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3-Mercapto-1,2,4-triazoles and N-acylated thiosemicarbazides as metallo-β-lactamase inhibitors

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ABSTRACT

The production of β-lactamases is an effective strategy by which pathogenic bacteria can develop resistance against β-lactam antibiotics. While inhibitors of serine-β-lactamases are widely used in combination therapy with β-lactam antibiotics, there are no clinically available inhibitors of metallo-β-lactamases (MBLs), and so there is a need for the development of such inhibitors. This work describes the optimisation of a lead inhibitor previously identified by fragment screening of a compound library. We also report that thiosemicarbazide intermediates in the syntheses of these compounds are also moderately potent inhibitors of the IMP-1 MBL from *Pseudomonas aeruginosa*. The interactions of these inhibitors with the active site of IMP-1 were examined using in silico methods.

Many pathogenic bacteria have developed resistance against β-lactam antibiotics via mechanisms such as reduced cell-wall permeability, efflux of antibiotics and drug degradation mediated by β-lactamases. β-Lactamases are enzymes that inactivate β-lactam antibiotics by hydrolysing the key four-membered lactam ring of these drugs.[1] Class B β-lactamases are zinc-containing metalloenzymes (metallo-β-lactamases, MBLs) which use a metal-bound hydroxyl group as the nucleophile[2] and are able to promote the hydrolysis of a broad range of antibiotics, including penicillins, cephalosporins and carbapenems.[3] While clavulanic acid effectively inhibits serine β-lactamases,[4] there are no clinically available inhibitors of MBLs. Therefore, there is an urgent need to develop such compounds since multi-drug resistant pathogens such as *Pseudomonas aeruginosa* and *Acinetobacter* spp. produce clinically relevant levels of MBLs.

The imipenemase (IMP-1) MBL from *P. aeruginosa* has been identified in many reported cases of antibiotic resistance in medical facilities world-wide, leading to diseases such as pneumonia, bacteriemia, urosepsis and wound infections.[5] MBL-mediated antibiotic resistance has also been observed in clinical isolates of *Serratia marcescens*, *Klebsiella pneumoniae*, and *Citrobacter freundii*,[6] which arises because mobile genetic elements allow such resistance to spread to unrelated bacterial species.

Although no inhibitors of MBLs are clinically approved, a number of MBL inhibitors have been reported, including phthalic acid derivatives,[7] maleic acid derivatives,[8] succinic acid derivatives[9] and trifluoromethyl ketones.[10] Irreversible thiol-containing inhibitors of MBLs have also been described.[11]

We recently reported the discovery, by fragment-based screening of a 500 compound Maybridge™ library, of several new classes of lead inhibitors against the IMP-1 MBL. All of these compounds displayed K_i values of around 1 millimolar.[12] Of the small fragments identified in that work, we considered that 4-methyl-5-(trifluoromethyl)-4*H*-1,2,4-triazole-3-thiol (**1**) was the most promising for further study as kinetic assays indicated that its mode of inhibition was purely competitive, that is, showing no component due to uncompetitive inhibition. Herein, we report our efforts for elaborating this ring system in attempts to improve the potency of this compound, and our finding that intermediates in these syntheses, N-acylated thiosemicarbazides, are also potent inhibitors of the IMP-1 MBL.

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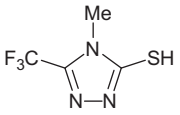


1, K_{ic} = 0.97 ± 0.60 mM

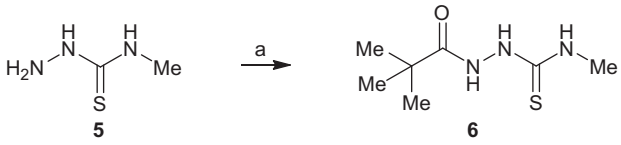
1,2,4-Triazole-3-thiols **4** were prepared in most cases from acylated thiosemicarbazides **3** on treatment with strong base, either aqueous sodium hydroxide solution or sodium ethoxide in ethanol. Acylated thiosemicarbazides **3** were prepared by a variety of methods, either direct thermal condensation of thiosemicarbazide **2** with carboxylic acids or by acylation of thiosemicarbazide **2** with acid chlorides or acid anhydrides. In some cases the intermediate acylated thiosemicarbazides **3** could not be isolated, and the 1,2,4-triazole-3-thiol products (**4o** and **4p**) instead formed directly (Scheme 1).

4-Methyl-1-pivaloylthiosemicarbazide **6** was prepared by the acylation reaction of 4-methylthiosemicarbazide **5** shown in Scheme 2.

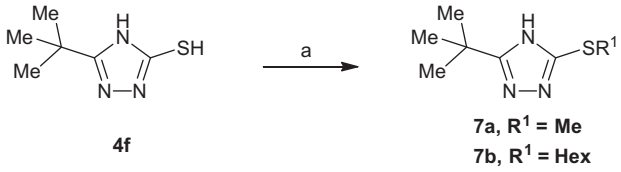
Two S-alkylated 1,2,4-triazole-3-thiols, **7a** and **7b**, were prepared by chemoselective alkylation of **4f** with the appropriate alkyl halide and base in hot acetonitrile (Scheme 3). Desulfurisation of **4a** gave 3-methyl-4*H*-1,2,4-triazole (**8**) according to Ainsworth's general method[13] (Scheme 4).

A number of substituted benzoic acids were required for this work. Their syntheses are outlined in Schemes 5–7. Starting with 4-benzoylbenzoic acid **9**, hydrogenolysis gave 4-benzylbenzoic acid **10** (Scheme 5).

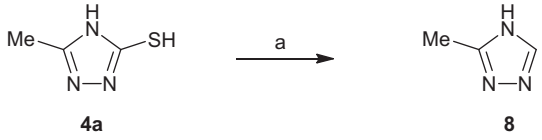
4-Benzoylbenzoic acids **13**, were prepared form the appropriate substituted benzoic acids **11** by regioselective Friedel–Crafts acylation of toluene promoted by phosphorus pentoxide adsorbed onto silica, as described by Zarei et al.,[14] followed by benzylic oxidation of **12** using Jones reagent in hot acetic acid (Scheme 6).



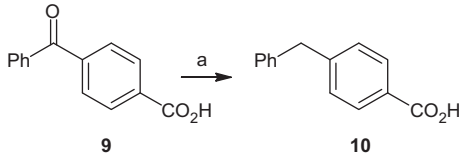
Scheme 2. Reagents and conditions: (a) CMe_3COCl, pyr, THF, Δ, 3 h, 44%.



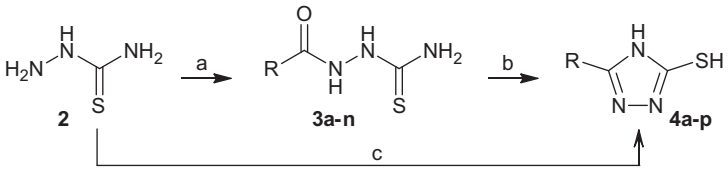
Scheme 3. Reagents and conditions: (a) R^1-X, Et_3N, MeCN, Δ, 2 h, 37% (**7a**); 37% (**7b**).

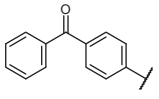
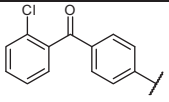
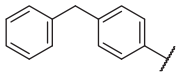
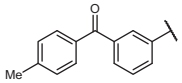
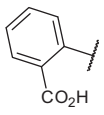
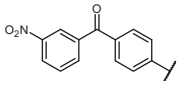
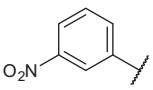
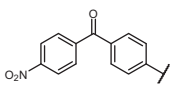


Scheme 4. Reagents and conditions: (a) $NaNO_2$, HNO_3, 45 °C, 30 min, 36%.

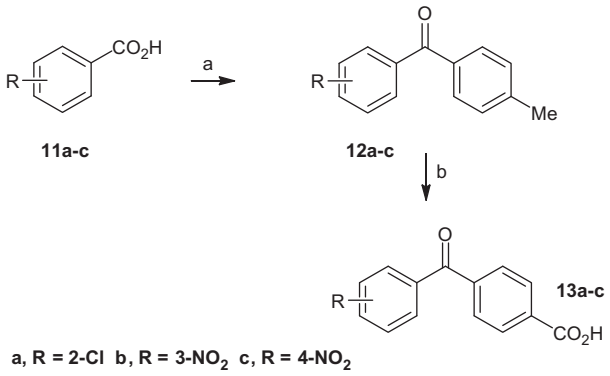


Scheme 5. Reagents and conditions: (a) H_2/Pd/C, AcOH, 65 °C, 44 h, 64%.



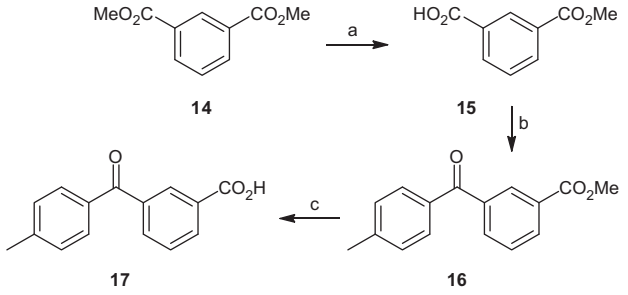
a	**Me**	**e**	$HO_2C(CH_2)_3$-	**i**		**m**	
b	**Et**	**f**	CMe_3	**j**		**n**	
c		**g**	**Ph**	**k**		**o**	CF_3
d	$HO_2C(CH_2)_2$-	**h**		**l**		**p**	$CH_3(CH_2)_5$-

Scheme 1. Reagents and conditions: (a) (i) RCO_2H, Δ, 2 h, 60% (**3a**); 53% (**3b**) or (ii) $(RCO)_2O$, Δ, 2 h, MeCN, 36% (**3c**); 66% (**3d**); 92% (**3e**) or (iii) RCOCl, pyr, rt, 24–72 h, 25% (**3f**); 71% (**3g**); 43% (**3h**); 76% (**3i**); 55% (**3j**); 26% (**3k**); 50% (**31**); 41% (**3m**); 39% (**3n**); (b) (i) Na, EtOH, Δ, o/n, 73% (**4a**); 63% (**4b**); 50% (**4g**); 47% (**4h**) or (ii) 10% aq NaOH, Δ, 3 h, 76% (**4c**); 56% (**4d**); 72% (**4e**); 37% (**4f**); 88% (**4i**); (c) RCO_2H, Δ, 6 h, 24% (**4o**); 17% (**4p**).



Scheme 6. Reagents and conditions: (a) PhMe, P_2O_5/SiO_2, Δ, 3 h, 51% (**12a**); 63% (**12b**); 66% (**12c**); (b) Jones reagent, AcOH, Δ, 4 h, 30% (**13a**); 64% (**13b**); 60% (**13c**).

The final benzoylbenzoic acid **17** used in this study was prepared as shown in Scheme 7. Dimethyl isophthalate **14** was hydrolysed to the monoacid **15**[15] which was then subjected to the Friedel–Crafts acylation with toluene and phosphorus pentoxide on silica gel to yield the ester **16**. Finally, saponification of **16** gave the required carboxylic acid **17**.



Scheme 7. Reagents and conditions: (a) NaOH, MeOH, rt, 18 h, 83%; (b) PhMe, P_2O_5/SiO_2, Δ, 3 h, 35%; (c) NaOH, H_2O, Δ, 2 h, 80%.

The inhibitory effects of 1,2,4-triazole-3-thiols **4**, **7** and **8** against the IMP-1 MBL were performed as previously described.[12] Briefly, kinetic studies were performed using the chromogenic substrate CENTA (see Supplementary data)[16] and a 96-well plate reader at pH 7.00 and monitoring the development of the chromophore 4-nitrothiophenolate at 405 nm. The results are shown in Table 1.

In examining structure–activity relationships for the structural elements of the starting 4-methyl-5-(trifluoromethyl)-4*H*-1,2,4-triazole-3-thiol (**1**), a number of general features are apparent:

Table 1
Inhibitory activities of 1,2,4-triazole-3-thiols against IMP-1

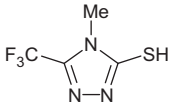
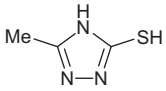
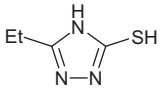
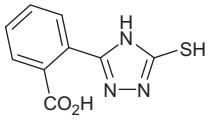
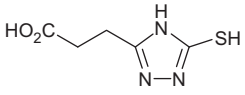
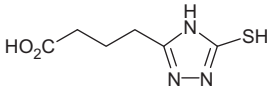
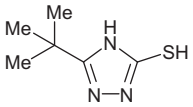
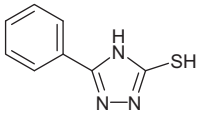
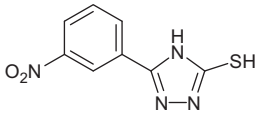
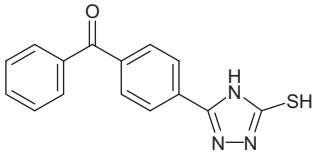
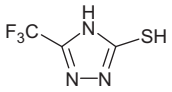
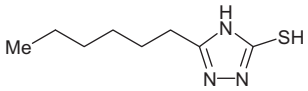
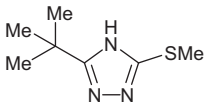
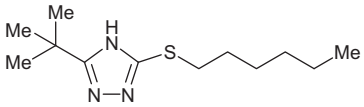
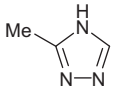
Compound	Structure	Inhibition % (1000 μM)	Inhibition % (100 μM)	Inhibition % (10 μM)
1		51	–	–
4a		33	–	–
4b		32	–	–
4c		–	7	0
4d		–	10	10
4e		–	5	0
4f		32	–	–
4g		0	–	–
4h		–	–	Insoluble

Table 1 (*continued*)

Compound	Structure	Inhibition % (1000 μM)	Inhibition % (100 μM)	Inhibition % (10 μM)
4i		–	44	10
4o		45	–	–
4p		–	–	Insoluble
7a		10	–	–
7b		–	–	Insoluble
8		0	–	–

– Not determined.

1. The *N*-methyl group of **1** is not necessary for potency, as compound **4o**, lacking this group, has very similar activity.
2. The thiol is a requirement for activity. Compound **8**, in which the thiol group has been removed, has no activity. Compound **7a**, in which the thiol group has been methylated, has somewhat lower activity than its non-methylated analogue **4f**.
3. Alkyl groups can substitute for the trifluoromethyl group of **1** without significant loss of activity. The 5-methyl- (**4a**) and 5-ethyl- (**4b**) derivatives retain much of the potency of **1**, as does the bulky 5-*tert*-butyl-derivative **4f**, although anionic alkyl side chains (**4d** and **4e**) exhibited diminished activity.
4. While simple 5-aryl-derivatives such as 5-phenyl (**4g**) and 5-(2-carboxyphenyl)- (**4c**) derivatives showed low inhibitory activities, a significant improvement in potency was observed for the 5-(4-benzoylphenyl)-derivative **4i**.

Although analysis of the structure–activity relationships of the compounds listed in Table 1 was instructive, it nonetheless did not lead to any large improvements in potencies relative to the starting triazole-thiol **1**. A fortuitous discovery was that many of the acylated thiosemicarbazide synthetic precursors **3** (Scheme 1) of the triazole-thiols **4** did themselves possess high potencies against the IMP-1 MBL (Table 2)

The structure–activity data in Table 2 shows that acylation of thiosemicarbazide with the bulky pivaloyl group led to derivatives with no activity (compounds **3f** and **6**) whereas anionic alkyl side chains gave modest inhibition at 10 μM (**3d** and **3e**). A sharp increase in potency was observed when the thiosemicarbazide was acylated with aromatic groups. With the exception of the 2-carboxylbenzoyl compound (**3c**) which exhibited no inhibition at 10 μM, all other aromatic substituents showed strong inhibition at 10 μM. The most potent compounds in this series included 4-(benzoyl)benzoyl derivatives, particularly **3i** and **3k–n**. Removal of the oxygen atom of the linking diaryl ketone group by reduction to the corresponding diaryl methane resulted in minimal decrease in potency (compare **3i** with **3j**), suggesting that the carbonyl group was unimportant for potency.

Based on the encouraging findings of the compounds listed in Table 2, several inhibitors were selected for more careful kinetic analyses to determine K_i values and their modes of inhibition (competitive versus uncompetitive). These results are summarised in Table 3. For comparison, the K_{ic} value for the known competitive MBL inhibitor L-captopril[17] is included in Table 3.

The results in Table 3 indicate that the 1,2,4-triazole-3-thiol (**4i**) and the acylated thiosemicarbazides (**3**) exhibit mixed inhibition (both competitive and uncompetitive inhibition modes were observed). This mixed inhibition has been observed previously by us for small fragments binding to IMP-1 MBL,[12] and also for inhibitors of another binuclear metallohydrolase, purple acid phosphatase.[18] We interpret this mixed inhibition mode to indicate that these inhibitors are capable of both binding in the active site of IMP-1 (competitive inhibition) and also of forming a ternary enzyme–substrate-inhibitor (ESI) complex which inhibits hydrolysis of the substrate (uncompetitive inhibition). Both of these possible binding modes may offer insights for the future design of more potent inhibitors.

To gain insight into the possible binding modes of these inhibitors, in silico docking of the most potent inhibitor, **3l** (K_{ic} 11 ± 4 μM), in the active site of the IMP-1 MBL was examined using Molegro Virtual Docker.[19] The lowest energy binding orientation of **3l** is shown in Figure 1. While we had anticipated that the sulfur atom of **3l** would bind to one or both of the metal ions in the active site, modelling unexpectedly suggested that the oxygen atoms of the nitro group were interacting with the zinc ions, twisting the nitro group out of planarity with the aromatic ring to allow oxygen–metal distances of 3.3 Å (Zn1) and 2.0 Å (Zn2) to be attained. The nitro-aromatic ring of inhibitor **3l** makes a close contact with the indolic side-chain of Trp 64 on the flexible loop of IMP-1 (Fig. 1),

Table 2
Inhibitory activities of acylated thiosemicarbazides **3** against IMP-1

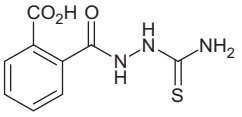
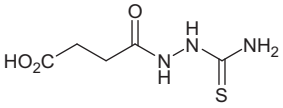
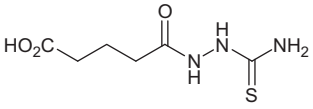
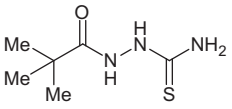
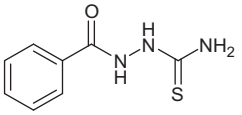
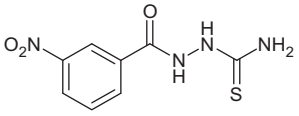
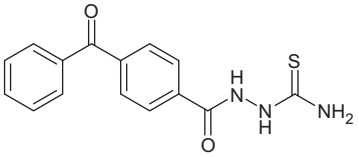
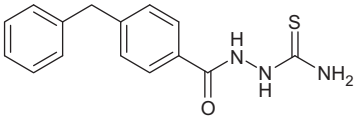
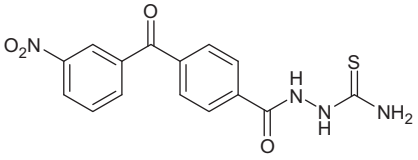
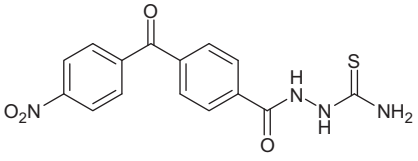
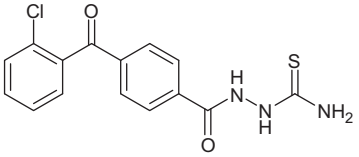
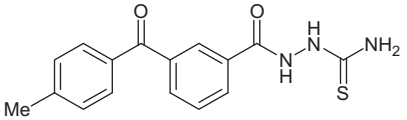
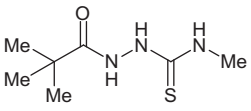
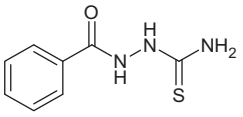
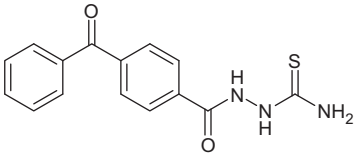
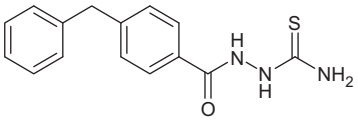
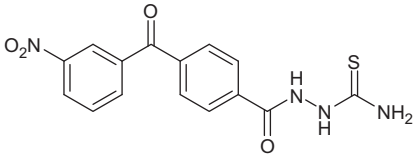
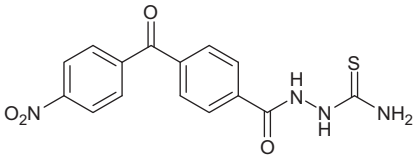
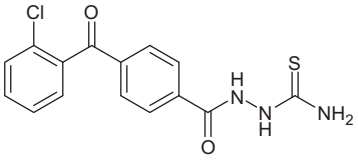
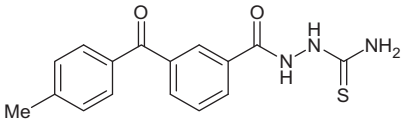
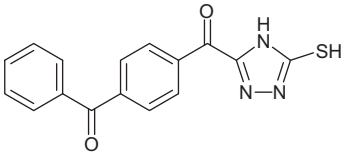
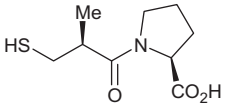
Compound	Structure	Inhibition % (10 μM)
3c		0
3d		13
3e		8
3f		0
3g		22
3h		30
3i		34
3j		26
3k		45
3l		45
3m		51
3n		38
6		0

Table 3
Kinetic data for selected compounds against IMP-1

Compound	Structure	K_{ic} (μM)	K_{iuc} (μM)
3g		19 ± 8	30 ± 8
3i		13 ± 4	26 ± 7
3j		18 ± 10	37 ± 15
3k		16 ± 7	15 ± 5
3l		11 ± 4	20 ± 5
3m		14 ± 4	13 ± 2
3n		41 ± 24	20 ± 4
4i		75 ± 30	56 ± 10
L-Captopril		12.5 ± 2.4[12]	—

— Indicates result >2 mM.

and two N-H bonds on the terminal thiourea group of **3l** form hydrogen bonds to the carbonyl oxygen on the backbone of Tyr 227 (not shown).

In contrast, modelling of inhibitor **3g** (K_{ic} 19 ± 8 μM), in the active site of the IMP-1 MBL indicated that the sulfur atom of the thiosemicarbazide moiety was binding to the two metal ions in an expected manner, with sulfur–metal distances of 2.3 Å (Zn1) and 2.0 Å (Zn2) (Fig. 2). This compares well with crystallographic data from a thiol inhibitor in complex with IMP-1, which shows sulfur-zinc lengths of 2.4 Å and 2.2 Å, respectively.[20] The terminal aromatic ring in **3g** interacts with a hydrophobic patch in the surface of the IMP-1 enzyme, formed by methylene groups of the side-chains of Lys 224 and His 263.

In conclusion, we have generated a number of analogues of our lead compound **1** and conducted structure–activity studies of these derivatives against the metallo-β-lactamase IMP-1. While a number of structural features of these 1,2,4-triazole-3-thiols have been shown to be important for strong binding, only modest improvements in potency (from **1**: K_i ~1 mM to **4i**: K_i ~70 μM) were obtained. In contrast, optimisation of acylated thiosemicarbazides **3** has led to several compounds with K_i values as low as 11 μM, comparable with the potency of L-captopril (12.5 μM). All of the newly

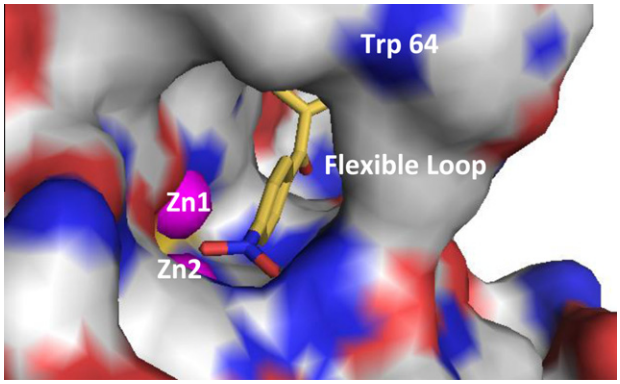


Figure 1. The predicted binding mode of inhibitor **3l** in the active site of the IMP-1 MBL. Atom colours are as follows: blue—nitrogen, red—oxygen, white—carbon (on IMP-1), yellow—carbon (on inhibitor), magenta—zinc active site metals.

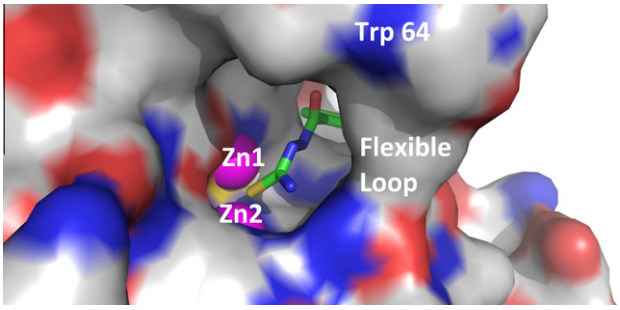


Figure 2. The predicted binding mode of inhibitor **3g** in the active site of the IMP-1 MBL. Atom colours are as follows: blue—nitrogen, red—oxygen, white—carbon (on IMP-1), green—carbon, yellow—sulfur (on inhibitor), magenta—zinc active site metals.

developed acylated thiosemicarbazides exhibited mixed mode inhibition kinetics against IMP-1, as we have previously observed for other inhibitors of this enzyme.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.10.116.

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