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Bioorganic & Medicinal Chemistry Letters 16 (2006) 5169–5175
Bioorganic & Medicinal Chemistry Letters

Homo-cysteinyl peptide inhibitors of the L1 metallo-β-lactamase, and SAR as determined by combinatorial library synthesis

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Received 10 June 2006; revised 30 June 2006; accepted 5 July 2006
Available online 27 July 2006

Abstract—Homo-cysteinyl peptides were found to be more active than cysteinyl peptides toward L1 metallo-β-lactamase as reversible competitive inhibitors. A combinatorial library of more than 90 homo-cysteinyl peptides was synthesized and screened for their inhibitory activity toward the L1 enzyme. A systematic structure–activity relationship analysis has revealed the preferred interaction groups for L1 conserved binding sites of β-lactam substrates. The most active compound **95b**, had a K_i of 2.1 nM.

Metallo-β-lactamases (MBLs) present a serious threat to the clinical utility of the most common, least expensive and highly effective β-lactam antibiotics. This is particularly so in view of their ability to hydrolyze most β-lactam antibiotics, especially the carbapenems that are noted for their broad-spectrum activity and stability to serine β-lactamases.[1] Mechanism based inactivators for serine enzymes such as clavulanic acid, sulbactam, and tazobactam are ineffective against Zn (II)-dependent MBLs.[2] Presently, there is no clinically useful inhibitor for MBLs, although several classes of experimental MBL inhibitors have been reported. These include trifluoromethyl alcohol and ketone,[3] hydroxamate,[4] phenazines,[5] thioesters,[6–9] biphenyl tetrazoles,[10,11] thiols,[9,12–17] cysteinyl peptides,[18] cysteinyl peptide mimetics,[19–21] 2,3-disubstituted succinic acid derivatives,[22] tricyclic natural products,[23] sulfonyl hydrazones,[24] carbapenem derivatives,[25,26] and 6-(mercaptomethyl)penicillinates.[27]

A clinically useful MBL inhibitor needs to meet several challenging requirements, including broad-spectrum activity, a good synergistic effect with antibiotics, and low toxicity. Although several of the reported inhibitors showed promising properties, none of them met all the above requirements. In order to optimize current leads or to design an alternative class of compounds as clinically useful inhibitors, a full understanding of the target enzyme structures, particularly with respect to the active sites of MBLs, is important.

Cysteinyl peptide inhibitor **1** was reported as a reversible competitive inhibitor for *Bacillus cereus* metallo-β-lactamase with a K_i of 3 μM.[18] Cysteinyl peptide mimicking inhibitor **2** was also found to be a competitive inhibitor for the IMP-1 enzyme with an IC_{50} of 90 nM.[20] The activity for both compounds was proposed to result from their ability to mimic antibiotic substrates such as penicillin **3** (Fig. 1). In addition, cysteinyl peptide mimicking inhibitor **2** was shown to bind to the IMP-1 enzyme at the conserved binding site for β-lactam

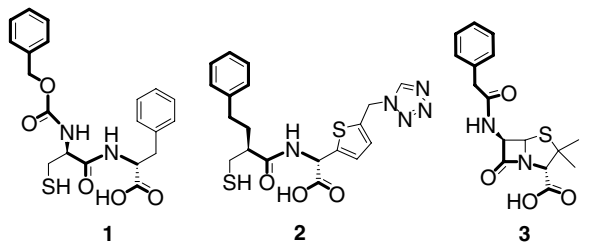

Figure 1. Structures of cysteinyl peptide inhibitor (*N*-carbobenzoxy-D-cysteinyl-D-phenylalanine) **1**, cysteinyl peptide mimicking inhibitor 2-[5-(1-tetrazolylmethyl) thien-3-yl]-*N*-[2-(mercaptomethyl)-4-(phenylbutyrylglycine)] **2**, and antibiotic substrate penicillin **3**. The bold lines highlight the similarities between the inhibitors and the substrate, and the stereoconfiguration that produces maximal inhibition is included.[18,19]

Keywords: Metallo-β-lactamase inhibitor; L1 enzyme; Cysteinyl peptide inhibitor; Homo-cysteinyl peptide inhibitor.
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0960-894X/$ - see front matter © 2006 Published by Elsevier Ltd.
doi:10.1016/j.bmcl.2006.07.001

substrates that comprises Zn metal ions, a hydrophobic pocket, and a conserved lysine.[18,19] These inhibitors demonstrated both broad-spectrum activity and high selectivity for MBLs with respect to other metallo enzymes, and are therefore candidates for further optimization.[20,21] Examination of these inhibitors suggested that chemical modification at several positions was feasible (Fig. 2), thereby allowing for a more systematic probing of the active sites of MBLs.

Combinatorial chemistry has demonstrated a high efficiency for synthesis of peptides or peptide mimetics on solid phase and provides an effective way to obtain large numbers of analogs for a given core structure. This work describes the library synthesis of analogs based on the structure of inhibitors **1** and **2**. It focuses on the determination of the structure–activity relationship (SAR) for the R^1, R^2, and R^3 positions in the context of their putative interaction with the three conserved binding sites of β-lactam substrate in the L1 enzyme: Zn metal ions, hydrophobic pocket, and serine. R^4 was fixed as a benzyl group for the purpose of this study and will be further examined in the future. The metallo-β-lactamase L1 used herein was isolated from *Stenotrophomonas maltophilia*, a significant hospital-acquired pathogen responsible for complications such as bacteremia, endocarditis, respiratory tract infection, central nervous system infection, and urinary tract infection.[28]

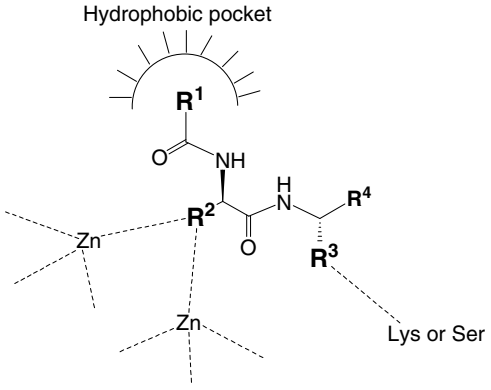


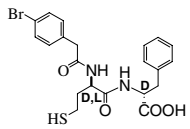
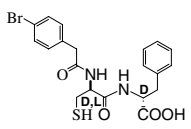
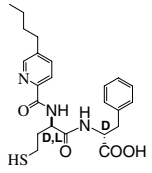
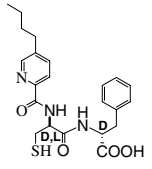
Figure 2. Potential positions for modification. R^1, R^2, and R^3 interact with the conserved β-lactam substrate binding sites including hydrophobic pocket, Zn metal ions, and serine in L1 or lysine in most other MBLs.

Stereochemistry at the two chiral centers. For cysteinyl peptide inhibitor **1** and cysteinyl peptide mimicking inhibitor **2**, the D,D configuration at the two chiral centers showed maximal inhibitory activity to both BCII and IMP-1 enzymes and also the highest selectivity against other metal enzymes.[18–20] To maximize the likelihood of developing a broad-spectrum inhibitor, the D,D configuration was used as the basis (where applicable) for all of the compounds synthesized within this study. Homo-cysteinyl peptides were synthesized from commercially available racemic homo-cysteine. Thus, all the homo-cysteinyl peptide products were tested as the unresolved diastereomer L,D and D,D pair.

Structure–activity at the R^2 position. The sulfhydryl group from cysteinyl peptide or cysteinyl peptide mimicking inhibitors is suggested to interact with the pair of Zn (II) metal ions of the active site of MBLs. As determined from the X-ray structure of the complex between IMP-1 and the cysteinyl peptide mimicking inhibitor **2**[19], the sulfhydryl group appears to interact with both zinc atoms as the sulfur to zinc distances found were 2.19 Å and 2.38 Å, both distances comparable with Zn–sulfur distances found in inorganic complexes.[29] Analogs with varying metal-ion coordinating groups were synthesized and tested for their inhibitory activity toward the L1 enzyme. Substitution of cysteine by tyrosine, lysine, histidine, aspartic acid, glutamic acid, serine, methionine, arginine, ornithine or homo-serine resulted in a total loss of inhibition when tested in a preliminary assay,[30] whereas the homo-cysteinyl analog gave comparable inhibition to the parent cysteinyl compound (both showed better than 80% inhibition). Determination of K_is demonstrated that the homo-cysteinyl peptide **4** was more active than the cysteinyl peptide **5**. This trend, although significantly less so, was also found for another pair of analogs 6 and 7 (Table 1). It is further noted that as racemic homo-cysteine was used, the actual activity of the preferred isomer may likely be higher.

Based on these results, homo-cysteine was selected for the R^2 position in subsequent analogs, for optimization of both the R^1 and R^3 positions.

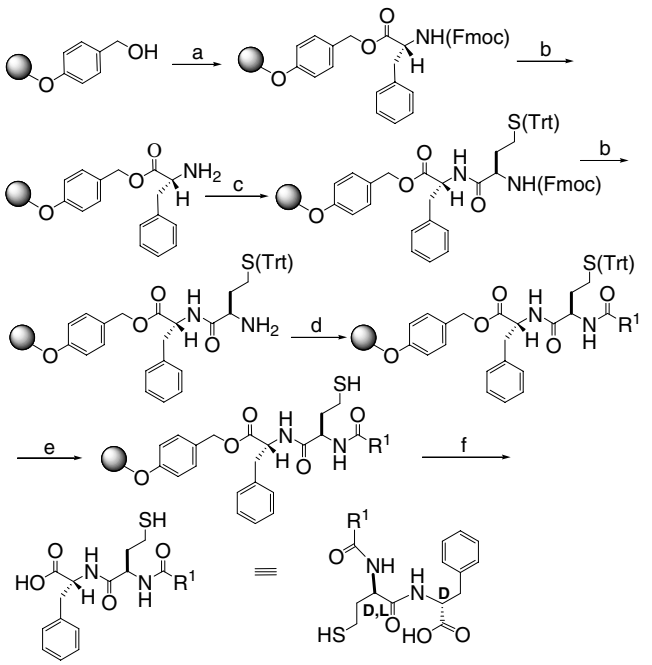
Table 1. Homo-cysteinyl and cysteinyl peptide inhibitory activity to L1 enzyme

Compound	Homo-cysteinyl peptide	K_i to L1 (μM)	Compound	Cysteinyl peptide	K_i to L1 (μM)
4		0.35 ± 0.07	**5**		3.67 ± 0.38
6		0.23 ± 0.04	**7**		0.88 ± 0.09

Structure–activity at the R^1 position. In the development of β-lactam antibiotics, most of the structural diversity occurs at the 6β position for penicillin derivatives and the 7β position for cephalosporin derivatives. These modifications probe interactions with the hydrophobic pocket of MBLs. Considering the broad substrate profiles of MBLs, it was decided to explore in detail the molecular basis for the interaction of groups at the R^1 position with the hydrophobic pocket. To this end, a homo-cysteinyl-based peptide library of 85 analogs varying at the R^1 position was synthesized and tested for inhibition of the L1 enzyme. As shown in Scheme. 1, a solid phase synthesis protocol using Wang resin (*p*-benyloxybenzyl alcohol resin) and standard peptide chemistry was used to produce a homo-cysteinyl peptide library.

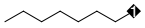
The synthesized compounds were tested in a L1 inhibitory assay without further purification. Based on these preliminary assay results, analogs were categorized into several groups for activity comparison (Table 2a–c). As seen, of the **21** diverse analogs categorized with an aliphatic group at the R^1 position, only 2 compounds, **27** and **28**, showed >80% inhibition.

Among those with an aromatic group at the R^1 position, structures associated with higher inhibition were: (a) indole scaffold (compounds **86**–**90**); or (b) phenyl scaffold with meta-carboxylic acid or meta-nitro-substitution (compounds **46**–**49** and **84**). When compared to the meta-carboxylic acid or meta-nitro substituted analogs, the comparable ortho- or para-substituted compounds showed a much lower inhibition in the preliminary assay (compounds **29**, **36**, and **37**).



Scheme 1. Reagents: (a) Fmoc-D-Phe-OH, DIC, DMAP, DCM/DMF; (b) 20% Pip/DMF; (c) Fmoc-D,L-Hcy(Trt)-OH, DIC, HOBt, DCM/DMF; (d) R^1COOH, DIC, HOBt, DCM/DMF; (e) 3% TFA/4% TES/DCM; (f) 60% TFA/20% TES/DCM.

Table 2. Preliminary assay results of inhibitory activity to L1 enzyme for all analogs at R^1 position

Compound	R^1
(***2a***) *Aliphatic analogs*	
0–20% inhibition	
8	
21–40% inhibition	
9	
41–60% inhibition	
10	
11	
12	
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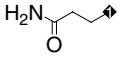
16	
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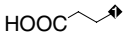
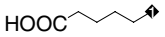
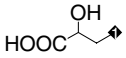
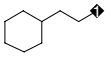
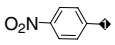
18	
19	
20	
61–80% inhibition	
21	
22	
23	
24	
25	
26	
81–100% inhibition	
27	
28	
(***2b***) *Non-hetero-aromatic analogs*	
41–60% inhibition	
29	
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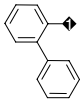
32	

(*continued on next page*)

Table 2 (*continued*)

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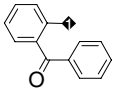
33	
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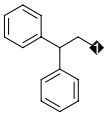
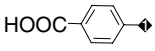
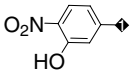
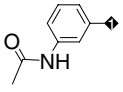
35	
61–80% inhibition	
36	
37	
38	
39	
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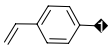
41	
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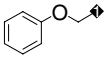
43	
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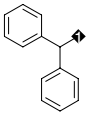
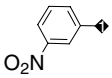
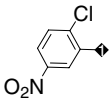
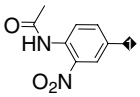
45	
81–100% inhibition	
46	
47	
48	

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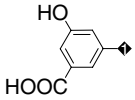
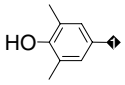
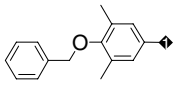
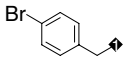
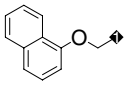
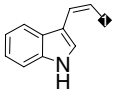
49	
50	
51	
52	
53	
(**2c**) *Hetero-aromatic analogs* 21–40% inhibition	
54	
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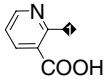
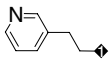
41–60% inhibition	
56	
57	
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Table 2 (*continued*)

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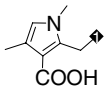
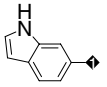
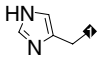
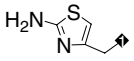
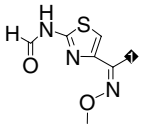
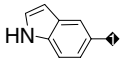
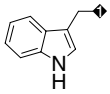
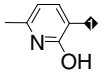
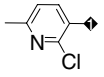
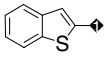
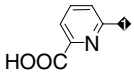
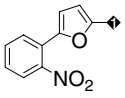
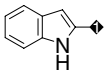
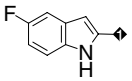
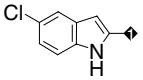
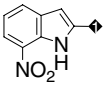
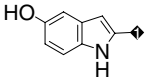
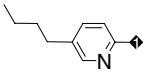
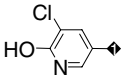
68	
69	
70	
71	
72	
61–80% inhibition	
73	
74	
75	
76	
77	
78	
79	
80	
81	
82	
83	
81–100% inhibition	
84	
85	
86	

Table 2 (*continued*)

Compound	R^1
87	
88	
89	
90	
91	
92	

Compounds showing over 80% inhibition in the preliminary assay were considered as hits. To obtain a more accurate SAR for the active compounds, all the hits were further purified and a full determination of the K_i values was done[31] (Table 3). When considering the indole scaffold, electron-withdrawing substitution (compounds **87**–**89**) leads to an increase of inhibitory activity and electron-donating substitution (compound **90**) leads to a decrease of inhibitory activity compared to the unsubstituted form (compound **86**). Meta-carboxylic acid-substituted phenyl scaffold (compounds **49** and **84**) is associated with the highest inhibitory activity amongst all the hits, which suggests that the meta-carboxylic acid group may have a special interaction with the active site of L1 enzyme.

Structure–activity at the R^3 position. The carboxylate attached to the 5- or 6-membered rings of the β-lactam antibiotics has been shown to participate in an electrostatic interaction with a conserved lysine present in most of the MBLs, L1 being an exception in that this position in the binding site is occupied by a serine residue. Conversion of the carboxylic acid (compounds **94** and **96**) to the corresponding amide (compounds **93** and **95**) significantly increases the inhibitory activity toward the L1 enzyme (Table 4). However, for MBLs with lysine in the binding site (IMP-1 and CcrA), this substitution (acid to amide) leads to a decrease in inhibition.

The most active compound **95** which incorporates the optimum structures found for the R^1, R^2, and R^3 positions, and tested as the unresolved L,D and D,D pair, exhibited competitive inhibition against L1 enzyme with a K_i of approximately 6 nM (Fig. 3).

These two diastereomers, designated as **95a** and **95b**, were separated by reversed-phase HPLC and when tested individually were found to have K_i of 0.13 ± 0.01 μM and 0.0021 ± 0.0002 μM, respectively.

Table 3. Homo-cysteinyl library hits structure and K_i value to L1 enzyme

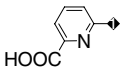
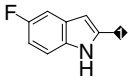
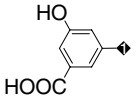
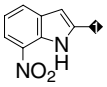
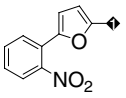
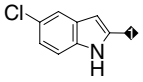
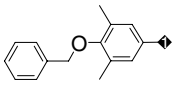
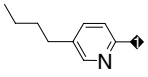
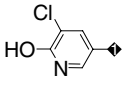
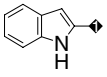
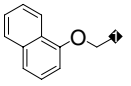
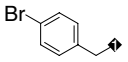
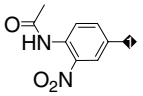
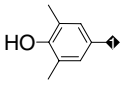
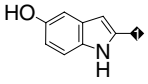
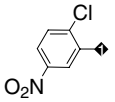
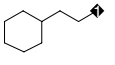
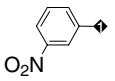
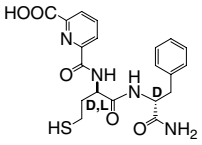
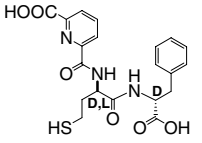
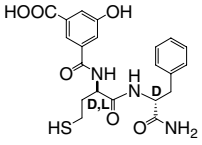
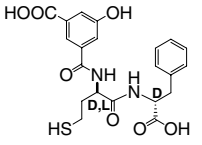
Compound	R^1	K_i to L1 (μM)
84		0.012 ± 0.02
87		0.012 ± 0.02
49		0.14 ± 0.01
89		0.15 ± 0.01
85		0.16 ± 0.02
88		0.20 ± 0.03
51		0.20 ± 0.03
91		0.23 ± 0.04
92		0.26 ± 0.07
86		0.29 ± 0.03
53		0.29 ± 0.02
52		0.35 ± 0.07
48		0.39 ± 0.05
27		0.52 ± 0.09
50		0.54 ± 0.09
90		0.57 ± 0.27
47		0.78 ± 0.05
28		0.78 ± 0.06
46		1.03 ± 0.11

Table 4. Amide and acid inhibitory activity to L1 enzyme

Compound	R^1	K_i to L1 (μM)
93		0.026 ± 0.005
94		0.12 ± 0.02
95		0.0058 ± 0.0004
96		0.14 ± 0.01

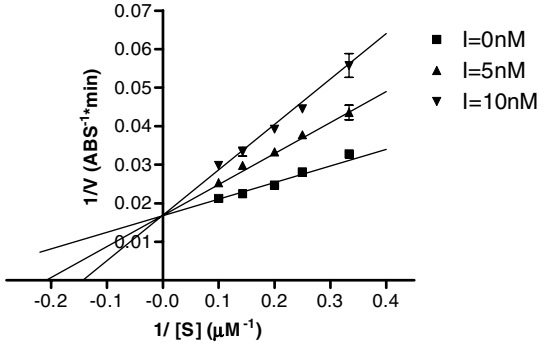


Figure 3. Lineweaver–Burk Plot reveals competitive L1 inhibition by compound **95**.

In summary, sub-structure optimization of peptide inhibitors of L1 enzyme demonstrated a preference for homo-cysteine over cysteine at position R^2. Substitution by other metal-ion ligands resulted in a full loss of activity in preliminary assay, confirming the essential requirement for the sulfhydryl group for the inhibition. In the absence of an absolute stereochemical determination at this position, by comparison with the preferred stereochemistry of the cysteine analogs, the stereochemistry of the more active isomer is most likely to be in the D form. If confirmed, this result suggests the real possibility of finding a broad-spectrum inhibitor for all three L1, IMP-1, and BCII enzymes. A detailed SAR (R^1) was determined for the hydrophobic binding pocket and a preference for the amide at the R^3 position is consistent with the loss of the basic residue (lysine to serine) in the active site of the L1 enzyme. The most active compound **95b**, with a K_i of 2.1 nM, is the first nanomolar inhibitor of L1 enzyme reported to date. The extension of this

research from L1 to other MBLs will provide more information about the active sites of different MBLs and will accelerate the process to find clinically useful inhibitors.

Acknowledgments

The authors thank Professor Michael W. Crowder from Department of Chemistry and Biochemistry, Miami University, Ohio, USA, for providing the L1 and CcrA enzymes and Professor James Spencer from Department of Cellular and Molecular Medicine, University of Bristol School of Medical Sciences, UK, for providing the IMP-1 enzyme. The authors also thank Mrs. Leslie J. Willingham for assistance with library synthesis and Mr. Charles K. Arrington for assistance with MS and HPLC analysis.

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30. Preliminary assay condition: 1.35 nM L1 enzyme, 50 μM nitrocefin substrate, 200 μM inhibitor, and 50 mM MOPS buffer, pH 7. The inhibition percentage was calculated based on the comparison of the initial velocity and EDTA was used as the internal control.
31. Steady-state enzyme kinetics were performed at 25 °C in 50 mM MOPS buffer, pH 7.0, in a 1 ml cuvette reaction volume in a Lambda 20 spectrophotometer. Hydrolysis of nitrocefin was monitored at 480 nm. L1 enzyme (final concentration of 1.35 nM) was pre-incubated with inhibitors for 1 min, and then the initial velocities were measured immediately after the addition of the substrate. Analysis of enzyme kinetic data was carried out using Prism 4.0 software for regression process of the data.