

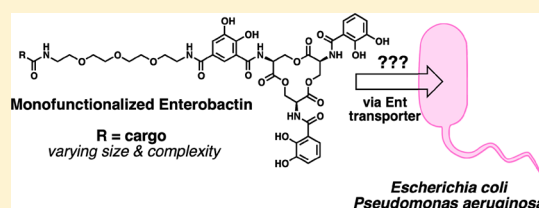
Siderophore-Mediated Cargo Delivery to the Cytoplasm of *Escherichia coli* and *Pseudomonas aeruginosa*: Syntheses of Monofunctionalized Enterobactin Scaffolds and Evaluation of Enterobactin–Cargo Conjugate Uptake

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S Supporting Information

ABSTRACT: The design and syntheses of monofunctionalized enterobactin (Ent, L- and D-isomers) scaffolds where one catechol moiety of enterobactin houses an alkene, aldehyde, or carboxylic acid at the C5 position are described. These molecules are key precursors to a family of 10 enterobactin–cargo conjugates presented in this work, which were designed to probe the extent to which the Gram-negative ferric enterobactin uptake and processing machinery recognizes, transports, and utilizes derivatized enterobactin scaffolds. A series of growth recovery assays employing enterobactin-deficient *E. coli* ATCC 33475 (*ent*[−]) revealed that six conjugates based on L-Ent having relatively small cargos promoted *E. coli* growth under iron-limiting conditions whereas negligible-to-no growth recovery was observed for four conjugates with relatively large cargos. No growth recovery was observed for the enterobactin receptor-deficient strain of *E. coli* H1187 (*fepA*[−]) or the enterobactin esterase-deficient derivative of *E. coli* K-12 JW0576 (*fes*[−]), or when the D-isomer of enterobactin was employed. These results demonstrate that the *E. coli* ferric enterobactin transport machinery identifies and delivers select cargo-modified scaffolds to the *E. coli* cytoplasm. *Pseudomonas aeruginosa* PAO1 K648 (*pvd*[−], *pch*[−]) exhibited greater promiscuity than that of *E. coli* for the uptake and utilization of the enterobactin–cargo conjugates, and growth promotion was observed for eight conjugates under iron-limiting conditions. Enterobactin may be utilized for delivering molecular cargos via its transport machinery to the cytoplasm of *E. coli* and *P. aeruginosa* thereby providing a means to overcome the Gram-negative outer membrane permeability barrier.



INTRODUCTION

Siderophores are low-molecular-weight high-affinity Fe(III) chelators that are biosynthesized and exported by bacteria, fungi, and plants during periods of nutrient limitation for acquiring this essential metal ion from the extracellular milieu.^{1,2} Both naturally occurring and synthetic siderophore mimics are useful for bioremediation,³ iron chelation therapies,^{4,5} antibiotic drug-delivery strategies,^{6–14} Fe(III) detection,^{15–18} protein identification,¹⁹ and pathogen capture.^{20,21} These types of applications benefit from or require siderophores amenable to facile and site-specific synthetic modification. In this work, we expand the current toolkit of site-specifically modifiable siderophore scaffolds to include triscatecholate enterobactin, and we report that various synthetic enterobactin–cargo conjugates are transported to the cytoplasm of the Gram-negative bacterial species *Escherichia coli* and *Pseudomonas aeruginosa* by the enterobactin uptake machinery.

Enterobactin (Ent, **1**, Figure 1A) is a canonical siderophore biosynthesized by Gram-negative species of *Enterobacteriaceae* that include *E. coli*, *Salmonella*, and *Klebsiella*.²² Decades of exploration pertaining to enterobactin biosynthesis and coordination chemistry, in addition to investigations of the proteins involved in its cellular transport and processing,

provide a detailed molecular and physiological understanding of how this chelator contributes to bacterial iron homeostasis and colonization.²² The enterobactin synthetase is comprised of four proteins, EntBDEF, and is responsible for the production of enterobactin from L-serine and 2,3-dihydroxybenzoic acid (DHB).²³ Following biosynthesis, Ent is exported into the extracellular space where it scavenges Fe(III). Enterobactin coordinates Fe(III) by its three catecholate groups with $K_a \approx 10^{49} \text{ M}^{-1}$.²⁴ In *E. coli*, the outer membrane transporter FepA (and to a lesser extent Cir and Fiu) recognizes and binds ferric enterobactin with sub-nanomolar affinity,^{25,26} and provides periplasmic entry where the siderophore forms a complex with the periplasmic binding protein FepB.²⁷ Subsequently, ferric Ent is transported into the cytosol, which requires the action of ExbBD, TonB, and FepCDG, the latter of which constitute the inner-membrane ATP-binding cassette (ABC) transporter system (Figure 1B).^{28–32} Fes, the cytosolic enterobactin esterase, catalyzes the hydrolysis of the [Fe(Ent)]³⁺ macro-lactone,³³ and the ferric reductase YgjH may subsequently assist in iron release such that the metal ion can be used metabolically.³⁴ Several pathogenic Gram-negative species

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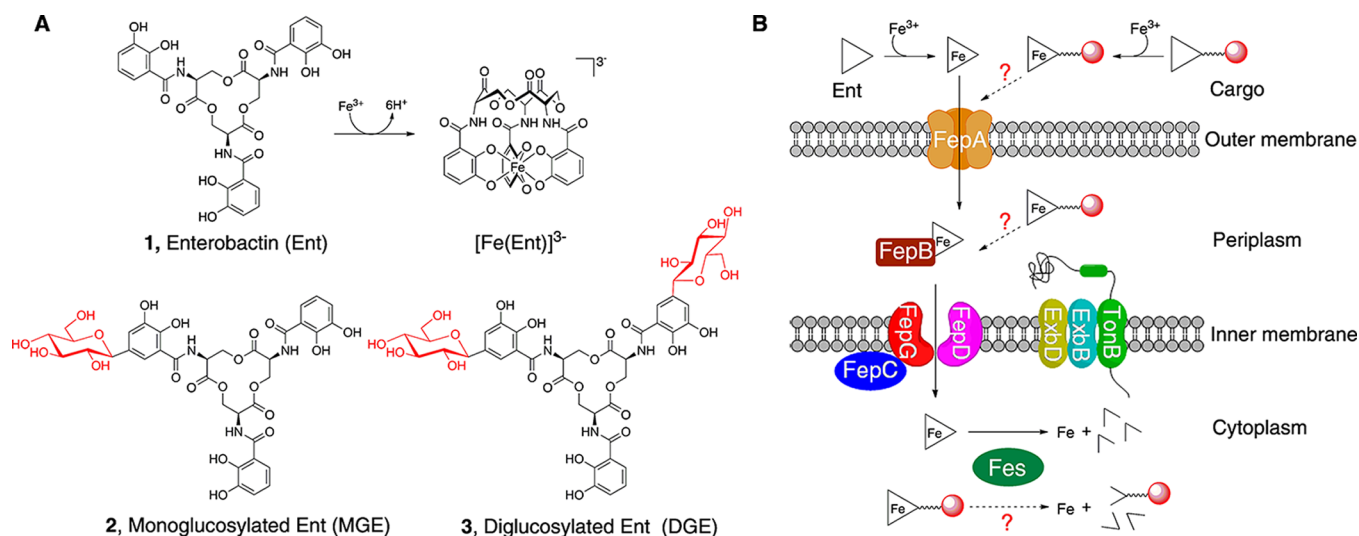


Figure 1. Siderophores and siderophore transport machinery relevant to this work. (A) Structures of enterobactin **1** and the salmochelins MGE **2** and DGE **3**. (B) Cartoon depiction of the enterobactin transport and processing machinery in *E. coli*.

harbor gene clusters (e.g., *iroA*, MccE492) responsible for post-assembly line modifications of the enterobactin scaffold to provide the salmochelins.^{33,35–38} Salmochelins are a family of glucosylated enterobactin derivatives where the sugar moieties are attached to the C5 position of one or more catechol rings (e.g., MGE **2** and DGE **3**, Figure 1A).³⁹

Gram-negative bacteria have an outer membrane that serves as a permeability barrier and prevents cellular entry of many molecules, including antibiotics (e.g., vancomycin). Siderophore uptake machinery provides one route to overcome this permeability barrier,^{6–14} and enterobactin and its transporter FepA have been identified as a desirable siderophore/receptor pair for cargo delivery to Gram-negative bacterial species.^{13,37} FepA-mediated uptake of the ribosomal peptide antibiotics colicin B⁴⁰ and MccE492m,⁴¹ in addition to bacteriophage,⁴² indicates that this receptor has the capacity to transport large molecules. Moreover, the catechol siderophore transporters of *E. coli* (e.g., Fiu, Cir) recognize synthetic catechol-modified β -lactam antibiotics;^{43–46} these serendipitous observations motivated early “Trojan horse” delivery strategies. Indeed, small-molecule antibiotics appended to siderophore-inspired di- and tricatechol platforms have been evaluated for antibacterial activity with mixed results.^{47–51} Most recently, amoxicillin and ampicillin, β -lactam antibiotics that act in the periplasm and target bacterial cell wall biosynthesis, were covalently linked to a tripodal catechol platform and remarkably afforded ca. 10^2 - to 10^3 -fold enhanced activity against *P. aeruginosa* PAO1 compared to the free drug.⁴⁹

The ability of FepABCDG and the TonB-ExbB-ExbD system of *E. coli*, as well as the enterobactin transport machinery of other bacterial species, to recognize and provide cytosolic transport of unnatural cargo appended to the native ligand remains unexplored. Enterobactin exhibits C_3 symmetry and houses no unique functional group for site-specific synthetic modification. Total syntheses of enterobactin,^{52–56} hydrolytically stable enterobactin analogues,^{57–60} and salmochelins⁶¹ have been reported. To the best of our knowledge, no enterobactin scaffold housing a useful site-specific synthetic handle has been presented. Such scaffolds are a prerequisite for employing enterobactin in a variety of paradigms that include

cargo delivery, iron and siderophore detection, and bacterial capture.

Herein we present a family of 10 enterobactin–cargo conjugates that are based on a monofunctionalized enterobactin scaffold. Inspired by the salmochelins, we have derivatized enterobactin at the C5 position of the catechol, which provides a point for site-specific modification without compromising the Fe(III)-binding groups or the macrolactone (Figure 2). Moreover, we report that the ferric enterobactin uptake machineries of *E. coli* and *P. aeruginosa* PAO1 deliver enterobactin-derivatized cargo to the cytoplasm of both species under iron-deficient conditions, and that cargo size is an important and species-specific parameter to evaluate in enterobactin conjugate design.

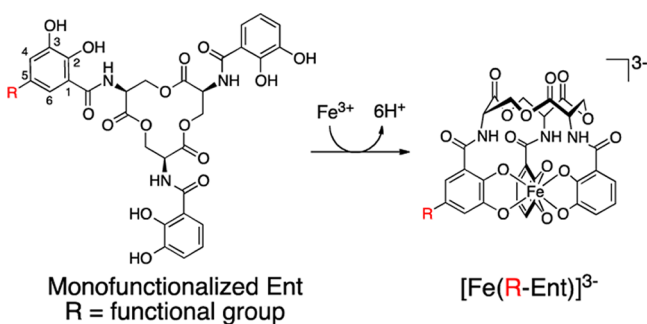


Figure 2. Enterobactin substituted at the C5 position.

EXPERIMENTAL SECTION

Reagents. Dimethylformamide (DMF) and dichloromethane (CH_2Cl_2) were dried over 4 Å molecular sieves or by using a VAC solvent purification system (Vacuum Atmospheres). Anhydrous dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich and used as received. The triserine lactone **4** and its D-isomer **5** were synthesized according to a literature procedure.⁵⁵ 2,3-Bis(benzyloxy)-benzoic acid **6**,⁶² vancomycin-alkyne **7**,⁶³ and *tert*-butyl(2-oxo-2-(prop-2-yn-1-ylamino)ethyl)carbamate **8**⁶³ were synthesized according to literature procedures. L-Ent **1** and its D-isomer **9** were synthesized as reported elsewhere.^{55,56} *tert*-Butyl-3-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)propanoate **10** was purchased from BOC Sciences (Shirley, NY), 11-azido-3,6,9-trioxaundecan-1-amine **11** was purchased from

Fluka, 6-((*tert*-butoxycarbonyl)amino)hexanoic acid **12** was purchased from Advanced Chem Tech, and Fmoc-PEG₃-CO₂H **13** was purchased from Chem-Impex International, Inc. The syntheses of the PEG-derivatized cargos **14**–**18** are provided as Supporting Information. Methyl 5-allyl-3-methoxysalicylate **19** was obtained from Sigma Aldrich. All other chemicals were purchased from Sigma-Aldrich, Alfa Aesar, or TCI in the highest available purity and used as received.

General Synthetic Materials and Methods. EMD TLC silica gel 60 F₂₅₄ plates were used for analytical thin-layer chromatography. EMD PLC silica gel 60 F₂₅₄ plates of 1-mm thickness were used for preparative TLC. Zeoprep 60HYD silica gel (40–63 μ m) obtained from Zeochem was used for flash chromatography. ¹H, ¹⁹F, and ¹³C NMR spectra were collected on a Varian 300 or 500 MHz spectrophotometer, which were operated at ambient probe temperature (293 K) and housed in the Department of Chemistry Instrumentation Facility. The ¹H and ¹³C NMR spectra were referenced to internal standards and ¹⁹F spectra were referenced to an external CFCl₃ standard. An Avatar FTIR instrument was used to acquire IR spectra. Optical absorption spectra were recorded on an Agilent 8453 diode array spectrophotometer (1-cm quartz cuvettes, Starna). General methods for high-performance liquid chromatography and mass spectrometry, ¹H and ¹³C NMR spectra, and IR spectroscopic data are provided as Supporting Information.

Methyl 5-Allyl-2,3-dihydroxybenzoate (20). Methyl 5-allyl-3-methoxysalicylate (**19**, 2.22 g, 10.0 mmol) and anhydrous *N,N*-diisopropylethylamine (DIPEA, 1.94 g, 15.0 mmol) were dissolved in 125 mL of dry CH₂Cl₂ and stirred at rt for five min. The solution was cooled to –78 °C in an acetone/dry ice bath, and boron tribromide (BBr₃, 1 M solution in CH₂Cl₂, 30 mL, 30 mmol) was added slowly over ca. 10 min via a syringe to afford a yellow solution. The reaction was stirred at –78 °C for 1 h, warmed to –30 °C over the course of 1 h, and subsequently warmed to rt and stirred for another 4.5 h. Water (200 mL) was added slowly to quench the reaction, and the organic phase was washed with saturated aqueous potassium carbonate (K₂CO₃, 3 $\times$ 100 mL). The organic phase was dried over sodium sulfate (Na₂SO₄), and the solvent was removed under reduced pressure to afford a brown oil. Flash chromatography on silica gel with a solvent gradient (100% hexanes to 20% EtOAc/hexanes) gave the product as a white solid (1.09 g, 53%). TLC *R*_f = 0.5 (silica, CH₂Cl₂); mp = 55–56 °C. ¹H NMR (CDCl₃, 500 MHz), δ 3.29 (2H, d, *J* = 7.0 Hz), 3.95 (3H, s), 5.05–5.10 (2H, m), 5.80 (1H, s), 5.91 (1H, m), 6.97 (1H, s), 7.18 (1H, s), 10.76 (1H, s). ¹³C NMR (CDCl₃, 125 MHz), δ 39.4, 52.3, 111.9, 116.0, 119.8, 120.4, 131.1, 137.0, 144.8, 147.2, 170.7. HRMS (DART): [M+Na]⁺ *m/z* calcd, 231.0628; found, 231.0637.

5-Allyl-2,3-bis(benzyloxy)benzoic Acid (21). Alkene **20** (2.18 g, 10.5 mmol), benzyl bromide (10.8 g, 60.3 mmol), and K₂CO₃ (24.5 g, 17.8 mmol) were combined in 200 mL of acetone at rt. The reaction was refluxed under N₂ for 16 h, which provided a yellow solution with white solids, and the mixture was cooled to rt and filtered. The filtrate was concentrated under reduced pressure to afford a yellow oil. The oil was dissolved in a 375-mL mixture of 4:1 MeOH/5 M NaOH (aq). The resulting solution was refluxed for 3.5 h and concentrated under reduced pressure to afford a white-yellow oil. Water (300 mL) was added to the oil, and the aqueous phase was washed with hexanes (4 $\times$ 100 mL). The pH of the aqueous phase was adjusted to ca. 1 by addition of 12 M HCl and the product precipitated as a white solid. A 100-mL portion of CH₂Cl₂ was added, and the resulting mixture was partitioned. The aqueous phase was extracted with additional CH₂Cl₂ (2 $\times$ 100 mL) and the combined organic layers were dried over Na₂SO₄ and concentrated under reduced pressure to yield **21** as a white solid (3.91 g, 99%). TLC *R*_f = 0.55 (silica, CH₂Cl₂); mp = 135–136 °C. ¹H NMR (CDCl₃, 300 MHz), δ 3.38 (2H, d, *J* = 6.6 Hz), 5.06–5.14 (2H, m), 5.17 (2H, s), 5.22 (2H, s), 5.92 (1H, m), 7.09 (1H, d, *J* = 2.1 Hz), 7.31–7.50 (10H, m), 7.58 (1H, m). ¹³C NMR (CDCl₃, 125 MHz), δ 39.6, 71.4, 76.9, 116.7, 119.3, 122.6, 123.9, 127.8, 128.4, 128.7, 128.7, 129.1, 129.2, 134.8, 135.8, 136.2, 137.2, 145.5, 151.2, 165.6. HRMS (DART): [M–H][–] *m/z* calcd, 373.1445; found, 373.1439.

(*E*)-2,3-Bis(benzyloxy)-5-(prop-1-en-1-yl)benzoic Acid (22). A 30-mL portion of methanol (MeOH) was degassed with N₂ for 4 h at rt and **21** (750 mg, 2.00 mmol) was subsequently added. The mixture was stirred at rt until **21** dissolved and PdCl₂ (58 mg, 0.32 mmol) was added to give a cloudy brown solution. The reaction was stirred at rt for 24 h and filtered. The filtrate was concentrated and purified by column chromatography using silica gel (1:4:5 EtOAc/hexanes/CH₂Cl₂) to yield **22** as a light yellow solid (666 mg, 89%). TLC *R*_f = 0.4 (40% EtOAc/hexanes); mp = 140–142 °C. ¹H NMR (CDCl₃, 300 MHz), δ 1.88–1.90 (3H, m), 5.19 (2H, s), 5.23 (2H, s), 6.25 (1H, dq, *J* = 15.9, 6.0 Hz), 6.32–6.38 (1H, m), 7.22 (1H, d, *J* = 2.1 Hz), 7.32–7.51 (10H, m), 7.69 (1H, d, *J* = 2.1 Hz). ¹³C NMR (CDCl₃, 125 MHz), δ 18.3, 71.4, 77.0, 115.8, 121.6, 122.7, 127.4, 127.7, 128.4, 128.7, 129.1, 129.2, 129.3, 134.7, 135.0, 135.9, 145.7, 151.3, 165.5. HRMS (DART): [M–H][–] *m/z* calcd, 373.1445; found, 373.1457.

***N,N'*-(3,5,7,11)-11-(2,3-Bis(benzyloxy)-5-(*E*-prop-1-en-1-yl)benzamido)-2,6,10-trioxo-1,5,9-trioxacyclododecane-3,7-diyl)bis(2,3-bis(benzyloxy)benzamide) (23).** Trilactone **4** (740 mg, 2.00 mmol) and DIPEA (2.58 g, 20.0 mmol) were mixed in dry DMSO (8 mL) and stirred for 10 min at rt to give a clear solution. PyAOP (3.13 g, 6.07 mmol), **22** (748 mg, 2.00 mmol), and **6** (1.00 g, 2.99 mmol) were dissolved in dry DMSO (10 mL) and added to the solution containing **4**, and the reaction turned yellow and became orange after stirring for 2 h at rt. The orange solution was mixed with EtOAc (50 mL) and water (50 mL) and partitioned. The organic phase was washed with brine (3 $\times$ 50 mL), dried over Na₂SO₄, and concentrated to afford a yellow oil. Flash chromatography on silica gel with a solvent gradient (10% EtOAc/hexanes to 55% EtOAc/hexanes) yielded the product as a white foam (931 mg, 37%). TLC *R*_f = 0.3 (50% EtOAc/hexanes); mp = 100–102 °C (dec.). ¹H NMR (CDCl₃, 300 MHz), δ 1.88–1.91 (3H, m), 4.01–4.11 (3H, m), 4.16–4.22 (3H, m), 4.91–4.98 (3H, m), 5.03–5.19 (12H, m), 6.17–6.40 (2H, m), 7.10–7.47 (32H, m), 7.66–7.71 (3H, m), 8.51–8.53 (3H, m). ¹³C NMR (CDCl₃, 125 MHz), δ 18.2, 40.6, 51.2, 63.9, 70.9, 76.0, 76.1, 114.2, 117.3, 120.4, 122.8, 124.1, 125.7, 126.1, 126.3, 127.4, 127.5, 127.9, 128.0, 128.2, 128.4, 128.4, 128.7, 128.7, 129.6, 134.1, 135.8, 135.8, 136.0, 136.0, 145.5, 146.7, 151.4, 151.4, 164.7, 168.8, 168.8. HRMS (DART): [M+H]⁺ *m/z* calcd, 1250.4645; found, 1250.4653.

***N,N'*-(3,5,7,11)-11-(2,3-Bis(benzyloxy)-5-formylbenzamido)-2,6,10-trioxo-1,5,9-trioxacyclododecane-3,7-diyl)bis(2,3-bis(benzyloxy)benzamide) (24).** A portion of compound **23** (285 mg, 0.228 mmol) was dissolved in 1,4-dioxane (9 mL) at rt, and water (3 mL) was added to give a colorless solution. Osmium tetroxide (OsO₄, 68 μ L of 2.5 wt % solution in 2-methyl-2-propanol, 6.7 μ mol) was added and the reaction was stirred for 0.5 h at rt, which afforded a light brown solution. Sodium periodate (NaIO₄, 76.5 mg, 0.570 mmol) was then added and the reaction was stirred for another 2 h at rt. The suspension was partitioned in water (20 mL) and EtOAc (50 mL). The organic phase was washed with 0.1 M sodium thiosulfate (Na₂S₂O₃, 3 $\times$ 20 mL) and brine (2 $\times$ 20 mL), and dried over Na₂SO₄. Flash chromatography on silica gel with a solvent gradient (20% EtOAc/hexanes to 65% EtOAc/hexanes) yielded the product as white solid (165 mg, 58%). TLC *R*_f = 0.6 (70% EtOAc/hexanes); mp = 74 °C (dec.). ¹H NMR (CDCl₃, 300 MHz), δ 4.03–4.11 (3H, m), 4.18–4.26 (3H, m), 4.90–4.96 (3H, m), 5.05–5.28 (12H, m), 7.09–7.44 (31H, m), 7.65–7.67 (2H, m), 8.14–8.15 (1H, m), 8.46–8.52 (3H, m), 9.86 (1H, s). ¹³C NMR (CDCl₃, 125 MHz), δ 51.4, 51.4, 51.7, 64.1, 64.2, 71.0, 71.2, 76.2, 76.2, 76.5, 113.1, 117.3, 117.4, 122.9, 123.0, 124.2, 126.2, 126.3, 126.5, 127.5, 127.6, 127.8, 128.1, 128.3, 128.4, 128.4, 128.5, 128.5, 128.5, 128.6, 128.8, 128.9, 132.1, 135.2, 135.3, 135.9, 135.9, 136.0, 146.7, 146.8, 151.5, 151.5, 151.7, 152.2, 163.7, 164.9, 164.9, 168.7, 168.9, 169.1, 190.6. HRMS (DART): [M+H]⁺ *m/z* calcd, 1238.4287; found, 1238.4279.

3,4-Bis(benzyloxy)-5-((3,5,7,11)-7,11-bis(2,3-bis(benzyloxy)benzamido)-2,6,10-trioxo-1,5,9-trioxacyclododecan-3-yl)carbamoyl)benzoic Acid (25). A portion of **24** (112 mg, 0.0903 mmol) was dissolved in 1,4-dioxane (3 mL) at rt. Sulfamic acid (NH₂SO₃H, 15.8 mg, 0.162 mmol) was dissolved in water (0.75 mL) and added to the dioxane solution. Sodium chlorite (NaClO₂,

14.7 mg, 0.163 mmol) was dissolved in 0.2 mL of water and the resulting solution was added to the reaction over the course of 10 min, and the reaction turned yellow. After stirring for 0.5 h at rt, the reaction was partitioned in water (10 mL) and EtOAc (20 mL), the aqueous phase was extracted with EtOAc (2 × 10 mL), and the combined organic phases were dried over Na₂SO₄. Flash chromatography on silica gel with a solvent gradient (CH₂Cl₂ to 10% MeOH/CH₂Cl₂) yielded the product as white solid (87 mg, 76%). TLC *R*_f = 0.5 (10% MeOH/CH₂Cl₂); mp = 128–129 °C (dec.). ¹H NMR (CDCl₃, 500 MHz), δ 4.05–4.08 (3H, m), 4.22–4.25 (3H, m), 4.93–4.98 (3H, m), 5.06–5.25 (12H, m), 7.06–7.47 (31H, m), 7.67–7.69 (2H, m), 7.86 (1H, s), 8.44–8.47 (2H, m), 8.54–8.57 (2H, m). ¹³C NMR (CDCl₃, 125 MHz), δ 51.4, 51.5, 51.6, 64.1, 71.1, 71.2, 76.2, 76.4, 117.5, 117.6, 123.0, 124.2, 125.4, 125.6, 126.2, 127.5, 127.6, 127.8, 128.1, 128.3, 128.4, 128.4, 128.5, 128.6, 128.7, 128.8, 128.9, 135.4, 135.6, 135.9, 136.1, 146.8, 150.7, 151.4, 151.5, 164.1, 165.0, 168.8, 168.9, 169.0, 169.3. HRMS (DART): [M+H]⁺ *m/z* calcd, 1254.4230; found, 1254.4204.

Enantiomers 26–28. The D-isomers of the enterobactin alkene 23, aldehyde 24, and acid 25 were synthesized as described for the L-isomers except that triserine lactone 5 was employed instead of 4. The synthetic procedures and characterization are provided as Supporting Information.

tert-Butyl-(1-(3-(((3S,7S,11S)-7,11-bis(2,3-dihydroxybenzamido)-2,6,10-trioxo-1,5,9-trioxacyclododecan-3-yl)-carbamoyl)-4,5-dihydroxyphenyl)-1-oxo-5,8,11-trioxa-2-azatridecan-13-yl)carbamate (29). Compound 25 (50 mg, 40 μmol), PyAOP (34 mg, 60 μmol) and DIPEA (15.2 μL, 160 μmol) were mixed in 2 mL of dry CH₂Cl₂ at rt. A portion of 7 (15 mg, 48 μmol) was then added and the resulting yellow solution was stirred for 4 h at rt. The crude reaction was washed with 0.01 N HCl (2 × 10 mL), dried over Na₂SO₄, and concentrated. The benzyl-protected product was purified by preparative TLC (10% MeOH/CH₂Cl₂) and obtained as a white viscous solid (46 mg, 75%). TLC *R*_f = 0.7 (10% MeOH/CH₂Cl₂). ¹H NMR (CDCl₃, 500 MHz), δ 1.42 (9H, s), 3.27–3.28 (2H, m), 3.50–3.52 (2H, m), 3.59–3.66 (12H, m), 4.02–4.07 (3H, m), 4.15–4.18 (3H, m), 4.90–4.94 (3H, m), 5.03–5.20 (12H, m), 7.10–7.45 (36H, m), 7.65–7.67 (2H, m), 7.85–7.85 (1H, m), 7.99 (1H, bs), 8.49–8.54 (3H, m). ¹³C NMR (CDCl₃, 125 MHz), δ 28.3, 39.9, 40.2, 51.3, 51.4, 63.9, 64.1, 69.7, 70.0, 70.2, 70.3, 70.4, 71.1, 71.2, 76.2, 76.3, 79.0, 116.7, 117.5, 120.3, 123.0, 124.2, 125.4, 126.1, 126.2, 127.6, 127.6, 127.8, 128.2, 128.3, 128.4, 128.4, 128.4, 128.5, 128.6, 128.6, 128.7, 128.8, 128.8, 129.0, 130.2, 135.4, 135.7, 135.9, 135.9, 136.1, 146.8, 146.9, 149.0, 151.5, 151.8, 155.9, 164.2, 164.8, 164.9, 165.8, 168.9, 169.0, 169.1. HRMS (ESI): [M+Na]⁺ *m/z* calcd, 1550.5942; found, 1550.5977.

This benzyl-protected product was dissolved in 2 mL of 1:1 EtOAc/EtOH, the reaction flask was purged with N₂, and 45 mg Pd/C (10 wt %) was added. The reaction was stirred under H₂ (1 atm) for 6 h at rt, and the Pd/C was removed by centrifugation (13,000 rpm, 10 min). The clear supernatant was decanted, concentrated, and re-dissolved in a 4:2:1 mixture of 1,4-dioxane/H₂O/MeOH, and purified by semipreparative HPLC (20% B for 5 min followed by 20–70% B over 15 min, 4 mL/min). The product eluted at 15.8 min and was lyophilized to give 29 as white solid (15 mg, 50%). The analytical HPLC trace of the purified product is reported as Supporting Information. HRMS (ESI): [M+Na]⁺ *m/z* calcd, 1010.3125; found, 1010.3173.

N³-((3S,7S,11S)-7,11-Bis(2,3-dihydroxybenzamido)-2,6,10-trioxo-1,5,9-trioxacyclododecan-3-yl)-N¹-(1-cyclohexyl-1-oxo-5,8,11-trioxa-2-azatridecan-13-yl)-4,5-dihydroxyisophthalamide (30). Compound 30 was synthesized as described for 29 except that 14 (13.6 mg, 45.0 μmol) was used instead of 7. After purification by preparative TLC (10% MeOH/CH₂Cl₂), the benzyl-protected precursor of 30 was obtained as a white viscous solid (37 mg, 60%). TLC *R*_f = 0.6 (10% MeOH/CH₂Cl₂). ¹H NMR (CDCl₃, 500 MHz), δ 1.17–1.21 (3H, m), 1.37–1.43 (2H, m), 1.62–1.63 (1H, m), 1.72–1.74 (2H, m), 1.78–1.81 (2H, m), 2.00–2.06 (1H, m), 3.39–3.42 (2H, m), 3.51–3.53 (2H, m), 3.59–3.61 (2H, m), 3.64–3.65 (10H, m), 4.01–4.06 (3H, m), 4.13–4.17 (3H, m), 4.88–4.93

(3H, m), 5.04–5.21 (12H, m), 6.23–6.25 (1H, m), 7.09–7.45 (35H, m), 7.64–7.66 (2H, m), 7.86 (1H, d, *J* = 2.0 Hz), 8.02 (1H, d, *J* = 2.0 Hz), 8.49–8.54 (3H, m). ¹³C NMR (CDCl₃, 125 MHz), δ 25.6, 29.5, 38.8, 40.0, 45.3, 51.3, 51.4, 63.9, 64.1, 69.8, 69.8, 70.0, 70.3, 70.4, 70.4, 71.2, 71.2, 76.2, 76.3, 116.8, 117.5, 120.4, 123.0, 124.3, 125.4, 126.1, 126.2, 127.6, 127.6, 127.9, 128.2, 128.3, 128.4, 128.4, 128.5, 128.5, 128.6, 128.6, 128.8, 128.8, 128.9, 129.0, 130.1, 135.4, 135.7, 135.9, 136.0, 136.1, 146.8, 146.9, 149.1, 151.6, 151.8, 164.3, 164.9, 164.9, 165.8, 168.9, 169.0, 169.1, 176.2. HRMS (ESI): [M+Na]⁺ *m/z* calcd, 1560.6150; found, 1560.6269. Compound 30 was purified by semipreparative HPLC (20% B for 5 min followed by 20–70% B over 15 min, 4 mL/min). The product eluted at 15.1 min and was obtained as white solid (20 mg, 58%). The analytical HPLC trace of the purified product is reported as Supporting Information. HRMS (ESI): [M+Na]⁺ *m/z* calcd, 1020.3333; found, 1020.3346.

N³-((3R,7R,11R)-7,11-Bis(2,3-dihydroxybenzamido)-2,6,10-trioxo-1,5,9-trioxacyclododecan-3-yl)-N¹-(1-cyclohexyl-1-oxo-5,8,11-trioxa-2-azatridecan-13-yl)-4,5-dihydroxyisophthalamide (31). Compound 31 was synthesized as described for 30 except that 28 (36 mg, 29 μmol) was used instead of 25. After purification by preparative TLC (10% MeOH/CH₂Cl₂), the benzyl-protected precursor of 31 was obtained as a white oily solid (29 mg, 65%). TLC *R*_f = 0.6 (10% MeOH/CH₂Cl₂). ¹H NMR (CDCl₃, 500 MHz), δ 1.17–1.25 (3H, m), 1.38–1.44 (2H, m), 1.63 (1H, m), 1.72–1.81 (4H, m), 2.01–2.06 (1H, m), 3.40–3.41 (2H, m), 3.39–3.42 (2H, m), 3.51–3.53 (2H, m), 3.58–3.65 (12H, m), 4.01–4.06 (3H, m), 4.13–4.16 (3H, m), 4.87–4.95 (3H, m), 5.03–5.21 (12H, m), 6.22–6.23 (1H, m), 7.09–7.45 (35H, m), 7.65–7.66 (2H, m), 7.86 (1H, s), 8.02 (1H, s), 8.49–8.54 (3H, m). ¹³C NMR (CDCl₃, 125 MHz), δ 25.6, 29.5, 38.8, 40.0, 45.3, 51.3, 51.4, 63.9, 64.1, 69.8, 69.8, 70.0, 70.3, 70.4, 70.4, 71.2, 71.2, 76.2, 76.3, 116.8, 117.5, 120.4, 123.0, 124.3, 125.4, 126.1, 126.2, 127.6, 127.6, 127.9, 128.2, 128.3, 128.4, 128.4, 128.5, 128.5, 128.6, 128.6, 128.8, 128.8, 128.9, 129.0, 130.1, 135.4, 135.7, 135.9, 136.0, 136.1, 146.8, 146.9, 149.1, 151.6, 151.8, 164.3, 164.9, 164.9, 165.8, 168.9, 169.0, 169.1, 176.2. HRMS (ESI): [M+Na]⁺ *m/z* calcd, 1560.6150; found, 1560.6141. Compound 31 was purified by semipreparative HPLC (20% B for 5 min followed by 20–70% B over 15 min, 4 mL/min). The product eluted at 14.8 min and was obtained as white solid (5.1 mg, 27%). The analytical HPLC trace of the purified product is reported as Supporting Information. HRMS (ESI): [M+Na]⁺ *m/z* calcd, 1020.3333; found, 1020.3328.

N³-((3S,7S,11S)-7,11-Bis(2,3-dihydroxybenzamido)-2,6,10-trioxo-1,5,9-trioxacyclododecan-3-yl)-4,5-dihydroxy-N¹-(1-naphthalen-2-yl)-1-oxo-5,8,11-trioxa-2-azatridecan-13-yl)-isophthalamide (32). Compound 32 was synthesized as described for 29 except that 15 (20 mg, 44 μmol) was used instead of 7. After purification by preparative TLC (5% MeOH/CH₂Cl₂), the benzyl-protected precursor of 32 was obtained as a white-yellow oily solid (37 mg, 59%). TLC *R*_f = 0.6 (10% MeOH/CH₂Cl₂). ¹H NMR (CDCl₃, 500 MHz), δ 3.44–3.74 (16H, m), 3.94–4.08 (4H, m), 4.12–4.16 (2H, m), 4.78–4.82 (1H, m), 4.87–4.92 (2H, m), 5.02–5.17 (12H, m), 7.01–7.52 (39H, m), 7.58–7.59 (1H, m), 7.64–7.66 (2H, m), 7.79–7.84 (3H, m), 7.94–7.94 (1H, m), 8.29–8.31 (1H, m), 8.47–8.50 (3H, m). ¹³C NMR (CDCl₃, 125 MHz), δ 39.6, 39.9, 51.4, 51.4, 63.9, 64.1, 69.6, 69.7, 70.2, 70.4, 71.1, 71.2, 71.2, 76.2, 76.3, 76.3, 116.7, 117.5, 120.3, 123.1, 124.3, 124.6, 125.0, 125.2, 125.4, 126.1, 126.2, 126.2, 126.9, 127.6, 127.6, 127.9, 128.1, 128.2, 128.4, 128.4, 128.5, 128.6, 128.6, 128.8, 128.9, 129.0, 130.0, 130.1, 130.3, 133.5, 134.5, 135.4, 135.7, 135.9, 136.0, 136.2, 146.9, 146.9, 149.0, 151.6, 151.7, 164.2, 164.9, 164.9, 165.7, 168.9, 169.0, 169.1, 169.6. HRMS (ESI): [M+Na]⁺ *m/z* calcd, 1604.5837; found, 1604.5964. Compound 32 was purified by semipreparative HPLC (20% B for 5 min followed by 30–55% B over 10 min, 4 mL/min) and eluted at 12.7 min. The isolated product was lyophilized and obtained as a white solid (4.4 mg, 18%). The analytical HPLC trace of the purified product is provided as Supporting Information. HRMS (ESI): [M+Na]⁺ *m/z* calcd, 1064.3020; found, 1064.3084. Mass spectrometric analysis of the crude reaction indicated M+4 in addition to the desired product 32 and suggested partial reduction of the naphthalene cargo under the

deprotection conditions. From analysis of HPLC peak areas, the ratio of **32** and the partial reduction product is ca. 4:1.

N¹-(1-(3-Benzylphenyl)-1-oxo-5,8,11-trioxa-2-azatridecan-13-yl)-N³-((3S,7S,11S)-7,11-bis(2,3-dihydroxybenzamido)-2,6,10-trioxo-1,5,9-trioxacyclododecan-3-yl)-4,5-dihydroxyisophthalamide (33). Compound **33** was synthesized as described for **29** except that **16** (24 mg, 62 μ mol) was used instead of **7**. Partial purification by preparative TLC (10% MeOH/CH₂Cl₂) afforded the benzyl-protected precursor of **33** as a white-yellow solid with a grease contamination (43 mg, 67%). TLC R_f = 0.6 (10% MeOH/CH₂Cl₂). ¹H NMR (CDCl₃, 500 MHz), δ 3.57–3.61 (12H, m), 3.94–3.95 (2H, d, J = 6.0), 3.97–4.05 (3H, m), 4.07–4.15 (3H, m), 4.85–4.90 (3H, m), 5.01–5.17 (12H, m), 7.01–7.40 (30H, m), 7.62–7.70 (3H, m), 7.82 (1H, d, J = 2.0), 7.99–8.00 (1H, d, J = 2.0), 8.47–8.51 (3H, m). HRMS (ESI): [M+Na]⁺ m/z calcd, 1644.6150; found, 1644.6105. A portion (32.5 mg, 20.0 μ mol) of this material was carried on without further purification or characterization. Compound **33** was purified by semipreparative HPLC (20% B for 5 min followed by 20–70% B over 15 min, 4 mL/min). The product eluted at 15.8 min and was obtained as white solid (13.5 mg, 62%). The analytical HPLC trace of the purified product is provided as Supporting Information. HRMS (ESI): [M+Na]⁺ m/z calcd, 1104.3333; found, 1104.3305.

N³-((3S,7S,11S)-7,11-Bis(2,3-dihydroxybenzamido)-2,6,10-trioxo-1,5,9-trioxacyclododecan-3-yl)-4,5-dihydroxy-N¹-(1-oxo-1-(11-oxo-2,3,5,6,7,11-hexahydro-1H-pyrano[2,3-f]pyrido[3,2,1-*ij*]quinolin-10-yl)-5,8,11-trioxa-2-azatridecan-13-yl)isophthalamide (34). Compound **34** was synthesized as described for **29** except that **17** (18 mg, 39 μ mol) was used instead of **7**. After purification by preparative TLC (10% MeOH/CH₂Cl₂) the benzyl-protected precursor of **34** was obtained as an orange oily solid (18 mg, 26%). TLC R_f = 0.7 (10% MeOH/CH₂Cl₂). ¹H NMR (CDCl₃, 500 MHz), δ 1.93–1.95 (4H, m), 2.71–2.83 (4H, m), 3.26–3.32 (4H, m), 3.56–3.69 (16H, m), 3.99–4.18 (6H, m), 4.88–4.94 (3H, m), 5.01–5.18 (12H, m), 6.94 (1H, s), 7.06–7.43 (35H, m), 7.62–7.66 (2H, m), 7.80–7.80 (1H, m), 7.97–7.97 (1H, m), 8.47–8.53 (4H, m), 9.02–9.03 (1H, m). ¹³C NMR (CDCl₃, 125 MHz), δ 19.9, 20.0, 21.0, 27.3, 39.4, 40.1, 49.7, 50.2, 51.5, 64.1, 69.9, 71.1, 71.2, 76.3, 105.4, 108.1, 115.9, 117.5, 119.8, 123.0, 124.3, 125.7, 126.3, 127.2, 127.6, 127.6, 127.8, 128.1, 128.2, 128.5, 128.5, 128.6, 128.9, 128.9, 129.0, 130.0, 135.7, 136.0, 136.2, 146.9, 148.2, 148.3, 149.0, 151.6, 151.7, 152.6, 162.9, 164.4, 165.0, 165.0, 168.9, 169.1. HRMS (ESI): [M+Na]⁺ m/z calcd, 1717.6313; found, 1717.6287. Compound **34** was purified by semipreparative HPLC (20% B for 5 min followed by 20–70% B over 15 min, 4 mL/min). The product eluted at 17.1 min and was obtained as an orange solid (4.5 mg, 48%). The analytical HPLC trace of the purified product is provided as Supporting Information. HRMS (ESI): [M+Na]⁺ m/z calcd, 1177.3496; found, 1177.3540.

7-(4-(1-(3-(((3S,7S,11S)-7,11-Bis(2,3-dihydroxybenzamido)-2,6,10-trioxo-1,5,9-trioxacyclododecan-3-yl)carbamoyl)-4,5-dihydroxyphenyl)-1-oxo-5,8,11-trioxa-2-azatetradecan-14-oyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic Acid (35). Compound **35** was synthesized as described for **29** except that **18** (26 mg, 48 μ mol) was used instead of **7**, and TMSCl (10 μ L, 79 μ mol) and DIPEA (15 μ L, 0.16 mmol) were mixed with **18** before addition to the solution containing **25**. After purification by preparative TLC (10% MeOH/CH₂Cl₂), the benzyl-protected precursor of **35** was obtained as a yellow oily solid (46 mg, 65%). TLC R_f = 0.65 (10% MeOH/CH₂Cl₂). ¹H NMR (CDCl₃, 500 MHz), δ 1.13 (2H, bs), 1.33 (2H, bs), 2.64 (2H, bs), 3.23–3.30 (4H, m), 3.51 (1H, bs), 3.63 (14H, bs), 3.79 (4H, bs), 3.99–4.04 (3H, m), 4.11–4.14 (3H, m), 4.86–4.91 (3H, m), 5.01–5.19 (12H, m), 7.06–7.43 (39H, m), 7.59–7.61 (2H, m), 7.83 (1H, s), 7.97–7.99 (2H, m), 8.45–8.49 (3H, m), 8.69 (1H, s). ¹³C NMR (CDCl₃, 125 MHz), δ 8.2, 33.4, 35.4, 40.0, 41.1, 45.3, 49.3, 50.0, 51.3, 51.4, 51.4, 63.9, 64.1, 67.1, 69.7, 70.2, 70.3, 70.4, 70.5, 71.2, 71.3, 76.2, 76.3, 105.2, 108.0, 112.3, 112.4, 116.7, 117.5, 120.0, 120.0, 120.5, 123.0, 124.3, 125.6, 126.1, 126.1, 127.6, 127.6, 127.8, 128.2, 128.3, 128.4, 128.4, 128.5, 128.6, 128.6, 128.8, 128.8, 129.0, 130.2, 135.5, 135.7, 135.9, 136.0, 136.1, 138.9, 145.2, 145.3, 146.8, 146.8, 147.4, 149.0, 151.6, 151.6, 151.8, 152.4, 154.4, 164.2, 164.9, 164.9, 165.8, 166.9, 168.9, 169.0, 169.1, 169.7, 176.9. ¹⁹F NMR (CDCl₃, 282

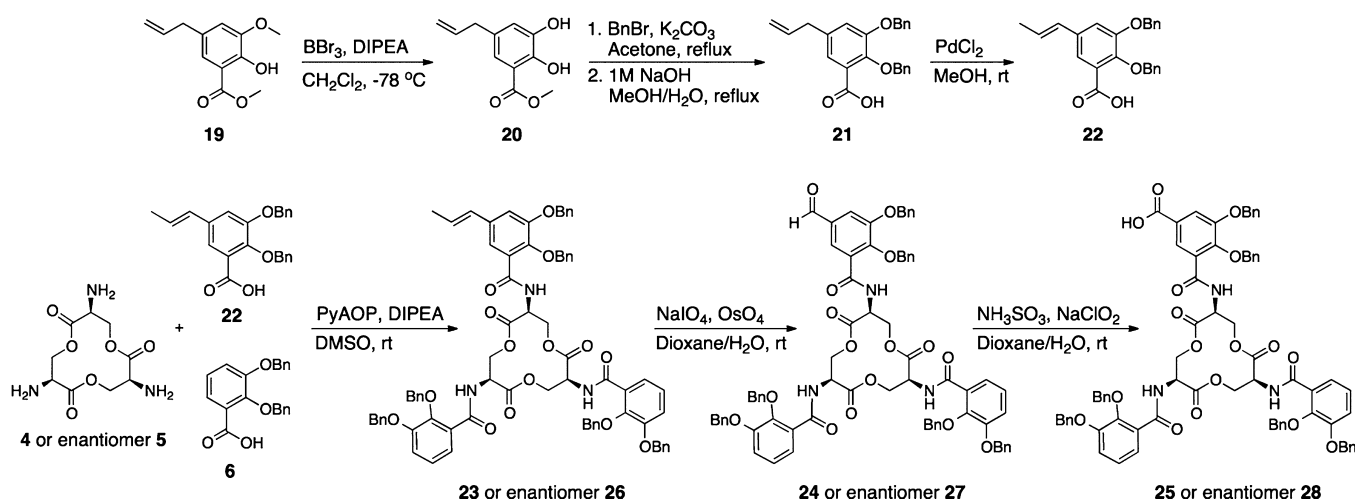
MHz) δ –121.3. HRMS (ESI): [M+Na]⁺ m/z calcd, 1792.6434; found, 1792.6337. Compound **35** was purified by semipreparative HPLC (20% B for 5 min followed by 20–70% B over 10 min, 4 mL/min) and eluted at 15.2 min. The isolated product was lyophilized and obtained as a white solid (2.5 mg, 9%). The HPLC trace of the purified product is provided as Supporting Information. HRMS (ESI): [M+Na]⁺ m/z calcd, 1252.3617; found, 1252.3633.

7-(4-(6-Aminohexanoyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic Acid (36). Ciprofloxacin (**37**, 331 mg, 1.00 mmol) and DIPEA (1.0 mL, 5.7 mmol) were mixed in 6 mL of dry CH₂Cl₂, and TMSCl (370 μ L, 2.91 mmol) was added to give a clear yellow solution. 6-((*tert*-Butoxycarbonyl)-amino)hexanoic acid (**12**, 346 mg, 1.50 mmol), PyAOP (834 mg, 1.60 mmol), and DIPEA (700 μ L, 4.02 mmol) were dissolved in 4 mL of dry CH₂Cl₂, and the two solutions were combined and stirred overnight at rt. The reaction was quenched with MeOH (10 mL), and the resulting solution was concentrated to dryness, and the crude product was redissolved in 40 mL of EtOAc. The organic phase was washed with 10 mM HCl (2 $\times$ 40 mL) and saturated aqueous NaHCO₃ (2 $\times$ 40 mL), dried over Na₂SO₄, and purified by flash chromatography on silica gel (3% MeOH/CH₂Cl₂) to give **38** as yellow solid (243 mg, 45%). TLC R_f = 0.7 (5% MeOH/CH₂Cl₂). ¹H NMR (CDCl₃, 300 MHz), δ 1.14–1.20 (2H, m), 1.32–1.53 (13H, m), 1.59–1.69 (2H, m), 2.36 (2H, t, J = 6.0 Hz), 3.08 (2H, dt, J = 6.3, 6.3 Hz), 3.26–3.56 (4H, m), 3.51–3.59 (1H, m), 3.69–3.82 (4H, m), 4.68 (1H, bs), 7.32 (1H, d, J = 7.2 Hz), 7.82 (1H, d, J = 12.9 Hz), 8.60 (1H, s), 14.9 (1H, bs). ¹³C NMR (CDCl₃, 125 MHz), δ 8.1, 24.7, 26.4, 28.3, 29.8, 32.9, 35.3, 40.2, 41.0, 45.1, 49.3, 49.9, 78.9, 105.0, 107.7, 111.9, 112.1, 119.6, 119.7, 138.8, 145.2, 145.3, 147.3, 152.4, 154.4, 155.9, 166.6, 171.4, 176.7. ¹⁹F NMR (CDCl₃, 282 MHz), δ –121.1. HRMS (ESI): [M+H]⁺ m/z calcd, 545.2775; found, 545.2768.

The TFA salt of **36** (202 mg, 98%) was obtained as a yellow solid from **38** (201 mg, 0.369 mmol) by stirring **38** in 40% TFA/CH₂Cl₂ at rt for 3 h and removing the solvent. TLC R_f = 0.1 (10% MeOH/CH₂Cl₂). ¹H NMR (CD₃OD, 300 MHz), δ 1.41–1.52 (4H, m), 1.65–1.77 (4H, m), 2.52 (2H, t, J = 7.2 Hz), 2.96 (2H, t, J = 7.2 Hz), 3.34–3.43 (4H, m), 3.82 (5H, m), 7.57 (1H, d, J = 7.5 Hz), 7.85 (1H, d, J = 13.2 Hz), 8.76 (1H, s). ¹³C NMR (CDCl₃, 125 MHz), δ 7.8, 23.8, 25.4, 26.5, 26.6, 32.2, 35.4, 39.0, 39.1, 41.0, 45.0, 48.1, 48.3, 48.5, 48.6, 48.8, 49.0, 49.1, 49.5, 105.0, 107.0, 111.6, 111.8, 119.3, 119.4, 138.8, 145.1, 145.2, 147.4, 152.3, 154.3, 167.3, 171.8, 176.5. ¹⁹F NMR (CDCl₃, 282 MHz), δ –76.0, –120.9. HRMS (ESI): [M+H]⁺ m/z calcd, 445.2251; found, 445.2255.

7-(4-(6-(3-(((3S,7S,11S)-7,11-Bis(2,3-dihydroxybenzamido)-2,6,10-trioxo-1,5,9-trioxacyclododecan-3-yl)carbamoyl)-4,5-dihydroxybenzamido)hexanoyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic Acid (40). Compound **40** was synthesized as described for **35** except that DMSO (1.5 mL) was used as the solvent and compound **36** (19.4 mg, 34.8 μ mol) was used instead of **18**. After preparative TLC purification (10% MeOH/CH₂Cl₂), **39** was obtained as white viscous solid (17 mg, 74%). TLC R_f = 0.6 (10% MeOH/CH₂Cl₂). ¹H NMR (CDCl₃, 300 MHz), δ 1.17–1.83 (10H, m), 2.40 (2H, bs), 3.29–3.44 (6H, m), 3.70–3.86 (5H, m), 4.02–4.17 (6H, m), 4.86–4.93 (3H, m), 5.04–5.21 (12H, m), 7.07–7.42 (33H, m), 7.60–7.64 (2H, m), 7.85–8.05 (3H, m), 8.47–8.50 (3H, m), 8.74 (1H, bs), 15.0 (1H, bs). ¹³C NMR (CDCl₃, 125 MHz), δ 8.1, 12.3, 17.2, 18.6, 24.4, 26.3, 26.4, 26.5, 29.0, 32.8, 34.7, 39.7, 41.2, 45.3, 46.2, 46.3, 51.4, 51.5, 51.5, 52.0, 54.8, 63.9, 64.1, 64.2, 71.1, 71.2, 71.2, 76.2, 76.3, 76.3, 105.2, 109.5, 113.0, 113.2, 116.6, 117.5, 120.1, 123.0, 124.3, 125.5, 126.1, 127.6, 127.8, 128.2, 128.3, 128.4, 128.4, 128.5, 128.6, 128.6, 128.6, 128.8, 128.9, 128.9, 129.0, 130.3, 135.5, 135.8, 135.9, 136.0, 136.1, 138.1, 145.4, 146.8, 148.4, 149.0, 151.6, 151.8, 152.3, 164.4, 164.9, 165.0, 165.8, 166.1, 168.8, 169.0, 169.1, 171.5. HRMS (ESI): [M+H]⁺ m/z calcd, 1680.6303; found, 1680.6352. Compound **40** was purified by semipreparative HPLC (20% B for 5 min followed by 20–70% B over 15 min, 4 mL/min) and eluted at 16.1 min. The isolated product was lyophilized and obtained as a white-yellow solid (6.7 mg, 59%). The HPLC trace of the purified product is provided as Supporting

Scheme 1. Syntheses of 22 (Top) and Monofunctionalized Enterobactin Scaffolds (Bottom)



Information. HRMS (ESI): $[M+H]^+$ m/z calcd, 1140.3486; found, 1140.3482.

N¹-(2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethyl)-N³-((3S,7S,11S)-7,11-bis(2,3-dihydroxybenzamido)-2,6,10-trioxo-1,5,9-trioxacyclododecan-3-yl)-4,5-dihydroxyisophthalamide (41). 11-Azido-3,6,9-trioxaundecan-1-amine (11, 8.2 μ L, 42 μ mol) and 25 (40 mg, 32 μ mol) were dissolved in 1 mL of dry CH_2Cl_2 . PyAOP (33.2 mg, 63.8 μ mol) and DIPEA (22.2 μ L, 128 μ mol) were added to give a light yellow solution. The reaction was stirred for 4 h at rt and concentrated, and the crude product was purified by preparative TLC (50% EtOAc/ CH_2Cl_2) to afford 41 as a light yellow oil (31 mg, 68%). TLC R_f = 0.3 (50% EtOAc/ CH_2Cl_2). 1H NMR ($CDCl_3$, 300 MHz), δ 3.34 (2H, t, J = 5.1 Hz), 3.61–3.69 (14H, m), 3.97–4.18 (6H, m), 4.88–4.94 (3H, m), 5.02–5.22 (12H, m), 7.08–7.46 (34H, m), 7.64–7.67 (2H, m), 7.85 (1H, d, J = 1.8 Hz), 7.99 (1H, d, J = 2.1 Hz), 8.48–8.52 (3H, m). ^{13}C NMR ($CDCl_3$, 125 MHz), δ 40.0, 50.6, 51.4, 64.0, 64.1, 69.7, 69.9, 70.3, 70.6, 71.2, 71.2, 76.3, 116.7, 117.5, 120.4, 123.1, 124.3, 125.5, 126.2, 126.2, 127.6, 127.6, 127.9, 128.3, 128.4, 128.4, 128.5, 128.6, 128.7, 128.8, 128.9, 129.0, 130.2, 135.5, 135.8, 136.0, 136.0, 136.2, 146.9, 146.9, 149.1, 151.6, 151.8, 164.2, 164.9, 164.9, 165.9, 168.9, 169.1, 169.1. HRMS (ESI): $[M+Na]^+$ m/z calcd, 1476.5323; found, 1476.5345.

Vancomycin-PEG-Ent (42). A DMSO solution of 41 (19 mg/mL, 1.3 mM, 250 μ L), an aqueous solution of 8 (20 mg/mL, 1.3 mM, 250 μ L), a DMF solution of benzoic acid (49 mg/mL, 450 mM, 50 μ L), and an aqueous solution of $CuSO_4$ (10 mg/mL, 45 mM, 50 μ L) were mixed together, and an additional 400 μ L of DMSO was added to yield a clear light blue solution. An aqueous solution of sodium ascorbate (NaAsc, 18 mg/mL, 90 mM, 50 μ L) was subsequently added. The reaction turned from colorless to yellow and was stirred at rt for 15 min, at which time another 50 μ L of aqueous NaAsc was added. After stirring for 15 min, the crude reaction was frozen and lyophilized to give a brown oil. The oil was dissolved in a 2:1:1 ratio of dioxane/MeOH/ H_2O and purified by semipreparative HPLC (50% B for 5 min followed by 50–100% B over 11 min, 4 mL/min). The benzyl-protected precursor of 42 eluted at 13 min and was obtained as white solid after lyophilization (3.5 mg, 36%). HRMS (ESI): $[M+2Na]^{2+}/2$ m/z calcd, 1520.5030; found, 1520.5171.

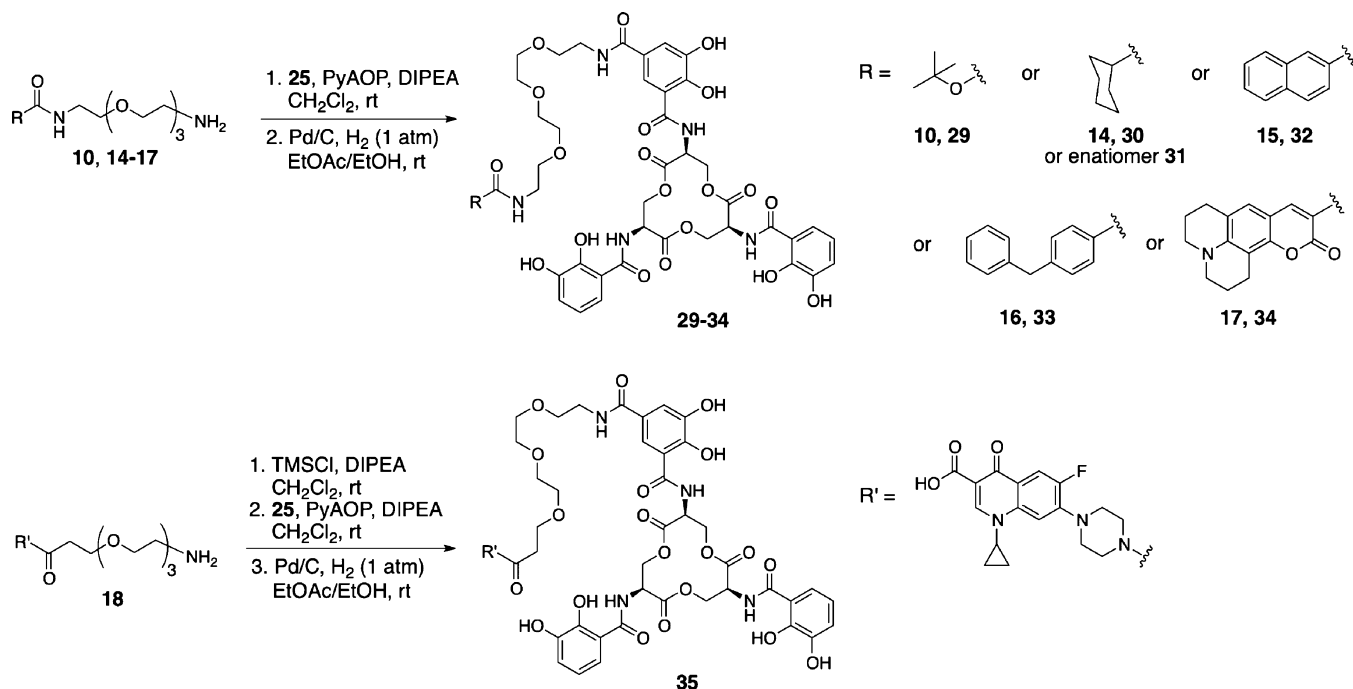
A portion of this precursor (14 mg, 4.7 μ mol; obtained from four 250- μ L scale click reactions) was dissolved in 30% H_2O /MeOH, the flask was purged with N_2 , and 16 mg of Pd/C (10 wt %) was added. The reaction was stirred under H_2 (1 atm) for 24 h at rt, and the Pd/C was removed by centrifugation (13,000 rpm, 10 min). The supernatant was concentrated by lyophilization and the resulting residue was dissolved in a 2:1:1 mixture of dioxane/MeOH/ H_2O . HPLC purification (20% B for 5 min followed by 20–46% B over 8 min, 4 mL/min) gave 43 as white solid (6.3 mg, 55%). The HPLC trace of the purified product is reported in Supporting Information. HRMS (ESI): $[M+2H]^{2+}/2$ m/z calcd, 1228.3796; found, 1228.3796.

tert-Butyl(2-(((1-(3-(((3S,7S,11S)-7,11-bis(2,3-dihydroxybenzamido)-2,6,10-trioxo-1,5,9-trioxacyclododecan-3-yl)-1-oxo-5,8,11-trioxa-2-azatridec-13-yl)-1H-1,2,3-triazol-4-yl)methyl)amino)-2-oxoethyl)carbamate (43). Compound 43 was synthesized as described for 42 except that a DMSO solution of 7 (2.8 mg/mL, 13 mM, 25 μ L) was used instead of 8. HPLC purification gave 3.3 mg of the benzyl-protected precursor of 43 as a white solid (58%). HRMS (ESI): $[M+H]^+$ m/z calcd, 1688.6489; found, 1688.6421. Compound 43 (3.3 mg, 33%) was obtained from the precursor (13.3 mg, 7.88 μ mol; obtained from four 25- μ L scale click reactions) following the same procedure as synthesizing 42. HPLC purification (0% B for 5 min followed by 0–45% B over 8 min, 4 mL/min) afforded 43 as a white solid with a retention time of 12.8 min. The HPLC trace of the purified product is reported as Supporting Information. HRMS (ESI): $[M+H]^+$ m/z calcd, 1126.3853; found, 1126.3832.

Growth Recovery Assays. General microbiology methods are included as Supporting Information. Overnight cultures were prepared by inoculating 5 mL of LB (*E. coli*) or LB base supplemented with 2.5 g/L NaCl (*P. aeruginosa*) with the appropriate freezer stocks and the cultures were incubated at 37 $^{\circ}C$ in a tabletop incubator shaker set at 150 rpm. The overnight culture was diluted 1:100 into 5 mL of fresh media with or without 200 μ M 2,2'-dipyridyl (DP) and incubated at 37 $^{\circ}C$ with shaking at 150 rpm until the optical density at 600 nm (OD_{600}) reached 0.6. The cultures were diluted to an OD_{600} value of 0.001 in 50% reduced MHB medium (10.5 g/L) with or without 200 μ M (*E. coli*) or 600 μ M DP (*P. aeruginosa*). A 90- μ L aliquot of the diluted culture was mixed with a 10- μ L aliquot of a 10x solution of the siderophore or siderophore–cargo conjugate in a 96-well plate, which was wrapped in parafilm and incubated at 30 $^{\circ}C$ with shaking at 150 rpm for 19 h. Bacterial growth was assayed by measuring OD_{600} using a BioTek Synergy HT plate reader. Each well condition was prepared in duplicate, and three independent replicates of each assay were conducted on different days. The resulting mean OD_{600} are reported, and the error bars are the standard deviation of the mean obtained from the three independent replicates.

RESULTS AND DISCUSSION

Design and Synthesis of Monofunctionalized Enterobactin Platforms. We present a stepwise synthesis to monofunctionalized enterobactin scaffolds in Scheme 1. Guided by the structures of MGE and DGE (Figure 1A), we chose to install functional groups amenable to synthetic modification at the C5 position of one enterobactin catechol ring. This position is remote from the iron-binding hydroxyl groups in addition to the macrolactone (Figure 2). Prior studies of the salmochelins indicate that modification at this site compromises neither

Scheme 2. Syntheses of Enterobactin–Cargo Conjugates 29–35^a

^aThe syntheses of 14–18 are provided as Supporting Information.

Fe(III) complexation nor the esterase-catalyzed hydrolysis of the macrolactone.^{33,64} The structure of the antibiotic–side-phore conjugate MccE492m (Figure S1),⁶⁵ which exhibits a monoglucosylated enterobactin derivative attached to a ribosomal peptide, also influenced our decision to prepare monofunctionalized enterobactin platforms. We selected methyl 5-allyl-3-methoxysalicylate **19** as a starting material because of its commercial availability. This precursor was demethylated using BBr_3 in the presence of DIPEA to prevent HBr addition to the alkene moiety, and **20** was obtained in 53% yield as a white powder. Benzyl protection of **20** and subsequent hydrolysis of the methyl ester in refluxing sodium hydroxide were performed following a literature protocol⁶² for catecholate protection of DHB and **21** was obtained in 99% yield as a white powder. Palladium-catalyzed isomerization of the alkene was achieved by using PdCl_2 in degassed methanol and **22** was obtained in 89% yield as a light yellow solid following workup. Next, a one-pot coupling reaction between the enterobactin trilactone **4**, **6**, and **22** was performed with a 1:1.5:1 ratio and PyAOP as the coupling reagent. This reaction provided a mixture of **23**, its di- and trisubstituted analogues, and unmodified Ent. These products were separated by flash chromatography and afforded **23** in 37% yield as a white foam. The 1:1.5:1 ratio of **4/6/22** was chosen based on several optimization trials and this ratio provided the highest yield of the desired monosubstituted product. Oxidation of alkene **23** by using OsO_4 and NaIO_4 in mixed 1,4-dioxane/water afforded **24** as a white foam in 58% yield. Further oxidation of **24** under mild conditions provided carboxylic acid **25** in 76% yield as a white powder. This stepwise synthesis provides gram quantities of **23**–**25** (L-isomers) in high purity, and these molecules are stable when stored as dry solids at 4 °C. The stepwise coupling and oxidations were also performed using triserine lactone **5** to afford the D-enantiomers alkene **26**, aldehyde **27**, and acid **28** (Supporting Information). The D-enantiomer of Ent is

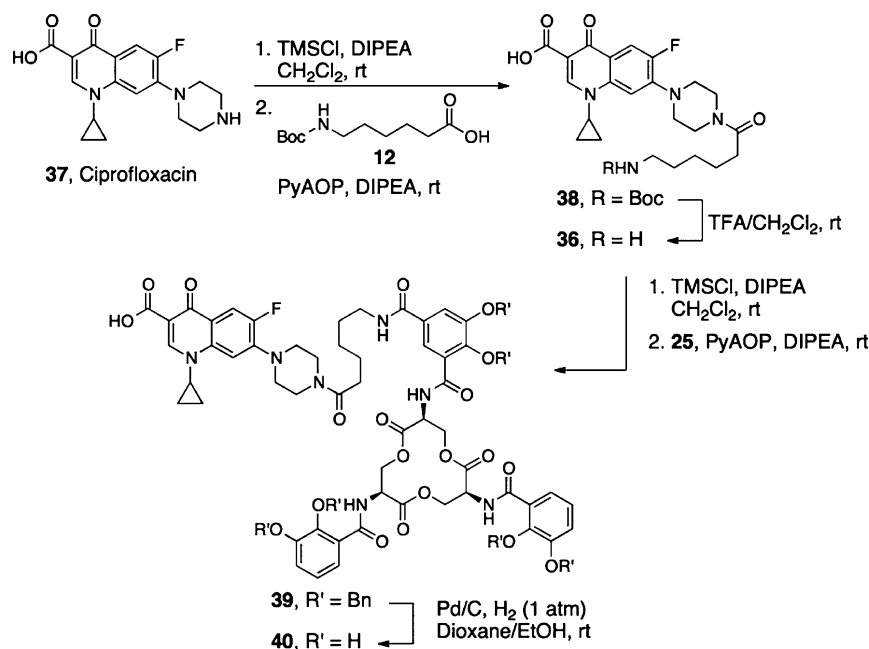
transported into *E. coli* by FepA, but it is not a substrate for the enterobactin esterase Fes.⁶⁶ We therefore reasoned that conjugates based on D-Ent would provide useful controls for conjugate uptake studies, and that this enantiomer may also be advantageous in antibacterial drug delivery applications because it provides an iron-starvation effect.

This synthesis provides a family of enterobactin scaffolds amenable to functionalization. For instance, alkene **23** may be employed in olefin cross metathesis,⁶⁷ aldehyde **24** in reductive amination, and acid **25** in peptide coupling reactions. Moreover, other organic transformations of **22** or **23** may provide additional versatile functional groups (e.g., hydroxyl), affording more synthetic possibilities for enterobactin derivatives that can be utilized in various applications.

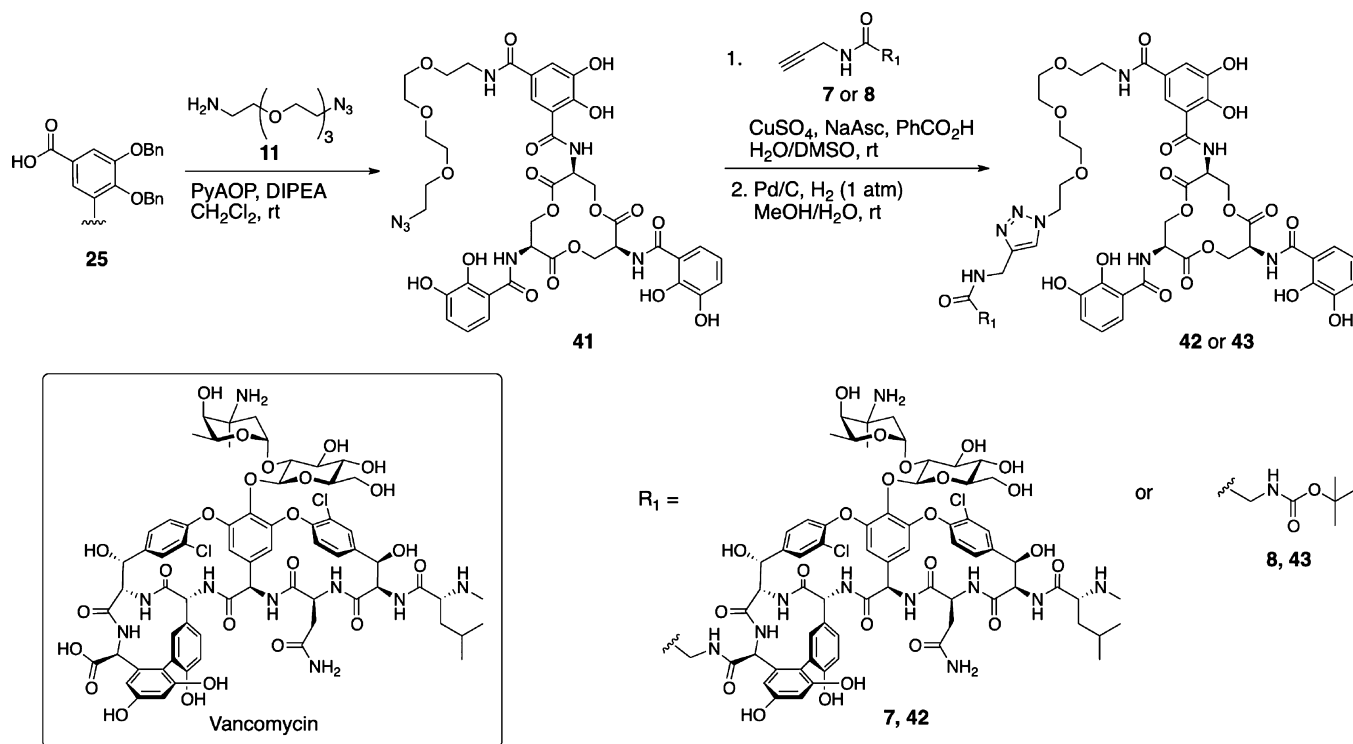
Design and Synthesis of Enterobactin–Cargo Conjugates. We selected carboxylic acid **25** as a key intermediate for the preparation of enterobactin–cargo conjugates, and evaluated two strategies for appending cargo to the enterobactin scaffold. In one thrust, standard peptide coupling chemistry was employed to link cargo to the enterobactin acid via an amide bond (Schemes 2 and 3). In the second approach, enterobactin-azide **41** was prepared and “click” chemistry utilized for cargo attachment (Scheme 4). In both cases, benzyl deprotection unmasked the enterobactin catecholates in the final step.

We selected a variety of commercially available molecules housing carboxylic acids as cargos. The cargos presented in Scheme 2 vary in size and shape and include a simple Boc protecting group, cyclohexane, naphthalene, phenylmethylbenzene, ciprofloxacin, and coumarin 343. This selection includes cargo expected to be nontoxic (e.g., Boc, cyclohexane) in addition to an antibiotic (ciprofloxacin) and a fluorophore (coumarin 343). Next, we selected PEG₃ as a stable and water-compatible linker. It provides ca. 14-Å separation between enterobactin and the cargo. The conjugates depicted in Scheme

Scheme 3. Synthesis of Enterobactin–Ciprofloxacin Conjugate 40



Scheme 4. Syntheses of Enterobactin–Cargo Conjugates by Click Chemistry



2 were prepared by coupling the PEG-derivatized cargo 10, 14–18 to 25 using PyAOP as the coupling reagent. The resulting benzyl-protected conjugates were purified by preparative TLC and obtained in yields ranging from 26% (Bn-34) to 75% (Bn-29). Benzyl deprotection reactions were performed by hydrogenation over Pd/C and the resulting enterobactin–cargo conjugates were purified by reverse-phase semipreparative HPLC. Conjugates 29–35 were obtained in milligram quantities and high purity judging by analytical HPLC (Figures S2–S11) and LC/MS analysis (Table S1). Conjugate 31

houses D-Ent and was prepared to probe the role of Fes-mediated hydrolysis in the bacterial growth recovery assays.

Scheme 3 exhibits the synthesis of enterobactin–ciprofloxacin 40 where the PEG linker is substituted by a C₅ alkyl chain to probe the consequences of variable linker composition and hydrophilicity. The synthesis of 40 was carried out by reacting ciprofloxacin with 6-Boc-aminohexanoic acid 12 followed by Boc deprotection, coupling of the resulting free amine to 25, and benzyl deprotection. The carboxylic acid of ciprofloxacin was protected *in situ* by using trimethylsilyl chloride (TMSCl)

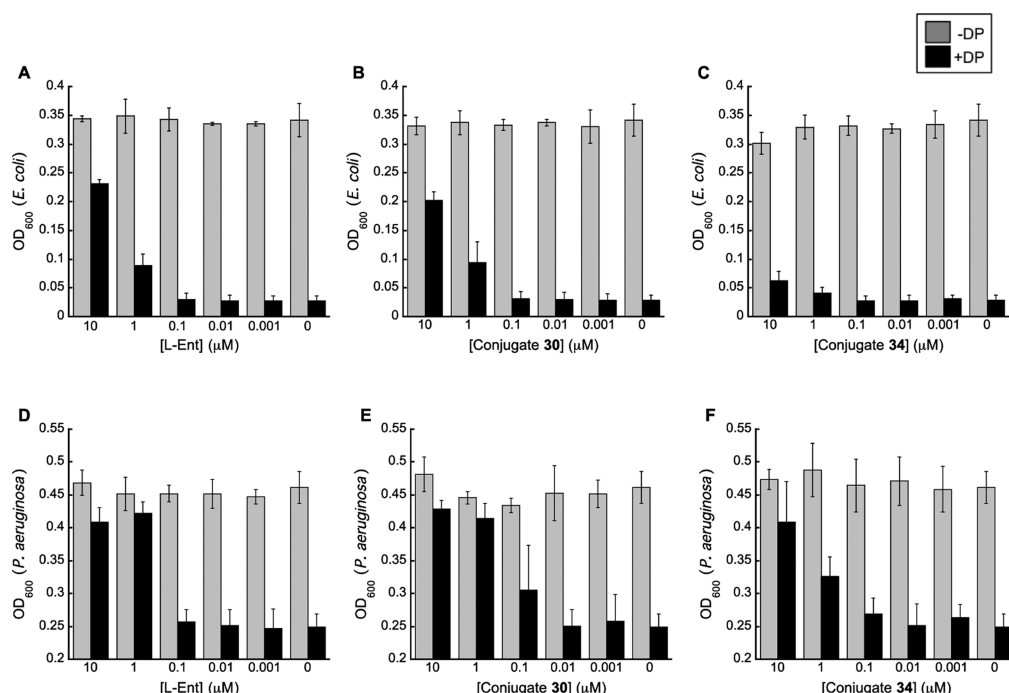


Figure 3. *E. coli* ATCC 33475 (*ent*-) and *P. aeruginosa* PAO1 (*pvd*-, *pch*-) growth recovery assays employing L-Ent and select enterobactin–cargo conjugates (50% MHB, $\pm$ 200 or 600 μ M DP, t = 19 h, 30 °C). Gray bars: OD₆₀₀ of bacteria cultured in the absence of DP. Black bars: OD₆₀₀ of bacteria cultured in the presence of 200 (*E. coli*) or 600 (*P. aeruginosa*) μ M DP. (A) L-Ent promotes growth recovery of *E. coli*. (B) Enterobactin conjugate 30 housing a cyclohexyl cargo affords growth recovery of *E. coli*. (C) Enterobactin conjugate 34 housing a coumarin moiety affords little-to-no growth recovery of *E. coli*. (D) L-Ent promotes growth recovery of *P. aeruginosa*. (E) Enterobactin conjugate 30 housing a cyclohexyl cargo affords growth recovery of *P. aeruginosa*. (F) Enterobactin conjugate 34 housing a coumarin moiety affords growth recovery of *P. aeruginosa*. Figures 4 and S15 contain the assay results for the other conjugates. Each bar indicates the average of three independent replicates (two wells per replicate) and the error bars are the standard deviation of the mean.

to prevent self-coupling in the syntheses of both 35 and 40. In this general approach of attaching a carboxylic acid cargo, the linkers were first coupled to the cargo rather than to the Ent scaffold because the Ent macrolactone degrades in the presence of primary amines and under acidic conditions such as those required to remove Boc protecting groups.

In Scheme 4, we present the synthesis of 42, an enterobactin–vancomycin conjugate assembled via click chemistry. Vancomycin is a nonribosomal peptide antibiotic active against Gram-positive organisms that inhibits cell wall biosynthesis by binding to the D-Ala-D-Ala of lipid II and blocking peptidoglycan cross-linking.⁶⁸ It exhibits poor activity against Gram-negative bacteria because it is too large to cross the outer membrane. Because modification of the C-terminal carboxylic acid with a PEG chain did not perturb its antibacterial activity,⁶⁹ we selected this site as a point of attachment. Moreover, we employed click chemistry for the conjugate assembly to avoid complications with the various functional groups exhibited by vancomycin. First, the azide-functionalized PEG linker 11 was coupled to 25 to afford the enterobactin–azide 41 in 68% yield. Copper(I)-catalyzed azide–alkyne cycloaddition of 41 with alkyne 8⁶³ subsequently afforded enterobactin–vancomycin 42 in 55% yield following hydrogenation and purification. This synthetic approach was extended to 43, a small analogue of 42 that houses a *tert*-butyl cargo, and the strategy is also applicable to other alkyne-substituted cargos that are compatible with the benzyl deprotection conditions.

Enterobactin–Cargo Conjugates Coordinate Fe(III). The optical absorption spectrum of each enterobactin–cargo

conjugate exhibited catecholate absorption at ca. 316 nm (MeOH, rt). With the exception of 34, which afforded a yellow solution because of the coumarin moiety, methanolic solutions of each conjugate turned from colorless to wine-colored following the addition of ca. one equiv of aqueous Fe(III), and the expected ligand-to-metal charge transfer (LMCT) bands were observed, indicating Fe(III) coordination to the enterobactin catecholates (Figures S12–S14).⁷⁰

Enterobactin–Cargo Conjugate Delivery to the *E. coli* Cytoplasm. We employed three nonpathogenic *E. coli* strains that are defective in enterobactin synthesis, enterobactin transport, or ferric enterobactin utilization in growth recovery assays (Table S2). *E. coli* ATCC 33475 (*ent*-) cannot biosynthesize enterobactin, but retains the capacity to import and metabolize the siderophore.⁷¹ *E. coli* H1187 (*fepA*-) lacks the outer membrane enterobactin receptor. *E. coli* K-12 JW0576 (*fes*-) can accumulate ferric enterobactin, but cannot release the iron because it is deficient in the enterobactin esterase Fes. As a result of these defects in iron metabolism, all three strains grow poorly under conditions of iron limitation.⁷¹ The iron chelator DP was used to generate iron-deficient conditions and promote expression of siderophore transport machinery in the growth recovery assays.

We first evaluated whether the enterobactin conjugates afforded growth recovery of *E. coli* (*ent*-) cultured under iron-deficient conditions (50% MHB, 200 μ M DP). *E. coli* (*ent*-) grew to OD₆₀₀ $\approx$ 0.35 in 50% MHB medium (30 °C, t = 19 h), and this value decreased to <0.05 when 200 μ M DP was added to the media. Low-micromolar concentrations of L-Ent restored growth, as expected,⁷⁰ and the *E. coli* cultures reached OD₆₀₀ $\approx$

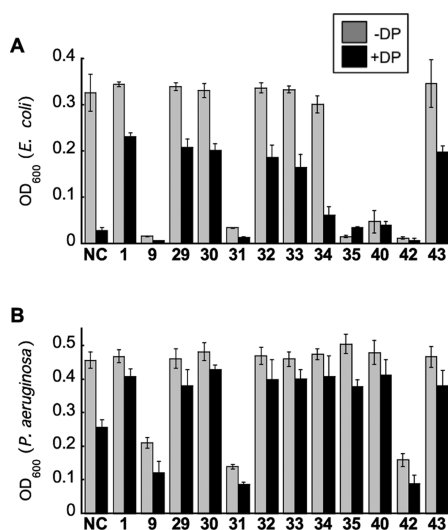


Figure 4. Comparative effects of enterobactin–cargo conjugates on bacterial cell growth. *E. coli* and *P. aeruginosa* were cultured in the presence of 10 μ M of L-Ent 1, D-Ent 9 and the enterobactin–cargo conjugates 29–35, 40, 42, 43 in the absence (gray bars) and presence (black bars) of DP (50% MHB, $T = 30\text{ }^{\circ}\text{C}$, $t = 19\text{ h}$). (A) *E. coli* ATCC 33475 (*ent*-) and the DP concentration was 200 μ M. (B) *P. aeruginosa* PAO1 (*pvd*-, *pch*-) and the DP concentration was 600 μ M. NC refers to a no-conjugate control.

0.2 in the presence of 10 μ M Ent (Figure 3). Likewise, low-micromolar concentrations of the enterobactin–cargo conjugates 29–33 and 43 exhibiting Boc (29, 43), cyclohexyl (30), naphthyl (32), and phenylmethylbenzyl (33) cargos afforded growth recovery to similar levels (Figures 4 and S15). No growth restoration was observed when *E. coli* (*fepA*-) or *E. coli* (*fes*-) were cultured with 29 or 30 (Figures 5 and S16), which supports the notion that the growth recovery of *E. coli* (*ent*-) results from FepA-mediated cytoplasmic transport and Fes-catalyzed hydrolysis of the enterobactin moiety to release iron. Moreover, the D-enantiomer of enterobactin, D-Ent 9, is not a substrate for Fes and does not provide growth recovery (Figures 4 and S17).⁶⁶ Indeed, no growth promotion occurred when *E. coli* (*ent*-) was treated with conjugate 31, the D-enantiomer of 30 (Figures 4 and S15). Taken together, these results demonstrate that the enterobactin transport machinery has the capacity to recognize and transport cargo-derivatized enterobactin scaffolds to the *E. coli* cytoplasm, and that these molecules are substrates for the cytoplasmic esterase Fes.

We observed no convincing evidence for marked uptake of larger cargos by *E. coli* ATCC 33475, which suggests that FepA of this *E. coli* strain has a cargo size limit. For instance, under iron limitation, negligible *E. coli* growth recovery and no toxicity was observed following treatment with the enterobactin–coumarin conjugate 34 (Figure 3), suggesting that *E. coli* (*ent*-) may not readily import 34. Moreover, no growth recovery occurred following treatment of *E. coli* with either ciprofloxacin 35 or 40 (Figures 4 and S15). In the absence of DP, these conjugates afforded a concentration-dependent inhibition of *E. coli* growth. Likewise, 10 μ M of vancomycin 42 inhibited the growth of *E. coli* ($\pm$ DP, Figures 4 and S15). This behavior contrasts that of unmodified vancomycin, which is inactive against *E. coli* over the concentration range employed in this study. Two possible origins for inhibitory activity of the ciprofloxacin and vancomycin conjugates are (i) enterobactin–antibiotic uptake and resulting antibacterial action or (ii) a lack

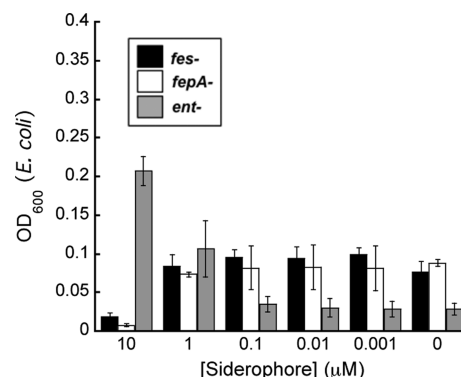


Figure 5. Comparison of growth recovery for *E. coli* (*ent*-), *E. coli* (*fepA*-), and *E. coli* (*fes*-) with conjugate 29 in the presence of 200 μ M DP. Black bars: *E. coli* (*fes*-) cultured with conjugate 29. White bars: *E. coli* (*fepA*-) cultured with conjugate 29. Gray bars: *E. coli* (*ent*-) cultured with conjugate 29 in the presence of 200 μ M DP. See Figures 4 and S16 for additional data.

of active transport into *E. coli*, resulting in extracellular iron chelation and hence nutrient deprivation. Taking all observations into account, including those for *P. aeruginosa* described below, we contend that the latter option is the most probable explanation.

Enterobactin–Cargo Conjugate Delivery to the *P. aeruginosa* Cytoplasm. We next sought to determine whether the enterobactin–cargo conjugates provided growth recovery for *P. aeruginosa* PAO1. This Gram-negative opportunistic human pathogen synthesizes and exports two siderophores, pyoverdine (*pvd*) and pyochelin (*pch*), and employs multiple additional mechanisms for iron acquisition.^{72,73} *P. aeruginosa* utilizes enterobactin as a xenosiderophore, and the genes *pfeA*^{74,75} and *pirA*⁷⁶ encode outer membrane enterobactin transporters. Similar to the *E. coli* experiments, we focused on using *P. aeruginosa* strains deficient in siderophore production or utilization in growth recovery assays. *P. aeruginosa* K648 (*pvd*-, *pch*-) is deficient in both pyoverdine and pyochelin biosynthesis and shows attenuated growth in iron-deficient conditions, whereas *P. aeruginosa* K407 (*pvd*-, *pFr*-) is deficient in pyoverdine biosynthesis and lacks the enterobactin transporter PfeA.⁷⁴

In 50% MHB medium, *P. aeruginosa* (*pvd*-, *pch*-) grew to $\text{OD}_{600} \approx 0.45$ ($30\text{ }^{\circ}\text{C}$, $t = 19\text{ h}$) and this value diminished to ca. 0.25 in the presence of 600 μ M DP. Supplementation of the iron-limiting growth medium with low-micromolar concentrations of L-Ent resulted in the restoration of *P. aeruginosa* growth to $\text{OD}_{600} \approx 0.40$ (Figure 3). Comparable growth recovery was observed for cultures treated with eight of the nine conjugates based on L-Ent (Figures 3, 4, and S18). Vancomycin 42, which exhibits the largest cargo, afforded a growth inhibitory effect ($\pm$ DP) as observed for *E. coli* (*ent*-). In contrast to its L-Ent analogue 30, conjugate 31 based on D-Ent was growth inhibitory as was D-Ent (Figures 4 and S18). This result demonstrates that *P. aeruginosa* also requires the L-isomer for iron utilization. Lastly, no growth enhancement of *P. aeruginosa* (*pvd*-, *pFr*-) was observed in the presence of L-Ent or conjugate 30 (600 μ M DP); instead, these siderophores caused growth inhibition at micromolar concentrations (Figure S19). These results demonstrate that PfeA is necessary for conjugate-mediated growth recovery, supporting its role as a transporter for the enterobactin conjugates. In total, these assays reveal that the enterobactin transport machinery of *P. aeruginosa*, and PfeA

in particular, recognizes and delivers various cargo-modified enterobactin scaffolds to the cytoplasm.

Ciprofloxacin is a fluoroquinolone antibiotic that acts in the cytoplasm and inhibits DNA gyrase.⁷⁷ The fact that ciprofloxacin conjugates **35** and **40** each restored *P. aeruginosa* growth indicated that the cargo was successfully delivered to the cytoplasm of this microbe with negligible impact of the variable linker composition, and that conjugation of ciprofloxacin to enterobactin attenuated its antibacterial activity. This observation is in general agreement with reports of pyoverdine–fluoroquinolone⁷⁸ and pyochelin–fluoroquinolone^{79,80} conjugates where the antibiotic was covalently attached to the siderophore and points to the need for appropriate linker design for fluoroquinolone delivery and release after cellular entry.⁸¹ These pyoverdine/pyochelin–antibiotic conjugates afforded no antipseudomonal activity or diminished activity relative to the unmodified drug, and the pyoverdine–fluoroquinolone antibiotic exhibited decreased *E. coli* gyrase inhibitory activity *in vitro*.⁷⁸

A comparison of the enterobactin–cargo growth recovery profiles for *E. coli* and *P. aeruginosa* (Figures 4, S15, and S18) reveals that these particular microbes have different capacities for internalizing enterobactin–cargo conjugates, and that cargo size is an important factor. Vancomycin has a rigid dome-like structure and a molecular weight of ca. 1.4 kDa, and the assays presented in this work suggest that this molecule is too big for enterobactin-mediated transport into *E. coli* or *P. aeruginosa*. In contrast, small and malleable cargos such as a Boc protecting group and cyclohexane afforded growth recovery comparable to that of L-Ent for both strains. A comparison of OD₆₀₀ values for bacterial cultures treated with such conjugates (e.g., **29**, **30**, **32**, **34**) shows that growth recovery to levels comparable to that of L-Ent occurs at a conjugate concentration of 1 μ M for *P. aeruginosa* whereas 10 μ M is required for *E. coli*. *P. aeruginosa* responds to lower Ent concentrations than *E. coli*, which indicates a higher uptake efficiency. Coumarin 343 is an example of a cargo that exhibits no signs of toxicity over the concentration range tested and affords markedly different results on microbial growth promotion for these two species. A comparison of the ciprofloxacin conjugate data for *E. coli* and *P. aeruginosa* also suggests differential uptake. For both the ciprofloxacin and coumarin cargo, the growth recovery assays indicate that the enterobactin transport machinery of *P. aeruginosa* imports these cargos whereas the *E. coli* system does not. These observations suggest that species-selective targeting may be possible with strategic cargo choice even when a siderophore is utilized by multiple microbial species.

SUMMARY AND PERSPECTIVES

We have designed and prepared a family of monofunctionalized enterobactin derivatives, and utilized these scaffolds for the preparation of enterobactin–cargo conjugates bearing cargos of varying size and complexity. Growth recovery assays employing *E. coli* and *P. aeruginosa* revealed that the enterobactin uptake machineries of these Gram-negative species recognize and transport enterobactin–cargo conjugates to the Gram-negative cytoplasm. These studies are significant in several respects. First, the notion of using siderophores for antibiotic delivery across the Gram-negative outer membrane, which serves as a permeability barrier, has achieved long-term interest.^{6–8,13} Such “Trojan horse” antibiotics are largely inspired by the sideromycins,^{11,12} a family of siderophore–antibiotic conjugates produced by the soil bacterium *Streptomyces*, and by early

observations that catechol-modified β -lactams were recognized by the iron-uptake machinery of Gram-negative microbes.^{43–46} Significant efforts have been made to prepare and characterize synthetic siderophore–antibiotic conjugates with the goal of targeting drug-resistant Gram-negative pathogens.^{13,14} Timely examples of siderophore–antibiotic conjugates with antimicrobial activity include a mycobactin–artemisinin conjugate that kills *Mycobacterium tuberculosis* and *Plasmodium falciparum*,⁸² and amoxicillin/ampicillin-appended tripodal triscatecholates that exhibit potent antipseudomonal activity relative to the parent β -lactam antibiotics.⁴⁹ One bottleneck with this general approach, and using siderophores in other applications, is that few synthetically tractable and modifiable native siderophores are available. DFO B and pyoverdine, which are readily obtained commercially (DFO B) or from bacterial cultures (pyoverdines), provide free amino groups useful for conjugation and are most commonly derivatized for application-based work.¹⁸ Syntheses of modified pyochelin,¹⁵ petrobactin,¹⁹ and mycobactin^{82,83} platforms that house functional groups amenable to site-specific elaboration have been reported, and these scaffolds are important contributions to the toolkit of siderophores that can be modified without compromising Fe(III) coordination in addition to recognition by siderophore-binding proteins. The syntheses described in this work provide enterobactin with a functional handle for versatile chemical modifications, and will allow strategic use of this canonical siderophore in a multitude of chemical biology and biotechnology initiatives.

Unanswered questions regarding the antibacterial activity and fate of reported synthetic siderophore–antibiotic conjugates exist. Whether a given conjugate is actively transported into the bacterial cell is oftentimes unclear. Because FepA recognizes relatively large biomolecules including MccE492m (84-aa) and colicin B (324-aa), it is tempting to predict that FepA may accommodate almost any cargo appended to an enterobactin or catecholate platform. The results presented in this work challenge this notion and indicate that cargo size is an important and species-specific parameter. Our assays indicate that *P. aeruginosa* PAO1 has a greater capacity to import enterobactin–cargo conjugates than *E. coli* ATCC 33475. It will be interesting to determine the cargo scope of other *E. coli* strains and bacterial species that utilize enterobactin for iron acquisition, and understand the molecular and physiological basis for such variations. Colicins are largely α -helical⁴⁰ and MccE492m shares some sequence homology with colicins.⁸⁴ It is likely that some enterobactin receptors have decreased propensity to transport synthetic small molecules or natural products with less structural malleability (i.e., vancomycin) than an α -helical peptide. Moreover, factors such as electrostatics and functional groups may also influence transport.

The mechanisms of iron release from siderophores, which vary tremendously for the myriad of siderophores produced by different bacterial species, are another important consideration in siderophore–cargo conjugate design. Guided by studies of chiral recognition in enterobactin transport, which demonstrated that D-Ent is transported into *E. coli* but cannot be hydrolyzed by Fes,⁷¹ we designed the monofunctionalized D-Ent scaffolds to probe cytosolic delivery. This design feature prevents esterase-catalyzed iron release from enterobactin-based conjugates in the cytoplasm and may have practical utility. From the standpoint of drug delivery, a tug-of-war may result from utilizing an iron-supplying siderophore that confers a growth advantage for delivering a toxic payload to a bacterial

cell, and preventing iron release may be beneficial. In other applications, siderophore-fluorophore conjugates are of interest for bacterial detection and diagnostics, and Fe(III) binding to and release from the siderophore will likely influence the photophysical properties of such molecules.

In summary, these investigations reveal that the enterobactin transport machineries of *E. coli* (e.g., FepABCDG and TonB-ExbB-ExbD) and *P. aeruginosa* (e.g., PfeA) will deliver enterobactin-modified cargo to the Gram-negative cytoplasm. Moreover, the preparative work affords a new siderophore platform amenable to synthetic elaboration and an entry route for employing the native enterobactin scaffold in a variety of application-based initiatives that include intracellular cargo delivery, iron sensing, siderophore labeling, protein and pathogen detection, and therapeutic development.

■ ASSOCIATED CONTENT

■ Supporting Information

Syntheses and characterization of 14–18 and 26–28, general liquid chromatography, mass spectrometry and microbiology methods, summary of enterobactin–cargo conjugate characterization (Table S1), summary of bacterial strains and sources (Table S2), structure of MccE492m (Figure S1), HPLC traces for the purified conjugates (Figures S2–S11), optical absorption spectra (Figures S12–S14), growth recovery assays (Figures S15–S19), ^1H and ^{13}C NMR spectra, and IR spectroscopic 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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