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Design, synthesis, and evaluation of efflux substrate–metal chelator conjugates as potential antimicrobial agents

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Abstract—Maintaining a proper balance of metal concentrations is critical to the survival of bacteria. We have designed and synthesized a series of conjugates of metal chelators and efflux transporter substrates aimed at disrupting bacterial metal homeostasis to achieve bacterial killing. Biological studies showed that two of the compounds had very significant antimicrobial effect with an MIC value of 7.8 μg/mL against Gram-positive *Bacillus subtilis*.

The emergence of drug-resistant bacteria and fungi presents a major problem in the medical field.[1–4] Consequently, there is an urgent need for the development of novel types of antimicrobial agents targeting unique mechanisms and pathways. Bacteria and fungi generally develop drug resistance in three ways: producing metabolizing enzymes for the degradation of the drugs, modifying their targets to render the drugs ineffective, and expressing a high level of efflux proteins that 'pump' the drug out in order to lower its concentration.[1,2,4–11] The expression of efflux proteins is an especially common phenomenon even in non-drug-resistant strains of bacteria. This is also the mechanism responsible for multi-drug resistance (MDR).

Conceivably, one can take advantage of the efflux proteins in developing novel antimicrobial agents. Indeed, the efflux transporters have been targeted for the development of therapeutic agents aimed at reversing MDR. This has been tested on both humans (cancer) and microbes with only limited success.[12–20] The less than desirable effect of these efflux inhibitors in clinical trials is most likely due to a combination of three factors. First, efflux-mediated resistance is not the sole mechanism through which bacteria or cancer develops resistance. Second, efflux (MDR) proteins are a family of proteins and therefore, inhibiting only one may not achieve the desired effect of increasing drug concentration. Third, both bacterial and cancer cells have ways to compensate for the inhibition of a particular efflux pump. Considering all these factors, we designed an approach that takes advantage of the very existence of the efflux transporters in microbes to develop new antimicrobial agents. This novel approach aims to turn the efflux transporters into 'suicide' machines, which cause disruption of metal homeostasis and consequently bacterial death. Aimed at testing the feasibility of this new approach, we have designed, synthesized, and evaluated a series of nine compounds. Two of these compounds (**1** and **6**) were shown to have very significant antimicrobial effect with an minimum inhibition concentration (MIC) of 7.8 μg/mL.

Design. Again, the goal of the study is to turn MDR-efflux transporters into 'suicide' machines to achieve disruption of metal homeostasis and consequently bacterial death. MDR transporters in bacteria belong to three major families of proteins. These include the major facilitator superfamily (MFS) and the resistance-nodulation-division family (RND)—both driven by the proton motive force,[21] and the ABC (ATP-binding cassette)

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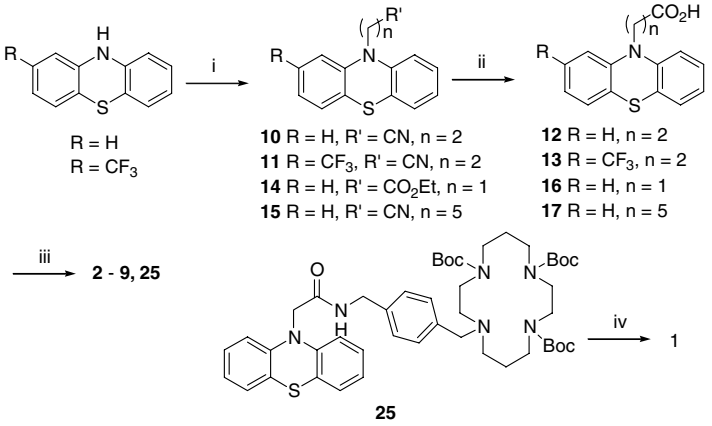
superfamily driven by ATP as a source of energy.[10,22,23] These proteins generally have broad substrate specificity and can transport a wide variety of antibiotics and other xenobiotics including tetracyclines, macrolides, β-lactams, fluoroquinolones, isoniazid, rifampicin, ethambutol, etc.[21] In our approach of disrupting metal homeostasis in bacteria, we plan to synthesize conjugates of metal chelators with efflux substrates as potential antimicrobial agents. If the intracellular concentration of the target metal is higher than that in the extracellular medium, one can envision a situation where the chelator conjugate can passively diffuse into bacteria in the free form and then bind to a metal in the intracellular environment. The recognition of the efflux substrate portion of the conjugate by the efflux transporter should result in the outward movement of the conjugate, which helps to 'drag' a metal ion with it. The rapid passive diffusion of the free conjugates into bacteria and their subsequent efflux after metal chelation essentially generate an active membrane 'pore' for the target metal and can be an efficient way of disrupting the metal homeostasis of bacteria, which can lead to cell death.

In designing the conjugates that can be used for the disruption of metal homeostasis in bacteria, one needs to consider the substrate(s) to use, the chelator(s) to use, and the linker position and length for conjugating the chelator to the substrate. Many antibiotics are known substrates for the efflux pumps in bacteria. However, to avoid complication in interpreting the antimicrobial results, we desire to choose something other than a known antimicrobial agent as the 'substrate' moiety. There have been recent reports indicating that phenothiazine can be recognized by the efflux systems of microorganisms.[24–33] Furthermore, the chemistry to conjugate phenothiazine to a metal chelator is relatively straightforward. Therefore, we chose phenothiazine as the substrate moiety. As for the chelators to use, in this preliminary study we desire to 'scan' a few well-known chelators to see which one would have the best effect. As for the linker length, we chose some medium-length linkers, which are unlikely to affect the substrate property of the phenothiazine unit, and yet not too long to have other kind of unintended consequences. For these reasons, we designed compounds **1**–**9**.

Synthesis. The conjugation chemistry used is either amidation or esterification. Phenothiazines with a carboxyl group tethered through a 1- to 5-carbon linker were first synthesized from phenothiazine or 2-trifluoromethylphenothiazine by either a Michael addition[34] followed by hydrolysis leading to **12**–**13** or alkylation[35–37] followed by hydrolysis[38] leading to **16**–**17** (Scheme 1). Coupling **16** with **24** followed by deprotection of the Boc group resulted in the formation of chelator **1**. Efflux substrate–metal chelator conjugates **2**–**9** were obtained by either amidation or esterification between **12**–**13**, **16**–**17**, and the appropriate chelators, **20**, **21**, and **26**, respectively (Fig. 1).

Chelators **20**, **21**, **24**, and **26** were synthesized as described in Scheme 2 following well-established literature procedures. Thus, benzylation[39] of 1,4,7,10-tetraoxa-13-aza-cyclopentadecane and bispyridin-2-ylmethylamine with *p*-cyanobenzylbromide gave compounds **18** and **19**, respectively, which were hydrogenated with Raney-nickel catalyst to afford chelators **20** and **21**, respectively. Chelator **24** was prepared from cyclam in three steps: benzylation[40,41] with *p*-cyanobenzylbromide, protection with di-*tert*-butyl dicarbonate, and hydrogenation. Bromination of 4′-(4-methylphenyl)-[2,2′:6′,2″]terpyridine with NBS and AIBN gave chelator **26**.

Antimicrobial tests. The antimicrobial activities of these conjugates were evaluated using two model systems: one Gram-positive (*Bacillus subtilis*) and one Gram-negative (*Escherichia coli*). The effects of these compounds were very different on Gram-positive and Gram-negative bacteria. Against Gram-positive *B. subtilis*, the tetraaza macrocycle conjugate **1** showed very significant activity with an MIC of 7.8 μg/mL (Table 1). The bis(2-picolyl)amine compounds **5**–**7** also showed good activities with MIC's in the range of 7.8–30 μg/mL. It



Scheme 1. Synthesis of efflux substrate-metal chelator conjugates. Reagents and conditions: (i)a—acrylonitrile, $Bu_4N^+OH^-$, 0 °C to rt (to give **10**, 55% and **11**, 59%); (i)b—ethyl bromoacetate, KOH, NaI, DMF, 50 °C (to give **14**, 46%); or c—5-bromovaleronitrile, KOH, NaI, DMF, 50 °C (to give **15**, 65%); (ii) KOH, MeOH, reflux, 75–97%; (iii)a—**20**,**21**, or **24**, EDCI, HOBT, DCM, rt (to give **2**–**7**, **25**, 40–77%); or b—**26**, $NaHCO_3$, DMF, rt (to give **8**, 50% and **9**, 60%); (iv) HCl, dioxane, rt, 67%.

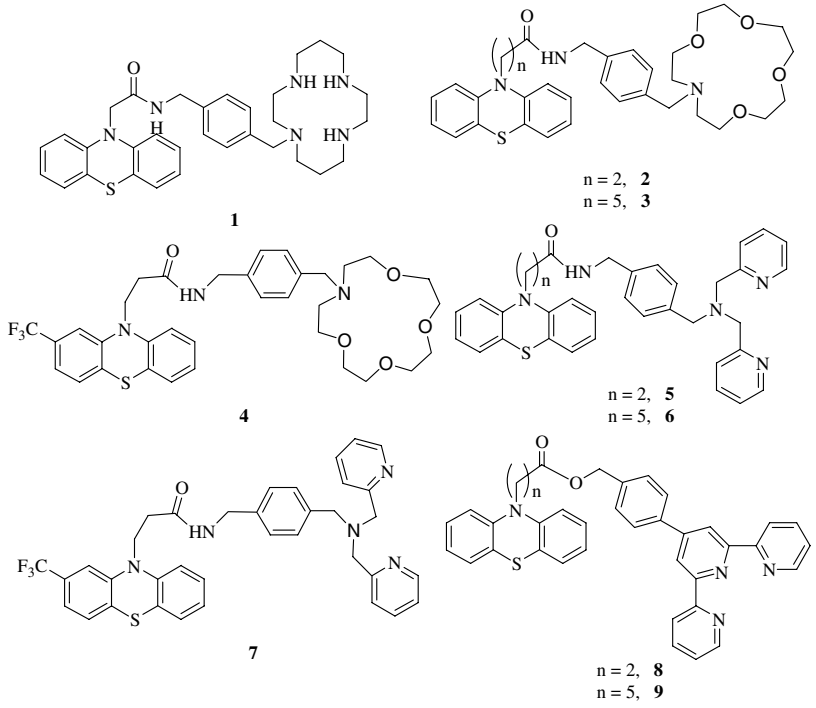
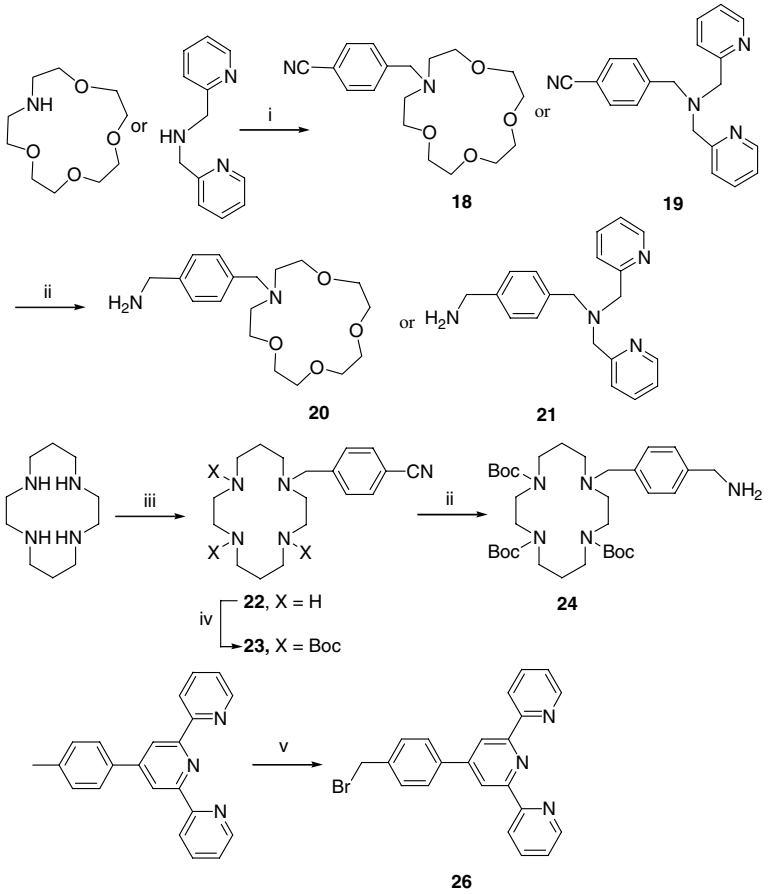


Figure 1. Structure of compounds **1**–**9**.



Scheme 2. Synthesis of chelators **20**, **21**, **24**, and **26**. Reagents and conditions: (i) *p*-cyanobenzyl bromide, K_2CO_3, DCM, rt, 48–50%; (ii) H_2/Raney-Ni, rt, 72–84%; (iii)a—$B[NH(CH_3)_2]_3$, toluene, reflux; b—*n*-BuLi, *p*-cyanobenzyl bromide, THF, −30 °C to rt, 51%; (iv) $(Boc)_2O$, $NaHCO_3$, THF, rt, 97%; (v) NBS, AIBN, CCl_4, *hν*, reflux, 60%.

Table 1. Antimicrobial effect[a] of efflux substrate–metal chelator conjugates **1**–**9**

Compound	Clog*P*	*E. coli* (*MC4100*) (mg/mL)	*B. subtilis* (mg/mL)
1	2.94	2	0.0078
2	3.53	X[b]	2
3	4.65	X[b]	0.06
4	4.45	X[b]	0.5
5	6.04	X[b]	0.03
6	7.15	X[b]	0.0078
7	6.96	X[b]	0.016
8	7.84	X[b]	X[b]
9	8.96	X[b]	0.5
Phenothiazine			0.5
Chelator of **1**			X[b]
Chelator of **5**			2
Ampicillin			0.025
Kanamycin			<0.003[c]

[a] Assay procedure: grow bacteria to lag phase; mix bacteria with soft agar (0.7%) and spread in normal LB plates (LB+1.5% agar); dissolve **1**–**9** in DMSO and conduct 2-fold serial dilutions; spot the solution in the bacterial plates.

[b] No inhibition zones were detected below 2 mg/mL.

[c] MIC was not obtained for Kanamycin, 0.003 mg/mL was the lowest concentration examined. DMSO was used as negative control (data not shown).

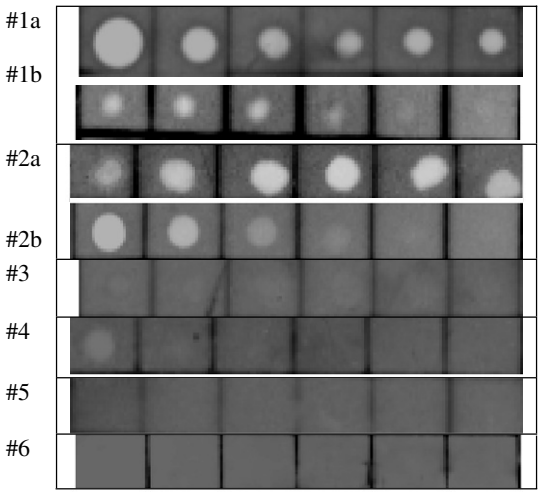


Figure 2. Rows 1a,b: compound **1**; rows 2a,b: compound **6**; row 3: compound **8**; row 4: phenothiazine; row 5: chelator portion of **1**; row 6: chelator portion of **6**. The starting concentrations for rows 1a, 2a, 3, 4, 5, and 6 were 2 mg/mL with serial dilutions of 5- and 4-fold for the first two, and 2-fold for subsequent tests. Rows 1b and 2b show the results of the subsequent 2-fold dilutions from rows 1a and 2a, respectively. The MIC was 7.8 μg/mL for **1** and **6**. *Bacillus subtilis* was the model organism.

is interesting to note that the compound with the longest linker (**6**) in the bis(2-picolyl)amine series seemed to have the best activity (7.8 μg/mL). The azacrown ether compounds **2** and **4** and the tripyridine compounds **8** and **9** showed poor or no activities with their MIC's generally over 0.5 mg/mL with the exception of **3**, which has an MIC of 60 μg/mL. Again, the compound (**3**) with the longest linker in the tripyridine series also seems to be most active. One can think of partition coefficient being a factor affecting the antimicrobial activities of these compounds because of the increased Clog*P* with increased linker length (Table 1), though it is hard to draw conclusions given the limited number of compounds studied.

Figure 2 also shows part of the inhibition test results with compounds **1** and **6** against Gram-positive *B. subtilis*. Again, compounds **1** and **6** showed very significant inhibitory activities with an MIC of 7.8 μg/mL (Fig. 2, rows 1a,b and 2a,b). The three control compounds, the phenothiazine portion and the chelator portions of **1** and **6**, did not show any activities at comparable concentrations (Fig. 2, rows 4–6). Specifically, the phenothiazine portion (row 4) did not show any inhibitory activity at or below 0.5 mg/mL and the chelator portions (rows 5 and 6) of **6** and **1**, respectively, did not show any activity at the highest concentration tested, 2 mg/mL. Row 3 shows the negative results for compound **8** as a comparison. Kanamycin and ampicillin were used as positive controls.

None of the compounds tested inhibited Gram-negative *E. coli* (Table 1). The lower activity against Gram-negative bacteria is consistent with the notion that the outer membrane of Gram-negative bacteria might limit the penetration of those compounds with molecular weight above the usual cutoff point of 500–600[42] (molecular weight: **1**, 558 and **6**, 613).

It is interesting to note that the chelators for the two most active compounds, **1** and **6**, are both Zn^{2+} binders.[43,44] Previous research using *E. coli* as a model has shown that bacteria concentrate zinc and iron by several orders of magnitude relative to the concentration in a typical growth medium until they reach a concentration of about 0.1 mM.[45] Therefore, theoretically $Zn^{2+}$is a good target for developing the intended chelator-based antimicrobial agents due to the expected differential concentrations of this metal ion inside and outside of bacteria.[43,46,47] However, more work is needed to elucidate the mechanism of action of these compounds.

In conclusion, metal ions play critical roles in bacteria. Consequently, intracellular metal concentrations are tightly regulated by various cellular machineries. Minor disruption of the metal-ion homeostasis can lead to a detrimental effect resulting in cell death. Intuitively, it is reasonable to think that disruption of the metal homeostasis would lead to cell stress or death. In this study, we have designed, synthesized, and evaluated a series of metal chelator–efflux substrate conjugates. Such conjugates are designed to disrupt the metal homeostasis of bacteria by turning MDR efflux transporters of bacteria into 'suicide' machines, which help to 'drag' metal ions through the cell membrane. Among the nine compounds synthesized, two (**1** and **6**) showed very significant antimicrobial activities, each with an MIC of 7.8 μg/mL. Much more biological work is needed to elucidate the mechanism of action. We also hope that the initial success of this novel approach will stimulate more work in this general area of targeting metal

homeostasis and efflux transporters for designing new antimicrobial agents.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.10.094.

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