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Conformationally-Restricted Analogues of Efflux Pump Inhibitors that Potentiate the Activity of Levofloxacin in *Pseudomonas aeruginosa*

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Abstract—Conformational restriction of the ornithine residue of the efflux pump inhibitor D-ornithine-D-homophenylalanine-3-aminoquinoline (MC-02,595, **2**) furnished bioisosteric proline derivatives that were less toxic in vivo and as active as the lead in potentiating the activity of the fluoroquinolone levofloxacin via the inhibition of efflux pumps in *Pseudomonas aeruginosa*.

We recently described the identification and optimization of a series of inhibitors of the RND efflux pumps in *Pseudomonas aeruginosa*.[1–4] The compounds, exemplified by L-Phe-L-Arg-β-naphthylamine (MC-207,110, **1**) and D-Orn-D-hPhe-3-aminoquinoline (MC-02,595, **2**) (Chart 1), produce an 8-fold reduction in the MIC of the fluoroquinolone levofloxacin (LVFX) at concentrations of the inhibitor as low as 5–10 μg/mL.[5,6]

As the optimization phase of the program progressed, it became apparent that, when administered via intravenous (iv) bolus injection, many of the key compounds displayed appreciable toxic effects in mice and rats. A concerted synthetic effort was undertaken to address this problem and we describe herein our efforts in this area. We demonstrate that conformational restriction of a key residue furnishes efflux pump inhibitors (EPIs) that are as potent as but significantly less toxic than both **1** and **2**.

The in vitro activity and in vivo toxicity in mice of the representative EPIs **1** and **2** are displayed in Table 1. Both analogues potentiate the activity of LVFX but are toxic to mice, with LD_{50}s < 25 mg/kg for each compound. The observed toxic effect was immediate following the iv bolus injection of the compounds. A variety of structurally diverse compounds that similarly potentiated the activity of LVFX in *P. aeruginosa*, such as the *N*-Me analogue **3**, the benzothiazole **4** and the *t*-butyl quinoline derivative **5**, also displayed acute toxicity profiles in mice similar to that observed for **1** and **2** (Table 1). To address the observed toxicity of the entire class of EPIs, we began to probe whether the structural features that elicited this effect could be uncoupled from the potentiation activity.

We first examined the role that routes of compound delivery had on toxicity and showed that iv infusion, intraperitoneal (ip) or subcutaneous (sc) administration significantly ameliorated the immediate toxic effects (Table 2). For instance, the MLD (mg/kg) of **2** was < 200 versus 25 when the compound was administered ip rather than iv, respectively. Less lipophilic analogues, such as the Leu derivative **6**, were less toxic but also less potent. The basic residue of the ornithine group was ultimately implicated as the major contributor to the observed toxicity (compare, for example, the toxicity profile of **7** with **2**). Realizing that a basic functional

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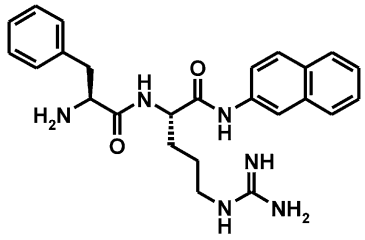
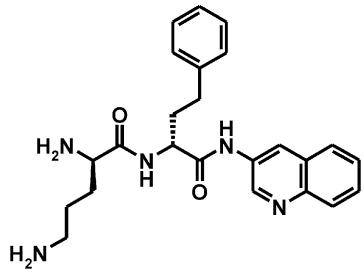


L-Phe-L-Arg-β-naphthylamine (1, MC-207,110)



D-Orn-D-hPhe-3-aminoquinoline (2, MC-02,595)

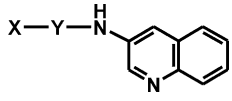
Chart 1.

Table 1. In vitro activity and in vivo toxicity of selected analogues[a]

Compd	MIC[b] (μg/mL)	MPC_8[c] (μg/mL)	MLD[d], iv (mg/kg)	LD_{50} (mg/kg)
1	>512	10	<25	<25
2	>512	10	<25	<25
3	256	5	<25	—
4	128	5	25	—
5	64	5	35	—
8	>512	5	50	—
9	>512	10	70	—
10	128	5	>150	—
11	>512	10	>100	—
LVFX	—	—	>100	>100

[a]See text and refs 5 and 6 for a complete description of the potentiation and toxicity testing.
[b]MIC, minimum concentration (μg/mL) of efflux pump inhibitor required to inhibit the growth of PAM 1723.
[c]MPC_8, minimum concentration (μg/mL) of efflux pump inhibitor required to reduce (potentiate) the MIC of levofloxacin 8-fold.
[d]MLD, minimum dose (mg/kg) causing lethality to ≥66% of the animals tested.

Table 2. The role of the di-amine in the acute toxicity[a]



Compd	X	Y	MLD[a] (mg/kg)		MPC_8[b] (μg/mL)
			iv	ip	
2	Orn	hPhe	<25	200	10
6	Orn	Leu	50	300	20
7	Ala	hPhe	125	—	>40

[a]MLD, minimum dose (mg/kg) causing lethality to ≥66% of the animals tested.
[b]MPC_8, minimum concentration (μg/mL) of efflux pump inhibitor required to reduce (potentiate) the MIC of levofloxacin 8-fold.

group was the primary cause of the observed toxic effects but knowing from our previous work[1] that this same group was crucial for potentiation activity led us to consider various appropriate replacements for ornithine.

We evaluated an assortment of structural modifications to address this problem, one of which was to conformationally restrict the amino group of ornithine to furnish appropriately substituted proline derivatives. Analogues such as these had been shown to be bioisosteres of arginine, which itself is an isostere of ornithine.[7] This approach led to the identification of the constrained derivative **8** (Fig. 1). The compound, which incorporated the two basic residues necessary for activity, was less toxic (MLD 50 mg/kg, iv bolus) and more active than **2**. The positive toxicity results observed for **8** led us to prepare many of the most compelling analogues from our previous studies[1–3] with the new, constrained proline piece. A sampling of the diverse set of compounds prepared in order to capitalize on this result is highlighted by compounds **9**–**11**. In general the original observation for **8** held; that is, analogues containing the new proline scaffold were typically less toxic than the corresponding compounds incorporating the arginine or ornithine residue.

One of the more compelling derivatives from this study was MC-04,124 (**11**), which was >4-fold less toxic in rodents but as potent as the earlier optimized lead **2**. Like **2**, **11** displayed no activity against a strain of *P. aeruginosa* lacking all three primary efflux pumps, confirming that it was acting via the inhibition of these pumps. The compound retained the broad-spectrum pump inhibitory characteristics of the entire series, which involves blockade of the MexAB-OprM, MexCD-OprJ and MexEF-OprN RND efflux pumps.[5,6] Pharmacological profiling showed that **11** had similar rat protein binding (≈65%) as **2** but improved rat pharmacokinetics [CL_{free} (L/h/kg) 1.7 vs 2.9, respectively]. In addition, **11** demonstrated, in combination with LVFX, efficacy in an in vivo model of infection similar to the results obtained and previously reported for **2**.[7]

Because of its interesting profile, an efficient synthesis of **11** was necessary so as to obtain sufficient material for extensive in vitro and in vivo biological and toxicological studies. As shown in Scheme 1, the proline moiety was prepared in seven steps using a modified literature procedure[8] and then was coupled to the free base of D-hPhe-3-aminoquinoline, which was prepared in two steps from *N*-Boc-D-hPhe. Deprotection and purification led to the desired **11** in good yields.

In summary, conformational restriction of a residue critical to the pharmacophore of this class of RND EPIs was successful in uncoupling the observed toxic effects in rats and mice from the potentiation activity. Results of the extensive pharmacokinetic bio-profiling of **11** and

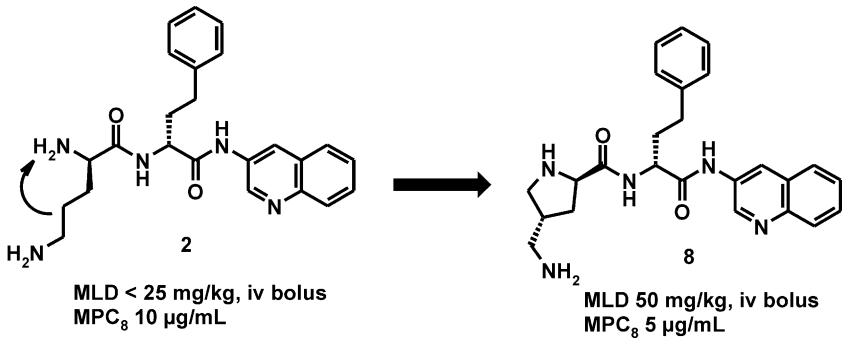
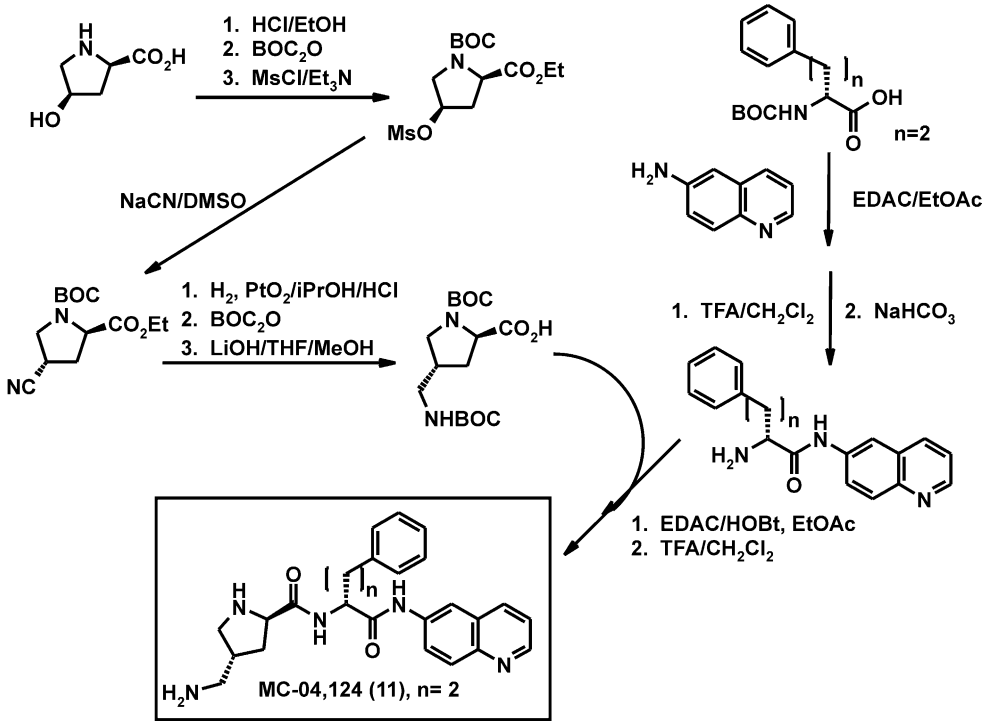


Figure 1. Conformational restriction of the ornithine group of **2**.



Scheme 1. Synthesis of MC-04,124 (**11**).

related proline-containing analogues will be the subject of a future publication.

Chemicals

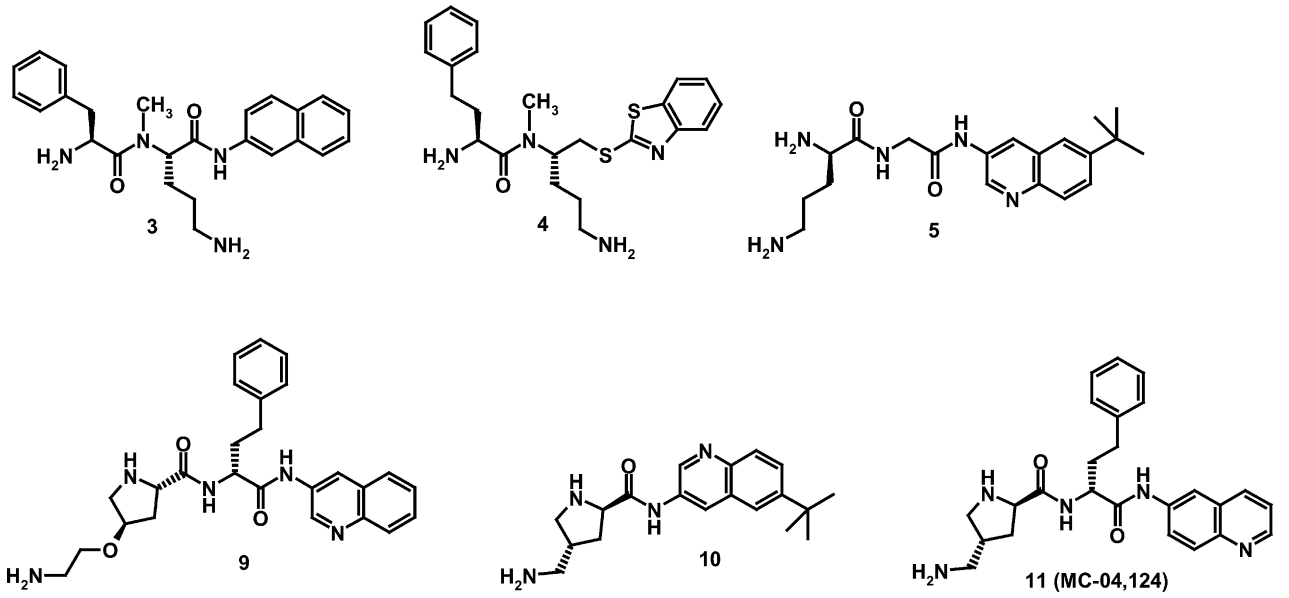
The preparation of **1** was described in ref 1, and **2** and **3** were reported in ref 2. The peptidomimetics **4** and **5** were reported in ref 3. All analogues were purified by reverse-phase MPLC and tested as their bis-TFA salts. The structural identity of each compound was confirmed by ^{1}H NMR and MS. LVFX was provided by Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan).

Toxicity Assays

LD_{50}s were determined using standard protocols. To minimize animal use, an alternative acute toxicity assay was developed whereby male Swiss Webster mice (3–5/dose) were treated iv or ip with the test compound. Endpoints were recorded as the Minimum Lethal Dose (MLD), which is defined as the minimum dose causing lethality to $\geq$66% of the animals tested.

In Vitro Potentiation assay

Compounds were assayed in the presence and absence of LVFX against PAM 1723, a laboratory strain of *P. aeruginosa* that overexpresses the MexAB-OprM pump. The activity of the inhibitors was quantified by the term MPC_8, which is the minimum concentration (μg/mL) of inhibitor required to decrease (potentiate) the MIC of LVFX 8-fold. The MIC (μg/mL) of each compound was also determined to ensure that the potentiation effect observed was not due to intrinsic antibacterial activity of the putative EPI. A more complete description of the methods utilized to monitor the biological activity of test compounds can be found in refs 5 and 6.



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