Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 663–667

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Addressing the Stability of *C*-Capped Dipeptide Efflux Pump Inhibitors that Potentiate the Activity of Levofloxacin in *Pseudomonas aeruginosa*

Thomas E. Renau,[a,*] Roger Léger,[a] Eric M. Flamme,[a] Miles W. She,[a] Carla L. Gannon,[a] Kristina M. Mathias,[a] Olga Lomovskaya,[a] Suzanne Chamberland,[a] Ving J. Lee,[a] Toshiharu Ohta,[b] Kiyoshi Nakayama[b] and Yohei Ishida[b]

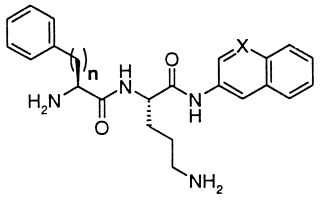
[a]*Microcide Pharmaceuticals, Inc., 850 Maude Ave., Mountain View, CA 94043, USA*
[b]*New Product Research Laboratories I, Daiichi Pharmaceutical Co. Ltd., 1-16-13, Kita-Kasai, Edogawa, Tokyo 134-8630, Japan*

Received 2 October 2000; accepted 3 January 2001

Abstract—Synthetic optimization of a biologically labile class of dipeptides that function as efflux pump inhibitors to potentiate the antibacterial agent levofloxacin in *Pseudomonas aeruginosa* has led to the discovery of a related series of compounds that are completely stable in a variety of biological matrices. Other than the stability profile, the in vitro profile of the new series is essentially identical to that observed with the original one. A prototypical compound from the new series demonstrates potentiation in an in vivo model of infection.

Efflux of antibacterial agents is increasingly being recognized as a major cause of microbial resistance.[1–4] Studies of clinical isolates that overexpress efflux pumps have demonstrated that nearly all antibacterials are substrates for one or more of these pumps. Consequently, several strategies to overcome efflux-mediated resistance have been initiated. While one approach has focused on identifying derivatives of known classes that are poor substrates for the efflux pumps,[5,6] another has targeted the inhibition of these pumps so as to enhance the activity of existing antibiotics.[7,8]

We have pursued the latter approach and recently reported the identification of a class of *C*-capped dipeptides that function as bacterial efflux pump inhibitors (EPIs) to potentiate the activity of the fluoroquinolone levofloxacin (LVFX) in *Pseudomonas aeruginosa*.[9,10] The compounds, exemplified by L-phenylalanine-L-ornithine-β-naphthylamine (L-Phe-L-Orn-β-Na, **1a**; Chart 1), were stable in growth media (e.g., Mueller Hinton Broth, MHB) and potentiated the activity of LVFX 8-fold at concentrations as low as 2.5 μg/mL but were unstable in several biological matrices such as mouse and rat human serum. These results were understandable given that the compounds contained natural (L) amino acids. Recognizing that overcoming this problem was of critical importance for the program, we devised a plan whereby we would continue our SAR studies using the original series as a template and simultaneously initiate an effort to address the instability of the entire series. The present report describes our efforts in this area. We show that following iterative structural modifications a class of stable, structurally related inhibitors was identified that



L-Phe-L-Orn-β-Na (1a): n= 1, X= CH
L-hPhe-L-Orn-3-NHQ (1b): n= 2, X= N

Chart 1.

*Corresponding author. Fax: +1-650-428-3550; e-mail: tomrenau@microcide.com

0960-894X/01/$ - see front matter
PII: S0960-894X(01)00033-6

maintains all of the favorable biological features of the original series.

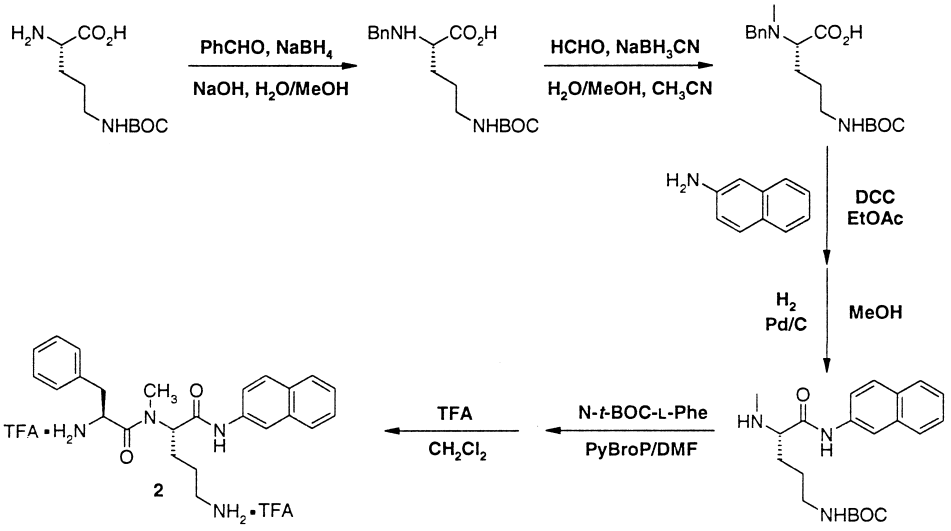
Compound **1a**, which exemplifies the original series,[9] potentiates the activity of LVFX 8-fold at 5 μg/mL but is unstable in the presence of rat serum (Table 1). The initial product of serum degradation was identified by LC–MS as L-Orn-β-Na (data not shown). This analogue had been prepared previously as part of our initial SAR studies and was shown not to potentiate LVFX.[9] To address the instability of the original series, we prepared the *N*-methyl derivative **2** according to Scheme 1. While compound **2** was as potent as **1a** in vitro ($MPC_8 = 5$ μg/mL) it was considerably more stable than **1a** in rat serum (Table 1); however, additional stability studies of **2** in Briton–Robinson buffer over a pH range of 4–8 demonstrated that the δ-amino group of ornithine readily cyclized to form a lactam, liberating β-naphthylamine (Fig. 1). The lactam, like L-Orn-β-Na, did not potentiate LVFX (data not shown).

The stability results with **2** led us to examine additional structural motifs that might retain potentiation activity. Replacing the ornithine was a logical first step, but we had previously demonstrated that although several groups could substitute for ornithine, their introduction typically led to a reduction in potency.[9] Standard approaches such as replacing the amide linkage of the capping group with an ether or hydroxyethylene moiety were considered. An alternative strategy involved

Table 1. Activity and stability profiles of study compounds

Compd	Stability in rat serum[a]; % recovery at indicated time					MIC[b] (μg/mL)	MPC_8[c] (μg/mL)
	1 min	3 min	5 min	10 min	2 h		
1a[d]	100	80	64	36	—	512	5
2	100	99	100	97	—	256	5
3	100	87	76	38	—	512	10
4d	100	98	98	100	95	>512	10
MC-207,110[e]	100	73	55	25	0	>512	10

[a]Stability of analogues was determined as described in the text. Values are the average of two or more experiments.
[b]MIC: minimum concentration (μg/mL) of efflux pump inhibitor required to inhibit the growth of PAM 1032, a laboratory strain of *Pseudomonas aeruginosa* that overexpresses the MexAB-OprM efflux pump.[12]
[c]MPC_8: minimum concentration (μg/mL) of efflux pump inhibitor required to reduce (potentiate) the MIC of levofloxacin 8-fold; analogues were evaluated in PAM 1032.
[d]See Chart 1. Analogue **1a** is referred to as compound **2** in ref 9.
[e]MC-207,110 is referred to as compound **1** in ref 9.



Scheme 1. Synthesis of compound **2**.

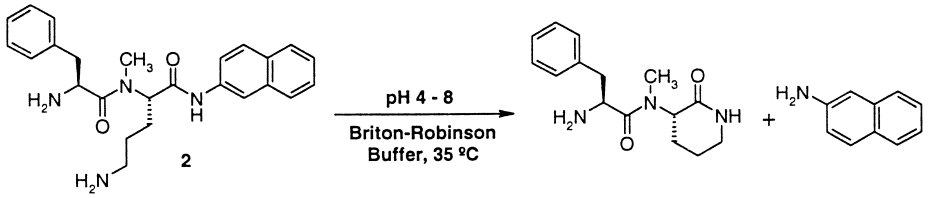


Figure 1. Lactam formation from **2**.

switching the relative positions of the two amino acids (Fig. 2). This approach furnished compounds such as L-Orn-L-Phe-β-Na (**3**), the synthesis of which is shown in Scheme 2.

Compound **3** did potentiate LVFX, although it was less potent than **1a** and **2** (Table 1). Serum stability studies of **3** demonstrated that it was unstable in rat serum (recovery @ 10 min = 38%, Table 1) but this result was not surprising given that **3** still contained two amino acids of the L-configuration. Notably, in a more extensive stability study compound **3** demonstrated no propensity to form a lactam.

We capitalized on this observation by exploring the SAR around **3**. Interestingly, various compounds from the new series were consistently 2- to 4-fold less potent when compared to their original series counterpart (such as **3** vs **1a**). Within the new series, however, the SAR profile of the compounds was essentially identical to the original series.[9] For instance, replacement of phenylalanine (such as in **3**) with homophenylalanine (hPhe) consistently gave a 2-fold improvement in potentiation activity. Similarly, replacement of β-naphthylamine with the 3-aminoquinoline (3-NHQ) moiety, to furnish compounds such as L-Orn-L-hPhe-3-NHQ (**4a**), gave derivatives having the best balance of potency and intrinsic antibacterial activity.

The effect of stereochemistry on the potentiation activity was explored by comparing the activity of the four isomers of L-Orn-L-hPhe-3-NHQ (**4a**). The results, displayed in Table 2, demonstrate that the activity of **4d** (MC-02,595, Chart 2) was essentially the same as its original series counterpart, L-hPhe-L-Orn-3-NHQ (**1b**; Chart 1). This was quite fortuitous since **4d**, which incorporated two unnatural (D) amino acids, was completely stable up to 24 h when incubated in mouse and rat serum (Table 1). In addition, **4d**, like **3**, did not form a lactam under basic conditions.

Compound **4d** demonstrated little to no potentiating activity (MPC_8 >40 μg/mL) against a strain of *P. aeruginosa* lacking all three primary efflux pumps (PAM 1626), confirming that it was acting by inhibition of the pumps. Similar results were observed for **1a**–**1b**.[9,10]

Further SAR studies supported the initial results observed for **4d**, demonstrating unequivocally that compounds of the new series that incorporated both D amino acids were stable under a variety of biological conditions and had essentially the same potentiation activity as compounds of the original series that contained the natural (L) amino acids. These results were extended in vivo where **4d**, in combination with LVFX, showed a significant effect on the growth of *P. aeruginosa* in a murine neutropenic thigh model. Both **4d** and LVFX alone resulted in growth similar to the untreated controls. The potentiation activity in vivo was shown not to be due to a pharmacokinetic interaction between **4d** and LVFX resulting in elevated LVFX concentrations (data not shown).

In summary, we have identified, via iterative synthetic manipulations, a class of EPIs that is completely stable to various biological matrices and that potentiate the activity of LVFX both in vitro and in vivo in a manner similar to that of the original series. The preparation

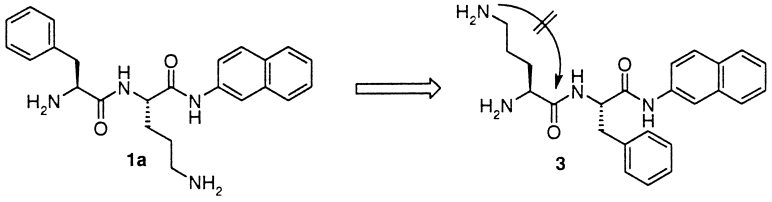
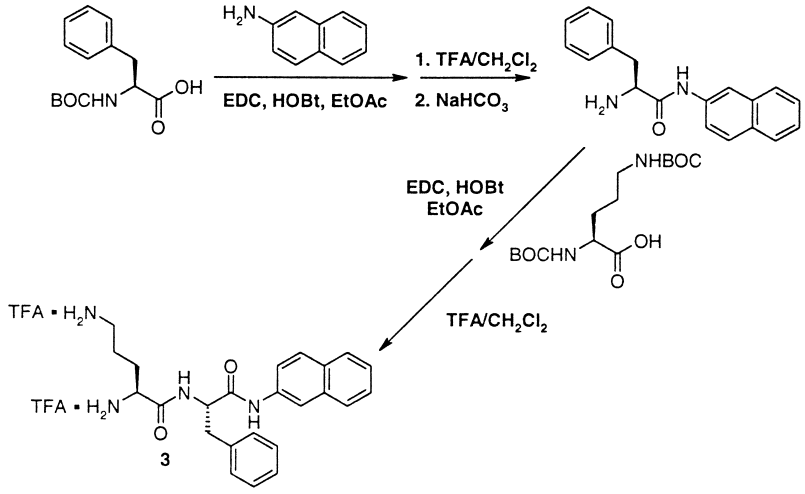
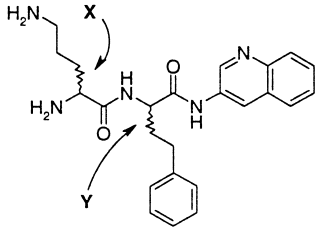


Figure 2. Switching the relative positions of the amino acids.



Scheme 2. Synthesis of compound **3**.

Table 2. Role of stereochemistry on levofloxacin potentiation



Compd	X	Y	MIC to levofloxacin (μg/mL in the presence of efflux pump inhibitor[a] Efflux pump inhibitor concentration (μg/mL) 0	5	10	20	40	MPC_8[b] (μg/mL)
4a	L	L	4	2	1	0.5	0.125	20
4b	D	L	4	4	2	0.125	0.125	20
4c	L	D	4	4	0.5	0.06	0.015	10
4d	D	D	4	1	0.125	0.03	0.015	10
1b[c]	—	—	4	1	0.06	0.03	0.03	10

[a]Compounds were evaluated in PAM 1032, a laboratory strain of *Pseudomonas aeruginosa* that overexpresses the MexAB-OprM efflux pump.[12]
[b]MPC_8: minimum concentration (μg/mL) of efflux pump inhibitor required to reduce (potentiate) the MIC of levofloxacin 8-fold.
[c]See Chart 1. Analogue **1b** is referred to as compound **12** in ref 9.

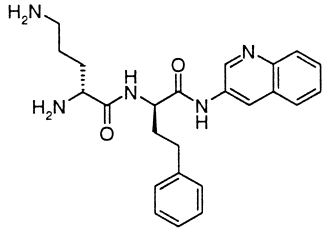
and evaluation of additional analogues of **4d**, such as various peptidomimetics, are underway. The results of these studies will be the subject of future reports.

Chemicals

Standard peptide couplings were used in the preparation of the final products. The preparation of **1a**–**1b** is described in ref 9. The *N*-methyl derivative **2** was prepared using the synthesis outlined in Scheme 1. Scheme 2 illustrates the route employed to synthesize compounds **3**,**4a**–**d**. All final compounds were purified by reverse-phase MPLC and tested as their bis-trifluoroacetic acid (TFA) salts. The structural identity of each compound was confirmed by ^1H NMR and MS. LVFX was provided by Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan).

Stability Assays

The test compounds were incubated at 37 °C in mouse or rat serum for periods ranging from 1 min to 24 h. At the appropriate time, 4 μL of trichloroacetic acid (TCA, 70%) and 200 μL CH_3CN were added dropwise to each sample. Samples were separated by centrifugation at 3600×*g* for 6 min at room temperature. Supernatants were subsequently removed and the samples filtered using a 4 mm nylon filter with a 0.45 μm pore size. In a parallel manner, test compounds were also incubated in H_2O. This control, in which all the compounds were stable, ensured that the stability of the samples was not affected by factors other than serum. Samples were analyzed by HPLC[11] and the results are recorded in Table 1 as % recovery of unchanged material. The initial lead,[9] L-phenylalanine-L-arginine-β-naphthylamine (MC-207,110), was used as a positive control. The stability of **2** over a pH range of 4–8 was also measured in Briton–Robinson buffer at 35 °C.



D-Orn-D-hPhe-3-NHQ (4d, MC-02,595)

Chart 2.

In Vitro Potentiation Assay

Compounds were assayed in the presence and absence of LVFX against PAM 1032, a laboratory strain of *P. aeruginosa* that overexpresses the MexAB-OprM pump.[12] 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.

Acknowledgements

The authors thank Scott Hecker, William Watkins and Mary Price for their review of the manuscript. We also thank Mary Price for analytical support.

References and Notes

1. Lawrence, L. E.; Barrett, J. F. *Exp. Opin. Invest. Drugs* **1998**, *7*, 199.

2. Van Bambeke, F.; Balzi, E.; Tulkens, P. M. *Biochem. Pharmacol.* **2000**, *60*, 457.
3. Poole, K. *Antimicrob. Agents Chemother.* **2000**, *44*, 2233.
4. Levy, S. B. *Antimicrob. Agents Chemother.* **1992**, *36*, 695.
5. Testa, R. T.; Petersen, P. J.; Jacobus, N. V.; Sum, P. E.; Lee, V. J.; Tally, F. P. *Antimicrob. Agents Chemother.* **1993**, *37*, 2270.
6. Brennan, L.; Duignan, J.; Petitpas, J.; Anderson, M.; Fu, W.; Retsema, J.; Rainville, J.; Smyth, K.; Su, W.; Sutcliffe, J. 38th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, CA, 24–27 September, 1998; American Society for Microbiology: Washington, DC, 1998; F-124.
7. Renau, T. E.; Hecker, S. J.; Lee, V. J. In *Ann. Rep. Med. Chem.*; Bristol, J. A., Ed.; Academic: New York, 1998; Vol. 33, pp 121–130.
8. Nelson, M. L.; Park, B. H.; Levy, S. B. *J. Med. Chem.* **1994**, *37*, 1355.
9. Renau, T. E.; Léger, R.; Flamme, E. M.; Sangalang, J.; She, M. W.; Yen, R.; Gannon, C. L.; Griffith, D.; Chamberland, S.; Lomovskaya, O.; Hecker, S. J.; Lee, V. J.; Ohta, T.; Nakayama, K. *J. Med. Chem.* **1999**, *42*, 4928.
10. Lomovskaya, O.; Warren, M. S.; Lee, A.; Galazzo, J.; Fronko, R.; Lee, M.; Blais, J.; Cho, D.; Chamberland, S.; Renau, T.; Léger, R.; Hecker, S.; Watkins, W.; Hoshino, K.; Ishida, H.; Lee, V. *Antimicrob. Agents Chemother.* **2001**, *45*, 105.
11. HPLC conditions: Column: PLRP-S100Å; 5 μm, 4.6 mm×15 cm. Flow rate: 1 mL/min. Mobile phase: 95% 0.1% TFA, 5% acetonitrile ramping to 70–73% buffer/organic over 20 min. Diode array UV detection wavelength: 225–245 and 240–280 nm.
12. Lomovskaya, O.; Lee, A.; Hoshino, K.; Ishida, H.; Mistry, A.; Warren, M. S.; Boyer, E.; Chamberland, S.; Lee, V. J. *Antimicrob. Agents Chemother.* **1999**, *43*, 1340.