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BRIEF COMMUNICATION

Labrenzbactin from a coral-associated bacterium *Labrenzia* sp.

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Abstract

A new catecholate-containing siderophore, labrenzbactin (**1**), was isolated from the fermentation broth of a coral-associated bacterium *Labrenzia* sp. The structure and absolute configuration of **1** was determined by spectroscopic methods and Marfey's analysis. Overall, **1** showed antimicrobial activity against *Ralstonia solanacearum* SUPP1541 and *Micrococcus luteus* ATCC9341 with MIC values of 25 and 50 μg ml^{-1}, respectively, and cytotoxicity against P388 murine leukemia cells with an IC_{50} of 13 μM.

Marine microorganisms are attracting much attention as a source of new compounds alternative to terrestrial microorganisms [1]. The number of new compounds from marine microorganisms is steadily increasing in recent years [1, 2]. A large portion of marine microorganisms are likely associated with host organisms, especially marine invertebrates, colonizing outside and/or inside of the host body and forming microbial communities unique to each host species [3]. Such host-associated microorganisms are suggested to have a beneficial role in protecting the host from invading or opportunistic pathogens, presumably by producing antimicrobial substances [4].

Labrenzia species are Gram-negative, aerobic, motile, and rod-shaped bacteria of the class *Alphaproteobacteria*. They require NaCl for growth and are isolated from marine habitats, in many reported cases, in association with marine invertebrates such as corals [5–7]. Although the species of *Labrenzia* possess biosynthetic genes for the production of polyketide, non-ribosomal peptide, and terpenoid [7], very little is known about their secondary metabolites. To date, a polyketide-derived pederin analog and two cyclopropane-containing fatty acids have been reported [8, 9].

In our investigation of secondary metabolite production by coral-associated bacteria, *Labrenzia* sp. C1-1 was found to produce a new catecholate-class siderophore designated labrenzbactin (**1**, Fig. 1). In this paper, we describe the isolation, characterization, and bioactivity of **1**.

The producing strain C1-1 was isolated from a cultured Scleractinian (stony) coral *Montipora* sp., and identified as a member of the genus *Labrenzia* by 16S rRNA gene sequence analysis. The whole culture broth of strain C1-1 cultured in A3M seawater medium was extracted with 1-butanol. After evaporation, the extract was fractionated by a sequence of chromatographies, and the final purification was achieved by reversed-phase HPLC to give labrenzbactin (**1**).

Compound **1** was obtained as a brown amorphous solid. The molecular formula of $C_{32}H_{36}N_4O_{10}$ with 17 degree of

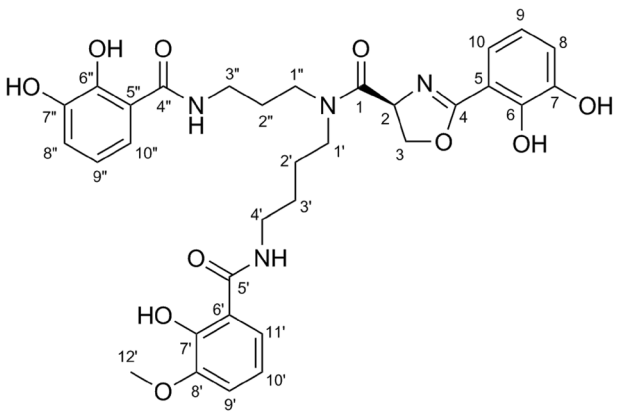

Fig. 1 Structure of labrenzbactin (**1**)

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unsaturation was determined by HRESITOFMS that gave a molecular ion $[M + Na]^+$ at *m/z* 659.2324 (calcd for $C_{32}H_{36}N_4O_{10}Na$, 659.2329) and NMR spectroscopic data (Table 1). The IR spectrum of **1** indicated the presence of OH and/or NH (3313 cm^{-1}) and carbonyl (1634 cm^{-1}) groups. Our in-house UV database indicated that the UV spectrum with the absorption bands at λ_{max} 208, 250, and 314 nm was similar to those for catechoserine (211, 248, and 319 nm) [10] or ulbactin F (204, 252, and 317 nm) [11], suggestive of the presence of a hydroxylated benzene ring conjugated with a double bond like a carbonyl group.

NMR spectroscopic data of **1** revealed the presence of 32 carbons attributable to four carbonyl-like deshielded sp^2 carbons, eighteen sp^2 carbons (nine are proton-bearing), eight methylenes, one methine, and one methoxy group (Table 1). COSY analysis identified proton resonances for three sets of 1,2,3-trisubstituted benzene rings, H8/H9/H10, H9′/H10′/H11′, and H8″/H9″/H10″ (Fig. 2). HMBC correlations from H8 and H10 to C6 and H9 to C5 and C7 established the 2,3-dihydroxyphenyl substructure. An oxazoline ring was deduced from the HMBC correlations from H2 and H3 to C4 and chemical shift consideration for H2, H3, and C4. The linkage between C4 and C5 and the attachment of C1 to C2 were determined by the observed HMBC correlations. Another 2,3-dihydroxyphenyl moiety consisting of the carbons from C5″ to C10″ and a 2-hydroxy-3-methoxyphenyl moiety comprising C6′ to C11′ were also established by analyzing HMBC correlation data. The position of the methoxy group was confirmed by NOE detected between H12′ and H9′. The COSY spectrum also provided two short methylene chains from H1′ to H4′ and from H1″ to H3″. Mutual HMBC correlations between H1′ and H1″ linked these carbon chains through a nitrogen atom, which was in turn connected with the carbonyl carbon C1 by HMBC correlations from H1′ and H1″ to C1. Finally, two aromatic parts were connected to the methylene chains via an amide bond, on the basis of HMBC correlations from H4′ and H11′ to C5′ and from H3″ and H10″ to C4″ to complete the planar structure of **1** (Fig. 2). This structure was further validated by analyzing the NMR spectral data obtained in DMSO-d_6 that gave a higher peak resolution for ^{1}H NMR spectrum (Supplementary Information). Some of the ^{1}H and ^{13}C NMR signals were observed as duplicated peaks, presumably due to the tautomeric isomerization of amide bonds, but all the signals could be assigned to its structure (Table 1). The absolute configuration of the oxazoline moiety derived from serine in **1** was determined by the advanced Marfey's analysis [12]. Following acid-catalyzed hydrolysis, **1** was derivatized using 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA) and retention time was compared with L-serine standard that was similarly derivatized with L-FDLA and 1-fluoro-2,4-dinitrophenyl-5-D-leucinamide (D-FDLA) using LC–MS. The derivatized L-serine standard with L-FDLA and D-FDLA eluted at 9.5 and 10.7 min, respectively, while the L-FDLA derivative of the acid hydrolysate of **1** eluted at 9.5 min. The serine residue was determined to have the L-configuration and thus the configuration of C2 was established to be *S* (Fig. 1).

Biological activity of labrenzbactin (**1**) was evaluated by cytotoxicity and antimicrobial assays. Overall, **1** showed moderate cytotoxicity against P388 murine leukemia cells at IC_{50} 13 μM (IC_{50} of a reference drug doxorubicin: 0.018 μM) and no appreciable activity in antimicrobial assay against *Bacillus subtilis* ATCC6633, *Staphylococcus aureus* FDA209P JC-1, *Rhizobium radiobacter* NBRC14554, and *Escherichia coli* NIHJ JC-2. However, it showed weak antibacterial activity against *Ralstonia solanacearum* SUPP1541 and *Micrococcus luteus* ATCC9341 with MIC values of 25 and 50 μg ml^{-1}, respectively.

In summary, our chemical investigation of a marine bacterium *Labrenzia* led to the discovery of labrenzbactin (**1**). It is a new member of bacterial siderophores in which a linear triamine, spermidine or norspermidine, is amidated with acyl groups containing 2,3-dihydroxyphenyl and/or 2-hydroxyphenyl units (Fig. 3). Several related compounds are reported: parabactin (**2**) [13] from *Paracoccus denitrificans*; agrobactin (**3**) [14] from a plant pathogen *Agrobacterium tumefaciens* (currently *Rhizobium radiobacter*); fluvibactin (**4**) [15], and vibriobactin (**5**) [16] from *Vibrio fluvialis* and *Vibrio cholerae*, respectively.

Experimental section

General experimental procedure

Optical rotation was measured using a JASCO DIP-3000 polarimeter. The UV spectra were recorded on a Shimadzu UV-1800 spectrophotometer. The IR spectrum was measured on a PerkinElmer Spectrum. NMR spectra were obtained on a Bruker AVANCE 500 spectrometer in a mixture of $CDCl_3$ and CD_3OH (9:1) due to the low solubility of **1** in a single solvent or in DMSO-d_6. The residual solvent signals ($CDCl_3$: δ_H 7.26 and δ_C 77.24; DMSO-d_6: δ_H 2.50 and δ_C 39.5) were used as internal standards. HRESITOFMS was recorded on a Bruker micrOTOF focus mass spectrometer.

Microorganism

Strain C1-1 was isolated from a cultured stony coral (*Montipora* sp.) purchased from an aquarium vendor in Nagasaki, Japan. The coral specimen was washed with 70%

Table 1 ^{1}H and ^{13}C NMR data for labrenzbactin (1) in $CDCl_3$–CD_3OH (9:1) and in DMSO-d_6

	$CDCl_3$–CD_3OH (9:1)			DMSO-d_6	
Position	δ_C[a]	δ_H mult (J in Hz)[b]	HMBC[b,c]	δ_C[a]	δ_H mult (J in Hz)[b]
1	169.8, C			168.5/168.6	
2	65.4, CH	5.16 t (8.4)	1, 3, 4	64.3	5.36 t (8.5)/5.37 t (8.5)
3	69.0, CH_2	4.48 t (8.9)	1, 2, 4	69.3	4.53 t (9.0)
		5.08 t (7.5)	1, 2, 4		4.76 t (7.3)
4	167.4, C			166.0	
5	110.2, C			110.1	
6	147.5, C			148.1	
7	145.0, C			145.7	
8	119.1, CH	6.99 d (7.2)	6, 10	119.5	6.96 d (7.6)
9	119.3, CH	6.75 t (7.3)	5, 7	118.8	6.74 t (8.0)
10	119.4, CH	7.15 d (7.9)	4, 6, 8	117.87	7.07 d (7.9)
1′	47.6, CH_2	3.62 m	1, 2′, 1″	45.1/46.8	3.32, 3.36 m/3.54, 3.63 m
2′	26.3, CH_2	1.82[d]	1′, 3′	26.1	1.63, 1.72 m
3′	27.0, CH_2	1.72 m	1′, 2′, 4′	24.5/26.2	1.51 m/1.50 m
4′	38.9, CH_2	3.52[e]	3′, 5′	38.5/38.6	3.36 m/3.28 m
5′	169.5, C			169.49/169.53	
6′	111.5, C			114.75/114.83	
7′	150.0, C			150.9/151.0	
8′	148.6, C			148.4	
9′	114.7, CH	6.87 d (7.9)	7′, 11′	115.3	7.06[f]
10′	118.7, CH	6.72 t (7.9)	6′, 8′	117.7	6.78 t (8.1)
11′	118.6, CH	7.20 d (7.7)	5′, 7′, 9′	118.45/118.50	7.39 d (8.6)/7.41 d (8.6)
12′	56.2, CH_3	3.82 s	8′	55.7	3.74 s/3.75 s
4′-NH		7.50 br.s	5′		8.82 t (5.4)/8.86 t (5.5)
1″	43.3, CH_2	3.46[e]	1, 1′, 2″, 3″	43.4/44.8	3.38 m/3.53, 3.69 m
2″	27.2, CH_2	1.82[d]	1″, 3″	27.1/28.5	1.77 m/1.95 m
3″	36.1, CH_2	3.29 m	1″, 2″, 4″	36.7	3.26 m, 3.39 m
4″	170.3, C			169.7/169.9	
5″	114.5, C			114.9/115.1	
6″	149.3, C			149.6/149.7	
7″	145.8, C			146.2	
8″	118.3, CH	6.95 d (7.5)	6″, 10″	118.7	6.89 d (7.8)
9″	118.8, CH	6.71 t (7.9)	5″, 7″	117.86	6.66 t (7.9)
10″	116.9, CH	7.10 d (7.9)	4″, 6″, 8″	117.0/117.2	7.24 d (8.0), 7.27 d (7.9)
3″-NH		8.03 br.s	4″		8.76 t (5.4)/8.85[g]
OH					9.23 br.s
OH					11.78 br.s
OH					12.77 br.s

[a]Recorded at 125 MHz (reference δ_C 77.2 for $CDCl_3$–CD_3OH; δ_C 39.5 for DMSO-d_6)

[b]Recorded at 500 MHz (reference δ_H 7.26 for $CDCl_3$–CD_3OH; δ_C 2.50 for DMSO-d_6)

[c]HMBC correlations are from proton(s) stated to the indicated carbon

[d–g]Overlapping signals

ethanol and then washed with sterile natural seawater. A piece of the coral (*ca*. 1 g) was homogenized by mortar and pestle with an equal amount of sterile natural seawater (1 ml), and the suspension was diluted up to 10^{-5} and 0.1 ml of each dilution liquid was spread on plates of marine agar 2216 (Difco). After cultivation at 23 °C for 2 days, a single

colony was repeatedly transferred onto the same agar medium to obtain the pure isolate of strain C1-1. The isolated strain was identified as a member of genus *Labrenzia* on the basis of 99.9% similarity in the 16 S rRNA gene sequence (1395 nucleotides; DDBJ accession number LC456786) to *Labrenzia aggregata* IAM 12614 ^{T} (accession number AAUW01000037).

Fermentation

Strain C1-1 cultured on marine agar 2216 (Difco) was inoculated into a 500-ml K-1 flask containing 100 ml of V-22 seed medium consisting of soluble starch 1%, glucose 0.5%, NZ case 0.3%, yeast extract 0.2%, Tryptone (Difco Laboratories, Sparks, MD, USA) 0.5%, K_2HPO_4 0.1%, $MgSO_4{\cdot}7H_2O$ 0.05%, and $CaCO_3$ 0.3% (pH 7.0) in natural seawater (collected from Toyama Bay, Japan). The flasks were placed on a rotary shaker (200 rpm) at 30 °C for 2 days. The seed culture (3 ml) was transferred into 500-ml K-1 flasks, each containing 100 ml of A-3M production medium consisting of glucose 0.5%, glycerol 2%, soluble starch 2%, Pharmamedia (Traders Protein, Memphis, TN, USA) 1.5%, yeast extract 0.3%, and Diaion HP-20 (Mitsubishi Chemical, Kanagawa, Japan) 1% in natural seawater. The pH of the medium was adjusted to 7.0 before sterilization. The inoculated flasks were placed on a rotary shaker (200 rpm) at 30 °C for 5 days.

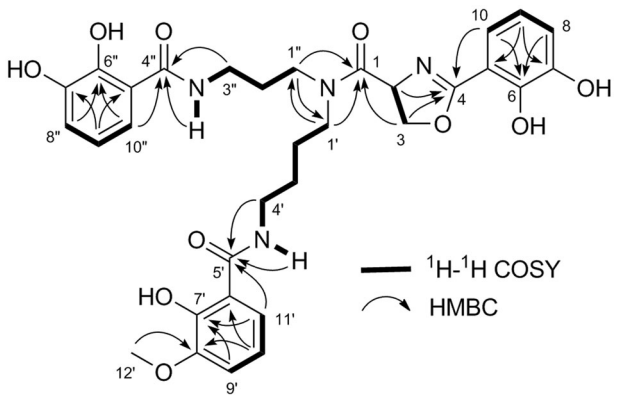

Fig. 2 COSY and key HMBC correlations for labrenzbactin (**1**)

Extraction and isolation

At the end of the fermentation period, 100 ml of 1-butanol was added to each flask, and the flasks were allowed to shake for 1 h. The mixture was centrifuged at 6000 rpm for 10 min, and the organic layer was separated from the aqueous layer. Evaporation of the solvent gave 5.0 g of crude extract from 2.0 l of culture. The crude extract was subjected to silica gel column chromatography with a step gradient of $CHCl_3$–MeOH (1:0, 20:1, 10:1, 4:1, 2:1, 1:1, and 0:1 v/v). Fraction 3 (10:1) was concentrated to provide a dark brown oil (1.0 g), which was then fractionated by reversed-phase ODS column chromatography with a gradient of MeCN–0.1% HCO_2H (2:8, 3:7, 4:6, 5:5, 6:4, 7:3, and 8:2 v/v). Fraction 4 (5:5) was concentrated and extracted with EtOAc. The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentered to give 96 mg of semi-pure material. The final purification was achieved by preparative HPLC (Cosmosil π-NAP, Nacalai Tesque Inc., 10 × 250 mm, 4 ml min^{-1}, UV detection at 254 nm) with 40% MeCN in 0.1% HCO_2H to give compound **1** (7.8 mg, t_R 22.6 min) from 24.0 mg of semi-pure compound.

Labrenzbactin (**1**): light brown amorphous solid; $[\alpha]_D^{24}$ + 6.0 (*c* 0.10, 9:1 $CHCl_3$/MeOH); UV (MeOH) λ_{max} (log ε) 208 (4.81), 250 (4.33), and 314 (3.87); (MeOH—0.01 M HCl (99:1)) 208 (4.74), 256 (4.23), and 313 (3.76); (MeOH —0.01 M NaOH (99:1)) 223 (4.78), 334 (4.03) nm; IR ν_{max} (ATR) 313, 1716 cm^{-1}; for ^{1}H and ^{13}C NMR data, see

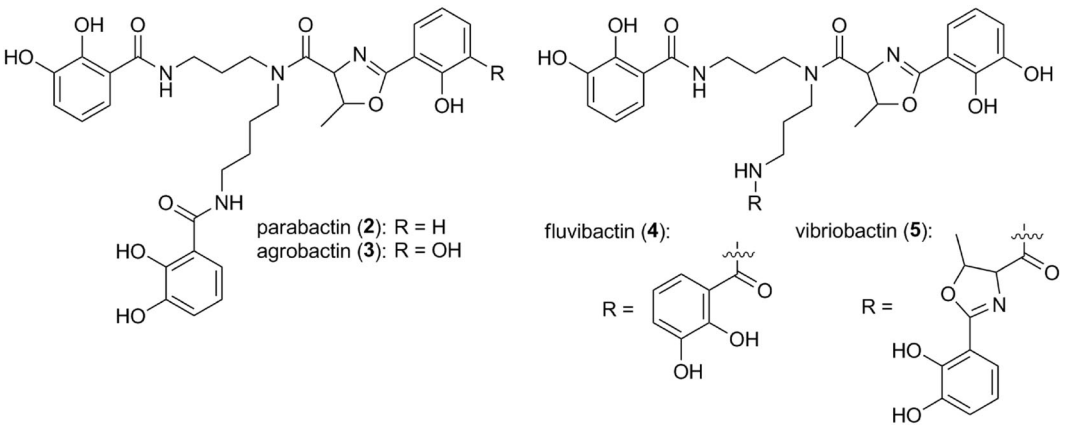

Fig. 3 Related metabolites from bacteria, parabactin (**2**), agrobactin (**3**), fluvibactin (**4**), and vibriobactin (**5**)

Table 1; HRESITOFMS $[M + Na]^+$ 659.2324 (calcd for $C_{32}H_{36}N_4O_{10}Na$, 659.2329).

Marfey's analysis

A portion of **1** (0.1 mg) was hydrolyzed at 105 °C with 5 M HCl (100 µl) overnight in sand bath, and the reaction mixture was evaporated to dryness. A 0.1 M $NaHCO_3$ solution (100 µl) was added to the dried hydrolysate of **1**, as well as to standards of L-serine (Ser). A solution of 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA) in acetone (0.05 mg in 50 µl) was added to each reaction tube and a solution of 1-fluoro-2,4-dinitrophenyl-5-D-leucinamide (D-FDLA) in acetone (0.05 mg in 50 µl) was added to L-serine reaction tube. Each tube was sealed and heated at 50 °C for 30 min. To quench reactions, 0.2 M HCl (50 µl) was added and then diluted with 50% MeCN/0.05% TFA (100 µl, 50:50). The Marfey's derivatives of the hydrolysate and standards were analyzed by LC–MS using a Cosmosil 5C18-AR-II column (Nacalai Tesque, 2 × 150 mm) eluted with MeCN–0.1% HCO_2H at a flow rate of 0.2 ml min^{-1}, monitoring at 340 nm in negative ion mode. The gradient elution was set as follows: 0–30 min (25–65% MeCN), 30–32 min (65–100% MeCN). Retention times for the amino acid standard were 9.5 min for L-Ser-L-FDLA and 10.7 min for L-Ser-D-FDLA, while the L-FDLA hydrolysate of **1** gave a peak at 9.5 min.

Antimicrobial assay

Antimicrobial activity was evaluated by the liquid microculture method using round-bottom 96-well microtiter plates against six bacteria, *Bacillus subtilis* ATCC6633, *M. luteus* ATCC9341, *S. aureus* FDA209P JC-1, *R. solanacearum* SUPP1541, *R. radiobacter* NBRC14554, and *E. coli* NIHJ JC-2 and two yeasts *Candida albicans* NBRC0197 and *Saccharomyces cerevisiae* S100 as indication strains. Tryptic Soy Broth (DIFCO Laboratories) and Potato Dextrose Broth (DIFCO Laboratories) were used for bacteria and yeasts, respectively. Compound **1** and reference drugs, kanamycin sulfate for bacteria and amphotericin B for yeasts, were made in twofold dilution series along the longer side of the plates by sequential transfer of 100-µl aliquots between the adjacent wells, to which the same amount of medium was pre-dispensed. To each well was added a 100-µl suspension of the indication strains prepared at 0.5 McFarland (~10^8 cfu ml^{-1}) from a culture at the logarithmic growth phase. The solvent vehicle added to the top rows was set at 0.5% of the final culture volume to avoid the effect on the growth of microbes. The plates were incubated for 48 h at 37 °C for bacteria and at 32 °C for yeasts. The tests were done in triplicate and the MIC values were read from the lowest drug concentrations at which no growth was observed.

Cytotoxicity assay

P388 murine leukemia cells were maintained in RPMI-1640 medium containing phenol red, L-glutamine, and HEPES (product no. 189-02145) supplemented with 10% fetal bovine serum and 0.1 mg ml^{-1} gentamicin sulfate. Compound **1** and doxorubicin as a reference were serially diluted by a factor of 3.16 (half-logarithmic dilution) in a 96-well round-bottom microtiter plate. To each well were seeded the cells at a final density of 1×10^4 cells ml^{-1}, and 200-µl cultures thus made were incubated for 48 h at 37 °C in an atmosphere of 5% CO_2 in air with 100% humidity. Viability of the cells was visualized by MTT, added to each well as a 50-µl solution in phosphate-buffered saline without Ca^{2+} prepared at 1 mg ml^{-1}. After incubating for 4 h at 37 °C, the medium was carefully removed by a suction aspirator, and formazan dye formed by respiratory reduction by living cells was solubilized by 100 µl of DMSO. The absorption at 540 nm was read by a microplate reader to calculate the rate of cell growth inhibition at each concentration, and the results of triplicate experiments were plotted on single-logarithmic charts to deduce IC_{50} values.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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