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MexAB-OprM-Specific Efflux Pump Inhibitors in *Pseudomonas aeruginosa*. Part 1: Discovery and Early Strategies for Lead Optimization

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Abstract—The identification of a series of compounds that specifically inhibit efflux by the MexAB-OprM pump system in *Pseudomonas aeruginosa* is described. Synthesis and in vitro structure–activity relationships (SARs) are outlined. Early leads lacked activity in animal models, and efforts to improve solubility and reduce serum protein binding by the introduction of polar groups are discussed.

The demand for the discovery of new antimicrobial drugs is continually fuelled by the emergence of resistance. The more an antibiotic is used for the treatment of infections, the greater the probability that resistance to it will develop. Even antibacterial drugs with novel mechanisms of action suffer the same fate, and a fundamental solution is required to break the vicious circle.[1–5] Active efflux of antimicrobial agents is increasingly being recognized as a major cause of antimicrobial resistance among a diverse set of antibacterial agents including β-lactams, macrolides, tetracyclines and fluoroquinolones.[6–10] A particularly problematic pathogen in the clinical setting is *Pseudomonas aeruginosa*, an opportunistic Gram-negative pathogen characterized by intrinsic resistance to a wide variety of antimicrobial agents.[11–13] This property has been attributed both to the impermeability of the outer membrane as well as to the activity of several efflux systems of the Resistance-Nodulation-Division (RND) superfamily, four of which have been identified to date: MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM.[14–17]

We have previously disclosed the discovery and optimization of compounds that inhibit all RND pumps in *P. aeruginosa* and thereby potentiate the activity of the fluoroquinolone Levofloxacin (LVFX).[18–20] Herein, we describe the first example of a MexAB-OprM-specific efflux pump inhibitor (EPI). Such an agent might be used for the potentiation of anti-pseudomonas β-lactams that are substrates of this pump (e.g., aztreonam (AZT), piperacillin, meropenem), as well as quinolones (Fig. 1). We also summarize in vitro SAR and initial attempts to optimize properties for use in vivo.

Compound **1** (Fig. 2) was identified in a high-throughput screen for LVFX potentiation using a MexAB-OprM over-expressing strain[21,22] of *P. aeruginosa* (PAM1032).[23]

Inhibitors of RND pumps in *P. aeruginosa* are not expected to display intrinsic antibacterial activity, as functioning pumps are not essential for survival of the organism in vitro. Neither **1** nor any analogues reported

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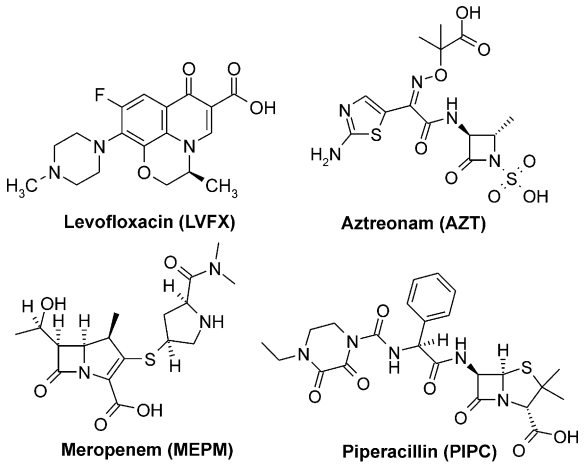


Figure 1. Levofloxacin and anti-pseudomonas β-lactams.

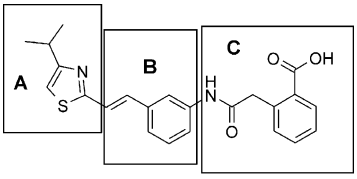
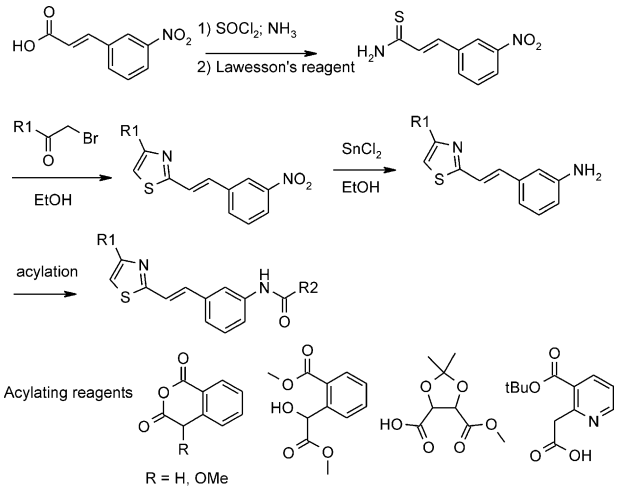


Figure 2. Structure of compound **1**.

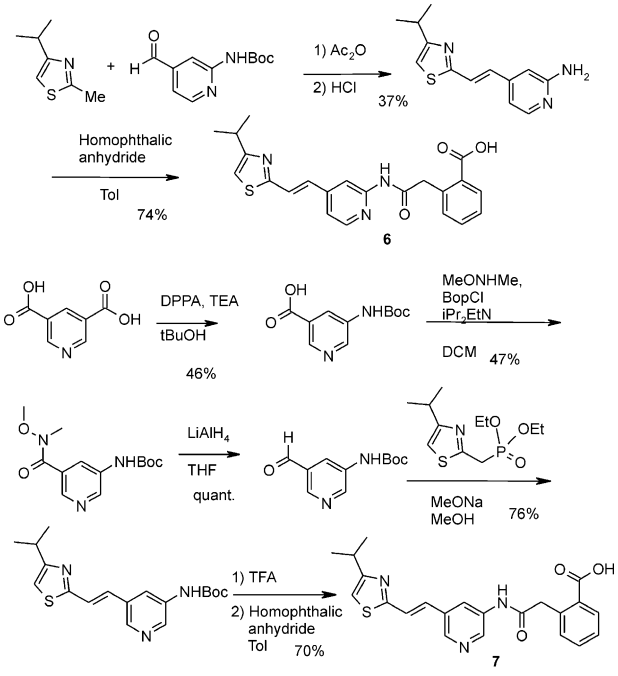
herein were antibacterials (MIC >320 μg/mL). When combined with antibiotics, the potentiation activity was conveniently expressed as the lowest concentration of EPI achieving an 8-fold reduction in antibiotic MIC (Minimum Potentiation Concentration, or MPC_8). By this measure, the activity of **1** as a potentiator of LVFX against PAM1032 and PAM1723 (a strain in which MexAB-OprM is overexpressed and MexCD-OprJ and MexEF-OprN are genetically disrupted)[21,22] was impressive (MPC_8 ≤0.63 μg/mL). However, it was devoid of potentiation activity against MexCD-OprJ, MexEF-OprN and MexXY-OprM over-expressing strains, suggesting that the action of inhibition is specific for the MexAB-OprM pump. Direct inhibition of a fluorescent substrate of this pump was also measured spectrophotometrically.

While the microbiological profile of **1** was attractive, the physicochemical properties were problematic. The aqueous solubility was poor (2.85 μg/mL in pH 6.8 buffer solution), the lipophilicity was high (log D>4), and the affinity for serum albumin was also high (>98% bound, K_d 0.28 μM). In addition, no significant potentiation of LVFX was seen in a murine sepsis model using a MexAB-OprM overexpressing strain of *P. aeruginosa* (data not shown). We wished to reduce the serum protein binding so as to allow an assessment of free drug clearance. In order to accomplish this we generated two strategies, one based upon polar group substitution and the other looking for alternative scaffolds. In this paper, we report the scope and limitation of the first strategy; the results of the search for alternative scaffolds will be the subject of subsequent publications.

For convenience, the structure of **1** was divided into three parts (A, B and C) as exemplified in Figure 2, and



Scheme 1. Synthesis of variants of Parts A and C.



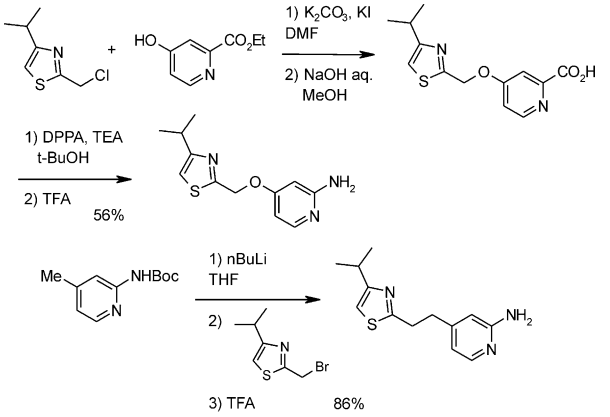
Scheme 2. Synthesis of variants of Part B.

the outcome of the incorporation of polar groups into each was examined. The syntheses of novel analogues are depicted in Schemes 1–3, and final structures are displayed in Figure 3. Thiazoles were synthesized in a conventional manner by the condensation of haloketones with the requisite thioamide.[24–26] Variants of the homophthalic acid moiety in **1** were generated by direct acylation of the key intermediate aniline with a variety of carboxylic acid derivatives.[27–30]

Analogues containing pyridyl surrogates for the central phenyl moiety (part B) were synthesized by a Knoevenagel-type condensation between a 2-methylthiazole and a 2-aminopyridine-4-carboxaldehyde derivative,[25,26] or by a Horner–Emmons reaction between the pyridine-aldehyde and a phosphonate (Scheme 2).[24] Ether tethers were conveniently constructed via alkylation of pyridones. The ethylene tethers resulted from

the coupling of the dianion of 2-(*t*-butyloxycarbonylamino)-4-methylpyridine with the same thiazole-containing electrophile (Scheme 3).[31]

New analogues were tested for the potentiation of LVFX and AZT using PAM1723.[32] Assays were conducted in the presence and absence of 10% horse serum



Scheme 3. Synthesis of ether and ethylene tethers.

in order to evaluate the effects of protein binding.[35] Entries 1–5 in Table 1 describe the modifications to the thiazole moiety A. Introduction of a carboxamide (as in **2**) reduced activity dramatically, although the influence of serum was also diminished; other more polar variants abolished activity completely. Replacement of the isopropyl substituent with pentafluoroethyl (**3**) gave somewhat reduced activity. *t*-Butyl or phenyl variants (**4**,**5**) could be accommodated with no deleterious effect on potency, but also no apparent impact on the effect of protein binding.

Next, the modification of styrene portion (B) was examined. Three pyridyl variants gave very different results, with **8** dramatically less active than the other two. The 3,5-disubstituted pyridine **7** gave good activity that was not greatly affected by the addition of serum. The LVFX potentiation of **6** was excellent, but its extreme insolubility confounded attempts to generate meaningful data with AZT. To address this problem, alternatives to the vinyl moiety were then evaluated. Molecules bearing methyloxy (**9**) and ethylene (**10**) tethers displayed favorable activity profiles similar to **7**.

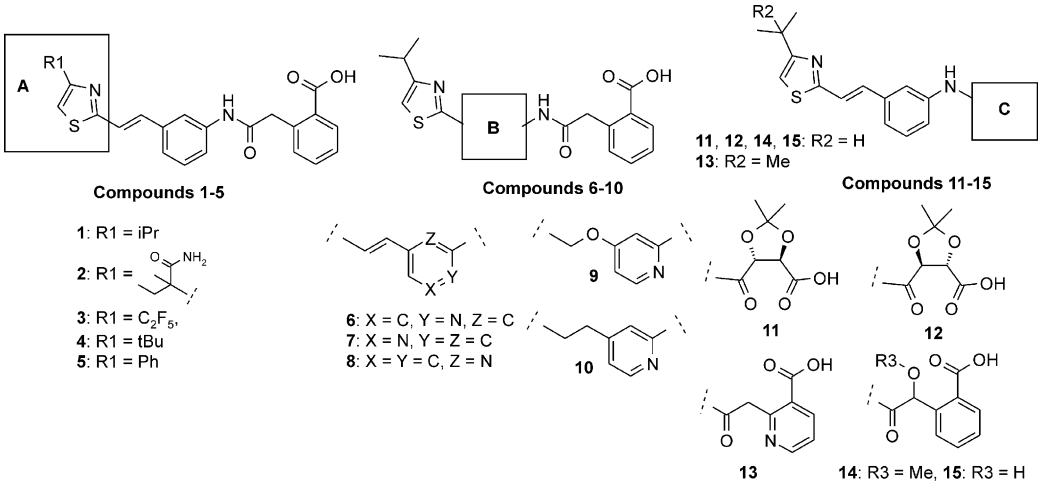


Figure 3. Structure of **1** and novel analogues.

Table 1. Potentiation activity of **1** and novel analogues[a]

	MPC_8 (AZT) (μg/mL)		Ratio MPC_8 (AZT) with/without serum)	MPC_8 (LVFX); (μg/mL)
	Without serum	With 10% horse serum		
1	≤0.63	10	>16	≤0.63
2	20	40	2	80
3	2.5	80	32	2.5
4	≤0.63	20	>32	0.63
5	1.25	40	32	1.25
6	>10[b]	10	—	0.31
7	5	10	2	2.5
8	20	80	4	>80
9	2.5	5	2	2.5
10	5	10	2	2.5
11	20	40	2	40
12	5	20	4	20
13	2.5	20	8	1.25
14	20	160	8	10
15	2.5	40	16	2.5

[a]All compounds lacked intrinsic antibacterial activity.
[b]The apparent lack of activity is attributable to precipitation of the compound.

Based upon the known structure of serum albumin and its interaction with acidic molecules,[33,34] we envisaged that the introduction of bulky or hydrophilic groups adjacent to the carboxylic acid moiety might reduce protein binding. Variants in part C were designed with this notion in mind. One enantiomer (**12**) of the dioxolane-containing pair showed more activity than the other (**11**), and the serum effect was indeed reduced. The reasonable potency of the pyridyl analogue (**13**) was compromised by the addition of serum; similarly, the incorporation of hydroxyl and methoxy groups in this region gave no improvement in serum effect (**14**, **15**), although the activity of the alcohol was encouraging.

Compounds with reasonable activity and little influence of serum in vitro were evaluated as potentiators of LVFX in murine neutropenic thigh or sepsis models. Marginal activity was seen for only one compound (**10**), which exhibited a slight effect in the sepsis model (data not shown). The protein binding of this compound, as measured by conventional ultra-filtration techniques, was 96.7% (K_d 12.3 μM), whereas for all the other analogues tested it was >98%. We concluded that lipophilic acids such as these exhibited extremely high affinities for serum albumin and that more radical structure manipulation would be required to reduce the binding significantly.

In summary, we synthesized several derivatives of **1**, the first known MexAB-OprM specific inhibitor of efflux in *P. aeruginosa*, in an attempt to lower serum protein binding. Pyridine variants with ether or ethylene tethers replacing the styrene moiety exhibited the most promising profile, but were still not efficacious in vivo. Incorporation of more polar groups simply abolished the activity. We therefore concluded that other strategies would be required to overcome the problem, and a successful alternative approach is reported in the following paper.

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