Syntheses of Siderophore–Drug Conjugates Using a Convergent Thiol–Maleimide System

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Ⓢ Supporting Information

ABSTRACT: Three siderophore–drug conjugates (sideromycins) were synthesized by preparation of a maleimide linked derivative of the siderophore desferrioxamine B and reacting the corresponding Ga^{3+}-complex with freshly prepared thiol-containing antibiotics: loracarbef, ciprofloxacin, and nadifloxacin. The conjugates and their synthetic precursors were tested against a broad panel of bacteria and were found to display Gram-positive selective, growth inhibitory activity (μM), indicating that this approach is suitable for the convergent syntheses and screening of novel sideromycins.

KEYWORDS: Antibiotic, drug-conjugate, siderophore
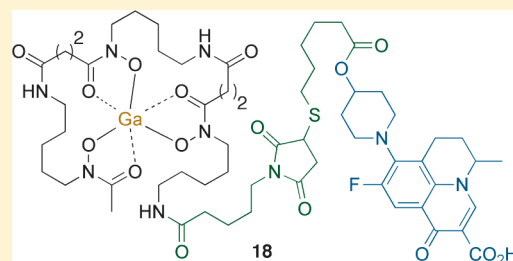
Siderophores are high affinity, Fe^{3+}-selective chelators produced by bacteria and fungi. Under iron deficient conditions, these molecules are secreted into the environment where they gather essential iron from diverse sources and deliver it to the producing organism.[1−6] The biological importance of iron has prompted bacteria to develop means for the recognition of exogenous siderophores in order to gain a competitive advantage, which in turn has led to the biosyntheses of naturally occurring siderophore-drug conjugates (sideromycins) such as the albomycins,[7,8] salmycins,[9] and microcins.[10,11] These conjugates are modified siderophores containing an antibiotic portion that is delivered into a microorganism upon acquisition of the siderophore–Fe^{3+} complex.[17−19] The inability to handle the actively transported drug might lead to cell death, thus combating siderophore thievery.

Exploiting iron uptake as a biological target has been an exciting field for the study and development of new methods that allow the transport of antibacterial agents into pathogenic cells, and a variety of synthetic sideromycins have been reported in the literature.[12−19] One of the advantages of using these chelators as carriers is the often selective recognition of ferri-siderophores by microorganisms. Drug-conjugates of these molecules have been demonstrated to be highly potent antibiotic agents, stressing the value of their syntheses and study.

The assembly of these molecules, however, is a chemically challenging effort that typically requires manipulation of protecting groups and tailoring of conditions suitable to the drug or compound of interest. With the limited availability of some siderophores, it is highly desirable to minimize the number of steps needed to accomplish the conjugation, and thus, a convergent approach would provide a new tool toward these fascinating molecules. An example of versatile side-rophore functionalization was the report by Koizumi and collaborators on the conjugation of desferrioxamine B (DfoB) with monoclonal antibody OST7 (IgG1) to deliver radio-gallium ^{67}Ga into tumors of mice,[20] for scintigraphic imaging, by employing three different strategies: (1) using glutaraldehyde as a linker, (2) acylation of both building blocks with 2-pyridyl disulfide and subsequent condensation via a disulfide bond, and (3) linking a maleimide to DfoB and conjugation with the thiol-containing antibody (**1**, Figure 1). In order to optimize the properties of the conjugates and reduce radionuclide localization within nontargeted organs, a metab-
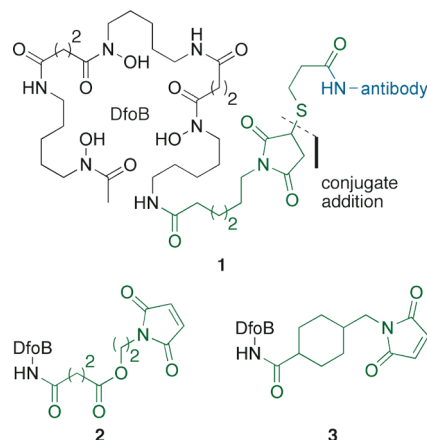

Figure 1. Use of maleimide linkers for the functionalization of DfoB.[20−22]

olizable maleimide linker (2) was later reported,[21] indicating that specific characteristics of the conjugates could be modified by the linker of choice. Tinianow also reported the use of a maleimide-containing DfoB (3) to deliver ^{89}Zr using the monoclonal antibody thiotrastuzumab, for positron emission tomography (PET) imaging.[22]

We are interested in the assembly of novel sideromycins through the previously described thiol-maleimide strategy, utilizing different siderophores. In this work, we explore the scope and limitations of this approach by conjugation of functionalized DfoB 12 (Figure 2) and thiol-containing antibiotics (e.g., 14) to afford siderophore−drug conjugates such as Ga-DfoB-ciprofloxacin 17.

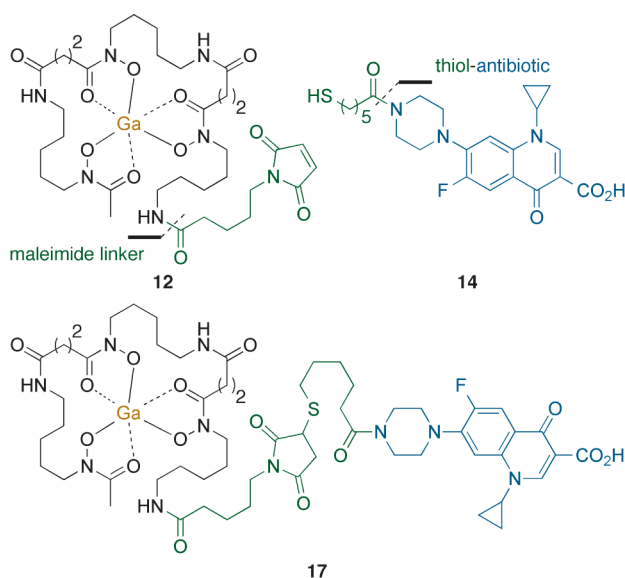**Figure 2.** Use of maleimide−DfoB 12 and thiol−ciprofloxacin 14 in the synthesis of sideromycin 17.

The attachment of a thiol-containing linker to the drugs of interest: ampicillin, loracarbef, ciprofloxacin, and nadifloxacin (representing drugs with different targets) was envisioned by the use of S-trityl protected acid 4[23,24] (Scheme 1), which was obtained from the condensation between 6-bromohexanoic acid and S-triphenylmethane thiol in the presence of DBU to afford the desired intermediate in 41% yield. Activated ester 5 was obtained from coupling fragment 4 with NHS using EDC·HCl. The resulting material could be purified by silica gel chromatography but was often found to be pure enough for

further use after aqueous workup. Ampicillin was acylated by reaction with 5 in THF/H$_2$O/Et$_3$N to give the desired adduct, 6, in 30% yield. Optimized conditions allowed the isolation of drug-derivatives loracarbef 7 (56% yield), ciprofloxacin 8 (51% yield), and nadifloxacin 9 (24% yield). The last was obtained from the EDC·HCl-mediated condensation between nadifloxacin and synthetic precursor 4.

The maleimide-functionalized siderophore was obtained by reacting commercially available DfoB mesylate salt and freshly prepared NHS ester 10 under buffered conditions to give 11 in 50% yield (Scheme 2). Ga^{3+}-complex 12 was isolated after refluxing the previous intermediate with Ga(acac)$_3$ in MeOH in 84% yield. We decided to use gallium as a protecting group for the hydroxamic chelators in DfoB. We were also interested in the potential role of gallium within our conjugates, since growth inhibition of *Pseudomonas aeruginosa* has been reported in the literature with Ga(NO$_3$)$_3$,[25] GaCl$_3$, and Ga-DfoB.[26]

The removal of the S-trityl protecting group from intermediates 7−9 was achieved with a mixture of TFA (20% v/v) and iPr$_3$SiH (2% v/v) in deoxygenated CH$_2$Cl$_2$ (Scheme 2). Sulfur deprotection was monitored by TLC, and the formation of the corresponding thiol-containing drugs 13−15 was confirmed by LCMS. We were unable to prove the formation of a thiol-ampicillin derivative from the deprotection of 6 or to detect any adduct from the conjugation with maleimide-Ga-DfoB 12. Ga-DfoB-loracarbef sideromycin 16 was obtained in 56% yield from the reaction between freshly prepared thiol 13, maleimide 12, and DIPEA in deoxygenated THF. The corresponding Ga-DfoB-ciprofloxacin 17 (24% yield) and Ga-DfoB-nadifloxacin 18 (21% yield) were prepared in a similar fashion. The solvent used in the drug conjugation influenced product formation. When the assembly of 17 was attempted in CH$_2$Cl$_2$, the desired sideromycin was not detected even after 70 h.

The novel conjugates and their synthetic precursors were screened for antibacterial activity against a panel of Gram-positive and Gram-negative bacteria in an agar diffusion assay[27,28] (Table S1 of the Supporting Information (SI)). The S-trityl protected antibiotics displayed broad growth inhibition of bacteria (2 mM), with the exception of ciprofloxacin derivative 8, where significant levels of precipitation were observed in aqueous media due to limited solubility. In general, *Mycobacterium smegmatis* and *Escherichia coli* were not affected by our compounds. To study the effect of gallium, we tested Ga(acac)$_3$ and Ga(NO$_3$)$_3$ but no growth inhibition (at 0.2 mM) was observed under the assay conditions. Maleimide-functionalized DfoB 11 and the

Scheme 1. Syntheses of Thiol-Containing Antibiotic Precursors
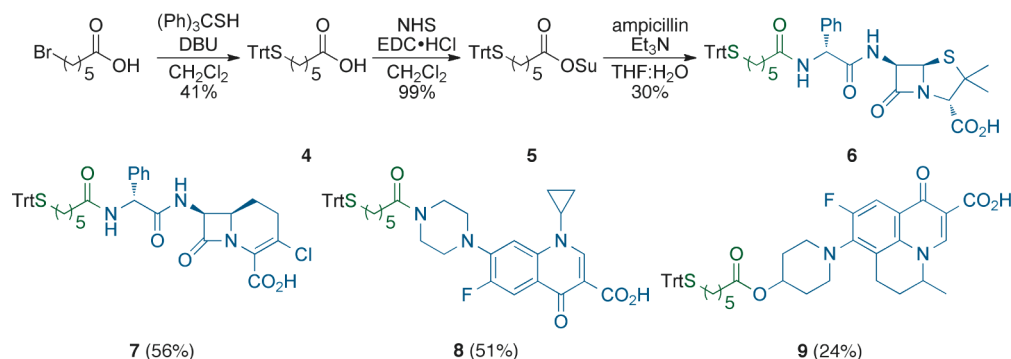
Scheme 2. Assembly of Ga-DfoB—Drug Conjugates
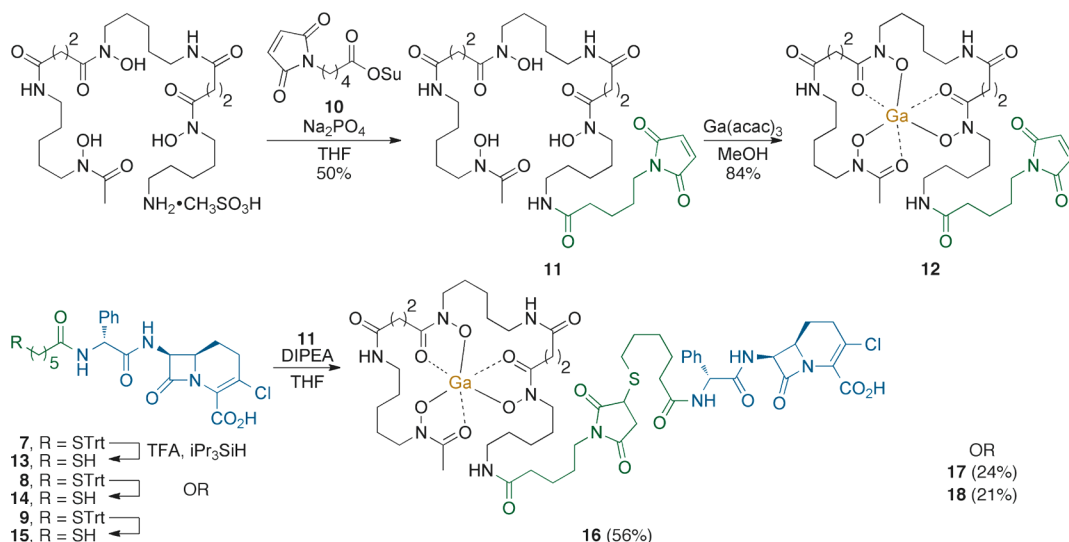
corresponding Ga-DfoB complex **12** did not display antibiotic properties. However, drug-conjugates **16–18** were potent growth inhibitors, particularly against Gram-positive bacteria and *Mycobacterium vaccae*.

After susceptible bacteria were identified, MIC values were determined in MHII media as well as iron-deficient conditions (MHII + 2,2′-bipyridine).[29] In MHII (Table 1), both *S*-trityl

Table 1. Minimum Inhibitory Concentration of Compounds (MIC, μM) Determined in MHII Media[a–d]

compd	*B. subtilis* ATCC 6633	*S. aureus* SG511	*M. luteus* ATCC 10240	*M. vaccae* IMET 10670	*P. aeruginosa* K799/wt
6[c]	2.15[b]	0.39[b]	1.19	>25	>25[b]
7[c]	3.13[b]	2.35[b]	1.71	6.25	>25[b]
8[c]	>25[b]P	>25[b]	>25[b]	>25 P	>25[b]
9[c]	3.13	25	>25	10.4	>25
Ga(NO₃)₃[c]	>25	>25	>25	>25	>25
12[c]	>50[b]	>50[b]	>50[b]	>50	>50[b]
16[c]	27	9.38[b]	0.001	>50	>50[b]
17[c]	>50[b]	31.25[b]	>50[b]	>50	>50[b]
18[c]	<0.1	12.5	50	3.13	>50
Lora[c]	0.46	0.78	0.39	>25	>25
Nadi[c]	0.008	0.06	0.20	0.06	>2
Cipro	0.06	0.47	7.54	0.24	0.94

[a]MICs were determined by the visual end point broth microdilution method following CLSI guidelines.[29] [b]Reported values are the average of two sets of duplicates (N = 4). [c]Reported values are the average of triplicates (N = 3). [d]P, observed precipitation.

protected β-lactams **6** and **7** displayed potent (MIC = 0.39–3.13 μM) antibiotic activity against *Bacillus subtilis*, *Staphylococcus aureus*, and *Micrococcus luteus*, comparable to that of loracarbef (MIC = 0.39–0.78 μM). In the case of *M. vaccae*, derivative **7** (6.25 μM) was more potent than the parent β-lactam (>25 μM), possibly due to increased lipophilicity.

The MIC values of ciprofloxacin analog **8** were not adequately determined because of its limited solubility in aqueous solution. However, nadifloxacin (Nadi) derivative **9** displayed improved solubility and a range of inhibitory activity

(MIC = 3.13 → 25 μM) without enhancing the potency of Nadi. Consistent with our findings in the agar diffusion assay, Ga(NO₃)₃ and Ga-DfoB **12** did not display growth inhibition (MIC >50 μM). *P. aeruginosa* (K799/wt) was not inhibited at the highest concentrations tested, in either MHII or iron-depleted media (MIC >50 μM, Table S2 of the SI).

Ga-DfoB-loracarbef conjugate **16** retained the antibiotic activity of the β-lactam drug, while displaying remarkable inhibition of *M. luteus* (MIC = 0.001 μM) enhancing the activity of loracarbef (MIC = 0.39 μM). While this result merits further study, the finding that *M. luteus* was strongly inhibited by **16** is consistent with the fact that it possesses a limited set of penicillin-binding proteins (PBPs) that render this Gram-positive bacteria particularly sensitive to β-lactam antibiotics.[30] Ga-DfoB-ciprofloxacin **17** displayed reduced inhibition when compared to **16** (MIC = 31.25 → 50 μM), but enhanced solubility and therefore improved antibiotic activity over the *S*-trityl protected precursor **8**. Ga-DfoB-nadifloxacin conjugate **18** (Figure 3) was a strong inhibitor of *B. subtilis* (MIC = <0.1 μM) and *M. vaccae* (MIC = 3.13 μM), consistent with the increased potency of Nadi when compared to Cipro.
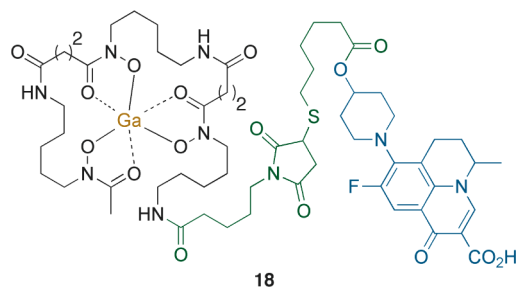

Figure 3. Ga-DfoB-nadifloxacin conjugate **18**.

Considering that Ga(NO₃)₃, Ga(acac)₃, and Ga-DfoB **12** did not display growth inhibition under the different conditions tested, the observed antibiotic activity of sideromycins **16–18** can be attributed to a functional drug moiety. Our results are also consistent with bacterial internalization of the conjugates, as reflected by the growth inhibition of cytoplasmic targets with fluoroquinolone-based sideromycins (**17** and **18**). Recently,

Rzhepishevska and collaborators reported the antibacterial properties of gallium citrate (Ga-Cit) and Ga-DfoB against Gram-positive and Gram-negative bacteria.[31] Growth inhibition by gallium was affected by the metal−ligand and the media composition. IC_{90} values for Ga-DfoB against several bacteria were higher (mM) than the concentrations screened in this work. To explore the effect of further iron-restricted conditions, we determined the MIC values in MHII media with the addition of metal-chelator 2,2′-bipyridine (Table S2 of the SI). In general, we observed comparable values between both assays, corroborating the displayed antibiotic activity of our synthetic compounds. Moderate enhancement (3-fold) was observed with loracarbef-conjugate 16 against *B. subtilis* and *S. aureus* (MIC = 3.9−7.8 μM). The iron-depleted media was not conducive for the culturing of *M. vaccae*, which failed to grow after two attempts.

In conclusion, we have developed a convergent approach for the assembly of siderophore−drug conjugates using a thiol−maleimide strategy. As anticipated, the novel sideromycins displayed selective antibacterial properties and provided us with valuable information suitable for the study of these molecules. Because the siderophore of choice often determines the spectrum of activity of these conjugates, the described methodology could be expanded to the design of Gram-negative antibacterials through the use of an appropriate chelator.

■ ASSOCIATED CONTENT

ⓈSupporting Information

Experimental procedures of chemistry and microbiology, and ^{1}H and ^{13}C NMR spectra of reported compounds. 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.

■ ACKNOWLEDGMENTS

■ ABBREVIATIONS

DBU, 1,8-diazabicycloundec-7-ene; DfoB, desferrioxamine B; Ga-DfoB, Ga^{3+}-complex of DfoB; EDC·HCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl; LCMS, liquid chromatography mass spectrometry; MHII, Mueller−Hinton agar; MIC, minimum inhibitory concentration; NHS, *N*-hydroxysuccinimide; THF, tetrahydrofuran; TFA, trifluoroacetic acid; TLC, thin layer chromatography

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