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NOTE

Catechoserine, a new catecholate-type inhibitor of tumor cell invasion from *Streptomyces* sp.

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Microbial natural products represent the primary resource for drug discovery, accounting for >20 000 bioactive compounds discovered to date.[1] The majority of microbial drug discovery research has focused on actinomycetes, in particular strains of the chemically prolific genus *Streptomyces*. Drug discovery efforts from actinomycetes, however, have been in decline, in part because of the relatively high frequency of the isolation of known compounds. To overcome this problem, extensive attempts have been directed toward the development of effective strain selection methods to improve the efficiency of the discovery process.[2–4] Sequence-based strain selection is one of the approaches to eliminate the strains which might be studied previously. Although it is not clear whether the phylogenic novelty is associated with the production ability of new compounds, recent studies have shown the occurrence of new metabolites in *Streptomyces* species that have a low 16S rRNA gene sequence homology to the known species.[5–7] In our continuing investigation on chemical diversity within this group,[8–10] a *Streptomyces* strain that shared a low 16S rRNA gene identity (96.3%) with the nearest type strain for *S. albus* was found to produce a new catecholate derivative, catechoserine (**1**), along with two known biosynthetically related metabolites, *N*,*N*′-bis(2,3-dihydroxybenzoyl)-*O*-L-seryl L-serine[11] (**2**) and *N*,*N*′,*N*′-tris(2,3-dihydroxybenzoyl)-*O*-L-seryl-*O*-L-seryl L-serine[11] (**3**) (Figure 1).

The producing-strain TP-A0874 was isolated from a compost sample collected in Ishikawa, Japan, and identified as a member of the genus *Streptomyces* by 16S rRNA gene sequence analysis. The whole culture broth of strain TP-A0874 cultured in A-3M liquid medium was separated into the supernatant and the mycelium. The latter was then extracted with methanol, and the organic solvent was removed under reduced pressure. The remaining aqueous solution was combined with the supernatant and fractionated by HP-20 resin column chromatography. The fractions were further purified by reversed-phase HPLC to yield compounds **1**–**3**.

Catechoserine (**1**) was obtained as a colorless, amorphous solid, which had a molecular formula of $C_{14}H_{19}NO_6$ by interpretation of HR-ESITOFMS (high resolution electrospray ionization time-of-flight mass spectrometry) (obsd $(M+Na)^+$ at *m*/*z* 320.1101) and NMR data (Table 1). The IR spectrum of **1** indicated the presence of hydroxyl (3356 cm^{-1}) and carbonyl (1728, 1640 cm^{-1}) functionalities. The UV spectrum displayed absorption bands at λ_{max} 211, 248 and 319 nm, suggesting the presence of a hydroxylated benzoyl chromophore similar to myxochelin A.[12] NMR data of **1** revealed the presence of 14 carbons attributable to two carbonyl carbons, six aromatic carbons (three are proton bearing and two are oxygenated), four methylenes (two are oxygenated), one nitrogen-bearing methine and one methyl group. These data accounted for all of the observed NMR resonances except for four exchangeable protons. In the ^1H-^1H COSY spectrum, three fragments, H-4/H-5/H-6, H_2-8/H-9 and H_2-11/H_2-12/H_2-13/H_3-14 were detected (Figure 2). The first fragment showed a typical $^3J_{HH}$ coupling pattern for a 1,2,3-trisubstituted benzene ring. This was consistent with the HMBC correlations from H-4 to C-2 and C-3, H-5 to C-1 and C-3 and H-6 to C-2. Downfield shifts of C-2 (δ 148.1) and C-3 (δ 148.5) indicated that these carbons were oxygenated. An HMBC correlation from H-6 to C-7 (δ 169.3) allowed this carbonyl carbon being connected at C-1, establishing a 2,3-dihydroxybenzoyl substructure. The second fragment was expanded to include a carbonyl carbon C-10 (δ 170.6) on the basis of HMBC correlations from H_2-8 and H-9 to this carbon. Furthermore, both ^1H (δ 4.72) and ^{13}C (δ 52.2) chemical shifts at C-9 were characteristic of the α-position proton and carbon of *N*-acyl α-amino acids, consistent with an HMBC correlation from H-9 to another carbonyl carbon C-7 (δ 169.3). Thus, the second fragment was assigned as a serine residue connected to the first fragment through an amide linkage. The third fragment was connected to the serine carboxyl group through an ester linkage on the basis of an HMBC correlation from H_2-11 to C-10 to complete the planar structure of **1**. The absolute configuration of the

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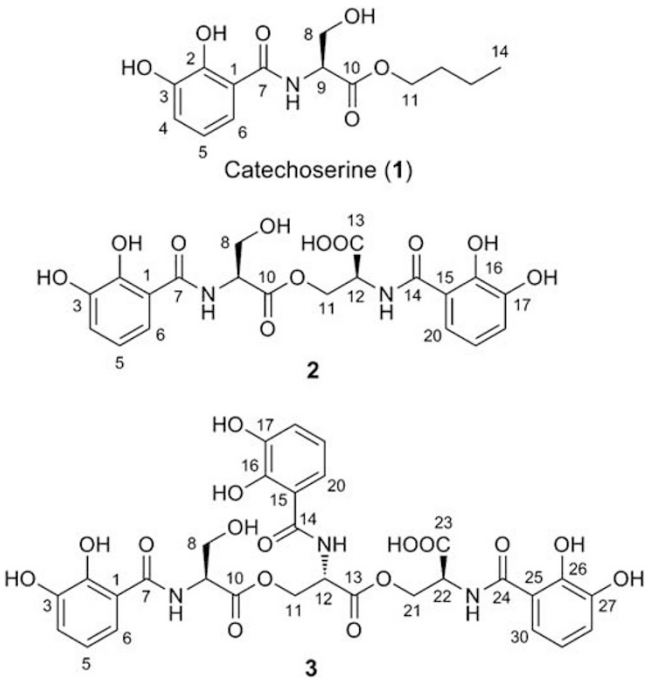

Figure 1 Structures of catechoserine (**1**) and related metabolities (**2** and **3**).

Table 1 ^{1}H and ^{13}C NMR data for catechoserine (1) in CD_3OD

Position	δ_C[a]	δ_H *mult (J in Hz)*[b]	*HMBC*[b,c]
1	115.8, qC		
2	148.1, qC		
3	148.5, qC		
4	118.3, CH[d]	6.96, dd (7.5, 1.2)	2, 3, 5, 6
5	118.4, CH[d]	6.76, dd (8.0, 7.5)	1, 2, 3, 4, 6, 7
6	118.4, CH[d]	7.35, dd (8.0, 1.2)	2, 4, 7
7	169.3, qC		
8	61.4, CH_2	4.02, dd (11.5, 4.2)	9, 10
		3.95, dd (11.5, 4.0)	9, 10
9	55.2, CH	4.72, t (4.0)	8, 7, 10
10	170.6, qC		
11	65.0, CH_2	4.20, t (6.5)	10, 12, 13
12	30.3, CH_2	1.66, tt (7.5, 6.5)	11, 13, 14
13	18.7, CH_2	1.42, qt (7.5, 7.5)	11, 12, 14
14	12.6, CH_3	0.94, t (7.5)	12, 13

[a]Recorded at 125 MHz.
[b]Recorded at 500 MHz.
[c]HMBC correlations are from proton(s) stated to the indicated carbon.
[d]Interchangeable signals.

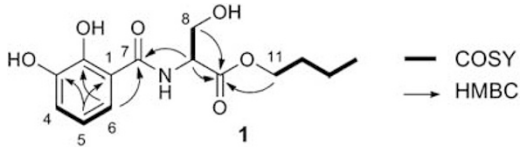

Figure 2 2D NMR correlations for catechoserine (**1**).

serine residue in **1** was determined using Marfey's analysis.[13] Following hydrolysis, **1** was derivatized using 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA) and compared with D- and L-serine standards that were similarly derivatized with L-FDLA. The serine residue was determined to have the L configuration.

Compounds **1–3** were evaluated in tumor cell invasion assay using a reconstituted extracellular matrix Matrigel since some natural and synthetic molecules containing hydroxybenzoyl moieties are known to be active in this system.[14–16] As expected, **1** inhibited the invasion of murine colon carcinoma 26-L5 cells with an IC_{50} value of 2.5 μM, while the IC_{50} values for **2** and **3** were 2.7 and 5.8 μM, respectively. Adhesion, enzymatic degradation of extracellular matrix proteins and migration are the major steps of invasion. Compounds **1–3** did not show any inhibitory effects on these events at 1–10 μM unlike myxochelins[14] that inhibit matrix metalloproteases or lupinacidins[17] that inhibit cell migration. Further investigation is being directed to identify their mode of action. Compounds **1–3** showed no appreciable antimicrobial activities against *Micrococcus luteus*, *Escherichia coli* and *Candida albicans* (MICs > 30 μg ml^{-1}).

Compounds **1–3** share an *N*-2,3-dihydroxybenzoyl-L-serine as a common structural unit. This unit is relatively rare in nature[11] while the 2,3-dihyroxybenzoyl group is common in siderophores produced by Gram-negative bacteria including human pathogens.[18,19] Compounds **2** and **3** are the partially degraded products of enterobactin, a cyclic trimer of *N*-2,3-dihydroxybenzoyl-L-serine responsible for virulence in some bacteria such as *Salmonella typhimurium* and *Klebsiella pneumoniae*.[11,19] To the best of our knowledge, this is the first report on the antiinvasive activities of **2** and **3**. The compounds isolated in this study comprise simple building blocks, providing a starting point for optimization of new antiinvasive agents through analog synthesis.[16,20]

EXPERIMENTAL PROCEDURE

General experimental procedures

Optical rotations were measured using a JASCO DIP-3000 polarimeter (JASCO Corporation, Tokyo, Japan). UV spectra were recorded on a Hitachi U-3210 spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan). IR spectra were measured on a Perkin-Elmer Spectrum 100 (Perkin-Elmer Co. Ltd., Fremont, CA, USA). NMR spectra were obtained on a Bruker AVANCE 500 spectrometer (Bruker, Rheinstetten, Germany) and referenced to the residual solvent signals (δ_H 3.30, δ_C 49.0 for CD_3OD; δ_H 2.49, δ_C 39.5 for DMSO-d_6). HR-ESITOFMS was recorded on a Bruker microTOF focus (Bruker). HPLC separation was performed using an XTerra RP_{18} (Waters Corporation, Milford, MA, USA, 7 μm, 19×300 mm) with a photodiode array detector.

Producing microorganism

The bacterial strain TP-A0874 was isolated from a compost sample collected in Ishikawa, Japan. The strain was identified as a member of the genus *Streptomyces* on the basis of 96.3% 16S rRNA gene sequence (1457 nucleotides; DDBJ accession number AB449812) identity with *Streptomyces albus* subsp. *coleimyceticus* NBRC 13840 (accession number AB184522).

Fermentation

Strain TP-A0874 cultured on a Bn-2 slant (soluble starch 0.5%, glucose 0.5%, meat extract (Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo, Japan) 0.1%, yeast extract (Difco Laboratories, Becton, Dickinson and Company, Sparks, MD, USA) 0.1%, NZ-case (Wako Chemicals USA Inc., Richmond, VA, USA) 0.2%, NaCl 0.2%, $CaCO_3$ 0.1% and agar 1.5%) was inoculated into 500 ml K-1 flasks each containing 100 ml of the V-22 seed medium consisting of soluble starch 1%, glucose 0.5%, NZ-case 0.3%, yeast extract 0.2%, tryptone (Difco Laboratories) 0.5%, K_2HPO_4 0.1%, $MgSO_4{\cdot}7H_2O$ 0.05% and $CaCO_3$ 0.3% (pH 7.0). The flasks were placed on a rotary shaker (200 r.p.m.) at 30 °C for 4 days. The seed culture (3 ml) was transferred into 500 ml K-1 flasks each containing 100 ml of the A-3M production medium consisting of glucose 0.5%, glycerol 2%, soluble starch 2%, Pharmamedia (Traders Protein, Lubbock, TX, USA) 1.5%, yeast extract 0.3% and Diaion HP-20 (Mitsubishi Chemical Co., Tokyo, Japan) 1%. The pH of the medium was adjusted to 7.0 before sterilization. The inoculated flasks were placed on a rotary shaker (200 r.p.m.) at 30 °C for 6 days.

Isolation

At the end of the fermentation period, the whole culture broth (2 l) was centrifuged at 6000 r.p.m. for 10 min, and the supernatant was separated. The mycelium was extracted with 1 l of MeOH at room temperature for 3 h, and the solution was recovered by filtration. After repeating this extraction process twice, the combined organic solution was concentrated under reduced pressure. The remaining aqueous solution was combined with the supernatant and charged onto a column of HP-20 resin, which was eluted with a step gradient of MeOH/distilled water (increasing MeOH by 20% from 0 to 100% MeOH). Compounds **1**, **2** and **3** were detected in fractions eluted with 100%, 60% and 80% MeOH, respectively, by HPLC. The 100% MeOH fraction was concentrated under reduced pressure to give crude brown solid (1.7 g), which was further purified by preparative HPLC with MeOH/0.1% HCO_2H (MeOH concentration: 30% for 0–8 min; 30–85% for 8–42 min) at 12 ml min^{-1}. The fraction containing **1** was pooled, concentrated under reduced pressure, and the remaining aqueous solution was lyophilized to yield catechoserine (**1**, t_R 29.9 min, 13 mg). Similarly, *N*,*N*′-bis(2,3-dihydroxybenzoyl)-*O*-seryl serine (**2**, t_R 23.2 min, 24 mg) and *N*,*N*′,*N*′-tris(2,3-dihydroxybenzoyl)-*O*-seryl-*O*-seryl serine (**3**, t_R 29.9 min, 20 mg) were obtained from the HP-20 fractions eluted with 60% and 80% MeOH, respectively.

Catechoserine (1)

Colorless, amorphous solid; $[\alpha]_D^{23}$+13 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 211 (4.62), 248 (4.17), 319 (3.78) nm; IR (ATR) ν_{max} 3356, 2960, 1728, 1640 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HR-ESITOFMS $(M+Na)^+$ 320.1101 (calcd for $C_{14}H_{19}NO_6Na$, 320.1105).

Compound 2

Colorless, amorphous solid; $[\alpha]_D^{23}$+13 (*c* 0.10, MeOH); ^1H NMR (500 MHz, DMSO-d_6) δ 3.78 (1H, dd, *J*=11.0, 3.7 Hz, H-8), 3.82 (1H, dd, *J*=11.0, 5.5 Hz, H-8), 4.41 (1H, dd, *J*=11.0, 6.2 Hz, H-11), 4.57 (1H, m, H-9), 4.62 (1H, dd, *J*=11.0, 3.5 Hz, H-11), 4.78 (1H, m, H-12), 6.70 (1H, t, *J*=7.5 Hz, H-5), 6.72 (1H, t, *J*=7.5 Hz, H-19), 6.94 (1H, d, *J*=7.0 Hz, H-4), 6.95 (1H, d, *J*=7.0 Hz, H-18), 7.33 (1H, d, *J*=8.0 Hz, H-20), 7.37 (1H, d, *J*=7.0 Hz, H-6), 8.94 (1H, d, *J*=7.5 Hz, 7-NH), 8.97 (1H, br s, 12-NH); ^{13}C NMR (125 MHz, DMSO-d_6) δ 51.7 (C-12), 55.2 (C-9), 60.7 (C-8), 63.7 (C-11), 115.5 (C-15), 115.9 (C-1), 118.1 (C-20), 118.21 (C-5)[a] 118.24 (C-19)[a] 118.6 (C-6), 118.8 (C-4), 118.9 (C-18), 146.0 (C-17), 146.1 (C-3), 148.2 (C-2), 148.6 (C-16), 168.5 (C-7), 168.7 (C-14), 169.9 (C-10), 170.3 (C-13); [a]assignment interchangeable. HR-ESITOFMS $(M-H)^-$ 463.0988 (calcd for $C_{20}H_{19}N_2O_{11}$, 463.0944).

Compound 3

Colorless, amorphous solid; $[\alpha]_D^{23}$+11 (*c* 0.10, MeOH); ^1H NMR (500 MHz, DMSO-d_6) δ 3.74 (1H, dd, *J*=11.0, 3.7 Hz, H-8), 3.80 (1H, dd, *J*=11.0, 6.0 Hz, H-8), 4.41 (1H, dd, *J*=11.0, 7.0 Hz, H-21), 4.44 (1H, dd, *J*=11.0, 6.5 Hz, H-11), 4.59 (2H, m, H-9 and H-21), 4.61 (1H, dd, *J*=11.0, 4.0 Hz, H-11), 4.74 (1H, br s, H-12), 4.90 (1H, ddd, *J*=7.0, 7.0, 4.5 Hz, H-22), 6.67 (1H, t, *J*=8.0 Hz, H-19), 6.68 (1H, t, *J*=7.5 Hz, H-29), 6.69 (1H, t, *J*=7.5 Hz, H-5), 6.93 (3H, m, H-4, H-18, and H-28), 7.28 (1H, d, *J*=8.0 Hz, H-30), 7.29 (1H, d, *J*=8.0 Hz, H-20), 7.37 (1H, d, *J*=8.0 Hz, H-6), 8.90 (1H, br s, 7-NH), 8.98 (1H, br s, 12-NH), 9.10 (1H, d, *J*=7.0 Hz, 22-NH); ^{13}C NMR (125 MHz, DMSO-d_6) δ 52.0 (C-22), 52.3 (C-12), 55.6 (C-9), 61.3 (C-8), 63.6 (C-21), 64.7 (C-11), 115.8 (C-15)[a] 116.0 (C-25)[a] 116.6 (C-1), 118.5 (C-5)[b] 118.6 (C-19)[b], 118.7 (C-20, C-29, C-30)[b,f], 119.1 (C-6), 119.3 (C-4)[c], 119.4 (C-18)[c], 119.5 (C-28)[c], 146.54 (C-3)[d], 146.56 (C-17)[d], 146.58 (C-27)[d], 148.7 (C-2), 149.2 (C-16)[e], 149.4 (C-26)[e], 168.8 (C-24), 169.1 (C-14), 169.3 (C-7), 169.5 (C-13), 170.3 (C-23), 170.8 (C-10); [a–e]assignment interchangeable; [f]overlapped signals. HR-ESITOFMS $(M-H)^-$ 686.1468 (calcd for $C_{30}H_{28}N_3O_{16}$, 686.1475).

Marfey's analysis

A portion of **1** (0.5 mg) was hydrolyzed at 110 °C with 6 N HCl (200 µl) for 15 h, 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-Ser and D-Ser. A solution of L-FDLA in acetone (0.05 mg in 50 µl) was added to each reaction tube. Each tube was sealed and incubated at 50 °C for 30 min. To quench reactions, 50 µl of 2 N HCl was added and then diluted with 100 µl of MeOH/0.2% HCO_2H (50 : 50). The Marfey's derivatives of the hydrolysate and standards were analyzed by HPLC using a Cosmosil 5C18-AR-II column (Nacalai Tesque, Kyoto, Japan, 4.6×250 mm) eluted with MeCN-2% HCO_2H in H_2O at a flow rate of 1.0 ml min^{-1}, monitoring at 340 nm. The gradient elution was set as follows: 0–5 min (25% MeCN), 5–45 min (25–55% MeCN). Retention times for the amino-acid standards were L-Ser-L-FDLA 22.0 min and D-Ser-L-FDLA 22.9 min, while the L-FDLA-hydrolysate of **1** gave a peak at 22.0 min.

Biological assays

Invasion and migration assays[14] and cytotoxic, cell adhesion and matrix metalloproteinase assays[16] were carried out according to the procedures previously described.

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