Siderophore-fluoroquinolone conjugates containing potential reduction-triggered linkers for drug release: synthesis and antibacterial activity

Cheng Ji and **Marvin J. Miller**[*]

Department of Chemistry and Biochemistry, 251 Nieuwland Science Hall, University of Notre Dame, Notre Dame, Indiana, 46556, United States

Abstract

Syntheses of two siderophore-fluoroquinolone conjugates with a potential reduction triggered linker for drug release are described. The "trimethyl lock" based linker incorporated in the conjugates was designed to be activated by taking advantage of the reductive pathway of bacterial iron metabolism. Electrochemical and LC-MS studies indicated that the linker is thermodynamically reducible by common biological reductants and the expected lactonization proceeds rapidly with concomitant release of the drug. Antibacterial activity assays revealed that conjugates with the reduction-triggered linker were more potent than their counterparts with a stable linker, which suggests that drug release occurs inside bacterial cells.

Keywords

Siderophores; Antibiotics; "Trojan Horse"; Iron transport; Drug delivery; Drug release

Introduction

The ever-growing bacterial resistance to antibiotics is emerging as a serious problem that poses great threat to public health (Boucher et al. 2009; Spellberg et al. 2008). Not only is the situation worsened by the lack of new antibiotics being created, but the increasing use and misuse of existing antibiotics are also significant contributors that promote resistance (Levy and Marshall 2004; Projan 2003). Of several modes of resistance mechanisms, the inability of some antibiotics to enter the cell via size-restricted porins is especially significant (Fernandez and Hancock 2012; Pages et al. 2008). Thus, there is always a dire need for improved drug-delivery processes to circumvent the permeability problem.

The idea of conjugation antibiotics to bacterial iron chelators, known as siderophores, to facilitate transport of antibiotics into the bacterial cell has attracted considerable attention (Ji et al. 2012a; Mislin and Schalk 2014; Möllmann et al. 2009; Page 2013; Zheng and Nolan 2014). Under iron-limited conditions, bacteria synthesize and secrete siderophores for

[*]To whom correspondence should be addressed: mmiller1@nd.edu, Telephone: 574 631 7571, Fax: 574 631 6652.

extracellular solubilization of otherwise insoluble iron. Fe(III)-siderophore complexes are recognized by specific bacterial membrane receptors, and then translocated across the cell membrane in the presence of certain ATP-dependent active transporters. Once inside the cell, the iron is released from the Fe(III)-siderophore complex for further use, usually via a reductive mechanism, to give Fe(II), to which siderophores bind much more weakly (Harrington and Crumbliss 2009). Since bacterial iron acquisition largely relies on siderophore pathways, especially under iron-limited growth conditions during infection of a host, the use of siderophore-drug conjugates has become an attractive way to bypass bacterial permeability barriers and thus improve the efficacy of the drug and reduce the chances of resistance development. Several naturally occurring sideromycins, siderophore-antibiotics, such as albomycins (Braun et al. 1983; Braun et al. 2009) and salmycins (Vértesy et al. 1995; Wencewicz and Miller 2013; Wencewicz et al. 2009), have been proven to use specific iron transport systems to enter targeted bacteria, and consequently are very effective natural antibacterial agents (Figure 1). Extensive studies of albomycins have revealed that the drug component, a toxic thionucleoside, is enzymatically released after the conjugate is transported (Braun et al. 1983). Salmycins, albeit less studied, are proposed to release the constituent aminoglycoside antibiotic via an intramolecular cyclization process triggered by iron reduction (Roosenberg and Miller 2000; Wencewicz et al. 2013; Wencewicz et al. 2009). In both cases, an intracellular drug release process, initiated after active assimilation by bacteria, is believed to play a key role for the activity of the conjugates.

Inspired by these natural examples, a series of synthetic siderophore-drug conjugates, properly designed to mimic natural siderophores, with potential drug release linkers have been reported. For example, pyoverdin and pyochelin, the two native siderophores of *Pseudomonas aeruginosa*, were conjugated to fluoroquinolones with both stable and hydrolysable methylenedioxy linkers to target the corresponding siderophore producing strain (Hennard et al. 2001; Noël et al. 2011; Rivault et al. 2007). Biological assays revealed that conjugates with a labile linker, that could induce release of the drug, displayed greatest activities. However, the methylenedioxy linker was non-specifically hydrolysable which could lead to premature release of the antibiotic in the extracellular media. Therefore, novel linkers with drug release function that could be selectively triggered inside microbial cells need to be further explored.

We recently reported use of a "trimethyl lock" induced lactonization triggered by the action of enzymes such as esterases/phosphatases for the drug release process in the design and syntheses of new siderophore-drug conjugates (Ji and Miller 2012). The chemical nature of the trimethyl lock system, **1**, is an *o*-hydroxycinnamic acid derivative in which unfavorable steric interactions between three methyl groups encourage rapid lactonization to form a hydrocoumarin (Scheme 1) (Levine and Raines 2012). The esterases/phosphatases triggered linkers, similar to the pyoverdin/pyochelin-fluoroquinolone examples mentioned above, also have the disadvantage of being hydrolyzed by some extracellular hydrolases secreted into the culture medium by bacteria. In contrast to the extracellular esterases/phosphatases which are commonly seen, ferric reductases, the enzymes responsible for iron release from Fe(III)-siderophore complexes, are found predominantly inside bacterial cells with few exceptions

(Schröder et al. 2003). Moreover, most ferric reductases lack substrate specificity (Miethke and Marahiel 2007; Schröder et al. 2003), suggesting that they may be suitable generalized targets for the development of drug release linkers incorporated in siderophore-drug conjugates. We therefore reasoned that the "trimethyl lock" **1** could provide an attractive reduction triggered linker which could release certain antibiotics in a controlled manner. In this approach, a quinone derivative was used as the precursor to the "trimethyl lock" structure, and the to-be-released drug was condensed with the carboxyl group. Upon active transport of the conjugate **2** into the microbe, it was expected that during the Fe(III) reduction process, the constituent quinone linker might also be reduced by hydride donors such as flavin or NAD(P)H used to reduce Fe(III) to Fe(II) for bacterial iron incorporation. The resulting hydroquinone **3** with the "trimethyl lock" structure only has a transient lifetime, and was anticipated to rapidly cyclize to form lactone **5** with concomitant release of the drug.

Two different siderophores, desferrioxamine B and a previously reported biscatecholate-monohydroxamate mixed-ligand siderophore (Scheme 2) were selected for appropriate modification to test our reduction triggered release design. Desferrioxamine B, a hydroxamate-containing siderophore, is utilized by a variety of Gram-positive and Gram-negative bacteria and some fungi (Müller and Raymond 1984). The artificial mixed-ligand siderophore containing both catecholate and hydroxamate ligands was designed to use multiple siderophore recognition processes to initiate active transport through the outer membrane of various Gram-negative species (Diarra et al. 1996; Ghosh et al. 1996; Wencewicz and Miller 2013). The drug linked to the siderophores was ciprofloxacin, a broad-spectrum fluoroquinolone antibiotic that targets bacterial DNA gyrases and topoisomerases (Mitscher 2005). Despite the aforementioned concerns about development of antibiotic resistance, ciprofloxacin is still active against many strains of pathogenic bacteria. However, previous attempts to demonstrate activity when conjugated to siderophores were unsuccessful apparently because, in contrast to β-lactam siderophore conjugates (Ji et al. 2012b; Möllmann et al. 2009; Zheng and Nolan 2014), attachment of the relatively large siderophore to ciprofloxacin interferes with target recognition of the modified fluoroquinolone (Wencewicz et al. 2009). Thus, herein we describe the syntheses of two siderophore-ciprofloxacin conjugates with and without the potential reduction triggered "trimethyl lock" linker, and a preliminary study of their biological activity. With the aim of assessing the feasibility of using the linker in siderophore-drug conjugates, electrochemical and LC-MS studies of linker activation are also reported. The results indicate that ciprofloxacin conjugates with the newly designed reduction triggered linker are notably more active than those without the releasable linker.

Results and discussion

Design and syntheses of conjugates

To initiate the syntheses of the conjugates, an effective linking strategy to attach siderophores to the reduction triggered linker had to be identified. We decided to incorporate a urea linkage because of its appreciable stability and synthetic accessibility. Thus, synthesis of the desferrioxamine conjugate (Scheme 3) started with bromo acid **6** which was

conveniently converted to its methyl ester **7** using thionyl chloride in methanol. Nucleophilic substitution of **7** with NaN_3 in aqueous methanol provided azide **8** in excellent yield. The azide was converted to vinylogous amide **9** in 66% yield by a triphenylphosphine-mediated reduction. Treatment of **9** with excess phosgene in toluene afforded an unstable intermediate with a proposed carbamoyl chloride structure **10a**. Interestingly, proton and carbon NMR spectra suggested that the intermediate predominantly existed as the cyclic chloro oxazoline structure **10b**, probably due to the electron deficiency of the quinone unit, which induces the intramolecular addition. Reaction of desferrioxamine with intermediate **10** in the presence of triethylamine gave ester **11** with the desired urea incorporated between the siderophore and the linker. Subsequent hydrolysis of the ester and coupling of the resulting acid **12** with ciprofloxacin using EDC and HOBt produced the desferrioxamine-ciprofloxacin conjugate **13** in moderate yield.

Synthesis of the mixed-ligand siderophore-ciprofloxacin conjugate, **23**, started with preparation of the biscatechol fragment **16** (Scheme 4). Selective acylation of the primary amino groups of spermidine with 2,3-diallyloxybenzoic acid **14** was achieved by using *N,N*-carbonyldiimidazole (CDI) as the activating reagent to give bis-acylated spermidine **15** in 87% yield. Treatment of amine **15** with succinic anhydride and a catalytic amount of DMAP generated acid **16** in 74% yield.

Since the carboxylic acid terminus in the mixed-ligand siderophore was not compatible with the chemistry of forming a urea linkage, an additional diamine spacer was needed. Therefore, using the same phosgene-based methodology, *N*-1-Boc-1,4-butanediamine was coupled with vinylogous amide **9** via a urea linkage, providing compound **17** in 91% yield (Scheme 5). After removal of the Boc protecting group from **17** with trifluoroacetic acid, the resulting amine was coupled with acid **19**, the hydroxamate component of the mixed-ligand siderophore, to afford compound **20** in 50% yield. Again, the Boc protecting group in compound **20** was cleaved with TFA and the resulting amine was coupled with acid **16**, to yield methyl ester **21**. Subsequent hydrolysis of the ester and coupling of the resulting acid with *O*-allylciprofloxacin afforded the fully protected conjugate **22**. Pd(0)-mediated global deallylation of **22** furnished the mixed-ligand siderophore-ciprofloxacin conjugate **23** in moderate yield.

In parallel, we also synthesized desferrioxamine and mixed-ligand siderophore ciprofloxacin conjugates with stable succinic linkers as two controls to be included in the biological assays (Scheme 6).

Electrochemical and LC-MS studies

Since our intent was to have the quinone "trimethyl lock" linker in conjugates **13** and **23** activated by reduction, determination of the reduction potential of the quinone "trimethyl lock" linker was important. Two model compounds **11Fe** and **11Ga** were synthesized from compound **11** with the corresponding metal salts and subjected to cyclic voltammetry studies. The voltammograms of both complexes (Figure 2) showed only a single irreversible reduction peak for each, consistent with the literature report for other quinone "trimethyl lock" analogs (Mendoza et al. 2012; Ong et al. 2008). At pH 7, **11Fe** and **11Ga** displayed

identical reduction peak potentials ($E_p = -412$ mV vs. SCE or -168 mV vs. NHE), demonstrating that they are thermodynamically reducible by common biological reductants such as NAD(P)H (Harrington and Crumbliss 2009). Complex **11Ga**, in which the metal center is redox-inert within the experimental potential window, has the same reduction potential as **11Fe**, suggesting that the measured potential is solely attributed to quinone reduction. The absence of an Fe(III) reduction wave of **11Fe** also indicated that the reduction of the quinone unit is thermodynamically easier than reduction of the siderophore-chelated Fe(III).

In order to examine whether the quinone "trimethyl lock" in the synthesized conjugates would induce release of the antibiotic under mild reduction conditions, conjugate **13** was treated with a ~20-fold excess of sodium dithionite ($Na_2S_2O_4$) and the mixture was incubated at 37 °C. The reaction progress was monitored by LC-MS (Figure 3). HPLC chromatography and ESI-MS analyses showed that, after 5 min, the peak of the conjugate $[(M+H)^+=1152.37]$ was largely diminished while the hydroquinone intermediate $[(M+H)^+=1154.22]$, was the predominant species. Meanwhile, as proposed, the lactone **26** $[(M+H)^+=822.85]$, and the released ciprofloxacin $[(M+H)^+=332.18]$ also started to appear and complete release of the ciprofloxacin was achieved after 25 min. This result clearly showed that ciprofloxacin could be cleaved within a short time range after the linker is activated by reduction. The combined results from the electrochemical and chemical reduction studies demonstrated the feasibility of using the quinone "trimethyl lock" as a reduction triggered drug release linker in siderophore-antibiotic conjugates.

Biological studies

The siderophore-ciprofloxacin conjugates **13** and **23** were evaluated for their ability to inhibit the growth of bacteria using standard agar diffusion assays. Representative Gram-positive strains included *Bacillus subtilis, Staphylococcus aureus* and *Micrococcus luteus*. The Gram-negative strains studied included *Pseudomonas aeruginosa* (a wild type K799/wt and a penetration mutant strain K799/61), *Escherichia coli*, and *Acinetobacter baumannii*. The parent drug ciprofloxacin and conjugates **24** and **25** each with a stable succinic linker were also included in the assay as controls.

The results of assays for antibacterial activity of the four conjugates are summarized in Table 1. At the same concentration (0.2 mM), conjugates with the quinone "trimethyl lock" linker (**13** and **23**) were more active than their counterparts (**24** and **25**, respectively) with a non-releasable succinic linker in all cases, as indicated by the larger inhibition zones (Figure 4). A further kinetic study also showed that conjugate **13** with the reductive sensitive linker was more potent than **24** without the reducible linker at the same concentration (0.1 μM) in inhibiting growth of *B. subtilis* (Figure 5). It is reasonable to assume that, if recognized by the microbial siderophore receptor, **13** and **24, 23** and **25** are transported at similar rates, as they possess similar molecular shapes and topology around the iron coordination site. The results therefore suggest that the "trimethyl lock" facilitated drug release process occurred after the conjugate was transported. Also notable is that mixed-ligand conjugates **23** and **25** showed a narrower spectrum of activity (Gram-negative) relative to the desferrioxamine conjugates, which is consistent with previous observations and suggests bacterial outer

membrane recognition selectivity as expected (Diarra et al. 1996; Ghosh et al. 1996; Wencewicz and Miller 2013). Although the anticipated drug release process might happen, all four conjugates are less active relative to the parent drug ciprofloxacin. This is possibly because 1) bacterial recognition of the chemically modified siderophore is affected; 2) activation efficiency of the linker is low inside the bacteria. Further investigation by attaching siderophores with better outer membrane receptor recognition to the linker could provide conjugates with enhanced activities. An artificial triscatecholate sideophore with linker attachment site remote from the site of iron binding is a promising candidate (Ji et al. 2012b). Improvement of the linker's activation efficiency might be achieved by introducing electron-withdrawing groups to the quinone moiety (e.g. replacing -CH_3 with -CF_3) to further increase its electron deficiency although it could be more synthetically challenging.

Conclusions

Two siderophore-ciprofloxacin conjugates with a potential reduction triggered linker for drug release were synthesized. The linker incorporated in the conjugates was designed to be activated by taking advantage of the reductive pathway of bacterial iron metabolism. Upon reduction, a rapid "trimethyl lock" facilitated lactonization was anticipated to release the antibiotic from the conjugate. Electrochemical studies indicated that the quinone moiety in the linker is thermodynamically reducible by common biological reductants and the expected lactonization is rapid and induces concomitant release of the drug as supported by LC-MS analysis. Furthermore, conjugates with the reduction triggered linker were more potent in antibacterial assays relative to their counterparts with the stable linker, which suggests the drug release occurred inside the bacterial cells. However, the activity of conjugates **13** and **23** was weaker than that of the parent drug ciprofloxacin, suggesting that either the siderophore recognition or linker activation efficiency or both factors require further optimization. Once optimized, the efficient synthetic strategy developed for attaching siderophores to the quinone "trimethyl lock" linker via a urea linkage can and will be used in the development of new generation of siderophore-drug conjugates with a drug release linker.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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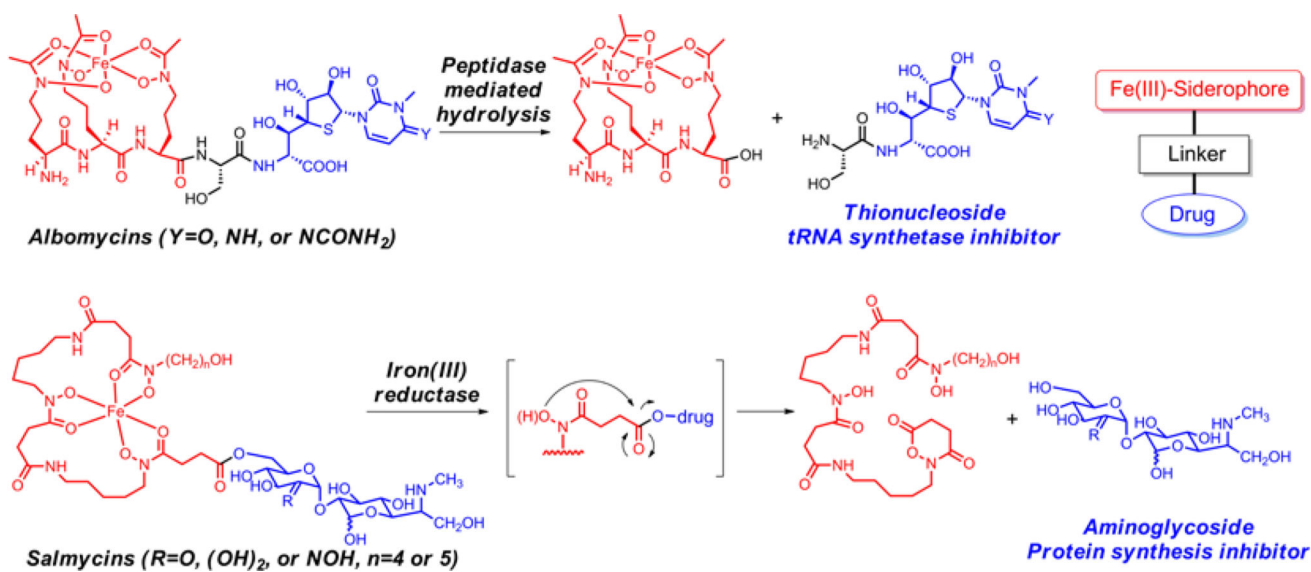**Figure 1.**
General sideromycins (siderophore drug conjugates) and two naturally occurring examples, albomycin and salmycin.
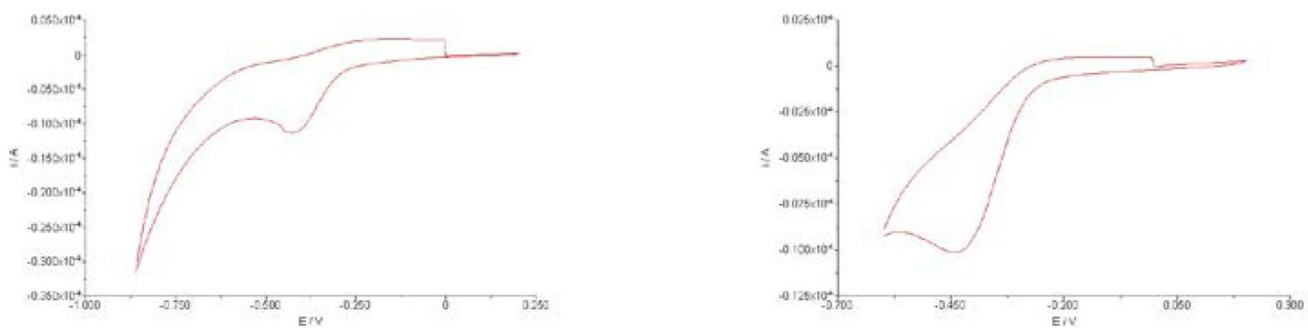
Figure 2.
Cyclic voltammograms of **11Fe** (left, 2 mM) and **11Ga** (right, 2 mM). Conditions: platinum working electrode; platinum wire auxiliary electrode; saturated calomel electrode (SCE) reference electrode; 0.1 M KCl as the background electrolyte in 0.05 M phosphate buffer pH 7.0 at ambient temperature; scan rate = 100 mV s^{-1}.
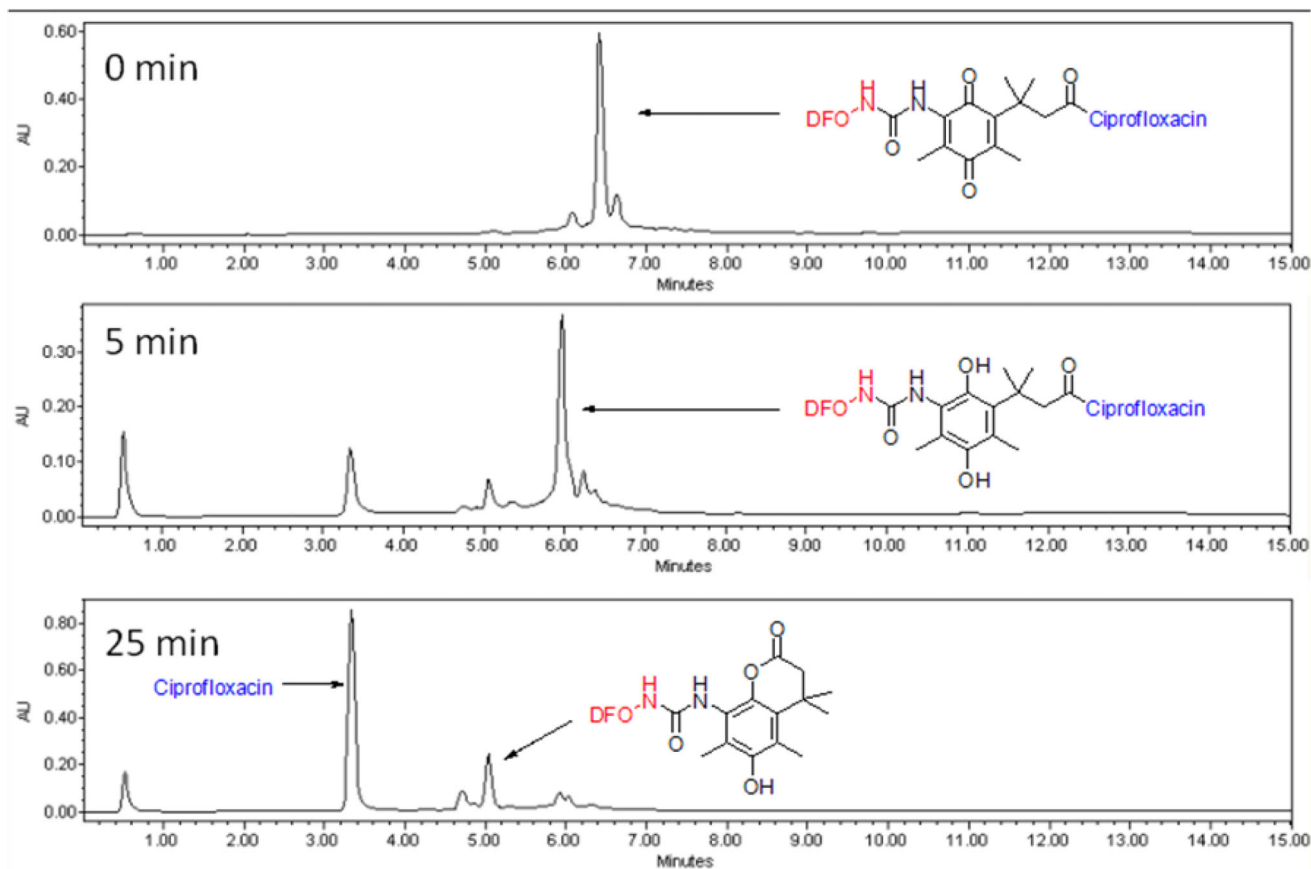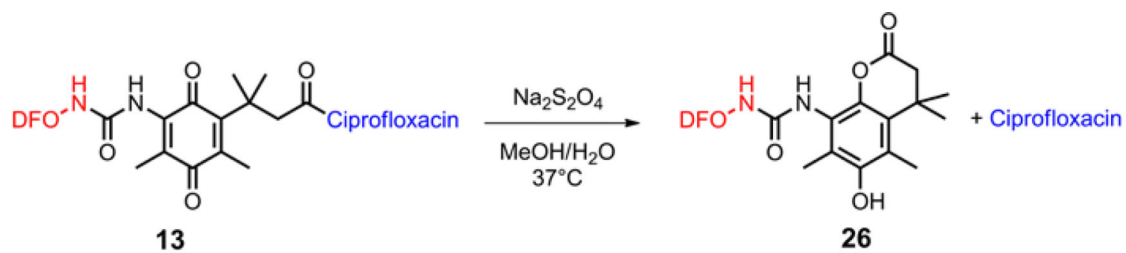
Figure 3.
LC-MS study of the chemical reduction of conjugate **13**
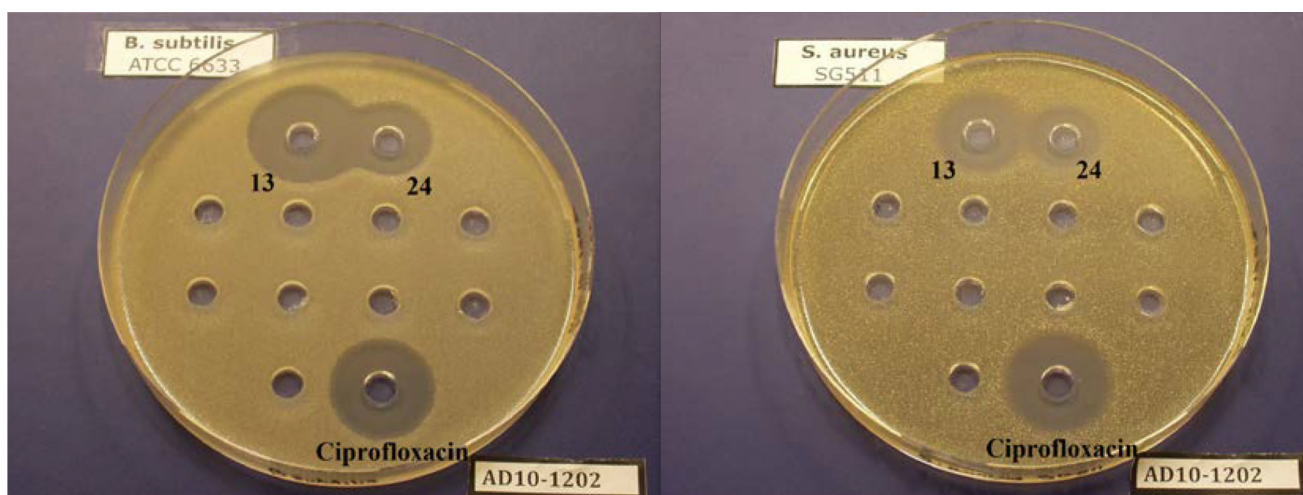
Figure 4.
Agar diffusion assay for conjugates **13** and **24** tested against *B. subtilis* ATCC 6633 (left)
and *S. aureus* SG511 (right).
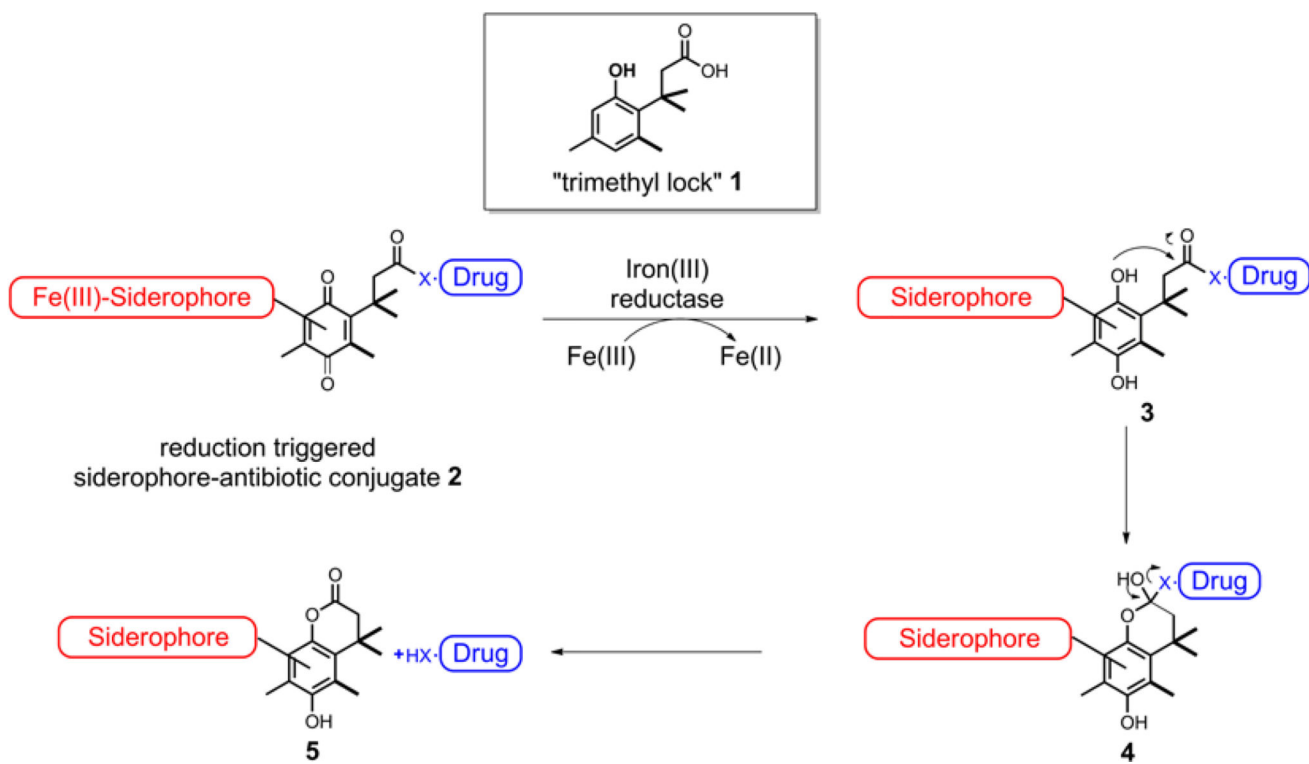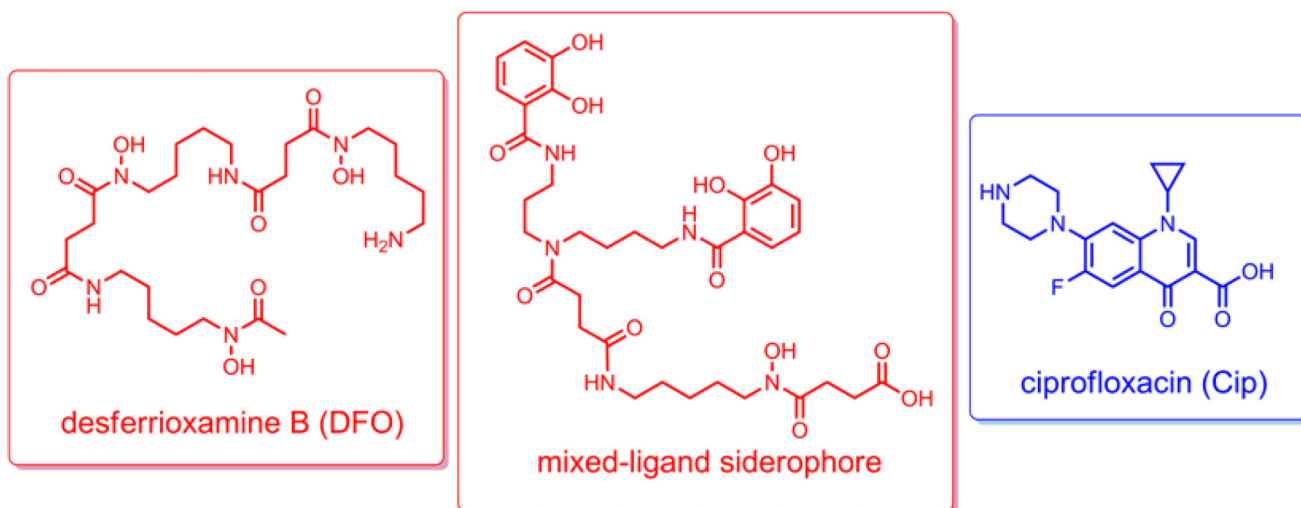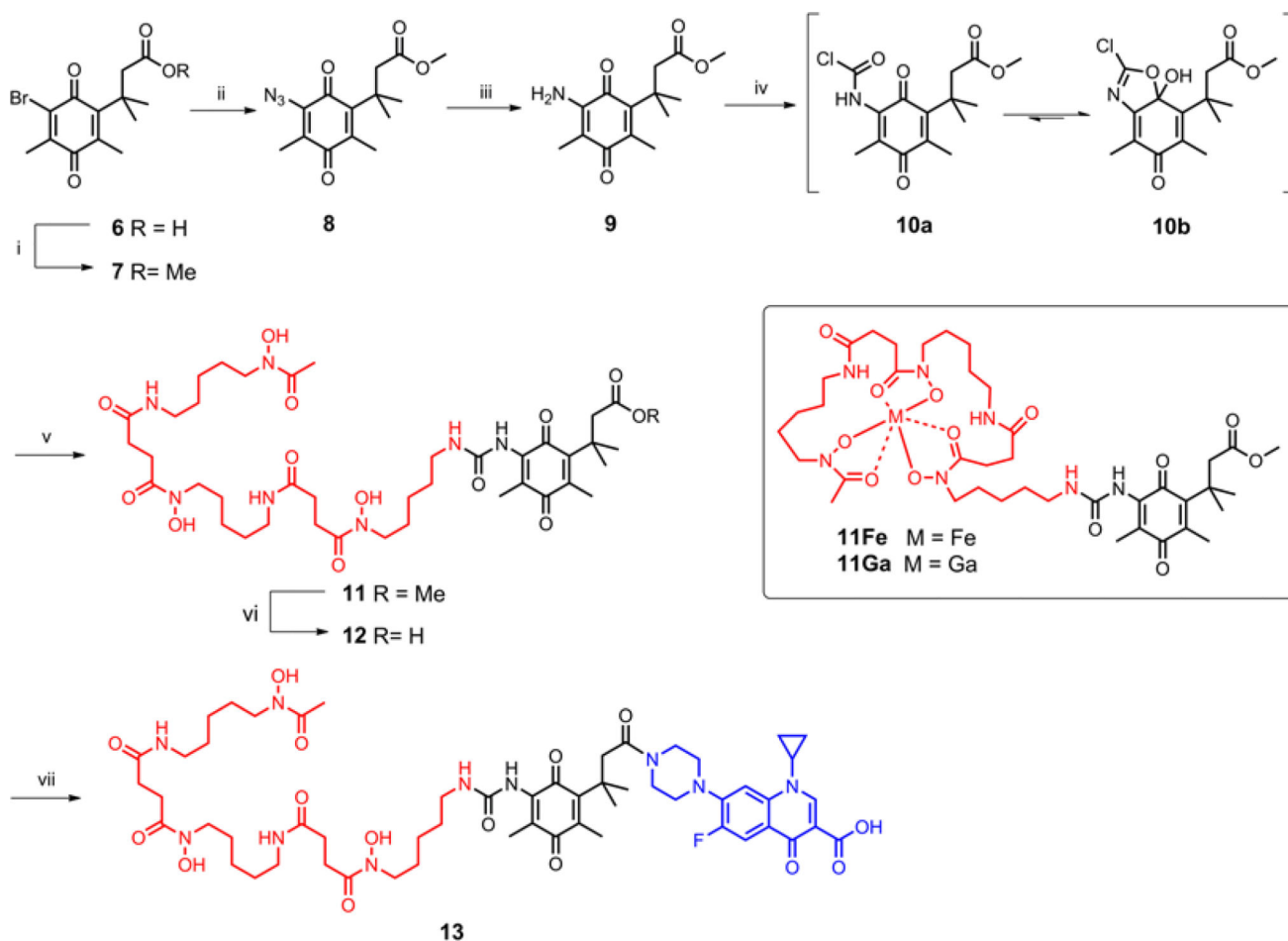
Growth curve of *B. subtilis* ATCC 6633
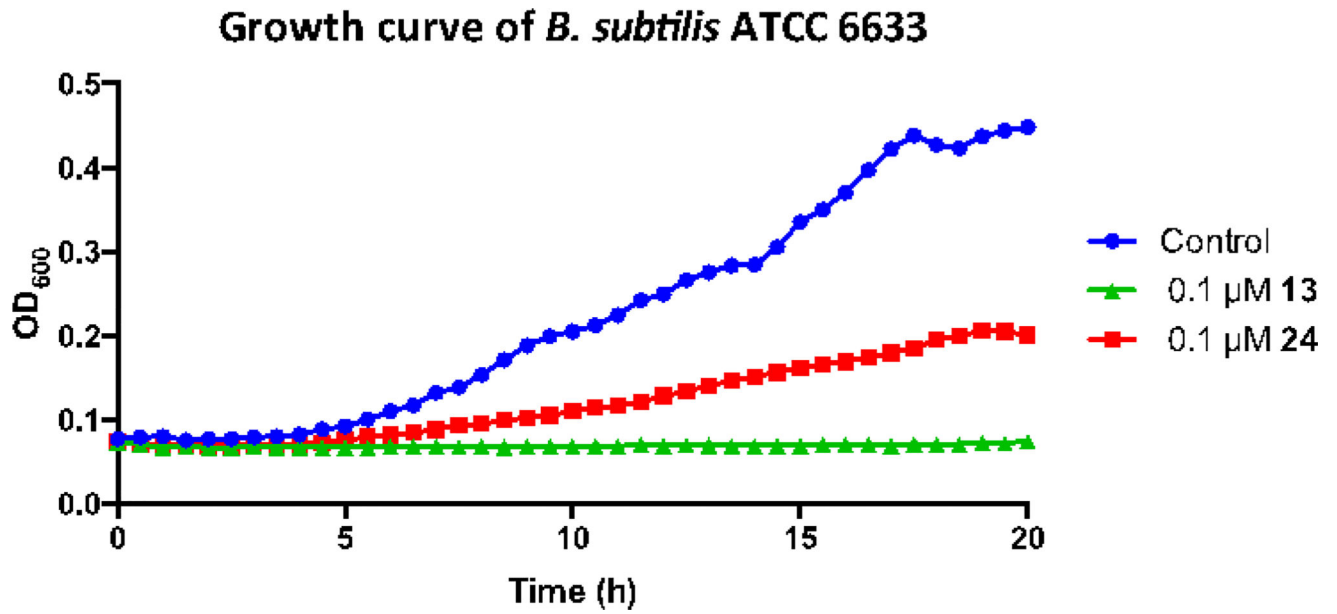
Figure 5.
Growth curve of *B. subtilis* in the presence of conjugates **13** and **24** under iron deficient conditions.
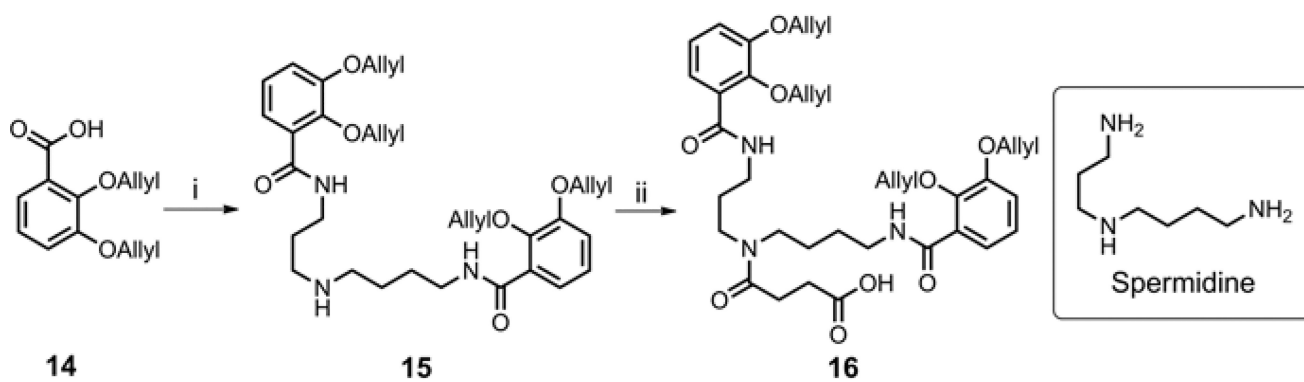
Scheme 1.
"trimethyl lock" derived siderophore-antibiotic conjugates.

Scheme 2.
Structures of desferrioxamine B, biscatecholate-monohydroxamate mixed-ligand siderophore and ciprofloxacin.
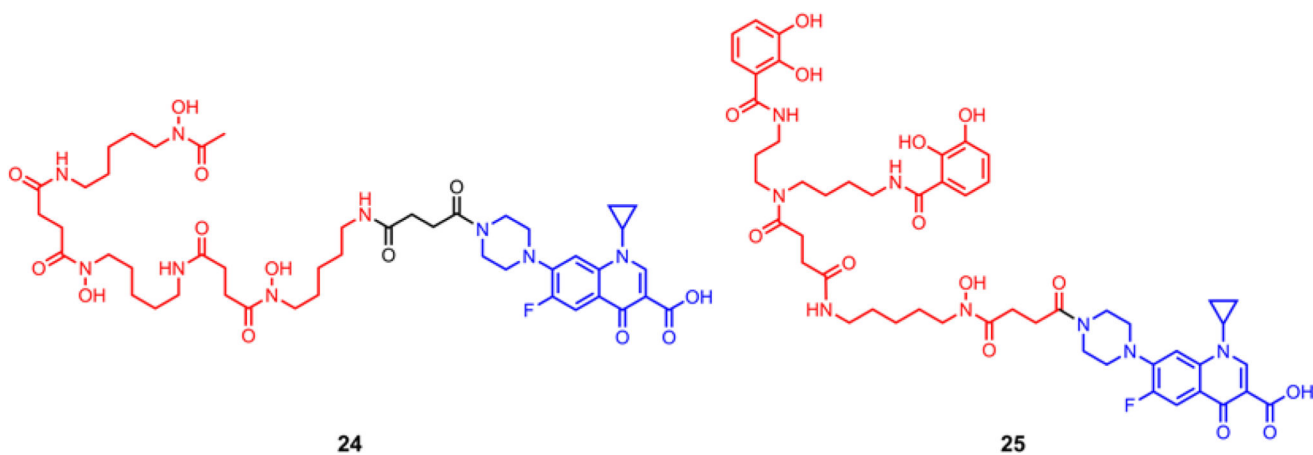
Scheme 3.
Synthesis of reduction triggered desferrioxamine-ciprofloxacin conjugate **13**. Reagents and conditions: (i) SOCl$_2$, MeOH, reflux, 77%; (ii) NaN$_3$, MeOH-H$_2$O, rt, 91%; (iii) 1. PPh$_3$, CH$_2$Cl$_2$, rt; 2. AcOH, THF-H$_2$O, reflux, 66%; (iv) 1. COCl$_2$, toluene, rt; (v) desferrioxamine mesylate, Et$_3$N, DMF, 0°C-rt, 74% for 2 steps; (vi) LiOH, MeOH-H$_2$O, rt, 90%; (vii) ciprofloxacin, EDC, HOBt, Et$_3$N, DMAP, DMF, 0°C-rt, 60%

Scheme 4.
Synthesis of the protected biscatechol siderophore fragment **16**. Reagents and conditions: (i) CDI, spermidine, CH$_2$Cl$_2$, 0°C-rt, 87%; (ii) succinic anhydride, DMAP, pyridine, 95°C, 74%
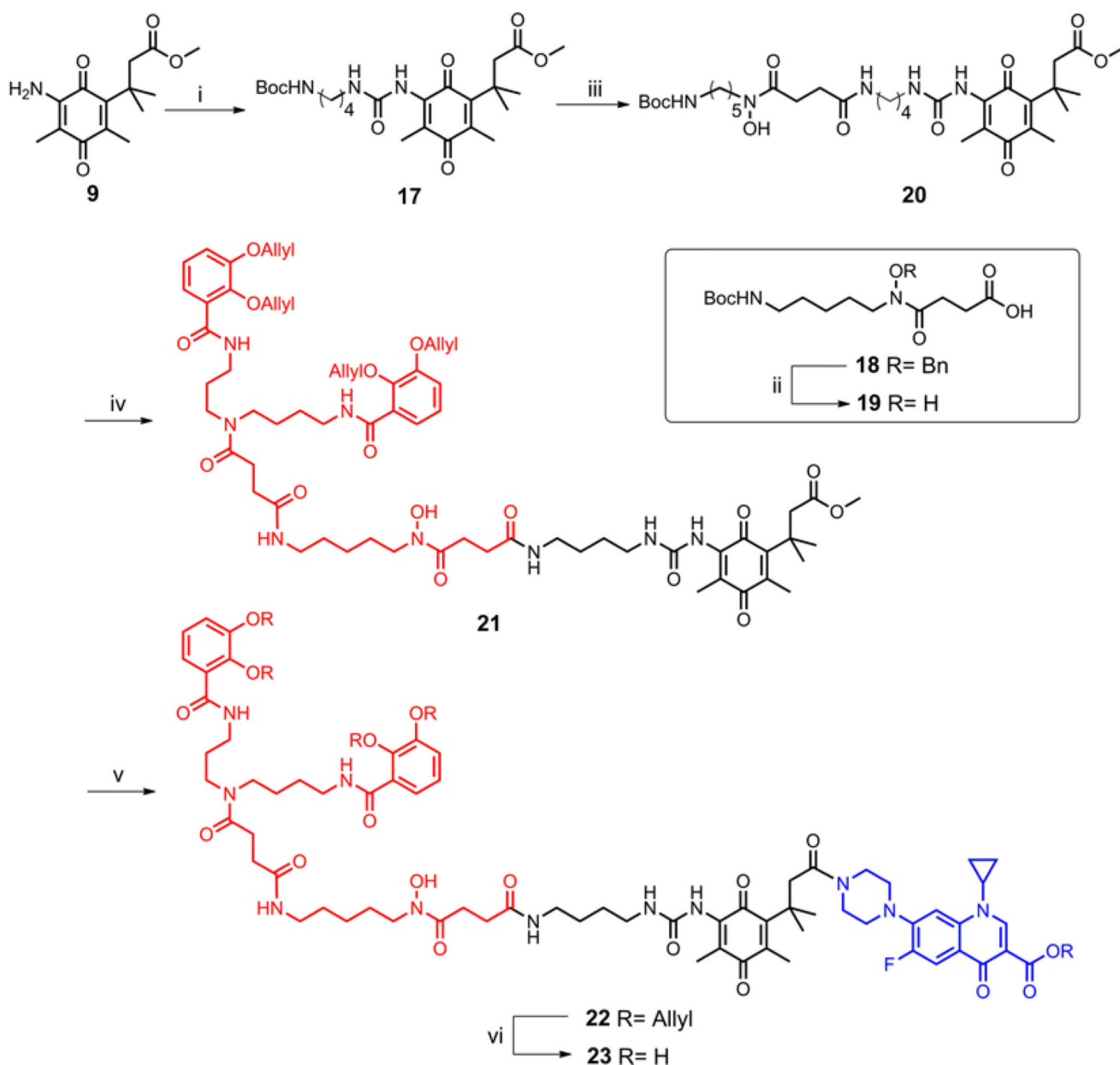
Scheme 5.
Synthesis of mixed-ligand siderophore-ciprofloxacin conjugate **23**. Reagents and conditions:
(i) 1. COCl$_2$, toluene, rt; 2. *N*-1-Boc-1,4-butanediamine, Et$_3$N, CH$_2$Cl$_2$, 0°C-rt, 91%; (ii) H$_2$,
Pd/C, MeOH, rt, 99%; (iii) 1. TFA, CH$_2$Cl$_2$, 0°C-rt; 2. Acid **19**, EDC, HOBt, Et$_3$N, DMAP,
CH$_2$Cl$_2$, 0°C-rt, 50%; (iv) 1. TFA, CH$_2$Cl$_2$, 0°C-rt; 2. Acid **16**, EDC, HOBt, Et$_3$N, DMAP,
CH$_2$Cl$_2$, 0°C-rt, 43%; (v) 1. LiOH, MeOH-H$_2$O, 0°C-rt; 2. *O*-Allylciprofloxacin, EDC,
HOBt, Et$_3$N, DMAP, CH$_2$Cl$_2$, 0°C-rt, 55%; (vi) Pd(PPh$_3$)$_4$, sodium benzenesulfinate,
AcOH, THF-MeOH, rt, 48%.

Scheme 6.
Siderophore-ciprofloxacin conjugates (desferrioxamine **24** and mixed-ligand siderophore **25**) with a stable linker.

Table 1

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

| Compds | Gram-positive bacteria | | | Gram-negative bacteria | | | |
| | B. subtilis | S. aureus | M. luteus | P. aeruginosa | | E. coli | A. baumannii |
	ATCC 6633	SG 511	ATCC 10240	K799/wt	K799/61	X580	ATCC 17961[a]
Ciprofloxacin	28[c]	28[b]	12P[b]	21[c]	24/32p[c]	31[d]	15p[b]
13	34	25	14	27/31P	31/46P	34/38P	NT
24	26	22	0	18P	19/26P	32/39P	NT
23	20/26p	13P	0	19	19/26p	27	20
25	0	0	0	14	0	21	18

p, partially clear inhibition zone/colonies in the inhibition zone; P, unclear inhibition zone/many colonies in the inhibition zone; NT, not tested

Exactly 50 μL of a 0.2 mM solution of each compound dissolved in 1:9 DMSO/MeOH was filled in 9 mm wells in agar media (Mueller Hinton II). Inhibition zones read after incubation at 37 °C for 24 h.

[a] A. baumannii was tested in the presence of 100 μM of 2,2′-bipyridine.

[b,c,d] Ciprofloxacin was tested at (b) 5 μg/mL, (c) 1.66 μg/mL, (d) 0.33 μg/mL in H_2O.