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Iron Transport-Mediated Drug Delivery: Practical Syntheses and In Vitro Antibacterial Studies of *Tris*-catecholate Sidero-phore-Aminopenicillin Conjugates Reveals Selectively Potent Anti-Pseudomonal Activity

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Abstract

An artificial *tris*-catecolate siderophore with a tripodal backbone and its conjugates with ampicillin and amoxicillin were synthesized. Both conjugates exhibited significantly enhanced *in vitro* antibacterial activities against Gram-negative species compared to the parent drugs, especially against *P. aeruginosa*. The conjugates appeared to be assimilated by an induced bacterial iron transport process as their activities were inversely related to iron concentration. The easily synthesized *tris*-catecolate siderophore has great potential for future development of various drug conjugates to target antibiotic-resistant Gram-negative bacteria.

The development of antimicrobial resistance among Gram-negative pathogens poses a serious threat to global public health.[1–2] Among several mechanisms of antibiotic resistance in Gram-negative bacteria, a major problem is the low permeability of their outer membrane which serves as a barrier to prevent antibiotic uptake by passive diffusion.[3] Thus, the development of methods to overcome this permeability-mediated resistance is an important therapeutic goal. During the course of infection, most microbes assimilate physiologically essential iron by synthesizing and utilizing high affinity ferric ion chelators, called siderophores. The Fe(III)-siderophore complexes are recognized and active transport through the bacterial cell membrane is intitiated via specific receptors.[4–5] Attachment of antibiotics to siderophores produces potential “Trojan Horse” conjugates that are anticipated to enter pathogenic bacteria via their iron uptake system, thereby circumventing the permeability-mediated drug resistance problem. While studies of both natural and artificial siderophore-drug conjugates (sideromycins) have demonstrated their potential for development of selective antimicrobial agents,[6–7] additional studies are needed. Herein, we report that synthetic conjugates of a relatively simple siderophore mimic and penicillins do, in fact, have selectively potent anti-pseudomonal activity, while the parent antibiotics, themselves, are inactive.

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium that endangers immunocompromised patients, including those with cystic fibrosis (CF),[8] cancer,[9] or AIDS.[10] Once the infection is established it is very difficult to eradicate since *P. aeruginosa* is intrinsically resistant to many of the existing antibiotics including β-lactams. Inadequate penetration through the cell envelope is a significant factor in the resistance of *P. aeruginosa*

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Supporting Information. Experimental procedures, copies of spectral data and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

to β-lactam antibiotics such as ampicillin and amoxicillin.[11–12] The use of drug conjugates of native siderophores of *P. aeruginosa*, namely pyoverdin and pyochelin, to promote active uptake of antibiotics has been explored.[13–17] Conjugates of aminopenicillin with artificial *bis*-, *tris*- and tetrakis-catecholate siderophores based on polyamino carboxylic acids have also been synthesized and found to effectively inhibit growth of *P. aeruginosa*.[18–21]

Like many other types of bacteria, strains of *P. aeruginosa* have developed receptors to recognize and transport Fe(III)-siderophore complexes from other species (xenosiderophores) in order to gain a competitive growth advantage.[22] Enterobactin (Figure 1) is a *tris*-catecholate siderophore primarily found in Gram-negative bacteria, such as *Escherichia coli* and *Salmonella typhimurium*.[23] Enterobactin can also promote iron uptake into *P. aeruginosa* and the uptake is specifically inducible by enterobactin under iron-limiting conditions.[24] Using enterobactin as a shuttle to deliver antibiotics into *P. aeruginosa* and other producing bacteria is an attractive strategy to pursue. However, it is also synthetically challenging as enterobactin has no functionality or site suitable for drug conjugation. Investigation of receptor binding and transport of Fe(III)-enterobactin has revealed that an unsubstituted triscatechol iron center and the coordinated catechol amide groups are essential for recognition as a siderophore while the trilactone backbone is not required and can be replaced with very different molecular scaffolds.[23] Building on this important information, and with the goal of developing practical syntheses of microbe-selective sideromycins, we anticipated that simplified symmetrical siderophore analogs like **2** with linkers remote from the site of iron binding would be suitable for conjugation to antibiotics.

The syntheses of triscatecholate siderophore **2** and its aminopenicillin conjugates (**10** and **11**) are summerized in Scheme 1. As the backbone of the artificial siderophore, tricarbamate **4** was synthesized from trinitrile **3** according to a published procedure[25] with minor modifications. In brief, trinitrile **3** was reduced with borane in THF and the resulting triamine was protected with Boc_2O to give tricarbamate **4**. Reduction of the nitro group in **4** was accomplished with $NaBH_4$ in the presence of a catalytic amount of $NiCl_2$ to give amine **5** in 92% yield. Treatment of amine **5** with methyl succinyl chloride provided a succinate derivative **6**, of which the carboxyl terminus later served as the coupling site with the aminopenicillins. Treatment of **6** with 6 N HCl effected simultaneous removal of the Boc protecting groups and hydrolysis of the methyl ester to give triamino acid **7** as its tris HCl salt in quantitative yield. As demonstrated by Möllmann *et. al.*,[21] using acylated catecholates as the siderophore components has the benefit of not only facilitating synthesis but also preventing pharmacological side effects of the catechol groups. The acylated catecholates are expected to serve as prodrugs to the required iron binding catechols while circumventing potential methylation by a catechol O-methyltransferase (COMT) that would lead to a permanent loss of effective iron binding ability necessary for siderophore activity.[26] Therefore, triamino acid **7** was acylated with 2,3-diacyloxybenzoyl chloride **8** in aqueous sodium bicarbonate to give tripodal siderophore **9**, which can serve as a common intermediate for attachment of various amino or hydroxyl-containing drugs via an amide or ester linkage. As a proof-of-principle study, two aminopencillins, ampicillin and amoxicillin, which by themselves are not active against wild type strains of *P. aeruginosa*, were chosen for the syntheses of conjugates. Thus, siderophore **9** was coupled via its mixed anhydride with ampicillin and amoxicillin to provide conjugates **10** and **11**, respectively.

Conjugates **10** and **11** were first evaluated for their antibacterial ability against a collection of strains of *P. aeruginosa* using the agar well diffusion test. The influence of iron concentration on antibacterial activity was probed as well by conducting assays in iron-rich and iron-deficient media. As shown in Table 1, the parent drugs, ampicillin and amoxicillin, were inactive or only weakly active against wild type strains of *P. aeruginosa* (KW799/wt,

PAO1, Pa4, and Pa6) due to low membrane permeability. This was confirmed by studies of inhibition of the *P. aeruginosa* permeable mutant K799/61 in the assay, where both drugs are highly active as indicated by the large zones of inhibition they induced. Thus, strains of *P. aeruginosa* have the target of these classical antibiotics, but they cannot normally be accessed by passive diffusion. Addition of the siderophore portion to ampicillin or amoxicillin significantly increased their antibiotic activity against wild type *P. aeruginosa* strains except Pa6, especially in iron-deficient media, which reflects the situation *in vivo* in the infected host. As two control samples, siderophore **9** and its conjugate with phenylglycinamide (an analog of **10** without the β-lactam fragment, see supporting information) did not show any inhibitory effect in the agar well diffusion test, clearly indicating that the observed activity of the conjugates **10** and **11** was totally due to the β-lactam warhead.

It is known that the expression of genes that encode siderophore transport systems is induced in bacteria by low iron availability, but repressed when iron is sufficient.[27] Therefore, the augmented activities of conjugates **10** and **11** most likely represent the increased expression of appropriate siderophore receptors at the pathogen cell surface under iron limited conditions. This was better demonstrated in assays with *P. aeruginosa* K648, a strain deficient for its native siderophore pyoverdin and pyochelin biosynthesis. While both conjugates *10* and *11* were inactive against the K648 strain in the iron-rich medium, the activities were largely elevated when tested in the iron-deficient medium, indicating that uptake of the conjugates was induced under iron-restricted conditions. Interestingly, both conjugates **10** and **11** showed no activity against a clinical isolate *P. aeruginosa* Pa6 even under iron-deficient conditions. It is known that Pa6 as well as PAO1 and Pa4 are different from each other in the type of pyoverdins they produce and utilize.[28] It will be of great interest to further investigate the relationship between xenosiderophore usage and native siderophore production in *P. aeruginosa*.

A further investigation of dependence of antibacterial activity on siderophore receptors was conducted with wild type *E. coli* H1443 and mutant H1876 which has defects in its enterobactin-mediated iron transport system (Table 1). Compared to the wild type strain H1443, the activities of both conjugates drastically decreased against the triple mutant H1876 (*fep*A-, *cir*-, *fiu*-, genes that encode the receptors of Fe(III)-enterobactin and its hydrolysis products in *E. coli*), clearly indicating that the conjugates use the enterobactin-mediated iron transport system to penetrate the bacterial outer membrane barrier.

Conjugates **10** and **11** were subjected to further assays to determine their minimum inhibitory concentrations (MIC) in both iron-rich and iron-deficient media (Table 2). Both conjugates exhibited excellent antibacterial activity against the various wild type strains of *P. aeruginosa* in iron-deficient medium, with MICs ranging from 0.05 to 0.39 μM while ampicillin and amoxicillin were generally inactive (>100 μM). The only exception was Pa6, against which both conjugates were inactive, consistent with the observation from the agar diffusion assay. In iron-rich media, the inhibitory activities of the conjugates were largely impaired, further demonstrating that iron concentration of the media regulates the expression of siderophore outer membrane receptors and is thus inversely related to the activity of siderophore-drug conjugates. Conjugates **10** and **11** were also tested against selected strains of *E. coli* and *Klebsiella pneumoniae,* both of which are able to synthesize and utilize enterobactin and its degraded product for iron uptake under iron-limited conditions.[29] When tested against *E. coli,* conjugate **10** showed an 8-fold increase relative to ampicillin while conjugate **11** was almost as equipotent as amoxicillin. In sharp contrast to the activity enhancement observed in *P. aeruginosa* strains, both conjugates were found to be inactive (>100 μM) against *K. pneumoniae.* It appears that *P. aeruginosa, E. coli* and *K. pneumoniae,* either induced or inherently, have different abilities to use triscatecholate **2** as a siderophore

for iron uptake. Another possibility for the high MICs from *K. pneumoniae* is that resistant mutants could developand proliferate in the time course of the assay. Similar phenomena were observed in studies of *E. coli* exposed to catechol-based siderophore-loracarbef conjugates[30] and *P. aeruginosa* exposed to pyoverdin-ampicillin conjugates.[14] Attempts to isolate mutants and studies of bacterial growth kinetics in the presence of the conjugates are currently under investigation.

In summary, an artificial *tris*-catecolate siderophore with a tripodal backbone and its conjugates with ampicillin and amoxicillin were synthesized and studied. Relative to the parent drugs, both conjugates exhibited significantly enhanced *in vitro* antibacterial activities against Gram-negative species, especially against *P. aeruginosa.* The conjugates use energy dependentactive bacterial iron uptake systems to bypass the Gram-negative outer membrane permeability barrier, which accounts for their increased activities. The easily synthesized *tris*-catecolate siderophore can and will be utilized in future development of new drug conjugates that have different cellular targets and modes of action against Gram-negative bacteria.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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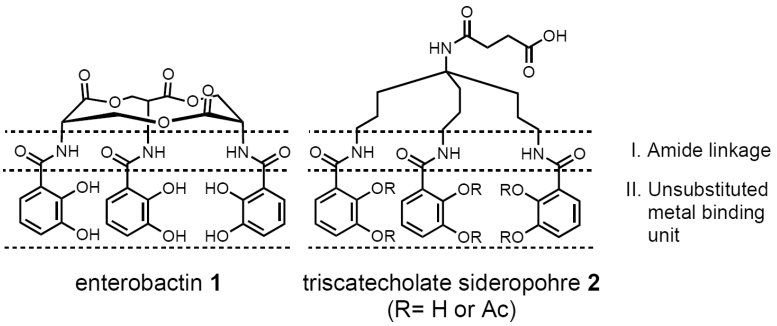
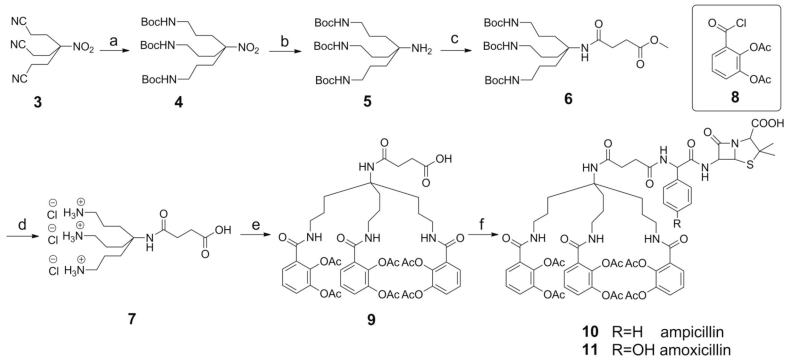


Figure 1.
Structures of enterobactin **1** and triscatecholate siderophore **2**.



Scheme 1. Syntheses of the *tris* -catecholate siderophore and its aminopenicillin conjugates 10 and 11

Reagents and conditions: (a) 1. BH_3-THF, THF, reflux; 2. Boc_2O, Et_3N, MeOH, reflux, 83% for 2 steps; (b) $NiCl_2$, $NaBH_4$, MeOH, sonication, rt, 92%; (c) Methyl succinyl chloride, Et_3N, CH_2Cl_2, 0 °C to rt, 75%; (d) 6 N HCl, reflux, 100%; (e) 2,3-Diacyloxybenzoyl chloride 8, aqueous $NaHCO_3$/THF, 0 °C to rt, 57%; (f) 1. Isobutyl chloroformate, *N*-methyl morpholine, THF, 0 °C; 2. ampicillin or amoxicillin, Et_3N, THF/H_2O, 0 °C to rt, 55% (for **10**), 50% (for **11**).

Table 1

Diameter of growth inhibition zones (mm) in the agar diffusion antibacterial susceptibility assay.

	10[a]		**11**		**Ampicillin**		**Amoxicillin**	
	+Fe	**−Fe**	**+Fe**	**−Fe**	**+Fe**	**−Fe**	**+Fe**	**−Fe**
P. aeruginosa KW799/wt	20	24	25	26		23p	27	18p
P. aeruginosa KW799/61	13P	21	13P	26	40	40	42	39
P. aeruginosa PAO1	0	16	0	19	0	0	0	0
P. aeruginosa Pa4	0	18	0	20	0	h	0	h
P. aeruginosa Pa6	0	0	0	0	0	0	0	0
P. aeruginosa K648 (*pvd−, pch–*)	0	15	0	17	0	0	0	0
E. coli H1443 (*fepA+, cir+, fiu+*)	0	19	0	21	19/23P	16/23p	19/24P	19/23p
E. coliH 1876 (*fepA−, cir−, fiu−*)	0	0	0	14P	0	0	0	0

p, partially clear inhibition zone/colonies in the inhibition zone; P, unclear inhibition zone/many colonies in the inhibition zone; h: indicates only a hint of growth inhibition detectable; Exactly 50 μL of a 0.2 mM solution of each compound dissolved in 1:9 DMSO/MeOH was added to 9 mm wells in agar media (Standard I Nutrient Agar, Serva or Mueller Hinton II Agar, Becton, Dickinson and Company); Inhibition zones were read after incubation at 3 7 °C for 24 h.

[a]Compound **10** was tested at 0.1 mM.

Table 2

In Vitro antibacterial activities of the siderophore-β-lactam conjugates 10 and 11 (MIC in μM).

	10		11		Ampicillin		Amoxcillin	
	+Fe	−Fe	+Fe	−Fe	+Fe	−Fe	+Fe	−Fe
P. aeruginosa KW799/wt	33	0.05	25	0.05	>200	>200	>200	>200
P. aeruginosa KW799/61	12.5	0.067	12.5	0.083	0.52	0.78	0.46	0.39
P. aeruginosa PAO1	50	0.39	50	0.39	>200	>100	>200	>100
P. aeruginosa Pa4	25	0.39	25	0.21	>200	>100	>200	>100
P. aeruginosa Pa6	>200	>200	>200	>200	>200	>200	>200	>200
E.coli ATCC 25922	150	1.56	100	6.25	16.7	12.5	4.17	4.17
K.pneumoniae ATCC 8303 ×68	>200	>100	>200	>100	>200	>100	100	>100

[a]MIC_{90} values (μM) were determined using the broth microdilution method in Mueller-Hinton broth No.2 (MHII) with visual end point analysis according to the CLSI guidelines.

[b]Each compound was tested in triplicate.