg) were adjusted to pH 2.0 and were then applied to a CHP-20 P column (110 ml). After washing with water, **2** was eluted with 50% MeOH. The eluate (451.4 mg) was chromatographed on a Sephadex LH-20 column (50 ml; GE Healthcare Bio-Science AB, Uppsala, Sweden), eluted isocratic with 50% MeOH. The selected fractions were concentrated *in vacuo* to give **2** as a colorless syrup (187.3 mg).

Antimicrobial activity of the compounds

Clostridium perfringens B-0866 was from the in-house collection of the Institute of Microbial Chemistry. *C. difficile* BAA-1382 was purchased from the American Type Culture Collection (Manassas, VA, USA). Other microbial strains were purchased from the Japan Collection of Microorganisms (Tsukuba, Japan). MICs were determined by the standard agar dilution method recommended by the Clinical and Laboratory Standards Institute guidelines.²⁴ Bacteria were incubated on ABCM agar (Eiken Chemical, Tokyo, Japan) at 37 °C under anaerobic culture conditions for 20 h. Moreover, MICs were also determined by the microbroth dilution method under the presence of the iron (III) chloride hexahydrate (Wako Pure Chemical Industries, Osaka, Japan) to confirm that the antimicrobial activities were attributed to chelating activity (Supplementary Table S1).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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