Genomics-driven discovery of chiral triscatechol siderophores with enantiomeric Fe(III) coordination†

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Ferric complexes of triscatechol siderophores may assume one of two enantiomeric configurations at the iron site. Chirality is known to be important in the iron uptake process, however an understanding of the molecular features directing stereospecific coordination remains ambiguous. Synthesis of the full suite of (DHB-Lys-Ser)3 macro lactone diastereomers, which includes the siderophore cyclic trichysobactin (CTC), enables the effects that the chirality of Lys and Ser residues exert on the configuration of the Fe(III) complex to be defined. Computationally optimized geometries indicate that the Δ/Δ configurational preferences are set by steric interactions between the Lys sidechains and the peptide backbone. The ability of each (DHB-Lys-Ser)3 diastereomer to form a stable Fe(III) complex prompted a genomic search for biosynthetic gene clusters (BGCs) encoding the synthesis of these diastereomers in microbes. The genome of the plant pathogen Dickeya chrysanthemi EC16 was sequenced and the genes responsible for the biosynthesis of CTC were identified. A related but distinct BGC was identified in the genome of the opportunistic pathogen Yersinia frederiksenii ATCC 33641; isolation of the siderophore from Y. frederiksenii ATCC 33641, named frederiksenibactin (FSB), revealed the triscatechol oligoester, linear-(DHB-Lys-Ser)3. Circular dichroism (CD) spectroscopy establishes that Fe(III)-CTC and Fe(III)-FSB are formed in opposite enantiomeric configuration, consistent with the results of the ferric complexes of the cyclic (DHB-Lys-Ser)3 diastereomers.

Introduction

Chirality is universally significant in biological reactions, including the essential microbial process of iron acquisition mediated by small-molecule chelators known as siderophores. The specific three-dimensional structure of an Fe(III)-siderophore complex plays a role in the ability of a bacterium to recognize, acquire, and extract iron from it.1,2 The triscatechol siderophores enterobactin (Ent) and bacillibactin (BB) each coordinate Fe(III) with three 2,3-dihydroxybenzoyl (DHB) ligands framed on a macro lactone derived from three Ser or Thr residues, respectively [ESI Fig. S1†]. Unlike Ent, BB also contains a glycine residue inserted between the macrolactone core and DHB. In stark contrast to Fe(III)-Ent3−, which forms exclusively in the Δ configuration,3,4 Fe(III)-BB3− adopts the opposing Δ configuration.4 Several related triscatechol siderophores are further distinguished from Ent and BB by the presence of a chiral amino acid inserted between DHB and the oligoester backbone, including cyclic trichysobactin [CTC; Dickeya chrysanthemi EC16] with D-Lys,5 as well as the linear tris-Ser scaffolds of trivanchrobactin with D-Arg,6 and turneribactin with D-Om.7 Structurally, the influence amino acids exert on the configuration at the Fe(III) site is incompletely understood; evolutionarily, these structural differences hint that chirality confers a competitive advantage in microbial iron uptake.

To understand the factors controlling stereospecific Fe(III) coordination of the expanded triscatechol–triserine siderophore CTC, (DHB-Lys-Ser)3, we synthesized the full suite of cyclic (DHB-Lys-Ser)3 diastereomers of CTC (Fig. 1). We report that circular dichroism (CD) spectroscopic measurements of the Fe(III) complexes of these ligands allow the relationship between siderophore chirality and the configuration at the Fe(III) site to be defined. Computational modeling of the ferric complexes reveals steric interactions between the Lys sidechains and the peptide backbone dictate the configurational preference. Fe(III) complexation by each (DHB-Lys-Ser)3 diastereomer prompted microbial genome mining that led to the discovery of a new triscatechol siderophore, frederiksenibactin (FSB). FSB features Lys residues inserted between...
Chiral triscatechol siderophores and synthetic analogs are capable of coordinating labile metal ions with a thermodynamic preference for a specific stereochemistry at the metal center. The presence of chirality at both the metal center and in the ligand renders the Δ and Λ stereoisomers diastereomeric and energetically inequivalent. To establish the relationship between the chirality at the amino acid adjacent to the catecholamide, the stereochemistry of the triserine macrolactone, and the stereochemistry at Fe(III), we synthesized the four C3-symmetric cyclic diastereomers (DHB^3-Lys^3-Ser)_3, (DHB^3-Lys^3-Ser)_3, (DHB^3-Lys^3-Ser)_3, and (DHB^3-Lys^3-Ser)_3 (Scheme S1†) of which (DHB^3-Lys^3-Ser)_3 is structurally identical to CTC. Well-established methodology to construct the cyclic triserine macrolactone (1 in Scheme S1†), provided a convenient synthetic platform to access CTC and related diastereomers (Fig. S2–S6, Tables S1 and S2†). In the absence of crystallographic information, circular dichroism (CD) spectroscopy can provide information on the stereochemical configuration of optically-active metal complexes. As expected, enantiomeric pairs of ligands, such as (DHB^3-Lys^3-Ser)_3 and (DHB^3-Lys^3-Ser)_3, coordinate Fe(III) with opposite handedness, as indicated by the CD spectra (Fig. 2). Two prominent CD bands at 435 nm and 545 nm arise from characteristic LMCT transitions and are therefore sensitive to the chirality at the iron center. When comparing diastereomeric ligands, we observed that the CD spectra of Fe([DHB^3-Lys^3-Ser]_3 and Fe([DHB^3-Lys^3-Ser]_3 are similar in both the sign and the intensity. The analogous correspondence was observed for the CD bands of Fe([DHB^3-Lys^3-Ser]_3 (i.e., CTC) and Fe([DHB^3-Lys^3-Ser]_3 (Fig. 2, Table 1).† Comparison of the signs of the Cotton effects for the Fe([DHB^3-Lys^3-Ser]_3 complexes with those of Fe(III)--Ent^− and Fe(III)--BB^3+, for which the chirality at the metal center is known, allows the configuration of the new complexes to be determined. (DHB^3-Lys^3-Ser)_3 and (DHB^3-Lys^3-Ser)_3 both form Δ complexes, whereas (DHB^3-Lys^3-Ser)_3 and (DHB^3-Lys^3-Ser)_3 both form Λ complexes. These results suggest that the handedness of metal-ion chelation is set by the chirality of the Lys unit, and not the chirality of the triserine macrolactone. In comparison, the Δ configuration of Fe(III)--Ent^− and Δ configuration of Fe(III)--enantioEnt^− has been attributed to nonbonding interactions within the chiral triserine macrolactone.†

### Results and discussion

#### Chirality of Fe(III)--([DHB^L-D,Lys^L-D,Ser]_3) diastereomers

Chiral triscatechol siderophores and synthetic analogs are capable of coordinating labile metal ions with a thermodynamic preference for a specific stereochemistry at the metal center. The presence of chirality at both the metal center and in the ligand renders the Δ and Λ stereoisomers diastereomeric and energetically inequivalent. To establish the relationship between the chirality at the amino acid adjacent to the catecholamide, the stereochemistry of the triserine macrolactone, and the stereochemistry at Fe(III), we synthesized the four C3-symmetric cyclic diastereomers (DHB^3-Lys^3-Ser)_3, (DHB^3-Lys^3-Ser)_3, (DHB^3-Lys^3-Ser)_3, and (DHB^3-Lys^3-Ser)_3 (Scheme S1†) of which (DHB^3-Lys^3-Ser)_3 is structurally identical to CTC. Well-established methodology to construct the cyclic triserine macrolactone (1 in Scheme S1†), provided a convenient synthetic platform to access CTC and related diastereomers (Fig. S2–S6, Tables S1 and S2†). In the absence of crystallographic information, circular dichroism (CD) spectroscopy can provide information on the stereochemical configuration of optically-active metal complexes. As expected, enantiomeric pairs of ligands, such as (DHB^3-Lys^3-Ser)_3 and (DHB^3-Lys^3-Ser)_3, coordinate Fe(III) with opposite handedness, as indicated by the CD spectra (Fig. 2). Two prominent CD bands at 435 nm and 545 nm arise from characteristic LMCT transitions and are therefore sensitive to the chirality at the iron center. When comparing diastereomeric ligands, we observed that the CD spectra of Fe([DHB^3-Lys^3-Ser]_3 and Fe([DHB^3-Lys^3-Ser]_3 are similar in both the sign and the intensity. The analogous correspondence was observed for the CD bands of Fe([DHB^3-Lys^3-Ser]_3 (i.e., CTC) and Fe([DHB^3-Lys^3-Ser]_3 (Fig. 2, Table 1).† Comparison of the signs of the Cotton effects for the Fe([DHB^3-Lys^3-Ser]_3 complexes with those of Fe(III)--Ent^− and Fe(III)--BB^3+, for which the chirality at the metal center is known, allows the configuration of the new complexes to be determined. (DHB^3-Lys^3-Ser)_3 and (DHB^3-Lys^3-Ser)_3 both form Δ complexes, whereas (DHB^3-Lys^3-Ser)_3 and (DHB^3-Lys^3-Ser)_3 both form Λ complexes. These results suggest that the handedness of metal-ion chelation is set by the chirality of the Lys unit, and not the chirality of the triserine macrolactone. In comparison, the Δ configuration of Fe(III)--Ent^− and Δ configuration of Fe(III)--enantioEnt^− has been attributed to nonbonding interactions within the chiral triserine macrolactone.†

#### Computational modeling

To better understand the mechanism by which amino acid chirality dictates the configurational preferences of the Fe(III)--([DHB^L-D,Lys^L-D,Ser]_3) complexes, the structures and energies of the four enantiomeric pairs of diastereomers were optimized computationally (PBE0/6-311++G(d,p)) (Fig. 3). Comparing the energies of the Fe(III) complexes of a given ligand with different handedness, we observe complete agreement with the CD spectroscopic results. For example, the energy of A-Fe(III)--([DHB^3-Lys^3-Ser]_3) is lower than that of Δ-Fe(III)--([DHB^3-Lys^3-Ser]_3), consistent with the formation of the Δ complex in aqueous solution (Fig. 2).

Insight into the origin of the differential stabilities of the Fe(III)--([DHB^L-D,Lys^L-D,Ser]_3) complexes comes directly from the

#### Table 1

| Ligand      | Cyclic (C) or linear (L) Configuration | C or L | Δmax (nm) | Δε (M⁻¹ cm⁻¹) |
|-------------|---------------------------------------|-------|----------|---------------|
| Ent = ([DHB^3-Ser]_3, ref. 3)          | C      | Δ        | 553      | -2.2          |
| BB = ([DHB-Gly^2-Thr])                | C      | Δ        | 545      | +1.7          |
| (DHB^3-Lys^3-Ser)_3                   | C      | Δ        | 563      | -2.3          |
| (DHB^3-Lys^3-Ser)                     | C      | Δ        | 559      | +2.2          |
| (DHB^3-Lys^3-Ser)                     | C      | Δ        | 561      | -2.3          |
| (DHB^3-Lys^3-Ser)                     | C      | Δ        | 558      | +2.1          |
| Frederiksenibactin (FSB) L            | L      | Δ        | 556      | -2.2          |

*a 40 μM Fe(III) complexes in citrate–phosphate buffer, pH 7.40.

b ([DHB^3-Lys^3-Ser]) is the cyclic analog of FSB. c Synthetic (DHB^3-Lys^3-Ser), shown here is indistinguishable from CTC isolated from *D. chrysanthemi* EC16.
optimized geometries (Fig. 4). In this molecular framework, the Lys sidechains are able to wrap around the complex so as to allow each terminal ammonium group to hydrogen-bond with the carbonyl of the DHB unit of an adjacent arm. We observe, however, that this interaction is present in all of the optimized geometries, preferred and non-preferred.

Closer analysis revealed that the prime influence of the Lys residue chirality is the impact that it has on $\psi$ (N-Carbonyl-C$_x$-N torsion angle). It is well established that certain values of $\psi$ are unfavorable for polypeptides, contributing, for example, to the characteristic distribution of protein dihedral angles in Ramachandran plots. Specifically, favorable $\psi$ values are those that prevent the amino acid side chain from eclipsing the adjacent carbonyl. In the Fe(III)–[(DHBDLys$^+$Ser)$_3$] complexes, combination of either $\Delta$ configuration at Fe(III) and $\delta$Lys, or $\Lambda$ and $\delta$Lys, produce $\psi$ angles near $\pm 60^\circ$, which introduces a steric clash between the carbonyl O atom and the Lys side chain (Fig. 5). In contrast, the combination of $\Lambda$ and $\delta$Lys, as occurs in CTC, produces no such clash.

It is noteworthy that, of all the diastereomeric combinations of metal chelation handedness and amino acid chirality, our calculations predict that the most stable structures are those assumed by $\Delta$-Fe(III)–[(DHBD$^+$Lys$^+$Ser)$_3$] and its enantiomer $\Lambda$-Fe(III)–[(DHBD$^-$Lys$^+$Ser)$_3$]. Organisms have adopted this stability by using (DHBP$^+$Lys$^+$Ser)$_3$, which is the siderophore CTC, for iron acquisition. The fact that the other diastereomers that we investigated also form Fe(III) complexes gives rise to the question of whether they too might be used biologically.

Genomic screen for catechol-based siderophores

Inspired by the discovery of other naturally occurring siderophores with $\delta$- and $\lambda$-amino acids – that is, trivanchrobactin ($\delta$-Arg), and turnerbactin ($\lambda$-Orn) – we initiated a search for biosynthetic gene clusters (BGCs) encoding diastereomers of CTC. The biosynthesis of chrysobactin (i.e., DHBDLys$^+$Ser) in D. dadantii 3937 requires genes encoding 2,3-DHB synthesis, as well as the non-ribosomal peptide synthetase (NRPS) CbsF with an epimerization, E, domain to convert $\lambda$Lys to $\delta$Lys. In contrast to D. dadantii 3937, the plant pathogen D. chrysanthemi EC16 produces not only the monocatechol chrysobactin, but also the triscatechol macrolactone CTC. We found that the genome of the D. chrysanthemi EC16 contains a BGC homologous to the cbs locus of D. dadantii 3937 (genome sequence reported herein; Tables S3 and S4†). Genome mining revealed similar but distinct BGCs in several Yersinia genomes, including the BGC freABCEF of opportunistic pathogen Yersinia frederiksenii ATCC 33641 (Tables S5 and S6†). The fre locus contains genes encoding 2,3-DHB synthesis, as well as the NRPS FreF with adenylation domains selecting for $\lambda$Lys and $\delta$Lys. However, FreF lacks an E domain, implicating biosynthesis of a siderophore comprised of DHBD$^+$Lys$^+$Ser units (Fig. 6).

**Frederiksenibactin and cyclic trichrysoibactin siderophores**

Siderophores from Y. frederiksenii ATCC 33641 were extracted and purified from a low-iron culture (Fig. S7†). Three compounds with m/z of 370.17, 721.31 and 1072.44 were identified by UPLC-ESIMS. These signals are equivalent to the [M + H]$^+$ signals for chrysobactin, linear dichrysobactin, and linear trichrysoibactin, which are DHBD$^+$Lys$^+$Ser, linear-(DHBD$^+$Lys$^+$Ser)$_2$, and linear-(DHBD$^+$Lys$^+$Ser)$_3$, respectively. In contrast to D. chrysanthemi EC16, which produces trichrysoibactin in both cyclic and linear forms, we have only been able...
to detect a linear triscatechol siderophore (m/z 1072.44; Fig. S8†) in the culture supernatant of *Y. frederiksenii* ATCC 33641. We have named this new siderophore frederiksenibactin (FSB). We note that the related triscatechol siderophores trivanchrobactin and turnerbactin are also linear and that their cyclic forms have not been detected in biological systems.  

Marfey’s analysis17 establishes the presence of ^1^Lys and ^3^Ser in FSB, consistent with the genomic prediction (Fig. S9†). The proposed structure of FSB was confirmed by ^1^H and ^13^C NMR spectroscopic data, which were assigned through ^1^H–^1^H COSY, ^1^H–^13^C HSQC, and ^1^H–^13^C HMBC NMR data (Fig. S10–S14†). While the NMR spectral data of FSB (Table S7†) are similar to those of CTC, several features confirm the mass spectrometric results indicating that FSB is a linear compound. Specifically, the three Ser residues are inequivalent (Fig. S11†). The Ser methylene protons involved in the backbone ester linkages, C16/C16′, at 4.25–4.46 ppm are shifted significantly downfield relative to the corresponding protons on C16 at 3.67 ppm and 3.78 ppm, which are adjacent to the unmodified Ser hydroxyl group. Additionally, the protons on the three methine carbons (C15, 4.59 ppm; C15′, 4.69 ppm; C15″, 4.41 ppm) are inequivalent, as are the protons on the chiral methine carbons derived from Lys (C9, C9′ and C9″, 4.50–4.65 ppm). The ^1^H NMR spectrum of FSB is consistent with related asymmetric linear triscatechol siderophores trivanchrobactin6 and turnerbactin.7 Thus, FSB is a novel siderophore and a natural diastereomer of linear trichrysobactin.

**Chirality of Fe(m)–FSB and Fe(m)–CTC**

The CD spectra of Fe(m)–CTC and Fe(m)–FSB (Fig. 7) appear as near mirror-images of each other, indicating an opposite configurational preference around iron. Through comparison to the CD spectra of Fe(m)–Ent3, Fe(m)–BB, and the Fe[m]–[[DHB/L/Lys/L/Ser]3] complexes (Table 1)3,4 Fe(m)–FSB is assigned a Δ configuration. The comparison of Fe(m)–FSB to Fe(m)–[[DHB/L/Lys/L/Ser]3] (Fig. 2, Table 1) also establishes that linearization of the trilactone does not significantly affect the configuration of the ferric complex. Earlier work revealed that linearization of Ent also does not invert its overall configurational preference, however, a small fraction of the Δ enantiomer is formed.11,13 Our earlier work with the cyclic Fe(m)–[[DHB/L/Lys/L/Ser]3] complexes suggests that the opposing chirality observed for ferric complexes of FSB and CTC is likely due to the stereochemistry of the Lys residue adjacent to the catecholamide and not due to the linear or cyclic nature of the triseryn backbone.

**Fe(m) exchange between FSB and CTC**

Surprisingly little is known about the exchange of Fe(m) among triscatechol siderophores. CD spectroscopy is uniquely poised to monitor Fe(m) exchange between optically-active siderophores. The intensity of the Δ–Fe(m)–CTC CD bands decrease upon addition of equimolar FSB as a result of formation of nearly equimolar Δ–Fe(m)–CTC and Δ–Fe(m)–FSB (Fig. 8A). Moreover the equivalent equilibration approached from reaction of Δ–Fe(m)–FSB with CTC is also observed (Fig. 8B). Interestingly, a weak negative band at 435 nm and a weak positive

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**Fig. 6** Biosynthetic gene clusters and structures of frederiksenibactin, FSB (*Y. frederiksenii* ATCC 33641) and cyclic trichrysobactin, CTC (*D. chrysanthemi* EC16).

**Fig. 7** CD spectra of 40 μM Fe(m)–FSB and 40 μM Fe(m)–CTC in citrate–phosphate buffer (pH 7.40).

**Fig. 8** Equilibration of Fe(m)–CTC and Fe(m)–FSB with FSB and CTC, respectively. (Top) Reaction of 100 μM Fe(m)–CTC with 100 μM FSB in 50 mM citrate–phosphate, pH 7.40. (Bottom) Reaction of 100 μM Fe(m)–FSB with 100 μM CTC in 50 mM citrate–phosphate, pH 7.40.
Conclusions

In sum, BGCs encoding synthesis of the triscatechol siderophores CTC and FSB were identified and the structure of FSB was elucidated. The opposing configurations of Δ-Fe(mn)-FSB and Δ-Fe(mn)-CTC are established by the stereochemistry at Lys. The most stable configuration by computational modeling is Δ-Fe(mn)-[(DHB<sup>+</sup>Lys<sup>−3</sup>Ser)<sub>3</sub>], which, strikingly, has been adopted for microbial iron acquisition as Fe(mn)-CTC. The next most stable conformation corresponds to the cyclic form of FSB, Δ-Fe(mn)-[(DHB<sup>+</sup>Lys<sup>−3</sup>Ser)<sub>3</sub>], raising questions about the predicted low energy conformations of the corresponding linear triscatechol siderophores.

The suite of cyclic and linear (DHB<sup>+</sup>Lys<sup>−3</sup>Ser)<sub>3</sub> siderophores and analogs raises further significant questions regarding the effect of a mismatched Δ- and Δ-Fe(mn) configuration on microbial iron uptake and growth. For example, discrimination at the outer membrane receptor protein could prevent uptake of the wrong Fe(mn)-enantiomer as has been observed with Fe(mn) complexes of pyochelin and enantio-pyochelin. If iron uptake is insensitive to the Fe(mn)-enantiomer chirality, discrimination could still occur at other points including the iron-release process, as is observed in Bacillus subtilis in the Fe(s)-catalyzed hydrolysis of the macroolactone of Fe(enantioEnt)<sub>3</sub> which is required for release of iron. Additionally, it may be possible for the relevant siderophore-interacting proteins to invert the configuration of a mismatched Fe(mn)-siderophore complex upon binding, as has been observed for the periplasmic binding protein CeuE of Campylobacter jejuni.

Siderophores are primarily extracellular metabolites and facile Fe(mn) exchange observed between triscatechol siderophores is likely of biological consequence within complex microbial communities. Certainly, the rate of release of the newly synthesized apo siderophores during growth of Y. frederiksenii ATCC 33641 and D. chrysanthemi EC16, which is occurring over the time scale of hours to days, could be exchanging Fe(mn) within hours with other triscatechol siderophores, as evinced by the CD results (Fig. 8). In fact Fe(mn) exchange between the triscatecholate siderophores is orders of magnitude faster than Fe(mn) exchange between hydroxamate siderophores or between hydroxamate and catecholate siderophores.

Experiments addressing the questions raised above are in progress, as well as the question of whether BGCs encoding the synthesis of the diastereomers of trivanchrobactin, (DHB<sup>+</sup>Lys<sup>−3</sup>Ser)<sub>3</sub>, and turnerbactin, (D HB<sup>+</sup>Orn<sup>−3</sup>Ser)<sub>3</sub>, are present in microbial genomes. The discovery of frederiksenibactin and its relationship to CTC exemplifies the structural variability of microbial siderophores and provides a natural system to determine the significance of chirality within siderophore-mediated microbial iron-uptake pathways.

Experimental

General experimental procedures

UV-visible absorbance and circular dichroism spectroscopy were measured on an Agilent Cary 300 UV Vis spectrophotometer and a Jasco J-1500 CD spectrophotometer, respectively. <sup>1</sup>C NMR spectroscopy was performed on a Bruker Advanced Neo 500 MHz spectrometer equipped with a probe cryoprobe at RT. All <sup>1</sup>H, COSY, HMBC, HSQC NMR spectroscopy was performed on a Varian Unity 600 MHz spectrometer at RT. Chemical shifts were referenced through residual solvent peaks <sup>1</sup>H (DMSO-d<sub>6</sub>) 2.50 ppm, <sup>1</sup>C (DMSO-d<sub>6</sub>) 39.51 ppm. Mass spectrometry analysis of Y. frederiksenii ATCC 33641 supernatant extracts and purified FSB was carried out on a Waters Xevo G2-XS QToF with positive mode electrospray ionization coupled to an ACQUITY UPLC H-Class system with a waters BEH C18 column. Y. frederiksenii ATCC 33641 culture extracts were analyzed using a linear gradient of 0–30% CH<sub>3</sub>CN (+0.1% formic acid) in ddH<sub>2</sub>O (+0.1% formic acid) over 10 min. For MS/MS analysis, a collision energy of 15 eV was employed. HR-ESIMS analysis of synthetic compounds was carried out on a Waters LCT Premier ESI TOF introduced into the ESI by direct infusion via a syringe pump.

Synthesis of the cyclic (DHB<sup>+</sup>Lys<sup>−3</sup>Ser)<sub>3</sub>, diastereomers

Established peptide coupling methodology was employed to construct the two key amide bonds in 4 (Scheme S1†). Reaction of chiral triamine 1 with HATU (3 eq.), Boc-Lys(Z)-OH (3 eq.), and DIPEA (9 eq.) cleanly affords intermediate 2 (Scheme S1†). Removal of the N<sub>E</sub>-Boc protecting groups (step b, Scheme S1†) and subsequent coupling to benzyl-protected 2,3-dihydroxybenzoic acid (step c, Scheme S1†) yields 3 in an 81% yield over two steps. Global deprotection by hydrogenolysis over 10% Pd/C (step d, Scheme S1†) yields (DHB<sup>+</sup>Lys<sup>−3</sup>Ser)<sub>3</sub>, (4) as an enantiopure product. Initial synthetic efforts in which the direction of peptide coupling was reversed were highly susceptible to epimerization at the Lys stereocenter, consistent with the observed chiral instability of N<sub>E</sub>-acylated amino acids upon
activation as a HOBT or HOAT ester.26 N2-Boc-Nc-Cbz-t-lysine was substituted for N2-Boc-Nc-Cbz-t-lysine in the synthesis of (DBH-Lys)3Ser1 and (DBH-Lys)3Ser2. N-Trietyl-l-serine was substituted for N-trityl-l-serine in the synthesis of 1 to yield (DBH-Lys)3Ser1 and (DBH-Lys)3Ser2.

Synthesis of N,N′,N′′-Tris[N2-Boc-Nc-Cbz-t-lysyl]cyclotri-seryl trilactone, 2. N2-Boc-Nc-Cbz-t-lysine (502 mg, 1.32 mmol) was dissolved in 10 mL of dry DMF under an argon atmosphere and cooled in an ice bath. HATU (502 mg, 1.32 mmol) and DIPEA (836 µL, 4.8 mmol) were added at 0 °C and the flask was subsequently taken out of the ice bath and stirred for 3 min. Trisericine triactone hydrochloride (148.5 mg, 0.4 mmol), prepared according to literature procedure,27 was added as a solid to the flask and the reaction was stirred overnight at RT. The solvent was removed in vacuo and the crude reaction mixture was brought up in DCM and rinsed quickly with 1 M HCl (30 mL, ×3) and brine (30 mL). The organic layer was concentrated and then loaded onto a silica column. Purification by flash chromatography using a gradient of 2–4% MeOH in DCM afforded 2 as a colorless solid. (76% yield).1H NMR (DMSO-d6, 25 °C): δ = 1.20–1.60 (m, 45H; CH2), 2.96 (m, 6H; CH2), 3.91 (td, J = 8.5, 4.5, 3H; CH), 4.10 (dd, J = 11.0, 4.5, 3H; CH2), 4.36 (t, J = 10.1, 3H; CH2), 4.59 (m, 3H; CH), 5.00 (s, 6H; CH2), 6.85 (d, J = 7.9, 3H; NH), 7.21 (t, J = 5.7, 3H; NH), 7.28–7.38 (m, 15H; Ar-H), 8.34 (d, J = 7.4; 3H) ppm. 13C NMR (DMSO-d6, 25 °C): δ = 22.7, 28.2, 29.1, 31.4, 38.2, 50.6, 54.0, 63.1, 65.1, 78.1, 127.7, 128.3, 137.3, 154.5, 156.0, 169.5, 172.6 ppm. HRMS (ESI) m/z calcd for C66H93N9O21 + Na+: 1370.6384 [M + Na]; found: 1370.6362.

Synthesis of N,N′,N′′-Tris[N2-Boc-Nc-Cbz-t-lysyl]cyclotri-seryl trilactone (BnDBHLys3Ser), 3. Compound 2 (404.6 mg, 0.3 mmol) was added to a dry flask under argon and dissolved in 6 mL dry DCM. The flask was cooled in an ice bath and 4 mL of TFA was added. After stirring for 1.5 h at RT, full deprotection of the boc groups was observed by TLC. Volatiles were removed in vacuo and the pale yellow oil was brought up in 5 mL of dry DMF. In a separate flask, 2,3-dibenzoyloxybenzoic acid (341 mg, 0.99 mmol), HATU (376 mg, 0.99 mmol), and DIPEA (627 µL, 3.6 mmol) were added to 5 mL of dry DMF under an argon atmosphere and stirred for 3 min at RT. The contents of the first flask were then transferred to the reaction mixture via syringe and the reaction was left to stir overnight at RT. The reaction mixture was concentrated, loaded onto a silica column, and then purified by flash chromatography using a gradient of 1–3% MeOH in DCM. Fractions were combined and concentrated to yield 3 as a white solid. (81% yield over 2 steps)1H NMR (DMSO-d6, 25 °C): δ = 1.28 (m, 6H; CH2), 1.33 (m, 6H; CH2), 1.47 (m, 3H; CH2), 1.61 (m, 3H; CH2), 2.93 (m, 6H; CH2), 4.13 (m, 3H; CH), 4.38 (t, J = 10.3, 3H; CH2), 4.49 (td, J = 8.3, 5.2, 3H; CH), 4.64 (m, 3H; CH), 4.96 (s, 6H; CH2), 4.99 (d, J = 10.6, 3H; CH), 5.08 (d, J = 10.6, 3H; CH2), 5.20 (s, 6H; CH2), 7.15 (m, 6H; NH, Ar-H), 7.20–7.43 (m, 45H; Ar-H), 7.50 (m, 6H; Ar-H), 8.43 (d, J = 7.7, 3H; NH), 8.63 (d, J = 7.1, 3H; NH) ppm.13C NMR (DMSO-d6, 25 °C): δ = 22.5, 26.7, 31.1, 38.7, 50.8, 52.6, 63.1, 115.9, 118.2, 118.4, 118.9, 146.1, 148.6, 169.5, 171.6 ppm. HRMS (ESI) m/z calcd for C46H61N19O23: 877.4224 [M + Na]; found: 877.4209.

Amino acid analysis of frederiksenibactin and synthetic cyclic (DBH-Lys)3Ser, analogs by Marfrey’s method

(DBH-Lys)3Ser, or FSB (2 mg) was dissolved in 2 M HCl and heated at 110 °C in a sealed glass ampule under argon for 24 h. The hydrolysis mixture was evaporated to dryness under a stream of air and redissolved in 100 µL ddH2O. 1-Fluoro-2,4-dinitrophenyl-l-alanine amide (FDAA, 1 M in acetone, 150 µL) and NaHCO3 (1 M, 20 µL) were added and the solution was briefly vortexed and placed on a heating block (40 °C) for 1 h. 10 µL of 2 M HCl was then added to quench the reaction and solutions were stored at −20 °C in the dark prior to analysis.

Amino acid standards were derivatized according to the same procedure. Derivatized hydrolysis products of FSB were separated by HPLC on a YMC 4.6 × 250 mm C18-AQ column using a gradient from 10% CH3CN in ddH2O (0.05% trifluoroacetic acid) to 40% CH3CN in ddH2O (0.05% trifluoroacetic acid) over 60 min. Derivatized hydrolysis products of (DBH-Lys)3Ser, were separated by HPLC on a YMC 4.6 × 250 mm C18-A column using a gradient from 10% CH3CN in TEAP buffer (50 mM, pH 3.00) to 40% CH3CN in TEAP buffer over 60 min. Derivatized hydrolysis products were co-injected with derivatized amino acid standards to determine the constituent amino acids of FSB and to determine the extent of epimerization during synthesis of synthetic (DBH-Lys)3Ser. Three peaks corresponding to FDAA-derivatized lysine were observed, corresponding to products derivatized at either the α-amine, ε-amine, or both amines.
FDAA-derivatized 5Ser co-eluted with 5Lys and 5Lys derivatized at the ε-amine under the conditions used for Marfey’s analysis of FSB (YMC C18-AQ column).

**Preparation of Fe(III)-complexes and circular dichroism spectroscopy**

Fe(III)-complexes of the [DHB1-Lys13Ser3] diastereomers and FSB for CD spectroscopy were prepared in citrate–phosphate buffer (50 mM, pH 7.40) by mixing a solution of FeCl3 [2.45 mM, 0.1 M HCl (aq)] with 1.0 equivalent of the desired apo-ligand. Formation of the Fe(III)-complex was tracked by UV-visible spectroscopy by observing the absorbance at 498 nm. The resulting solution was equilibrated for 30 min in the dark prior to analysis by CD spectroscopy.

Full CD spectra were acquired using the following parameters: 4 s D.I.T., 1 nm bandwidth, 50 nm s⁻¹ scanning speed, with 3 accumulations. Fe(III) exchange assays were performed by preparing pre-equilibrated Fe(III)-complexes of either FSB or CTC as described above. At time t = 0, an equimolar amount of the opposing apo-ligand was added to the Fe(III)-complex and the resulting solution was gently vortexed. CD spectra were acquired as a single accumulation at 20 min intervals using the following parameters: 400–600 nm; 2 s D.I.T., 1 nm bandwidth, and 100 nm s⁻¹ scan speed.

**Computational modeling**

Electronic structure calculations were performed using Gaussian 16. The structures of the following four complexes were optimized: \( \Delta \text{Fe(III)}-[\text{DHB1Lys5Ser3}] \), \( \Delta \text{Fe(III)}-[\text{DHB1Lys5Ser3}] \), \( \Delta \text{Fe(III)}-[\text{DHB1Lys5Ser3}] \), and \( \Delta \text{Fe(III)}-[\text{DHB1Lys5Ser3}] \). Note that the structures of the corresponding \( \Delta \) isomers were not optimized, because each is an enantiomer of one of the four \( \Delta \) complexes listed above, and therefore energetically equivalent. The input geometries were generated manually. The Fe(III) centers were treated as high-spin (S = 5/2) and the Lys residues were protonated to afford neutral complexes (z = 0). Optimizations were performed at the PBE0/6-311++G(d,p) level of theory with Grimme’s D3 empirical dispersion correction and tight convergence criteria. Implicit aqueous solvation was included using a conductor-like polarizable continuum model (CPCM). The energy values presented in Fig. 3 are electronic energies that have not been zero-point corrected. Optimized coordinates are collected in Table S3.† Taxonomic classification was determined by comparing average-nucleotide identity (ANI) against type strain Genbank sequences using OrthoANIu. By this metric, the previous assignment of strain EC16 as *D. chrysanthemi* is strongly supported, with a 99.97% ANI with *D. chrysanthemi* NCPPB 4027.

**Bacterial growth and siderophore isolation**

*Yersinia frederiksenii* ATCC 33641, obtained from the American Type Culture Collection (ATCC), was cultured on Diffco Luria Bertani (LB) Miller (BD biosciences) medium plates. A single colony of *Y. frederiksenii* ATCC 33641 was inoculated into 50 mL of Diffco LB Miller (BD biosciences) media and grown overnight at 30 °C, shaking at 180 rpm. A portion of the overnight culture (5 mL) was then inoculated into low-iron minimal media (2 L, pH 7.0) containing sodium succinate (4 g L⁻¹), KHPO4 (6 g L⁻¹), KH2PO4 (3 g L⁻¹), NH4Cl (1 g L⁻¹), CaCl2⋅2H2O (20 mg L⁻¹), and MgSO4⋅7H2O (200 mg L⁻¹) in an acid-washed 4 L Erlenmeyer flask. The culture was shaken at RT, 180 rpm for 72 h. Cultures were harvested in the late log phase of growth by centrifugation (SLA-3000 rotor, ThermoScientific) at 6000 rpm for 30 min at 4 °C. Culture supernatants were decanted into a clean, acid-washed Erlenmeyer flask containing 100 g of Amberlite XAD-4 polystyrene resin, which was shaken at 120 rpm for 4 h at 4 °C. The resin was filtered from the supernatant, rinsed with 100 mL of 90/10% ddH2O/MeOH, and then eluted with 250 mL of 95%: 5% MeOH/ddH2O. The eluent was concentrated under reduced pressure to a volume of 30 mL and stored at 4 °C prior to analysis. Frederiksenibactin and the related monocatechol and dicatechol compounds were purified by semi-preparative RP-HPLC on a YMC-Actus 20×250 mm C18 ODS-AQ column using a linear gradient of 15% MeOH in ddH2O (+0.1% trifluoroacetic acid) to 40% MeOH in ddH2O (+0.1% trifluoroacetic acid) over 25 min.

**Data availability**

The draft genome sequence of *Dickeya chrysanthemi* EC16 was deposited at NCBI under the BioProject ID PRJNA690813.
Author contributions

P. R. S. synthesized the compounds, isolated the FSB siderophore and carried out its structural and CD characterization; Z. L. R. carried out the bioinformatics analysis and biosynthetic gene cluster prediction; T. C. J. carried out the computational investigations. A. B. directed the project. All authors were involved in writing the manuscript and all authors provided feedback on the manuscript.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

‡ Two prominent low-energy CD bands at 435 nm and 545 nm arise from characteristic LMCT transitions and are therefore sensitive to the chirality at the Fe(III) center. The CD bands at 270 nm, 310 nm, and 360 nm are assigned to ligand-based transitions corresponding to the amide (270 nm) and ester (310 and 360 nm) carboxyls, in analogy to Fe[n]-BHx.
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