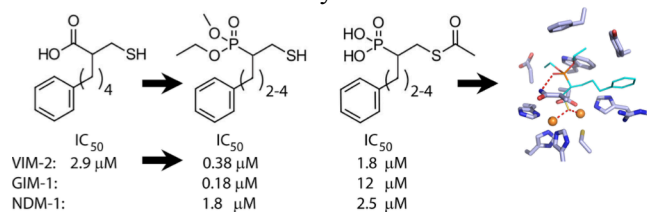


Graphical Abstract

Metallo- β -lactamase inhibitors by bioisosteric replacement: preparation, activity and binding

Leave this area blank for abstract info.

Susann Skagseth^{a,#}, Sundus Akhter^{b,#}, Marianne H. Paulsen^{b,†}, Zeeshan Muhammad^b, Silje Lauksund^c, Ørjan Samuelsen^{c,d}, Hanna-Kirsti S. Leiros^{a,*} and Annette Bayer^{b,*}



Metallo- β -lactamase inhibitors by bioisosteric replacement: preparation, activity and binding

Susann Skagseth^{a,#}, Sundus Akhter^{b,#}, Marianne H. Paulsen^{b,†}, Zeeshan Muhammad^b, Silje Lauksund^c, Ørjan Samuelsen^{c,d}, Hanna-Kirsti S. Leiros^{a,*} and Annette Bayer^{b,*}

^a The Norwegian Structural Biology Centre (NorStruct), Department of Chemistry, Faculty of Science and Technology, UiT The Arctic University of Norway, N-9037 Tromsø, Norway.

^b Department of Chemistry, Faculty of Science and Technology, UiT The Arctic University of Norway, N-9037 Tromsø, Norway.

^c Norwegian National Advisory Unit on Detection of Antimicrobial Resistance, Department of Microbiology and Infection Control, University Hospital of North Norway, N-9038 Tromsø, Norway.

^d Department of Pharmacy, UiT The Arctic University of Norway, N-9037 Tromsø, Norway.

* Corresponding authors: Annette Bayer, E-mail: annette.bayer@uit.no, Phone +47 77 64 40 69; Hanna-Kirsti S. Leiros, E-mail: hanna-kirsti.leiros@uit.no, Phone +47 77 64 57 06.

These authors have contributed equally to this work.

† Current address: Department of Pharmacy, UiT The Arctic University of Norway, N-9037 Tromsø, Norway.

ARTICLE INFO

Article history:

Received

Received in revised form

Accepted

Available online

Keywords:

Crystal structure;

Inhibition properties;

Carboxylate bioisosters;

Thiols;

Metallo- β -lactamase inhibitors

ABSTRACT

Bacterial resistance is compromising the use of β -lactam antibiotics including carbapenems. The main resistance mechanism against β -lactams is hydrolysis of the β -lactam ring mediated by serine- or metallo- β -lactamases (MBLs). Although several inhibitors of MBLs have been reported, none has been developed into a clinically useful inhibitor. Mercaptocarboxylic acids are among the most prominent scaffolds reported as MBL inhibitors. In this study, the carboxylate group of mercaptocarboxylic acids was replaced with bioisosteric groups like phosphonate esters, phosphonic acids and *MH*-tetrazoles. The influence of the replacement on the bioactivity and inhibitor binding was evaluated. A series of bioisosteres of previously reported inhibitors was synthesized and evaluated against the MBLs VIM-2, NDM-1 and GIM-1. The most active inhibitors combined a mercapto group and a phosphonate ester or acid, with two/three carbon chains connecting a phenyl group. Surprisingly, also compounds containing thioacetate groups instead of thiols showed low IC₅₀ values. High-resolution crystal structures of three inhibitors in complex with VIM-2 revealed hydrophobic interactions for the diethyl groups in the phosphonate ester (inhibitor **2b**), the mercapto bridging the two active site zinc ions, and tight stacking of the benzene ring to the inhibitor between Phe62, Tyr67, Arg228 and His263. The inhibitors show reduced enzyme activity in *Escherichia coli* cells harboring MBL. The obtained results will be useful for further structural guided design of MBL inhibitors.

2017 Elsevier Ltd. All rights reserved.

1. Introduction

Antibiotics are essential for modern medicine and as antibiotics are now becoming increasingly ineffective, effective treatment of an ever-increasing range of infections is threatened.[1, 2] As defence mechanism against β -lactam antibiotics, bacteria are able to produce β -lactamases that hydrolyse and inactivates the antibiotic.[3, 4] β -Lactamases are grouped into two super families, the serine dependent β -lactamases (SBLs; Amber class A, C, and D) and metallo- β -lactamases (MBLs; Amber class B).[5, 6] The MBLs are classified into three subclasses (B1, B2 and B3), with the majority of nosocomial MBLs belonging to the B1 subclass. This includes variants of the Verona integron-encoded metallo- β -

lactamase (VIM), the New Delhi metallo- β -lactamase (NDM), [7] and the German Imipenemase (GIM). The active site of the B1 MBLs is generally well preserved despite large variation in the second shell interacting residues. The active site holds two cationic zinc ions (Zn1, Zn2), as a metal co-factor of enzyme activity, which are coordinated by H116, H118 and H196 (Zn1) and D120, C221 and H263 (Zn2).[8, 9] Here and throughout the paper, the standard numbering scheme for class B β -lactamases is used. [10]. In general, the sequence identity among the class B1 MBLs is low. For the enzymes used in this study, the sequence identity is 32% between VIM-2 and NDM-1, 28% for VIM-2 versus GIM-1 and 24% between NDM-1 and GIM-1 (**Figure S1** in SI).

The MBLs hydrolyze a broad spectrum of β -lactam antibiotics [3, 8, 11, 12] including the last resort antibiotics; the carbapenems. In addition, genes encoding certain MBLs are associated with mobile genetic elements and can spread across different types of Gram-negative bacteria.[7, 11] Inhibitors of SBLs are available clinically, [13] including the broad spectrum inhibitor avibactam. [14] Unfortunately, all SBL inhibitors lack activity against MBLs. [15] Although the search for MBL inhibitors started from mid 1990s, and a wide variety of scaffolds has been reported [16-18], clinically useful MBL inhibitors remain an elusive goal.

In this study we decided to explore the use of thiol-based compounds as one of our strategies to gain increased knowledge on MBL inhibitors. Thiols have the potential to coordinate the zinc ions in the MBL active site, due to the thiophilic nature of zinc, and thereby preventing β -lactam hydrolysis. Some mercaptocarboxylic acids have been evaluated successfully against several MBLs [19-32], making this class of compounds an interesting starting point for further investigations. Our focus was to elucidate the effect of replacement of the carboxylate group in the promising mercaptocarboxylic acid scaffold with bioisosters on the bioactivity and enzyme-inhibitor binding. Bioisosteric replacement is a concept in medicinal chemistry to rationally improve the activity or physicochemical properties of biologically active compounds. [33] A structural element of a biological active compound is replaced with a substitute that maintain some of the properties of the parent structure, e.g. inhibitor binding to a biological target, while others are changed, e.g. lipophilicity or steric size. Typical bioisosters of carboxylic acids include among others phosphonates, NH-tetrazoles and sulphonamides. [34]

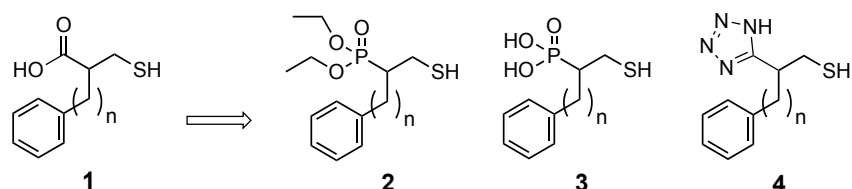
The target structures **2–4** (Scheme 1) were envisioned by substitution of the carboxylate group of the known mercaptocarboxylic acid inhibitor **1** [25, 35] with bioisosteric

groups like phosphonate esters, phosphonic acids or NH-tetrazoles, respectively. The carbon chain of the alkylphenyl substituent was varied ($n = 2-4$; Scheme 1) with the goal to evaluate the effect of chain length on hydrophobicity and bioactivity. The compounds and relevant intermediates from the synthetic pathway were simultaneously evaluated against the three MBLs VIM-2, NDM-1 and GIM-1 with the goal to find MBL inhibitors able to act on several different MBL subfamilies. For each target we have determined the IC_{50} value through enzyme inhibition assays and the effect of the inhibitors in whole cell *E. coli* assays with VIM-2, GIM-1 or NDM-1. Some inhibitors were also tested in a synergy assay with two or three clinical isolates from *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* or *Escherichia coli*. The enzyme-inhibitor complexes of the best inhibitors were further evaluated by X-ray analysis and the impact from second shell interacting residues on ligand binding were examined.

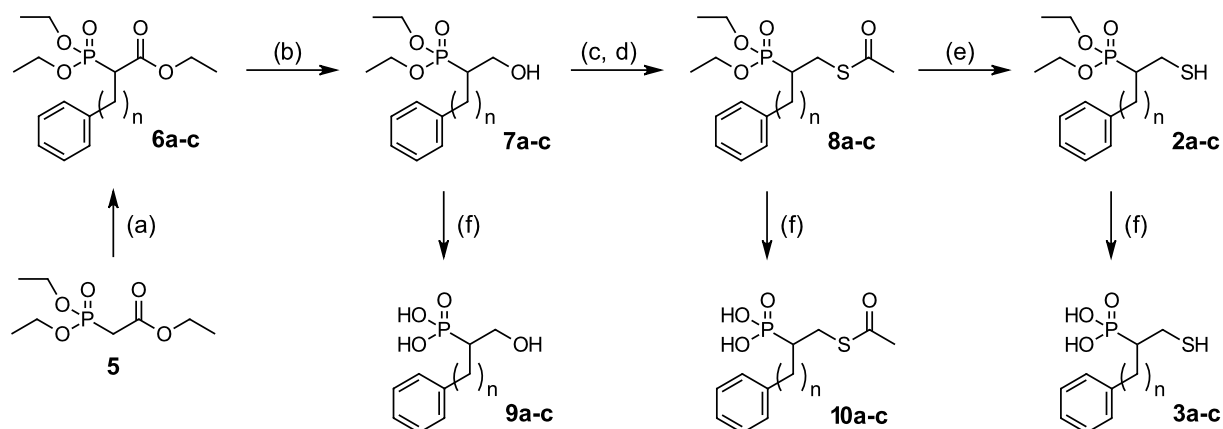
2. Results and discussion

2.1. Chemistry

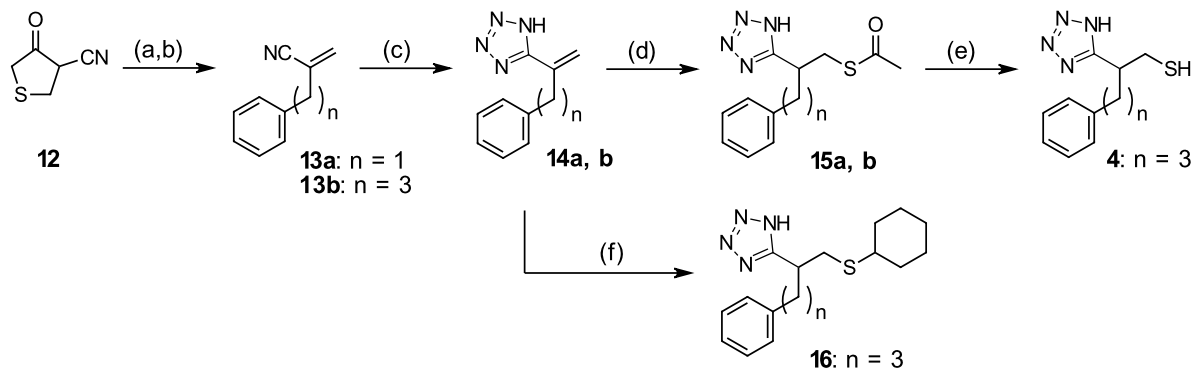
For the purpose of this work, all compounds (Scheme 2 and 3) were prepared as racemic mixtures. Phosphonate ester **2a–c** and phosphonic acid **3a–c** analogues with differing chain lengths ($n = 2-4$) were prepared according to the synthetic strategy presented in Scheme 2. Triethyl phosphonoacetate **5** was alkylated using potassium *tert*-butoxide (KO*t*Bu) as base to afford the mono-alkylated acetates **6a–c** in moderate yields (45–73%). Chemoselective reduction of the ester in presence of the phosphonate was obtained with lithium borohydride to provide the corresponding alcohols **7a–c**. Subsequent mesylation followed by substitution with potassium thioacetate gave the thioacetates **8a–c**. Several methods for deprotection of the thioacetates were evaluated. [36-38] Best results were obtained by treatment with sodium methylthiolate (NaSMe) providing the free thiols **2a–c** in



Scheme 1. Design of new MBL inhibitors through bioisosteric substitution of the carboxylate group.



Scheme 2. Synthesis of phosphonate and phosphonic acid containing thiol-based inhibitors. Reagents and conditions: **a**: $n = 2$; **b**: $n = 3$; **c**: $n = 4$; (a) R–Br, KO*t*Bu, DMF, 0 °C, **6a**: 62%, **6b**: 73%, **6c**: 45%; (b) LiBH₄, THF, MW 80 °C for 10 min, **7a**: 60%, **7b**: 95%, **7c**: 56%; (c) MsCl, Et₃N, DMAP, CH₂Cl₂, rt; (d) KSAc, DMF, rt; **8a**: 54%, **8b**: 34%, **8c**: 71% over two steps; (e) NaSMe, MeOH, –20 °C, **2a**: 30%, **2b**: 74%, **2c**: 77%; (f) TMSBr, CH₂Cl₂, then MeOH, rt., **3a**: 96%, **3b**: 90%, **3c**: 98%, **9a**: 76%, **9b**: 61%, **9c**: 55%, **10a**: 91%, **10b**: 71%, **10c**: 65%.



Scheme 3. Synthesis of NH-tetrazole containing thiol based inhibitors. Reagents and conditions: **a**: $n = 1$; **b**: $n = 3$; (a) R—Br, K_2CO_3 , acetone, reflux; (b) 5% aq. NaOH, **13a**: 51%, **13b**: 21%; (c) $TMSN_3$, $n-Bu_2SnO$ (20 mol%), 1,4-dioxane, MW 150 °C for 50 min, **14a**: 78%, **14b**: 74%; (d) HSAc, DMF, 60 °C, **15a**: 95%, **15b**: 98%; (e) NaSMe, MeOH, -20 °C, **4**: 74%; (f) cyclohexylthiol, DMF, 60 °C, **16**: 93%.

good yields (74–77%) and purity (>95% by HPLC). The phosphonate ester analogues **2**, **7** and **8** were purified by normal-phase flash column chromatography to >95% purity as determined by HPLC analysis. The diethyl phosphonates **2**, **7** and **8** were transferred to the corresponding phosphonic acid derivatives **3**, **9** and **10**, respectively, by treatment with trimethylsilyl bromide (TMSBr) followed by a MeOH quench. Purification of the phosphonic acid analogues **3**, **9** and **10** could be achieved by washing with EtOAc/pentane to provide the target compounds with moderate to good yields and purity >95% as determined by HPLC analysis.

The NH-tetrazole analogues **4**, **15** and **16** were prepared as shown in **Scheme 3**. α -Substituted acrylonitriles **13a** and **13b** with varying chain lengths ($n = 1, 3$) were prepared by a procedure based on the work of Baraldi et al. [39]. Microwave promoted reaction of the acrylonitriles with trimethylsilyl azide with dibutyltin oxide as catalyst (20 mol%) [40] gave the corresponding NH-tetrazoles **14a** and **14b** (74–78% yield), which proved to be excellent Michael acceptors. The addition of potassium thioacetate resulted in the tetrazolyl thioacetates **15a** and **15b** in good yields (> 95 %), while the addition of cyclohexanethiol gave **16** (93% yield). Deprotection of the thioacetates by treatment with sodium methylthiolate (NaSMe) provided the free thiol **4** in moderate yield (74%) and purity >96% as determined by HPLC analysis.

2.2. Characterization of inhibitor properties against VIM-2, GIM-1 and NDM-1

The inhibitory activities of the mercaptocarboxylic acid **1c** [25] and bioisosters **2a–c**, **3a–c** and **4**, as well as several intermediates from the synthesis (**7–10 a–c**, **15a**, **15b** and **16**) were determined by the half maximal inhibitory concentration (IC_{50}) values (**Table 1**). For VIM-2 and GIM-1, the IC_{50} values were measured using nitrocefin as a reporter substrate, while IC_{50} values for NDM-1 were measured with imipenem as reporter substrate. Nitrocefin is hydrolyzed by NDM-1 with a high catalytic efficiency and unsuitable as a reporter substrate for NDM-1 [41]. The influence of dimethyl sulfoxide (DMSO) on the enzyme activity was investigated in the assay, and a concentration of 2.5% DMSO was tolerated without influencing the enzyme activity (data not shown). The IC_{50} values were determined by measuring the initial rate of the reactions with inhibitors at different concentration in a 2-fold dilution series, and were fitted to a dose-response curve (IC_{50} curves are given in **Figure S2–S4** in SI).

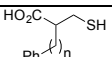
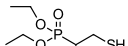
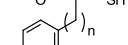
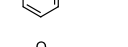
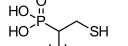
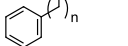
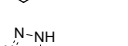
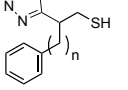
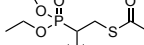
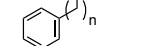

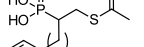
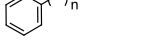
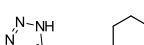
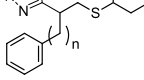
In order to validate our assay, the IC_{50} of the previously reported VIM-2 inhibitor **1c** [25] was measured against VIM-2. We obtained an IC_{50} of 2.9 μM , which is in the same range as the reported value of 1.1 μM (**Table 1**). The difference in IC_{50} values is most likely due to different assay buffers and protein constructs. The new compounds showed IC_{50} values ranging from 0.38 to 133 μM with VIM-2, 0.18 to >5000 μM with GIM-1 and 1.8 to 144 μM with NDM-1 (**Tables 1** and **S1** in SI). The synthetic intermediates **7–9 a–c**, **15a** and **15b** did not have inhibitory activity ($IC_{50} > 10 \mu M$, see **Table S1** in SI) against any of the MBLs. Compounds **2a–c**, **3a–c**, **4**, **10a–c** and **16** showed activity at varying levels. In general, the inhibitors showed better IC_{50} values towards VIM-2 compared to GIM-1 and NDM-1, with the exception of inhibitor **2a**, which had a ~five times lower IC_{50} for GIM-1 (0.18 μM) compared to VIM-2 (0.89 μM).

Inhibitors **2a** and **2b** showed IC_{50} values ranging from 0.18–2.2 μM for all three MBLs. The IC_{50} s were within the same range for VIM-2 and GIM-1, and slightly increased for NDM-1 (1.8–2.2 μM). Inhibitor **2c** precipitated from the buffer solution and was not evaluated further. The results indicate that for the mercaptophosphonates **2**, lengthening the side chain from two (**2a**) to three methylene groups (**2b**), did not have a great effect on the activity.

While the mercaptophosphonate esters **2** showed highest activity against GIM-1, the mercapto- and thioacetate phosphonic acids, **3** and **10**, were more active towards VIM-2 and NDM-1 than GIM-1. The IC_{50} values for thioacetate phosphonic acids **10a–c** ranged from 1.8 to 4.7 μM , and from 2.5 to 6.6 μM for VIM-2 and NDM-1, respectively. With GIM-1 the inhibitors **10a–c** showed slightly higher IC_{50} values of 12 to 26 μM . The same trend was seen with the mercaptophosphonic acids **3a** and **c**, where the IC_{50} values for VIM-2 and NDM-1 were in the same range (7.8 and 8.6 μM with VIM-2 and 5.9 and 8.5 μM with NDM-1, respectively), while the values for GIM-1 were 2–4 fold higher (23 and 16 μM , respectively). A possible explanation for this might be that GIM-1 has a narrower active site compared to other MBLs due to the aromatic side chains Trp228 and Tyr233. [42] However, this does not explain the low IC_{50} values for inhibitors **2a** and **2b**.

Substituting the carboxylic acid of compound **1** with a NH-tetrazole (**4**) had a deteriorating effect on the inhibitory activity, while substitution with phosphonate esters and phosphonic acid groups led to similar (**2** and **10**) or improved (**3**) activity. Comparing the inactive alcohols **7** and **9** with the corresponding active thiols and thioacetates **2**, **3** and **10** illustrates the

Table 1. Evaluation of compound **1** and bioisosteres thereof as inhibitors of VIM-2, GIM-1 or NDM-1 measured as inhibition concentrations (IC₅₀) against purified enzyme and percent inhibition in *E. coli* SNO3 bacterial whole cell experiments.

Compound	VIM-2 ^a		GIM-1 ^a		NDM-1 ^b	
	IC ₅₀ (μM)	% inhib	IC ₅₀ (μM)	% inhib	IC ₅₀ (μM)	% inhib
 1c (n = 4)	2.9 (1.1) ^c	-	-	-	56	-
 2a (n = 2)	0.89	78	0.18	64	2.2	33
 2b (n = 3)	0.38	79	0.31	48	1.8	62
 2c (n = 4)	p ^d	p ^d	p ^d	p ^d	p ^d	p ^d
 3a (n = 2)	7.8	92	23	37	5.9	42
 3b (n = 3)	33.2	95	28	46	7.1	45
 3c (n = 4)	8.6	94	16	31	8.5	42
 4 (n = 3)	28	67	68	25	12	16
 8a (n = 2)	133	17	18	25	nh ^e	i ^f
 8b (n = 3)	34	14	26	21	nh ^e	i ^f
 8c (n = 4)	20	12	13	25	nh ^e	i ^f
 10a (n = 2)	2.3	94	12	40	2.9	39
 10b (n = 3)	4.7	95	26	37	6.6	38
 10c (n = 4)	1.8	97	20	50	2.5	37
 16 (n = 3)	5	73	36	8	10	i ^f

^a Reporter substrate used was nitrocefin (NCF); ^b Reporter substrate used was imipenem (IPM); ^c Values in parentheses as reported by Jin et al. [25]; ^d p = precipitated in buffer and was not tested; ^e nh = no hydrolysis; ^f i = inactive.

importance of the sulfur atom on the β-carbon for the inhibitor activity. However, activity seem to depend on a subtle combination of the sulfur atom and a carboxylic acid bioisoster as revealed when comparing the mercapto and thioacetate substituted phosphonate esters and phosphonic acids **2**, **3**, **8** and **10**. The mercapto substituted phosphonate esters **3** were the overall most active inhibitors, while the thioacetate substituted phosphonate esters **8** showed the lowest activity of all four. Surprisingly, the same trend was not observed for the pair of phosphonic acids **2** and **10**. Here, the thiol **2** and the thioacetate **10** inhibited the MBLs at the same level, which may indicate that the interaction of the phosphonic acid with the enzyme is more important for the activity than the interaction with the thiol. This interpretation would be in agreement with the previously reported observation that a mercaptophosphonic acid coordinated the MBL CphA by the two oxygen atoms of the phosphonic acid and not by the sulfur atom of the thiol. [43]

The most active inhibitors showed activity in the low micromolar to high nano micromolar range for the racemic mixtures. In two recent studies, pure stereoisomers of the mercaptocarboxylic acids captopril [19] and bithiazolidine [21] were found to display 10–100 times differences in inhibitory activity between different stereoisomers. This will most likely be the case for the compounds prepared in this work, and for future work this can be pursued. A large variation in inhibitory activity towards three B1 MBLs was observed, which indicates that these inhibitors do not fulfill the requirements for a broad-spectrum MBL inhibitor.

2.3. Evaluation of inhibitors in bacterial cell assays

To investigate if the inhibitors were active against MBLs in bacterial cells, two different assays were used. Initially, the inhibitors were tested in a whole cell assay using *E. coli* SNO3 cells with *bla*_{VIM-2}, *bla*_{GIM-1} or *bla*_{NDM-1} in pET26b(+) (**Table 1**). The inhibitory activity were measured as percent inhibition calculated by measuring the slope of the initial hydrolysis of the reporter substrates for up to 60 minutes, and the slope of a control without inhibitor present, according to equation 1.

The percent inhibition for the most active compounds is shown in **Table 1**. High percent inhibition (>70%) was observed for several compounds, which indicates that the compounds pass the bacterial cell membrane of *E. coli*. The highest percent inhibition was observed for inhibitors **3a–c** and **10 a–c** in *E. coli* with VIM-2, with 92 to 95% and 94 to 97% inhibition, respectively. These results are in agreement with the low IC₅₀ values (1.8–8.6 and 33 μM). The inhibitory activity of **3a–c** and **10a–c** was less pronounced with GIM-1 and NDM-1 resulting in an inhibition of around 30 to 50%, although the IC₅₀ values against NDM-1 were in the same range as against VIM-2.

The inhibitors **2a** and **2b**, showing lowest IC₅₀ values, showed 78% and 79% inhibition with VIM-2, respectively. While the percent inhibition of inhibitor **2a** and **2b** were 64% and 48% for GIM-1 and 33% and 62% for NDM-1, respectively. The phosphonate esters **2** were found to be more hydrophobic at pH 7.1 (clogD = 3.4–3.8), which is the pH of the buffer medium, compared to the phosphonic acids **3** and **10** (clogD = -0.9–0.1), which probably results in poorer passive permeability [44] of the former explaining the lower % inhibition observed for these compounds. For NDM-1, inhibitor **2b** was the best inhibitor in terms of the lowest IC₅₀ and highest effect in the cell-based

screening assay. Modification of **2** to alter the physicochemical properties may be a direction for further inhibitor improvement.

Additionally, the inhibitors were tested for synergistic activity with meropenem against MBL-producing clinical strains, and effect from the inhibitors alone on the bacterial growth. The results are given in **Table S2** in the SI. Disappointingly, synergy testing of the inhibitors with meropenem using bacterial strains from *P. aeruginosa* or *K. pneumoniae* did not show a lowering of the MIC. However, more promising results were observed when **3b** and **10b** were tested against an *E. coli* strain with VIM-29. In these experiments, the MIC was lowered from 8–32 mg/L with only meropenem to 1 mg/L in presence of inhibitors (**Table S2** in SI). Since VIM-2 has 90% sequence identity to VIM-29 it is likely to believe that VIM-2 could also be inhibited in a similar assay. In addition, the tested compounds were not toxic at high concentrations (>500 μ M) to the three clinical cell lines included in this study (**Table S2** in SI).

2.4. Crystal structure complexes of VIM-2 with **2b**, **10b** and **10c**

To better understand the effects of bioisosteric substitution and to address the question of the coordinating properties of the phosphonic acid group compared to the thiol, we attempted to obtain complex structures of the new inhibitors. Initial attempts to soak native crystals were found unsuccessful, whereas our new protocol for a DMSO free co-crystallization method [45] was successful for inhibitors **2b**, **10b** and **10c**. Several more inhibitors were included in the experiment without providing satisfactory complexes. All the new VIM-2 complex structures have two protein molecules in the asymmetric unit, a $\alpha\beta/\beta\alpha$ fold with mobile loops adjacent to the active site, and there are two zinc ions in the active site, Zn1 bound to His116, His118 and His122 and Zn2 coordinated by Asp120, Cys221 and His263. For all structures, the interactions in the best-defined protein chain will be described, and only discrepancies for other chain will be mentioned.

The VIM-2_2b complex was the best-defined complex to 1.55 Å (**Table 2**) and this complex was used to investigate the two different enantiomers. Herein, the (*S*)-form of the inhibitor **2b** did not fit into the observed polder omit map (**Figure 1A**), whereas for the (*R*)-form (**Figure 1B**), the substituents at the stereogenic center are all defined within the polder omit map. Therefore, we anticipate that VIM-2 is selectively binding the (*R*)-enantiomer of **2b**, since the CH₂-SH group, the CH₂-CH₂ chain and the phosphonate ester are defined inside both the polder omit and the final 2Fo-Fc map (**Figure 1B, C**). The three-dimensional arrangement of substituents on the stereogenic centre of (*R*)-**2b** (**Figure 2**) is in agreement with the reported complex of VIM-2_1b [35] selectively bound to (*S*)-**1b**. Note that the difference in naming of the stereoisomers ((*S*)-**1b** versus (*R*)-**2b**) relates to differences in priority according to the Khan-Ingold-Prelog rules (for **1b**: CH₂SH > CO₂H while for **2b**: P(O)(OH)₂ > CH₂SH) not to inversion of the stereocenter. For both VIM-2_10b and **10c** structures, the (*R*)-configuration fits best in the polder omit and 2Fo-Fc maps (**Figure 1D–I**), but less convincing than for the highest resolved VIM-2_2b complex (**Figure 1A–C**). This indicates that for the structurally related compounds of this study the three-dimensional arrangement corresponding to the (*R*)-configuration probably leads to inhibitors with improved binding and inhibition properties.

Table 2. X-ray data collection and crystallographic refinement statistics for VIM-2 complexes of **2b**, **10b** and **10c**. Values in parenthesis are for the highest resolution shell.

	VIM-2_2b	VIM-2_10b	VIM-2_10c
PDB entry			
X-ray source	ID29, ESRF	ID29, ESRF	ID29, ESRF
Data collection statistics			
Space group	C2	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Unit cell (Å)			
a	101.11	45.78	45.72
b	79.07	90.87	91.07
c	67.47	124.08	122.92
β (°)	130.3		
Resolution (Å)	24.94-1.55 (1.61-1.55)	24.75-1.85 (1.92-1.85)	24.77-1.67 (1.73-1.67)
Wavelength (Å)	0.97625	0.97625	0.97625
No. unique reflections	54949 (5447)	45 000 (4421)	56573 (5666)
Multiplicity	2.0 (1.9)	6.5 (6.7)	6.4 (6.3)
Completeness (%)	93.58 (93.51)	99.93 (99.98)	94.12 (92.38)
Mean <I> / < σ _I >	8.16 (1.92)	14.60 (2.30)	10.80 (1.61)
R-merge ^a	0.064 (0.417)	0.073 (0.791)	0.102 (0.925)
CC _{1/2}	0.995 (0.679)	0.999 (0.785)	0.997 (0.634)
Resolution (Å)	24.94-1.55 (1.61-1.55)	24.75-1.85 (1.92-1.85)	24.77-1.67 (1.73-1.67)
Wavelength (Å)	0.97625	0.97625	0.97625
No. unique reflections	54949 (5447)	45 000(4 421)	56573 (5666)
Multiplicity	2.0 (1.9)	6.5 (6.7)	6.4 (6.3)
Completeness (%)	93.58 (93.51)	99.93 (99.98)	94.12 (92.38)
Mean <I> / < σ _I >	8.16 (1.92)	14.60 (2.30)	10.80 (1.61)
R-merge ^a	0.064 (0.417)	0.073 (0.791)	0.102 (0.925)
CC _{1/2}	0.995 (0.679)	0.999 (0.785)	0.997 (0.634)
Wilson B-factor (Å ²)	14.84	27.24	20.49
Refinement statistics			
Resolution (Å)	24.94-1.55	25-1.85	25-1.67
R-factor (all reflections)	0.1575	0.1662	0.1778
R-free ^b	0.2006	0.2039	0.2140
RMSD bond lengths (Å)	0.011	0.013	0.011
RMSD bond angles (°)	1.31	1.32	1.32
Ramachandran favored (%)	98.0	97.0	97.0
Ramachandran outliers (%)	0.46	0.47	0.95
Clashscore	5.50	3.00	5.62
Average B-factor (Å ²)			
All atoms	20.8	36.5	27.4
Chain A/B	20.6/20.6	32.6/37.2	25.3/26.18
Solvent	34.0	47.9	37.0
Ligand A /B	40.3/32.8	47.5/58.5	63.04/64.24
Mean occupancy			
Zn1 A/B	0.9/0.9	1.0/1.0	1.0/1.0
Zn2 A/B	0.75/1.0	0.6/0.7	0.7/0.5
Ligand A/B	1.0/0.9	0.7/0.8	0.9/0.9

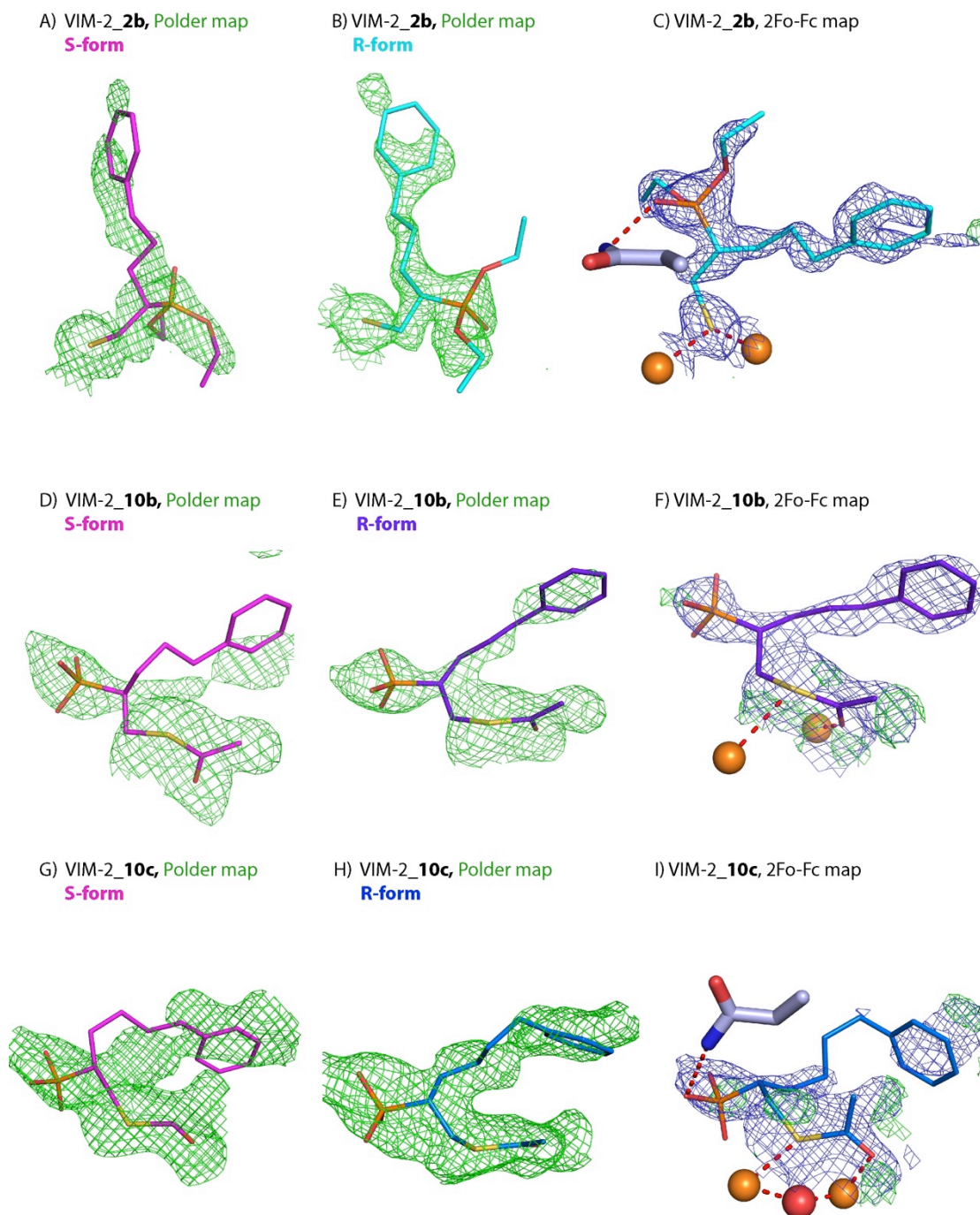


Figure 1: Observed electron density maps for the new inhibitors with polder omit maps depicted at 2.5σ (green), 2Fo-Fc maps at 1.0σ (blue) and Fourier difference maps (Fo-Fc) at 4.0σ (green) and -4.0σ (red). The polder omit maps for VIM-2 with the inhibitors **2b**, **10b** and **10c** in (*S*)-form (left panels), (*R*)-form (middle panels) and the final 2Fo-Fc map (right panels) are displayed. The zinc ions (orange) and adjacent water (red) are shown, and hydrogen bonds are shown as red dashed lines.

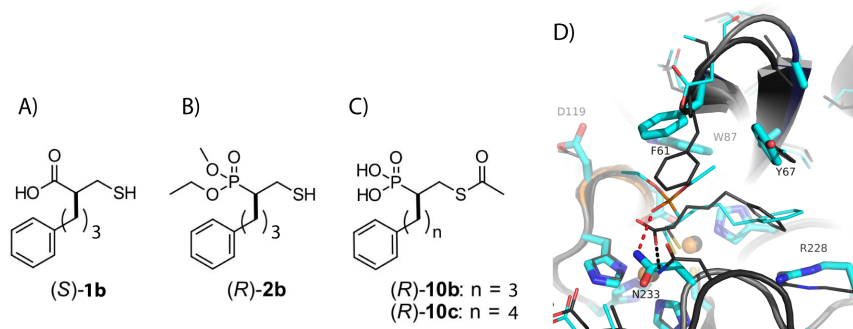


Figure 2: A-C) Stereochemical view of the enantiomeric form of **1b**, **2b**, **10b** and **10c** coordinated and bound to VIM-2. D) Overlay of VIM-2_2b (cyan) and previously reported VIM-2_1b [35] (grey).

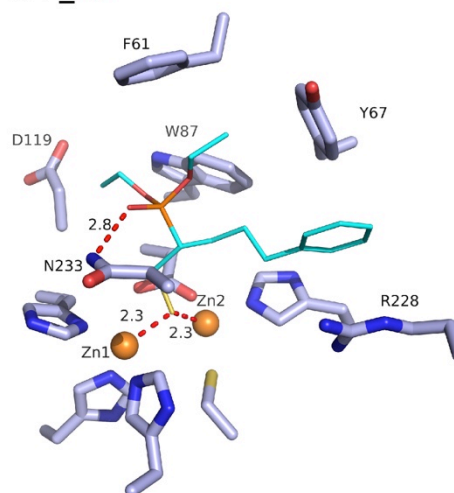
In the VIM-2_2b crystal structure there are hydrogen bonds from the OAE atom of the P=O double bond in compound 2b to the Asn233 sidechain ($d(\text{OAE}_{\text{ligand}} \dots \text{ND2}_{\text{N233}}) = 2.84 \text{ \AA}$) and from the SH group to both zinc ions ($d(\text{S}_{\text{ligand}} \dots \text{Zn1/Zn2}) = 2.30/2.30 \text{ \AA}$). Further, the benzene ring of compound 2b is sandwiched between Arg228 and Tyr67, making T-shaped π - π stacking interactions with Tyr67 and cation- π interactions with the positively charged Arg228 in a parallel manner. The benzene ring is also in a T-shaped stacking conformation with His263. Additionally, the ethyl groups of the phosphonate ester make hydrophobic interactions with Phe61, Tyr67, Trp87, and CA and CB carbon atoms of Asp119 (Figure 3A). These two ethyl groups are absent in the corresponding phosphonic acids 3, which display higher IC₅₀ values (7.8–33.2 μM) towards VIM-2 than the phosphonate esters 2 (0.38–1.7 μM) (Table 1), indicating that the phosphonic acid group is not involved in corresponding favorable interactions.

Comparison of the VIM-2_2b structure with the previously reported complex of VIM-2_1b [35] (Figure 2D) showed that the sulfur atom of thiol 2b is bridging the zinc(II) atoms of VIM-2 resembling the earlier reported complex of VIM-2_1b and numerous other thiol-B1 MBL complex structures. [19, 23, 28, 35, 46-49] Also, the propylphenyl chains of 1b and 2b occupy the same region in VIM-2. Still there is a T-shaped stacking for Phe61 and phenyl in our new 2b complex, and a face-to-face more parallel π - π stacking for 1b (PDB ID: 2YZ3) [35]. Both the carboxyl group of 1b and the phosphonate P=O of 2b are forming hydrogen bonds with the ND2 atom in the side chain of Asn233. The slightly different orientations of the inhibitors 1b and 2b may be attributed to the hydrophobic interactions of the two ethyl groups of 2b with residues Phe61, Tyr67, Trp87, and CA and CB carbon atoms in the Asp119 (Figure 3A).

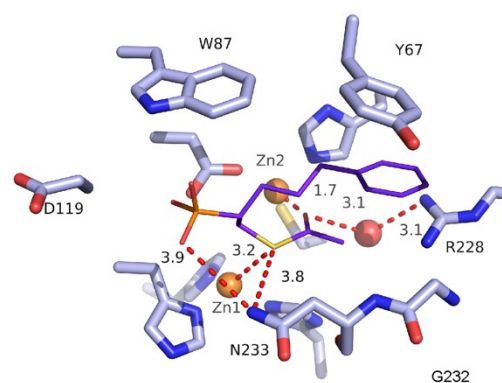
The resolution of the VIM-2_10b structure is 1.85 \AA , and this inhibitor was refined in the (*R*)-form since this fitted best in the observed electron density maps (Figure 1D, E). In the final electron density maps the orientation of the thioacetate group is described adequately but the width of the electron density is indicating flexibility and there is positive electron in the Fo-Fc map. This additional density could be ascribed to the hydroxyl ion between Zn1 and Zn2, but this could not be modeled and is thus left out in the final structure. Further, there is additional unmodeled density in elongation of the methyl group of the thioacetate of 10b. The complex of VIM-2_10c is very similar to VIM-2_10b. Still, for the VIM-2_10c structure, a hydroxyl ion bridging Zn1 and Zn2 in both chain A and chain B could be modeled, but the methylene chain is not well defined in the electron density and displays high B-factors (Table 2, Figure 1F and 1G).

From the VIM-2_10b and _10c complex structures, it is clear that the sulfur atom of the thioacetate is binding the zinc atoms differently when compared to typical thiol complexes e.g. the VIM-2_2b complex. In both the VIM-2_10b and 10c structures, the thioacetate interacts with Zn2 and the ND2 atom of Asn233 through the sulfur atom, with His263 and through a water molecule to the guanidinium group of Arg228 (Figure 3B and 3C, respectively). In VIM-2_10b the S-atom is close to both zinc ions ($d(\text{S}_{\text{ligand}} \dots \text{Zn1/Zn2}) = 2.96/3.22 \text{ \AA}$) (Figure 3B), while for VIM-2_10c, the S to zinc distances are longer ($d(\text{S}_{\text{ligand}} \dots \text{Zn1/Zn2}) = 3.29/4.02 \text{ \AA}$; Figure 3C). In both structures, the phosphonic acid is adjacent to Trp87 and interacting with the side chain of Asn233. These findings differ from the reported CphA-mercaptophosphonic acid complex, in which the Zn was coordinated by two oxygen atoms of the phosphonic acid and not

A) VIM-2_2b



B) VIM-2_10b



C) VIM-2_10c

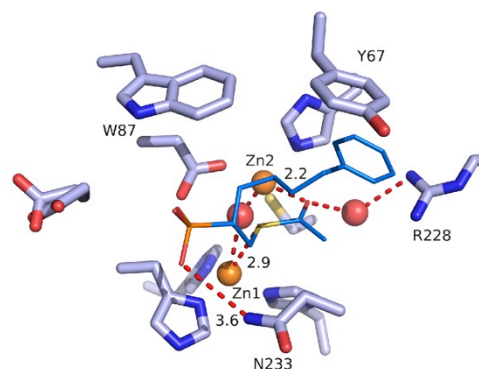


Figure 3. Interactions found in the VIM-2_2b (A), VIM-2_10b (B) and the VIM-2_10c (C) complexes. For the two latter (10b and 10c), residue Phe61 is situated above the paper plane and left out of the figure for simplification.

by the sulfur atom of the thiol. [43] However, CphA belongs to the B2 MBL subclass containing one zinc ion in the active site compared to two zinc ions for B1 MBLs, which may explain the observed differences. In addition, the benzene ring of compound 10b is more parallel to Tyr67 compared to VIM-2_2b structures, and both Arg228 and the main chain of Gly232 are stacking at the opposite side, and His263 in a T-shaped orientation.

Furthermore, the position of residue Phe61 differs significantly between complexes of 2b and 10b/c. In the complexes of 10b and 10c, the residue Phe61 is moved and closed down on the inhibitors interacting with the phosphonic

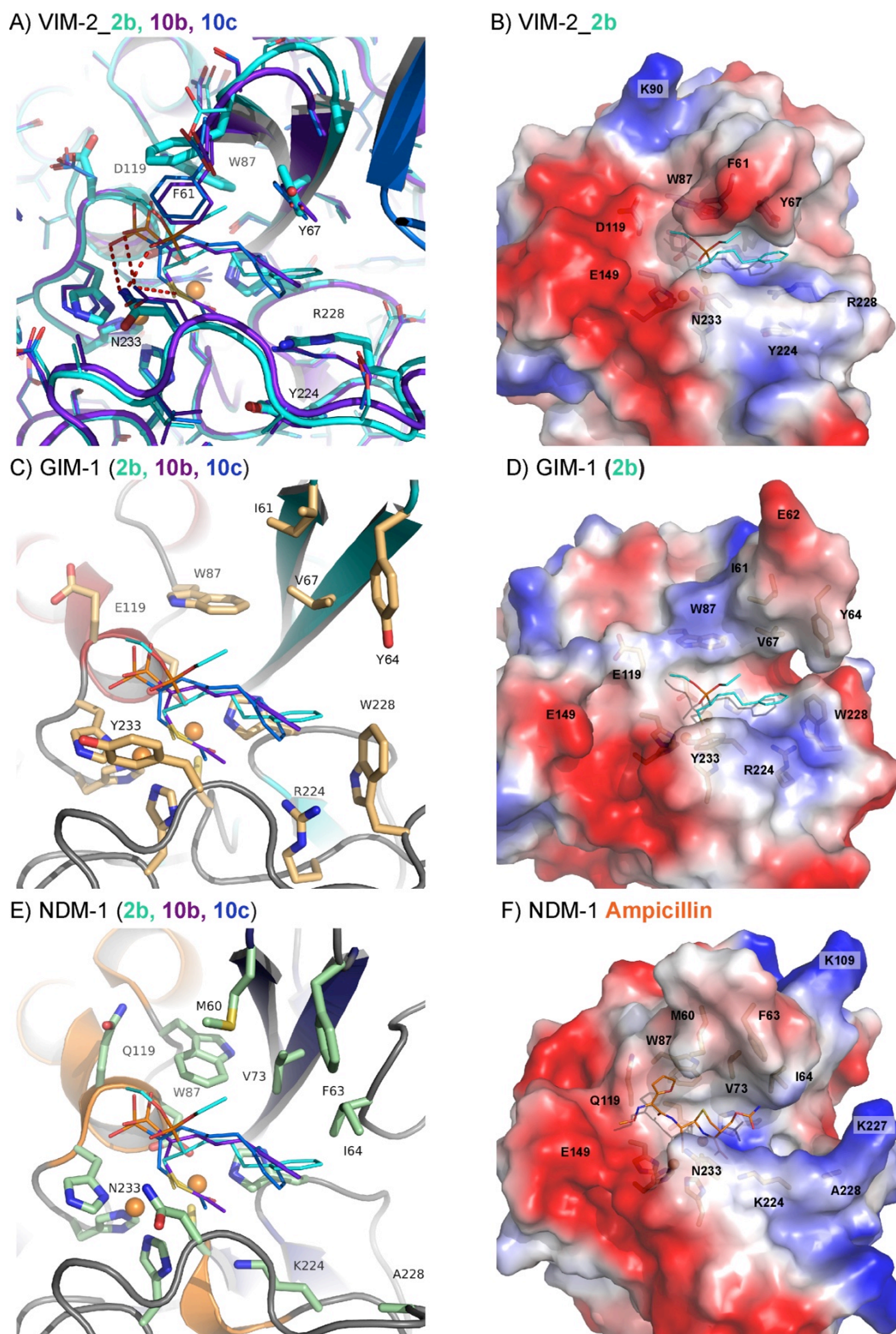


Figure 4. Ribbon diagrams (left panels) and calculated electrostatic surfaces (right panels) of VIM-2 (A, B), GIM-1 (C, D) and NDM-1 (E, F). In panel F, the structure of ampicillin (orange) in complex with NDM-1 (PDB ID: 4RLO) is given. Residues discussed or being highly charged are labeled.

acid group, whereas in the **2b** structure Phe61 is in a slight more open conformation interacting with the ethyl groups (**Figure 4A**).

2.5. Modeling of complexes with GIM-1 and NDM-1

The sequence identity between the three MBLs included in this study is 32% (VIM-2 versus NDM-1), 28% (VIM-2 versus GIM-1) or 24% (NDM-1 versus GIM-1) (**Figure S1**). Low sequence identity is also found between the second shell sphere residues that constitute the active site together with the six conserved zinc binding residues (116, 118, 196, 120, 221, 263). The impact from

these second shell interacting residues on ligand binding with GIM-1 and NDM-1 was examined by modeling the inhibitor binding based on the VIM-2 complexes obtained.

When superimposing the new VIM-2 complexes with inhibitors **2b**, **10c** and **10b** onto the GIM-1 structure (**Figure 4C, D**) (PDB ID: 2YNW; [50]), it becomes clear that the R2 binding sites accommodating the phenyl group of the inhibitors are very different. For GIM-1, both Trp228 and Tyr64 confine the R2 site making it smaller and more hydrophobic. Our reported IC_{50}

values for compounds **2** and **10** are not significantly different for the longest ($n = 4$) compared to the shortest ($n = 2$) inhibitors in a series (**Table 1**). This indicates that similar favorable T-shaped stacking from Trp228 and cation- π interactions from Arg224 to the phenyl groups of the inhibitors are possible for all compounds within a series. Several conformations are described for Trp228 in the native GIM-1 crystal structures, [50] which all can contribute to the described inhibitor interactions. Furthermore, residue 233 is a tyrosine in GIM-1 and the polar interactions observed in VIM-2_2b, 10b and 10c from Asn233 to the P=O group cannot be formed. Still the low IC_{50} of for **2a** of 0.18 μ M and 64% inhibition towards GIM-1 (**Table 1**) tells that it is possible to inhibit GIM-1 in a cell based assay.

The new synthesized inhibitors are less efficient towards *E. coli* SNO3 cell with NDM-1, but **2a**, **2b** and **10a-c** show low IC_{50} values with pure enzyme (1.8–6.6 μ M; **Table 1**). As with VIM-2 (and in contrast to GIM-1), NDM-1 has an Asn233 residue, which can bind the inhibitors through the P=O group as for VIM-2. However, hydrophobic stabilization of the phenyl rings of the inhibitors is limited for NDM-1 as the R2 binding site consists of Ala228 and Lys224, where the lysine residue is shorter than Arg228 (VIM-2) and Arg224 (GIM-1). Further, Phe63 in NDM-1 is too far away to reach down to the phenyl groups of the modelled **2b**, **10b** and **10c** inhibitor complexes (**Figure 4E**). In the reported complex structure of NDM-1 with hydrolyzed ampicillin (PDB ID: 3Q6X; **Figure 4F**), the substrate interacts with Ile64 and Val73 (in the R2 site), and the “R1” part of the ampicillin with Met60 and Gln119. The NDM-1 binding site is more hydrophobic and also different from both VIM-2 and GIM-1 with few conserved second shell residues, thus the design of an inhibitor hitting all three MBL targets is challenging.

3. Conclusion

Our bioisosteric approach on designing new metallo- β -lactamase inhibitors was found successful with low dose rate measurement for the **2** series of inhibitors ($IC_{50} = 0.18$ – 2.2 μ M) towards all three enzymes, VIM-2, GIM-1 and NDM-1. For the cell-based assay with a β -lactamase-negative *E. coli* SNO3 cells inducing expression of one MBL, the best inhibitors were the **3** and **10** series. The three new crystal structures of VIM-2 with **2b**, **10b** or **10c**, reveal energetic favorable cation- π interactions from Arg228, and stacking from Tyr67 and Phe61 with the phenyl ring of the inhibitors. Asn233 in VIM-2 is also contributing with polar interactions to the P=O group (**2b**) and sulphur atom (**10b**, **10c**). Disappointingly, no synergistic effect was seen in *P. aeruginosa* nor in *K. pneumoniae* bacterial strains, but some effects on a clinical isolate from *E. coli* with VIM-29 were observed for **3b** and **10b** showing reduced MIC. A broad inhibitor hitting VIM-2, GIM-1 and NDM-1 was not found, but one promising hit is the thioacetate phosphonic acid **10b**, which seems to target both VIM-2 and NDM-1. The reported VIM-2_10b structure is therefore a valuable starting point for a structure-guided design of an inhibitor targeting both VIM-2 and NDM-1 simultaneously.

4. Experimental

4.1. Chemistry

All reagents and solvents were purchased from commercial sources and used as supplied unless otherwise stated. Compounds **1c** and **17** were prepared according to the literature. [36] Reactions were monitored by thin-layer chromatography (TLC) with Merck pre-coated silica gel plates (60 F₂₅₄). Visualization was accomplished with either UV light or by immersion in potassium permanganate or phosphomolybdic acid (PMA) followed by light heating with a heating gun. Purification of

reactions was carried out by flash column chromatography using silica gel from Merck (Silica gel 60, 0.040 - 0.063 mm). Analytical HPLC was carried out on a Purity analysis was carried out on Waters Acquity UPLC® BEH C18 (1.7 μ m, 2.1 \times 100 mm) column on a Waters Acquity I-class UPLC with Photodiode Array Detector. NMR spectra were obtained on a 400 MHz Bruker Avance III HD equipped with a 5 mm SmartProbe BB/1H (BB = 19F, 31P-15N). Data are represented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet), coupling constant (J, Hz) and integration. Chemical shifts (δ) are reported in ppm relative to the residual solvent peak (CDCl₃: δ_H 7.26 and δ_C 77.16; Methanol-d₄: δ_H 3.31 and δ_C 49.00). ³¹P NMR spectra were recorded using an insertion NMR tube filled with PPh₃ ($\delta = -5.4$ ppm) solution in C₆D₆ as a reference. Positive ion electrospray ionization mass spectrometry was conducted on a Thermo electron LTQ Orbitrap XL spectrometer. IR spectra were recorded with Model Varian 7000e FT-IR spectrometer.

4.1.1. Preparation and spectroscopic data of 2-(Mercaptomethyl)-6-phenylhexanoic acid (**1c**):

The title compound **1c** was prepared as reported in the literature. [36] The final deacetylation was performed according to the general procedure (see chapter 4.1.5). The preceding thioacetate (1.67 mmol, 468 mg, 1 equiv) in methanol (6 mL) was treated with 1M NaSMe in MeOH (3.4 mmol, 3.4 mL, 2 equiv). Reaction time was 1.5 h. Column chromatography (6% MeOH: CH₂Cl₂) gave the product **1c** as a white solid (1.23 mmol, 290 mg, 74%) of high purity (>95% as determined by HPLC). ¹H NMR (400 MHz, Chloroform-*d*) δ 10.85 (s, 1H), 7.30-7.16 (m, 4H), 2.79-2.74 (m, 1H), 2.68-2.60 (m, 2H), 1.75-1.66 (m, 4H), 1.53 (t, $J = 8.5$ Hz, 1H), 1.44 (q, $J = 7.8$ Hz, 2H). ¹³C NMR (101 MHz, Chloroform-*d*) δ 180.7, 142.2, 128.4, 128.3, 125.8, 125.7, 49.0, 35.6, 31.2, 31.2, 30.9, 26.6, 25.6. HRMS (ESI): Calcd. for C₁₃H₁₇O₂S [M+H]⁺ 237.0954; found 237.0944. The NMR and MS data were in accordance with the literature. [36]

4.1.2. General procedure for the alkylation of triethyl phosphonoacetate (**6a-c**)

A solution of ethyl-2-(diethoxyphosphoryl) acetate **5** in DMF (25mL/mol) was stirred at 0 °C. The base was added and the solution was continued to stir for 20 min before the alkyl halide was added drop wise. The reaction mixture was allowed to warm to room temperature and then heated at 60 °C for 18 hours. After cooling the reaction mixture was acidified with 10% citric acid and extracted with diethyl ether (3 x 30 mL). The combined organic phases were washed with H₂O (3 x 30 mL), brine (1 x 30 mL) and dried over Na₂SO₄. The product **6a-c** was purified with column chromatography using 2:1 diethyl ether/pentane as eluent.

4.1.2.1. Ethyl 2-(diethoxyphosphoryl)-4-phenylbutanoate (**6a**):

Potassium *tert*-butoxide (0.028 mol, 3.15 g, 1 equiv), ethyl 2-(diethoxyphosphoryl) acetate (0.028 mol, 6.30 g, 1 equiv) and 1-bromo-2-phenylethane (0.028 mol, 5.18 g, 1 equiv) gave **6a** (0.018 mol, 5.76 g, 62%) as a pale yellow oil. ¹H NMR (400 MHz, Chloroform-*d*) δ 7.28-7.26 (m, 2H), 7.21-7.16 (m, 3H), 4.23-4.20 (m, 2H), 4.14-4.07 (m, 4H), 2.96 (ddd, $J = 22.9, 11.0, 3.6$ Hz, 1H), 2.79-2.69 (m, 1H), 2.64-2.56 (m, 1H), 2.35-2.26 (m), 2.18-2.12 (m, 1H), 1.30-1.28 (m, 9H). ¹³C NMR (101 MHz, Chloroform-*d*) δ 169.5 (d, $J = 5.0$ Hz), 140.9, 128.9 (d, $J = 10.7$ Hz), 126.7, 63.2 (dd, $J = 8.4, 6.7$ Hz), 61.9, 46.2, 44.9, 34.9 (d, $J = 15.4$ Hz), 29.1 (d, $J = 4.4$ Hz), 16.9 (dd, $J = 6.1, 2.3$ Hz), 14.7. ³¹P NMR (162 MHz, Chloroform-*d*): δ 23.0. HRMS (ESI): Calcd. for C₁₆H₂₆O₅P [M+H]⁺ 329.1512; found 329.1519.

4.1.2.2. Ethyl 2-(diethoxyphosphoryl)-5-phenylpentanoate (**6b**):

Potassium *tert*-butoxide (0.064 mol, 7.18 g, 0.8 equiv), ethyl 2-(diethoxyphosphoryl) acetate (0.08 mol, 20.01 g, 1 equiv) and 1-bromo-3-phenylpropane (0.064 mol, 12.71 g, 0.8 equiv) gave **6b** (0.046 mol, 15.89 g, 73%) as a pale yellow oil. ¹H NMR (400 MHz, Chloroform-*d*) δ 7.29-7.24 (m, 2H), 7.19-7.14 (m, 3H), 4.28-4.08 (m, 6H), 2.96 (ddd, *J* = 22.7, 10.9, 3.8 Hz, 1H), 2.63 (t, *J* = 7.8 Hz, 2H), 2.07-2.02 (m, 1H), 1.92-1.89 (m, 1H), 1.72-1.65 (m, 2H), 1.34-1.26 (m, 9H). ¹³C NMR (101 MHz, Chloroform-*d*) δ 169.1 (d, *J* = 4.8 Hz), 141.8 (d, *J* = 29.8 Hz), 128.3-128.2 (m), 125.8 (d, *J* = 29.8 Hz), 62.6 (dd, *J* = 9.1, 6.6 Hz), 61.3, 46.3, 44.9, 35.3, 30.1 (d, *J* = 14.9 Hz), 26.6 (d, *J* = 4.8 Hz), 16.3 (dd, *J* = 5.9, 2.7 Hz), 14.1. ³¹P NMR (162 MHz, Chloroform-*d*): δ 23.1. HRMS (ESI): Calcd. for C₁₇H₂₇O₅NaP [M+Na]⁺ 365.1488; found 365.1484.

4.1.2.3. Ethyl 2-(diethoxyphosphoryl)-6-phenylhexanoate (**6c**):

Potassium *tert*-butoxide (0.014 mol, 1.55 g, 0.7 equiv), ethyl 2-(diethoxyphosphoryl) acetate (0.019 mol, 4.25 g, 1 equiv) and 1-bromo-4-phenylbutane (0.013 mmol, 2.83 g, 0.7 equiv) gave **6c** (0.009 mol, 3.37g, 73%) as a yellow oil. ¹H NMR (400 MHz, Chloroform-*d*) δ 7.33-7.28 (m, 2H), 7.22-7.15 (m, 3H), 4.27-4.12 (m, 6H), 2.96 (ddd, *J* = 22.5, 11.0, 3.8 Hz, 1H), 2.65-2.58 (m, 2H), 1.72-1.68 (m, 1H), 1.68-1.65 (m, 1H), 1.38-1.36 (m, 2H) 1.3-1.25 (m, 11H). ¹³C NMR (101 MHz, Chloroform-*d*) δ 169.3 (d, *J* = 4.8 Hz), 142.2, 128.3 (d, *J* = 8.7 Hz), 125.7, 62.7(t, *J* = 6.6, 14.6 Hz), 61.3, 46.5, 45.2, 35.5, 30.9, 28.1 (d, *J* = 15.0 Hz), 26.9 (d, *J* = 4.9 Hz), 16.4 (dd, *J* = 6.1, 3.2 Hz), 14.2. ³¹P NMR (162 MHz, Chloroform-*d*): δ 23.3. HRMS (ESI): Calcd. for C₁₈H₃₀O₅P [M+H]⁺ 357.1825; found 357.1822.

4.1.3. General procedure for the reduction of alkylated triethyl phosphonoacetate (**7a-c**)

LiBH₄ was dissolved in THF (2 mL/ mmol) at 0 °C and slowly added to the ester **6a-c** in THF at 0 °C. The suspension was stirred at room temperature for 30 minutes and irradiated at 80 °C for 10 min. The mixture was cooled to RT and slowly diluted with MeOH and stirred for 30 min until excess of the LiBH₄ was quenched. The mixture was acidified with 10% citric acid, saturated with NaCl and the product was extracted with diethyl ether. The organics were dried over Na₂SO₄, filtered and concentrated in vacuum. The product **3a-c** was purified with column chromatography with 5% MeOH: CH₂Cl₂.

4.1.3.1. Diethyl 1-hydroxy-4-phenylbutan-2-ylphosphonate (**7a**):

Ethyl 2-(diethoxyphosphoryl)-4-phenylbutanoate **6a** (1.6 mmol, 0.53 g, 1 equiv) and LiBH₄ (4.8 mmol, 0.12 g, 3 equiv) gave **7a** (1.0 mmol, 0.28 g, 60%) as a colorless oil. ¹H NMR (400 MHz, Chloroform-*d*) δ 7.31-7.26 (m, 2H), 7.21-7.18 (m, 3H), 4.14-4.07 (m, 4H), 3.89-3.79 (m, 2H), 3.15-3.12 (m, 1H), 2.81 (ddd, *J* = 14.5, 9.2, 5.7 Hz, 1H), 2.69 (ddd, *J* = 13.8, 9.4, 6.6 Hz, 1H), 2.01-1.98 (m, 2H), 1.97-1.83 (m, 1H), 1.35-1.29 (m, 6H). ¹³C NMR (101 MHz, Chloroform-*d*) δ 141.1, 128.4 (d, *J* = 5.7 Hz), 126.1, 61.9 (t, *J* = 6.7 Hz), 60.7 (d, *J* = 5.5 Hz), 39.1, 37.7, 33.5 (d, *J* = 11.1 Hz), 27.0 (d, *J* = 3.5 Hz), 16.5 (t, *J* = 5.5 Hz). ³¹P NMR (162 MHz, Chloroform-*d*): δ 33.3. HRMS (ESI) Calcd. for C₁₄H₂₄O₄P [M+H]⁺ 287.1407; found 287.1408.

4.1.3.2. Diethyl 1-hydroxy-5-phenylpentane-2-ylphosphonate (**7b**):

Ethyl 2-(diethoxyphosphoryl)-5-phenylpentanoate **6b** (4.7 mmol, 1.60 g, 1 equiv) and LiBH₄ (0.012 mol, 255 mg, 2.5

equiv) gave **7b** (4.5mmol, 1.34g, 95%) as a colorless oil. ¹H NMR (400 MHz, Chloroform-*d*) δ 7.29-7.25 (m, 2H), 7.19-7.16 (m, 3H), 4.15-4.08 (m, 4H), 3.82-3.77 (m, 2H), 3.46 (s, 1H), 2.62 (t, *J* = 7.4 Hz, 2H), 1.81-1.71 (m, 1H), 1.68-1.66 (m, 2H), 1.66-1.50 (m, 1H), 1.31 (m, 6H). ¹³C NMR (101 MHz, Chloroform-*d*) δ 141.8, 128.3 (d, *J* = 5.7 Hz), 125.7, 61.8 (dd, *J* = 13.1, 6.8 Hz), 60.5 (d, *J* = 4.4 Hz), 39.9, 38.5, 35.6, 29.2 (d, *J* = 10.4 Hz), 25.1 (d, *J* = 3.7 Hz), 16.4 (d, *J* = 5.5 Hz). ³¹P NMR (162 MHz, Chloroform-*d*): δ 33.0. HRMS (ESI) Calcd. for C₁₅H₂₆O₄P [M+H]⁺ 301.1563; found 301.1566.

4.1.3.3. Diethyl 1-hydroxy-6-phenylhexane-2-ylphosphonate (**7c**):

Ethyl 2-(diethoxyphosphoryl)-6-phenylhexanoate **6c** (4.21 mmol, 1.5 g, 1 equiv) and LiBH₄ (10.53 mmol, 229 mg, 2.5 equiv) gave **7c** (2.34 mmol, 737 mg, 56%) as a colorless oil. ¹H NMR (400 MHz, Chloroform-*d*) δ 7.21-7.17 (m, 2H), 7.11-7.08 (m, 3H), 4.08-4.02 (m, 4H), 3.78-3.65 (m, 2H), 2.56-2.52 (m, 2H), 1.91-1.87 (m, 1H), 1.63-1.53 (m, 3H), 1.46-1.44 (m, 2H), 1.35-1.34 (m, 1H), 1.25 (t, *J* = 7.1 Hz, 6H). ¹³C NMR (101 MHz, Chloroform-*d*) δ 142.4, 128.5-128.4 (m), 125.9, 62.1 (dd, *J* = 13.6, 6.9 Hz), 60.9 (d, *J* = 5.7 Hz), 40.0, 38.7, 35.8, 31.4, 27.3 (d, *J* = 11.0 Hz), 25.3 (d, *J* = 3.8 Hz), 16.6. ³¹P NMR (162 MHz, Chloroform-*d*): δ 33.3. HRMS (ESI): Calcd. for C₁₆H₂₈O₄P [M+H]⁺ 315.1720; found 315.1723.

4.1.4. General procedure for thioacetylation (**8a-c**)

Compound **7a-c** (1 equiv) was dissolved in CH₂Cl₂ (5 mL/mmol) and added triethylamine (1.05 equiv) and a catalytic amount of 4-dimethylaminopyridine (DMAP). The mixture was stirred for 5 minutes at RT, before methanesulfonyl chloride (1.05 equiv) was slowly added. The reaction mixture was stirred for 3–24 hr at RT. The solvent was evaporated, and the remaining crude was quenched with 100mL aq. NH₄Cl and extracted with diethyl ether, dried over Na₂SO₄, filtered, and evaporated. To the crude was added an excess (7-10 equiv) of 2 M potassium thioacetate in DMF and stirred overnight at RT. The brown suspension was quenched with aq. NH₄Cl (100 mL) and the product was extracted with diethyl ether (3 x 30 mL). The combined organic layers were washed with H₂O (3 x 50 mL), dried over Na₂SO₄ and concentrate to yield an orange oil. The crude was purified with column chromatography using 5–15% MeOH:CH₂Cl₂ as eluent.

4.1.4.1. *S*-2-(Diethoxyphosphoryl)-4-phenylbutyl ethanethioate (**8a**):

Diethyl 1-hydroxy-4-phenylbutan-2-ylphosphonate **7a** (2.8 mmol, 825 mg, 1 equiv), triethylamine (2.9 mmol, 355 mg, 1.05 equiv), methanesulfonyl chloride (2.9 mmol, 355 mg, 1.05 equiv), DMAP (catalytic amount) and potassium thioacetate (20.3 mmol, 2.31 g, 7 equiv) gave **8a** (1.5 mmol, 520 mg, 54%) as an orange oil. ¹H NMR (400 MHz, Chloroform-*d*) δ 7.33-7.30 (m, 2H), 7.25-7.22 (m, 3H), 4.23-4.13 (m, 4H), 3.44-3.39 (m, 1H), 3.08-3.05 (m, 1H), 2.85-2.81 (m, 2H), 2.37 (s, 3H), 2.10-2.05 (m, 2H), 1.93-1.91 (m, 1H), 1.41-1.36 (m, 6H). ¹³C NMR (101 MHz, Chloroform-*d*) δ 195.3, 141.4, 128.5 (d, *J* = 12.7 Hz), 126.0, 61.9 (dd, *J* = 6.8, 2.5 Hz), 36.4, 35.1, 33.5 (d, *J* = 7.5 Hz), 30.6, 29.6 (d, *J* = 3.3 Hz), 28.3 (d, *J* = 2.0 Hz), 16.5 (d, *J* = 5.9 Hz). ³¹P NMR (162 MHz, Chloroform-*d*): δ 30.3. HRMS (ESI): Calcd. for C₁₆H₂₆O₄PS [M+H]⁺ 345.1285; found 345.1289.

4.1.4.2. *S*-2-(diethoxyphosphoryl)-5-phenylpentyl ethanethioate (**8b**):

Diethyl 1-hydroxy-5-phenylpentane-2-ylphosphonate **7b** (0.93 mmol, 289 mg, 1 equiv), triethylamine (0.98 mmol, 111 mg, 1.05 equiv), methanesulfonyl chloride (0.98 mmol, 111 mg, 1.05

equiv), DMAP (catalytic amount) and potassium thioacetate (9.3 mmol, 1.06 g, 10 equiv) gave **8b** (0.32 mmol, 114 mg, 34%) as an orange oil. ¹H NMR (400 MHz, Chloroform-*d*) δ 7.22-7.18 (m, 2H), 7.12-7.08 (m, 3H), 4.07-4.00 (m, 4H), 3.26 (ddd, *J* = 15.2, 13.9, 5.1 Hz, 1H), 2.92 (ddd, *J* = 13.9, 12.0, 8.2 Hz, 1H), 2.54 (t, *J* = 7.3 Hz, 2H), 2.25 (s, 3H), 1.99-1.86 (m, 1H), 1.76-1.70 (m, 3H), 1.63-1.43 (m, 1H), 1.27-1.23 (m, 6H). ¹³C NMR (101 MHz, Chloroform-*d*) δ 195.1, 141.8, 128.3 (dd, *J* = 16.7, 1.9 Hz), 125.7, 61.8 (t, *J* = 6.9 Hz), 37.0, 35.7-35.4 (m), 30.4 (d, *J* = 2.5 Hz), 28.9 (dd, *J* = 7.3, 2.3 Hz), 28.3 (d, *J* = 1.9 Hz), 27.4 (d, *J* = 3.7 Hz), 16.4 (d, *J* = 5.8 Hz). ³¹P NMR (162 MHz, Chloroform-*d*): δ 30.5. HRMS (ESI): Calcd. for C₁₇H₂₈O₄PS [M+H]⁺ 359.1440; found 359.1449.

4.1.4.3. *S*-2-(diethoxyphosphoryl)-6-phenylhexyl ethanethioate (**8c**):

Diethyl 1-hydroxy-6-phenylhexane-2-ylphosphonate **7c** (13 mmol, 4.20 g, 1 equiv), triethylamine (0.014 mmol, 1.42 g, 1.05 equiv), methanesulfonyl chloride (1.34 mmol, 152 mg, 1.05 equiv), DMAP (catalytic amount) and potassium thioacetate (0.14 mmol, 15.98 g, 10 equiv) gave **8c** (9.4 mmol, 3.502 g, 71%) as an orange oil. ¹H NMR (400 MHz, Chloroform-*d*) δ 7.34-7.30 (m, 2H), 7.24-7.21 (m, 3H), 4.18-4.15 (m, 4H), 3.38 (ddd, *J* = 15.2, 13.8, 5.0 Hz, 1H), 3.03 (ddd, *J* = 13.9, 11.9, 8.2 Hz, 1H), 2.67 (t, *J* = 7.6 Hz, 2H), 2.38 (s, 3H), 2.06-1.97 (m, 1H), 1.83-1.77 (m, 1H), 1.69-1.62 (m, 3H), 1.62-1.56 (m, 2H), 1.40-1.36 (m, 6H). ¹³C NMR (101 MHz, Chloroform-*d*) δ 195.4, 142.5, 128.4 (d, *J* = 13.2 Hz), 125.7, 61.9 (dd, *J* = 6.9, 5.0 Hz), 37.2, 35.7 (d, *J* = 8.9 Hz), 31.5, 30.6, 28.5 (d, *J* = 1.8 Hz), 27.9 (d, *J* = 3.5 Hz), 27.1 (d, *J* = 7.4 Hz), 16.5 (d, *J* = 6.0 Hz). ³¹P NMR (162 MHz, Chloroform-*d*): δ 31.1. HRMS (ESI): calcd. For C₁₈H₂₆O₄PS [M+H]⁺ 373.1602; found 373.1289.

4.1.5. Deacetylation of thioacetates (preparation of **1c**, **2a-c** and **4**):

Except for **2a**, the following general procedure was applied. To a stirred solution of thioacetate (**8** or **15**) (1 equiv) in methanol (6 mL) at -20 °C was added sodiumthiomethoxide, NaSMe (1 M solution in MeOH, 1 equiv). The reaction mixture was stirred for 30 minutes at -20 °C, before aq. HCl (0.1 M) was added. The reaction mixture was extracted with DCM (3 x 30 mL), the combined organic layers were washed with brine, dried over MgSO₄, filtered and concentrate. The resulting crude was purified by column chromatography.

4.1.5.1. Diethyl 1-mercapto-4-phenylbutan-2-ylphosphonate (**2a**):

According to the reported procedure, [38] a 0.1 M NaOMe/MeOH solution (3 mmol, 3 mL, 2 equiv) was added to thioacetate **8a** (1.5 mmol, 0.52g, 1 equiv) at 0 °C. The resulting mixture was stirred for 15 min at rt under H₂ and concentrated. Aq. NH₄Cl (30 mL) and 0.1 M HCl (10 mL) were added to the crude and the product was extracted with diethyl ether. The combined organics were dried and concentrated. Repeated column chromatography (5–20% MeOH: CH₂Cl₂ and 3:1 EtOAc:Et₂O) gave **2a** (0.46 mmol, 139 mg, 30%) as a slightly orange oil. ¹H NMR (400 MHz, Methanol-*d*₄) δ 7.33-7.29 (m, 2H), 7.26-7.20 (m, 3H), 4.18-4.10 (m, 4H), 3.04-2.92 (m, 1H), 2.86-2.72 (m, 4H), 2.15-2.01 (m, 3H), 1.71 (t, *J* = 8.3 Hz, 1H), 1.37-1.33 (m, 6H). ¹³C NMR (101 MHz, Methanol-*d*₄) δ 142.9, 129.7, 127.3, 63.7 (dd, *J* = 7.1, 1.9 Hz), 40.5, 39.1, 34.4 (d, *J* = 8.2 Hz), 30.2 (d, *J* = 3.2 Hz), 23.9 (d, *J* = 2.6 Hz), 16.9 (d, *J* = 5.8 Hz). ³¹P NMR (162 MHz, Chloroform-*d*) δ 32.9. HRMS (ESI): Calcd. for C₁₆H₂₈O₃PS [M+H]⁺ 331.1491; found 331.1494.

4.1.5.2. Diethyl 1-mercapto-5-phenylpentan-2-ylphosphonate (**2b**):

Thioacetate **8b** (0.56 mmol, 0.20 g, 1 equiv) in methanol and 1 M NaSMe in MeOH (0.6 mmol, 0.6 mL, 1 equiv) gave **2b** (0.41 mmol, 130 mg, 74%) as white oil after purification with column chromatography (5-20% MeOH: CH₂Cl₂). ¹H NMR (400 MHz, Chloroform-*d*) δ 7.29-7.25 (m, 2H), 7.19-7.15 (m, 3H), 4.12-4.04 (m, 4H), 2.96-2.85 (m, 1H), 2.69-2.62 (m, 3H), 1.98-1.93 (m, 1H), 1.80-1.76 (m, 4H), 1.65 (t, *J* = 8.4 Hz, 1H), 1.32-1.28 (m, 6H). ¹³C NMR (101 MHz, Chloroform-*d*) δ 142.0, 128.5 (d, *J* = 8.8 Hz), 125.9, 61.9 (dd, *J* = 7.3, 3.1 Hz), 40.4, 38.9, 35.9, 28.9 (d, *J* = 8.2 Hz), 26.7 (d, *J* = 3.6 Hz), 23.7 (d, *J* = 2.7 Hz), 16.6 (d, *J* = 6.2 Hz). ³¹P NMR (162 MHz, Chloroform-*d*) δ 31.9. HRMS (ESI): Calcd. for C₁₅H₂₆O₃PS [M+H]⁺ 317.1335; found 317.1337.

4.1.5.3. Diethyl 1-mercapto-6-phenylhexan-2-ylphosphonate (**2c**):

Thioacetate **8c** (1.07 mmol, 400 mg, 1 equiv) in methanol and 1M NaSMe in MeOH (1.1 mmol, 1.1 mL, 1 equiv) gave **2c** (0.82 mmol, 270 mg, 77%) as a colorless oil after purification with column chromatography (5–20% MeOH: CH₂Cl₂). ¹H NMR (400 MHz, Chloroform-*d*) δ 7.28-7.23 (m, 2H), 7.17-7.15 (d, *J* = 6.8 Hz, 3H), 4.12-4.05 (m, 4H), 2.94-2.83 (m, 1H), 2.70-2.60 (m, 3H), 1.98-1.80 (m, 1H), 1.76-1.66 (m, 5H), 1.60-1.43 (m, 2H), 1.30 (t, *J* = 7.0 Hz, 6H). ¹³C NMR (101 MHz, Chloroform-*d*) δ 142.5, 128.5 (d, *J* = 11.5 Hz), 125.8, 61.8 (d, *J* = 6.9 Hz), 40.3, 38.9, 35.7 (d, *J* = 1.9 Hz), 31.4, 26.9-26.8 (m), 23.6 (d, *J* = 2.3 Hz), 16.6 (d, *J* = 5.9 Hz). ³¹P NMR (162 MHz, Chloroform-*d*) δ 31.5. HRMS (ESI): Calcd. for C₁₆H₂₈O₃PS [M+H]⁺ 331.1486; found 331.1491.

4.1.5.4. 5-Phenyl-2-(1H-tetrazol-5-yl)pentane-1-thiol (**4**):

Thioacetate **15b** (0.1 mmol, 30 mg, 1 equiv) in methanol was treated with 1 M NaSMe in MeOH (0.2 mmol, 0.2 mL, 2 equiv). The reaction time was 1.5 h at RT. Column chromatography (3% MeOH: CH₂Cl₂) provided **4** as white solid (0.073 mmol, 18.1 mg, 73%) with high purity (> 95% as determined by HPLC). ¹H NMR (400 MHz, Methanol-*d*₄) δ 7.19-7.04 (m, 5H), 3.45-3.43 (m, 1H), 3.04-2.95 (m, 2H), 2.56 (q, *J* = 7.6 Hz, 2H), 1.82 (ddd, *J* = 35.0, 8.0, 4.1 Hz, 2H), 1.75-1.72 (m, 2H), 1.49-1.36 (m, 2H). ¹³C NMR (101 MHz, Methanol-*d*₄) δ 158.3, 141.5, 127.9, 125.5, 41.1, 35.3, 34.8, 32.7, 28.5. HRMS (ESI): Calcd. for C₁₂H₁₇N₄S [M+H]⁺ 249.1170; found 249.1168.

4.1.6. General procedure for the hydrolysis of phosphonic esters (preparation of **3**, **9** and **10**)

Bromotrimethylsilane (3 equiv) was added drop wise to **2**, **7** or **8** (1 equiv) in 10 mL dry CH₂Cl₂ under argon. The mixture was stirred overnight at room temperature. The solvent and excess TMSBr were removed under reduced pressure, MeOH (0.2 mL/mmol of substrate) was added and the reaction mixture was stirred for 1 h at RT. The reaction mixture was evaporated to dryness. The crude **3**, **9** or **10** was obtained as viscous oil. Crude product was dissolved in minimal volume of EtOAc and cold pentane was added resulting in the separation of compound as oily or crystalline material. The pentane layer was discarded and the remaining compound was dried under vacuum.

4.1.6.1. 1-Hydroxy-4-phenylbutan-2-ylphosphonic acid (**9a**)

Diethyl 1-hydroxy-4-phenylbutyl-2-ylphosphonate **7a** (0.31 mmol, 95 mg, 1 equiv) and TMSBr (140 mg) gave crude **9a** (77 mg) as a colorless oil. A small batch (30 mg) was dissolved in 2-3 drops of EtOAc and washed with cold pentane, afforded the pure product **9a** (0.086 mmol, 20 mg, 76%). ¹H NMR (400 MHz, Chloroform-*d*) δ 8.65 (s, 2H), 7.15-7.08 (m, 5H), 3.84-3.80 (m, 2H), 2.69-2.58 (m, 2H), 2.02-1.80 (m, 2H), 1.78-1.73 (m, 1H).

^{13}C NMR (101 MHz, Methanol- d_4) δ 142.0, 128.0 (d, $J = 13.0$ Hz), 125.5, 60.3, 40.5, 39.2, 33.6 (d, $J = 8.3$ Hz), 28.1 (d, $J = 3.2$ Hz), 22.8. ^{31}P NMR (162 MHz, Methanol- d_4) δ 26.1. HRMS (ESI): Calcd. for $\text{C}_{10}\text{H}_{16}\text{O}_4\text{P}$ $[\text{M}+\text{H}]^+$ 231.0781; found: 231.0779.

4.1.6.2. 1-Hydroxy-5-phenylpentane-2-ylphosphonic acid (**9b**):

Diethyl 1-hydroxy-5-phenylpentan-2-ylphosphonate **7b** (0.36 mmol, 0.1 g, 1 equiv) and TMSBr (1.06 mmol, 0.16 g, 3 equiv) gave crude **9b** (110 mg) as a colourless oil. A small batch (64 mg) was dissolved in 2-3 drops of EtOAc and washed with cold pentane, afforded the pure product **9b** (0.16 mmol, 39 mg, 61%). ^1H NMR (400 MHz, Methanol- d_4) δ 7.29-7.15 (m, 5H), 3.92 (ddd, $J = 14.3, 11.0, 4.9$ Hz, 1H), 3.73-3.64 (m, 1H), 2.68-2.63 (m, 2H), 1.98-1.69 (m, 5H). ^{13}C NMR (101 MHz, Methanol- d_4) δ 142.2, 127.9 (d, $J = 15.9$ Hz), 125.3, 60.4, 40.9, 39.5, 35.7, 29.6 (d, $J = 8.3$ Hz), 25.8 (d, $J = 3.4$ Hz). ^{31}P NMR (162 MHz, Methanol- d_4) δ 31.1. HRMS (ESI): Calcd. for $\text{C}_{11}\text{H}_{18}\text{O}_4\text{P}$ $[\text{M}+\text{H}]^+$ 245.0937; found 245.0936.

4.1.6.3. 1-Hydroxy-5-phenylpentane-2-ylphosphonic acid (**9c**):

Diethyl 1-hydroxy-5-phenylpentan-2-ylphosphonate **7c** (0.36 mmol, 100 mg, 1 equiv) and TMSBr (0.95 mmol, 150 mg, 3 equiv) gave crude **9c** (110 mg) as colorless oil. A small batch (38 mg) was dissolved in 2-3 drops of EtOAc and washed with cold pentane, afforded the pure product product **9c** (21 mg, 0.081 mmol, 55%). ^1H NMR (400 MHz, Chloroform- d) δ 8.22 (s, 2H), 7.27-7.20 (m, 2H), 7.15-7.10 (m, 3H), 3.91-3.74 (m, 2H), 2.57-2.53 (m, 2H), 1.94-1.73 (m, 1H), 1.69-1.56 (m, 1H), 1.55-1.38 (m, 5H). ^{13}C NMR (101 MHz, Methanol- d_4) δ 142.4, 127.9 (d, $J = 13.5$ Hz), 125.2, 60.3, 40.9, 39.5, 35.3, 31.5, 27.2 (d, $J = 8.5$ Hz), 25.7 (d, $J = 3.4$ Hz). ^{31}P NMR (162 MHz, Methanol- d_4) δ 32.0. HRMS (ESI): Calcd. for $\text{C}_{12}\text{H}_{19}\text{O}_4\text{PNa}$ $[\text{M}+\text{H}]^+$ 281.0916; found 281.0913.

4.1.6.4. 1-Acetylthio-4-phenylbutan-2-ylphosphonic acid, (**10a**):

S-2-(diethoxyphosphoryl)-4-phenylbutyl ethanethioate **8a** (0.77 mmol, 268 mg, 1 equiv) and TMSBr (2.31 mmol, 353 mg, 3 equiv) gave crude **10a** (232 mg) as an orange oil. The crude was dissolved in 2-3 drops of EtOAc and washed with cold pentane, afforded the pure product **10a** (0.69 mmol, 201 mg, 91%). ^1H NMR (400 MHz, Methanol- d_4) δ 7.26-7.22 (m, 2H), 7.19-7.14 (m, 3H), 3.44-3.36 (m, 1H), 3.04 (ddd, $J = 13.8, 10.4, 8.4$ Hz, 1H), 2.79-2.76 (m, 2H), 2.30 (s, 3H), 2.07-1.81 (m, 3H). ^{13}C NMR (101 MHz, Methanol- d_4) δ 195.5, 141.8 (d, $J = 17.5$ Hz), 128.1-128.0 (m), 125.5, 40.4, 39.0, 37.2, 35.8, 33.1 (t, $J = 8.0$ Hz), 29.7 (d, $J = 2.9$ Hz), 28.9 (dd, $J = 10.7, 2.5$ Hz), 28.0, 22.9. ^{31}P NMR (162 MHz, Methanol- d_4) δ 30.4. HRMS (ESI): Calcd. for $\text{C}_{12}\text{H}_{18}\text{O}_4\text{PS}$ $[\text{M}+\text{H}]^+$ 289.0658; found 289.0660.

4.1.6.5. 1-(Acetylthio)-5-phenylpentan-2-ylphosphonic acid, (**10b**):

S-2-(diethoxyphosphoryl)-5-phenylpentan ethanethioate **8b** (0.53 mmol, 190 mg, 1 equiv) and TMSBr (1.58 mmol, 242 mg, 3 equiv) gave crude **10b** (207 g) as an orange oil. The crude was dissolved in 2-3 drops of EtOAc and washed with cold pentane, afforded the pure product **10b** (0.37 mmol, 113 mg, 71%). ^1H NMR (400 MHz, Methanol- d_4) δ 7.25-7.13 (m, 5H), 3.39-3.30 (m, 1H), 3.00 (ddd, $J = 14.0, 10.9, 8.5$ Hz, 1H), 2.58 (t, $J = 7.3$ Hz, 2H), 2.27 (s, 3H), 1.94-1.56 (m, 6H). ^{13}C NMR (101 MHz, Methanol- d_4) δ 195.4, 141.9, 128.0 (d, $J = 18.7$ Hz), 125.4, 37.8, 36.4, 35.4, 29.0 (d, $J = 7.4$ Hz), 28.0, 27.5 (d, $J = 3.1$ Hz). ^{31}P

NMR (162 MHz, Methanol- d_4) δ 30.8. HRMS (ESI): Calcd. for $\text{C}_{13}\text{H}_{20}\text{O}_4\text{PS}$ $[\text{M}+\text{H}]^+$ 303.0814; found 303.0818.

4.1.6.6. 1-(Acetylthio)-6-phenylhexan-2-ylphosphonic acid, (**10c**):

S-2-(diethoxyphosphoryl)-6-phenylhexyl ethanethioate, **8c** (0.478 mmol, 0.178 g, 1 equiv) and TMSBr (1.434 mmol, 0.219 g, 3 equiv) gave crude **10c** (113 mg) as an orange oil. The crude was dissolved in 2-3 drops of EtOAc and washed with cold pentane, afforded the pure product **10c** (0.31 mmol, 98 mg, 65%). ^1H NMR (400 MHz, Methanol- d_4) δ 7.26-7.20 (m, 3H), 7.19-7.12 (m, 2H), 3.39-3.33 (m, 1H), 3.04 (ddd, $J = 13.8, 10.8, 8.6$ Hz, 1H), 2.65-2.58 (m, 3H), 2.30 (s, 3H), 1.96-1.89 (m, 1H), 1.82-1.72 (m, 4H), 1.64-1.59 (m, 1H). ^{13}C NMR (101 MHz, Methanol- d_4) δ 195.5, 141.9, 128.1-127.8 (m), 125.3, 40.9, 39.6, 37.8, 36.4, 35.5 (d, $J = 11.7$ Hz), 29.2-28.8 (m), 28.1, 27.5 (d, $J = 3.1$ Hz), 26.7 (d, $J = 3.0$ Hz), 23.0. ^{31}P NMR (162 MHz, Methanol- d_4) δ 31.5. HRMS (ESI): Calcd. for $\text{C}_{14}\text{H}_{20}\text{O}_4\text{PS}[\text{M}+\text{H}]^+$ 315.0820; found 315.0817.

4.1.6.7. 1-Sulfanyl-4-phenylbutan-2-ylphosphonic acid (**3a**):

Diethyl 1-sulfanyl-4-phenylbutan-2-ylphosphonate **2a** (0.14 mmol, 43 mg, 1 equiv) and TMSBr (0.43 mmol, 65 mg, 3 equiv) gave crude **3a** (38 mg) as an orange oil. The crude was dissolved in 2-3 drops of EtOAc and washed with cold pentane, afforded the pure product **3a** (0.13 mmol, 33 mg, 96%). ^1H NMR (400 MHz, Chloroform- d) δ 9.05 (s, 2H), 7.28-7.19 (m, 5H), 3.05-2.94 (m, 1H), 2.78-2.74 (m, 3H), 2.14-2.07 (m, 3H), 1.56 (t, $J = 8.4$ Hz, 1H). ^{13}C NMR (101 MHz, Methanol- d_4) δ 141.8, 128.1 (d, $J = 12.3$ Hz), 125.6, 40.4, 39.0, 33.1 (d, $J = 7.5$ Hz), 28.9, 22.9. ^{31}P NMR (162 MHz, Methanol- d_4) δ 30.43. HRMS (ESI): Calcd. for $\text{C}_{10}\text{H}_{16}\text{O}_3\text{PS}$ $[\text{M}+\text{H}]^+$ 247.0552; found 247.0552.

4.1.6.8. 1-sulfanyl-5-phenylpentan-2-ylphosphonic acid (**3b**):

Diethyl 1-sulfanyl-5-phenylpentan-2-ylphosphonate **2b** (0.05 mmol, 15 mg, 1 equiv) and TMSBr (0.14 mmol, 22 mg, 3 equiv) gave crude **3b** as an orange oil. The crude was dissolved in 2-3 drops of EtOAc and washed with cold pentane, afforded the pure product **3b** (0.04 mmol, 11 mg, 90%). ^1H NMR (400 MHz, Methanol- d_4) δ 7.27-7.18 (m, 4H), 7.16-7.12 (m, 1H), 2.97 (ddd, $J = 17.8, 13.7, 3.8$ Hz, 1H), 2.66-2.60 (m, 3H), 1.91-1.78 (m, 5H). ^{13}C NMR (101 MHz, Methanol- d_4) δ 142.9, 128.8 (d, $J = 16.9$ Hz), 126.1 (d, $J = 3.3$ Hz), 41.8, 40.4, 36.3, 29.9 (d, $J = 7.4$ Hz), 27.5 (d, $J = 2.5$ Hz), 23.8. ^{31}P NMR (162 MHz, Methanol- d_4) δ 30.12. HRMS (ESI): Calcd. for $\text{C}_{11}\text{H}_{18}\text{O}_3\text{PS}$ $[\text{M}+\text{H}]^+$ 261.0709; found 261.0712.

4.1.6.9. 1-sulfanyl-6-phenylhexan-2-ylphosphonic acid, (**3c**):

Diethyl 1-sulfanyl-6-phenylhexan-2-ylphosphonate **2c** (0.30 mmol, 100 mg, 1 equiv) and TMSBr (0.91 mmol, 144 mg, 3 equiv) gave crude **3c** as an orange oil. The crude was dissolved in 2-3 drops of EtOAc and washed with cold pentane, afforded the pure product **3c** (0.29 mmol, 81 mg, 98%). ^1H NMR (400 MHz, Chloroform- d) δ 8.59 (s, 2H), 7.29-7.25 (m, 2H), 7.19-7.16 (m, 3H), 2.98 (ddd, $J = 20.0, 13.9, 4.6$ Hz, 1H), 2.75-2.63 (m, 1H), 2.62 (dd, $J = 8.8, 6.4$ Hz, 2H), 2.11-2.02 (m, 1H), 1.83 (ddd, $J = 16.5, 9.4, 6.2$ Hz, 2H), 1.67-1.63 (m, 2H), 1.49-1.47 (m, 2H), 1.34 (t, $J = 7.1$ Hz, 1H). ^{13}C NMR (101 MHz, Methanol- d_4) δ 142.5 (d, $J = 3.2$ Hz), 128.1-127.9 (m), 125.2, 41.3 (d, $J = 134.8$ Hz), 35.3, 31.5-31.4 (m), 26.9-26.9 (m), 23.3. ^{31}P NMR (162 MHz, Methanol- d_4) δ 30.5. HRMS (ESI): Calcd. for $\text{C}_{12}\text{H}_{19}\text{O}_3\text{NaPS}$ $[\text{M}+\text{H}]^+$ 297.0690; found 297.0685.

4.1.7. General procedure for the synthesis of acrylonitriles (**13a** and **13b**)

Compound **13a** and **13b** were prepared by a modified procedure based on the work of Baraldi *et al.* [39] K_2CO_3 (3 equiv) and 4-cyano-3-oxotetrahydrothiophene **12** (1 equiv) were stirred in dry acetone (20 mL). To this suspension alkyl halide (1 equiv) was added and the mixture was refluxed for 5 h. The residue was dissolved in ether and stirred vigorously with 5% NaOH aq. (10 mL). The solution was kept at RT until complete fragmentation of the intermediate adduct was observed by TLC. The organic phase was separated and dried with sodium sulfate. The crude oil was further purified by column chromatography using 20% diethyl ether in pentane as eluent.

4.1.7.1. 2-Benzylacrylonitrile (**13a**):

K_2CO_3 (11.7 mmol, 1.62 g, 3 equiv), compound 4-cyano-3-oxotetrahydrothiophene (3.9 mmol, 0.5 g, 1 equiv), and benzyl bromide (3.9 mmol, 0.673 g, 1 equiv), gave **13a** as an oil (1.99 mmol, 284 mg, 51%). 1H NMR (400 MHz, Chloroform-*d*) δ 7.40 (t, J = 8.0 Hz, 2H), 7.33 (d, J = 7.3 Hz, 1H), 7.26 (d, J = 7.4 Hz, 2H), 5.95 (s, 1H), 5.73 (s, 1H), 3.59 (s, 2H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 135.6, 131.1, 129.2, 128.9, 128.9, 128.6, 128.6, 128.4, 127.4, 122.7, 118.5, 40.8. HRMS (ESI): Calcd. for $C_{10}H_{10}N$ $[M+H]^+$ 144.0808; found 144.0808.

4.1.7.2. 2-Methylene-5-phenylpentanenitrile (**13b**):

K_2CO_3 (47.1 mmol, 6.51 g, 3 equiv), 4-cyano-3-oxotetrahydrothiophene (15.7 mmol, 2.00 g, 1 equiv), 1-bromo-3-phenylpropane (15.7 mmol, 3.13 g, 1 equiv), gave **13b** as an oil (3.29 mmol, 564 mg, 21%). 1H NMR (400 MHz, Chloroform-*d*) δ 7.34 (dd, J = 8.1, 6.7 Hz, 2H), 7.24-7.19 (m, 3H), 5.87 (s, 1H), 5.71 (d, J = 1.6 Hz, 1H), 2.68 (t, J = 7.6 Hz, 2H), 2.29-2.27 (m, 2H), 1.96-1.90 (m, 2H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 141.1, 130.5, 128.5, 128.5, 128.4, 126.1, 122.9, 118.6, 34.7, 33.9, 29.1. HRMS (ESI): Calcd. for $C_{12}H_{14}N_4$ $[M+H]^+$ 172.1114; found 172.1121.

4.1.7.3. 4.1.8 General procedure for the preparation of tetrazoles (**14a** and **14b**)

Dibutyltin oxide (0.2 equiv) and trimethylsilyl azide (2 equiv) were added to a solution of **13** (1 equiv) in anhydrous 1,4-dioxane (2 mL/mmol). The reaction mixture was subjected to microwave irradiation in a tightly sealed vessel for 50 min at 150 $^{\circ}C$, then cooled to room temperature. The solvent was removed under reduced pressure. The residue was dissolved in diethyl ether (10 mL) and extracted with 2 M aq. NaOH (3 x 10 mL). The aqueous layer was acidified with 4 M aq. HCl to pH 1 and extracted with ethyl acetate (4 x 10 mL). The organic extract was washed with brine (10 mL), dried over $MgSO_4$, and evaporated under reduced pressure to give the target tetrazoles, which were recrystallized from EtOAc.

4.1.7.4. 5-(3-phenylprop-1-en-2-yl)-1H-tetrazole (**14a**):

Dibutyltin oxide (0.07 mmol, 0.017 g, 0.2 equiv), trimethylsilyl azide (1.4 mmol, 0.161 g, 2 equiv) and **13a** (0.100 g, 0.7 mmol, 1 equiv) gave **14a** (0.55 mmol, 102 mg, 78%). 1H NMR (400 MHz, Methanol-*d*₄) δ 7.35-7.18 (m, 5H), 6.04 (s, 1H), 5.47 (s, 1H), 4.92 (s, 1H), 3.92 (s, 2H). ^{13}C NMR (101 MHz, Methanol-*d*₄) δ 137.5, 133.2, 128.7, 128.1, 126.3, 120.3, 38.9. HRMS (ESI): Calcd. for $C_{10}H_{10}N_4$ $[M+H]^+$ 187.0981; found 187.0978.

4.1.7.5. 5-(5-Phenylpent-1-en-2-yl)-1H-tetrazole (**14b**):

Dibutyltin oxide (0.58 mmol, 145.39mg, 0.2 equiv), trimethylsilyl azide (5.84 mmol, 0.672 g, 2 equiv) and **13b** (2.92 mmol, 1 equiv) gave **14b** (2.16 mmol, 462 mg, 74%). 1H NMR (400 MHz, Chloroform-*d*) δ 7.14-7.00 (m, 5H), 5.87 (s, 1H), 5.47 (t, J = 1.4 Hz, 1H), 4.84-4.83 (m, 1H), 2.58 (dd, J = 15.7, 7.8 Hz, 4H), 1.78 (m, 2H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 157.3, 143.1, 134.7, 129.4, 129.4, 126.9, 120.6, 36.2, 34.0, 30.9. HRMS (ESI): Calcd. for $C_{12}H_{15}N_4$ $[M+H]^+$ 215.1292; found 215.1291.

4.1.8. General procedure for the Michael addition to vinylic tetrazoles (preparation of **15** and **16**)

Vinylic tetrazole **14** (1 equiv) and thioacetic acid (26 equiv) or thiol (4 equiv) were dissolved in anhydrous DMF. The reaction mixture was subjected to heating at 60 $^{\circ}C$ in a tightly sealed vessel for 22 h with stirring. The solvent was removed under reduced pressure and the crude was subjected to column chromatography using 5% MeOH in CH_2Cl_2 .

4.1.8.1. S-(3-Phenyl-2-(1H-tetrazol-5-yl)propyl) ethanethioate (**15a**)

14a (0.32 mmol, 0.060 g, 1 equiv), and thioacetic acid (8.6 mmol, 0.662 g, 26 equiv) gave **15a** (0.30 mmol, 79 mg, 95%) as a colorless oil. 1H NMR (400 MHz, Chloroform-*d*) δ 7.20-7.16 (m, 3H), 7.08-7.06 (m, 2H), 3.86 (q, J = 10.5, 7.7 Hz, 1H), 3.44-3.40 (m, 1H), 3.33 (d, J = 14.0 Hz, 1H), 3.23 (d, J = 7.6 Hz, 2H), 2.27 (s, 3H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 195.9, 157.9, 137.0, 128.9, 128.9, 128.7, 127.0, 39.7, 38.0, 31.8, 30.6. HRMS (ESI): Calcd. for $C_{12}H_{15}ON_4S$ $[M+H]^+$ 263.0964; found 263.0961.

4.1.8.2. S-(5-phenyl-2-(1H-tetrazol-5-yl)pentyl) ethanethioate (**15b**)

14b (0.466 mmol, 0.100 mg, 1 equiv), and thioacetic acid (12.1 mmol, 0.925 g, 26 equiv) gave **15b** (0.456 mmol, 132 mg, 98%) as a colorless oil. 1H NMR (400 MHz, Methanol-*d*₄) δ 7.22-7.11 (m, 5H), 4.93 (s, 1H), 3.38-3.30 (m, 2H), 3.16 (dd, J = 15.2, 10.3 Hz, 1H), 2.58 (t, J = 7.5 Hz, 2H), 2.24 (s, 3H), 1.91-1.77 (m, 2H), 1.61-1.45 (m, 2H). ^{13}C NMR (101 MHz, Methanol-*d*₄) δ 196.2, 159.6, 142.9, 129.4, 126.9, 37.2, 36.2, 33.9, 33.3, 30.4, 29.9. HRMS (ESI): Calcd. for $C_{14}H_{19}ON_4S$ $[M+H]^+$ 291.1284; found 291.1274.

4.1.8.3. '5-(1-(cyclohexylthio)-5-phenylpentan-2-yl)-1H-tetrazole (**16**)

14a (0.23 mmol, 0.050 g, 1 equiv), and cyclohexyl thiol (0.933 mmol, 0.108 g, 4 equiv) gave **16** (0.216 mmol, 71.50 mg, 93%). 1H NMR (400 MHz, Methanol-*d*₄) δ 7.23-7.09 (m, 5H), 2.95-2.82 (m, 1H), 2.58-2.55 (m, 2H), 1.86-1.68 (m, 6H), 1.59-1.18 (m, 10H). ^{13}C NMR (101 MHz, Methanol-*d*₄) δ 158.94, 141.6, 127.9, 127.9, 125.5, 43.6, 36.7, 34.9, 33.6, 33.4, 33.3, 32.8, 28.6, 25.6, 25.5. HRMS (ESI): Calcd. for $C_{18}H_{27}N_4S$ $[M+H]^+$ 331.1950; found 331.1951.

4.2. Biological activity

4.2.1. Gene Constructs of VIM-2, NDM-1 and GIM-1

In this study two types of gene constructs were used. The first included the native leader sequence to allow the proteins to be transported to the periplasm, for the three enzymes VIM-2 from *Pseudomonas aeruginosa* strain 301-5473 (GenBank no Q9K2N0; [51]), GIM-1 from *P. aeruginosa* (GenBank no Q704V1; [50, 52]) and NDM-1 (GenBank no. E9NWK5, e.g [53, 54]), where the latter bla_{NDM-1} gene is reported from several organisms. Cloning of bla_{NDM-1} or bla_{GIM-1} genes into the *Escherichia coli* pET26b(+) vector (Novagen) was performed using the Primers listed in **Table S3**, and by restriction cutting as

described for VIM-26. [55] Cloning of *bla*_{VIM-2} into pET26b(+) are described previously. [56] The obtained *E. coli* pET-26b(+) MBL constructs were further used in the whole cell-based inhibitor assays.

In the second gene constructs used for the recombinant gene expression, the native leader sequence was removed and replaced with a hexa-His tag and a TEV cleavage site as reported earlier for VIM-2 (residues V27-E268; [57]) and GIM-1 (residues Q19-D250; [50]) both in pDEST14. NDM-1 used a codon optimized synthetic gene (Life Technologies, Thermo Fisher Scientific), with a TEV cleavage site with sequence ENLYFQG and residues G36-R280 in NDM-1 transformed in pDONR™221, and further sub cloned into pDEST17 with carries an N-terminal hexa His-tag, yielding pDest17-NDM-1. Herein the residue numbering is the class B β-lactamase numbering scheme will be applied [10].

4.2.2. Recombinant protein expression and purification of VIM-2, GIM-1 and NDM-1

The three proteins were expressed and purified following this protocol. pDest17-NDM-1 was transformed into *E. coli* BL21 Star (DE3) pLysS (Invitrogen), and pDEST14 plasmids with VIM-2 or GIM-1 were transformed into in-house modified *E. coli* BL21 Star (DE3) pLysS (Invitrogen) cells with the pRARE plasmid (Novagen) in order to allow expression of genes encoding tRNAs for rare codons [58]. Precultures grown in Terrific Broth (TB) media with 100 µg/ml ampicillin (Sigma-Aldrich) and 34 µg/ml chloramphenicol (Sigma-Aldrich). The precultures were inoculated to 2 L Luria-Bertani (LB) media containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol and grown at 37 °C to reach an optical density (OD₆₀₀ nm) of 0.5-1.0 before induced expression with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich). The induced cultures were grown overnight at 20 °C before collecting the cells by centrifugation (8,900 X g, 30 min, 4 °C). Buffer A containing 50 mM HEPES pH 7.2, 100 µM ZnCl₂ and 150 mM NaCl was used to resuspend the cell pellets, following sonication and collecting the supernatants by centrifugation (3000 g, 40 min, 4 °C). The recombinant proteins were affinity purified using a 1 ml or 5 ml His-Trap HP column (GE Healthcare) in buffer A washed with 5% buffer B (50 mM HEPES pH 7.2, 100 µM ZnCl₂, 150 mM NaCl and 1 M imidazole), before eluted in a gradient of 5 to 100% buffer B. Peak fractions were investigated using 4–20% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad) [59]. The peaks containing MBL protein was added in-house-made His-tagged TEV protease in a 1:100 mg ratio of TEV:protein and dialyzed at 4 °C overnight using 10-kDa cutoff Snakeskin (Pierce) in buffer C (50 mM HEPES pH 7.2, 150 mM NaCl, 1 mM EDTA and 1 mM β-mercaptoethanol). To remove uncleaved protein and TEV protease a second His-Trap purification was performed. SDS-PAGE analysis was used to estimate a purity of ~95% of the fractions containing protein, which then were pooled and dialyzed in buffer A overnight.

4.2.3. Co-crystallization of VIM-2 with 2b, 10b and 10c

Co-crystallization of VIM-2 was successful with inhibitors **2b**, **10b** and **10c**, when applying our new protocol for DMSO free co-crystallization [45]. MRC 96-wells were pre-coated with 3 µL inhibitor (100 mM) dissolved in DMSO, and DMSO evaporated after leaving the plates in the fume hood for 24 hours. 60 µL reservoir solution consisting of 22-27% PEG 3350 and 0.2 M magnesium formate was added to each well. The crystallization plates were then shaken for 24 hours to allow the inhibitors to dissolve, followed by manual mixing of hanging drops in a 1:1 ratio of VIM-2 (9.4 mg/ml) and reservoir. The crystals were cryoprotected in 25% PEG 3350, 0.2 M MgCl₂, 15% ethylene

glycol and 50 mM HEPES pH 7.2, before they were flash cooled in liquid nitrogen and X-ray data collected at ESRF, ID29.

Coordinate and structure factor files for the VIM-2_2b (PDB accession number 5MM9), VIM-2_10b (PDB accession number 5NHZ) and VIM-2_10c (PDB accession number 5NI0) were deposited in the Protein Data Bank (PDB).

4.2.4. Dose Rate Inhibition Studies for IC₅₀ Determination

The half-maximal inhibitory concentration (IC₅₀) against the VIM-2, NDM-1 and GIM-1 enzymes were determined by using sixteen different concentration of inhibitor compounds ranging from 0 µM to 250 µM. A 100 µl solution with 50 mM HEPES buffer (pH 7.2), 100 µM ZnCl₂, purified enzyme (10 nM VIM-2, NDM-1 or GIM-1) and 2.5-0 mM inhibitor was incubated in a 96 well plate at 25 °C for 5 min. In addition, the enzyme buffer contained 400 µg/ml Bovine Serum Albumin (BSA) to prevent protein unfolding and loss of activity due to low concentrations [60, 61]. 100 µM of the reporter substrate nitrocefin (VIM-2, GIM-1) or imipenem (NDM-1) was added to the enzyme-inhibitor solution and the increase in absorbance at 482 nm (nitrocefin) or 260 nm (imipenem) was recorded on a Spectramax M2e spectrophotometer (Molecular Devices). Each data point was performed in triplicates and the initial velocity for each inhibitor concentration was analyzed by a log [inhibitor] vs. response curve fitting to calculate IC₅₀ in GraphPad Prism 5.0 software.

4.2.5. Cell-based screening assay of the inhibition potential

The inhibitory activity of the inhibitors was investigated in a cell-based assay using a β-lactamase-negative *E. coli* SNO3 (*ampA1 ampC8 pyrB recA rpsL*) (24) transformed with pET26b(+) containing *bla*_{VIM-2}, *bla*_{GIM-1} or *bla*_{NDM-1}. The screen was conducted in 96-well plates (Corning) in duplicates. One µl inhibitor (final concentration 250 µM), 50 µl overnight culture (adjusted to an OD₆₀₀ of 1 in LB broth) of *E. coli* SNO3 containing one of the MBLs and 50 µl LB media with final concentration 0.8 mM Isopropyl β-D-1-thiogalactopyranoside (Sigma, IPTG) was added to each well. The plate was incubated at 37 °C for 20 min with shaking to induce the expression of the MBL. Subsequently, 50 µl nitrocefin (diluted in 50 mM HEPES pH 7.2 and 100 µM ZnCl₂ to give a final concentration of 1.6 mM in the assay) was added. Nitrocefin hydrolysis was measured at OD₄₈₂ every minute for 3 hours with shaking (47 seconds) in between reads using a Spectramax M2e spectrophotometer (Molecular Devices). EDTA (250 µM concentration) was used as positive control and wells containing no inhibitor as negative controls. The percent inhibition was calculated according to equation 1.

$$\% \text{ inhibition} = (\text{Slope}_{\text{sample}} - \text{Slope}_{\text{Average negative control}}) \times 100 \% \quad (1)$$

The synergistic effect of the inhibitors with meropenem was tested against selected clinical bacterial strains containing MBLs. The bacterial strains were plated on lactose agar plates with 100 mg/L ampicillin and lactose agar and incubated overnight at 37 °C. The inhibitors were diluted to a final concentration of 125 µM in Mueller Hinton (MH) broth. In order to monitor the effect of the DMSO in the assay, a DMSO control was included with a concentration of 5%. Meropenem was diluted in MH broth in a 2-fold dilution series with final concentrations of 256 µg/mL – 0.0625 µg/mL. The microtiter plates were inoculated with a 0.5 McFarland suspension of the bacterial strain in 0.85% NaCl, which were diluted in MH broth. A quality check of bacterial suspension in 0.85% NaCl in a 1:100 ratio was incubated on MH

agar plates overnight at 37 °C. The final CFU/mL inoculum were calculated and compared to a standard. The microtiter plates were incubated for 20 hours at 37 °C. The minimum inhibitory concentrations (MIC) were read visually of the plates the next day.

Acknowledgments

This work has been funded by the research Council of Norway (FRIMEDBIO 2011; 213808) and this is highly acknowledged. The provision of beam time at ID29, ESRF is highly valued. We acknowledge Trine Josefine O. Carlsen for purification and

References and notes

- [1] World Health Organization (WHO), Antimicrobial resistance: global report on surveillance, (2014).
- [2] World Health Organization (WHO), The evolving threat of antimicrobial resistance : options for action, (2012).
- [3] A.M. Queenan, K. Bush, *Clin. Microbiol. Rev.*, 20 (2007) 440-458.
- [4] J.D. Pitout, *Drugs*, 70 (2010) 313-333.
- [5] R.P. Ambler, *Philos. Trans. R. Soc., B*, 289 (1980) 321-331.
- [6] K. Bush, G.A. Jacoby, *Antimicrob. Agents Chemother.*, 54 (2010) 969-976.
- [7] T.R. Walsh, *Int. J. Antimicrob. Agents*, 36 (2010) S8-S14.
- [8] T. Palzkill, *Ann. N. Y. Acad. Sci.*, 1277 (2013) 91-104.
- [9] J. Jeon, J. Lee, J. Lee, K. Park, A. Karim, C.-R. Lee, B. Jeong, S. Lee, *Int. J. Mol. Sci.*, 16 (2015) 9654-9692.
- [10] G. Garau, I. García-Sáez, C. Bebrone, C. Anne, P. Mercuri, M. Galleni, J.M. Frère, O. Dideberg, *Antimicrob. Agents Chemother.*, 48 (2004) 2347-2349.
- [11] T.R. Walsh, M.A. Toleman, L. Poirel, P. Nordmann, *Clin. Microbiol. Rev.*, 18 (2005) 306-325.
- [12] G. Patel, R. Bonomo, *Front. Microbiol.*, 4 (2013) 1-17.
- [13] J.D. Buynak, *Biochem. Pharmacol.*, 71 (2006) 930-940.
- [14] J.L. Liscio, M.V. Mahoney, E.B. Hirsch, *Int. J. Antimicrob. Agents*, 46 (2015) 266-271.
- [15] S.M. Drawz, K.M. Papp-Wallace, R.A. Bonomo, *Antimicrob. Agents Chemother.*, 58 (2014) 1835-1846.
- [16] M.M. González, A.J. Vila, in: Springer Berlin Heidelberg, Berlin, Heidelberg, 2016, pp. 1-34.
- [17] W. Jing-Fang, C. Kuo-Chen, *Curr. Top. Med. Chem.*, 13 (2013) 1242-1253.
- [18] W. Fast, L.D. Sutton, *Biochim. Biophys. Acta*, (2013) 1648-1659.
- [19] J. Brem, S.S. van Berkel, D. Zollman, S.Y. Lee, O. Gileadi, P.J. McHugh, T.R. Walsh, M.A. McDonough, C.J. Schofield, *Antimicrob. Agents Chemother.*, 60 (2016) 142-150.
- [20] F.-M. Klingler, T.A. Wichelhaus, D. Frank, J. Cuesta-Bernal, J. El-Delik, H.F. Müller, H. Sjuts, S. Göttig, A. Koenigs, K.M. Pos, D. Pogoryelov, E. Proschak, *J. Med. Chem.*, 58 (2015) 3626-3630.
- [21] M.M. González, M. Kosmopoulou, M.F. Mojica, V. Castillo, P. Hinchliffe, I. Pettinati, J. Brem, C.J. Schofield, G. Mahler, R.A. Bonomo, L.I. Llarull, J. Spencer, A.J. Vila, *ACS Infect. Dis.*, 1 (2015) 544-554.
- [22] N. Li, Y. Xu, Q. Xia, C. Bai, T. Wang, L. Wang, D. He, N. Xie, L. Li, J. Wang, H.-G. Zhou, F. Xu, C. Yang, Q. Zhang, Z. Yin, Y. Guo, Y. Chen, *Bioorg. Med. Chem. Lett.*, 24 (2014) 386-389.
- [23] B.M.R. Liénard, G. Garau, L. Horsfall, A.I. Karsisiotis, C. Damblon, P. Lassaux, C. Papamicael, G.C.K. Roberts, M. Galleni, O. Dideberg, J.M. Frère, C.J. Schofield, *Org. Biomol. Chem.*, 6 (2008) 2282-2294.
- [24] Q. Sun, A. Law, M.W. Crowder, H.M. Geysen, *Bioorg. Med. Chem. Lett.*, 16 (2006) 5169-5175.
- [25] W.C. Jin, Y. Arakawa, H. Yasuzawa, T. Taki, R. Hashiguchi, K. Mitsutani, A. Shoga, Y. Yamaguchi, H. Kurosaki, N. Shibata, M. Ohta, M. Goto, *Biol. Pharm. Bull.*, 27 (2004) 851-856.

crystallization of proteins and Kinga Leszczak for contributions to the synthetic work.

Supplementary Material

Supplementary data related to this article can be found at:

Coordinate and structure factor files for the VIM-2_2b (PDB accession number 5MM9), VIM-2_10b (PDB accession number 5NHZ) and VIM-2_10c (PDB accession number 5NI0) were deposited in the Protein Data Bank (PDB).

- [26] U. Heinz, R. Bauer, S. Wommer, W. Meyer-Klaucke, C. Papamichaels, J. Bateson, H.W. Adolph, *J. Biol. Chem.*, 278 (2003) 20659-20666.
- [27] C. Mollard, C. Moali, C. Papamicael, C. Damblon, S. Vessilier, G. Amicosante, C.J. Schofield, M. Galleni, J.-M. Frère, G.C.K. Roberts, *J. Biol. Chem.*, 276 (2001) 45015-45023.
- [28] 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-US*, 39 (2000) 4288-4298.
- [29] M.I. Page, A.P. Laws, *Chem. Commun.*, (1998) 1609-1617.
- [30] S. Bounaga, A.P. Laws, M. Galleni, M.I. Page, *Biochem. J.*, 331 (1998) 703-711.
- [31] M. Goto, T. Takahashi, F. Yamashita, A. Koreeda, H. Mori, M. Ohta, Y. Arakawa, *Biol. Pharm. Bull.*, 20 (1997) 1136-1140.
- [32] J. Brem, S.S. van Berkel, W. Aik, A.M. Rydzik, M.B. Avison, I. Pettinati, K.-D. Umland, A. Kawamura, J. Spencer, T.D.W. Claridge, M.A. McDonough, C.J. Schofield, *Nat Chem*, 6 (2014) 1084-1090.
- [33] G.A. Patani, E.J. LaVoie, *Chem. Rev.*, 96 (1996) 3147-3176.
- [34] C. Ballatore, D.M. Hury, A.B. Smith, *ChemMedChem*, 8 (2013) 385-395.
- [35] Y. Yamaguchi, W. Jin, K. Matsunaga, S. Ikemizu, Y. Yamagata, J.I. Wachino, N. Shibata, Y. Arakawa, H. Kurosaki, *J. Med. Chem.*, 50 (2007) 6647-6653.
- [36] J.D. Park, D.H. Kim, *J. Med. Chem.*, 45 (2002) 911-918.
- [37] R.V. Bikbulatov, F. Yan, B.L. Roth, J.K. Zjawiony, *Bioorg. Med. Chem. Lett.*, 17 (2007) 2229-2232.
- [38] L. Zervas, I. Photaki, N. Ghelis, *J. Am. Chem. Soc.*, 85 (1963) 1337-1341.
- [39] P.G. Baraldi, G.P. Pollini, V. Zanirato, A. Barco, S. Benetti, *Synthesis*, (1985) 969-970.
- [40] S.M. Lukyanov, I.V. Bliznets, S.V. Shorshnev, G.G. Aleksandrov, A.E. Stepanov, A.A. Vasil'ev, *Tetrahedron*, 62 (2006) 1849-1863.
- [41] T. Christopheit, H.K. Leiros, *Bioorg. Med. Chem. Lett.*, 26 (2016) 1973-1977.
- [42] S. Skagseth, T.J. Carlsen, G.E. Bjerga, J. Spencer, Ø. Samuelsen, H.K.S. Leiros, *Antimicrob. Agents Chemother.*, 60 (2015) 990-1002.
- [43] 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.*, 53 (2010) 4862-4876.
- [44] H. Nikaido, *Microbiol. Mol. Biol. Rev.*, 67 (2003) 593-656.
- [45] T. Christopheit, K.-W. Yang, S.-K. Yang, H.-K.S. Leiros, *Acta Cryst.*, F72 (2016) 813-819.
- [46] H. Kurosaki, Y. Yamaguchi, H. Yasuzawa, W.C. Jin, Y. Yamagata, Y. Arakawa, *ChemMedChem*, 1 (2006) 969-972.
- [47] P. Hinchliffe, M.M. González, M.F. Mojica, J.M. González, V. Castillo, C. Saiz, M. Kosmopoulou, C.L. Tooke, L.I. Llarull, G. Mahler, R.A. Bonomo, A.J. Vila, J. Spencer, *Proc. Natl. Acad. Sci. U. S. A.*, 113 (2016) E3745-E3754.
- [48] M.F. Mojica, S.G. Mahler, C.R. Bethel, M.A. Taracila, M. Kosmopoulou, K.M. Papp-Wallace, L.I. Llarull, B.M. Wilson, S.H. Marshall, C.J. Wallace, M.V. Villegas, M.E. Harris, A.J. Vila, J. Spencer, R.A. Bonomo, *Biochemistry-US*, 54 (2015) 3183-3196.
- [49] D.T. King, L.J. Worrall, R. Gruninger, N.C.J. Strynadka, *J. Am. Chem. Soc.*, 134 (2012) 11362-11365.

- [50] P.S. Borra, Ø. Samuelsen, J. Spencer, T.R. Walsh, M.S. Lorentzen, H.-K.S. Leiros, *Antimicrob. Agents Chemother.*, 57 (2013) 848-854.
- [51] T. Christopeit, T.J. Carlsen, R. Helland, H.-K.S. Leiros, *J. Med. Chem.*, (2015) 8671-8682.
- [52] M. Castanheira, M.A. Toleman, R.N. Jones, F.J. Schmidt, T.R. Walsh, *Antimicrob. Agents Chemother.*, 48 (2004) 4654-4661.
- [53] D. Yong, M.A. Toleman, C.G. Giske, H.S. Cho, K. Sundman, K. Lee, T.R. Walsh, *Antimicrob. Agents Chemother.*, 53 (2009) 5046-5054.
- [54] K.K. Kumarasamy, M.A. Toleman, T.R. Walsh, J. Bagaria, F. Butt, R. Balakrishnan, U. Chaudhary, M. Doumith, C.G. Giske, S. Irfan, P. Krishnan, A.V. Kumar, S. Maharjan, S. Mushtaq, T. Noorie, D.L. Paterson, A. Pearson, C. Perry, R. Pike, B. Rao, U. Ray, J.B. Sarma, M. Sharma, E. Sheridan, M.A. Thirunarayan, J. Turton, S. Upadhyay, M. Warner, W. Welfare, D.M. Livermore, N. Woodford, *Lancet Infect. Dis.*, 10 (2010) 597-602.
- [55] H.-K.S. Leiros, K.S.W. Edvardsen, G.E.K. Bjerga, Ø. Samuelsen, *FEBS Journal*, 282 (2015) 1031-1042.
- [56] Ø. Samuelsen, M. Castanheira, T.R. Walsh, J. Spencer, *Antimicrob. Agents Chemother.*, 52 (2008) 2905-2908.
- [57] T. Christopeit, T.J. Carlsen, R. Helland, H.-K.S. Leiros, *J. Med. Chem.*, (2015) In press.
- [58] E.A. Rogulin, T.A. Perevyazova, L.A. Zheleznaya, N.I. Matvienko, *Biochemistry-Moscow+*, 69 (2004) 1123-1127.
- [59] U.K. Laemmli, *Nature*, 227 (1970) 680-685.
- [60] N. Laraki, N. Franceschini, G.M. Rossolini, P. Santucci, C. Meunier, E. de Pauw, G. Amicosante, J.M. Frère, M. Galleni, *Antimicrob. Agents Chemother.*, 43 (1999) 902-906.
- [61] S. Siemann, D.P. Evanoff, L. Marrone, A.J. Clarke, T. Viswanatha, G.I. Dmitrienko, *Antimicrob. Agents Chemother.*, 46 (2002) 2450-2457.