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Chlorocatechelins A and B from *Streptomyces* sp.: New Siderophores Containing Chlorinated Catecholate Groups and an Acylguanidine Structure

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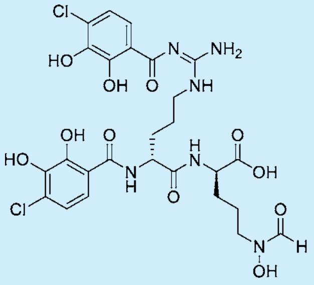
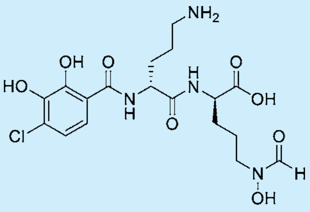
Ⓢ *Supporting Information*

ABSTRACT: Two novel siderophores, chlorocatechelins A and B, were isolated from a culture broth of *Streptomyces* sp. Their structures were determined by spectroscopic analysis and degradation study. They contain chloro-substituted catecholate that has not been reported in natural products, whereas this group was conjugated to guanidine to form acylguanidine in chlorocatechelin A. This acylguanidine decomposed in weakly acidic solutions to furnish a less potent siderophore chlorocatechelin B. Chemical and biological insights into acylguanidine are also discussed.



chlorocatechelin A (**1**)



chlorocatechelin B (**2**)

Iron is an essential element for organisms as it is requisite in various biological processes including primary metabolism. However, most iron exists as insoluble Fe(III) under aerobic conditions at neutral pH and is hard to be taken up by organisms. To overcome this problem, microbes produce and secrete low molecular weight compounds called siderophores which can capture and solubilize Fe(III).[1] Siderophores, in many cases, possess three bidentate ligands in one molecule to form a stable octahedral complex with Fe(III). The bidentate groups are usually catecholate, hydroxamate, or α-hydroxycarboxylate.[2] On the other hand, the backbone structures to which bidentate groups are attached vary among siderophores. Variation of the backbone and combination of the bidentate groups give structural diversity to siderophores, which can benefit their producers on iron availability. For example, amychelin,[3] produced by *Amycolatopsis* sp. AA4, seems to frustrate "siderophore piracy": amychelin inhibited growth of microbes that utilize siderophores secreted by other organisms.[4]

Once Fe(III)-siderophore complexes are formed and incorporated into cells, microbes need to extract Fe(III) from the stable complex. The most common way to release iron is to convert the coordinated Fe(III) to Fe(II) by ferric reductase.[5] The redox potentials (vs the normal hydrogen electrode (NHE)) of siderophore-coordinated Fe(III) range from −82 mV to −750 mV (enterobactin),[6] which are reported to have good relationships with the affinity of siderophore for Fe(III) in physiological conditions.[7,8] Fe(III)-siderophore complexes with relatively high redox potentials such as ferrioxamine B (−454 mV)[6] can be reduced directly with ferric reductases. However, when the complex exhibits much lower redox potentials such as Fe(III)-enterobactin, the siderophore needs to be partially degraded before reduction.[9]

During the course of our screening for siderophores from microbial extracts, we discovered novel siderophores named chlorocatechelin A (**1**) and B (**2**) (Figure 1). The backbone structure of metabolite **1** was a dipeptide, to which two sets of 4-chloro-2,3-dihydroxy-benzoic acid (CDB) were attached. CDB has never been found in natural products. In addition, one CDB unit was condensed with guanidine to form an acyl guanidine structure, which has been reported only in a few natural products, such as heterobactin (**3**).[10,11] Herein we report isolation, structure elucidation, and iron-binding properties of chlorocatechelins. We also discuss the lability of acylguanidine found in chlorocatechelin A (**1**).

Broth extracts of microorganisms (∼1100) were screened based on Fe(III)-chelating activities using chrome azurol S (CAS) assay.[12] An extract of a culture broth of *Streptomyces* sp. ML93-86F2 showed a rapid decrease of absorbance at 620 nm that corresponds to the CAS-Fe(III) complex, indicating that the extract contained Fe(III)-chelating substances. Addition of a ferric or ferrous ion to the culture apparently suppressed production of some metabolites, suggesting that the chelating

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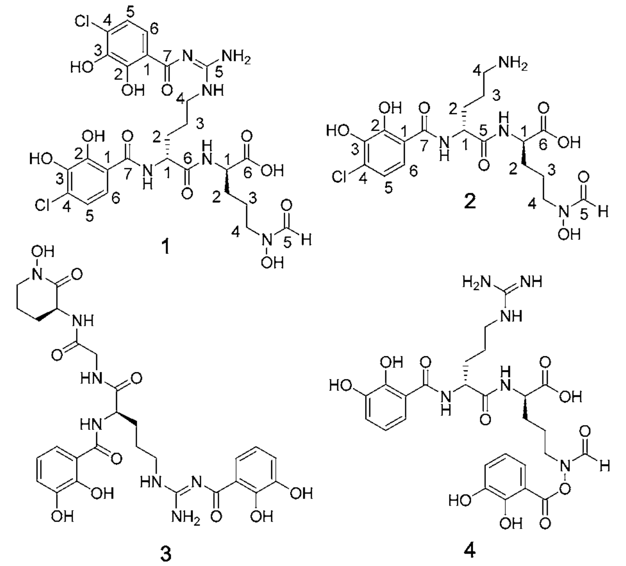


Figure 1. Chemical structures of chlorocatechelins A (**1**) and B (**2**), and structurally related compounds heterobactin (**3**) and mirubactin (**4**).

substances may work as siderophores under physiological conditions (Figure S1). For isolating the siderophores, a scale-up culture (1400 mL) of this microorganism was prepared and extracted with 2240 mL of EtOH. A half portion of this extract was subjected to a series of solvent partition followed by ODS column chromatography using stepwise elution with aqueous MeOH (0−100% MeOH). CAS-positive fractions eluted with 30−90% MeOH were combined, which was fractionated by reversed-phase HPLC to yield chlorocatechelins A (**1**) (75.6 mg)[13] and B (**2**) (12.4 mg).[14]

The LR-MS spectra of **1** showed a characteristic isotope pattern; the intensity ratio of signals for *m/z* 673, 675, and 677 was 9:6:1, indicating that **1** contains two chlorine atoms. The molecular formula of **1** was established as $C_{26}H_{30}Cl_2N_6O_{11}$ on the basis of HR-ESIMS (*m/z* 673.1432 $[M + H]^+$, calcd 673.1422). The ^1H NMR spectrum exhibited several exchangeable protons from 9.5 to 8.3 ppm and two α protons at 4.55 (1H, m) and 4.20 ppm (1H, m), indicating the presence of a peptide moiety. Four aromatic protons at 7.48 (1H, d, J = 8.5 Hz), 7.24 (1H, d, J = 8.2 Hz), 6.86 (1H, d, J = 8.5 Hz), and 6.60 ppm (1H, d, J = 8.2 Hz) were observed, whereas the ^{13}C NMR spectrum contained 12 aromatic carbons at 153.6, 151.1, 143.0, 142.6, 123.5, 121.8, 119.4, 118.4, 118.2, 116.8, 116.0, and 113.6 ppm, suggesting the presence of two aromatic rings. A detailed analysis of the 2D NMR data including DQF-COSY, HMQC, and HMBC spectra revealed that **1** was composed of one arginine (Arg), one *N*-δ-hydroxy-*N*-δ-formyl ornithine (hfOrn), and two trisubstituted benzoic acid residues. The benzoic acids were revealed to be 4-chloro-2,3-dihydroxy benzoic acids (CDB) on the basis of the molecular formula and the extensive analysis of NMR and UV absorption spectra.[15,16] The *N*-hydroxy-*N*-formyl group in hfOrn and two CDB residues were suggested to be putative iron-chelating moieties (Figure 2a). Two sets of ^1H and ^{13}C signals were observed in the hfOrn moiety due to the geometrical isomerization of the *N*-hydroxy-*N*-formyl group.

The order of the backbone in compound **1** was determined by an HMBC correlation from H1 of hfOrn to C6 of Arg. The connectivity of CDB1 to Arg was also determined by HMBC correlations from H1 and amide NH of Arg to C7 of CDB1. However, we could not determine the location of CDB2 because of the lack of HMBC correlations. Next, compound **1** was subjected to MS/MS analysis. The $[M + H]^+$ ion of **1** was fragmented by collision-induced dissociation (CID) to give various product ions including fragments associated with CDB-Arg-hfOrn (*m/z* 503.17), 2CDB-Arg (*m/z* 497.07), CDB-Arg (*m/z* 327.08), and CDB-Orn (*m/z* 285.06)[17] (Figure S2). The observation of the 2CDB-Arg fragment ion excluded the possibility that CDB2 was connected to hfOrn as reported for mirubactin (**4**).[18] We next measured the ^{13}C NMR spectra of **1** in 600 μL of DMSO-d_6 and 10 μL of H_2O or D_2O. Low-field shifts due to hydrogen−deuterium exchange were observed for signals at C2 and C3 of CDB1, suggesting that the hydroxyl groups of CDB1 existed as free forms (Figure 3). Taken together, we deduced the connectivity of CDB2 and the guanidino group of Arg. The absolute configuration of **1** was determined by an advanced Marfey method.[19] An acid hydrolysate of **1** was reacted with 1-fluoro-2,4-dinitrophenyl-5-L/D-leucinamide (L- or D-FDLA) and analyzed by LC-MS to reveal the presence of D-Arg and D-Orn. Hence, the structure of **1** was determined as shown in Figure 1.

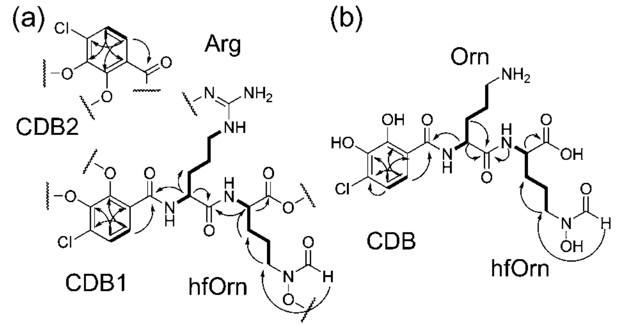


Figure 2. HMBC (arrow) and DQF-COSY (bold) correlations of (a) **1** and (b) **2**.

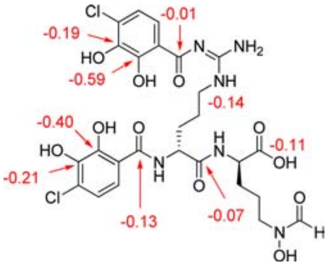


Figure 3. Key deuterium shift values $(\Delta\delta = \delta_{(+D_2O)} - \delta_{(+H_2O)})$ in ^{13}C NMR spectra of **1** in DMSO-d_6.

The molecular formula of chlorocatechelin B (**2**) was established as $C_{18}H_{25}ClN_4O_8$ on the basis of HR-ESIMS (*m/z* 461.1433 $[M + H]^+$, calcd 461.1434). Metabolite **2** gave ^1H and ^{13}C NMR spectra that were simpler than those of **1**. The planar structure of **2** was determined by 2D NMR analysis (Figure 2b) to be composed of one unit each of CDB, Orn, and hfOrn. The connectivity of CDB to Orn was determined by an HMBC correlation from H1 of Orn to C7 of CDB. No HMBC correlation between Orn and hfOrn was observed in CD_3OD; hence, we next recorded the NMR spectra in CD_3OH. Two amide protons at 8.67 (1H, br m) and 8.47 ppm (1H, br m) were observed in the ^1H NMR spectrum, and these were assigned as 1-NH of Orn and hfOrn, respectively, based on a

DQF-COSY experiment. An HMBC correlation from 1-NH of hfOrn to C5 of Orn was observed, and thus hfOrn was connected to Orn. Advanced Marfey analysis[19] revealed the absolute stereochemistries of both Orn and hfOrn were D, and thus the chemical structure of **2** was determined as shown in Figure 1.

Characteristic features of the chemical structures of chlorocatechelins implied that they are siderophores. In fact, chlorocatechelins A (**1**) and B (**2**) in cell culture were rarely detected in the presence of iron (Figure S1). To investigate the iron binding property of **1**, we first examined a spectrophotometric titration experiment of compound **1** with $FeCl_3$ in 50 mM BisTris buffer (pH 7.0, I = 0.1 M, Figure 4a, S3a). An increasing absorbance at 516 nm was observed until 1 equiv of $FeCl_3$ was added, indicating that **1** formed a complex with Fe(III) at a 1:1 ratio. Next, the redox potential of the Fe(III)-**1** complex was measured in cyclic voltammetry (CV) experiments. The mixture of **1** (100 μM) and $FeCl_3$ (60 μM) in 5 mM BisTris buffer (pH 7.0, I = 0.1 M, Figure 4b) gave a reversible voltammogram with a half wave potential ($E_{1/2}$) of −578 mV (vs NHE). Under our conditions, the well-studied Fe(III)-enterobactin complex exhibited irreversible voltammograms, whereas ferrioxamine B showed a reversible voltammogram with an $E_{1/2}$ of −440 mV (Figure S4a). These results indicated that the Fe(III)-binding affinity of chlorocatechelin A (**1**), possessing two CDB groups and one *N*-hydroxy-*N*-formyl group, is higher than that of desferrioxamine B, a siderophore with three hydroxamate groups, and lower than that of the catecholate-type-siderophore enterobactin (reported $E_{1/2}$ = −750 mV[6]).

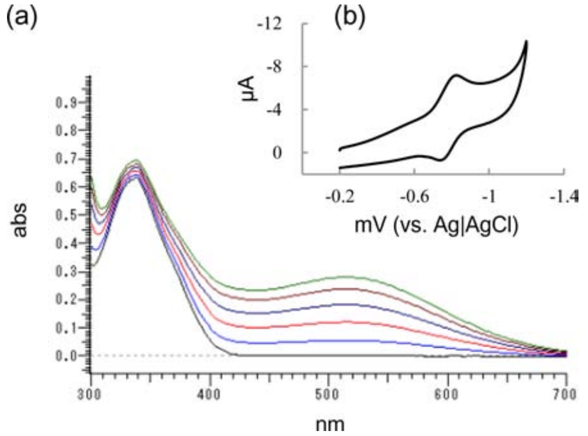
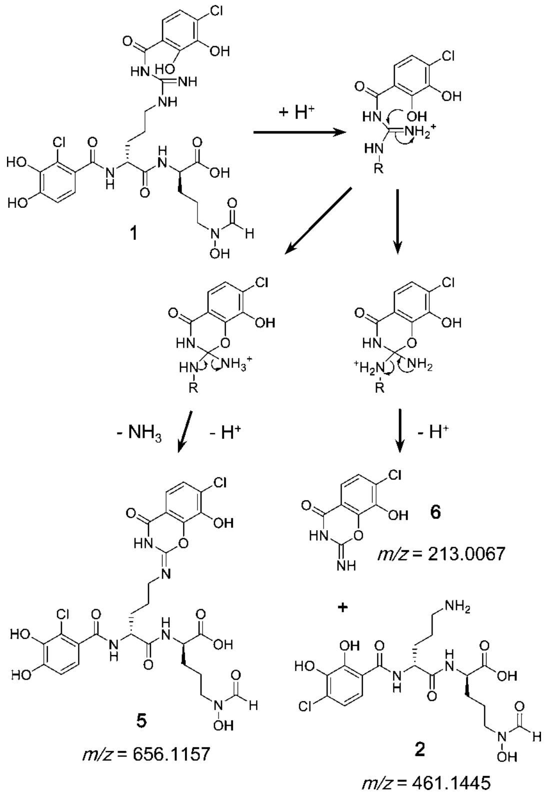


Figure 4. Iron binding properties of **1**. (a) Spectrophotometric titration of **1** with $FeCl_3$ (every 0.2 equiv from 0 to 1.0 equiv, black to green) in 50 mM Bis-Tris buffer (pH 7.0, I = 0.1 M with NaCl). (b) Cyclic voltammogram of Fe(III)-**1** complex (100 μM of **1** and 60 μM of $FeCl_3$) in 5 mM BisTris buffer (pH 7.0, I = 0.1 M with NaCl).

The molar ratio in the complex of chlorocatechelin B (**2**) and Fe(III) was also investigated by measuring the UV−vis spectral changes, revealing that three molecules of **2** can bind one molecule of Fe(III) (Figure S3b, S3c). To compare the redox potential of Fe(III)-**1** and that of Fe(III)-**2**, CV analysis of Fe(III)-**2** was conducted under various conditions. However, a reversible voltammogram was not obtained; the cathodic peak potential (E_{pa} = −450 mV) suggested the $E_{1/2}$ of Fe(III)-**2** was higher than −450 mV (Figure S4b). These data revealed that the redox potential of Fe(III)-**2** is much higher than that of Fe(III)-**1**, indicating that compound **2** had weaker affinity for Fe(III) than compound **1**.

During purification procedures, chlorocatechelin A (**1**) was always accompanied by a small amount of chlorocatechelin B (**2**) in the LC-MS analysis. We wondered if **1** was spontaneously converted to **2**. To test this possibility, the stability of **1** under various pH conditions (buffered with sodium phosphate) was tested. Compound **1** was stable in neutral and basic solutions (pH 7−11) for 24 h. On the other hand, **1** gave three compounds when incubated in acidic conditions (pH 3−5) (Figures S5, S6). One of them showed the same retention time, HRMS, and MS/MS fragmentation pattern as those of **2** in an LC-MS analysis, indicating this newly formed compound was **2**. The other two peaks had an *m*/*z* of 213.0067 and 656.1157, respectively. Judging from the molecular formula, the compound with an *m*/*z* of 213.0067 (corresponding to $C_8H_6ClN_2O_3$, Δmmu = +0.6) might be a fragment **6** that can be produced when compound **1** is decomposed to **2**. Elimination of NH_3 instead of **6** from compound **1** should give compound **5** with *m*/*z* 656.1157 (corresponding to $C_{26}H_{28}Cl_2N_5O_{11}$, Δmmu = 0.0) (Scheme 1).

Scheme 1. Proposed Mechanism for Decomposition of 1 and Production of 2



Considering the fact that compound **2** showed much lower affinity for Fe(III) than **1**, the labile CDB-guanidine function likely contributes to inactivation of **1**. One of the well-known inactivation systems of siderophores is hydrolysis by an esterase; one of three ester bonds in enterobactin is hydrolyzed by esterase when enterobactin forms a complex with Fe(III), which facilitates reduction of Fe(III) catalyzed by ferric reductase.[9] In our proposed model for degradation of **1** to **2**, nucleophilic attack by 2-OH of CDB2 is requisite (Scheme 1). Since this hydroxyl group may coordinate to Fe(III) in the complex of Fe(III)-**1**, degradation of Fe(III)-**1** might not occur.[20] Therefore, degradation of **1** might occur, not for releasing Fe(III), but for other purposes, e.g. inactivating an excess amount of the free siderophore.

In this study, two novel siderophores chlorocatechelins A (**1**) and B (**2**) were isolated and their structures were determined by spectroscopic analysis and degradation study. Chlorocatechelins **1** and **2** were the first natural products that contain CDB units. In addition, **1** possessed a rare acylguanidine structure. The acylguanidine structure is essential for the potent Fe(III)-chelating ability of chlorocatechelin A (**1**). Decomposition of the acylguanidine structure in acidic conditions furnished a lower-affinity siderophore chlorocatechelin B (**2**). Producing labile compounds leads to a waste of energy for microbes unless the decomposition plays any productive role. Detailed analysis of the metabolism of Fe(III)-**1** and Fe(III)-**2** in and around the producer and surrounding organisms would reveal new functions of siderophores.

ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedure, spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(13) Chlorocatechelin A (**1**): brown amorphous solid; $[\alpha]^{20}_{D}$ +2.6 (*c* 0.63, MeOH); UV (MeOH) λ_{max} (log ε) 212 (4.62), 261 (4.29), 315 (3.60) nm; IR (neat) 3297 (br), 2987, 1659, 1645, 1592, 1543, 1439, 1315, 1219 cm^{-1}; HRMS (ESI) *m/z* 673.1432 $[M + H]^+$ calcd for $C_{26}H_{31}Cl_2N_6O_{11}$, 673.1422; for ^{1}H NMR and ^{13}C NMR, see Table S1

(14) Chlorocatechelin B (**2**): brown amorphous solid: $[\alpha]^{20}_{D}$ −7.7 (*c* 0.70, MeOH); UV (MeOH) λ_{max} (log ε) 210 (4.38), 256 (4.01), 309 (3.33) nm; IR (neat) 3162 (br), 3085, 2936, 1669, 1653, 1558, 1541, 1437 cm^{-1}; HRMS (ESI) *m/z* 461.1433 $[M + H]^+$ calcd for $C_{18}H_{26}ClN_4O_8$, 461.1434; for ^{1}H NMR and ^{13}C NMR, see Table S2.

(15) The molecular formula and NMR spectra suggested two possibilities, 4-chloro-2,3-dihydroxybenzoic acid and 2-chloro-3,4-dihydroxybenzoic acid. The UV spectra of **1** and **2** indicated that they contained the former ones.[16]

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(17) This fragment peak might be produced by cleavage of the guanidine carbon and 5-NH in Arg as shown in Scheme 1, in addition to the amide bond fragmentation between Arg and hfOrn.

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(20) Although we tried to verify our hypothesis, we could not test it since Fe(III)-**1** precipitated in acidic conditions.