# Iron transport-mediated drug delivery using mixed-ligand siderophore–β-lactam conjugates

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**Background:** Assimilation of iron is essential for microbial growth. Most microbes synthesize and excrete low molecular weight iron chelators called siderophores to sequester and deliver iron by active transport processes. Specific outer membrane proteins recognize, bind and initiate transport of species-selective ferric siderophore complexes. Organisms most often have specific receptors for multiple types of siderophores, presumably to ensure adequate acquisition of the iron that is essential for their growth. Conjugation of drugs to synthetic hydroxamate or catechol siderophore components can facilitate active iron-transport-mediated drug delivery. While resistance to the siderophore–drug conjugates frequently occurs by selection of mutants deficient in the corresponding siderophore-selective outer membrane receptor, the mutants are less able to survive under iron-deficient conditions and *in vivo*. We anticipated that synthesis of mixed ligand siderophore–drug conjugates would allow active drug delivery by multiple iron receptor recognition and transport processes, further reducing the likelihood that resistant mutants would be viable.

**Results:** Mixed ligand siderophore–drug conjugates were synthesized by combining hydroxamate and catechol components in a single compound that could chelate iron, and that also contained a covalent linkage to carbacephalosporins, as representative drugs. The new conjugates appear to be assimilated by multiple active iron-transport processes both in wild type microbes and in selected mutants that are deficient in some outer membrane iron-transport receptors.

**Conclusions:** The concept of active iron-transport-mediated drug delivery can now be extended to drug conjugates that can enter the cell through multiple outer membrane receptors. Mutants that are resistant to such conjugates should be severely impaired in iron uptake, and therefore particularly prone to iron starvation.

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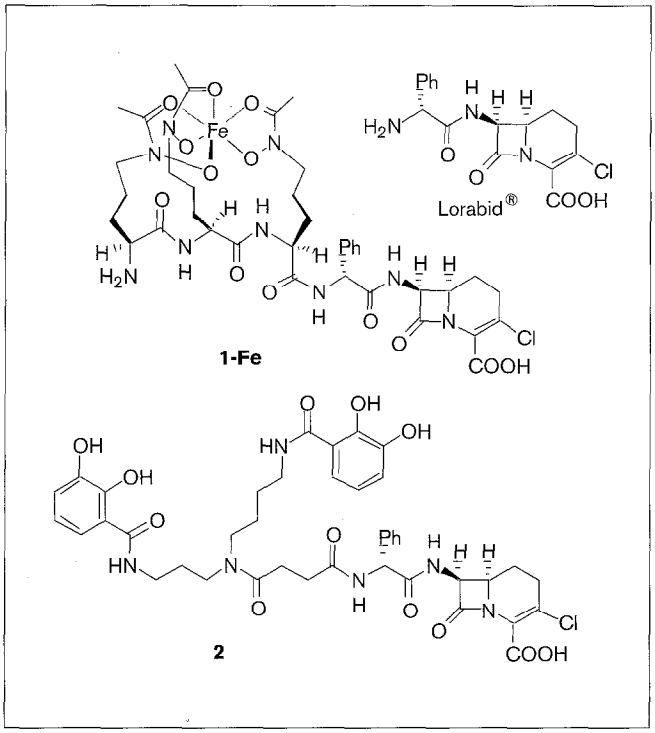
## Introduction

Resistance to individual or even whole classes of drugs is emerging as a major threat to antibiotic therapy [1,2]. The overuse of antibiotics promotes microbial resistance, which can arise from changes in microbial permeability barriers [3] or drug-binding sites [4], or from the acquisition of enzymes that destroy the antimicrobial agents [5]. Alternative drug-delivery processes have been sought to circumvent the problem of reduced permeability, which results from a limitation of passive diffusion through size-restricted porins.

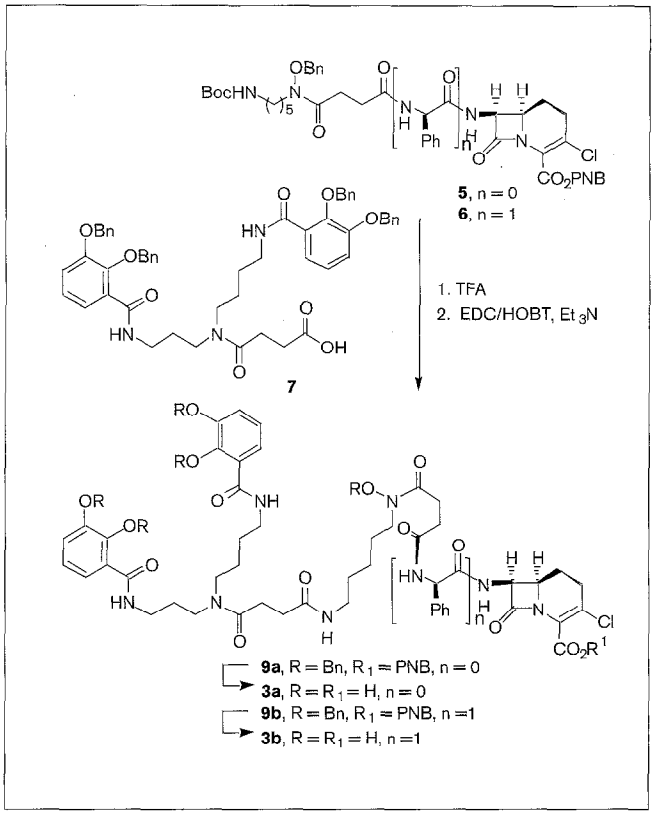
Most microbes assimilate physiologically essential iron by synthesizing and utilizing high affinity ferric ion chelators, called siderophores [6]. Proteins in the outer membrane then recognize the ferric complexes and initiate their active transport. The idea that conjugation of antibiotics to siderophores might facilitate active drug transport by siderophore-mediated drug delivery was therefore an attractive one. Indeed, there are several natural examples of antibiotic-containing siderophores (e.g. albomycins and ferrimycin A) that are taken up into bacterial cells via active iron-transport systems [7,8]. We previously reported the synthesis and antimicrobial activity of artificial siderophore–drug conjugates (compounds **1** [9] and **2** [10], Fig. 1) consisting of carbacephalosporins with separate hydroxamic-acid-based and catechol-based siderophore components [11,12]. As expected, biological assays revealed that these conjugates used different outer membrane receptor proteins to initiate entry into the cell [13,14]. Although strains that lack the outer membrane proteins that recognize and mediate uptake of compound **1** or **2** were rapidly selected *in vitro* and were resistant to the conjugates they were originally selected with, they were still susceptible to the other conjugate [14], showing that the defect in conjugate uptake was specific for the appropriate uptake system. Preliminary *in vivo* studies revealed that the selected mutants were not parhogenic, presumably because the mutant lacked a full complement of iron-assimilation mechanisms and is therefore at a growth

**Figure 1**




Artificial siderophore–drug conjugates: compounds **1** [9] and **2** [10].

**Figure 2**




Synthetic pathway for the formation of compounds **3a** and **3b** [16]. The protected hydroxymate derivatives (compounds **5** and **6**) are coupled with the protected biscatechol-containing spermidine derivative **7**.

disadvantage in serum. In addition, when both conjugates were used together the inhibition of microbial growth was more profound than when either conjugate was used alone. We therefore postulated that a drug conjugate that could be recognized by multiple siderophore-uptake systems, for example one containing both catechol and hydroxamate components, might show enhanced inhibition of microbial growth and minimize the development of resistance by selection of mutants defective in one type of siderophore recognition. Any strains resistant to such a drug would be expected to have defects in more than one iron-uptake system and therefore be especially prone to iron starvation. Here we describe the synthesis of the first mixed hydroxamate and catechol-containing siderophore-like conjugates of carbacephalosporins, and a preliminary study of their biological activity.

## Results and discussion

### Chemical synthesis

We anticipated that separate coupling of protected hydroxamate derivatives (compounds **5** and **6**) [15], of both the parent carbacephem nucleus and Lorabid® (Loracarbef) [16] with protected biscatechol-containing spermidine derivative **7** would provide a direct route to compounds **3a** and **3b** (Fig. 2). Alternatively, coupling of protected forms of δ-*N*-hydroxyornithyl carbacephalosporin **8a** or the corresponding tripeptide derivative **8b** [9] with **7** would produce compounds **4a** and **4b** (Fig. 3). Indeed, removal of the *t*-butoxycarbonyl (Boc) protecting groups from compounds **5** and **6** with trifluoroacetic acid (TFA) gave the corresponding amine salts, which were separately coupled to compound **7** using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), *N*-hydroxybenzotriazole (HOBT) and triethylamine to give compounds **9a** and **9b**. Similarly, removal of the carbobenzyloxy (Cbz) group (achieved using HBr/HOAc) from our previously described δ-*N*-hydroxy-L-ornithine derivatives **10** and **11** [9,17] and reprotection with the Boc group gave compounds **12** and **13**. Subsequent coupling with the *p*-nitrobenzyl (pNB) ester of Lorabid®, generated from Boc precursor **14**, produced protected hydroxamate conjugates **8a** and **8b**. Brief treatment of these conjugates with TFA to remove the *N*-terminal Boc groups was followed by separate reaction with the preformed *N*-hydroxysuccinimide (NHS) ester of compound **7** to provide protected conjugates **15a** and **15b**. Deprotection of compounds **9a,b** and **15a,b** under carefully selected conditions (see Materials and methods), gave fully deprotected conjugates **3a,b** and **4a,b**, respectively, in near-quantitative yield.

### Biological studies

Several assays of bacterial inhibition by conjugates **3a, 3b, 4a** and **4b** were performed (Tables 1–3). Previous studies of siderophore–antibiotic conjugates, using only the standard broth or agar dilution assays to determine the minimum inhibitory concentrations (MICs), were found to be misleading, as resistant mutants can develop and proliferate even though most of the population is sensitive to the drug [13]. We therefore performed agar diffusion assays of compounds **3a** and **3b** with select microbes. These studies suggested that compound **3b**, containing the important phenylglycyl side chain of Lorabid®, was a more effective growth inhibitor than compound **3a**. Not surprisingly, in the preliminary antibiotic susceptibility tests performed by serial dilutions in agar (Table 1), compound **3a** displayed moderate activity against some Gram-positive and Gram-negative bacteria. Compound **3b**, however, displayed only mild antibacterial activity against some Gram-positive and Gram-negative species, and showed outstanding activity against *Acinetobacter* (MIC = 0.03 $\mu g\ ml^{-1}$, whereas MIC = 64 $\mu g\ ml^{-1}$ for Lorabid® itself). The MIC data determined by the standard agar dilution method for compounds **4a** and **4b** also appeared disappointing for most organisms tested, with essentially no activity against Gram-positive bacteria and only slight activity against Gram-negative strains, although **4a** appeared the more effective of the two. The activities of compounds **3a, 3b** and **4a** were increased when they were tested in the presence of sulbactam, a β-lactamase inhibitor [18]. Growth inhibition of *Escherichia coli* and *Pseudomonas aeruginosa* wildtype strains (DC0, K799/WT) and mutants with diminished permeability barriers (DC2, K799/61), (Table 2) indicates that penetration of the intact outer membrane is not sufficient to reach full target activity. As described earlier [14], MIC determinations using standard dilution assays do not differentiate growth of the parent strain from selection and growth of mutants lacking specific siderophore receptors (thus giving misleadingly high MIC values). We therefore performed more detailed kinetic growth studies in broth with the β-lactam hypersensitive *E. coli* strain X580 [14]. Conjugates **3a,b** and **4a,b** each induced significant inhibitory effects, as microbial growth was significantly delayed compared to the control (data not shown). The bacteria that eventually did grow were separately incubated again in the presence of each of the test compounds. No delay of growth relative to the control was observed in this reinoculation experiment. In our previous experiments with siderophore–antibiotic conjugates [13,14] growth of the wild-type organism was completely inhibited until selection of resistant mutants from the parent *E. coli* strain occurred. As noted above, the resulting mutants are probably deficient in a specific form of iron transport, since growth was thereafter observed in the presence of the same conjugates that inhibit growth of the original organism, but not in the presence of other conjugates (see below). Similarly, the mutants that are resistant to the mixed-ligand conjugates are presumably deficient in one or more iron-transport systems. We therefore undertook a preliminary characterization of these mutants.

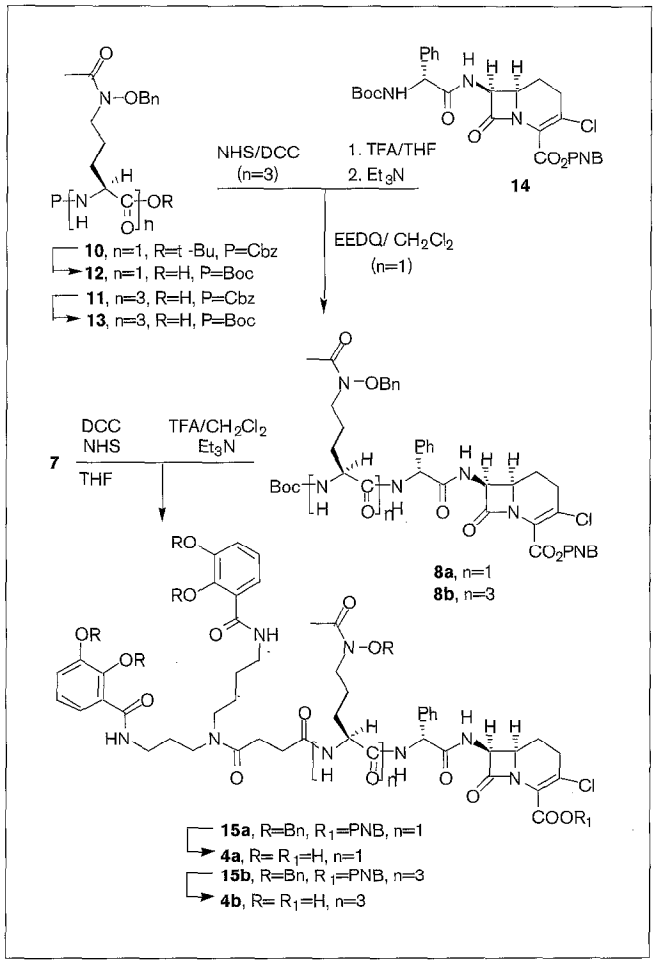
We previously found [13] that iron-transport-deficient mutants selected from previous exposure to siderophore-antibiotic conjugates such as compounds **1** and **2** were greatly impaired in their ability to grow in mammals. This was presumably because they had a decreased ability to assimilate iron, even though they were deficient in only one of several siderophore outer membrane receptors. The new mixed-ligand conjugates would be expected to be taken up by more than one receptor, indicating that mutants unable to recognize the conjugates might be missing more than one siderophore receptor and be especially prone to iron starvation under physiological conditions. Compounds **3a** and **3b** were tested for their ability to inhibit the growth of mutants previously isolated from the exposure of *E. coli* strain X580 to catechol conjugate **2**, which had been shown to lack the Cir protein [13] (Cir and Fiu are the two proteins in *E. coli* known to recognize and initiate the uptake of iron complexes of the monocatecholic enterochelin (enterobactin) precursor dihydroxybenzoic acid and the enterochelin (enterobactin) degradation product dihydroxybenzoylserine [19]). Both **3a** and **3b** delayed the growth of the Cir mutants relative to the control; the delay period was similar to that observed with unselected *E. coli* X580. This suggested that these compounds either do not exclusively use the Cir protein for transport, or do not use the Cir protein at all. Incubation with compounds **4a** and **4b** gave little change relative to the growth of the control, perhaps indicating that these conjugates do require the Cir protein for transport.

The mixed-ligand complexes (either alone or as the iron complex) were next tested for inhibition of the growth of mutants that had been previously isolated from the exposure of *E. coli* X580 to hydroxamate conjugate **1**, and which had been shown to lack the outer membrane triornithylhydroxamate receptor protein (FhuA) [13]. Growth was significantly inhibited by compounds **3b** and **4a**, again suggesting that these conjugates either do not require just FhuA for transport or do not use FhuA at all. The effect of compound **4b** on this mutant was less dramatic. Repetition of the growth inhibition/delay studies with *E. coli* X580 in the presence of 10 μM of one of the mixed-ligand conjugates and EDDA (ethylenediamine *bis*(*o*-hydroxyphenyl)acetic acid, used to simulate an iron-deficient medium similar to mammalian serum) resulted in an extension of the growth delay caused by compound **3a**, and complete inhibition of growth in the presence of **3b**, **4a** or **4b**. These results suggested that conjugates **3b** and **4a** may use different receptor transport systems from the original conjugates **1** and **2**, either instead of or in addition to the original system, whereas compound **4b** might not be as versatile. The enhanced

effect of the conjugates on inhibition of growth in the presence of EDDA (iron limited conditions) might also be due to the full expression of the siderophore transport systems in this severely iron-limited medium. Indeed, further studies with previously selected and characterized TonB mutants as well as Cir and Fiu single and double mutants from different strains of *E. coli* (Table 3) indicate that the activities of all four conjugates (**3a**, **3b**, **4a** and **4b**) are dependent on the iron recognition and transport proteins. The generally higher activity of compound **3b** allowed us to determine that it shows a clear preference for Fiu over Cir. Similar preferences have been noted for compounds **3a** and **4a** (U. Moellmann and R. Reissbrodt, personal communication).

Additional studies with a variety of organisms have been performed that confirm the versatility of the new mixed ligand conjugates, which will be reported elsewhere [20]. In general, the inhibitory activity of the conjugates is associated with the ability of the tested bacteria to use the individual siderophore portion of the molecules for growth promotion in tests excluding the antibiotic component. Assays of mixed ligand conjugates with a variety of mutants lacking one or more siderophore outer membrane receptor or transport proteins also confirm the suggestion from our work that in many cases these new conjugates can use more than one iron-assimilation pathway. Interestingly, growth of some microbes is inhibited by either the siderophore components alone or the corresponding conjugates even if the microbe does not recognize or use the relevant siderophore. In these cases, the inhibitory effect may be due to the ability of the siderophore component to withhold iron from certain bacteria, indicating that siderophore conjugates may in principle have two modes of action, inhibiting microbial growth through either active transport-mediated drug delivery or iron starvation.

**Figure 3**




Synthesis of compounds **4a** and **4b** [9]. The protected forms of δ-*N*-hydroxyornithyl carbacephalosporin, **8a**, or the corresponding derivative **8b** are coupled with compound **7**.

## Significance

The new mixed-ligand siderophore conjugates reported here appear to be able to use a variety of active transport processes to deliver antibiotics to, and inhibit the growth of, parent strains of pathogenic bacteria. Although iron-transport-deficient mutants were selected during treatment, they were iron-starved and, based on precedent [13], are expected to be less virulent than the wild type. These results suggest that it is possible to design, synthesize and use drug conjugates of siderophores and analogs that can be actively transported into microbial cells by multiple pathways. An appropriate choice of siderophore, linker and drug may allow selective targeting and destruction of pathogenic organisms, providing a significant therapeutic advantage during the development of new antimicrobial agents. By facilitating active transport, siderophore conjugation may also make drugs to which resistance has developed by microbial alteration of membrane permeability useful once more. Finally, siderophore components themselves or drug conjugates may limit microbial vitality by withholding the iron that is essential for their growth.

## Materials and methods

### *General methods*

Melting points were determined on a Thomas Hoover capillary melting point apparatus and were uncorrected. Infrared spectra (IR) were recorded on a Perkin-Elmer 1240 spectrophotometer, and were obtained either as thin film (neat) or as a KBr pellets. $^1$H and $^{13}$C NMR were performed on a General Electric GN-300 spectrometer using TMS ($CDCl_3$, $CD_3OD$ for $^1$H NMR) or solvent $CDCl_3$ ($CD_3OD$ for $^{13}$C NMR) as internal standards. Dioxane was used as the internal standard when $D_2O$ was used as the NMR solvent. Peak assignments for $^{13}$C NMR were made with the assistance of the distortionless enhanced polarization transfer (DEPT) pulse program [21]. Mass spectral data (EI, CI, FAB) were obtained on a Finnegan MAT Model 8430 spectrometer. Silica Gel PF254 (EM Science) was used for flash chromatography. Radial chromatography or preparative thin layer chromatography was performed with Kieselgel 60 PF254 (EM Science). Elemental analysis were performed by M-H-W laboratories. Solvents used were dried and purified by standard methods [22]. The term 'dried' refers to the drying

**Table 1**

**Selected minimum inhibitory concentration of Lorabid® and mixed-ligand–siderophore conjugates.**

| Organism | Strain | Lorabid® | 3a | 3b | 4a | 4b |
|---|---|---|---|---|---|---|
| *Staphylococcus aureus* | X1 | 1 | >128 | >128 | 16 | >128 |
| | X41 | 8 | >128 | >128 | 4 | >128 |
| | X400 | >128 | >128 | >128 | 8 | >128 |
| | S132 | >128 | >128 | >128 | 4 | >128 |
| *Staphylococcus epidermidis* | 270 | 4 | >128 | >128 | 2 | >128 |
| | 222 | 4 | >128 | >128 | 32 | >128 |
| *Streptococcus* A | C203 | 0.03 | 64 | 16 | 32 | 4 |
| *Streptococcus pneumonia* | PARK | 0.25 | 64 | 32 | 64 | 64 |
| *Enterococcus* sp. | 2041 | 64 | 128 | 128 | 64 | >128 |
| *Klebsiella* sp. | X26 | 0.25 | >128 | 16 | >128 | >128 |
| *Klebsiella* sp. | 68 | 0.5 | >128 | 64 | >128 | >128 |
| *Enterobacter aerogenes* | C32 | >128 | 128 | 64 | >128 | >128 |
| *Enterobacter cloacae* | EB5 | 16 | 128 | 32 | >128 | >128 |
| *Salmonella* sp. | X514 | 0.125 | 128 | 16 | >128 | >128 |
| *Serratia* | X99 | 16 | 128 | 64 | 128 | 128 |
| *Shigella sonnei* | NO | 2 | 128 | 32 | 128 | 128 |
| *Acinetobacter* | AC12 | 64 | 128 | 0.03 | 128 | 128 |

Inhibitory concentrations are given as μg ml$^{-1}$. The assay was performed at Eli Lilly and Co. using the standard agar dilution method [21].

of an organic layer over anhydrous magnesium or sodium sulfate. All reactions were performed under a nitrogen atmosphere.

*4-Nitrobenzyl-7β-[[[[N$^5$-[[N$^1$,N$^8$-bis[2,3-bis(benzyloxy) benzoyl]-spermidine-N$^4$-yl]succinyl]-N$^1$-(benzyloxy)-1,5-diaminopentyl]-N$^1$-yl]succinyl] amino]-1-carba-3-chloro-3-cephem-4-carboxylate (compound **9a**)*

Boc-protected amine **5** [15] (137 mg, 0.185 mmol) was dissolved in dry methylene chloride (1 ml) under nitrogen. The solution was cooled to 0 °C (ice bath) and TFA (1 ml) was added slowly. The reaction mixture was allowed to warm up to room temperature and further stirred for 30 min. The solvent and TFA were removed under a vacuum. Traces of TFA were removed by evaporating the oily residue with toluene to provide the TFA salt of the deprotected amine of compound **5** as a yellow oil. The TFA salt and acid **7** (167 mg, 0.190 mmol) were dissolved in methylene chloride and triethylamine (19 mg, 0.185 mmol) was added. The reaction mixture was stirred for 5 min, cooled (ice bath) and additional triethylamine (28 mg, 0.277 mmol) was added followed immediately by *N*-hydroxybenzotriazole (HOBT, 25 mg, 0.185 mmol) and EDC (53 mg, 0.277 mmol). The solution was warmed to room temperature and stirred overnight. The solvent was evaporated and the residue was dissolved in EtOAc. The EtOAc layer was washed with 0.5 N HCl, water and brine. Drying and removal of

**Table 2**

**Antibacterial activity in the agar diffusion assay against wild type strains and penetration mutants.**

| Test organism | 3a | 3a+S | 3b | 3b+S | 4a | 4a+S | 4b |
|---|---|---|---|---|---|---|---|
| *Bacillus subtilis* ATCC 6633 | 12P | 16 | 0 | 12.5P | 0 | 0 | 0 |
| *Micrococcus flavus* ATCC 10240 | 13 | 15 | 0 | 14 | 0 | 0 | 0 |
| *Salmonella gallinarum* ATCC 9184 | 22.5 | 21.5 | 0 | 17.5 | 0 | 12.5 | 0 |
| *Escherichia coli* DC 0[†] | 14 | 16.5 | 0 | 13p | 0 | 12p | 0 |
| *Escherichia coli* DC2[†] | 23.5 | 22.5 | 0 | 16 | 0 | 13.5 | 0 |
| *Pseudomonas aeruginosa* K 799/WT[‡] | 12P | 11/20P[§] | A | A18 | 15P | 17P | 0 |
| *Pseudomonas aeruginosa* K 799/61[‡] | 29 | 29 | 13 | 21 | 22 | 24.5 | 21 |

The table shows the size (in mm) of the inhibition zone seen when the indicated compounds were present at a concentration of 100 μg ml$^{-1}$. +S: with 0.5 mM sulbactam. [§]11/20P indicates a clear inhibition zone of 11 mm and an inhibition zone of 20 mm containing many resistant colonies., p: colonies within the inhibition zone, P: many colonies within the inhibition zone, A: indication of inhibition.The samples were applied in 50-μl aliquots onto 9-mm diameter agar wells. Strains were reported in the following references: [†]Richmond *et al.* [24]; [‡]Zimmerman [25].

**Table 3**

**Susceptibility of *E. coli* K-12 iron transport and porin mutants in the agar diffusion assay.**

| Strain | Relevant genotype | 3a | 3a+S | 3b | 3b+S | 4a | 4a+S | 4b |
|---|---|---|---|---|---|---|---|---|
| AB 2847 | aro B | 13 | 15.5 | 13P | 12.5 | 12P | 11.5 | 0 |
| BR 158 | ton B | 12 | 11.5 | 0 | 0 | 0 | 0 | 0 |
| H 1443 | aro B | 17.5 | 18 | 13/16P | 14 | 13/16P | 13.5 | 0 |
| H 1728 | cir, fiu | 0 | 11.5 | 0 | 0 | 0 | 0 | 0 |
| H 1875 | fep A, cir | 12.5 | 17 | 0 | 14.5 | 10.5p | 14 | 0 |
| H 1876 | fep A, cir, fiu | 0 | 12P | 0 | 0 | 0 | 0 | 0 |
| H 1877 | fep A, fiu | 0 | 13.5 | 0 | 11 | 0 | 0 | 0 |
| H 873 | fep A | 15 | 15.5 | 12.5p/15P | 14 | 12.5p/15P | 13 | 0 |

The zone of inhibition in mm is shown for the indicated compounds at a concentration of 100 μg $ml^{-1}$. The samples were applied as 50 μl aliquots in 9 mm diameter agar wells. +S: with 0.5 mM sulbactam, p: colonies within the inhibition zone, P: many colonies within the inhibition zone. fepA: enterochelin receptor [6,20], aroB: blocked aromate (dihydroxybenzoate and enterochelin biosynthesis [19,26].

solvent afforded a yellow oil that was purified by radial chromatography using MeOH/EtOAc (1:10) giving compound **9a** (155 mg, 56 %): IR (neat) 3420–2860 (br), 1775, 1730, 1650, 1520 $cm^{-1}$; $^1H$ NMR (20 % DMSO-$d_6$ in $CDCl_3$) δ 1.10–1.90 (m, 14H, $CH_2$), 2.21–2.75 (m, 10H, $CH_2$ including allylic $CH_2$), 2.97 (q, $J$ = 6.0 Hz, 2H, $CH_2$), 3.05–3.25 (m, 8H, $CH_2$), 3.40–3.60 (m, 2H, $CH_2$ obscured by solvent peak), 3.87 (m, 1H, C8$CH_2$C*H*), 4.86 (s, 2H, benzylic H), 5.01 (s, 4H, benzylic H), 5.17 (s, 4H, benzylic H), 5.35–5.45 (m, containing s at 5.41, benzylic H and C7NHC*H*), 7.05–7.55 (m, 31H, aromatic H), 7.69 (d, $J$ = 8.48 Hz, 2H, aromatic H), 7.77 (m, 1H, NH), 8.12–8.30 (m, containing d at 8.23, $J$ = 8.5 Hz, total 4H, aromatic H and NH), 8.72 (d, $J$ = 8.64 Hz, 1H, NH); $^{13}C$ NMR (20 % DMSO-$d_6$ in $CDCl_3$) δ 21.4, 23.6, 24.8, 25.9, 26.2, 26.4, 26.5, 27.0, 27.5, 27.8, 28.4, 28.8, 29.4, 30.7 and 31.0 (allylic), 36.7, 38.5, 38.8, 42.7, 44.6 (m), 46.7, 52.0, 58.0, 65.9, 70.2, 75.1, 75.2, 75.5, 115.8, 120.7, 120.9, 123.6, 123.7, 124.2, 127.8, 128.1, 128.3, 128.4, 128.5, 128.7, 128.8, 129.1, 129.3, 131.0, 131.0, 131.3, 134.9, 136.8, 137.1, 137.1, 137.2, 143.0, 145.2, 145.2, 147.2, 151.7, 160.1, 165.6, 165.7, 166.0, 166.5, 170.8, 171.1, 171.4, 172.0, 172.6; MS (FAB) 1501.7 ($MH^+$). Anal. calc'd for $C_{84}H_{89}N_8O_{16}Cl$: C, 67.17; H, 5.97; N, 7.46. Found: C, 66.98; H, 6.07; N, 7.18.

### *7β-[[[[N⁵-[[N¹,N⁸-bis[2,3-bis(hydroxy)benzoyl]spermidine-N⁴-yl]succinyl]-N¹-(hydroxy)-1,5-diaminopentyl]-N¹-yl]succinyl]amino]-1-carba-3-chloro-3-cephem-4-carboxylic acid (compound **3a**)*

Concentrated HCl (13.3 μl, 0.153 mmol, 300 mol %) of and 15.4 mg (20 % w/w) of Pd-C was added to a solution of compound **9a** (77 mg, 0.051 mmol) in 1.0 ml of 5 % aqueous dimethylformamide (DMF; made from deionized distilled water and HPLC-grade DMF). This mixture was exposed to hydrogen at atmospheric pressure for 36 h. The catalyst was removed by filtration. The DMF/water mixture was removed by evaporation under high vacuum. The residue was redissolved in MeOH and was evaporated. This process was repeated in an attempt to remove any residual DMF. However, the presence of traces of solvent was evident ($^1H$ NMR) due to the relatively high affinity of these compounds towards DMF. The semisolid product was filtered through a column of Sephadex LH 20 gel (3.0 g; 10 % MeOH/EtOAc) to provide compound **3a** in 99 % yield as an amber oil. $FeCl_3$ positive (purple); IR (TF) 3580–2600 (br), 1765, 1650 $cm^{-1}$; 1H NMR ($CD_3OD$) δ 1.20–2.05 (m, 14H, $CH_2$), 2.30–3.20 (m, 12 H, CH2 including allylic $CH_2$ obscured by solvent peak), 3.30–3.50 (m, 8H, $CH_2$), 3.55–3.75 (m, 2H, $CH_2$), 3.82–3.95 (m, 1H, C8$CH_2$C*H*), 5.25 (m, 1H, C7C*H*NH), 6.72–7.49 (m, 10H, ArH and NH); $^{13}C$ NMR (75 MHz, $CD_3OD$, all signals at 25 °C reported) δ 21.0, 23.2, 24.7, 24.8, 26.0, 27.0, 27.6, 28.4, 29.3, 29.5, 29.7, 29.8, 30.7, 32.0, 32.3 (allylic), 37.6, 38.0, 39.8, 40.0, 40.2, 44.3, 44.6 (m), 53.7, 59.5, 67.9, 116.5, 116.6, 118.5, 118.6, 121.0, 123.8, 129.5 (m), 136.1, 138.3, 147.2, 150.3 (m), 164.4, 167.3, 171.4 (m), 174.3, 174.4, 174.7. MS (FAB) *m/z* 916.3 ($MH^+$).

### *4-Nitrobenzyl-7β-[[[[[N⁵-[[N¹,N⁸-bis[2,3-bis(benzyloxy)-benzoyl]spermidine-N⁴-yl]succinyl]-N¹-(benzyloxy)-1,5-diaminopentyl]-N¹-yl]succinyl]-D-phenylglycyl]amino]-1-carba-3-chloro-3-cephem-4-carboxylate (compound **9b**)*

Protected conjugate **9b** was obtained from compound **6** as an oil in 64 % yield in the same manner as **9a** was obtained from compound **5**. IR (neat) 3420–2860 (br), 1775, 1730, 1650, 1520 $cm^{-1}$; $^1H$ NMR (20 % DMSO-$d_6$ in $CDCl_3$) δ 1.11–1.71 (m, 14H, $CH_2$), 2.22–2.76 (m, 10H, $CH_2$ including allylic $CH_2$), 2.95 (q, $J$ = 6.0 Hz, 2H, $CH_2$), 3.04–3.24 (m, 8H, $CH_2$), 3.46–3.60 (m, 2H, $CH_2$), 3.81 (m, 1H, C8$CH_2$C*H*), 4.84 (s, 2H, benzylic H), 5.00 (s, 4H, benzylic H), 5.16 (s, 4H, benzylic H), 5.35–5.45 (m, containing s at 5.40, benzylic H and C7NHC*H*), 5.52 (d, $J$ = 7.64 Hz, 1H, benzylic H), 7.05–7.53 (m, 36H, aromatic H), 7.68 (d, $J$ = 8.61 Hz, 2H, aromatic H), 7.75 (m, 1H, NH), 8.12–8.25 (m, containing d at 8.22, $J$ = 8.71 Hz, total 4H, aromatic H and NH), 8.68 (d, $J$ = 7.39 Hz, 1H, NH), 9.15 (d, $J$ = 8.82 Hz, 1H, NH); $^{13}C$ NMR (20 % DMSO-$d_6$ in $CDCl_3$) δ 21.1, 23.6, 24.8, 25.9, 26.2, 26.4, 26.5, 27.2, 27.5, 27.8, 28.4, 28.8, 29.5, 30.6 and 30.9 (allylic), 36.7, 38.5, 38.8, 42.7, 44.6 (m), 46.7, 52.0, 56.6, 57.7, 65.8, 70.2, 75.1, 75.2, 75.5, 115.8, 120.7, 120.7, 120.9, 122.8, 123.5, 123.7, 124.2, 127.3, 127.8, 128.0, 128.2, 128.2, 128.3, 128.5, 128.7, 128.8, 129.0, 129.3, 131.0, 131.0, 131.1, 131.3, 134.9, 136.8, 137.1, 137.1, 137.2, 143.0, 145.2, 145.2, 147.2, 151.6, 160.0, 165.5, 165.7, 165.6, 165.8, 165.9, 170.7, 170.7, 171.1, 171.3, 171.5, 174.6; MS (FAB) *m/z* 1501.7 ($MH^+$). Anal. calc'd for $C_{92}H_{96}N_9O_{17}Cl$: C, 67.57; H, 5.92; N, 7.71. Found: C, 67.47; H, 6.06; N, 7.49.

### *7β-[[[[[N⁵-[[N¹,N⁸-bis[2,3-bis(Hydroxy)benzoyl]spermidine-N⁴-yl]succinyl]-N¹-(hydroxy)-1,5-diaminopentyl]-N¹-yl]-succinyl]-D-phenylglycyl]amino]-1-carba-3-chloro-3-cephem-4-carboxylic acid (compound **3b**)*

Hydrogenation of compound **9b** was carried out using the same conditions as for **9a** to furnish compound **3b** in 99 % yield as a light amber oil: $FeCl_3$ positive (purple); IR (neat) 3560–2500 (br), 1760, 1650 $cm^{-1}$; 1H NMR ($CD_3OD$) δ 1.20–2.00 (m, 14H, $CH_2$), 2.30–3.15 (m, 12H, $CH_2$ including allylic $CH_2$ obscured by solvent peak), 3.25–3.45 (m, 8H, $CH_2$), 3.50–3.75 (m, 2H, $CH_2$), 3.80–3.90 (m, 1H, C8$CH_2$CH), 5.30–5.50 (m, 2H, benzylic CH and C7CHNH), 6.65–7.50 (m, 15H, ArH and NH); $^{13}C$ NMR (75 MHz, $CD_3OD$, all signals at 25 °C reported) δ 20.9, 22.8, 24.6, 24.8, 26.0, 27.0, 27.6, 28.4, 29.3, 29.5, 29.8, 29.8, 31.1, 32.0, 32.3 (allylic), 37.6, 37.9, 39.8, 40.0, 40.2, 44.3, 46.6 (m), 53.8, 59.3, 59.5, 67.9, 116.5, 116.6, 118.5, 118.6, 121.0, 123.9, 129.8

(m), 135.9, 138.1, 140.2, 147.2, 150.3 (m), 164.4, 167.4, 171.5 (m), 174.3, 174.4, 174.6, 175.2. MS (FAB) *m/z* 1049.4 ($MH^+$); HRMS (FAB) calc'd for $C_{50}H_{61}N_8O_{15}ClNa$: 1071.3843 ($MNa^+$). Found: 1071.3903.

### *$N^5$-Acetyl-$N_5$-(benzyloxy)-$N_2$-(tert-butoxycarbonyl)-L-ornithine (compound **12**)*

To a solution of compound **10** [9] (0.2 g, 0.42 mmol) in 5 ml of anhydrous methylene chloride was added a 33 % solution of HBr in glacial acetic acid (5 ml). The resulting solution was stirred for 15 min at room temperature. The methylene chloride was evaporated. The residue was diluted with $H_2O$ (5 ml), and neutralized with solid $NaHCO_3$. The solid residue after evaporation of the solvent was dissolved in 10 ml of tetrahydrofuran (THF): $H_2O$ (1:1), and the pH of the reaction mixture was maintained at 9. To the above, a solution of $Boc_2O$ (0.370 g, 1.69 mmol) in THF (3 ml) was added dropwise, and stirred overnight at room temperature. The THF was evaporated and the residue was extracted with ethyl acetate to remove excess $Boc_2O$. The organic layer was washed with water, and the combined aqueous layer, after acidification with saturated citric acid solution, was extracted repeatedly with ethyl acetate. The organic layer was washed with $H_2O$, brine, dried ($Na_2SO_4$), and filtered. Evaporation of the solvent, and flash chromatography (silica gel) using $CHCl_3$:MeOH:acetic acid (90:10:1) afforded **12** (140 mg, 86 %) as an oil. IR (neat): 3300 (br), 2980, 1720, 1700, 1660 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 300 MHz) δ 1.39 (s, 9H), 1.52–1.9 (m, 4H), 2.09 (s, 3H), 3.5–4.3 (m, 3H), 4.80 (s, 2H), 5.2–6.3 (m, 1H), 7.36 (s, 5H); $^{13}C$ NMR ($CDCl_3$) δ 21.2, 22.7, 28.2, 29.7, 44.6, 53.5, 76.7, 79.2, 128.6, 128.8, 129.1, 134.0, 155.7, 172.9, 178.9; MS (CI, isobutane) *m/z* 281 (M+1−100).

### *4-Nitrobenzyl-7β-[[$N^5$-acetyl-$N^5$-(benzyloxy)-$N^2$-(tert-butoxycarbonyl)-L-ornithyl-D-phenylglycyl]amino]-1-carba-3-chloro-3-cephem-4-carboxylate (compound **8a**)*

*N*-Boc protected Lorabid®, compound **14** (0.162 g, 0.277 mmol), was dissolved in 2 ml of dry methylene chloride under nitrogen. The solution was cooled to 0 °C (ice-bath) and 2 ml of TFA was added slowly. The reaction mixture was allowed to warm up to room temperature, and stirred for 30 min. The solvent and excess TFA were removed under reduced pressure. Traces of additional TFA were removed by azeotropic evaporation with benzene to provide the TFA salt as a solid. The TFA salt was dissolved in dry methylene chloride (6 ml), and dry triethylamine (38 ml, 0.277 mmol) was added to the solution to neutralize the salt. To the above solution, compound **12** (0.105 g, 0.277 mmol) in methylene chloride (4 ml) followed by 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ, 0.089 g, 0.360 mol) was added rapidly. The reaction mixture was stirred overnight at room temperature. The volatile components were evaporated and the residue was diluted with ethyl acetate (20 ml). The organic layers were washed with 0.5 N HCl, water, brine, dried ($Na_2SO_4$) and filtered. After evaporation of the solvent, the crude compound was purified by chromatography eluting with 1:1 EtOAc/hexanes to provide 0.164 g (70 %) of **8a** as a foamy solid: mp 140–142 °C; IR (KBr) 1765, 1625 (br), 1420 $cm^{-1}$; $^1H$ NMR ($CDCl_3$) δ 1.35–1.45 (m, containing s at 1.39, 11H), 1.50–1.80 (m, 5H), 1.99 (s, 3H), 2.42–2.52 (m, 2H), 3.40–4.00 (m, 3H), 4.2–4.4 (m, 1H), 4.77 (s, 2H), 5.23–5.40 (m, 3H), 5.73–5.92 (m, 1H), 7.2–7.45 (m, 10H), 7.57 (d, $J$ = 8.4 Hz, 2H), 7.87 (d, $J$ = 7.5 Hz, 1H), 8.15 (d, $J$ = 8.7 Hz, 2H), 8.44 (d, $J$ = 5.4 Hz, 1H); $^{13}C$ NMR ($CDCl_3$) δ 172.5, 172.4, 170.7, 164.6, 159.7, 155.9, 147.5, 142.2, 137.4, 134.0, 131.6, 129.1, 128.8, 128.7, 128.1, 123.5, 122.7, 79.7, 76.0, 65.9, 58.5, 56.7, 53.2, 52.3, 43.6, 31.5, 29.3, 28.1, 23.0, 21.2, 20.1; FAB HRMS Calc'd for $C_{42}H_{48}N_6O_{11}Cl$, 847.3070 ($MH^+$), found 847.3065. Anal. calc'd for $C_{42}H_{48}N_6O_{11}Cl$ C, 59.48; H, 5.71; N, 9.92. Found: C, 59.32; H. 5.61; N, 9.69.

### *4-Nitrobenzyl-7β-[[$N^5$-Acetyl-$N^5$-(benzyloxy)-$N^2$-[[$N^1$,$N^8$-bis[2,3-bis(benzyloxy)benzoyl]spermidine-$N^4$-yl]succinyl]-L-ornithyl-D-phenylglycyl]amino]-1-carba-3-chloro-3-cephem-4-carboxylate (**15a**)*

To a solution of compound **8a** (0.120 g, 0.141 mmol) in 1.0 ml of anhydrous methylene chloride under nitrogen at 0 °C was added 0.5 ml of TFA. The solution was stirred for 30 min, then concentrated under reduced pressure. Excess TFA was removed by repeated evaporations from benzene. The TFA salt was dissolved in 2 ml of methylene chloride, and dry triethylamine (20 ml, 0.141 mmol) was added. A solution of the active ester (obtained from overnight stirring of compound **7** [10] (0.124 g, 0.141 mmol), with dicyclohexylcarbodiimide (0.034 g, 0.169 mmol), and *N*-hydroxysuccinimide (0.019 g, 0.169 mmol)) in dry THF under nitrogen was added to the above solution of the free amine, and stirred overnight at room temperature. The solvent was removed under reduced pressure. The organic residue was diluted with ethyl acetate and washed with brine, dried, filtered, and concentrated to give a white foam. Compound **15a** was isolated by silica gel chromatography eluting with 10 % methanol/ethyl acetate to afford 0.143 g (80 %) of product as a foamy solid: mp 73–75 °C; IR (KBr) 1765, 1685–1600, 1425 $cm^{-1}$; $^1H$ NMR ($CDCl_3$) δ 1.1–1.9 (m, 13H),1.98 (s, 3H), 2.2–2.7 (m, 6H), 2.85–3.30 (m, 8H), 3.5–3.85 (m, 3H), 4.15–4.45 (m, 1H), 4.7–4.75 (m, 2H), 5.0–5.7 (m, 12H), 7.0–7.7 (m, 38H), 7.9–8.05 (m, 3H), 8.09 (d, $J$ = 9.0 Hz, 2H), 8.45–8.65 (m, 1H); $^{13}C$ NMR ($CDCl_3$) δ 173.8, 172.2, 171.6, 171.0, 165.3, 165.3, 165.2, 160.1, 151.7, 151.7, 151.6, 147.5, 146.7, 146.6, 146.6, 146.3, 142.3, 142.3, 136.6, 136.3, 136.3, 136.3, 134.2, 131.0, 129.2, 129.1, 128.9, 128.9, 128.7, 128.6, 128.5, 128.2, 127.7, 127.6, 124.4, 124.4, 123.6, 123.0 (m), 116.9, 116.8, 116.4, 77.2, 76.3 (m), 71.1, 71.1, 65.93, 58.5, 57.6, 53.5, 52.6, 52.5, 47.1, 45.0, 44.7, 42.8, 39.3, 39.0, 37.0, 36.9, 31.8, 31.6, 28.5, 28.4, 28.3, 27.6, 27.6, 27.5, 27.2, 26.6, 26.4, 26.0, 24.6, 23.5, 23.4, 21.4, 20.3; FAB HRMS calc'd for $C_{90}H_{93}N_9O_{17}Cl$, 1606.6378 ($MH^+$), found 1606.6431. Anal. calc'd for $C_{90}H_{93}N_9O_{17}Cl$: C, 67.22; H, 5.83; N, 7.84. Found: C, 67.12; H, 6.02; N, 7.66.

### *7β-[[$N^5$-acetyl-$N^5$-hydroxy-$N^2$-[[$N^1$,$N^8$-bis[2,3-bis(hydroxy)benzoyl]spermidine-$N^4$-yl]succinyl]-L-ornithyl-D-phenylglycyl]amino]-1-carba-3-chloro-3-cephem-4-carboxylic acid (compound **4a**)*

To a solution of compound **15a** (0.09 g, 0.084 mmol) in 1.0 ml of HPLC-grade DMF and 50 ml of distilled deionized water was added 20.8 μl of concentrated HCl (300 mol %) and 0.018 g of 10 % palladium on carbon. The mixture was exposed to hydrogen at atmospheric pressure for 42 h. The catalyst was removed by filtration with methanol and the solvents were removed under reduced pressure. $^1H$ NMR showed traces of DMF despite attempts to remove it. The product was obtained as an amber oil in near quantitative yield: $FeCl_3$ positive (purple); IR (neat) 3400–2600, 1760 (br), 1700–1610 (br) $cm^{-1}$; $^1H$ NMR ($CD_3OD$) δ 1.25–1.85 (m, 13H), 2.02 (br s, 3H), 2.1–2.9 (m, obscured by residual DMF, 6H), 3.1–3.8 (m, 11H), 4.2–4.4 (m, 1H), 4.6–5.0 (m, obscured by solvent peak, 2H), 6.7 (m, 1H), 6.95 (m, 1H), 7.15–7.50 (m, 11H), 7.95–8.25 (br m, exchangeable); $^{13}C$ NMR ($CD_3OD$) δ 172.8, 171.9, 171.1, 170.9, 168.0, 164.4, 164.3, 163.9, 163.8, 150.7, 147.5, 139.4, 137.1, 134.0, 131.2, 129.2, 128.5, 128.4, 121.7, 120.2, 119.4, 119.4, 118.7, 118.7, 118.6, 116.4, 73.8, 67.3, 58.0, 53.7, 47.9, 45.2, 44.2, 38.0, 37.2, 35.3, 33.7, 32.2 (allylic), 29.3, 28.7, 27.7, 27.6, 27.3, 25.4, 25.4, 24.5, 21.7. MS (FAB) *m/z* 1021 ($MH^+$), 1043 ($MNa^+$), and 1058.4 ($MK^+$).

### *$N^5$-acetyl-$N^5$-(benzyloxy)-$N^2$-(tert-butoxycarbonyl)-L-ornithyl-$N^5$-acetyl-$N^5$-(benzyloxy)-L-ornithyl-$N^5$-acetyl-$N^5$-(benzyloxy)-L-ornithine (compound **13**)*

Following the procedure described for the synthesis of compound **12**, compound **11** (0.2 g, 0.213 mmol) was exposed to a 33 % solution of HBr in glacial acetic acid and after workup gave compound **13** (0.15 g, 78 %) as an oil. IR (neat): 3300 (brm), 2940–3000 (br), 1720, 1665 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 300 MHz) δ 1.37 (s, 9H), 1.4–1.85 (m, 12H), 1.97, 2.04, 2.05 (3s, 9H), 3.4–3.9 (m, 6H), 4.2–4.6 (m, 3H), 4.77 (s, 6H), 7.3–7.5 (m, containing s at 7.39, 15H); $^{13}C$ NMR ($CDCl_3$) δ 172.8, 155.7, 155.7, 134.3, 134.2, 134.2, 129.3, 129.2, 128.9, 128.7, 79.4, 77.2, 76.2, 67.9, 55.7, 53.2 (m), 44.7 (m), 30.3 (m), 28.3, 23.2, 20.3; MS FAB *m/z* 905 ($MH^+$), 805 ($MH^+$−100).

*4-Nitrobenzyl-7β-[[$N^5$-acetyl-$N^5$-(benzyloxy)-$N^2$-(tert-butoxy-carbonyl)-L-ornithyl-$N^5$-acetyl-$N^5$-(benzyloxy)-L-ornithyl-$N^5$-acetyl-$N^5$-(benzyloxy)-L-ornithyl-D-phenylglycyl]amino]-1-carba-3-chloro-3-cephem-4-carboxylate (compound **8b**)*

*N*-Boc protected Lorabid®, compound **14** (0.043 g, 0.0745 mmol) was converted to the TFA salt as described earlier. The TFA salt was dissolved in 2 ml of anhydrous methylene chloride, and 28.5 μl of dry triethylamine was added to the resulting solution. A THF solution of the active ester prepared from compound 13 (0.067 g, 0.0745 mmol), NHS (0.01 g, 0.0894 mmol), and dicyclohexylcarbodiimide (DCC, 0.018 g, 0.0894 mmol) was added, and the reaction mixture was stirred overnight at room temperature. Workup and purification afforded 0.1 g of compound **8b** as a foamy solid (0.081 g, 90 %): mp 80–82 °C; IR (KBr) 1770, 1680–1610 $cm^{-1}$; $^1H$ NMR ($CDCl_3$) δ 1.32–1.42 (m, containing s at 1.40, 11H), 1.5–1.8 (m, 13H), 1.91 (s, 3H), 2.04 (s, 3H). 2.06 (s, 3H), 2.45–2.85 (m, 2H), 3.4–4.1 (m, 7H), 4.2–4.65 (m, 3H), 4.7–4.9 (m, 6H), 5.2–5.65 (m, 6H), 7.25–7.45 (m, 2H), 7.57 (d, $J = 8.4$ Hz, 2H), 7.65–7.75 (m, 1H), 8.16 (d, $J = 8.7$ Hz, 2H), 8.35–8.45 (m, 1H) ; $^{13}C$ NMR ($CDCl_3$) δ 172.7, 172.6, 172.3, 172.5, 171.3, 170.7, 164.4, 160.1, 155.5, 147.4, 141.9, 136.4, 133.4, 129.7, 128.7, 128.5, 128.9, 128.4, 128.0, 127.4, 123.4, 122.9, 79.3, 77.0, 75.9, 66.0, 58.9, 57.5, 53.6, 52.8, 52.5, 51.5, 48.7, 43.3, 33.5, 31.2, 29.8, 29.1, 28.0, 27.7, 26.0, 24.6, 23.6, 22.8, 22.8, 21.0, 20.2, 19.9; MS (FAB) *m/z* 1371 ($MH^+$), 1271 ($MH^+$–100).

*4-Nitrobenzyl-7β-[[$N^5$-acetyl-$N^5$-(benzyloxy)-$N^2$-[[$N^1$,$N^8$-bis[2,3-bis(benzyloxy)benzoyl]spermidine-$N^4$-yl]succinyl]-L-ornithyl-$N^5$-acetyl-$N^5$-(benzyloxy)-L-ornithyl-$N^5$-acetyl-$N^5$-(benzyloxy)-L-ornithyl-D-phenylglycyl]amino]-1-carba-3-chloro-3-cephem-4-carboxylate (compound **15b**)*

Compound **15b** was obtained (0.122 g, 74 %) from compound **8b** (0.098 g, 0.076 mmol) and compound **7** (0.067 g, 0.076 mmol) following the same coupling procedure as described for compound **15a**: IR (KBr) 1765, 1670–1610 $cm^{-1}$. $^1H$ NMR ($CDCl_3$) δ 1.0-1.85 (m, 21H), 1.9–2.0 (m, 9H), 2.06–2.08 (m, 3H), 2.3–2.7 (m, 6H), 3.0–4.9 (m, 15H), 4.0–4.45 (m, 3H), 4.65–4.85 (m, 6H), 5.0–5.6 (m, 14H), 7.0–8.4 (m, 54H); $^{13}C$ NMR ($CDCl_3$) δ 175.9, 175.7, 173.7, 172.5, 172.2, 172.1, 171.9, 171.7, 171.3, 165.4, 165.2, 165.2, 160.3, 160.3, 156.9, 151.7, 151.6, 147.6, 146.5, 142.4, 156.9, 151.7, 147.6, 147.6, 146.7, 146.6, 146.6, 146.5, 142.4, 142.4, 136.3, 129.2, 129.2, 128.6, 128.2, 127.6, 124.6, 124.4, 123.7, 123.6, 123.0, 122.9, 122.9, 116.7, 77.2, 76.4, 76.2, 71.1, 66.1, 66.0, 59.5, 58.3, 52.7, 52.7, 52.7, 51.6, 45.4, 45.3, 44.5, 39.0, 38.1, 36.9, 31.6, 31.5, 29.6, 28.8, 28.8, 27.8, 27.1, 26.7, 25.5, 24.9, 23.4, 20.4; MS (FAB) *m/z* 2131 ($MH^+$).

*7β-[[$N^5$-Acetyl-$N^5$-hydroxy-$N^2$-[[$N^1$,$N^8$-bis[2,3-bis(hydroxy) benzoyl] spermidine-$N^4$-yl]succinyl]-L-ornithyl-$N^5$-acetyl-$N^5$-(hydroxy)-L-ornithyl-$N^5$-acetyl-$N^5$-(hydroxy)-L-ornithyl-D-phenylglycyl]amino]-1-carba-3-chloro-3-cephem-4-carboxylic acid (compound **4b**)*

Following the procedure described for the synthesis of **4a**, a solution of **15b** (0.057 mg, 0.026 mmol) in 1 ml of 5 % aqueous DMF was hydrogenated in the presence of 10 % Pd/C (11.5 mg, 20 % w/w) and 6.6 ml (0.078 mmol, 3 equivalents) of concentrated HCl for 24 h. Workup provided compound **4b** as a light amber semisolid: $FeCl_3$ positive (purple); IR (neat) 3400–2600, 1760 (br), 1700–1610 (br) $cm^{-1}$; $^1H$ NMR ($CD_3OD$) δ 0.8–1.0 (m, 2H), 1.05–1.40 (m, 13H), 1.45–1.90 (m, 6H), 2.00–2.15 (m, 9H), 2.4–2.9 (m, obscured by residual DMF), 3.25–3.65 (m, 15H), 4.6–4.95 (m, obscured by solvent peak), 5.4 (m, 1H), 6.7 (m, 1H), 6.95 (m, 1H), 7.20–7.50 (m, 11H), 7.95–8.30 (br m, exchangeable); MS (FAB) *m/z* 1365.51 ($MH^+$), 1387.49 ($MNa^+$).

*Antibiotic agar dilution tests*

Antibiotic MICs were determined using a standard agar serial dilution test in Müller–Hinton medium [23]. Inocula of test organisms were adjusted to ~$10^5$ colony-forming units (CFU) per ml. After strains were incubated for 18–20 h at 37 °C, the MICs were read as the lowest dilution of antibiotic allowing no visible growth.

*Bacterial growth inhibition studies*

Growth kinetics in broth: the basic procedures described for assays of compounds **1** and **2** were followed [14]. The test compounds were prepared as 5 mM stock solutions in DMF and were added to sterile Luria broth or sterile Luria broth containing EDDA (100 μg $ml^{-1}$) to give 1.0 or 10 μM final concentration of the conjugates. 10 μl of a 24-h-old culture of *E. coli* X 580 in Luria broth was added per 50 ml of Luria broth. The bacteria were added immediately after addition of the conjugates or after preincubation of the test compound in broth for 12 h at 37 °C. The culture flasks were then shaken at 37 °C at 300 rpm. Aliquots were removed every 2 h and the culture turbidity was measured at 600 nm. All the assays were performed in duplicate.

*Antibiotic disc diffusion tests*

The ability of bacteria to use individual unconjugated siderophores, or the susceptibility of bacteria to siderophore drug conjugates, was tested by antibiotic disc diffusion tests under both iron-restricted and iron-sufficient conditions. Agar plates containing exactly 20 ml of Müller Hinton medium with or without EDDA at 100 μg $ml^{-1}$ were inoculated with a sterile cotton swab dipped in a bacterial suspension in saline (~$10^8$ CFU $ml^{-1}$) and used to streak the surface of the plates. Discs (6.0 mm) containing 0.04 mmole of each siderophore or siderophore-antibiotic conjugate were placed on the surface of agar plates to allow growth inhibition or promotion. Alternatively (Tables 2 and 3), 9-mm agar wells were cut out of the plate and separately filled with 50 μl of test solution in a concentration of 100 μl $ml^{-1}$. Plates were incubated at 37 °C for 24 h. *E. coli* strains DC0 and DC2 (Table 2) were obtained from Richmond [24]. *Pseudomonas aeruginosa* strains K799/WT and K 799/61 were obtained from Zimmermann [25]. *E. coli* K-12 strains were kindly provided by K. Hantke, University of Tübingen, Germany.

Growth promotion assays of separate siderophore components were performed by the procedures described by Reissbrodt [26].

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