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MexAB-OprM specific efflux pump inhibitors in *Pseudomonas aeruginosa*. Part 3: Optimization of potency in the pyridopyrimidine series through the application of a pharmacophore model

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Abstract—The addition of substituents to the pyridopyrimidine scaffold of MexAB-OprM specific efflux pump inhibitors was explored. As predicted by a pharmacophore model, the incorporation substituents at the 2-position improved potency. Piperidines were found to be optimal, and further introduction of polar groups without compromising the activity was shown to be feasible. Careful positioning of the essential acidic moiety of the pharmacophore relative to the scaffold led to the discovery of vinyl tetrazoles with still greater potency.

Peudomonas aeruginosa is an opportunistic pathogen whose intrinsic resistance to a wide variety of antimicrobial agents (including quinolones, β-lactams, aminoglycosides, macrolides and tetracyclines) is largely attributable to the expression of several efflux pumps, four of which have been identified to date: MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM.[1–4] We recently reported the discovery and initial optimization[5,6] of agents that are specific for the inhibition of MexAB-OprM. Our initial efforts were directed towards the improvement of physicochemical properties of the initial lead **1** (Fig. 1) so as to achieve in vivo activity. Two main strategies were explored: the reduction of high protein binding and increase in aqueous solubility through the incorporation of polar groups into the structure,[5] or redesign of the molecule by utilizing alternative scaffolds.[6]

The latter strategy culminated in the discovery of a series of pyridopyrimidines (typified by **2**) whose protein binding was greatly improved and which were efficacious in the potentiation of the quinolones Levofloxacin (LVFX) and Sitafloxacin (STFX) in vivo. The design of **2** was based upon the pharmocophore model depicted in Figure 2.[6] It is apparent from inspection of this model that **1** bears a hydrophobic moiety that is not present in **2**, and that the introduction of 2-substituents on the pyridopyrimidine scaffold might furnish more potent analogues. Herein, we report the results of SAR studies on the pyridopyrimidine scaffold designed to exploit

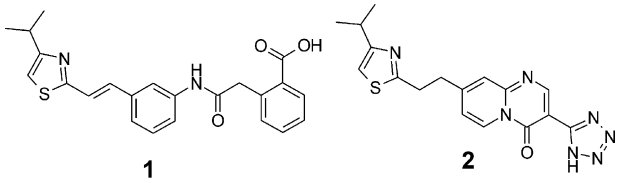

Figure 1. Structures of the first generation of MexAB-OprM specific efflux pump inhibitors.

Keywords: Efflux pump inhibitor; drug resistance; MexA,B-OprM efflux pump.
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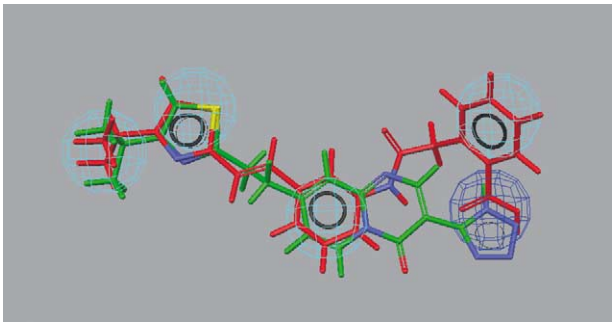


Figure 2. Pharmacophore model generated by CATALYST™[7] and overlaid structures of compound **1** (red) and **2** (green). Light blue spheres are hydrophobic sites, and deep blue for acidic ionizable site.

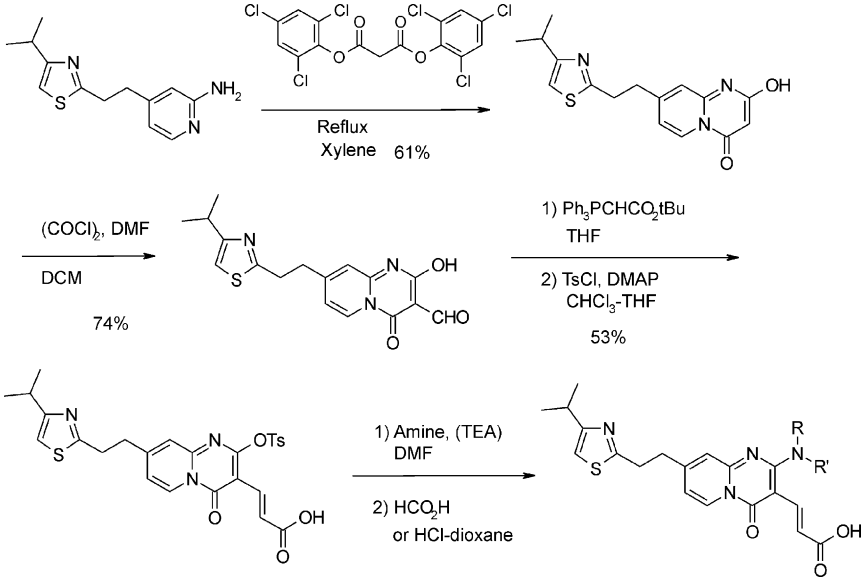
the extra hydrophobic binding pocket, together with optimization of the acidic moiety, leading to molecules possessing high MexAB-OprM pump inhibitory activity.

In a brief initial study to explore the optimum position of the acidic moiety relative to the scaffold,[6] we discovered that analogues in which the tetrazole in **2** was replaced by an acrylic acid moiety displayed increased potency (data not shown). Despite poor stability due to extremely facile photoisomerization of the olefin[8] the potency benefit, coupled with ease of synthesis, drove the decision to incorporate this change at the outset of subsequent efforts to add extra hydrophobic substituents on the scaffold. The ethylene tether between the aromatic entities in **2** was retained in this exercise in order to keep the protein binding as low as possible.[6]

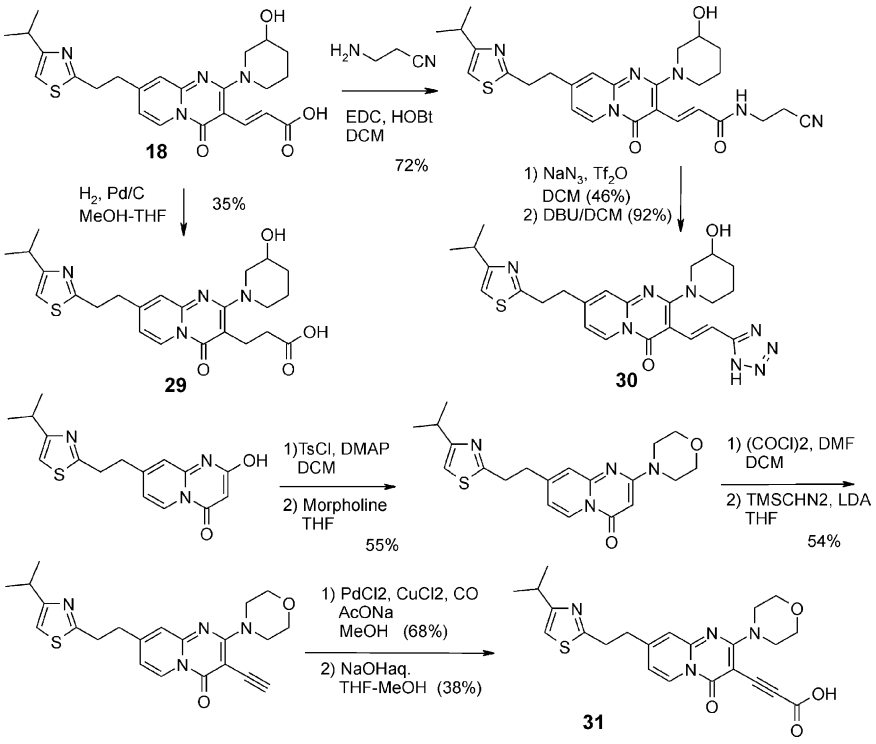
Scheme 1 depicts the route by which the unsaturated acidic analogues were made.[9] The use of activated malonic acid derivatives in the generation of the pyridopyrimidine was strategically convenient for two reasons. Formylation at the 3-position was facilitated by the presence of the 2-OH group, and after tosylation, introduction of hydrophobic 2-substituents by nucleophilic displacement was straightforward. More than 80 cyclic, acyclic, mono- and di-alkylamines were introduced in this manner. Representative examples are shown in Figure 3.

The activity of the efflux pump inhibitors (EPIs) was assessed using PAM1723,[10] an experimental strain of *P. aeruginosa* in which the MexAB-OprM pump is overexpressed and MexCD-OprJ and MexEF-OprN are genetically disrupted. Data for the potentiation of two MexAB-OprM substrates, LVFX and aztreonam (AZT), in the presence or absence of human serum albumin (HSA) is displayed in Table 1. To quantify the activity of the inhibitors, we defined the term MPC_n as the minimum concentration (μg/mL) of inhibitor required to reduce (potentiate) the activity of antibacterial drug *n*-fold. The protein binding was measured for those analogues that displayed reasonable activity that was not dramatically affected by the addition of HSA.

6-Membered cyclic amines furnished the most active compounds (Table 1, **16**–**26**); smaller (**13**–**15**) or larger (**28**) rings resulted in less potent or inactive analogues. Some acyclic alkylamino derivatives were active (**6** and **12**), but the potency was less than that of the cyclic amines (e.g., **13**, **16**). Among the 6-membered cyclic amines, piperidine (**16**, **18**, **19**, **21**, **22**, **25**, **26**) and morpholine (**17**) derivatives provided the best results. Interestingly, incorporation of neutral polar residues such as hydroxyl (**18**, **19**, **21**, **22** versus **16**) or carbamoyloxy groups (**25**, **26** versus **16**) on the 3- or 4-position of piperidine did not affect the activity, although amino substituents at these positions were not tolerated (**23**, **24**). 3-Substituted piperidines were more soluble than the 4-substituted isomers (data not shown). In general, the protein binding of novel active analogues was similar to that of **2**. Although its activity was moderate compared to the piperidines, the lower protein binding of the piperazine variant (**27**) was notable.



Scheme 1. Synthesis of 2-substituted analogues.



Scheme 2. Synthesis of acrylic acid variants.

Table 1. In vitro potentiation activity of novel EPIs[a]

	MPC_8 (AZT) μg/mL[b]		MPC_4 (LVFX) μg/mL[b]	MPC_8 (LVFX) μg/mL[b]	Serum protein binding[d]
	Without HSA	With 0.125% HSA			(%)
1	2	16	1	2	>98
2	16	16	>16[c]	>16[c]	81.5
3	>32[c]	32	8	>32[c]	—
4	64	64	8	64	82.1
5	128	128	32	>128[c]	—
6	32	32	2	16	—
7	>64[c]	>64	8	>64[c]	—
8	>64[c]	>64	16	>64[c]	—
9	>32[c]	>32	8	>32[c]	—
10	64	64	8	64	—
11	32	32	16	128	—
12	16	32	8	64	—
13	8	8	2	8	—
14	32	32	4	32	—
15	128	64	8	128	69.3
16	4	8	0.5	4	—
17	16	32	2	16	92
18	8	16	0.5	8	82.1
19	16	16	1	8	—
20	64	64	8	64	—
21	16	16	2	16	91.5
22	16	16	1	8	—
23	>128[c]	128	8	128	—
24	128	128	32	>128[c]	—
25	32	16	1	8	91.2
26	16	16	2	16	
27	16	32	8	32	67.8
28	128	128	8	128	—

[a] Versus PAM1723.[10] All the analogues were devoid of intrinsic antibacterial activity (MIC >320 μg/mL).
[b] Values are for 100% growth inhibition.
[c] Compound precipitation observed.
[d] Rat serum was used.

Table 2. In vitro potentiation activity of acrylic acid surrogates[a]

	MPC_8 (AZT); μg/mL[b]		MPC_4 (LVFX); μg/mL[b]	MPC_8 (LVFX); μg/mL[b]
	Without HSA	With 0.125% HSA		
17	16	32	2	16
18	8	16	0.5	8
29	>128[c]	>128[c]	32	>128[c]
30	4	4	0.5	4
31	8	16	2	16

[a] Versus PAM1723.[10] All the analogues were devoid of intrinsic antibacterial activity (MIC >320 μg/mL).
[b] Values are for 100% growth inhibition.
[c] Compound precipitation observed.

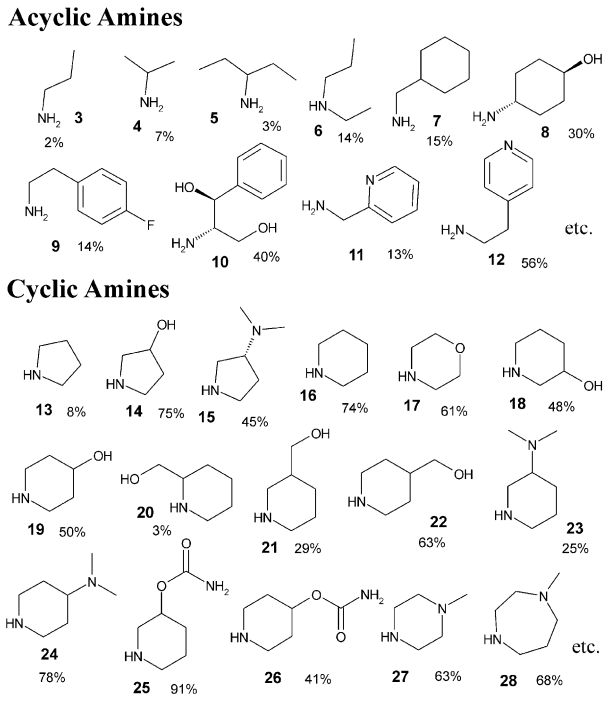


Figure 3. Representative amines introduced to 2-position of pyridopyrimidine scaffold. The number under the structure represents the compound number after attachment to the scaffold. Yields for the last two steps (Scheme 1) are also shown below the structure.

Next, further optimization of the acid surrogate, involving both exploration of the linker between the scaffold and the acidic moiety itself, was addressed. The synthesis of the derivatives is displayed in Scheme 2. Simple hydrogenation of **18** provided the saturated variant (**29**). The requisite tetrazole was synthesized via the cyanoethylamide, followed by treatment with triflic anhydride and sodium azide and deprotection under basic conditions. Finally, the acetylene-linked carboxylic acid was generated utilizing a Colvin rearrangement[11] and Pd-mediated carbonylation[12] as the key steps.

The in vitro activity of the acrylic acid surrogates along with the parent compounds (**17** and **18**) is displayed in Table 2. Saturation of the double bond (**29**) resulted in almost complete loss of activity, whereas the potency of the acetylene variant was similar to that of the vinyl parent (**17** versus **31**), although the stability was found to be much worse. Notable was the activity of the tetrazole variant (**30**), which displayed very high potency along with no apparent influence of the addition of HSA. However, the solubility in aqueous media was inferior to the carboxylic acid counterpart (**18**).

Of the novel analogues, we confirmed that **17** and **18**, like **2**,[6] potentiated LVFX in murine models of infection with *P. aeruginosa*. Details of the in vivo experiments will be reported elsewhere.

In conclusion, the in vitro activity of **2** was significantly enhanced by the incorporation of a hydrophobic group at the 2-position of the pyridopyrimidine scaffold, consistent with the pharmacophore model for this series of MexAB-OprM-selective EPIs. In addition, we have discovered that hydrophilic substitution was feasible on a piperidine moiety at the 2-position without compromising activity, providing a potential avenue for the enhancement of aqueous solubility. The inclusion of an olefin spacer between the tetrazole and pyridopyrimidine scaffold in **2** gave a further boost in potency, although the structural motif was prone to photochemical isomerization.[8] Efforts to address this issue will be reported in due course.

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