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Azolylthioacetamide: A Highly Promising Scaffold for the Development of Metallo-β-lactamase Inhibitors

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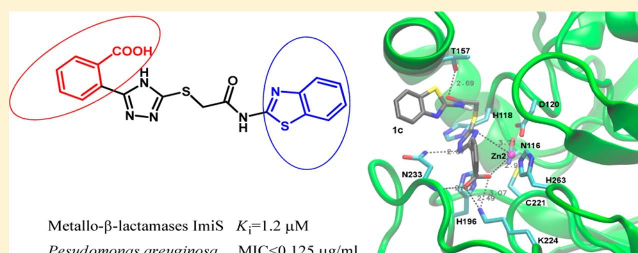
Supporting Information

ABSTRACT: A new scaffold, azolylthioacetamide, was constructed and assayed against metallo-β-lactamases (MβLs). The obtained molecules specifically inhibited MβL ImiS, and **1c** was found to be the most potent inhibitor, with a K_i = 1.2 μM using imipenem as substrate. Structure−activity relationships reveal that the aromatic carboxyl improves inhibitory activity of the inhibitors, but the aliphatic carboxyl does not. Compounds **1c−d** and **1h−i** showed the best antibacterial activities against *E. coli* BL21(DE3) cells producing CcrA or ImiS, resulting in 32- and 8-fold reduction in MIC values, respectively; **1c** and **1f−j** resulted in a reduction in MIC against *P. aeruginosa*. Docking studies revealed that **1a**, **1c**, and **1d** fit tightly into the substrate binding site of CphA as a proxy for ImiS with the aromatic carboxylate forming interactions with Lys224, the Zn(II) ion, the backbone of Asn233, and hydrophobic portions of the inhibitors aligning with hydrophobic patches of the protein surface.



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The development of β-lactam antibiotics over the past 70 years has led to the availability of drugs to treat a wide range of Gram-positive and Gram-negative bacterial infections. However, overuse of antibiotics has resulted in a large number of bacteria that produce β-lactamases,[1] and the resulting bacteria are resistant to the most commonly used antibiotics including penicillins, cephalosporins, and carbapenems. β-Lactamases are enzymes that inactivate β-lactam antibiotics by breaking the C−N bond of the β-lactam ring and render the drugs ineffective.[2]

There have been more than 1000 distinct β-lactamases identified, and these enzymes have been categorized into classes A to D, based on their amino acid sequence homologies.[3] Class A, C, and D enzymes are collectively called serine β-lactamases. These enzymes use a common catalytic mechanism in which an active-site serine nucleophilically attacks the β-lactam carbonyl, ultimately leading to a cleaved β-lactam ring. Class B enzymes, also called metallo-β-lactamases (MβLs), utilize either 1 or 2 equiv of Zn(II) to catalyze the β-lactam hydrolysis.[4] MβLs have been further subgrouped into subclasses B1 to B3, based on amino acid sequence homologies and Zn(II) content.[3] MβLs belonging to the B1 and B3 subclasses can hydrolyze almost all known β-lactam antibiotics. In contrast, the B2 subclass enzymes have a narrow substrate profile including carbapenems, which have been called "last resort" antibiotics.[5] There are no inhibitors of the MβLs available in the clinic to date.[6]

In a search for agents that could prevent the hydrolysis of antibiotics by MβLs, a large amount of effort has been made toward identifying novel inhibitors of these enzymes. However, the absence of a highly populated metastable covalent intermediate in the catalytic mechanism (as observed in serine β-lactamases) and the subtle but significant variations in MβLs' active site architectures make it difficult to search for clinically useful inhibitors that act on all three subclasses of MβLs or even on all the enzymes within the same subclass.[7] Toney et al. reported biphenyl tetrazoles and succinic acids to be effective inhibitors of the MβL IMP-1.[8] Mercaptocarboxylate,[9] 3-subsutituted phthalic acid derivatives,[10] and *N*-arylsulfonyl hydrazones[11] also displayed inhibitory activities on IMP-1. A 2-picolinic acid-based inhibitor was found to inhibit the B2 subclass CphA.[12] Also some broad-spectrum inhibitors of the MβLs have been reported, including mercaptophosphonate compounds, thiomandelic acid, and thiols.[13]

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The MβL ImiS is a representative of the B2 subclass enzymes and exhibits maximal activity with only one Zn(II) bound.[14] There has been a large amount of effort in structural, spectroscopic, mechanistic, steady-state kinetic, and inhibition studies of this enzyme.[15] The crystal structures of several MβLs, including CcrA[9] and L1,[16] which are B1 and B3 subclass enzymes, respectively, have been solved. Although no structure of ImiS is available to date, crystal structures of the very closely related B2 enzyme CphA[13] and the more distantly related Sfh-I[17] have been reported. Spectroscopic studies indicate that the catalytic activity of ImiS requires that Zn(II) or Co(II) (in substituted ImiS) is bound by a cysteine and a histidine residue and the metal ion can adopt 4- or 5-fold coordination.[18] Our studies showed that the reaction of ImiS with imipenem leads to a product-bound species, coordinated to Zn(II) via a carboxylate group;[19] EPR studies revealed that the metal ion in Co(II)-ImiS is 4-coordinate.[20] Site-directed mutagenesis studies indicated that Lys224 plays a catalytic role in ImiS, while the side chain of Asn233 does not play a role in binding or catalysis.[21] Steady-state kinetic studies revealed that Co(II)-ImiS exhibited the catalytic constants k_{cat} of 255 s^{-1} and K_m of 99 μM when using imipenem as substrate.[21] Inhibition studies revealed that *N*-heterocyclic dicarboxylic acid is a competitive inhibitor of ImiS with a K_i value of 7.1 μM.[15]

Azoles such as thiadiazoles, oxadiazoles, and triazoles[22] have been found to possess broad-spectrum antimicrobial activity and MβL inhibition. The exploration of new heterocycles that have potency to multiple biological targets remains an intriguing scientific endeavor. Recently, we reported diaryl-substituted azolylthioacetamides as broad-spectrum inhibitors of MβLs.[23] Our goal is to develop the specific or even broad-spectrum inhibitors of MβLs and to use these inhibitors as drug/inhibitor combinations to combat bacterial infections in which the bacteria produce a MβL. Toward this goal, novel azolylthioacetamides were synthesized and characterized (Figure 1). These compounds were tested as inhibitors against the purified MβLs CcrA, NDM-1, ImiS, and L1, which are representative enzymes belonging to the B1a, B1b, B2, and B3 subclasses of MβLs, respectively.[24] Furthermore, antimicrobial activities of these inhibitors in combination with existing antibiotics against antibiotic resistant strains were evaluated.

Twenty azolylthioacetamides (Figure 1) were synthesized as shown in Scheme 1. First, thiosemicarbazide was refluxed respectively with phthalic anhydride, succinic anhydride, or glutaric anhydride in acetonitrile to give substituted thiosemicarbazides (**4**−**6**). These intermediates were then refluxed in NaOH aqueous solution and acidified with dilute HCl to offer thiol triazoles (**7**−**9**).[25] Anilines with various substituents were acylated with chloroacetyl chloride to get α-chloroacetamides (**a**−**j**). Finally, under alkaline conditions, the thiol triazoles and α-chloroacetamides were cross-linked by a nucleophilic substitution reaction to afford the target products azolylthioacetamides (**1a**−**j**, **2a**−**e**, and **3a**−**e**).[26] All these azolylthioacetamides were characterized by ^{1}H and ^{13}C NMR and confirmed by MS.

To test whether these azolylthioacetamides were specific or even broad-spectrum inhibitors of the MβLs, the MβLs from subclasses B1a (CcrA), B1b (NDM-1), B2 (ImiS), and B3 (L1) were overexpressed and purified as previously described, respectively.[27−30] The inhibition experiments of the azolylthioacetamides as inhibitors against MβLs were conducted on an Agilent UV8453 spectrometer. The initial reaction rates were determined in triplicate, and the average value was recorded.

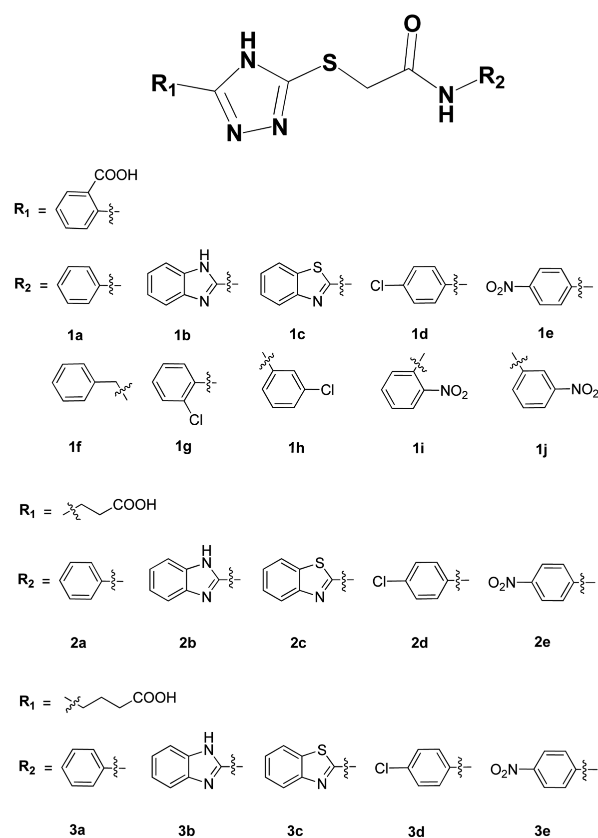

Figure 1. Structures of the synthesized azolylthioacetamides.

Scheme 1. Synthetic Route of the Azolylthioacetamides

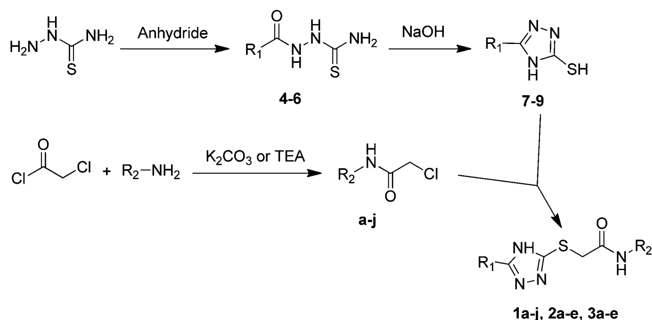

Table 1. Inhibition Constants of Metallo-β-lactamase ImiS by Azolylthioacetamides[a]

compd	K_i (μM)	compd	K_i (μM)
1a	1.8 ± 0.3	**1i**	2.7 ± 0.3
1b	3.6 ± 0.4	**1j**	1.9 ± 0.2
1c	1.2 ± 0.1	**2a**	>5000
1d	1.4 ± 0.5	**2b**	>2500
1e	2.2 ± 0.2	**2c**	
1f		**2d**	>5000
1g	2.3 ± 0.2	**3b**	
1h	2.4 ± 0.4		

[a]K_i values were determined using imipenem as substrate and ImiS as the enzyme in 50 mM Tris, pH 7.0, at 25 °C. The inhibitor concentrations were varied between 0 and 10 μM; empty cells showed no inhibition.

Kinetic studies were performed using cephalexin V as substrate of CcrA, NDM-1, and L1, and imipenem as substrate of ImiS.

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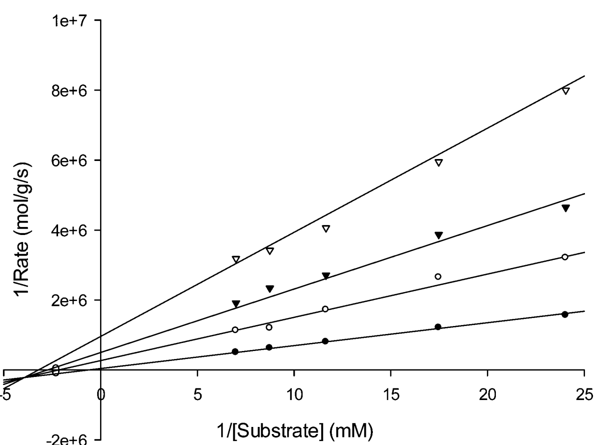

Figure 2. Lineweaver–Burk plots of inhibition of ImiS-catalyzed hydrolysis activity by azolylthioacetamide **1c**. Imipenem was used as substrate of ImiS. Data points show inhibitor **1c** concentrations of 0 (●), 2.5 (○), 5 (▼), and 10 (▽) μM.

The substrate concentrations were varied between 24 and 140 μM, and inhibitor concentrations were varied between 0 and 10 μM. The mode of inhibition was determined by generating Lineweaver–Burk plots of the data, and the K_i values for the inhibitors were determined by fitting initial velocity versus substrate concentration at each inhibitor concentration. Hydrolysis of substrate was monitored at 262 and 300 nm, respectively.

The inhibition studies indicated that azolylthioacetamides had specific inhibition activity against ImiS, but no activity was observed against CcrA, NDM-1, and L1 at inhibitor concentrations of up to no more than 10 μM. The inhibitory constants (K_i) of azolylthioacetamides against ImiS are listed in Table 1. It is clear to be observed that **1a–e** and **1g–j** exhibited high inhibitory activities against ImiS with a K_i value range of 1.2–3.6 μM. Among these azolylthioacetamides, **1c** showed the lowest K_i value of 1.2 μM. The fact that **1f** did not inhibit ImiS indicates that an aromatic amine exhibits better inhibition efficiency than an aliphatic amine. However, the azolylthioacetamides **2a–b** and **2d** with an aliphatic carboxyl at R_1 position showed little inhibitory activity against ImiS, exhibiting K_i > 2.5 mM, and **2c** and **3b** had no inhibitory activities, implying that the aliphatic carboxyl at R_1 position weakens inhibition efficiency of the inhibitors.

The above data of K_i values reveal a structure–activity relationship, which is that the aromatic carboxyl at the triazole ring (R_1 position) improves the inhibitory activity of azolylthioacetamide against ImiS. It was of interest to explore the pontential role of an aromatic carboxyl at the R_1 position. We will develop more azolylthioacetamides with various substituents for further structure–activity relationship studies.

The Lineweaver–Burk plots with azolylthioacetamide inhibitors indicated that **1a–e** and **1g–j** are mixed inhibitors of ImiS. This inhibition mode is the same as that of the previously reported diaryl-substituted azolylthioacetamide derivatives.[23] Inhibitor **1c** was taken as an example to show its inhibition mode against ImiS in Figure 2.

The antibacterial activities of the azolylthioacetamides were investigated by determining the minimum inhibitory concentrations (MICs) of existing antibiotics in the presence and absence of **1a–j**.[31] Two clinical bacteria *P. aeruginosa* and *K. pneumoniae* and four bacterial strains of *E. coli* BL21(DE3) containing plasmids pMSZ02-CcrA, pET26b-NDM-1, pET26b-

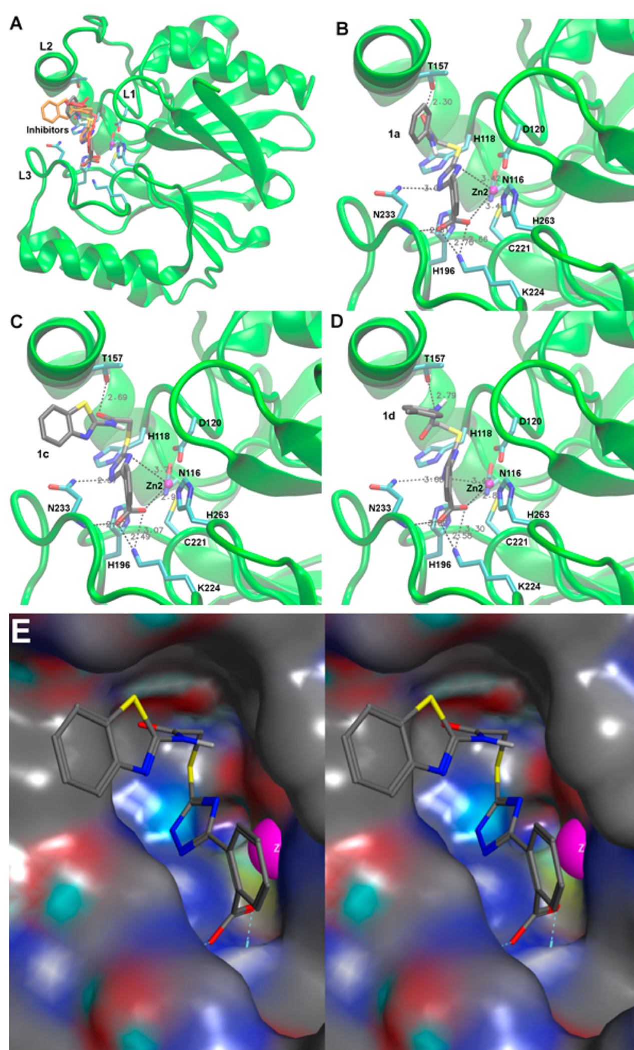

Figure 3. Low energy conformations of **1a**, **1c**, and **1d** docked into the active site of CphA (PDB code 2QDS[13]). (A) Overview of the entire enzyme with loops L1, L2, and L3 (labeling according to Garau et al.[33]) flanking the active site. The enzyme backbone is shown as a cartoon in green and selected residues are shown as sticks colored by atom (C, cyan; N, blue; O, red; S, yellow) and labeled at Cα. The Zn(II) ion is shown as a magenta sphere. All three inhibitors are superimposed: **1a** as sticks colored by atom (same colors as for protein residues except C in gray), **1c** as orange sticks, and **1d** as red sticks. (B–D) Detailed views of **1a**, **1c**, and **1d**, respectively, with key residues displayed and interactions between the inhibitors and protein residues indicated by dashed lines. Panels A–D were generated with VMD.[34] (E) Stereo view of **1c** (same coloring and conformation as in panel C) with the enzyme represented as a surface with neutral areas in gray, positively charged areas in blue, and negatively charged areas in red. Zn2 is shown as a magenta sphere. The figure was generated with MOE.[35]

ImiS, and pET26b-L1 were used to assess these inhibitors (Table 2). While this strain of *K. pneumoniae* does not produce a metallo-β-lactamase, it is known to produce extended spectrum β-lactamases (ESBLs);[32] therefore, the use of this strain in the MIC experiments allowed for us to evaluate the biological activity of the azolylthioacetamides toward other (serine) β-lactamases. *P. aeruginosa* is known to produce metallo-β-lactamase IMP-1;[9] therefore, the use of this strain in the MIC experiments allowed for us to evaluate the biological

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Table 2. Antibacterial Activities (MICs in μg/mL) of β-Lactam Antibiotics in the Presence of the Azolylthioacetamides 1a−j at a Concentration of 16 μg/mL

		azolylthioacetamides									
antibiotic-resistant bacteria	inhibitor blank	1a	1b	1c	1d	1e	1f	1g	1h	1i	1j
E. coli-L1[a]	8	8	8	8	8	16	16	64	64	>128	8
E. coli-NDM-1[a]	4	4	4	4	4	4	4	4	4	4	4
E. coli-CcrA[a]	4	4	4	<0.125	<0.125	2	4	2	0.5	0.25	2
E. coli-ImiS[b]	1	1	1	1	0.5	0.5	1	0.25	<0.125	<0.125	0.25
P. aeruginosa[b]	0.25	0.25	0.25	<0.125	0.25	0.25	<0.125	<0.125	<0.125	<0.125	<0.125
K. pneumoniae[b]	0.25	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

[a]Antibiotic used: cefalexin V. [b]Antibiotic used: imipenem.

activity of the azolylthioacetamide derivatives toward this clinically important strain.

MIC data indicate that some of the inhibitors **1c−j** increase the antimicrobial effect of antibiotics on Gram-negative *P. aeruginosa* and/or *E. coli* BL21(DE3) expressing ImiS or CcrA, and the largest effect was observed with *E. coli* BL21(DE3) expressing CcrA. However, these compounds showed no effect on *K. pneumonia* and on *E. coli* BL21(DE3) producing L1 and NDM-1 (Table 2). For *K. pneumoniae* this observation could be explained with decreased cell permeability but more likely with the compounds being ineffective against serine β-lactamases. Also for *E. coli* expressing L1 and NDM-1, permeability issues are unlikely because these cells are identical to those expressing CcrA and ImiS. More likely, it is a result of low binding affinity of these inhibitors as observed in the *in vitro* inhibition studies. It is not clear why the azolylthioacetamides **1e−i** increase MIC values of existing antibiotics for *E. coli* containing the L1 plasmid and *K. pneumoniae*. This could be due to an allosteric effect of the compounds on L1 and the ESBL expressed in *E. coli* and *K. pneumoniae*, respectively, as has been suggested recently for the activation of BcII and ImiS by a β-phospholactam.[36] Inclusion of compounds **1c−e** and **1g−j** resulted in 2−32-fold reduction in MIC for *E. coli* BL21(DE3) expressing CcrA, **1d−e** and **1g−j** resulted in 2−8-fold reduction in MIC for *E. coli* BL21(DE3) expressing ImiS, and **1c** and **1f−j** resulted in a 2-fold reduction in MIC for Gram-negative *P. aeruginosa*, but these three strains were not affected by **1a−b**.

The compounds presented here differ from those of our previous study[23] mostly in a negatively charged carboxylate group in R_1. In some compounds that had an *ortho*-hydroxyl phenyl group at this position, the hydroxyl formed a hydrogen bond with Lys224, while the triazole coordinated the Zn(II) ion(s) in docking calculations.[23] Introducing a carboxylate at the same position was expected to strengthen the interaction between this moiety and Lys224 through a salt bridge. The docking analysis procedure (see Supporting Information) resulted in 14, 13, and 10 conformations with average binding energies of −11.3, −11.8, and −11.7 kcal/mol for **1a**, **1c**, and **1d**, respectively. These correspond to theoretical K_is in the micromolar range, in good agreement with the experimental K_i values (1.2−2.7 μM). The differences between clusters within 0.5 kcal/mol are not significant, consistent with the values that deviate by less than an order of magnitude. The conformations shown in Figure 3 are the highest ranked (lowest energy; between −12.8 and −11.6 kcal/mol) conformations of those clusters. As expected, these docking calculations reveal that in compounds **1a**, **1c**, and **1d**, which exhibited the lowest K_i values with ImiS, the carboxylate forms a salt bridge with Lys224 of CphA, which serves as a proxy of ImiS. In addition, it interacts with Zn2 and the backbone amide of Asn233, thus tightly anchoring these compounds in the active site. Interestingly, the carboxylate is in an orientation perpendicular to the long axis of the binding site as well as to the phenyl ring it is attached to (Figure 3). The R_2 thioacetamides, which are similar to the R group of penicillins and R_1 of cephalosporins, are located in the area that is expected to be occupied by these antibiotic groups. R_2 (phenyl in **1a**, Figure 3B; benzylthiazolyl in **1c**, Figure 3C; *para*-chlorophenyl in **1d**, Figure 3D) binds in a hydrophobic area of the substrate binding site (Figure 3E), while either the carbonyl (Figure 3B,C) or amide (Figure 3D) of the thioacetamide interacts with Thr157. Somewhat unexpectedly, the triazole ring did not directly coordinate Zn2 edge-on, but rather interacted face-on with Zn2 at a distance of 3.4 to 3.7 Å, resembling a π electron−cation interaction. There are two possible explanations for this observation: (i) the tight binding of the carboxylphenyl ring to Lys224, Zn2, and Asn233 and resonance between the two rings does not allow the triazole ring to turn enough for an edge-on Zn2 coordination; (ii) there is also a hydrogen bond between the triazole ring and the Asn233 side chain, which could keep the triazole distant from Zn2. Figure 3E shows that compound **1c** fits very tightly into the substrate binding site of CphA, taking advantage of hydrophobic interactions between R_1 and R_2 and protein residues as well as electrostatic interactions, most importantly the salt bridge between the carboxylate and Lys224 and those between the triazole and Zn2 as well as the Asn233 side chain. This image may also serve as a guide to further improve these molecules, perhaps by further exploring hydrophobic interactions between R_1 and R_2 and the protein.

In addition to the tight fit of the compounds into the CphA binding site, we provide the following rationale for the preferential binding of the inhibitors studied here to ImiS (and CcrA in MIC assays), but not NDM-1 and L1. To that end, the different crystal structures used for docking here and previously[23] as well as the chemical structures of the compounds were analyzed. The aromatic carboxylate in R_1 of the **1a−j** series of the compounds studied here may need a certain width of the binding site to be accommodated in the perpendicular orientation mentioned above, which is provided in CcrA and CphA, but not in NDM-1 and L1. In addition, Zn2 and the bound inhibitor are shifted toward the empty Zn1 binding site in CphA, which is occupied by a Zn(II) ion in CcrA, NDM-1, and L1. In CphA, the triazole ring is located at the position that corresponds to Zn1's position in CcrA, NDM-1, and L1, which may explain why this favorable conformation cannot be accommodated in these enzymes.

In summary, 20 azolylthioacetamides **1a−j**, **2a−e**, and **3a−e** were synthesized and characterized by ^{1}H and ^{13}C NMR and MS. Several azolylthioacetamides were tested as inhibitors of

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the MβLs CcrA, NDM-1, ImiS, and L1. Nine azolylthioacetamides inhibited ImiS, exhibiting K_i values of 1.2−3.6 μM using imipenem as substrate. All compounds tested did not inhibit CcrA, NDM-1, and L1 *in vitro*. Structure−activity relationship studies reveal that the aromatic carboxyl at the triazole ring of azolylthioacetamides improves inhibitory activity of the inhibitors against ImiS and that an aliphatic carboxyl does not. The azolylthioacetamides were tested for antibacterial activity by examining the MIC values for existing antibiotics in the presence/absence of these compounds. The inclusion of eight azolylthioacetamides resulted in lower MIC values when using *E. coli* BL21(DE3) cells expressing CcrA, ImiS, or *P. aeruginosa*. The best inhibitors of ImiS, compounds **1a**, **1c**, and **1d** fit tightly into the substrate binding site of CphA as a proxy for ImiS with the aromatic carboxylate forming interactions with Lys224, Zn(II), and the backbone of Asn233, the triazole interacting with Zn(II) and the Asn233 side chain, and hydrophobic portions of the inhibitors occupying hydrophobic pockets. These studies demonstrate that the aromatic carboxyl-substituted azolylthioacetamides are good scaffolds for future inhibitors of the MβL ImiS and potentially other B2 subclass enzymes.

■ ASSOCIATED CONTENT

Supporting Information

Detailed synthesis procedure, NMR and ESI mass data for all target compounds, the methods for enzyme expression and purification, assay of inhibitory activity, determination of MIC, and docking study. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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