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Streptobactin, a Tricatechol-Type Siderophore from Marine-Derived *Streptomyces* sp. YM5-799

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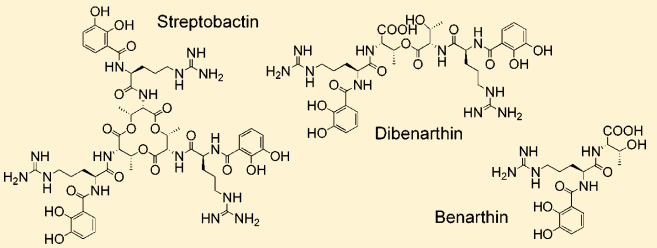
Supporting Information

ABSTRACT: A new catechol-type siderophore, streptobactin (**1**), was isolated from a culture broth of the marine-derived actinomycete *Streptomyces* sp. YM5-799. The structure of streptobactin was determined by NMR and MS analyses and ESIMS/MS experiments to be a cyclic trimer of benarthin. A dibenarthin (**2**), a tribenarthin (**3**), and benarthin (**4**) were also obtained. The production of **1** was regulated by an iron concentration in the culture. The iron-chelating activity of the compounds was evaluated by the chrome azurol sulfonate assay.



Iron is an essential element for most organisms because of its various roles in important biological processes, such as nucleic acid synthesis and repair, respiration, photosynthetic transport, nitrate reduction, nitrogen fixation, and detoxification of free radicals.[3] In spite of its high abundance in Earth's crust, the dissolved iron concentration is particularly low (<0.4 μM) in the surface waters of the open oceans.[4] Under such iron-limited conditions, marine bacteria utilize siderophores, relatively low molecular weight compounds that have a high-affinity iron-chelating ability, for their vital activity.[5] A wide range of structures have been reported for the siderophores produced by terrestrial and enteric bacteria.[6] Several siderophores have recently been isolated from marine-derived bacteria including actinomycetes.[7] We have also been collecting bacteria from various marine environments, particularly those producing siderophores detectable by the chrome azurol sulfonate (CAS) assay,[8] and have reported new catechol-type siderophores, pseudoalterobactins A and B, from the marine bacterium *Pseudoalteromonas* sp. KP20-4[9] and hydroxamate siderophores, tenacibactins A–D, from *Tenacibaculum* sp. A4K-17.[10] During our subsequent screening for siderophores from marine bacteria, we found that the marine-derived actinomycete *Streptomyces* sp. YM5-799 produced a new catechol-type siderophore along with its related compounds. Herein we describe the fermentation, isolation, structural determination including absolute configuration, and iron-binding activity of streptobactin[1] (**1**) and its related compounds (**2**, **3**) together with benarthin[2] (**4**). Benarthin was isolated from the culture broth of *Streptomyces xanthophaeus* MJ244-SF1 as an inhibitor of pyroglutamyl peptidase and determined as L-*N*-(2,3-dihydroxybenzoyl)arginyl-L-threonine.[2a]

RESULTS AND DISCUSSION

Strain YM5-799, producing siderophores **1**–**3** and benarthin (**4**), was isolated from the surface of algae collected from Hokkaido in north Japan. Morphological, physiological, and phylogenetic properties enabled YM5-799 to be identified as the genus *Streptomyces*. The siderophores, produced in the culture supernatant of *Streptomyces* sp. YM5-799, were isolated by chromatography on a porous-polymer resin Diaion HP20 column with a stepwise gradient of aqueous MeOH under acidic conditions. Using CAS assay-guided fractionation, the active fractions were further purified by preparative RP-HPLC to afford three new siderophores, streptobactin (**1**), dibenarthin (**2**), and tribenarthin (**3**), and a known compound, benarthin (**4**).

Streptobactin (**1**) was obtained as a colorless gum, the molecular formula being established as $C_{51}H_{69}N_{15}O_{18}$ on the basis of HR-FABMS data [found *m*/*z* 1180.5004, calcd for

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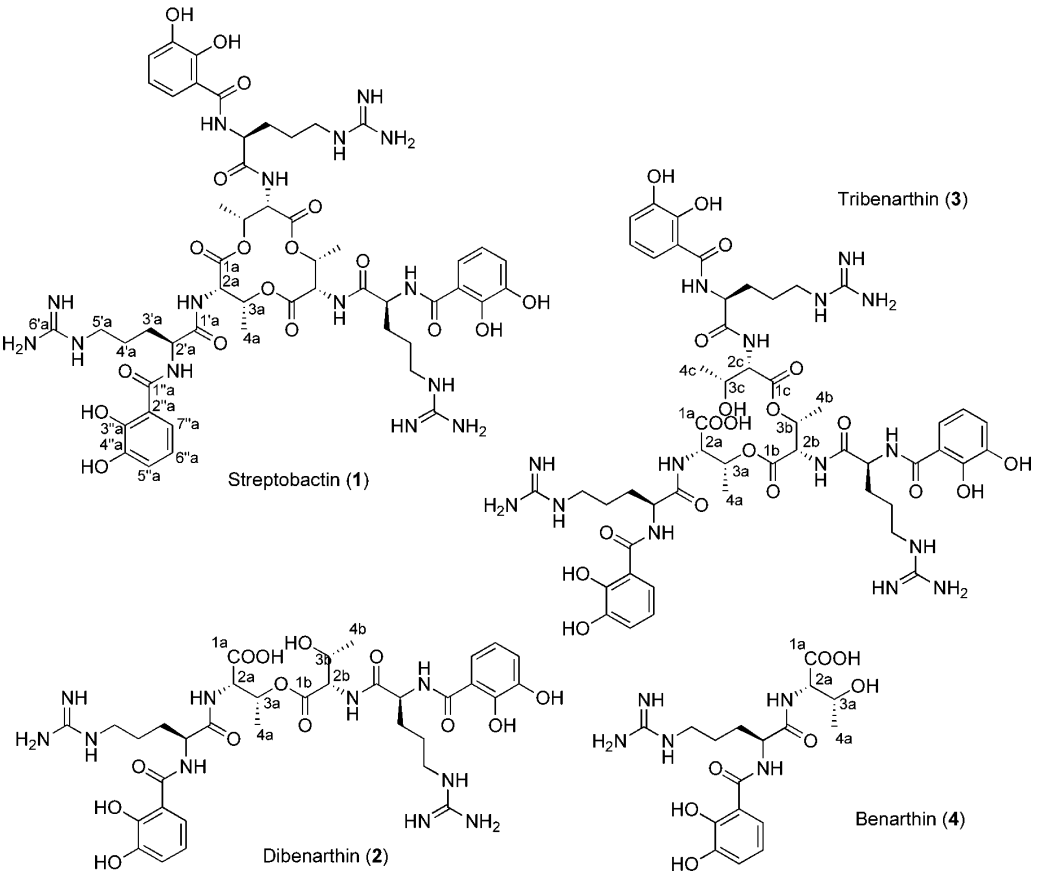
Chart 1



$[C_{51}H_{69}N_{15}O_{18} + H]^+$ 1180.5023]. The ^1H and ^{13}C NMR spectra of compound **1** were uncomplicated considering its molecular weight. Thus compound **1** was presumed to be a structure with high symmetry. The ^{13}C NMR and HSQC spectra revealed the presence of 17 carbon signals. Therefore, compound **1** should have a C_3 symmetry axis in its structure, suggesting a trimer of formula $C_{17}H_{23}N_5O_6$. The ^1H and ^{13}C NMR and HSQC spectra revealed the presence of one methyl (δ_C 16.2, δ_H 1.15), three broad methylene (δ_C 24.9, δ_H 1.58 and 1.63; δ_C 28.9, δ_H 1.80 and 1.90; δ_C 40.6, δ_H 3.10 and 3.20), six methine (δ_C 52.6, δ_H 4.78; δ_C 55.4, δ_H 4.88; δ_C 70.8, δ_H 5.43; δ_C 118.2, δ_H 6.70; δ_C 118.6, δ_H 6.93; δ_C 118.8, δ_H 7.40), and seven quaternary carbons (δ_C 116.5; 145.9; 147.8; 156.7; 168.07; 168.10; 172.3). These physiological properties and NMR data suggested that **1** was related to a catechol-type siderophore such as enterobactin,[11] bacillibactin,[12] vanchrobactin,[13] or JBIR-16.[14]

A partial structure was elucidated by the interpretation of data from $^1H-^1H$ COSY and $^1H-^{13}C$ HMBC experiments (see SI, Figure SI-1). A threonine moiety was determined by the spin network from the methyl to the amide proton (δ_H 8.79). The COSY and HMBC signals also revealed the 2,3-dihydroxybenzoyl (DHBA) part. The COSY spectrum did not sufficiently provide signals derived from three broad methylenes, but the distinctive quaternary carbon signal (δ_C 156.7), NH-substituted methylene carbon signal (δ_C 40.6) and its spin network ($-NH-CH_2-$), and methine-NH spin signal ($-NH-CH<$) suggested the existence of an arginine moiety in the structure of **1**. Connectivities of the moieties were determined by HMBC correlations. The HMBC signals from an amide proton in Thr (δ 8.79) to the carbonyl carbon in Arg (δ 172.3), and an amide proton in Arg (δ 8.80) to the carbonyl carbon in DHBA (δ 168.10 or 168.07), were revealed as a partial structure (-Thr-Arg-DHBA). Compound **1** has the formula $C_{51}H_{69}N_{15}O_{18}$ and a C_3 symmetry axis. Furthermore, a partial structure (-Thr-Arg-DHBA) has the formula $C_{17}H_{23}N_5O_6$; therefore, streptobactin possessed a catechol-bonded arginine-substituted siderophore framed on a cyclic triester backbone of threonine. NMR data of streptobactin (**1**) and benarthin (**4**), isolated in this study, are summarized in Table 1. The spectroscopic data of **4** corresponded well with literature data[2b] and also streptobactin (**1**), except for ^1H NMR signals in relation to the Thr moiety. The ^1H NMR signals of C-2a (δ 4.88), C-2a-N*H* (δ 8.80), and C-3a (δ 4.53) of **1** were shifted downfield compared to **2** at δ 4.23, 7.95, and 4.17, respectively. These downfield shifts indicated that **1** has a cyclic triester-bonded backbone similar to that in the enterobactin[11b] and dimeric 2,3-dihydroxybenzoylserine case.[8b]

Compound **2** was obtained as a colorless gum, the molecular formula being determined as $C_{34}H_{48}N_{10}O_{13}$ on the basis of HR-FABMS data [found m/z 805.3454 $[M + H]^+$, calcd 805.3481]. The ^{13}C NMR spectrum showed 32 resolved signals corresponding to 34 carbons. The molecular formula of compound **2** suggested that two benarthin units are combined via an ester bond $[2(C_{17}H_{25}N_5O_7)-H_2O]$.

A partial structure of compound **2** was elucidated by the interpretation of data from COSY, TOCSY, and HMBC experiments (see SI, Figure SI-9). In the same manner as for streptobactin (**1**), two benarthin units were constructed from 2D NMR analysis. As mentioned above, the ^1H NMR signals

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Table 1. NMR Data (750 MHz, DMSO-d_6) of Streptobactin (1) and Benarthin (4)

	streptobactin (1)		benarthin (4)	
position	^{13}C,[a] m[b]	^{1}H (J in Hz)[c]	^{13}C,[a] m[b]	^{1}H (J in Hz)[c]
Thr-a				
1a	168.10 or 168.07[f], C		171.8, C	
2a	55.4, CH	4.88, d (10.0)	57.5, CH	4.23, dd (3.2, 8.7)
2a-N*H*		8.79, br		7.95, d (8.7)
3a	70.8, CH	5.43, m	66.2, CH	4.17, m
4a	16.2, CH_3	1.15, d (5.8)	20.3, CH_3	1.18, d (6.4)
Arg-a				
1′a	172.3, C		171.5, C	
2′a	52.6, CH	4.78, m	52.4, CH	4.68, m
2′a-N*H*		8.80, br		8.79, d (8.0)
3′a	28.9, CH_2	1.80, 1.90, m	28.9, CH_2	1.73, 1.86, m
4′a	24.9, CH_2	1.58, 1.63, m	25.1, CH_2	1.57, m
5′a	40.6, CH_2	3.10, 3.20, m	40.4, CH_2	3.13, m
5′a-N*H*		7.47, t (5.3)		7.47, t (5.5)
6′a	156.7, C		156.6, C	
DHBA[d]-a				
1″a	168.10 or 168.07,[f] C		168.5, C	
2″a	116.5, C		115.9, C	
3″a	147.8, C		148.4, C	
4″a	145.9, C		146.0, C	
5″a	118.6, CH	6.93, d (7.7)	118.7, CH	6.93, dd (1.6, 8.0)
6″a	118.2, CH	6.70, t (8.0)	118.1, CH	6.70, dd (8.0, 8.0)
7″a	118.8, CH	7.40, d (8.0)	118.3, CH	7.38, dd (1.6, 8.0)
3″a-O*H*		11.55, br		11.80, s
4″a-O*H*		nd[e]		nd[e]

[a]δ in ppm (187.5 MHz). [b]Multiplicity. [c]δ in ppm (750 MHz). [d]2,3-Dihydroxybenzoic acid. [e]Not detected. [f]Indistinguishable.

around the ester bond were deshielded. The ^{1}H NMR signals of C-2a (δ 4.64), C-2a-N*H* (δ 8.42), and C-3a (δ 5.32) of **2** were shifted downfield compared to C-2b (δ 4.36), C-2b-N*H* (δ 8.08), and C-3b (δ 4.29), respectively. Therefore, a hydroxy group at C-3a should be connected to a carboxylic group at C-1b by an ester bond (see SI, Figure SI-9-b). ESIMS/MS analysis of compound **2** supported the structure (see SI). The ^{1}H and ^{13}C NMR assigned data are summarized in Table 2.

Compound **3** was also isolated as a colorless gum, the molecular formula being determined as $C_{51}H_{71}N_{15}O_{19}$ on the basis of HR-FABMS data [found m/z 1198.5100 $[M + H]^+$, calcd 1198.5129]. This formula indicates that three benarthin units are linearly connected via two ester bonds [3-($C_{17}H_{25}N_5O_7$)−$2H_2O$]. The ^{1}H NMR, COSY, and TOCSY spectra revealed three overlapped DHBAs, three overlapped arginines, and three threonine moieties (see SI, Figure SI-18). Because of sample limitations, the ^{13}C NMR spectra only permitted assignment of chemical shifts estimated from HSQC and HMBC spectra. As for dibenarthin (**2**), a C-3a hydroxy group should be connected to a C-1b carboxylic group, and a C-3b hydroxy group should be connected to a C-1c carboxylic group via two ester bonds. ESIMS/MS analysis of compound **3** also supported the linear structure of tribenarthin (see SI). The ESIMS/MS fragmentation spectrum is similar to that of trivanchrobactin.[13] The ^{1}H and ^{13}C NMR assigned data are summarized in Table 3.

Table 2. NMR Data (750 MHz, DMSO-d_6) of Dibenarthin (2)

position	^{13}C,[a] m[b]	^{1}H (J in Hz)[c]
Thr-a		
1a	170.5, C	
2a	54.8, CH	4.64, dd (2.4, 8.9)
2a-N*H*		8.42, br d (9.0)
3a	70.5, CH	5.32, m
4a	16.2, CH_3	1.15, d (6.1)
Arg-a		
1′a	171.7 or 171.9,[e] C	
2′a	52.5, CH	4.70, m
2′a-N*H*		8.79 or 8.81,[e] d (8.0)
3′a	29.0 or 29.1,[e] CH_2	1.75, 1.86, m
4′a	25.2 CH_2	1.58, m
5′a	40.4 or 40.5,[e] CH_2	3.14, m
5′a-N*H*		7.50, m or 7.52,[e] t (5.8)
6′a	156.7, C	
DHBA[d]-a		
1″a	168.3 or 168.6,[e] C	
2″a	115.9 or 116.1,[e] C	
3″a	148.2 or 148.4,[e] C	
4″a	145.99 or 146.03,[e] C	
5″a	118.7 or 118.8,[e] CH	6.94, dd (1.6, 8.0)
6″a	118.1, CH	6.71, dd (7.7, 8.0)
7″a	118.5 or 118.6,[e] CH	7.40, dd (1.6, 8.0)
3″a-O*H*		11.65 or 11.80,[e] br
4″a-O*H*		9.36, br
Thr-b		
1b	169.3, C	
2b	57.2, CH	4.36, dd (2.9, 9.0)
2b-N*H*		8.08, d (8.7)
3b	66.2, CH	4.29, m
4b	20.1, CH_3	1.08, d (6.1)
Arg-b		
1′b	171.7 or 171.9,[e] C	
2′b	52.5, CH	4.70, m
2′b-N*H*		8.79 or 8.81,[e] d (8.0)
3′b	29.0 or 29.1,[e] CH_2	1.75, 1.86, m
4′b	25.2, CH_2	1.58, m
5′b	40.4 or 40.5,[e] CH_2	3.14, m
5′b-N*H*		7.50, m or 7.52,[e] t (5.8)
6′b	156.7, C	
DHBA[d]-b		
1″b	168.3 or 168.6,[e] C	
2″b	115.9 or 116.1,[e] C	
3″b	148.2 or 148.4,[e] C	
4″b	145.99 or 146.03,[e] C	
5″b	118.7 or 118.8,[e] CH	6.94, dd (1.6, 8.0)
6″b	118.1, CH	6.71, dd (7.7, 8.0)
7″b	118.5 or 118.6,[e] CH	7.40, dd (1.6, 8.0)
3″b-O*H*		11.65 or 11.80,[e] br
4″b-O*H*		9.36, br

[a]δ in ppm (187.5 MHz). [b]Multiplicity. [c]δ in ppm (750 MHz). [d]2,3-Dihydroxybenzoic acid. [e]Indistinguishable.

The absolute configuration of streptobactin (**1**), dibenarthin (**2**), and benarthin (**4**) was determined by Marfey's analysis.[15] HPLC analysis of the FDAA derivatives of the acid hydrolysates of **1**, **2**, and **4** revealed that the constituent amino acids of these compounds were L-Arg and L-Thr.

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Table 3. NMR Data (750 MHz, DMSO-d_6) of Tribenarthin (3)

position	^{13}C,[a] m[b,g]	^{1}H (J in Hz)[c]	position	^{13}C,[a] m[b,g]	^{1}H (J in Hz)[c]
Thr-a			DHBA[d]-b		
1a	nd[e]		1″b	169.7,[g] C	
2a	54.6,[g] CH	4.56, m	2″b	116.4,[g] C	
2a-N*H*		8.44, m	3″b	149.0,[g] C	
3a	70.2,[g] CH	5.31, m	4″b	146.4,[g] C	
4a	15.9,[g] CH_3	1.08, d (6.0)	5″b	118.0,[g] CH	6.93, m
Arg-a			6″b	118.2,[g] CH	6.70, m
1′a	nd[e]		7″b	118.7,[g] CH	7.39, m
2′a	52.1,[g] CH	4.76, m	3″b-O*H*		11.57 or 11.66 or 11.81,[f] br
2′a-N*H*		8.76, m			9.30 or 9.36 or 9.38,[f] br
3′a	29.0,[g] CH_2	1.74, 1.85, m	Thr-c		
4′a	24.4,[g] CH_2	1.57, m	1c	nd[e]	
5′a	40.1,[g] CH_2	3.12, m	2c	56.9,[g] CH	4.36, dd (3.0, 9.0)
5′a-N*H*		7.48, m	2c-N*H*		8.06, d (9.0)
6′a	157.3,[g] C		3c	66.0,[g] CH	4.25, m
DHBA[d]-a			4c	20.0,[g] CH_3	1.08, d (6.0)
1″a	169.7,[g] C		Arg-c		
2″a	116.4,[g] C		1′c	nd[e]	
3″a	149.0,[g] C		2′c	52.1,[g] CH	4.76, m
4″a	146.4,[g] C		2′c-N*H*		8.76, m
5″a	118.0,[g] CH	6.93, m	3′c	29.0,[g] CH_2	1.74, 1.85, m
6″a	118.2,[g] CH	6.70, m	4′c	24.4,[g] CH_2	1.57, m
7″a	118.7,[g] CH	7.39, m	5′c	40.1,[g] CH_2	3.12, m
3″a-O*H*		11.57 or 11.66 or 11.81,[f] br	5′c-N*H*		7.48, m
4″a-O*H*		9.30 or 9.36 or 9.38,[f] br	6′c	157.3,[g] C	
Thr-b			DHBA[d]-c		
1b	nd[e]		1″c	169.7,[g] C	
2b	54.3,[g] CH	4.76, dd (3.0, 9.0)	2″c	116.4,[g] C	
2b-N*H*		8.52, d (9.0)	3″c	149.0,[g] C	
3b	70.2,[g] CH	5.41, m	4″c	146.4,[g] C	
4b	15.9,[g] CH_3	1.15, d (6.8)	5″c	118.0,[g] CH	6.93, m
Arg-b			6″c	118.2,[g] CH	6.70, m
1′b	nd[e]		7″c	118.7,[g] CH	7.39, m
2′b	52.1,[g] CH	4.76, m	3″c-O*H*		11.57 or 11.66 or 11.81,[f] br
2′b-N*H*		8.76, m	4″c-O*H*		9.30 or 9.36 or 9.38,[f] br
3′b	29.0,[g] CH_2	1.74, 1.85, m			
4′b	24.4,[g] CH_2	1.57, m			
5′b	40.1,[g] CH_2	3.12, m			
5′b-N*H*		7.48, m			
6′b	157.3,[g] C				

[a]δ in ppm (187.5 MHz). [b]Multiplicity. [c]δ in ppm (750 MHz). [d]2,3-Dihydroxybenzoic acid. [e]Not detected. [f]Indistinguishable. [g]Estimated values from HSQC and HMBC spectra.

Streptobactin (**1**), dibenarthin (**2**), and benarthin (**4**) possessed Fe-chelating activity comparable to that of deferoxamine mesylate by a CAS assay. The ED_{50} values (quadruplicate mean values) for streptobactin (**1**) and dibenarthin (**2**) were 156 and 117 μM, slightly stronger than that of deferoxamine mesylate (ED_{50} = 195 μM), while benarthin (**4**) showed a weak color change (ED_{50} = 937 μM) under our assay conditions. These siderophores are considered to have played an important role in the survival strategy of *Streptomyces* sp. strain YM5-799 under iron-limited conditions.

Patzer and Braun recently reported on the MS detection of "griseobactin" as a cyclic trilactone of DHBA-arginine-threonine from the unpurified fraction of *Streptomyces* sp. ATCC 700974.[16] They reported that analysis of related biosynthetic gene clusters of the strain ATCC 700974 and some *S. griseus* species indicated that streptobactin and "griseobactin" may be the same compound.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured with a Horiba SEPA-300 digital polarimeter. UV spectra were recorded with a Beckman DU 640 spectrometer and IR spectra with a JASCO FT/IR-430 instrument. The ^{1}H, ^{13}C, and all 2D NMR spectra were recorded with a Varian Unity INOVA 750 instrument. Chemical shifts are referenced to the solvent peaks of δ_H 2.49 and δ_C 39.5 for DMSO-d_6. Low- and high-resolution MS data were obtained on a JEOL JMS700 spectrometer. ESIMS/MS spectra were measured with a ThermoFinigan LCQ Advantage instrument.

Producing Microorganism and Taxonomy. The producing microorganism, strain YM5-799, was isolated from a brown alga [*Analipus japonicus* (Harvey) Wynne] collected off Charatsunai beach (Muroran, Japan, 42°18′18″ N, 140°59′16″ E) in June 2003. Detailed strain isolation procedures were previously described.[17] The algal sample was repeatedly washed with sterile seawater and homogenized with a glass rod in 5 mL of sterile seawater. A 50 μL sample of homogenate was plated onto 1/10 marine agar plates (10% marine broth 2216 (Becton Dickinson, MD) in seawater, solidified with 1.5%

agar) containing 50 mg/L of cycloheximide and incubated at 25 °C for 2 weeks. The bacterial colonies that had developed on the 1/10 marine agar plates were further purified by single colony isolation. A pure culture of strain YM5-799 was preserved in sterile seawater containing 10% glycerol at −80 °C. The strain was grown at 30 °C for 7 days on MB2216 agar and ISP medium No. 2 plate for maintenance and taxonomic characterization. Strain YM5-799 has branched aerial mycelia, forming spores and shapes brownish striated-banded, 1−2 mm in diameter colonies on ISP medium No. 2 plate. Strain YM5-799 produces grayish aerial and substrate mycelia on ISP media No. 3, No. 4, and No. 5 media. Strain YM5-799 has moderate salt tolerance capabilities, and thereby this strain has been fully acclimated to the marine environment. Other taxonomic characteristic features of this strain are summarized in Table 4. Genomic DNA was purified from

Table 4. Taxonomic Characteristics of *Streptomyces* sp. YM5-799

	strain YM5-799
liquefaction of gelatin	−
hydrolysis of starch	+
nitrate reduction	+
peptonization of skim milk	−
coagunation of skim milk	−
growth temperature range	20 °C (+), 25 °C (+), 30 °C (+), 37 °C (−)
salt tolerance	1% (+), 2% (+), 3% (+), 4% (+), 5% (−)
Carbon Utilization	
ISP medium No. 9 (negative control)	−
glucose (positive control)	+
L-rhamnose	−
D-mannitol	+
D-fructose	+
L-arabinose	−
raffinose	−
sucrose	−
D-xylose	−
inositol	−
Melanin Formation	
ISP medium No. 6	+
ISP medium No. 7	−

+: utilization, −: no utilization

the strain YM5-799 by using the QIAGEN Genomic-tip and buffer set (Qiagen). The 16S rRNA gene fragment was amplified by using universal primers corresponding to positions 8−27 as the forward primer and 1492−1510 as the reverse primer.[18] On the basis of the 1462 bp long 16S rRNA gene sequences, phylogenetically related bacteria were searched for by using the BLAST program[19] against the GenBank/EMBL/DDBJ database. The 16S rDNA sequence analysis enabled YM5-799 to be identified as the genus *Streptomyces* with high similarity, and the strain was tentatively designated as *Streptomyces* sp. YM5-799 [GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence is AB534176].

Fermentation of *Streptomyces* sp. YM5-799. The culture for the production of siderophores was carried out under iron-limited conditions. The strain YM5-799 produced siderophores in ASG medium containing 0.1 μM $FeCl_3$, but no production was observed in ASG medium containing 100 μM $FeCl_3$ or in marine broth 2216 (ca. 400 μM as $FeCl_3$) (data not shown). All glassware used for the culture and isolation of the siderophore was washed with 6 N HCl and then rinsed with Milli Q H_2O to avoid any iron contamination. The strain YM5-799 was cultured in 5 L of an ASG medium containing casamino acid (5 g/L), glycerol (3 g/L), glycerophosphate (0.1 g/L), NaCl (15.5 g/L), KCl (0.8 g/L), $MgSO_4 \cdot 7H_2O$ (12.4 g/L), $CaCl_2 \cdot 2H_2O$ (2.9 g/L), NH_3Cl (1.0 g/L), HEPES (2.6 g/L), $NaHCO_3$ (0.17 g/L), and 10 mM $FeCl_3$ (10 μL/L, final concentration of 0.1 μM) at pH 6.8 (before autoclaving) for 72 h at 30 °C with rotary shaking at 100 rpm.

Extraction and Isolation of Siderophores. The culture broth (5 L) was centrifuged at 8000*g* for 15 min at 4 °C. The resulting supernatant was acidified to pH 3 with 6 N HCl and applied to a column of Diaion HP20 (Mitsubishi Chemical Co.). The column was eluted with a stepwise gradient of aqueous MeOH (H_2O, 50% MeOH, and 100% MeOH, pH 3). The Fe-binding fraction was eluted with 50% MeOH using the CAS assay as the index. The 50% MeOH fraction was purified by HPLC (TSK gel ODS 80Ts, 2.0 × 25 cm, 10 mL/min, UV detection at 210 nm) with 5% MeCN in H_2O for 5 min and then a linear gradient up to 50% over 50 min containing 0.1% TFA to furnish four active fractions. The final purification was performed by RP-HPLC (TSK gel ODS 80Ts, ϕ2.0 × 25 cm, 10 mL/min, UV detection at 210 nm) eluted with a linear gradient from 20% to 50% aqueous MeCN containing 0.1% TFA in 30 min to yield pure benarthin (**4**, 3.4 mg, t_R = 8 min), benarthin-linear-dimer (**2**, 2.5 mg, t_R = 11 min), linear benarthin-linear-trimer (**3**, 0.4 mg, t_R = 14 min), and streptobactin (**1**, 18.8 mg, t_R = 16 min).

*Streptobactin (**1**):* colorless gum; $[\alpha]^{23}_D$ +16 (*c* 0.1, MeOH); UV λ_{max} (MeOH) (log ε) 246 (3.08), 320 (2.62), 357 (1.98) nm; IR (KBr) ν_{max} 3422, 1751, 1675, 1543, 1265, 1203, 1137 cm^{-1}; ^1H and ^{13}C NMR data (DMSO-d_6), see Table 1; HR-FABMS *m*/*z* 1180.5004 (calcd for $[C_{51}H_{69}N_{15}O_{18} + H]^+$ 1180.5023).

*Dibenarthin (**2**):* colorless gum; $[\alpha]^{23}_D$ +13 (*c* 0.1, MeOH); UV λ_{max} (MeOH) (log ε) 247 (3.99), 317 (3.65), 357 (3.44) nm; IR (KBr) ν_{max} 3423, 1638, 1543, 1386, 1262, 1205, 1132 cm^{-1}; ^1H and ^{13}C NMR data (DMSO-d_6), see Table 2; HR-FABMS *m*/*z* 805.3454 (calcd for $[C_{34}H_{48}N_{10}O_{13} + H]^+$ 805.3481).

*Tribenarthin (**3**):* colorless gum; $[\alpha]^{23}_D$ +29 (*c* 0.03, MeOH); UV λ_{max} (MeOH) (log ε) 247 (2.91), 317 (2.63), 359 (2.40) nm; IR (KBr) ν_{max} 3422, 1629, 1459, 1358, 1206, 1124 cm^{-1}; ^1H and ^{13}C NMR data (DMSO-d_6), see Table 3; HR-FABMS *m*/*z* 1198.5100 (calcd for $[C_{51}H_{71}N_{15}O_{19} + H]^+$ 1198.5129).

CAS Assay. Siderophore activity was evaluated by the chrome azurol sulfonate assay.[20] The principle of the assay is based on the color change of CAS from blue to orange, resulting from the removal of iron from CAS. Standard curves relating the CAS reactivity to iron-binding ligands were determined by using deferoxamine mesylate. The ED_{50} value is defined as the concentration of a compound that reduced the absorbance at 630 nm of the CAS solution by 50% in 4 h under our assay conditions.

Marfey's Analysis.[15] Approximately 0.2 mg of **1** was hydrolyzed with 100 μL of 6 N HCl at 110 °C for 14 h. The acid hydrolysate was freeze-dried and dissolved in 100 μL of 0.1 N HCl. To 50 μL of the acidic solution were added 20 μL of 1 N $NaHCO_3$ and 100 μL of a 1% 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (FDAA, Marfey's reagent, Pierce) solution in acetone, and the mixture was heated at 40 °C for 1 h. The mixture was cooled to room temperature, neutralized with 1 N HCl (20 μL), and evaporated to dryness. The residue was dissolved in 100 μL of MeCN, and 20 μL of the FDAA derivatives was analyzed by HPLC. The HPLC conditions were as follows: Develosil C30-UG5 column (ϕ4.6 × 250 mm, Muromachi Chemical Co.) maintained at 30 °C, photodiode array detector (primary UV detection at 340 nm), flow rate of 1.0 mL/min, linear gradient elution from 20% MeOH−0.1% TFA containing 10% MeCN to 20% MeOH−0.08% TFA containing 50% MeCN for 25 min. The FDAA derivatives of the acid hydrolysate were identified by comparing the retention time with FDAA derivatized from authentic amino acids. Compounds **2** and **4** were also analyzed in the same procedures. The retention times of the acid hydrolysate were 16.85 (L-Arg; D-Arg, 15.79 min) and 19.12 (L-Thr; L-*allo*-Thr, 20.93; D-*allo*-Thr, 22.53, and D-Thr, 23.89 min).

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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