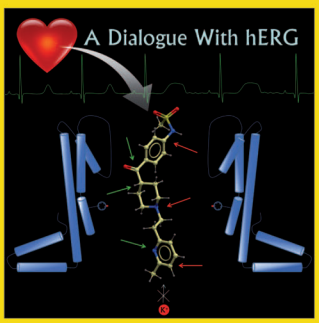
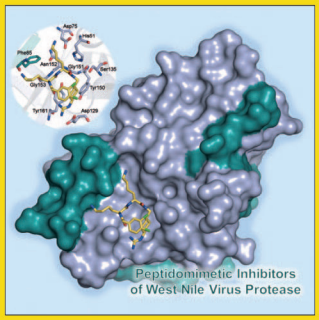
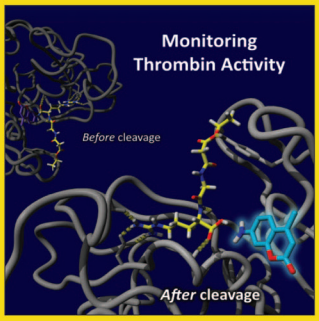
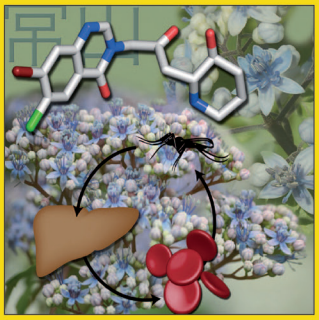
CHEMMEDCHEM

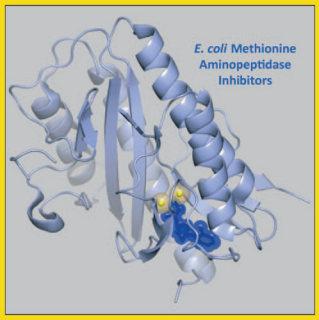
CHEMISTRY ENABLING DRUG DISCOVERY

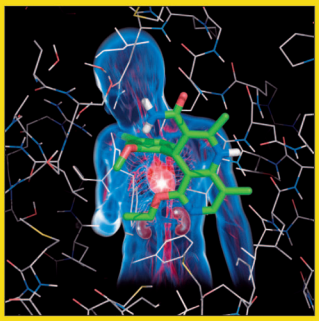


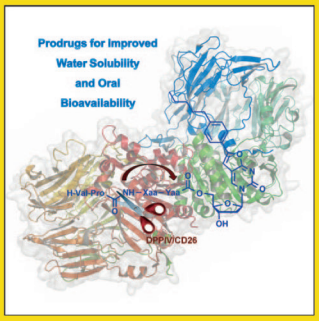


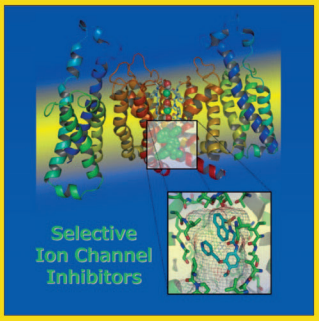


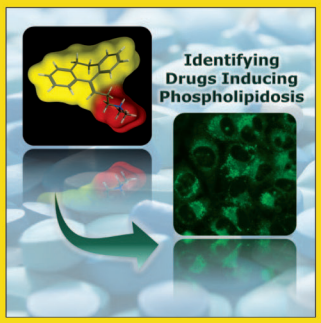












Reprint

A Journal of

ChemPubSoc Europe

WILEY-VCH

www.chemmedchem.org

ChemPubSoc Europe

DOI: 10.1002/cmdc.201700186

CHEMMEDCHEM Full Papers

SPECIAL ISSUE

1,2,4-Triazole-3-thione Compounds as Inhibitors of Dizinc Metallo-β-lactamases

Laurent Sevaille,[a] Laurent Gavara,*[a] Carine Bebrone,[b, j] Filomena De Luca,[c] Lionel Nauton,[d, k] Maud Achard,[e, l] Paola Mercuri,[b] Silvia Tanfoni,[c] Luisa Borgianni,[c] Carole Guyon,[a] Pauline Lonjon,[a, m] Gülhan Turan-Zitouni,[f] Julia Dzieciolowski,[g] Katja Becker,[g] Lionel Bénard,[h] Ciaran Condon,[i] Ludovic Maillard,[a] Jean Martinez,[a] Jean-Marie Frère,[b] Otto Dideberg,[d] Moreno Galleni,[b] Jean-Denis Docquier,[c] and Jean-François Hernandez*[a]

Metallo-β-lactamases (MBLs) cause resistance of Gram-negative bacteria to β-lactam antibiotics and are of serious concern, because they can inactivate the last-resort carbapenems and because MBL inhibitors of clinical value are still lacking. We previously identified the original binding mode of 4-amino-2,4-dihydro-5-(2-methylphenyl)-3*H*-1,2,4-triazole-3-thione (compound IIIA) within the dizinc active site of the L1 MBL. Herein we present the crystallographic structure of a complex of L1 with the corresponding non-amino compound IIIB (1,2-dihydro-5-(2-methylphenyl)-3*H*-1,2,4-triazole-3-thione). Unexpectedly, the binding mode of IIIB was similar but reverse to that of IIIA. The 3D structures suggested that the triazole–thione scaffold was suitable to bind to the catalytic site of dizinc metalloenzymes. On the basis of these results, we synthesized 54 analogues of IIIA or IIIB. Nineteen showed IC_{50} values in the micromolar range toward at least one of five representative MBLs (i.e., L1, VIM-4, VIM-2, NDM-1, and IMP-1). Five of these exhibited a significant inhibition of at least four enzymes, including NDM-1, VIM-2, and IMP-1. Active compounds mainly featured either halogen or bulky bicyclic aryl substituents. Finally, some compounds were also tested on several microbial dinuclear zinc-dependent hydrolases belonging to the MBL-fold superfamily (i.e., endonucleases and glyoxalase II) to explore their activity toward structurally similar but functionally distinct enzymes. Whereas the bacterial tRNases were not inhibited, the best IC_{50} values toward plasmodial glyoxalase II were in the 10 μM range.

Introduction

The emergence and spread of antibiotic-resistant bacteria have become an urgent threat to human health owing to the emergence of pan-drug-resistant Gram-negative pathogens.[1] In developed countries, the highest proportion of multidrug-resist-

[a] *L. Sevaille, Dr. L. Gavara, Dr. C. Guyon, P. Lonjon, Dr. L. Maillard, Prof. J. Martinez, Dr. J.-F. Hernandez*
Institut des Biomolécules Max Mousseron, UMR5247 CNRS, Université de Montpellier, ENSCM, Faculté de Pharmacie, 15 avenue Charles Flahault, 34093 Montpellier cedex 5 (France)
E-mail: laurent.gavara@umontpellier.fr
jean-francois.hernandez@umontpellier.fr

[b] *Dr. C. Bebrone, Dr. P. Mercuri, Prof. J.-M. Frère, Prof. M. Galleni*
Laboratoire de Macromolécules Biologiques, Centre d'Ingénierie des Protéines, Université de Liège, Allée du 6 août B6, Sart-Tilman, 4000 Liège (Belgium)

[c] *Dr. F. De Luca, S. Tanfoni, L. Borgianni, Prof. J.-D. Docquier*
Dipartimento di Biotecnologie Mediche, Università di Siena, 53100 Siena (Italy)

[d] *L. Nauton, Dr. O. Dideberg*
Institut de Biologie Structurale—Jean-Pierre Ebel, UMR5075 CNRS, CEA, Université Joseph Fourier, 41 rue Jules Horowitz, 38027 Grenoble cedex 1 (France)

[e] *Dr. M. Achard*
EMBL Outstation c/o DESY, Notkestrasse 85, 22603 Hamburg (Germany)

[f] *Dr. G. Turan-Zitouni*
Department of Pharmaceutical Chemistry Anadolu University, Faculty of Pharmacy, 26470 Eskisehir (Turkey)

[g] *Dr. J. Dzieciolowski, Prof. K. Becker*
Chair of Biochemistry and Molecular Biology, Interdisciplinary Research Center, Justus Liebig University, Heinrich-Buff-Ring 26-32, 35392 Giessen (Germany)

[h] *Dr. L. Bénard*
UMR8226, CNRS, Université Pierre et Marie Curie, Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, 75005 Paris (France)

[i] *Dr. C. Condon*
UMR8261, CNRS, Université Paris-Diderot, Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, 75005 Paris (France)

[j] *Dr. C. Bebrone*
Present address: Symbiose Biomaterials S.A., GIGA Bât. B34, 1 avenue de l'Hôpital, 4000 Liège (Belgium)

[k] *L. Nauton*
Present address: Institut de Chimie de Clermont-Ferrand, UMR6296 CNRS, Université Clermont Auvergne, 63000 Clermont-Ferrand (France)

[l] *Dr. M. Achard*
Present address: School of Chemistry and Molecular Bioscience, University of Queensland, St. Lucia, Brisbane, QLD 4072 (Australia)

[m] *P. Lonjon*
Present address: CERN, HSE/SEE/SI, 1211 Geneva 23 (Switzerland)

Supporting Information and the ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/cmdc.201700186.

SPECIAL ISSUE This article is part of a Special Issue on the 52rd International Conference on Medicinal Chemistry (RICT 2016, Caen, France). To view the complete issue, visit: http://onlinelibrary.wiley.com/doi/10.1002/cmdc.v12.12/issuetoc.

ant bacteria is found in hospitals. Indeed, approximately 70% of bacteria now causing hospital-acquired infections are resistant to at least one of the commonly used drugs. This leads to prolonged illness and increased mortality.

The most worrying resistance concerns the β-lactam antibiotics, the most widely used class of antibacterial agents that includes penicillins, cephalosporins, carbapenems, and monobactams.[2] Among them, carbapenems have the broadest spectrum of activity and represent last-resort drugs to treat life-threatening nosocomial infections. These drugs can be inactivated by hydrolysis of their β-lactam ring by bacterial enzymes called β-lactamases. These enzymes are grouped into four molecular classes. Classes A, C, and D act through a serine-based mechanism, whereas class B enzymes, called metallo-β-lactamases (MBLs), require one or two Zn atom(s) for β-lactam hydrolysis. Whereas combination therapies (treatment with a β-lactamase inhibitor such as clavulanic acid or tazobactam to prevent inactivation of β-lactam antibiotics) are useful to deal with bacteria producing active-site serine β-lactamases, MBL inhibitors are not currently available for clinical applications. There are three reasons why this is of particular concern: one, MBLs can degrade all classes of β-lactams (except monobactams), including carbapenems, which are stable toward the large majority of serine-β-lactamases; two, MBLs are produced by important Gram-negative pathogens (e.g., *Klebsiella pneumoniae, Pseudomonas aeruginosa*, *Aeromonas*, *Enterobacteriaceae*, *Acinetobacter*, and *Serratia marcescens*); three, many MBL genes are also present in environmental species constituting reservoirs of β-lactam resistance genes. MBLs are now regarded as a major therapeutic challenge.[3–5]

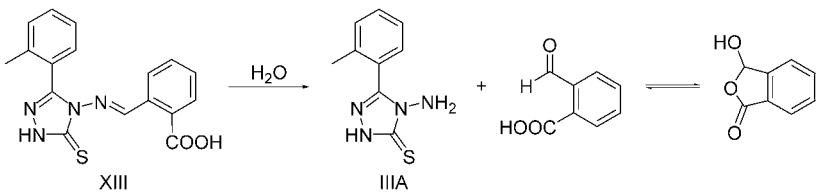
MBLs are classified into three subclasses, B1, B2, and B3, on the basis of subtle but significant variations in their active-site architectures.[3,4,6–9] Subclass B1 includes the most clinically relevant acquired MBLs (e.g., IMP-, VIM-, and NDM-types). Subclass B2 is represented by CphA and includes enzymes produced by different species of *Aeromonas*, whereas subclass B3 contains L1, GOB-1, FEZ-1, and other mostly resident enzymes, except for AIM-1 and SMB-1, which are apparently encoded by mobile genetic elements. Enzymes of subclasses B1 and B3 have a broad substrate profile and generally exhibit maximum activity as dizinc species, whereas MBLs of subclass B2 are monozinc enzymes and narrow-spectrum carbapenemases, with poor or no activity toward penicillins and cephalosporins.

This large structural and functional heterogeneity makes the design of potentially clinically useful and broad-spectrum MBL inhibitors very challenging.[10–13] Furthermore, the usual approach to inhibit metalloenzymes is to develop compounds possessing a zinc-coordinating group (e.g., thiol group). This approach can only have a clinical application if the inhibitor is highly MBL specific (i.e., does not inhibit human metalloenzymes). To date, several chemical families have been reported to inhibit MBLs. They include biphenyltetrazoles,[14] various thiol- and thioester-containing compounds,[15–27] di-[28–31] and polycarboxylate compounds,[32–34] hydroxamates,[35] arylsulfonyl-containing compounds,[36–40] trifluoromethyl alcohols and ketones,[41] tricyclic natural compounds,[42] and cyclic boronates.[43] Approaches to covalent MBL inhibitors have also been considered.[19,44,45]

Nevertheless, among all these series, only a limited number of compounds are active (K_i values between 10^{-6} and 10^{-8} M) on several MBLs from the three subclasses, or even on several of the most clinically relevant enzymes, and small and simple molecules with a zinc-coordinating thiol appear among the most promising in terms of affinity.[21–23] However, their flexibility and the strong Zn-coordinating properties of the thiol group might compromise their development as highly selective MBL inhibitors.

In a previous crystallographic study,[46] we obtained the structure of the L1 MBL (a dizinc subclass B3 enzyme from *Stenotrophomonas maltophilia*) in complex with a published inhibitor (XIII,[47] Scheme 1). Unexpectedly, the enzyme active site contained a fragment (named IIIA, Scheme 1) derived from the hydrolysis of the hydrazone-like bond of XIII. The same structure (PDB ID: 2HB9) was obtained after soaking a crystal of L1 in a solution of synthetic IIIA. Additionally, the distinctive feature of the L1–IIIA complex was the as-yet-unreported double coordination of the two Zn ions by two atoms of the 1,2,4-triazole-3-thione moiety (i.e., N^2 for Zn1 and S^3 for Zn2, Figure 1). It is noteworthy that a similar binding mode was recently demonstrated for a small triazole–thione compound in complex with VIM-2.[48]

Triazole–thiones are commonly reported as synthetic intermediates and in biological evaluations. Although they have mainly been screened for antibacterial, antifungal, anti-inflammatory, and antiproliferative activity,[49–53] only a few studies describe their use as metalloenzyme inhibitors such as the dicopper dopamine-β-hydroxylase,[54] the TNF-α converting enzyme,[55] ADAMTS-5,[56] and urease.[57] A few triazole–thione analogues with no amino group at the 4-position were reported to be modest inhibitors of the IMP-1 MBL[58,59] or were shown to be inactive against the CcrA, ImiS, and L1 MBLs at 50 μM.[60] Other triazole–thione compounds with an alkylated sulfur atom have also been published more recently,[61,62] and



Scheme 1. Hydrolysis of compound XIII[47] into compound IIIA[46] and 2-carboxybenzaldehyde in equilibrium with the cyclic lactol.

the structure of the complex formed by one of these compounds with VIM-2 showed that the two zinc atoms were coordinated by the nitrogen atoms at the 1- and 2-positions of the heterocycle.[63]

Following our findings, we investigated the potential of the triazole–thione heterocycle as a scaffold for MBL inhibition. This study describes the synthesis and evaluation of compound IIIA and its analogues as inhibitors of MBL enzymes representative of the three subclasses.

Results and Discussion

Binding mode of 1,2,4-triazole-3-thione to dizinc enzymes: The case of the L1 MBL

The 3D crystallographic structures of several important MBLs were previously obtained alone and in complex with an inhibitor, and they provided critical information on the binding modes of various inhibitors (e.g., IMP-1,[64] VIM-2,[65] VIM-4,[66] NDM-1,[67] CphA,[68] and L1).[46,69] As mentioned, compound IIIA (**1 a**) simultaneously coordinates the two Zn ions of the L1 active site through two atoms of the 1,2,4-triazole-3-thione moiety: N^2 for Zn1 and S^3 for Zn2.[46] Moreover, an interaction is formed between the 4-amino group and Asp120, one of the Zn2 ligands (Figure 1), and the phenyl ring has a weak CH–π interaction with a methyl group of Ile162. To explore the role of the amino group at the 4-position of the heterocycle, we synthesized IIIB (**1 b**, see Table 1), which differs from IIIA by the absence of this amino group. The 3D structure of L1 in complex with IIIB was obtained after soaking native L1 crystals in a solution of the inhibitor, as previously described (see Table S1 in the Supporting Information).[46] The structure (PDB ID: 5DPX) shows that IIIB is also able to coordinate the two Zn ions but, surprisingly, with a pattern different from that of IIIA. Indeed, the 1,2,4-triazole-3-thione heterocycle interacts with the Zn atoms through N^4 for Zn1 and S^3 for Zn2 (Figure 1). Compound IIIA cannot adopt a similar binding mode owing to the presence of an amino substituent on the N^4 atom of the triazole ring. The different orientation of the triazole ring results in binding of the aromatic group in an opposite area to the enzyme active site, and this leads to the formation of a strongly stabilizing π-stacking interaction with His118 (Figures 2 and 3). According to the $2F_o-F_c$ electron density map (Figure 2), the 2-toluoyl moiety can adopt two different orientations, which result from a 180° rotation of the phenyl ring around the C–C bond between the two cycles. In the case of the slightly major orientation shown in Figure 3, the methyl group of IIIB (**1 b**) is located in a hydrophobic pocket constituted by Pro227 and Phe156. Superimposition of the two complex structures with that of free L1 shows that side-chain rearrangements occur in the active site to adapt to the phenyl group of the two compounds. This adaptation can be explained by the high rigidity of the compounds and by the constraint associated with the simultaneous double coordination (Figure 4). In particular, the side chain of Tyr33 is completely retracted in the case of IIIA,[46] whereas that of Phe156 is more or less shifted by IIIB at the opposite side of the binding site depending on the orientation of its methyl group. This variable motion of the Phe156 side chain is supported by its poor electron density (Figure 2) and a B-factor higher than average (Figure S1) in the 5DPX.pdb structure. The marked movement of the Tyr33 side chain required for IIIA binding is probably more demanding than Phe156 shifting and might explain why IIIB does not bind as IIIA. Finally, a striking feature shared by the two structures is the increase in the Zn–Zn distance from 3.5 to 4.6 Å. Such a movement was already observed, although to a lesser extent, during the hydrolysis of nitrocefin by L1.[70]

IIIA and IIIB show modest activities toward L1, and IIIA displays slightly lower affinity than IIIB [$K_i = (108 \pm 9)$ and (35 ± 2) μM, respectively), which indicates that the 4-amino group is here less favorable for L1 inhibition. On the basis of these results that showed the high interest in the triazole–thione nucleus as a ligand for dizinc MBLs, we started the synthesis of analogues of IIIA and IIIB.

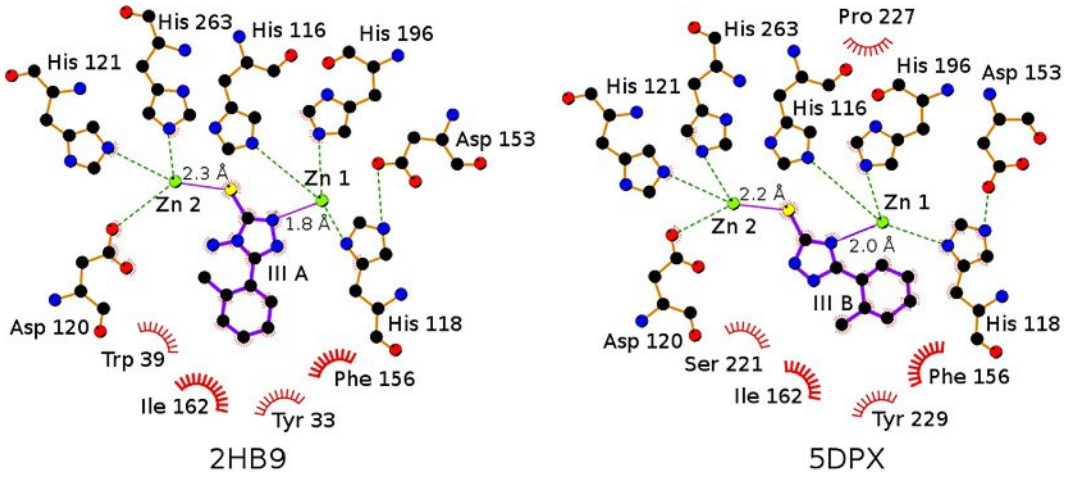


Figure 1. Binding[71a] mode of the 1,2,4-triazole-3-thione moiety of compounds IIIA (**1 a**) and IIIB (**1 b**) in the dizinc active site of L1. The structure of the L1–IIIA complex, 2HB9.pdb, was reported in Ref. [46].

Table 1. Inhibitory activity of 1,2,4-triazole-3-thiones against various MBLs.

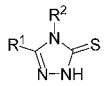
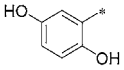
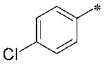
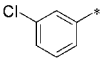
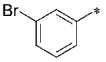
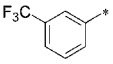
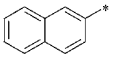
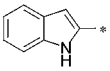
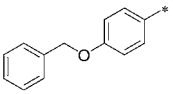
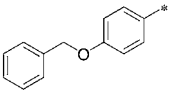
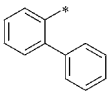
Compd			IC_{50} [μM] or (inhibition at 100 μM [%])[a]				
	R^1	R^2	L1	VIM-4	VIM-2	NDM-1	IMP-1
1a (IIIA)		NH_2	(24)	NI	NI	(20)	NI
1b (IIIB)		H	(23)	NI	NI	(18)	(57)
2a	CH_3	NH_2	NI	NI	NI	(11)	NI
2b	CH_3	H	NI	NI	(36)	(21)	(45)
3	CF_3	NH_2	NI	NI	NI	NI	NI
4		NH_2	NI	NI	NI	(47)	NI
5		NH_2	NI	NI	(13)	NI	(48)
6		NH_2	(14)	44.0±0.6	(13)	(57)	(41)
7		NH_2	(45)	(29)	NI	(37)	(≤10)
8		NH_2	(31)	(33)	(16)	(36)	13±1
9		NH_2	(27)	(31)	(27)	24±1	18±4
10		NH_2	(29)	NI	NI	(56)	NI
11		NH_2	NI	(53)	(53)	13±1	1.7±0.2
12		NH_2	NI	(12)	(14)	(18)	13±3
13		NH_2	(20)	(38)	(40)	29±2	25±0.3
14a		NH_2	(60)	101±2	(38)	36±1	23±1
14b		H	NI	(45)	NI	(41)	(43)
15		NH_2	(11)	NI	NI	(16)	8.1±0.2
16		NH_2	32±1	19±2	14±1	34±3	1.4±0.1
17a		NH_2	(43)	14±2	17±1	(52)	7.2±0.3
17b		H	97±13	25±1	49±6	11±1	1.8±0.2
18		NH_2	5.3±0.5	60±5	63±1	104±11	75±18

Table 1. (Continued)

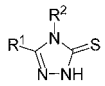
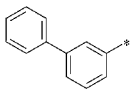
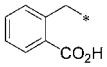
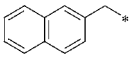
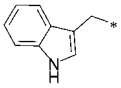
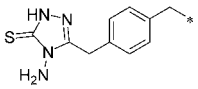
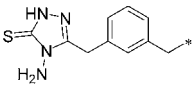
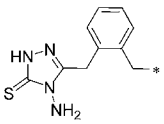
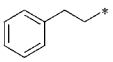
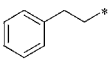
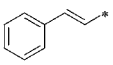
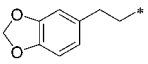
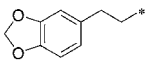
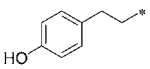
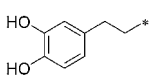
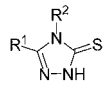
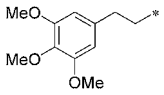
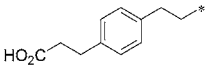
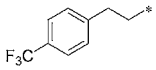
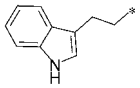
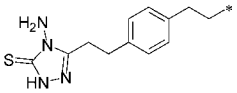
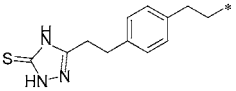
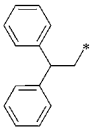
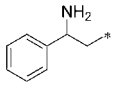
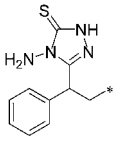
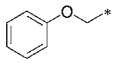
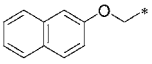
Compd			IC$_{50}$ [μM] or (inhibition at 100 μM [%])[a]				
	R^1	R^2	L1	VIM-4	VIM-2	NDM-1	IMP-1
19		NH_2	(16)	44 ± 2	51 ± 1	13 ± 1	5.6 ± 0.3
20		NH_2	NI	NI	NI	NI	NI
21		NH_2	NI	NI	NI	NI	NI
22		NH_2	NI	NI	NI	NI	NI
23 a		NH_2	(45)	(14)	NI	(53)	NI
23 b		H	(18)	NI	NI	(24)	(66)
24		NH_2	(31)	NI	NI	NI	NI
25		NH_2	(63)	NI	(11)	(15)	(31)
26		NH_2	(23)	46 ± 1	49 ± 2	12 ± 1	5.0 ± 0.2
27		NH_2	33 ± 2	31 ± 2	36 ± 1	54 ± 16	11 ± 1
28		NH_2	NI	(14)	NI	(27)	(60)
29		NH_2	(62)	(28)	NI	(41)	(33)
30		NH_2	(25)	NI	NI	(27)	NI
31		NH_2	NI	NI	NI	NI	(23)
32 a		NH_2	NI	(23)	(27)	(14)	(45)
32 b		H	NI	NI	NI	(34)	5.4 ± 0.9
33		H	NI	(12)	NI	(27)	8.0 ± 0.8
34 a		NH_2	NI	(44)	(41)	44 ± 3	27 ± 5
34 b		H	NI	NI	NI	(44)	(53)
35		H	NI	NI	NI	NI	(42)
36		NH_2	NI	NI	NI	NI	NI

Table 1. (Continued)

Compd			IC$_{50}$ [μM] or (inhibition at 100 μM [%])[a]				
	R^1	R^2	L1	VIM-4	VIM-2	NDM-1	IMP-1
37		NH_2	NI	NI	NI	NI	NI
38 b		H	NI	NI	NI	NI	NI
39		NH_2	(26)	NI	NI	(15)	(33)
40		NH_2	NI	(65)	(64)	26 ± 2	14 ± 0.1
41 a		NH_2	(63)	(33)	(13)	NI	(56)
41 b		H	(45)	NI	NI	(30)	NI
42		NH_2	NI	(53)	(45)	NI	5.1 ± 0.7
43		NH_2	(13)	NI	NI	(20)	NI
44		NH_2	(25)	NI	NI	(15)	NI
45		NH_2	(55)	46 ± 7	39 ± 1	42 ± 3	25 ± 3
46		NH_2	(32)	(10)	(20)	ND	(26)

[a] Assays were performed in triplicate by using nitrocefin as the substrate after 30 min pre-incubation of the compounds with the enzymes. Kinetic monitored at 30 °C by following the absorbance variation at $\lambda = 480$ nm. IC$_{50}$ values were determined if inhibition at 100 μM was > 70%. NI: no inhibition (≤ 10% inhibition at 100 μM). ND: not determined.

Synthesis

Two series of compounds, analogues of IIIA (**1 a**) and those of its 4-desamino derivative IIIB (**1 b**) (Table 1), were prepared as described in Scheme 2. The analogues of IIIA were synthesized from the corresponding carboxylic acids (RCOOH) in one step (R = alkyl; Beyer's method, Scheme 2 A) and in four steps (R = aryl, Scheme 2 B).[72] The analogues of IIIB were prepared in three steps (Scheme 2 C) by reaction of the acyl chloride with thiosemicarbazide followed by cyclization under basic conditions.

Most of the synthesized 1,2,4-triazole-3-thione derivatives were substituted at the 5-position by a (hetero)aromatic moiety and were directly linked to the heterocycle (as in **1 a**, **1 b**, and **5**–**22**), separated by one methylene bridge (as in **23 a**–**31**), separated by two methylene bridges (as in **32 a**–**44**), or separated by one methylene bridge and an oxygen atom (as in **45** and **46**). Starting from dicarboxylic precursors, we prepared six molecules containing two 1,2,4-triazole-3-thione moieties (i.e., compounds **29**–**31**, **41 a**, **41 b**, and **44**). Finally, four compounds (i.e., **2 a**, **2 b**, **3**, and **4**) displayed an alkyl substituent (CH_3, CF_3, cyclohexyl) at the 5-position.

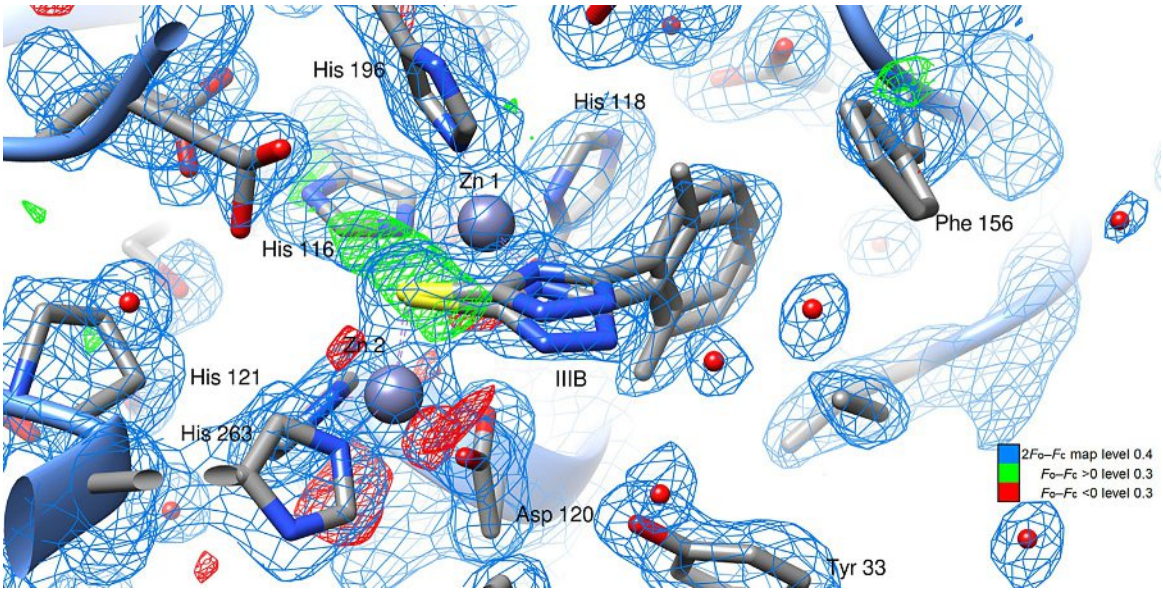


Figure 2. Electron[71b] density map for the structure of compound IIIB (**1 b**) binding in the active site of L1 (5DPX.pdb). In blue, the $2F_o-F_c$ map at 1.2σ contour level; in green and red, F_o-F_c map at 3.5σ contour level.

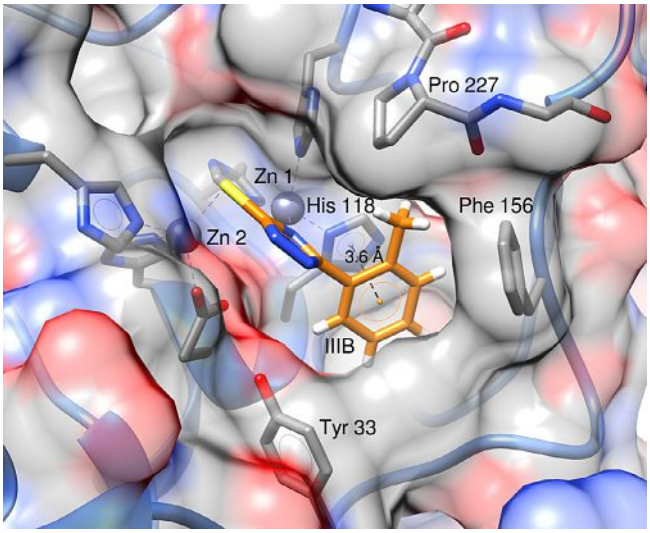


Figure 3. Compound[71b] IIIB (**1 b**) in the active site of L1 from 5DPX.pdb. The image shows the interactions established with the two Zn ions and π stacking with His118.

Evaluation of the inhibition of purified MBLs

The 1,2,4-triazole-3-thiones were first tested at a concentration of 100 μM for their potential inhibition of five representative MBLs, namely, L1, VIM-4, VIM-2, NDM-1, and IMP-1. Enzyme assays were performed in 96-well microtiter plates by using nitrocefin as the substrate. Its hydrolysis was monitored by following the absorbance variation at $\lambda = 480$ nm at $T = 30\,^{\circ}C$. IC_{50} values were then determined for the best compounds (inhibition at 100 μM >70%) by fitting raw absorbance data with the four-parameter Hill equation describing a sigmoidal dose–response curve. The inhibitory potencies are listed in Table 1.

In this single-concentration screening assay, the two parent analogues, IIIA (compound **1 a**) and IIIB (compound **1 b**), appeared either inactive or as weak inhibitors (inhibition <60% at 100 μM). In addition to **1 a** and **1 b**, a few other compounds allowed the activities of analogues of IIIA (4-amino) and IIIB (4-H) displaying the same 5-substituent (i.e., compounds **14 a** and **14 b**, **17 a** and **17 b**, **23 a** and **23 b**, **32 a** and **32 b**, **34 a** and **34 b**, **41 a** and **41 b**) to be compared. Overall, the enzymes did not show any specific preference for a particular series.

Analogues with small alkyl substituents at the 5-position (i.e., compounds **2 a** and **2 b** with a methyl group and compound **3** with a trifluoromethyl group), which were prepared to assess the contribution of the 1,2,4-triazole-3-thione moiety to binding, were also poorly active. These results demonstrated that, although essential for targeting the Zn-containing active sites, the heterocyclic scaffold had very low affinity by itself. Compound **4** with a larger alkyl substituent (i.e., R^1 = cyclohexyl) also did not show significant activity.

Compounds possessing an aryl substituent at the 5-position could be divided into three series according to the distance between the triazole–thione ring and the substituent. Overall, aryl compounds having IC_{50} values in the 1–50 μM range toward at least one MBL were obtained in the three series.

In the case of the series with an aromatic group directly linked to the triazole ring, poor inhibition was measured for compound **5** (R^1 = phenyl), a close analogue of IIIA, and for more hydrophilic analogues. Indeed, compounds **6** and **7** possessing a phenyl group substituted by one and two hydroxy groups, respectively, were only moderate inhibitors, with the exception of **6**, which inhibited VIM-4 with an IC_{50} value of 44 μM. Furthermore, changing the phenyl ring to a more hydrophilic pyridine (as in compounds **20** and **21**) or pyrazine (as in compound **22**) ring was not at all favorable.

More interesting results were obtained if the aromatic ring was substituted with either a halogen atom or a second cyclic

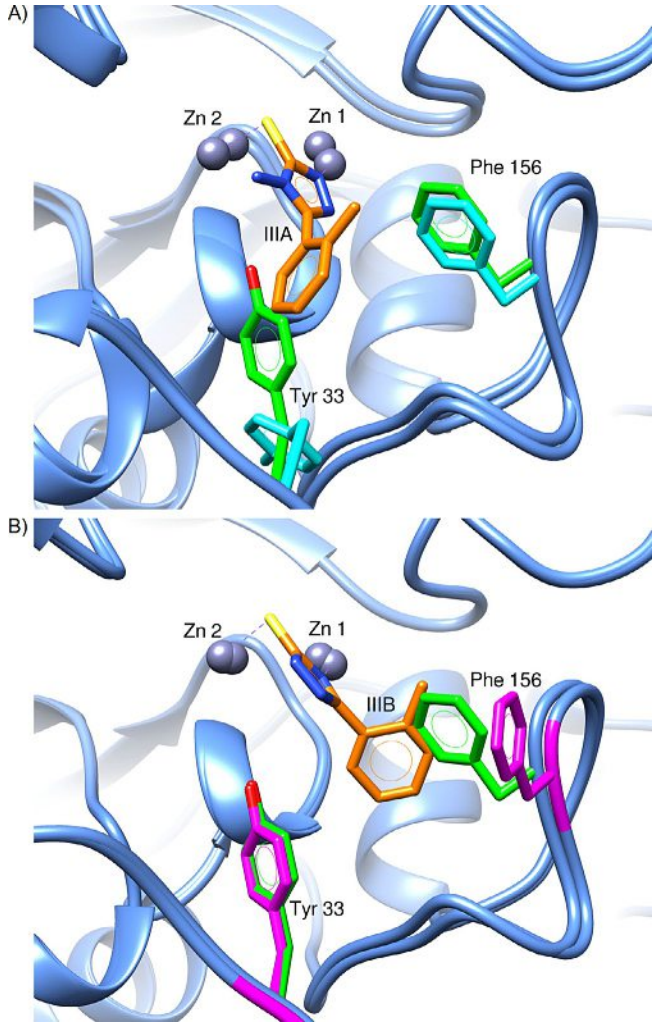
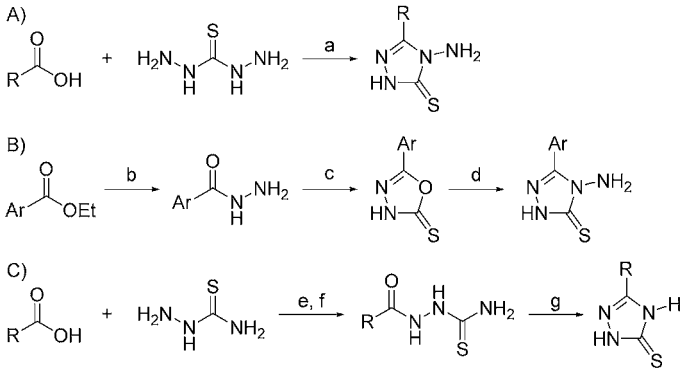


Figure 4. Superimposition[71b] of the 3D structures of free L1 (green side chains for Tyr33 and Phe156, from 2FM6.pdb)[46] and L1 complexed to A) IIIA (blue side chains, from 2HB9.pdb)[46] and B) IIIB (purple side chains, from 5DPX.pdb). The carbon atoms of the inhibitors are shown in orange. The Zn ions are depicted in magenta.



Scheme 2. Synthesis of 1,2,4-triazole-3-thione derivatives. A) 4-Amino derivatives with R≠aryl. B) 4-Amino derivatives with R=aryl (Ar). C) 4-H derivatives. *Reagents and conditions*: a) neat, 160 °C; b) hydrazine hydrate, neat, 120 °C, sealed tube; c) CS_2, KOH, EtOH, reflux; d) hydrazine hydrate, EtOH, 100 °C, sealed tube; e) $SOCl_2$, toluene, reflux; f) pyridine, toluene, RT; g) aqueous NaOH, reflux.

moiety that was either fused or separated. Indeed, with the exception of compound **10** (R^1=2-bromophenyl), all analogues bearing a halogen in the 3- or 4-position of the phenyl ring (i.e., compounds **8**, **9**, and **11**–**13**) inhibited IMP-1 in the micromolar range (IC_{50}=1.7 μM for **11** to 25 μM for **13**). Some of the compounds (i.e., **9**, R^1=3-Cl; **11**, R^1=3-Br; **13**, R^1=3-CF_3) were also active toward NDM-1 (IC_{50} values between 10 and 30 μM). Interestingly, the inhibition potency on IMP-1 and NDM-1 seemed to be correlated to the size of the halogen atom, and 3-bromophenyl analogue **11** was more favorable than 3-chlorophenyl and 3-fluorophenyl derivatives **9** and **12**. Furthermore, bicyclic analogues of IIIA {i.e., **14a**, R^1=1-naphthyl; **15**, R^1=2-naphthyl; **16**, R^1=1*H*-indol-2-yl; **17a**, R^1=4-(benzyloxy)-phenyl; **18**, R^1=[1,1′-biphenyl]-2-yl; **19**, R^1=[1,1′-biphenyl]-3-yl} and IIIB [i.e., **17b**, R^1=(4-benzyloxy)phenyl; but not **14b**, R^1=1-naphthyl] also significantly inhibited IMP-1 (IC_{50}=1.4 μM for **16** to 75 μM for **18**). Interestingly, compounds **16** (R^1=1*H*-indol-2-yl), **17a**/**17b** [R^1=(4-benzyloxy)phenyl], and **19** (R^1=[1,1′-biphenyl]-3-yl) showed wide activity spectra, as they significantly inhibited at least three out of the five tested MBLs. In particular, compounds **16**, **17b**, and **19** inhibited the three most clinically relevant MBLs, that is, NDM-1, VIM-2, and IMP-1, with IC_{50} values below or close to 50 μM.

Similar trends were observed if a CH_2 group was introduced between the aromatic ring and the 1,2,4-triazole-3-thione scaffold. Indeed, the simple arylmethyl compounds displaying a benzyl (i.e., compounds **23a** and **23b**) or a thienylmethyl (i.e., compound **25**) group, as well as a more hydrophilic 2-carboxybenzyl substituent (i.e., compound **24**) showed either no or moderate activity toward all enzymes. Again, replacing the phenyl group by a bulkier and hydrophobic bicyclic moiety such as the (5,6,7,8-tetrahydronaphthalen-1-yl)methyl group in compound **26** or the (naphthalen-2-yl)methyl group in compound **27** increased the inhibitory potency as well as the activity spectrum. However, compound **28** with an indolylmethyl group was either an inactive or only a moderate inhibitor of the tested MBLs, which indicated that separation of the indole group from the triazole cycle by one CH_2 unit and/or the substituted position (compared with **16**) was detrimental for recognition by all enzyme active sites.

In the phenylethyl series, extension of the chain by a second CH_2 group was mainly beneficial to IMP-1 inhibition. Whereas **32a** as the simple phenylethyl analogue of IIIA was poorly active, its IIIB counterpart **32b** was the best in this series and exhibited an IC_{50} value against IMP-1 in the micromolar range (5.4 μM). Similar activity against IMP-1 was measured for compound **33** (IC_{50}=8.1 μM), the unsaturated analogue of **32b**. Compound **39** with a 4-(trifluoromethyl)phenethyl moiety was poorly active against all enzymes. Bicycle-containing analogues were also significant inhibitors of IMP-1, albeit at a lower level: **34a** (but not **34b**) with a benzodioxole ring, **40** with an (1*H*-indol-3-yl)ethyl moiety, and **42** with a branched 2,2-diphenylethylene unit exhibited IC_{50} values in the micromolar range. Compounds **34a** and **40** also inhibited NDM-1 with an IC_{50} below

50 μM and moderately inhibited VIM-2 and VIM-4. As observed in other series, a more polar chain such as 4,(3)-(di)hydroxyphenethyl (as in compounds **35** and **36**), 3,4,5-trimethoxyphenethyl (as in compound **37**), 4-(3-carboxy-propion-1-yl)phenethyl (as in compound **38**), or 2-amino-2-phenylethyl (as in compound **43**) was detrimental for inhibition of all enzymes.

In a closely related series (CH_2CH_2 replaced by OCH_2), aryloxymethyl analogues **45** and **46** showed opposite behaviors. Whereas naphthyloxy-containing compound **46** was found to be poorly active on all enzymes, phenyloxy derivative **45** showed moderate inhibitory potencies toward VIM-4, VIM-2, NDM-1, and IMP-1. This was a surprising result, as the closest analogue of **45**, compound **32a**, was poorly active, and the presence of a bulky aromatic substituent was favorable in other series. This result suggested that the oxygen atom replacing the CH_2 group in **32a** significantly changed the conformation and/or contributed to hydrogen bonding with active-site residues of the MBL.

Finally, compounds **29**–**31**, **41a**, **41b**, and **44** with a double triazole–thione head and belonging to the benzyl and phenylethyl series were inactive or only moderate inhibitors of all tested MBLs. These results were not that surprising, as most of the active compounds in the present study possessed bulky and hydrophobic moieties at the 5-position of the triazole–thione heterocycle, and the second triazole–thione moiety could be considered as a polar substituent.

Some compounds (i.e., **4**, **7**–**9**, **13**, **14a**, **14b**, **17a**, **17b**, **20**–**22**, **24**–**26**, **28**–**31**, and **33**–**46**) were tested against the B2 enzyme CphA. Most of them showed less than 50% inhibition at 100 μM. Only **26**, **28**, and **37** significantly inhibited CphA with IC_{50} values of 25, 30, and 33 μM, respectively, which indicated that triazole–thione compounds could also inhibit this monozinc MBL.

Overall, compounds with either a large aromatic or halogenated aromatic substituent and no hydrophilic group were the most potent inhibitors found in this study. These results were reflected by in silico parameters calculated for all analogues (Table S3). Indeed, compounds with the largest polar surface area (PSA $\geq$ 200 Å^2) were found inactive, with the exception of compounds **16**, **34a**, and **40**, whereas a log*P* value of $\geq$1.2 was calculated for all compounds showing significant activity toward at least one MBL. This was not unexpected, as MBL inhibitors generally feature a hydrophobic moiety that binds in a predominantly hydrophobic pocket of the MBL binding sites. Moreover, the majority of the significant inhibitory potencies were obtained against IMP-1 with 19 compounds (i.e., compounds **8**, **9**, **11**–**14a**, **15**–**17b**, **19**, **26**, **27**, **32b**–**34a**, **40**, **42**, and **45**) showing IC_{50} values below 30 μM and 10 (i.e., compounds **11**, **15**–**17b**, **19**, **26**, **32b**, **33**, and **42**) showing IC_{50} values below 10 μM [compounds **11**, **16**, **32b**, and **33** showed ligand efficiency (LE) values $\geq$0.5, see Table S4]. A summary of the structure–activity relationship (SAR) for IMP-1 is presented in Figure 5. Among these compounds, some showed wider spectra of activity toward NDM-1 (i.e., compounds **9**, **11**, **13**, **14a**, **16**, **17b**, **19**, **26**, **34a**, **40**, and **45**) and VIM-2/VIM-4/L1 (i.e., compounds **16**, **17a**, **17b**, **19**, **26**, **27**, and **45**). Notably, none of the halogenated compounds significantly inhibited

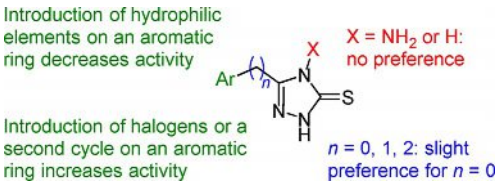


Figure 5. Summary of the SARs of the 1,2,4-triazole-3-thione derivatives for IMP-1 inhibition. These SARs are valid for all enzymes except that VIM-2, VIM-4, and L1 are not or are only poorly inhibited by halogenated analogues.

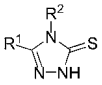
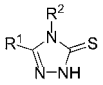
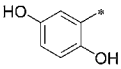
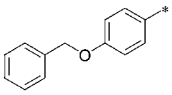
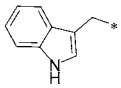
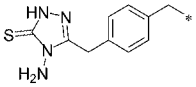
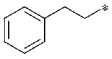
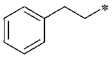
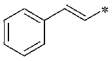
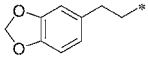
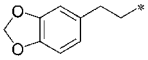
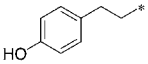
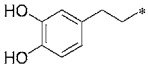
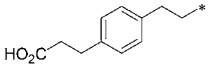
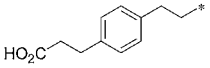
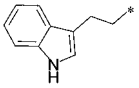
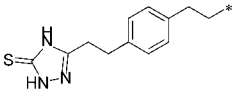
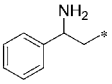
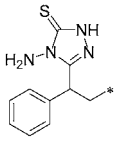
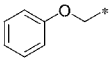
VIM-2. These different behaviors between the B1 enzymes IMP-1, NDM-1, and VIM-2 probably reflect the variability in the structures of the loops involved in inhibitor binding.[73] In this context, it is not surprising that VIM-2 and VIM-4 here shared a very close inhibition profile. Finally, compounds **16** (R^1 = 1*H*-indol-2-yl), **17b** [R^1 = 4-(benzyloxy)phenyl], **19** (R^1 = [1,1′-biphenyl]-3-yl), **26** [R^1 = (5,6,7,8-tetrahydronaphthalen-1-yl)methyl], and **27** [R^1 = (naphthalen-2-yl)methyl] were the most interesting MBL inhibitors reported in this study.

Evaluation of triazole–thione analogues as inhibitors of other dizinc enzymes of the MBL superfamily

MBLs belong to the so-called MBL superfamily, which contains numerous other binuclear Zn-dependent hydrolases with similar protein folds but with different biological functions, such as glyoxalase II, several nucleases, the quorum-quenching *N*-acyl-L-homoserine lactone hydrolase, or the bacterial desuccinylase DapE.[3,74] We evaluated the potential inhibitory effect of some of the triazole–thione compounds on three of these enzymes: glyoxalase II from *Plasmodium falciparum* (Table 2) and the exo/endoribonucleases tRNase Z from *Escherichia coli* and RNase J1 from *Bacillus subtilis*. In the case of the ribonucleases, none of the tested compounds (i.e., **1a**, **1b**, **2a**, **2b**, **3**, **5**, **23a**, **23b**, **32a**, and **32b**) showed inhibitory activity against RNase J1 at 100 μM or against tRNase Z at 1 mM, with the exception of IIIA analogue **2a**, which had a K_i value of 0.2 mM against tRNase Z. Given that this compound possesses a methyl group, the smallest substituent in the series, these results suggested that a bigger substituent at this position could not be accommodated by the enzyme. Corresponding non-amino compound **2b** was inactive. Therefore, the 1,2,4-triazole-3-thione scaffold did not appear to be suitable for the inhibition of dizinc ribonucleases.

A representative panel of triazole–thione compounds (i.e., **1a**–**5**, **7**, **14a**, **17a**, **23a**/**23b**, **25**, **26**, **28**, **29**, **32a**–**36**, **38a**, **38b**, **40**, **41b**, and **43**–**45**) was tested on glyoxalase II from *P. falciparum* (Table 2). A few of them showed modest inhibition with IC_{50} values in the 10–60 μM range, and the best inhibitor was compound **1a** (IIIA). If comparison was possible, the 4-amino derivatives were generally more potent than their non-amino counterparts (i.e., **1a**/**1b**, **2a**/**2b**, **23a**/**23b**, **32a**/**32b**, and **34a**/**34b**).

Table 2. Inhibitory activities of 1,2,4-triazole-3-thiones against glyoxalase II from *P. falciparum*.

Compd	 R¹	R²	IC_{50} [μM][a]	Compd	 R¹	R²	IC_{50} [μM][a]
1a (IIIA)		NH_2	10	**1b** (IIIB)		H	22
2a	CH_3	NH_2	>60 (14)	**2b**	CH_3	H	>60 (8)
3	CF_3	NH_2	60	**4**		NH_2	>40 (10)
5		NH_2	22	**7**		NH_2	>40 (0)
14a		NH_2	>40 (33)	**17a**		NH_2	20
23a		NH_2	60	**23b**		H	>40 (2)
25		NH_2	>20 (0)	**26**		NH_2	60
28		NH_2	>40 (0)	**29**		NH_2	>20 (0)
32a		NH_2	30	**32b**		H	>40 (22)
				33		H	>20 (0)
34a		NH_2	19	**34b**		H	>20 (25)
35		H	>60 (23)	**36**		NH_2	>20 (0)
38a		NH_2	>20 (25)	**38b**		H	60
40		NH_2	>40 (11)	**41b**		H	>30 (0)
43		NH_2	>40 (0)	**44**		NH_2	>20 (30)
45		NH_2	>20 (23)				

[a] If no IC_{50} has been measured, percent inhibition at the highest tested concentration is given in parentheses. Owing to the high absorbance of the compounds at $\lambda = 240$ nm, only limited concentrations could be tested in the enzymatic assay. For example, >60 (40%) implies that the highest concentration tested was 60 μM; at this concentration, the enzyme was inhibited by 40%. Therefore, $IC_{50} > 60$ μM.

Conclusions

Overall this study confirmed the potential of the 1,2,4-triazole-3-thione moiety (2,4-dihydro-3*H*-1,2,4-triazole-3-thione) as a metal ligand, as it is well adapted to target the dizinc active sites of metallo-β-lactamases. A total of 54 analogues of compounds IIIA (**1a**) and IIIB (**1b**) differing in their side chain at the 5-position of the heterocycle were prepared. We first demonstrated that L1, VIM-4, VIM-2, NDM-1, and IMP-1 did not show any specific preference for one of the 4*H*-1,2,4-triazole-3-thione or 4-amino-1,2,4-triazole-3-thione series. By exploring the pattern at the 5-position, we identified several inhibitors with an IC_{50} value in the micromolar range toward at least one metallo-β-lactamase (MBL; i.e., L1, VIM-4, VIM-2, NDM-1, or IMP-1). Of high interest, several compounds significantly inhibited the three most clinically relevant MBLs, that is, NDM-1,

IMP-1, and VIM-2. In particular, inhibitor **16** (4-amino-2,4-dihydro-5-(2-indolyl)-3*H*-1,2,4-triazole-3-thione) showed IC_{50} values below 50 μM toward these three enzymes as well as against VIM-4 and L1 (Table 1). This indicated that broad-spectrum inhibitors could be obtained from this series. In addition, a few compounds showed significant inhibitory activity toward the B2 enzyme CphA, which suggested that the heterocycle-thione could also target monozinc MBLs.

Screening some compounds against other enzymes of the MBL superfamily showed that triazole–thione analogues were nonfunctional for ribonucleases, which did not accept substituents larger than a methyl group at the 5-position. Higher activity was observed against the *Plasmodium* glyoxalase II. The size of the triazole–thione moiety, the constraint associated to the double coordination, and the moderate affinity for metals resulting in a very low potency for small analogues **2a**–**3** might offer a greater probability of sparing human metalloenzymes. On the basis of these results, further developments toward more potent triazole–thione analogues are in progress.

Experimental Section

General methods

^{1}H NMR and ^{13}C NMR spectra were recorded with a 300 or 400 MHz instrument in [D_6]DMSO solutions unless otherwise indicated. Splitting patterns in the ^{1}H NMR spectra are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Samples for reverse-phase (RP) HPLC were prepared in an acetonitrile/water (50:50 *v/v*) mixture. Compounds insoluble in this mixture were solubilized in DMSO. RP HPLC analyses were performed with a Chromolith SpeedRod C_{18} column (0.46×5 cm) by means of a linear gradient (0–100%) of 0.1% trifluoroacetic acid (TFA)/acetonitrile in 0.1% aqueous TFA over 5 min at a flow rate of 3 mL min^{-1}. LC–MS analysis was performed with a Waters Alliance 2690 HPLC coupled to a Waters-Micromass ZQ spectrometer (electrospray ionization mode, ESI+). All analyses were performed by using a RP C_{18} monolithic Onyx Phenomenex 2.5×0.46 cm column. The flow rate was set to 3 mL min^{-1} with eluent A (water/0.1% formic acid), and a gradient of 0→100% of eluent B (acetonitrile/0.1% formic acid) over 3 min was then used. Positive-ion electrospray mass spectra were acquired at a solvent flow rate of 100–500 μL min^{-1}. Nitrogen was used as both the nebulizing and drying gas. The data were obtained in a scan mode in 0.1 s intervals; 10 scans were summed up to get the final spectrum. Thin-layer chromatography was performed on aluminum-backed sheets of silica gel F_{254} (0.2 mm), which were visualized under $\lambda = 254$ nm light and by spraying with a 2% EtOH solution of ninhydrin followed by heating or by charring with an aqueous solution of ammonium sulfate and sulfuric acid [200 g $(NH_4)_2SO_4$ and 40 mL concentrated sulfuric acid in 1 L of water]. Column chromatography was performed by using Merck silica gel 60 of particle size 40–63 μm.

Chemistry

General procedure for the preparation of 4-amino-5-substituted-triazole-3-thiones

Route A (R≠aryl): The carboxylic acid (1 equiv) and thiocarbohydrazide (1 or 2 equiv) were ground, mixed together in a mortar, and then heated between 140 and 160 °C until fusion occurred. After cooling, a dilute aqueous solution of Na_2CO_3 (20 mL g^{-1}) was added, and the mixture was heated at 70 °C for 30 min. Generally, a precipitate appeared upon cooling, and it was collected by filtration and washed with water. Otherwise, water was evaporated. Compounds were either purified by crystallization in ethanol or by silica gel column chromatography.

Route B (R=aryl): **CAUTION:** *Hydrazine and carbon disulfide should be handled with care.* The carboxylic acid (1 equiv) was first converted into its ethyl ester by heating at reflux in absolute ethanol (15 mL g^{-1}) in the presence of a few drops of H_2SO_4. The ester was then treated overnight with hydrazine hydrate (2–4 equiv) without solvent at 120 °C. Evaporation of the excess amount of hydrazine gave the corresponding hydrazide compound. The hydrazide, solubilized in absolute ethanol (20 mL g^{-1}), was treated with CS_2 (5 equiv) in the presence of KOH (1.7 equiv) at 85 °C for 3 h. Water (20 mL g^{-1}) was added, and the pH was adjusted to 2–3 with 1 N HCl. The formed precipitate was collected by filtration and was washed with water to yield the 1,3,4-oxadiazolthione, which was used without further purification. Finally, the preceding compound (1 equiv) was treated with hydrazine hydrate (10 equiv) in absolute ethanol at 100 °C overnight in a sealed tube. After evaporation of the excess amount of hydrazine, the residue was purified by silica gel column chromatography to yield the final compound.

General procedure for the preparation of non-amino 5-substituted-triazole-3-thiones

Thionyl chloride (10 equiv) was added to a solution of the corresponding carboxylic acid (1 equiv) in toluene (5 mL g^{-1}), and the mixture was heated at reflux for 5 h. After removal of the solvent under vacuum, the residue was taken up in toluene (20 mL g^{-1}), and the solution was added to a suspension of thiosemicarbazide (1 equiv) in pyridine (20 mL g^{-1}) cooled in an ice/salt bath. The mixture was stirred at −5 °C for 1 h and at RT overnight. After filtration, the solvent was evaporated, and 2 N aqueous NaOH (50 mL g^{-1}) was added. The mixture was heated at reflux for 4 h and then acidified to pH 2 with concentrated aqueous HCl. The formed precipitate was then recovered by filtration, washed with 0.1 N HCl, and purified by silica gel column chromatography. The physical data and spectral information for all compounds are provided in the Supporting Information.

Enzymology

Metallo-β-lactamase inhibition assay: The 1,2,4-triazole-3-thiones were screened for inhibition against six representative metallo-β-lactamases, namely, the acquired IMP-1, VIM-2, VIM-4 and NDM-1, CphA from *Aeromonas hydrophila* and L1 from *Stenotrophomonas maltophilia*. Purified preparations of these enzymes were already available and were used in enzyme assays. Buffers were prepared with Milli-Q water and Zn^{II} residual concentrations were below 0.4 μM.

NDM-1, VIM-2, VIM-4, IMP-1, and L1 assays: Nitrocefin was solubilized at a concentration of 10 mM in DMSO and was then diluted to 128, 400, 480, 720, and 64 μM, respectively, in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) buffer solution pH 7.0 containing 50 μM $ZnSO_4$. The 1,2,4-triazole-3-thiones were solubilized at 10 mM in DMSO and were then diluted to 133 μM in the same buffer. NDM-1, VIM-2, VIM-4, IMP-1, and L1 enzymes were diluted to 80, 0.8, 0.8, 48, and 48 nM, respectively, in

50 mM HEPES buffer solution pH 7.0 containing 50 μM $ZnSO_4$ and 20 μg mL^{-1} bovine serum albumin (BSA).

Enzyme inhibition assays were performed in nonsterile acrylic 96-well microtiter plates with UV-transparent flat bottoms (Corning) in a reaction volume of 200 μL. The final DMSO concentration in the assay did not exceed 1%. The enzymes (25 μL stock solution) were mixed with the 1,2,4-triazole-3-thiones (150 μL) so that the final concentration of the compound reached 100 μM. Pre-incubation was performed at 30 °C for 30 min, and then the reaction was initiated by adding nitrocefin solution (25 μL). The final concentrations of nitrocefin were 16, 50, 60, 90, and 8 μM for NDM-1, VIM-2, VIM-4, IMP-1, and L1, respectively, which corresponded to three to four times the K_M values [measured K_M values were (4.4 ± 0.5), (16.5 ± 0.8), (18.9 ± 0.4), (31 ± 10), and (2.0 ± 0.3) μM for NDM-1, VIM-2, VIM-4, IMP-1, and L1, respectively]. The final concentrations of NDM-1, VIM-2, VIM-4, IMP-1, and L1 were 10, 0.1, 0.1, 0.6, and 0.6 nM, respectively. Nitrocefin hydrolysis was monitored at 30 °C by following the absorbance variation at $\lambda = 480$ nm by using a Victor 3V microplate reader. Initial velocities were obtained from plots of absorbance versus time by using data points from the linear portion of the hydrolysis curve. Inhibition percentages were calculated from the ratio of initial velocities with and without inhibitor.

IC_{50} values were determined for compounds exhibiting >70% inhibition at 100 μM. Final concentrations of 1,2,4-triazole-3-thiones were adjusted to 0–200 μM by 1:2 serial dilutions of the stock solutions. IC_{50} values were determined by fitting raw absorbance data with the four-parameter Hill equation describing a sigmoidal dose–response curve by using Microsoft Office Excel 2016. The assays were performed in triplicate; errors were less than 5%.

The quality of these enzyme assays was determined by calculating their Z' factors[75] with the following equation [Eq. (1)]:

$$Z'\text{factor} = 1 - \left(\frac{3\sigma_p + 3\sigma_n}{|\mu_p - \mu_n|}\right) \quad (1)$$

in which σ is the standard deviation and μ is the mean initial velocity for positive (p) and negative (n) controls. The Z' factors for the NDM-1, VIM-2, VIM-4, IMP-1, and L1 inhibition tests were found to be 0.70, 0.70, 0.79, 0.62, and 0.74, respectively.

CphA assays: Compounds were prepared as 10 mM solutions in DMSO before dilution with 15 mM Cacodylate pH 6.5 buffer. The enzyme was used at a final concentration of 0.8 nM. The enzyme and the inhibitor (100 μM) were pre-incubated for 30 min in a volume of 495 μL at 21 °C. Then, 10 mM imipenem (5 μL) was added, and the hydrolysis of imipenem was monitored by following the absorbance variation at $\lambda = 300$ nm by using an UvikonXL spectrophotometer connected to a computer through an RS232 serial interface. Experiments were performed at 30 °C, and initial rate conditions were used to study the inhibition with imipenem. The activity was tested by measuring the initial rates in three samples without inhibitor, which allowed determination of the percentage of residual β-lactamase activity in the presence of inhibitors. If the residual activity in the presence of 100 μM inhibitor was <30%, the initial rate conditions were used to study the inhibition in the presence of increasing concentrations of compounds (from 1 to 100 μM) and to determine the IC_{50} values. The assays were performed in triplicate; errors were less than 5%.

Test of RNase J1 ribonuclease activity: *B. subtilis* RNase J1 was pre-incubated with inhibitors for 10 min on ice in 10 μL reaction buffer [20 mM Tris·HCl pH 8.0, 8 mM $MgCl_2$, 100 mM NH_4Cl, 0.1 mM dithiothreitol (DTT)]. The reaction was started by adding quenched RNA (10 μL, ACUAGA-FAM-CAUACA-TAMRA) in the same buffer. The enzyme was assayed at 0.3 μM, and inhibitors were tested at final concentrations of 10, 20, and 100 μM. Release of FAM (fluorescein amidite) fluorescence ($\lambda = 520$ nm) following cleavage between FAM and TAMRA (tetramethylrhodamine) moieties was assayed at 30 °C in a Rotor Gene RT-PCR apparatus, as described.[76]

Kinetic measurements with *E. coli* tRNase Z: The *E. coli* tRNase Z was purified as previously described.[77] Phosphodiesterase activity was measured by using bis(*p*-nitrophenyl)phosphate (bpNPP) as a substrate.[78] Reactions were performed in 20 mM Tris·HCl pH 7.4, 100 mM NaCl at 22 °C. After 15 min incubation of tRNase Z with the inhibitor, reactions were initiated by adding bpNPP and were monitored by measuring the release of *p*-nitrophenol (pNP) at $\lambda =$ 405 nm for 2 min. Catalytic activities were expressed in μmol of pNP generated per minute per active subunit at 22 °C. All measurements were performed in triplicate, and experiments were repeated with at least two independent enzyme preparations.

Test of *P. falciparum* cytosolic glyoxalase II (PfcGloII) activity: The recombinant enzyme was produced in *E. coli* and purified as described.[79] To measure the activity of GloII, the decrease in absorbance resulting from *S*-D-lactoylglutathione ($\varepsilon_{240\,nm} = 3.1$ $mM^{-1}cm^{-1}$) hydrolysis was measured at 25 °C in 100 mM 4-morpholinopropane sulfonate (MOPS) buffer, pH 7.2, in a total volume of 0.5 mL. *S*-D-Lactoylglutathione was obtained from Sigma. Inhibitors were dissolved in DMSO and were added to the enzyme assay mixture before the reaction was started with enzyme (5–25 nM PfcGloII). For each assay, background absorbances induced by the inhibitors were taken into account. All experiments were performed in duplicate. Results are provided as mean values that differed by less than 15%.

Acknowledgements

We thank Mr. Pierre Sanchez for mass spectrometry analyses and Wolfram Meyer-Klaucke for advice about tRNase Z. Part of this work was supported by the Agence Nationale de la Recherche ("ANTIMBL", ANR-14-CE16-0028-01, including a fellowship to L.S.), the Deutsche Forschungsgemeinschaft (BE1540/15-2 within SPP 1710 to K.B.), and Agence Nationale de la Recherche ("subtilRNA", ANR-06-BLAN-0086) to C.C.

Conflict of interest

The authors declare no conflict of interest.

Keywords: antibiotics · bacterial resistance · lactams · metalloenzymes · nitrogen heterocycles

[1] a) T. R. Walsh, M. A. Toleman, *J. Antimicrob. Chemother.* **2012**, *67*, 1–3; b) G. M. Rossolini, F. Arena, P. Pecile, S. Pollini, *Curr. Opin. Pharmacol.* **2014**, *18*, 56–60.
[2] P. Nordmann, *Med. Mal. Inf.* **2014**, *44*, 51–56.
[3] C. Bebrone, *Biochem. Pharmacol.* **2007**, *74*, 1686–1701.
[4] T. R. Walsh, M. A. Toleman, L. Poirel, P. Nordmann, *Clin. Microbiol. Rev.* **2005**, *18*, 306–325.
[5] E. K. Phelan, M. Miraula, C. Selleck, D. L. Ollis, G. Schenk, N. Mitic, *Am. J. Mol. Biol.* **2014**, *4*, 89–104.
[6] V. P. Sandanayaka, A. S. Prashad, *Curr. Med. Chem.* **2002**, *9*, 1145–1165.

[7] M. Babic, A. M. Hujer, R. A. Bonomo, *Drug Resist. Updates* **2006**, *9*, 142 – 156.
[8] P. Nordmann, T. Naas, L. Poirel, *Emerging Infect. Dis.* **2011**, *17*, 1791 – 1798.
[9] T. Palzkill, *Ann. N. Y. Acad. Sci.* **2013**, *1277*, 91 – 104.
[10] C. Bebrone, P. Lassaux, L. Vercheval, J. S. Sohier, A. Jehaes, E. Sauvage, M. Galleni, *Drugs* **2010**, *70*, 651 – 669.
[11] S. M. Drawz, R. A. Bonomo, *Clin. Microbiol. Rev.* **2010**, *23*, 160 – 201.
[12] P. Oelschlaeger, N. Ai, K. T. Duprez, W. J. Welsh, J. H. Toney, *J. Med. Chem.* **2010**, *53*, 3013 – 3027.
[13] Z. Guo, S. Ma, *Curr. Drug Targets* **2014**, *15*, 689 – 702.
[14] J. H. Toney, P. M. Fitzgerald, N. Grover-Sharma, S. H. Olson, W. J. May, J. G. Sundelof, D. E. Vanderwall, K. A. Cleary, S. K. Grant, J. K. Wu, J. W. Kozarich, D. L. Pompliano, G. G. Hammond, *Chem. Biol.* **1998**, *5*, 185 – 196.
[15] S. Bounaga, A. P. Laws, M. Galleni, M. I. Page, *Biochem. J.* **1998**, *331*, 703 – 711.
[16] D. J. Payne, J. H. Bateson, B. C. Gasson, D. Proctor, T. Khushi, T. H. Farmer, D. A. Tolson, D. Bell, P. W. Skett, A. C. Marshall, R. Reid, L. Ghosez, Y. Combret, J. Marchand-Brynaert, *Antimicrob. Agents Chemother.* **1997**, *41*, 135 – 140.
[17] C. Mollard, C. Moali, C. Papamicael, C. Damblon, S. Vessilier, G. Amicosante, C. J. Schofield, M. Galleni, J.-M. Frère, G. C. Roberts, *J. Biol. Chem.* **2001**, *276*, 45015 – 45023.
[18] S. Siemann, A. J. Clarke, T. Viswanatha, G. I. Dmitrienko, *Biochemistry* **2003**, *42*, 1673 – 1683.
[19] H. Kurosaki, Y. Yamaguchi, T. Higashi, K. Soga, S. Matsueda, H. Yumoto, S. Misumi, Y. Yamagata, Y. Arakawa, M. Goto, *Angew. Chem. Int. Ed.* **2005**, *44*, 3861 – 3864; *Angew. Chem.* **2005**, *117*, 3929 – 3932.
[20] Q. Sun, A. Law, M. W. Crowder, H. M. Geysen, *Bioorg. Med. Chem. Lett.* **2006**, *16*, 5169 – 5175.
[21] B. M. R. Liénard, R. Hüting, P. Lassaux, M. Galleni, J.-M. Frère, C. J. Schofield, *J. Med. Chem.* **2008**, *51*, 684 – 688.
[22] B. M. R. Liénard, G. Garau, L. Horsfall, A. I. Karsisiotis, C. Damblon, P. Lassaux, C. Papamicael, G. C. Roberts, M. Galleni, O. Dideberg, J.-M. Frère, C. J. Schofield, *Org. Biomol. Chem.* **2008**, *6*, 2282 – 2294.
[23] P. Lassaux, M. Hamel, M. Gulea, H. Delbrück, P. S. Mercuri, L. Horsfall, D. Dehareng, M. Kupper, J.-M. Frère, K. Hoffmann, M. Galleni, C. Bebrone, *J. Med. Chem.* **2010**, *53*, 4862 – 4876.
[24] X.-L. Liu, Y. Shi, J. S. Kang, P. Oelschlaeger, K.-W. Yang, *ACS Med. Chem. Lett.* **2015**, *6*, 660 – 664.
[25] F.-M. Klingler, T. A. Wichelhaus, D. Frank, J. C. Bernal, J. El-Delik, H. F. Müller, H. Sjuts, S. Göttig, A. Koenigs, K. M. Pos, D. Pogoryelov, E. Proschak, *J. Med. Chem.* **2015**, *58*, 3626 – 3630.
[26] O. K. Arjomandi, W. M. Hussein, P. Vella, Y. Yusof, H. E. Sidjabat, G. Schenk, R. P. McGeary, *Eur. J. Med. Chem.* **2016**, *114*, 318 – 327.
[27] P. Hinchliffe, M. M. Gonzalez, M. F. Mojica, J. M. Gonzalez, V. Castillo, C. Saiz, M. Kosmopoulou, C. L. Tooke, L. I. Llarrull, G. Mahler, R. A. Bonomo, A. J. Vila, J. Spencer, *Proc. Natl. Acad. Sci. USA* **2016**, *113*, E3745 – E3754.
[28] J. H. Toney, G. G. Hammond, P. M. Fitzgerald, N. Sharma, J. M. Balkovec, G. P. Rouen, S. H. Olson, M. L. Hammond, M. L. Greenlee, Y. D. Gao, *J. Biol. Chem.* **2001**, *276*, 31913 – 31918.
[29] L. E. Horsfall, G. Garau, B. M. Liénard, O. Dideberg, C. J. Schofield, J.-M. Frère, M. Galleni, *Antimicrob. Agents Chemother.* **2007**, *51*, 2136 – 2142.
[30] Y. Hiraiwa, A. Morinaka, T. Fukushima, T. Kudo, *Bioorg. Med. Chem. Lett.* **2009**, *19*, 5162 – 5165.
[31] Y. Ishii, M. Eto, Y. Mano, K. Tateda, K. Yamaguchi, *Antimicrob. Agents Chemother.* **2010**, *54*, 3625 – 3629.
[32] A. M. King, S. A. Reid-Yu, W. Wang, D. T. King, G. De Pascale, N. C. Strynadka, T. R. Walsh, B. K. Coombes, G. D. Wright, *Nature* **2014**, *510*, 503 – 506.
[33] F. von Nussbaum, G. Schiffer, *Angew. Chem. Int. Ed.* **2014**, *53*, 11696 – 11698; *Angew. Chem.* **2014**, *126*, 11884 – 11887.
[34] A. M. Somboro, D. Tiwari, L. A. Bester, R. Parboosing, L. Chonco, H. G. Kruger, P. I. Arvidsson, T. Govender, T. Naicker, S. Y. Essack, *J. Antimicrob. Chemother.* **2015**, *70*, 1594 – 1596.
[35] B. M. R. Liénard, L. E. Horsfall, M. Galleni, J.-M. Frère, C. J. Schofield, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 964 – 968.
[36] S. Siemann, D. P. Evanoff, L. Marrone, A. J. Clarke, T. Viswanatha, G. I. Dmitrienko, *Antimicrob. Agents Chemother.* **2002**, *46*, 2450 – 2457.
[37] D. Minond, S. A. Saldanha, P. Subramaniam, M. Spaargaren, T. Spicer, J. R. Fotsing, T. Weide, V. V. Fokin, K. B. Sharpless, M. Galleni, C. Bebrone, P. Lassaux, P. Hodder, *Bioorg. Med. Chem.* **2009**, *17*, 5027 – 5037.
[38] T. Weide, S. A. Saldanha, D. Minond, T. P. Spicer, J. R. Fotsing, M. Spaargaren, J.-F. Frère, C. Bebrone, K. B. Sharpless, P. Hodder, V. V. Fokin, *ACS Med. Chem. Lett.* **2010**, *1*, 150 – 154.
[39] X. Wang, M. Lu, Y. Shi, Y. Ou, X. Cheng, *PLoS One* **2015**, *10*, e0118290.
[40] M. Brindisi, S. Brogi, S. Giovani, S. Gemma, S. Lamponi, F. De Luca, E. Novellino, G. Campiani, J. D. Docquier, S. Butini, *J. Enzyme Inhib. Med. Chem.* **2016**, *31*, 98 – 109.
[41] M. W. Walter, A. Felici, M. Galleni, R. P. Soto, R. M. Adlington, J. E. Baldwin, J.-F. Frère, M. Gololobov, C. J. Schofield, *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2455 – 2458.
[42] D. J. Payne, J. A. Hueso-Rodríguez, H. Boyd, N. O. Concha, C. A. Janson, M. Gilpin, J. H. Bateson, C. Cheever, N. L. Niconovich, S. Pearson, S. Rittenhouse, D. Tew, E. Diez, P. Perez, J. De La Fuente, M. Rees, A. Rivera-Sagredo, *Antimicrob. Agents Chemother.* **2002**, *46*, 1880 – 1886.
[43] J. Brem, R. Cain, S. Cahill, M. A. McDonough, I. J. Clifton, J. C. Jimenez-Castellanos, M. B. Avison, J. Spencer, C. W. G. Fishwick, C. J. Schofield, *Nat. Commun.* **2016**, *7*, 12406.
[44] J. Spencer, T. R. Walsh, *Angew. Chem. Int. Ed.* **2006**, *45*, 1022 – 1026; *Angew. Chem.* **2006**, *118*, 1038 – 1042.
[45] T. Christopeit, A. Albert, H.-K. S. Leiros, *Bioorg. Med. Chem.* **2016**, *24*, 2947 – 2953.
[46] L. Nauton, R. Kahn, G. Garau, J.-F. Hernandez, O. Dideberg, *J. Mol. Biol.* **2008**, *375*, 257 – 269.
[47] L. Olsen, S. Jost, H. W. Adolph, I. Pettersson, L. Hemmingsen, F. S. Jørgensen, *Bioorg. Med. Chem.* **2006**, *14*, 2627 – 2636.
[48] T. Christopeit, T. J. O. Carlsen, R. Helland, H.-K. S. Leiros, *J. Med. Chem.* **2015**, *58*, 8671 – 8682.
[49] N. F. Eweiss, A. A. Bahajaj, E. A. Elsherbini, *J. Heterocycl. Chem.* **1986**, *23*, 1451 – 1458.
[50] M. Ashok, B. S. Holla, B. Poojary, *Eur. J. Med. Chem.* **2007**, *42*, 1095 – 1101.
[51] B. Tozkoparan, N. Gökhan, G. Aktay, E. Yeşilada, M. Ertan, *Eur. J. Med. Chem.* **2000**, *35*, 743 – 750.
[52] A. Ts. Mavrova, D. Wesselinova, Y. A. Tsenov, P. Denkova, *Eur. J. Med. Chem.* **2009**, *44*, 63 – 69.
[53] Z. Li, Z. Gu, K. Yin, R. Zhang, Q. Deng, J. Xiang, *Eur. J. Med. Chem.* **2009**, *44*, 4716 – 4720.
[54] L. I. Kruse, C. Kaiser, W. E. DeWolf, J. A. Finkelstein, J. S. Frazee, E. L. Hilbert, S. T. Ross, K. E. Flaim, J. L. Sawyer, *J. Med. Chem.* **1990**, *33*, 781 – 789.
[55] J. L. Gilmore, B. W. King, N. Asakawa, K. Harrison, A. Tebben, J. E. Sheppeck II, R. Q. Liu, M. Covington, J. J. Duan, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 4678 – 4682.
[56] L. Maingot, F. Leroux, V. Landry, J. Dumont, H. Nagase, B. Villoutreix, O. Sperandio, R. Deprez-Poulain, B. Deprez, *Bioorg. Med. Chem. Lett.* **2010**, *20*, 6213 – 6216.
[57] Z. Amtul, M. Rasheed, M. I. Choudhary, R. Supino, K. M. Khan, Atta-Ur-Rahman, *Biochem. Biophys. Res. Commun.* **2004**, *319*, 1053 – 1063.
[58] P. Vella, W. M. Hussein, E. W. W. Leung, D. Clayton, D. L. Ollis, N. Mitic, G. Schenk, R. P. McGeary, *Bioorg. Med. Chem. Lett.* **2011**, *21*, 3282 – 3285.
[59] Faridoon, W. M. Hussein, P. Vella, N. U. Islam, D. L. Ollis, G. Schenk, R. P. McGeary, *Bioorg. Med. Chem. Lett.* **2012**, *22*, 380 – 386.
[60] L. Feng, K.-W. Yang, L.-S. Zhou, J.-M. Xiao, X. Yang, L. Zhai, Y.-L. Zhang, M. W. Crowder, *Bioorg. Med. Chem. Lett.* **2012**, *22*, 5185 – 5189.
[61] Y.-L. Zhang, K. W. Yang, Y. J. Zhou, A. E. LaCuran, P. Oelschlaeger, M. W. Crowder, *ChemMedChem* **2014**, *9*, 2445 – 2448.
[62] S.-K. Yang, J. S. Kang, P. Oelschlaeger, K. W. Yang, *ACS Med. Chem. Lett.* **2015**, *6*, 455 – 460.
[63] T. Christopeit, K.-W. Yang, S.-K. Yang, H.-K. S. Leiros, *Acta Crystallogr. Sect. F* **2016**, *72*, 813 – 819.
[64] N. O. Concha, C. A. Janson, P. Rowling, S. Pearson, C. A. Cheever, B. P. Clarke, C. Lewis, M. Galleni, J.-M. Frère, D. J. Payne, J. H. Bateson, S. S. Abdel-Meguid, *Biochemistry* **2000**, *39*, 4288 – 4298.
[65] I. Garcia-Saez, J.-D. Docquier, G. M. Rossolini, O. Dideberg, *J. Mol. Biol.* **2008**, *375*, 604 – 611.
[66] P. Lassaux, D. A. Traoré, E. Loisel, A. Favier, J.-D. Docquier, J. S. Sohier, C. Laurent, C. Bebrone, J.-M. Frère, J.-L. Ferrer, M. Galleni, *Antimicrob. Agents Chemother.* **2011**, *55*, 1248 – 1255.

[67] H. M. Zhang, Q. Hao, *FASEB J.* **2011**, *25*, 2574 – 2582.
[68] G. Garau, C. Bebrone, C. Anne, M. Galleni, J.-M. Frère, O. Dideberg, *J. Mol. Biol.* **2005**, *345*, 785 – 795.
[69] J. H. Ullah, T. R. Walsh, I. A. Taylor, D. C. Emery, C. S. Verma, S. J. Gamblin, J. Spencer, *J. Mol. Biol.* **1998**, *284*, 125 – 136.
[70] R. M. Breece, Z. Hu, B. Bennett, M. W. Crowder, D. L. Tierney, *J. Am. Chem. Soc.* **2009**, *131*, 11642 – 11643.
[71] a) Figure 1 was drawn using LigPlot: A. C. Wallace, R. A. Laskowski, J. M. Thornton, *Protein Eng.* **1995**, *8*, 127 – 134; b) Figures 2 – 4 were drawn using UCSF Chimera software: E. F. Pettersen, T. D. Goddard, C. C. Huang, G. S. Couch, D. M. Greenblatt, E. C. Meng, T. E. Ferrin, *J. Comput. Chem.* **2004**, *25*, 1605 – 1612.
[72] G. Turan-Zitouni, Z. A. Kaplancikli, K. Erol, F. S. Kiliç, *Il Farmaco* **1999**, *54*, 218 – 223.
[73] E. Cadag, E. Vitalis, K. P. Lennox, C. L. E. Zhou, A. T. Zemla, *BMC Res. Notes* **2012**, *5*, 96.
[74] F. Baier, N. Tokuriki, *J. Mol. Biol.* **2014**, *426*, 2442 – 2456.
[75] J. H. Zhang, T. D. Chung, K. R. Oldenburg, *J. Biomol. Screening* **1999**, *4*, 67 – 73.
[76] F. Sinturel, O. Pellegrini, S. Xiang, L. Tong, C. Condon, L. Bénard, *RNA* **2009**, *15*, 2057 – 2062.
[77] B. Kostelecky, E. Pohl, A. Vogel, O. Schilling, W. Meyer-Klaucke, *J. Bacteriol.* **2006**, *188*, 1607 – 1614.
[78] A. Vogel, O. Schilling, M. Niecke, J. Bettmer, W. Meyer-Klaucke, *J. Biol. Chem.* **2002**, *277*, 29078 – 29085.
[79] M. Akoachere, R. Iozef, S. Rahlfs, M. Deponte, B. Mannervik, D. J. Creighton, R. H. Schirmer, K. Becker, *Biol. Chem.* **2005**, *386*, 41 – 52.

Manuscript received: March 24, 2017
Revised manuscript received: May 12, 2017
Accepted manuscript online: May 15, 2017
Version of record online: June 12, 2017