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Optimization of amino acid thioesters as inhibitors of metallo-β-lactamase L1

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ABSTRACT

The emergence of antibiotic resistance caused by metallo-β-lactamases (MβLs) is a global public health problem. Recently, we found amino acid thioesters to be a highly promising scaffold for inhibitors of the MβL L1. In order to optimize this series of inhibitors, nine new amino acid thioesters were developed by modifying the substituents on the N-terminus of the thioesters and the groups representing the amino acid side chain. Biological activity assays indicate that all of them are very potent inhibitors of L1 with an IC_{50} value range of 20–600 nM, lower than those of most of the previously reported inhibitors of this scaffold. Analysis of structure–activity relationship reveals that big hydrophobic substituents on the N-terminus and a methionine amino acid side chain improves inhibitory activity of the thioesters. All these inhibitors are able to restore antibacterial activity of a β-lactam antibiotic against *Escherichia coli* BL21(DE3) cells producing L1 to that against *E. coli* cells lacking a β-lactamase. Docking studies reveal that a large N-terminal hydrophobic group results in a slightly different binding mode than smaller hydrophobic groups at the same position.

β-Lactam-containing drugs, such as, penicillins, cephalosporins and carbapenems are among the most important and frequently used antimicrobial agents, which prevent transpeptidase-mediated cross-linking of adjacent peptidoglycan strands to inhibit cell wall biosynthesis.[1,2] Over the past 70 years, the overuse of β-lactam-containing antibiotics has resulted in a large number of bacteria that are resistant to almost all antibiotics. Most commonly, bacteria become resistant to β-lactam antibiotics by producing β-lactamases, which hydrolyze the C–N bond in the four-membered ring of the β-lactam antibiotics.[3] β-Lactamases consist of four classes of enzymes (A–D) based upon DNA sequence similarity. The groups A, C and D are called serine β-lactamases (SβLs), which exhibit biological activity by utilizing an active-site serine as a nucleophile to attack the β-lactam carbonyl.[4] The group B enzymes require 1–2 Zn(II) ions to hydrolyze β-lactams and thus are called metallo-β-lactamases (MβLs). Now MβLs are of increasing clinical concern, because they hydrolyze almost all β-lactam antibiotics.[5] However, to date there are no inhibitors of the MβLs available in the clinic.[6,7] MβLs have been further subdivided into three subclasses, B1, B2, and B3. The MβL L1, a representative of B3 subclass enzymes, requires two metal ions in the active site for full activity: Zn1 has His116, His118, His196, and a bridging hydroxide as ligands; Zn2 has Asp120, His121, His263, the bridging hydroxide, and a terminally bound H_2O as ligands.[8]

Facing the increasing challenge of antibiotic resistance, a number of MβL inhibitors have been reported, including β-lactam derivatives,[9–12] thiol carboxylates,[13–15] and sulfonamides.[16] Also, aspergillomarasmine A (AMA)[5] and ebselen[17] as inhibitors of New Delhi metallo-β-lactmase-1 (NDM-1) have been described. Recently, we found that mercaptoacetic acid thioester, found in compounds **A**, **B**, and **C** (Fig. 1), is a highly promising scaffold for the development of specific L1 inhibitors.[18] In order to further increase the inhibitory activity of these compounds against L1, our idea was to change the substituent R^1 into a non-polar group as shown in Figure 1. In addition, the structure–activity relationship gained in our recent work implies that methionine and tryptophan side chains as R^2 groups are favorable for inhibitory activity,[18] suggesting to retain these amino acid side chains. Based on these ideas, we designed and synthesized compounds **1**–**3** and **7**–**9**. The series **4**–**6** with R^1 groups corresponding to those in **1**–**3**, but with a phenyl group derived from phenylalanine as the R^2 group was hypothesized to exhibit less inhibition. Thus, these compounds were synthesized and tested for comparison. Thioester

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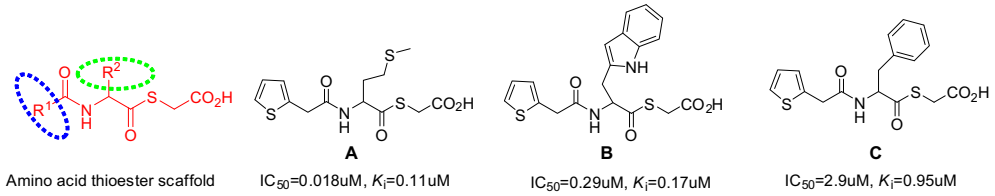


Figure 1. Amino acid thioester scaffold and structures of specific compounds **A**–**C** reported previously.[18]

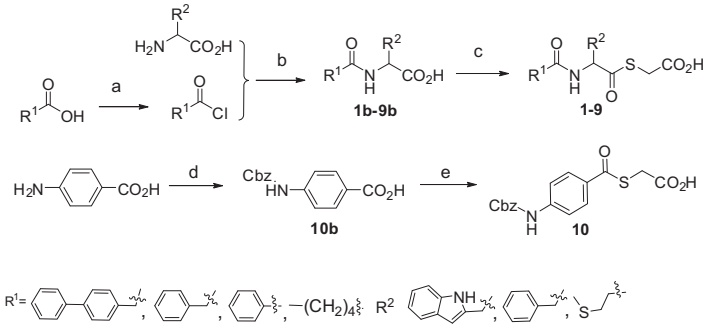
10 lacking an amino acid side chain was also included as a reference.

The synthetic pathway of the ten thioesters is shown in Scheme 1. Firstly, hexanoic acid, phenylacetic acid, 4-biphenylacetic acid, and benzoic acid, respectively, were refluxed with sulfur dichloride to get the corresponding acyl chlorides.[19,20] Secondly, racemic mixtures of amino acids reacted respectively with the four kinds of acyl chloride to give amides **1b**–**9b**. Finally, the amides reacted with mercaptoacetic acid to give the target products mercaptoacetic acid thioesters **1**–**9**. The synthetic process of **10b** and **10** is similar to that of **1b**–**9b** and **1**–**9**. The structures of the synthesized mercaptoacetic acid thioesters are shown in Figure 2. These synthesized thioesters were all characterized by ^{1}H and ^{13}C NMR and confirmed by MS (see Supporting information). Due to racemic mixtures of amino acids being used, the products are not expected to be chiral.

To test whether the inhibitory activities of these mercaptoacetic acid thioesters against L1 have improved, L1 was over-expressed and purified as previously described.[21] In vitro, the inhibitory activities of all compounds prepared were tested against L1 on an Agilent UV8453 spectrometer as described by Bush et al. using cefazolin as the substrate.[22] The substrate concentration was 60 μM, and inhibitor concentrations were varied between 0.01 and 1 μM. Enzyme and inhibitor were pre-incubated for 30 min before starting the kinetic assays. The inhibitor concentrations causing 50% decrease of enzyme activity (IC_{50}) were calculated based on the kinetic data. The IC_{50} values of the ten compounds against L1 with cefazolin as substrate are listed in Table 1. It can be observed that the thioesters **1**–**9** exhibited strong inhibition of L1 with an IC_{50} value range of 0.02–0.6 μM, while compound **10** had no inhibitory activity. A possible reason is that the amino acid in its structure was replaced by 4-aminobenzoic acid. Clearly, the IC_{50} values of **1**, **4** and **7** are smaller than those of **2**, **5** and **8**, respectively, revealing that the 4-diphenyl methyl as R^1 results in stronger inhibitory activity against L1 than the benzyl. IC_{50} values of **2** and **5** are lower than the corresponding values of **3** and **6**, respectively, indicating that the aromatic R^1 improves inhibitory activity compared to the aliphatic pentyl group. This activity relationship is related to the space sizes of these substitutes, that is, the 4-diphenyl methyl is larger than the benzyl, and the benzyl is larger than the pentyl group. Within the series with a constant 4-diphenyl methyl R^1 group, **1** and **7** are more potent than **4**, while in the series with a constant benzyl R^1 group, **2** and **8** are more potent than **5**, implying that the tryptophan and methionine amino acid side chains (R^2) are more favorable than the phenylalanine side chain, which is in agreement with previous results.[18] With the aliphatic pentyl R^1 group, the tryptophan side chain as R^2 in **3** did not result in higher potency than the phenylalanine side chain in **6**. **9** had almost identical potency as **8**, indicating that the phenyl R^1 group is nearly equivalent to the benzyl R^1 group. Furthermore, compared with the previously reported compounds **B** and **C**,[18] the inhibitory activities of **1** and **4** are increased, indicating that the 4-diphenyl methyl R^1 group is superior to the 2-thiophenyl methyl group.

The capacity of the mercaptoacetic acid thioesters to restore the antibacterial activity of cefazolin against *Escherichia coli* cells expressing L1 was investigated by determining the minimum inhibitory concentrations (MICs) of cefazolin in the absence and presence of 16 μg/mL **1**–**10**. MIC values were determined by using the Clinical and Laboratory Standards Institute (CLSI) macrodilution (tube) broth method.[23] The bacterial strain of *E. coli* BL21(DE3) containing plasmids pET26b-L1, as well as an *E. coli* BL21(DE3) control without plasmid, were used to assess these inhibitors. The final concentration of inhibitor was 16 μg/mL. The data listed in Table 2 shows that inhibitors **1**–**9** resulted in at least 4-fold reduction of MIC for *E. coli* BL21(DE3) expressing L1, effectively restoring the MIC observed with *E. coli* not expressing L1, indicating successful inhibition of L1 in vivo (inside the bacterial cells). Inhibitor **10** did not decrease the MIC relative to the blank control, indicating no inhibition of L1, which is consistent with the absence of any inhibition in vitro.

In order to clarify why the introduction of 4-biphenyl methyl as R^1 can enhance inhibitory activity, **4**, **5** and **6** were docked into the active site of the L1 crystal structure (PDB code 2AIO)[24] using the same procedure as reported previously.[25] We chose these compounds because of a significant improvement of their inhibitory effect compared to inhibitor **C** (IC_{50} = 2.9 μM).[18] Low-energy conformations (the top ranked conformations) of **4**, **5**, and **6** docked



Scheme 1. Synthetic route of mercaptoacetic acid thioesters. Reagents and conditions: (a) $SOCl_2$, DMF, 80 °C, 3 h; (b) NaOH, diethyl ether, HCl; (c) mercaptoacetic acid, ethyl chloroformate, Et_3N, AcOEt/DMF; (d) CbzCl, NaOH, diethyl ether, HCl; (e) mercaptoacetic acid, ethyl chloroformate, Et_3N, DMF.

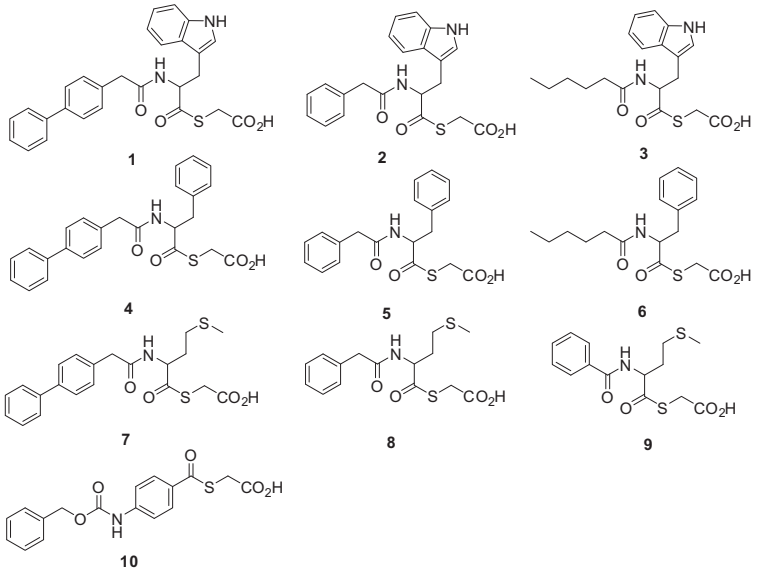


Figure 2. Structures of the synthesized mercaptoacetic acid thioesters.

Table 1
Inhibitory activities of mercaptoacetic acid thioesters against metallo-β-lactamase L1

Inhibitor	IC_{50} (μM)	Inhibitor	IC_{50} (μM)
1	0.08	**6**	0.33
2	0.21	**7**	0.02
3	0.60	**8**	0.09
4	0.14	**9**	0.08
5	0.25	**10**	–

Cefazolin was used as substrate; inhibitor concentrations were varied between 0.01 and 1 μM.
– No inhibition observed at the concentrations tested.

into the L1 active site with binding energies of −12.9, −12.3 and −11.5 kcal mol^{-1}, are shown in Figure 3, panels (B), (C), and (D), respectively. These binding energies showed a trend corresponding to the experimental IC_{50} values (0.14, 0.25, and 0.33 μM, respectively). The binding mode of **5** and **6** is the same as that of the previously docked compounds **A** and **B**, which is that one of the carboxylate oxygens bridges the two Zn(II) ions, while the other oxygen coordinates Zn2 and hydrogen bonds with Ser221, while the amide nitrogens form hydrogen bonds with the Tyr32 side chain (Fig. 3C and D).[18] The phenylalanine side chain (R^2) is located in a position corresponding to that of the methionine and tryptophan side chains of compounds **A** and **B**[18] and the 4-hydroxyphenyl ring of a co-crystalized moxalactam hydrolysis product (Fig. 3A).[24] The R^1 groups of **5** and **6** as well as **A** and **B**[18] are located in a space unoccupied by hydrolyzed moxalactam next to Tyr32. For inhibitor **4**, the big 4-biphenyl group changes the binding mode slightly. The position of the carboxylate is similar to that of **5** and **6**, but the amide oxygen hydrogen bonds with Tyr32 rather than the amide nitrogen and the thioester oxygen interacts with Zn1 (Fig. 3B). The R^1 group occupies the same position as R^1 in compounds **5** and **6**; however, the central portion of the molecule seems to be less extended and rather folded, probably due to the larger R^1 group. As a result, the orientation of the amide resembles more that of the amide in hydrolyzed moxalactam, including the hydrogen bond with Tyr32 via the amide oxygen rather than the nitrogen (Fig. 3A and B).

Table 2
MICs of cefazolin (μg/mL) in the presence and absence of mercaptoacetic acid thioester inhibitors at a concentration of 16 μg/mL observed in *E. coli* cells expressing L1 or no β-lactamase

Inhibitor	*E. coli*	*E. coli*-L1	Inhibitor	*E. coli*	*E. coli*-L1
Blank	4	16	**6**	4	2
1	4	4	**7**	4	4
2	4	4	**8**	4	4
3	4	4	**9**	4	4
4	4	2	**10**	4	16
5	4	2			

In conclusion, in further optimizing the inhibitor scaffold against L1, ten new mercaptoacetic acid thioesters were designed and synthesized. Biological activity assays indicate that nine thioesters containing amino acids exhibited IC_{50} values ranging from 0.02 to 0.6 μM, which are lower than those of the inhibitors reported previously.[18] All these inhibitors were able to decrease MIC values of cefazolin using *E. coli* BL21(DE3) cells producing L1 to levels when using the same cells not expressing any β-lactamase, thus effectively restoring the antimicrobial activity of the antibiotic by inhibiting L1. Analysis of structure–activity relationship reveals that replacement of 2-thiophenyl methyl[18] or benzyl R^1 groups by 4-diphenyl methyl improves the inhibitory activity of the thioesters. Docking studies revealed that the introduction of 4-diphenyl methyl as R^1 altered the orientation of the inhibitor slightly, resulting in a binding fashion that is more similar to that of a moxalactam hydrolysis product co-crystallized with L1.[24] As previously observed,[18] using methionine in the amino acid thioesters yielded the highest L1 binding affinity.

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Supplementary data

Supplementary data (detailed synthesis procedure, NMR and ESI mass data for target compounds; methods for enzyme overexpression and purification; inhibition assay; determination of MIC and IC_{50}; and docking study) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.08.048.

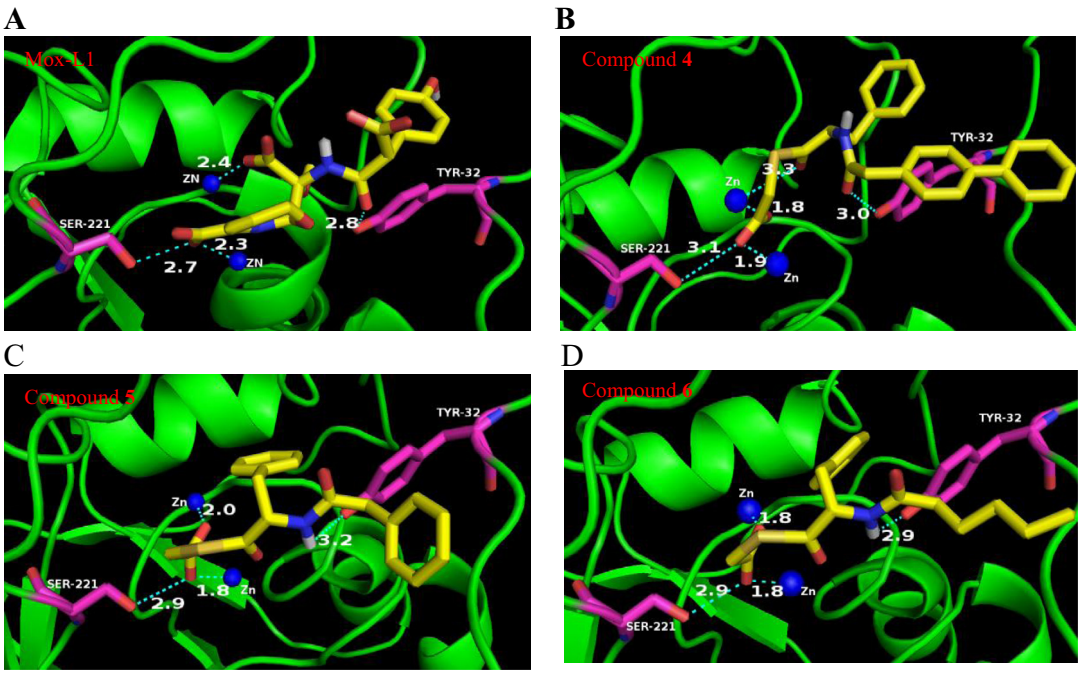

Figure 3. Hydrolyzed moxalactam co-crystallized with L1 (A).[24] Low energy conformations of **4** (B), **5** (C) and **6** (D) docked into the active site of L1. The enzyme backbone is shown as a cartoon in green and selected residues are shown as sticks colored by atom (C, magenta; N, blue; O, red). The Zn(II) ions are shown as blue spheres. Hydrolyzed moxalactam and three inhibitors are shown as sticks colored by atom (C, yellow; N, blue; O, red; S, pale yellow). Key interactions between the inhibitors and protein residues are indicated by dashed lines. The upper one is Zn1 and the lower one is Zn2. These figures were generated with PyMOL.

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