

# Discovery of an $\alpha$ -Aminophosphonic acid VIM-2 Inhibitor

**Mate Erdelyi**

`mate.erdelyi@kemi.uu.se`

Uppsala University <https://orcid.org/0000-0003-0359-5970>

**Kinga Gulyas**

Uppsala University

**Kim Bartels**

University Medical Center Hamburg Eppendorf

**Gurleen Kaur**

**Sahar Najam**

**Zoltán Palczert**

**Viola Tamasi**

Semmelweis University <https://orcid.org/0000-0001-7419-5603>

**Karina Tapia**

**Anna Andersson Rasmussen**

Lund Protein Production Platform LP3

**Máté Simon**

**Johannes Thoma**

University of Gothenburg <https://orcid.org/0000-0003-3584-8274>

**Miklós Csala**

<https://orcid.org/0000-0002-3829-4361>

**Christopher Schofield**

University of Oxford <https://orcid.org/0000-0002-0290-6565>

**Eike Schulz**

University Medical Center Hamburg Eppendorf <https://orcid.org/0000-0002-1685-8605>

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## Article

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# Abstract

The rise of antimicrobial resistance is a health crisis that severely compromises treatment options for bacterial infections. Metallo- $\beta$ -lactamases (MBLs) are zinc-dependent hydrolases that are an increasingly important contributor to this crisis by inactivating near all  $\beta$ -lactam antibiotics, including carbapenems. The WHO has identified the widespread dissemination of MBL-producing pathogens as a priority threat. The Verona integron-encoded metallo- $\beta$ -lactamase-2 (VIM-2) is one of the most prevalent and broadly disseminated MBLs, conferring high-level carbapenem resistance in Gram-negative pathogens. Here we report the  $\alpha$ -aminophosphonic acid group as a new scaffold that effectively targets VIM-2 providing a new avenue for restoring the clinical utility of  $\beta$ -lactams.  $\alpha$ -Aminophosphonic acid derivatives can be produced in 2–3 steps from commercially available starting materials. The VIM-2 inhibition activity, cytotoxicity, membrane permeability and the NMR and X-ray based characterisation of their VIM-2 binding mode are described. The overall results identify aminophosphonic acid derivatives as a new mode of MBL inhibition.

## Introduction

The global health threat of antimicrobial resistance (AMR) is endangering lives regardless of borders, resources, or public health infrastructure.<sup>1–4</sup> The growth of AMR is not only a matter of pathogen spread, but also a reflection of the limited chemical space presently available to defeat evolved bacterial enzymes.<sup>5,6</sup> The latter reflects a general lack of medicinal chemistry in the AMR field in recent decades, in particular at the early discovery stage, where new chemical scaffolds are required.<sup>7</sup>

The  $\beta$ -lactams remain the most important antibacterials, but as bacteria acquire enzymes capable of inactivating near all widely used  $\beta$ -lactam antibiotics, the effectiveness of this vital drug class is becoming increasingly compromised.<sup>8–11</sup> There are two structural/mechanistic classes of  $\beta$ -lactamases – the nucleophilic serine  $\beta$ -lactamases (SBLs, Ambler class A, C, D) and the metallo  $\beta$ -lactamases (MBLs, Ambler class B).<sup>12–14</sup> There are three subfamilies of MBLs (Ambler subfamilies B1-B3), of which the B1 subfamily is the most clinically relevant.<sup>10,15–18</sup>

The mechanism of the B1 MBLs employs a dinuclear zinc active site to hydrolyse diverse  $\beta$ -lactam scaffolds.<sup>12–14,19,20</sup> The Verona integron-encoded metallo- $\beta$ -lactamases (VIM) are among the clinically most important B1 MBLs and are widely disseminated among Gram-negative pathogens, particularly *Pseudomonas aeruginosa* and *Enterobacteriales*.<sup>21–24</sup> VIM-2 is globally distributed, including Europe, the Mediterranean region, East Asia and South America.<sup>25,26</sup> VIM-2 catalyses the hydrolysis of most  $\beta$ -lactam antibiotics, leaving few effective treatment options. The rapid global spread of VIM-2-producing strains has contributed significantly to the emerging antimicrobial resistance crisis and represents a major public health concern.<sup>27</sup>

Whilst SBL inhibitors, including clavulanic acid and avibactam, are widely used in combination therapies to protect  $\beta$ -lactam antibiotics, despite extensive efforts, no selective MBL inhibitors have yet been

approved for clinical use.<sup>28,29</sup> This is a concern as resistance mediated by MBLs is growing rapidly.<sup>19</sup> Classical  $\beta$ -lactam containing SBL inhibitors, such as clavulanic acid, are ineffective against MBLs (reflecting mechanistic differences between SBLs and MBLs), in some cases being substrates for them.<sup>30</sup>

Consequently, there is a pressing need to develop novel inhibitor chemotypes capable of selectively and potently inhibiting MBLs such as VIM-2, potentially through mechanisms that combine metal coordination with productive interactions across the extended and flexible MBL active-site surface. Such new inhibitor classes will be useful for restoring the clinical utility of  $\beta$ -lactam antibiotics against VIM-2-producing pathogens and for slowing the progression of resistance.

Over the past two decades, various strategies have been explored to inhibit VIM-2, yielding several compound classes that have validated the enzyme as a druggable target.<sup>20,29,31–33</sup> Amongst potent VIM-2 inhibitors are thiol-based inhibitors, which coordinate directly to the active-site zinc ions and often display nanomolar to low-micromolar potency.<sup>20,34</sup> However, their susceptibility to oxidation and limited pharmacokinetic properties have restricted their further development.<sup>20</sup> Boronic acid derivatives have also emerged as promising VIM-2 inhibitors by acting as transition-state mimics of  $\beta$ -lactam hydrolysis, with several examples demonstrating broad-spectrum  $\beta$ -lactamase activity.<sup>14,35</sup>

Phosphorous-containing compounds have shown promising properties for MBL inhibition by coordinating the zinc ions while providing a stable tetrahedral geometry that resembles the transition-state.<sup>36–40</sup> These, in general, have low cytotoxicity and promising drug-like properties.<sup>38,40,41</sup> Further approaches, including hydroxamate-based chelators, carboxylate-containing scaffolds, and heterocyclic metal-binding groups have been identified; however, none of these structures has yet been developed for clinical application.<sup>20,42</sup> Nevertheless, these developments demonstrate that VIM-2 inhibition is achievable. Most known VIM-2 inhibitors suffer from limited selectivity, stability, permeability or toxicity.<sup>20,43</sup> In order to reach clinical utility, the development of new MBL inhibitor scaffolds that combine metal coordination with optimized protein-binding elements, membrane permeability, low cytotoxicity and chemical and metabolic stability is desirable. Here we report on the synthesis of  $\alpha$ -aminophosphonic acid scaffold derivatives that inhibit VIM-2.

## Results & Discussion

Aromatic phosphonic acids have been reported to inhibit metallo- $\beta$ -lactamases.<sup>40</sup> Their phosphonic acid moiety (Fig. 1) was shown to coordinate to the two zinc ions at the VIM-2 active site, with an *ortho*-hydroxyphenyl substituent contributing to binding by hydrogen bonding to Asp118 (Asp120, BBL numbering<sup>40,44</sup>) or Asn210 (Asn233, BBL) as well as  $\pi$ - $\pi$  stacking with Phe62 (Phe61, BBL), Tyr67 (Tyr67, BBL) or Trp87 (Trp87, BBL). Some compounds with an amide link between the phosphonic acid and an aromatic ring were shown to inhibit several MBLs.<sup>37,40</sup>

**Design.** Here we report on the synthesis of  $\alpha$ -aminophosphonic acids (Fig. 1) and their ability to inhibit MBLs. Compound **4** was designed to evaluate the need for a benzylic substituent for VIM-2 inhibition by phosphonic acid derivatives. The analogous aromatic rings of reported phosphonic acid inhibitors appeared dynamic in the VIM-2 binding pocket, in general being solvent exposed.<sup>40</sup> Compound **5** was designed to evaluate whether an amide to secondary amine substitution influences VIM-2 inhibition activity. Finally, compound **6** was designed to explore the necessity of a phenol functionality by its substitution with a pyridine moiety. Similar to inhibitor **5j** (Fig. 1) and its analogues, compounds **4–6** were designed to be stereodynamic.<sup>40</sup> Accordingly, they are inseparable mixtures of their interconverting enantiomers.

**Synthesis.** The racemic  $\alpha$ -aminophosphonate diethyl esters **1–2** were synthesized via Kabachnik-Fields reaction of diethyl phosphite, benzyl amine and 2-methoxybenzaldehyde or pyridine-2-carbaldehyde using hafnium chloride as a catalyst in ethanol at 60°C for 18–48 h in 65% yield. Compounds **1–2** were subsequently reacted with boron tribromide to obtain the corresponding free phosphonic acids **5** and **6**, respectively. The hydrolysis of **1** was carried out in toluene at 60°C for 48 h yielding compound **5** in 55% after preparative HPLC purification. The reaction of compound **2** was performed in dichloromethane at room temperature for 24 h giving compound **6** in 46% purified yield. Alternatively,  $\alpha$ -aminophosphonate diethyl ester **1** was debenzylated with hydrogen gas in the presence of palladium hydroxide on carbon as catalyst in ethanol at room temperature in an overnight reaction (97% yield), followed by boron tribromide phosphonic ester hydrolysis in toluene (60°C, 36 h) to obtain final compound **4** in 72% yield after purification.

**Metallo- $\beta$ -lactamase inhibition.** To evaluate the MBL inhibitory potency of compounds **4–6**, their half-maximal inhibitory concentrations ( $IC_{50}$ s) against the VIM-1, VIM-2, NDM-1, IMP-1 and GIM-1 MBLs were measured (Table 1) using the fluorogenic substrate FC5.<sup>45</sup> VIM-1 was most potently inhibited by  $\alpha$ -aminophosphonate **5** with an  $IC_{50}$  of 36  $\mu$ M, with activity being lost upon a phenol to pyridine (**6**) substitution or debenzylation (**5**). In contrast, all three  $\alpha$ -aminophosphonates moderately inhibited VIM-2, suggesting that the phosphonic acid moiety is a critical structural motif for binding to VIM-2. The best VIM-2 inhibitory activity was shown by **5**, with an  $IC_{50}$  8.6  $\mu$ M, whereas debenzylation (**5**) or phenol to pyridine (**6**) substitution resulted in 17 to 25-fold decreases in activity, respectively. Despite the activity drop not being as severe as in the case of VIM-1, these observations suggest that the *ortho*-methoxy group and the amine attached benzyl moiety may be involved into interaction with the VIM-2 active site. The activity of **5** against both VIM-1 and VIM-2 makes it a promising lead towards novel inhibitors against VIM-variants.

Table 1

**Inhibitory activities ( $IC_{50}$ ) of the  $\alpha$ -aminophosphonic acids.**

The results reveal that the  $\alpha$ -aminophosphonic acids show promise for successful inhibition of the VIM-type MBLs. The enzymes were used at the following concentrations: VIM-1 at 500 pM, VIM-2 at 100 pM, NDM-1 at 50 pM, IMP-1 at 5 pM and GIM-1 at 1.25 nM.

| Inhibitor | $IC_{50}$ [ $\mu$ M] |       |        |        |       |
|-----------|----------------------|-------|--------|--------|-------|
|           | VIM-1                | VIM-2 | NDM-1  | IMP-1  | GIM-1 |
| <b>4</b>  | > 1000               | 145   | > 1000 | > 1000 | > 100 |
| <b>5</b>  | 36                   | 8.6   | > 1000 | 537    | > 100 |
| <b>6</b>  | > 1000               | 214   | > 1000 | 676    | > 100 |

None of the  $\alpha$ -aminophosphonates showed potent inhibition of NDM-1, IMP-1, or GIM-1. The weak inhibition of IMP-1 by **5** and **6** suggests that  $\alpha$ -aminophosphonates might have a potential to be optimized for IMP-1 inhibition. This is of significance as few potent IMP-1 inhibitors have been reported.

Phosphonate-type MBL inhibitors are under-explored with the few known examples having overall comparable activities to the compounds reported here ( $IC_{50}$  0.3 - >400  $\mu$ M). The reported compounds are, however, in contrast to **4–6**, not selective for specific MBLs.<sup>38</sup>

The  $\alpha$ -aminophosphonic acids **5–6** showed no cytotoxicity on HepG2 cells, indicating that they are a promising direction for further optimization into a clinically applicable inhibitor (**Supplementary Table S5**, Supplementary Information). The membrane permeability of compounds **5** and **6** was assessed using outer membrane vesicles (OMVs) containing VIM-2 or GIM-1 inside their lumen. These vesicles provide an *in situ* model of the bacterial outer membrane, preserving its native lipid and protein composition.<sup>40,46</sup> Enzyme activity within the vesicles was quantified measuring nitrocefin hydrolysis, with *L*-captopril serving as a positive control. The observed activities in intact OMVs were compared to those obtained following permeabilization of the OMVs with Triton X-100, as well as to measurements using purified VIM-2 and GIM-1.

It should be noted that these measurements used different substrate and assay conditions compared to the isolated protein assay, and hence the two measurements are not directly comparable. Nearly identical relative activities were obtained under all conditions, with no significant differences, indicating that **5** and **6** access the vesicle lumen without detectable restriction (Fig. 2a-b). This conclusion was supported by dose-response experiments measured at matched enzyme concentrations using OMVs containing VIM-2, as well as permeabilized OMVs and purified VIM-2. The resulting inhibition curves were superimposable across the full concentration range and yielded similar  $IC_{50}$  values, further supporting the proposal that both **5** and **6** readily traverse the bacterial membrane (Fig. 2c-d). Compound **5** is predicted to have favorable ADME features (**Supplementary Tables S11–13**, Supplementary

Information) including high aqueous solubility, low plasma protein binding and increased gastrointestinal absorption.

**Solution NMR binding studies.** The binding of the most potent VIM-2 inhibitor  $\alpha$ -aminophosphonic acid **5** was investigated with solution NMR. Protein-observed solution NMR titration enabled detection of the enzyme-ligand interaction and identification of the amino acids involved into binding under near-physiological conditions. Up to 10 molar equivalents of inhibitor **5** were titrated to the solution of the  $^{15}\text{N}$ -labeled enzyme recording a series of  $^1\text{H}$ ,  $^{15}\text{N}$  HSQC spectra. The weighted average of the  $^1\text{H}$  and  $^{15}\text{N}$  chemical shift changes of the VIM-2 backbone resonances were followed to pinpoint the amino acid residues that are affected by inhibitor binding (**Table S6**, Supporting Information). Residues most affected by the binding of the inhibitor were located in  $\beta$ -3 (residues 67Y-72L) and in loop-10 (196G-216L) (Fig. 3). The substantial chemical shift perturbations observed for residues in  $\beta$ -3 are most likely attributable to a  $\pi$ - $\pi$  interaction between Tyr67 and the inhibitor's *ortho*-hydroxyphenyl ring, with Tyr67 inducing local dynamic changes propagated along this secondary structural element. No significant chemical shift perturbations were observed for residues in loop-3 connecting  $\beta$ -2 and  $\beta$ -3. For compound **5**, the chemical shift perturbations expand over  $\beta$ -3, suggesting that the inhibitor structure is shifted towards this  $\beta$ -strand and the Zn2 site (Asp/Cys-site) of the binding pocket as compared to the previous phosphonic acid inhibitors that possessed an amide- instead of an amine-linker.<sup>40</sup> Extensive chemical shift perturbations in loop-10 indicate that the binding of inhibitor **5** is accommodated by this flexible loop region of VIM-2. Residues around the Zn2 coordination site (237I-242G) in loop-12 also showed notable chemical shift changes further pinpointing the location of inhibitor **5**.

**X-ray diffraction analysis of inhibitor 5 in complex with VIM-2.** VIM-2 crystallised in space group C2 with two molecules, in a *face-to-back* orientation, in each asymmetric unit (ASU), as reported.<sup>40</sup> The overall root mean square deviation (RMSD) between the two molecules in the asymmetric unit is 0.18 Å, indicating their high similarity. After soaking VIM-2 crystals with racemic **5**, they diffracted to  $\sim 1.4$  Å and allowed refinement of the structure to  $R_{\text{work}}$  and  $R_{\text{free}}$  values of 16.4% and 18.3%, respectively (**Supplementary Table S7**, Supplementary Information). The overall differences between the structure of VIM-2 in complex with **5** and apo-VIM-2 were minor, with RMSD 0.12 Å and 0.14 Å for chains A and B, respectively (**Supplementary Table S8**, Supplementary Information). After molecular replacement, strong positive difference electron density was observed in the active site of both subunits, indicating complex formation (Fig. 4).

In chain A, the quality of the electron density enabled modelling of only (*R*)-**5** with a real space correlation coefficient (RSCC) to the electron density of 0.94. Besides the electron density modelled with the inhibitor, additional electron density was detected close to the phosphonic group of the inhibitor and the zinc ions of the active site. This electron density could be fitted with the phosphonic acid group from an additional molecule of **5**, which was modelled as a phosphate ion, with other parts of the molecule being flexible. As observed in structural studies with VIM-2 and **5j** (Fig. 1),<sup>40</sup> this additional molecule could be

(S)-**5**; however, considering the 4.1 Å shift between the two phosphorous atoms, it could also reflect a second (R)-**5**. For readability, we henceforth refer to this second molecule as PO<sub>4</sub>-**5**. We note here that phosphate ions were neither added to the buffer during purification nor at crystallisation. As a control, we solved a structure of the apo-crystals fished from the same drop, and the non-soaked apo crystals did not show any 'phosphate' density in the same spot as observed for crystals soaked with **5**. In addition, we confirmed the absence of a phosphate ion in the sample by <sup>31</sup>P NMR, and ruled out the possibility that **5** would degrade to give a phosphate ion under the crystallographic conditions (**Supplementary Figure S20**, Supplementary Information).

In contrast, to chain A, where only the (R)-enantiomer alone was modelled, in chain B, the density of **5** was best fitted with a mixture of the (R)- and (S)-enantiomers of **5**, with an RSCC of 0.80 and 0.80, respectively (**Supplementary Table S9**, Supplementary Information). Overall, the observed densities suggest both enantiomers of **5** binding to the same chain B active site, as previously reported for phosphonic acid type inhibitor **5j** (PDB-ID 9F0S).<sup>40</sup> Again, residual positive difference density could be observed between the phosphonic acid group and the zinc-ions in active site B, however, this residual density could not be refined and was consequently left unfitted.

Although the overall difference between the apo and inhibitor bound state of VIM-2, as judged by the RMSD value, is small, some protein backbone shifts are seen around the inhibitor binding pocket. In chain A as well as chain B, residues Phe62 and Asp63 move by 0.5-1.0 Å to accommodate the inhibitor in the active site. In addition, in chain A, residues Gly209, Asn210 and Ala212 move by 0.5–0.6 Å (**Supplementary Table S10**, Supplementary Information). This additional shift may be explained by the presence of PO<sub>4</sub>-**5** on this site of the binding pocket.

Compound **5** binds to the active site of VIM-2 almost exclusively via its phosphonate group, which forms hydrogen bonds with the side chain of Arg205, the backbone amide proton of Asn210, and coordinates to the Zn<sup>2+</sup> ion of the active site. The phosphonate group of **5** is positioned to hydrogen bond to the side chain of His240 in the case of the (R)-enantiomer in

chain B (Fig. 5). The oxygen of the methylphenyl ether of **5** forms a weak hydrogen bond (2.9 and 3.2 Å) to the main-chain of Asn210 in subunit B, restricting its mobility. In active site A, this distance is 3.2 Å. The low inhibitory potency of **6** against VIM-1 and VIM-2 may be rationalized by the loss of hydrogen bonding to Asn210 when compared to the binding of **5** (**Supplementary Figure S18**, Supplementary Information). Similar amino acids are involved in the binding of **5** in the VIM-2 active site (Fig. 5) as observed for a previously published phosphonic acid-type inhibitor **5j**.<sup>40</sup> Clear exceptions are His114 and His116, which coordinate the second zinc ion in the previously determined structure of **5j**.<sup>40</sup> Neither of these is involved in the VIM-2 binding of **5** since the second zinc ion is not part of the inhibitor's coordination network. Arg205 and Gly209 interact with **5** but not with **5j**,<sup>40</sup> yet are involved in the binding of some analogues of **5j**.<sup>40</sup>

**Comparison to other phosphonic acid-type and boronate based inhibitors, and to hydrolysed  $\beta$ -lactam structures.** While the phosphonic acid group of **5j** (PDB-ID 9F0S) is situated between both zinc ions of the active site,<sup>40</sup> **5** coordinates only to one of the two zinc ions, Zn2, of the VIM-2 active site, in both chains A and B. This leads to a noticeable shift of the phosphonic group of **5**, relative to the phosphonic acid-type inhibitors described earlier. The phosphorus of the phosphonic group of **5** is shifted as compared to the structure with **5j**<sup>40</sup> by 3.5 Å in chain A, and by 2.6 Å in chain B, respectively (Fig. 6a). Thus, despite their overall similarity, the phosphonic acid group of **5** does not occupy the same position as that of the previously determined inhibitors, such as **5j**.<sup>40</sup> The position of the phosphorous atom of **5** differs by ~ 0.8 Å between chains A and chain B. We further observe that the benzyl group of **5** overlaps with the *ortho*-hydroxyphenyl core of previously determined phosphonate inhibitors,<sup>40</sup> whereas its *ortho*-hydroxyphenyl core with the thiophene ring of the previously published compounds. Hence, despite the dissimilar coordination of their phosphonate groups, the overall binding mode of **5** to VIM-2 resembles that of the previous inhibitors.

The recently disclosed 6-phosphonomethylpyridine-2-carboxylates (PMPCs) interact with the Zn2 of the B1 MBL binding site (IMP-1) via their carboxylate and pyridine nitrogen whereas their phosphonate group does neither interact with Zn1 or Zn2.<sup>47</sup> Interestingly, the phosphonate of PMPCs replaces Zn2 of the B3 MBL L1, forming a mono-zinc enzyme in which Zn(II) binding at the Zn1, but not the Zn2, site is observed. The binding mode of PMPCs is thus very different from that of **5** to the B1 MBL VIM-2. Similarly to **5**, heteroaryl phosphonate inhibitors also bind the Zn2 ion of VIM-2 (6NY7 and 6DD0),<sup>48</sup> however, these are somewhat less shifted towards Zn2 in the binding site (**Supplementary Figure S19**, **Supplementary Information**).

Structures of VIM-2 in complex with the faropenem (PDB-ID 7A60), and biapenem (PDB-ID 6Y6J), hydrolysis products of  $\beta$ -lactam antibiotics,<sup>49</sup> and with taniborbactam (PDB-ID 6SP7), a boronate-based inhibitor,<sup>35</sup> have been solved (Fig. 6b-d). The phosphonic acid of **5** occupies a similar position to the carboxylate group of the pyrroline ring of faropenem and biapenem (Fig. 6b-c). Interestingly, the PO<sub>4</sub>-**5** in chain A occupies the place of the carboxylate group of the opened  $\beta$ -lactam ring (Fig. 6d), suggesting that **5** mimics a product complex. This finding is corroborated by the comparison of the binding mode of **5** to that of taniborbactam. The phosphonate group of **5** is thus in a similar position to the carboxylate group of taniborbactam in both chains A and B, and the phosphonate of PO<sub>4</sub>-**5** in chain A occupies the same position as the boronate group.

The binding mode of **5** to VIM-2 is different from those of taniborbactam and of the previously reported phosphonic acid-based inhibitors, which resemble the tetrahedral transition state of  $\beta$ -lactam hydrolysis.<sup>35,40</sup> That of **5**, in contrast, on the one hand resembles the stereodynamic behaviour<sup>40</sup> of previous phosphonic acid-based inhibitors, as both enantiomers can bind to the active site of chain B. On the other hand, **5** interacts only with a single zinc ion, and rather resembles the “product complex” of hydrolysed antibiotics.

## Conclusion

Although phosphonic acids are established enzyme inhibitors, there has been little work on  $\alpha$ -aminophosphonic acids inhibitors, in particular of metallo-enzymes. We report the discovery of an  $\alpha$ -aminophosphonic acid-type VIM-2 inhibitor, widening the available chemical space for targeting MBLs. This new scaffold addresses challenges associated with previous inhibitor scaffolds, including effective penetration through the Gram-negative outer membrane, absence of detectable cytotoxicity, and a concise, modular synthetic route that will enable further efficient analogue development for structure–activity relationship exploration. The  $\alpha$ -aminophosphonic inhibitor presented herein displays a highly dynamic interaction within the active site, pointing to conformational flexibility and possible exchange between multiple binding modes playing an important role. Such a behavior highlights a non-classical inhibition mechanism that may differ from that of rigid, high-affinity chelators, which could be advantageous for modulating metallo- $\beta$ -lactamase activity while avoiding strong metal sequestration. The observation that the  $\alpha$ -aminophosphonic acids selectively inhibit the clinically relevant VIM subfamily MBLs is notable. Combined with previous results,<sup>50</sup> the work presented here shows the  $\alpha$ -aminophosphonic acid scaffold can be fine-tuned for the inhibition of different sets of MBLs. It will also be of interest to explore  $\alpha$ -aminophosphonic acid derivatives as inhibitors of human MBL fold enzymes, in particular nucleases involved in DNA damage repair, which are drug targets for cancer.<sup>51</sup> Overall, the results show the  $\alpha$ -aminophosphonic acid scaffold as a promising new hit for optimization, which will be enabled by the crystallographic and NMR studies provided here.

## Methods

**Chemistry.** The synthesis, work-up and purification procedures were performed with commercially available chemicals (Sigma-Aldrich, VWR, Merck, Fluorochem, Thermo Fisher Scientific, Solveco without further purification. Reactions and the analytical purity of the final compounds were monitored using LC-MS (Agilent 1260 Infinity II) machine equipped with an ESI-Q detector (Infinitylab LC-MSD) and an Agilent InfinityLab Poroshell 120 C18 column (2.7  $\mu$ m,  $\phi$  2.1 mm L 50 mm or 2.7  $\mu$ m,  $\phi$  4.6 mm L 50 mm) or a Kinetex C18 column (5  $\mu$ m, 100 Å,  $\phi$  3.0 mm L 150 mm). The final product purification was performed with preparative RP-HPLC (VWR LaPrep P110) with single wavelength detection (254 nm), using a Kinetex C18 column (5  $\mu$ m, F5, 100 Å, 250 x 21.2 mm) and gradients of CH<sub>3</sub>CN/H<sub>2</sub>O as the mobile phase at 10 mL/min flow rate. NMR spectra were recorded using a Varian Agilent MR400-DD2 400MHz spectrometer equipped with a OneNMR probe or on a Bruker Avance Neo 500 MHz spectrometer equipped with a cryogenic TXO probe. The chemical shifts are reported in ppm using the residual solvent signal as an indirect reference to TMS.

**Diethyl ((benzylamino)(2-methoxyphenyl)methyl)phosphonate (1).** <sup>40,52</sup> Compound **1** was synthesized according to the previously reported procedure<sup>40</sup> from 2-methoxybenzaldehyde (1.75 g, 12.8 mmol), diethyl phosphite (1.86 g, 13.5 mmol, 1.1 equiv.) and benzylamine (1.38 g, 12.8 mmol, 1.0 equiv.) in EtOH (13 mL) using HfCl<sub>4</sub> (82 mg, 0.26 mmol, 2 mol%) as the catalyst. The reaction was stirred at 60°C

overnight. The solvent was removed under reduced pressure and the crude product was purified by flash column chromatography (SiO<sub>2</sub>, gradient EtOAc in hexane, 20% to 100%). Compound **1** was obtained as a white solid (3.01 g, 8.3 mmol, 65%). MS ESI+ (*m/z*): 364.2 [M + H]<sup>+</sup> (364.16 calcd. for C<sub>19</sub>H<sub>27</sub>NO<sub>4</sub>P). <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD) δ 7.51 (dd, *J* = 7.6, 2.1 Hz, 1H, H-7), 7.32 (ddd, *J* = 7.9, 6.8, 2.1 Hz, 1H, H-5), 7.31–7.27 (m, 2H, H-11 and H-15), 7.25–7.20 (m, 3H, H-12, H-13 and H-14), 7.05–6.98 (m, 2H, H-6 and H-4), 4.58 (d, <sup>2</sup>*J*<sub>HP</sub> = 22.0 Hz, 1H, H-1), 4.10 (dq, *J* = 10.1, 7.1 Hz, 1H, OCH<sub>2</sub>), 3.90 (dq, *J* = 10.1, 7.1 Hz, 1H, OCH<sub>2</sub>), 3.80–3.72 (m, 4H, OCH<sub>2</sub> and OCH<sub>3</sub>), 3.75 (d, <sup>2</sup>*J*<sub>HH</sub> = 13.0 Hz, 1H, H-9a), 3.47 (d, <sup>2</sup>*J*<sub>HH</sub> = 13.0 Hz, 1H, H-9b), 1.28 (t, *J* = 7.1 Hz, 3H, CH<sub>3</sub>), 1.08 (t, *J* = 7.1 Hz, 3H, CH<sub>3</sub>). <sup>13</sup>C NMR (126 MHz, CD<sub>3</sub>OD) δ 159.4 (d, <sup>3</sup>*J*<sub>CP</sub> = 6.8 Hz, C-3), 140.6 (C-10), 130.3 (d, <sup>5</sup>*J*<sub>CP</sub> = 3.0 Hz, C-5), 129.9 (d, <sup>3</sup>*J*<sub>CP</sub> = 4.9 Hz, C-7), 129.7 (C-11 and C-15), 129.3 (C-12 and C-14), 128.2 (C-13), 124.9 (C-2), 121.8 (d, <sup>4</sup>*J*<sub>CP</sub> = 2.8 Hz, C-6), 111.9 (d, <sup>4</sup>*J*<sub>CP</sub> = 2.2 Hz, C-4), 64.3 (d, <sup>2</sup>*J*<sub>CP</sub> = 7.2 Hz, OCH<sub>2</sub>), 64.1 (d, <sup>2</sup>*J*<sub>CP</sub> = 7.2 Hz, OCH<sub>2</sub>), 56.0 (OCH<sub>3</sub>), 52.1 (d, <sup>1</sup>*J*<sub>CP</sub> = 158.4 Hz, C-1) 52.0 (d, <sup>3</sup>*J*<sub>CP</sub> = 17.2 Hz, C-9), 16.7 (d, <sup>3</sup>*J*<sub>CP</sub> = 5.9 Hz, CH<sub>3</sub>), 16.5 (d, <sup>3</sup>*J*<sub>CP</sub> = 5.9 Hz, CH<sub>3</sub>). <sup>31</sup>P NMR (162 MHz, CD<sub>3</sub>OD) δ 24.2.

**Diethyl ((benzylamino)(pyridin-2-yl)methyl)phosphonate (2).** <sup>53</sup> The synthetic procedure was adapted from that described previously.<sup>40</sup> Pyridine-2-carbaldehyde (3.55 mL, 37.3 mmol), benzylamine (4.16 mL, 38.1 mmol, 1.0 equiv.) and diethyl phosphite (5.20 mL, 39.6 mmol, 1.1 equiv.) were dissolved in absolute EtOH (32 mL). HfCl<sub>4</sub> (82 mg, 0.7 mmol, 2 mol%) was added in one portion and the reaction was stirred at 60°C for 48 h. The solvent was removed under reduced pressure and the crude product was purified by manual column chromatography (SiO<sub>2</sub>, 80% EtOAc 20% hexane), *R*<sub>f</sub> (EtOAc:Hex 6:1) = 0.35. The product **2** was obtained as an orange oil (8.05 g, 24.1 mmol, 65%). MS ESI+ (*m/z*): 335.2 [M + H]<sup>+</sup> (335.15 calcd for C<sub>17</sub>H<sub>24</sub>N<sub>2</sub>O<sub>3</sub>P). <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD) δ 8.55 (ddd, *J* = 5.0, 1.7, 0.9 Hz, 1H, H-4), 7.84 (ddd, *J* = 7.8, 7.3, 1.7 Hz, 1H, H-6), 7.55 (ddd, *J* = 7.8, 2.0, 0.9 Hz, 1H, H-7), 7.36 (ddd, *J* = 7.3, 5.0, 2.0 Hz, 1H, H-5), 7.31–7.21 (m, 5H, H-11, H-12, H-13, H-14 and H-15), 4.23 (d, <sup>2</sup>*J*<sub>HP</sub> = 22.2 Hz, 1H, H-1), 4.14 (m, 2H, OCH<sub>2</sub>), 4.01 (dq, *J* = 10.2, 7.0 Hz, 1H, OCH<sub>2</sub>), 3.90 (dq, *J* = 10.2, 7.0 Hz, 1H, OCH<sub>2</sub>), 3.79 (d, <sup>2</sup>*J*<sub>HH</sub> = 13.2 Hz, 1H, H-9a), 3.55 (d, <sup>2</sup>*J*<sub>HH</sub> = 13.2 Hz, 1H, H-9b), 1.29 (td, *J* = 7.0, 0.6 (<sup>4</sup>*J*<sub>HP</sub>) Hz, 3H, CH<sub>3</sub>), 1.15 (td, *J* = 7.0, 0.6 (<sup>4</sup>*J*<sub>HP</sub>) Hz, 3H, CH<sub>3</sub>). <sup>13</sup>C NMR (101 MHz, CD<sub>3</sub>OD) δ 157.1 (d, <sup>2</sup>*J*<sub>CP</sub> = 1.3 Hz, C-2), 150.1 (d, <sup>4</sup>*J*<sub>CP</sub> = 2.3 Hz, C-4), 140.4 (C-10), 138.4 (d, <sup>4</sup>*J*<sub>CP</sub> = 2.3 Hz, C-6), 129.6 (C-11 and C-15 or C-12 and C-14), 129.4 (C-11 and C-15 or C-12 and C-14), 128.3 (C-13), 125.4 (d, <sup>3</sup>*J*<sub>CP</sub> = 4.9 Hz, C-7), 124.4 (d, <sup>5</sup>*J*<sub>CP</sub> = 3.0 Hz, C-5), 64.8 (d, <sup>2</sup>*J*<sub>CP</sub> = 7.2 Hz, OCH<sub>2</sub>), 64.3 (d, <sup>2</sup>*J*<sub>CP</sub> = 7.2 Hz, OCH<sub>2</sub>), 61.7 (d, <sup>1</sup>*J*<sub>CP</sub> = 154.3 Hz, C-1), 52.7 (d, <sup>3</sup>*J*<sub>CP</sub> = 17.2 Hz, C-9), 16.7 (d, <sup>3</sup>*J*<sub>CP</sub> = 5.8 Hz, CH<sub>3</sub>), 16.6 (d, <sup>3</sup>*J*<sub>CP</sub> = 5.8 Hz, CH<sub>3</sub>). <sup>31</sup>P NMR (162 MHz, CD<sub>3</sub>OD) δ 22.3.

**Diethyl (amino(2-methoxyphenyl)methyl)phosphonate (3).** <sup>40</sup> Compound **3** was synthesized according to the reported procedure<sup>40</sup> from compound **1** (2.80 g, 7.7 mmol) and Pd(OH)<sub>2</sub>/C, (20% w/w, nominally 50% water, 1.64 g, 1.2 mmol, 0.15 equiv.) and H<sub>2</sub> (atm. pressure) in EtOH (200 mL). The reaction was run at r.t. overnight, filtered through a syringe filter and the solvent was removed under reduced pressure.

The product was obtained as a colorless liquid (2.03 g, 7.4 mmol, 97%). MS ESI+ ( $m/z$ ): 274.2  $[M + H]^+$  (274.12 calcd for  $C_{12}H_{21}NO_4P$ ).  $^1H$  NMR (400 MHz,  $CD_3OD$ )  $\delta$  7.47 (dd,  $J = 7.7, 1.2$  Hz, 1H, H-7), 7.30 (ddd,  $J = 8.3, 7.4, 1.9$  Hz, 1H, H-5), 7.00 (d,  $J = 8.4$  Hz, 1H, H-4), 6.99 (dd,  $J = 7.6, 7.6$  Hz, 1H, H-6), 4.73 (d,  $^2J_{HP} = 18.0$  Hz, 1H, H-1), 4.10 (dq,  $J = 10.1, 7.1$  Hz, 2H,  $OCH_2$ ), 3.94 (dq,  $J = 10.1, 7.1$  Hz, 1H,  $OCH_2$ ), 3.86 (s, 3H,  $OCH_3$ ), 3.83 (m, 1H,  $OCH_2$ ), 1.31 (t,  $J = 7.1$  Hz, 3H,  $CH_3$ ), 1.15 (t,  $J = 7.1$  Hz, 3H,  $CH_3$ ).  $^{13}C$  NMR (126 MHz,  $CD_3OD$ )  $\delta$  158.6 (d,  $^3J_{CP} = 6.2$  Hz, C-3), 131.8 (d,  $^5J_{CP} = 2.9$  Hz, C-5), 130.1 (d,  $^3J_{CP} = 4.7$  Hz, C-7), 130.0 (C-2), 122.0 (d,  $^4J_{CP} = 2.4$  Hz, C-6), 112.4 (d,  $^4J_{CP} = 2.1$  Hz, C-4), 65.2 (d,  $^2J_{CP} = 7.1$  Hz,  $OCH_2$ ), 64.9 (d,  $^2J_{CP} = 7.2$  Hz,  $OCH_2$ ), 56.3 ( $OCH_3$ ), 47.0 (d,  $^1J_{CP} = 155.1$  Hz, C-1), 16.7 (d,  $^3J_{CP} = 5.5$  Hz,  $CH_3$ ), 16.5 (d,  $^3J_{CP} = 5.6$  Hz,  $CH_3$ ).  $^{31}P$  NMR (162 MHz,  $CD_3OD$ )  $\delta$  25.9.

**General procedure A for synthesis of compounds 4–6.** The general synthetic procedure for preparation of 4–6 followed our reported protocol.<sup>40</sup>  $BBr_3$  (5 equiv., 1M in DCM) was added dropwise to the solution of compound 1–3 in dry toluene or DCM at  $-78^\circ C$  and the reaction mixture was stirred for 1 h. The mixture was allowed to warm to r.t. and was stirred for 24 - 48 h either at r.t. or at  $60^\circ C$ . An excess of MeOH was added at r.t. to the reaction mixture and stirred for 30 min. The solvents were removed under reduced pressure. The crude product was washed with toluene (3x) and was purified by preparative HPLC (C18 column, MeCN:H<sub>2</sub>O).

**(Amino(2-hydroxyphenyl)methyl)phosphonic acid (4).**<sup>54</sup> The synthesis was performed according to general procedure A for 36 h, compound 3 (80 mg, 0.29 mmol, in 10 mL toluene) gave the product 4 as an orange solid (43 mg, 0.21 mmol, 72%). MS ESI- ( $m/z$ ): 201.9  $[M-H]^-$  (202.03 calcd for  $C_7H_9NO_4P$ ).  $^1H$  NMR (500 MHz,  $D_2O$ )  $\delta$  7.36 (dd,  $J = 7.7, 1.7$  Hz, 1H, H-7), 7.28 (ddd,  $J = 7.8, 7.5, 1.7$  Hz, 1H, H-5), 6.98 (ddd,  $J = 7.7, 7.5, 1.2$  Hz, 1H, H-6), 6.94 (dd,  $J = 7.8, 1.2$  Hz, 1H, H-4), 4.68 (d,  $^2J_{HP} = 16.7$  Hz, 1H, H-1).  $^{13}C$  NMR (126 MHz,  $D_2O$ )  $\delta$  154.0 (d,  $^3J_{CP} = 5.1$  Hz, C-3), 130.2 (d,  $^5J_{CP} = 2.2$  Hz, C-5), 129.3 (d,  $^3J_{CP} = 4.6$  Hz, C-7), 120.6 (d,  $^4J_{CP} = 1.8$  Hz, C-6), 119.2 (d,  $^2J_{CP} = 4.3$  Hz, C-2), 116.4 (d,  $^4J_{CP} = 1.7$  Hz, C-4), 49.3 (d,  $^1J_{CP} = 141.0$  Hz, C-1).  $^{31}P$  NMR (162 MHz,  $D_2O$ )  $\delta$  11.0.

**((Benzylamino)(2-methoxyphenyl)methyl)phosphonic acid (5).**<sup>52</sup> The synthesis was performed according to general procedure A for 48 h, compound 1 (48 mg, 0.14 mmol, in 5 mL toluene) gave the product 5 as a white solid (24 mg, 0.08 mmol, 55%). MS ESI+ ( $m/z$ ): 308.7  $[M + H]^+$  (308.11 calcd for  $C_{15}H_{19}NO_4P$ ).  $^1H$  NMR (500 MHz,  $D_2O$ )  $\delta$  7.47–7.38 (m, 4H, H-5, H-12, H-13 and H-14), 7.36 (dd,  $J = 7.7, 1.8$  Hz, 1H, H-7), 7.29 (dd,  $J = 6.9, 2.7$  Hz, 2H, H-11 and H-15), 7.07 (d,  $J = 7.5$  Hz, 1H, H-4), 7.06 (dd,  $J = 8.0, 7.7$  Hz, 1H, H-6), 4.59 (d,  $^2J_{HP} = 17.0$  Hz, 1H, H-1), 4.22 (d,  $^2J_{HH} = 13.3$  Hz, 1H, H-9a), 4.13 (d,  $^2J_{HH} = 13.3$  Hz, 1H, H-9b), 3.79 (s, 3H,  $OCH_3$ ).  $^{13}C$  NMR (126 MHz,  $D_2O$ )  $\delta$  157.2 (d,  $^3J_{CP} = 4.8$  Hz, C-3), 130.7 (d,  $^5J_{CP} = 2.2$  Hz, C-5), 130.1 (C-10), 130.0 (C-11 and C-15), 129.9 (d,  $^3J_{CP} = 3.4$  Hz, C-7), 129.6 (C-13), 129.0 (C-12 and C-14), 121.1 (d,  $^4J_{CP} = 2.0$  Hz, C-6), 118.3 (d,  $^2J_{CP} = 4.6$  Hz, C-2), 111.7 (d,  $^4J_{CP} = 1.8$  Hz, C-4), 55.7 (d,  $^1J_{CP} = 135.6$  Hz, C-1), 55.4 ( $OCH_3$ ), 50.3 (d,  $^3J_{CP} = 6.6$  Hz, C-9).  $^{31}P$  NMR (162 MHz,  $D_2O$ )  $\delta$  9.1.

**((Benzylamino)(pyridin-2-yl)methyl)phosphonic acid (6).**<sup>55,56</sup> The synthesis was performed according to general procedure A for 24 h, compound **2** (85 mg, 0.25 mmol, in 10 mL DCM) gave the product **6** as a white solid (33 mg, 0.12 mmol, 46%). MS ESI +  $m/z$  279.3  $[M + H]^+$  (279.09 calcd for  $C_{13}H_{16}N_2O_3P$ ).  $^1H$  NMR (400 MHz,  $D_2O$ )  $\delta$  8.56 (d,  $J = 4.9$  Hz, 1H, H-4), 7.85 (dd,  $J = 7.8, 7.8$  Hz, 1H, H-6), 7.44–7.37 (m, 5H, H-5, H-7, H-12, H-13 and H-14), 7.37–7.32 (m, 2H, H-11 and H-15), 4.48 (d,  $^2J_{HP} = 16.6$  Hz, 1H, H-1), 4.29 (d,  $^2J_{HH} = 13.2$  Hz, 1H, H-9a), 4.25 (d,  $^2J_{HH} = 13.2$  Hz, 1H, H-9b).  $^{13}C$  NMR (126 MHz,  $D_2O$ )  $\delta$  150.2 (d,  $^2J_{CP} = 5.5$  Hz, C-2), 148.8 (d,  $^4J_{CP} = 2.1$  Hz, C-4), 138.2 (d,  $^4J_{CP} = 1.9$  Hz, C-6), 130.2 (C-10), 130.0 (C-11 and C-15), 129.6 (C-13), 129.1 (C-12 and C-14), 124.3 (d,  $^3J_{CP} = 3.6$  Hz, C-7), 123.9 (d,  $^5J_{CP} = 2.4$  Hz, C-5), 59.9 (d,  $^1J_{CP} = 129.2$  Hz, C-1), 50.9 (d,  $^3J_{CP} = 6.5$  Hz, C-9).  $^{31}P$  NMR (162 MHz,  $D_2O$ )  $\delta$  7.6.

**Enzymatic metallo- $\beta$ -lactamase assays.** The  $pI_{C_{50}}$  values of VIM-1, VIM-2, NDM-1 and IMP-1 were measured according to the reported method using substrate FC5.<sup>45</sup> To apply the previously reported fluorescence-based assay<sup>45</sup> to GIM-1, a Michaelis–Menten constant ( $K_M$ ) of 14  $\mu M$  was determined for the fluorogenic substrate FC5. Inhibition assays ( $pI_{C_{50}}$ ) for compounds **4–6** against GIM-1 were performed in a final reaction volume of 25  $\mu L$  using 28  $\mu M$  FC5, corresponding to saturating substrate conditions. Inhibitors were tested over a concentration range of 1  $\mu M$  to 1 mM and dispensed into non-binding black 384-well plates using a CyBio liquid handling system. Compounds were incubated with GIM-1 at a final concentration of 1.25 nM, prepared from a 2X enzyme stock in buffer A (50 mM HEPES, pH 7.0, 100  $\mu M$   $ZnCl_2$ , 0.01% Triton X-100, 1  $\mu g \cdot mL^{-1}$  BSA). Enzyme solution was added to all the wells containing compound and DMSO (positive controls) using an INTEGRA WELLJET. Buffer alone was added to negative control wells. Following a 10-min pre-incubation, FC5 (28  $\mu M$ ) was added to initiate the reaction.

Fluorescence was monitored using a BMG Labtech CLARIOstar or PHERAstar plate reader. Data analysis was performed using MARS software by defining positive and negative controls and selecting cycles corresponding to the linear reaction phase to calculate initial reaction velocities. Slopes ( $RFU \cdot min^{-1}$ ) were determined using the built-in slope calculation wizard and normalized by subtracting the negative control signal and scaling relative to the positive control. Dose–response curves were generated by fitting normalized data to a nonlinear sigmoidal inhibition model using GraphPad Prism to derive  $pI_{C_{50}}$  values.

**Recombinant protein production and purification.** VIM-1, VIM-2, NDM-1 and IMP-1 used for the  $pI_{C_{50}}$  measurements were expressed as previously described.<sup>45,47</sup> Production of His-tagged GIM-1 was performed in 9 x 1L LB medium at 25°C, 250 rpm in TunAir cultivation flasks. At  $OD_{600} = 0.44$ , the temperature was lowered to 18°C, and at  $OD_{600} = 0.91$ , Isopropyl- $\beta$ -D-1-thiogalaktopyranosid (IPTG) was added at a final concentration of 0.4 mM. Expression was allowed to continue for 19 h before the cells were harvested. After preparation of soluble extract from the pellet, using French press followed by ultracentrifugation, GIM-1 was purified on two serially connected 5 ml HisTrap HP columns. Peak fractions were pooled and then the protein sample was digested with tobacco etch virus (TEV) protease.

The TEV and tag were then removed by reverse IMAC. The flow through and wash fractions were collected, concentrated and further purified on a HiLoad 26/600 75pg gel filtration column in 20 mM HEPES, 0.1 mM ZnCl<sub>2</sub>, pH 7.0. The expression and purification yielded 143 mg GIM-1 batch 2. The final purity was estimated to be > 90% using SDS-PAGE analysis (**Figure S17**, Supporting Information).

**MTT cytotoxicity assays.** Cytotoxicity was evaluated using the MTT colorimetric assay originally described by Mossman,<sup>57</sup> using the Colorimetric (MTT) Kit for Cell Survival and Proliferation (Merck/Millipore, Darmstadt, Germany). HepG2 cells (human hepatocellular carcinoma cell line) were seeded into 96-well plates and allowed to adhere overnight in culture medium lacking penicillin/streptomycin. The following day, cells were exposed to the compounds at the indicated concentrations for 24 h. After the incubation period, the MTT reagent was added to each well and the plates were incubated at 37°C for another 3 h to allow viable cells to convert MTT into insoluble formazan crystals. Subsequently, the culture medium was carefully aspirated, and the crystals were dissolved in acidified isopropanol (0.1 mL of isopropanol containing 0.04 M HCl). Absorbance was measured using a multiscan spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA) at a test wavelength of 530 nm and a reference wavelength of 630 nm. Cell viability was calculated as a percentage relative to untreated control cells. Half-maximal inhibitory concentration (CC<sub>50</sub>) values were calculated using GraphPad Prism software (version 9.3.1) (**Table S5**, Supplementary Information).

**Membrane permeability.** OMVs carrying active VIM-2 or GIM-1 inside their lumen were prepared as described.<sup>40,46</sup> *E. coli* strain BL21(DE3)ΔompA<sup>58</sup> carrying plasmid pY326 or pY340 were grown at 37°C in LB media supplemented with 10 μM ZnCl<sub>2</sub> and 50 μg/mL kanamycin. When cultures reached an optical density (600 nm) of 0.4 β-lactamase production was induced by addition of 10 μM IPTG and growth was maintained for 2.5 h. Cells were removed by centrifugation at 10'000 xg for 10 min and cleared supernatants were filtered using 0.45 μm bottle top filters. OMVs were collected by centrifugation at 38'400 xg for 2 h, washed in buffer (Dulbecco's PBS, pH 7.4, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 136.9 mM NaCl, 2.7 mM KCl, supplemented with 0.9 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, and 10 μM ZnSO<sub>4</sub>), filtered using 0.45 μm syringe filters, recollected by centrifugation at 74 500 xg for 45 min, then resuspended in buffer. The total protein concentration was determined spectroscopically using spectra corrected for the light scattering contribution of the OMVs and assuming 1 Abs = 1 mg/ml as described.<sup>46</sup>

VIM-2 and GIM-1 were recombinantly produced and purified following standard protocols. *E. coli* strain BL21(DE3) carrying plasmid pSN02 or pSN03 were grown at 37°C in LB media supplemented with 10 μM ZnSO<sub>4</sub> and 50 μg/mL kanamycin. When cultures reached an optical density at 600 nm of ~ 0.4, the temperature was reduced to 20°C, after 1 h protein production was induced by addition of 10 μM IPTG and cultures were grown overnight at 20°C. Cells were harvested by centrifugation at 5,000 xg for 12 min and cell pellets were resuspended in 20 mL buffer (Dulbecco's PBS, pH 7.4, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 136.9 mM NaCl, 2.7 mM KCl, supplemented with 0.9 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, and 10 μM ZnSO<sub>4</sub>) supplemented with lysozyme and 1 tablet of EDTA-free protease inhibitor (Roche). Cells were disrupted by sonication, DNaseI (Thermo Fisher) was added, and cell debris was removed by

centrifugation at 75,000 xg for 30 min. The cleared lysate was bound to 1 ml Ni-NTA resin (Thermo Fisher) washed with buffer containing 10 mM imidazole and eluted with buffer containing 400 mM imidazole. Eluates were concentrated and further purified by size-exclusion chromatography using a Superdex 200 Increase column (Cytiva) and stored at -80°C until further use.

The activity of soluble  $\beta$ -lactamases as well as  $\beta$ -lactamases encapsulated in OMVs in presence of the compounds, Captopril (Sigma Aldrich), or buffer was determined by monitoring the hydrolysis of nitrocefin in clear flat bottom half area 96 well microplates (Corning) by measuring the increase in absorbance at 486 nm on an Infinite 200 Pro plate reader (Tecan) for 5 minutes. The final concentrations of the  $\beta$ -lactamases was adjusted to 1 nM for VIM-2 and 2 nM for GIM-1 and BSA was added to a final concentration of 400  $\mu$ g/mL. Concentrations of the OMV suspensions were adjusted to match those of the soluble enzymes by gauging the hydrolysis rate of OMVs suspensions solubilized with 0.1% (v/v) Triton X-100. 50  $\mu$ L of enzyme or OMV suspension were incubated with 1  $\mu$ L of compound (10 mM in 10% DMSO, unless otherwise indicated) for 5 min prior to the addition of 50  $\mu$ L nitrocefin solution (20  $\mu$ g/mL in buffer). For each compound the relative activity was determined based on the hydrolysis rate of the first 5 min of the reaction as  $(\partial \text{Abs}/\partial t)_{\text{compound}}/(\partial \text{Abs}/\partial t)_{\text{buffer}}$ . To determine the relative  $\beta$ -lactamase activity in absence of the bacterial membrane, OMVs were permeabilized by 15-fold dilution in buffer containing 0.1% (v/v) Triton X-100. For each condition three independent replicates were measured.

**NMR titrations.** NMR titration experiments were performed as previously described.<sup>40</sup> The spectra were recorded on a Bruker Avance Neo 600 MHz spectrometer equipped with a 5 mm TCI cryogenic probe at 25°C. Samples of inhibitor **5** and VIM-2 were prepared in buffer consisting of 20 mM KPO<sub>4</sub> buffer at pH 7.2, 100  $\mu$ M ZnSO<sub>4</sub> and 15% (v/v) D<sub>2</sub>O. The final protein concentration was 250  $\mu$ M. Spectra were measured using solvent suppression, 1024 points in f1, 16 transients, 512 increments in f2 and a relaxation delay of 0.2 s. The titration of compound **5** was carried out with 0, 0.50, 1.00, 1.50, 2.00, 2.50, 3.50, 4.50, 6.00, 10.0 molar equivalents of the inhibitor. The uniformly <sup>15</sup>N-labelled VIM-2 used for the NMR titration was expressed and purified as previously described.<sup>40,59</sup>

**Protein production and purification for X-ray diffraction.** Expression and purification were carried out as previously described.<sup>60</sup> In brief, the pDEST14\_VIM-2 plasmid,<sup>60</sup> containing the VIM-2 gene sequence for residues Val25-Glu300 with a N-terminal His<sub>6</sub>-tag cleavable by TEV protease, was transformed into chemically competent *Escherichia coli* BL21 (DE3). Bacteria were grown at 37°C in LB medium supplemented with 100  $\mu$ g/mL ampicillin. At an OD<sub>600</sub> of 1.0 protein expression was induced by addition of 0.4 mM IPTG and the cells were subsequently incubated at 20°C overnight. After harvesting the cells by centrifugation at 5,500 xg for 10 min, they were resuspended in lysis buffer (50 mM Tris/HCl, pH 7.2, 150 mM NaCl, 100  $\mu$ M ZnCl<sub>2</sub>) and finally lysed by sonication. The lysates were applied onto a 5 mL HisTrap FF column (Cytiva). After washing the column with washing buffer (50 mM Tris/HCl, pH 7.2, 150 mM NaCl, 100  $\mu$ M ZnCl<sub>2</sub>, 25 mM imidazole) the protein was eluted with a linear gradient from 25–500 mM imidazole (50 mM Tris/HCl, pH 7.2, 150 mM NaCl, 100  $\mu$ M ZnCl<sub>2</sub>, 25–500 mM imidazole). VIM-2

containing fractions were pooled and TEV protease was added (1:15 molar ratio) to cleave the His<sub>6</sub>-tag during overnight dialysis against lysis buffer. After removal of the His<sub>6</sub>-tag, uncleaved protein and the His-tagged TEV protease by reverse nickel-affinity chromatography, the buffer was exchanged to IEX A buffer (50 mM Tris/HCl, pH 7.2, 100 µM ZnCl<sub>2</sub>) using a 10 kDa cut-off centrifugal concentrator (Vivaspin, Sartorius). The sample was applied to a 5 mL HiTrap Q FF 5 mL column (Cytiva), washed with IEX A buffer, and eluted with a linear gradient of IEX buffer B (50 mM Tris/HCl, pH 7.2, 1M NaCl, 100 µM ZnCl<sub>2</sub>) from 0–100%. Purified VIM-2 was concentrated to 10.2 mg/mL using a 10 kDa cut-off centrifugal concentrator (Vivaspin, Sartorius), and finally flash-frozen in liquid nitrogen and stored at -80°C, prior to crystallization.

**VIM-2 crystallisation and inhibitor soaking for X-ray diffraction.** Crystals were grown as previously described.<sup>60</sup> For sitting-drop crystallisation (MRC maxi 48-well plates, Swissci), VIM-2 protein solution (10.2 mg/mL in 50 mM Tris/HCl, pH 7.2, 235 mM NaCl, 100 µM ZnCl<sub>2</sub>) was mixed in a 1:1 ratio with precipitant solution (27% (w/v) polyethylene glycol (PEG) 3350, 0.1 M magnesium formate) and equilibrated against 200 µL of the precipitant solution in the reservoir at 20°C. Native VIM-2 crystals were crushed, diluted in 50 µL precipitant solution, and vortexed with glass beads for 6-times 30 s to generate a seed stock for streak seeding. With seeding crystals grew after 1 day. The inhibitor was dissolved in cryoprotectant (25% (w/v) PEG3350, 0.2 M MgCl<sub>2</sub>, 50 mM HEPES pH 7.2, 15% (v/v) ethylene glycol) at a final concentration of 20 mM. For soaking experiments, crystals were placed into cryoprotectant containing 20 mM of the inhibitor for 4 h. After soaking the crystals were manually flash frozen in liquid nitrogen.

**X-ray data collection and processing.** X-ray data were collected at 100 K on beamline P13 (EMBL-Hamburg, Germany) using an Eiger 16 M detector (Dectris, Switzerland). RADDPOSE3D<sup>61</sup> was used to calculate the dose absorbed by the crystal. Data processing was performed using XDS<sup>62–64</sup> and AutoPROC with StarAniso.<sup>65,66</sup> The phase problem was solved by molecular replacement with phaser,<sup>67</sup> using a previously determined VIM-2 structure as search model (PDB-ID: 5YD7). The structure was refined using several rounds of *phenix.refine*<sup>68</sup> and manual model building with COOT,<sup>69</sup> *Phenix\_elbow*<sup>68</sup> was used to generate the geometry restraints for the inhibitor and POLDER-OMIT maps were generated in *phenix.polder*<sup>68</sup>. Figures were generated using PyMol (Schrödinger LLC) and LigPlot+.<sup>70</sup> Statistics for data collection and refinement as well as the Protein Data Bank (PDB) accession codes are shown in **Table S7**, Supporting Information.

## Declarations

## Competing interests

The authors declare no competing interests.

# Funding

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## Author contributions

K.V.G. planned the project, the design and the synthesis. K.V.G. synthesized and purified the final compounds **4–5**, analyzed and characterized all compounds, and did the NMR binding studies including analysis. M.S. synthesized compound **6**. A.A.R. and K.V.G. expressed and purified GIM-1 and <sup>15</sup>N-labelled VIM-2. G.K. and K.C.T. performed the *IC*<sub>50</sub> enzymatic assays. Z.P., V.T. and M.Cs. measured the cytotoxicity. S.N. and J.T. carried out the membrane permeability studies. K.B. purified and soaked VIM-2 with the inhibitor, collected the data, solved the structures and prepared the figures. K.B., E.C.S. and C.J.S. analyzed the data. M.E. conceived and supervised the project. All authors contributed to the writing.

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## Data availability.

The datasets generated and/or analysed during the current study (FIDs, crystallographic CIF files) are available in the Zenodo repository, DOI:10.5281/zenodo.19502937. The atomic coordinates and experimental data for the X-ray structure of the co-crystal of VIM-2 with **5** (PDB-ID: 29GW) have been deposited to the Protein Data Bank ([www.pdb.org](http://www.pdb.org)). Synthetic procedures, NMR spectra and analytical data for the synthesized compounds details of the X-ray crystallographic analyses and the cytotoxicity, enzyme inhibition, membrane permeability and bacterial assays are given as Supplementary Information.

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## Scheme 1

Scheme 1 is available in the Supplementary Files section.

## Figures

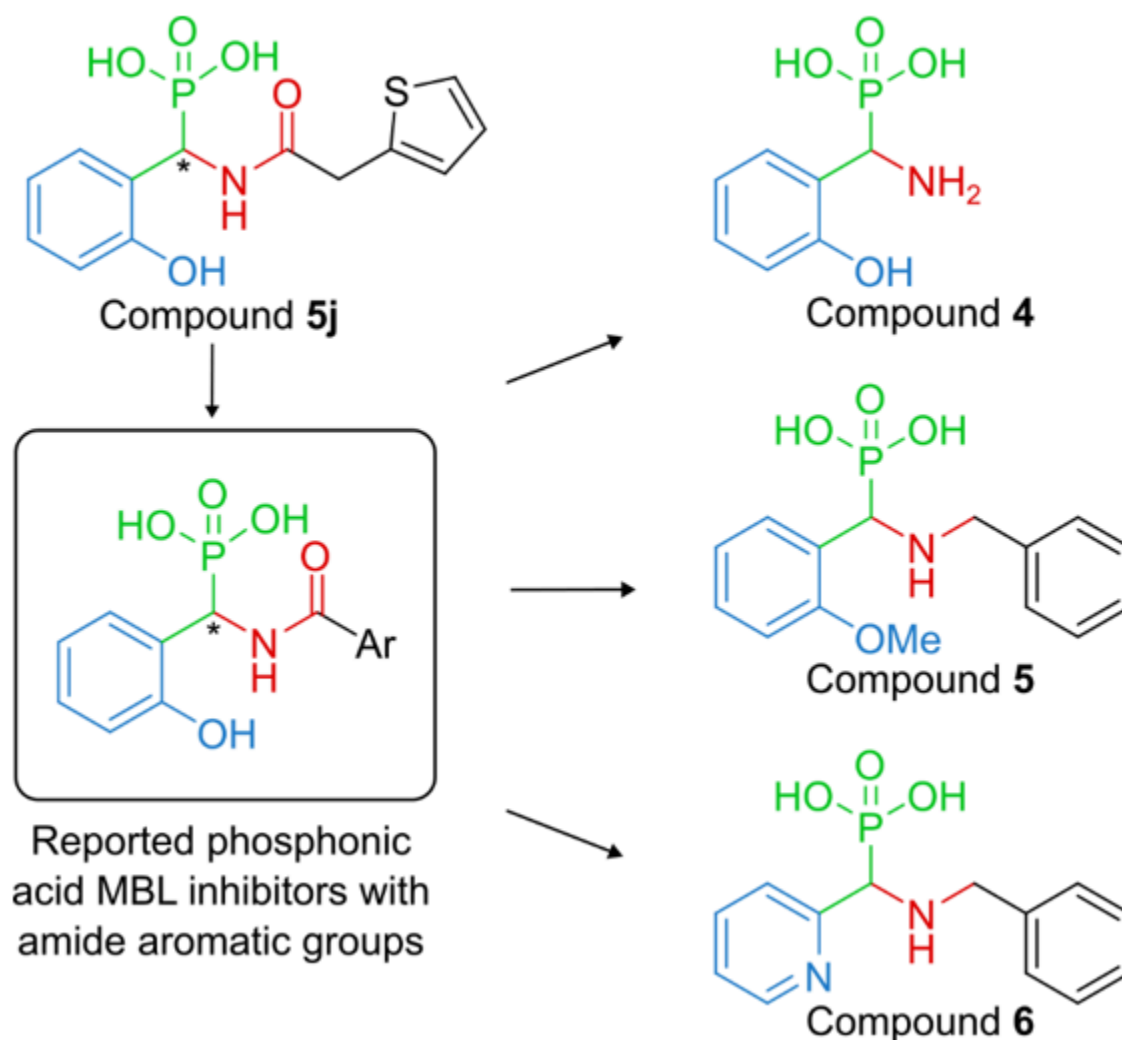


Figure 1

**$\alpha$ -Aminophosphonic acids are analogues of reported phosphonate-based metallo- $\beta$ -lactamase inhibitors<sup>40</sup> (boxed).** The importance of a benzyl substituent (**4**), amide to amine substitution (**5**), and a phenol group (**6**) was investigated.

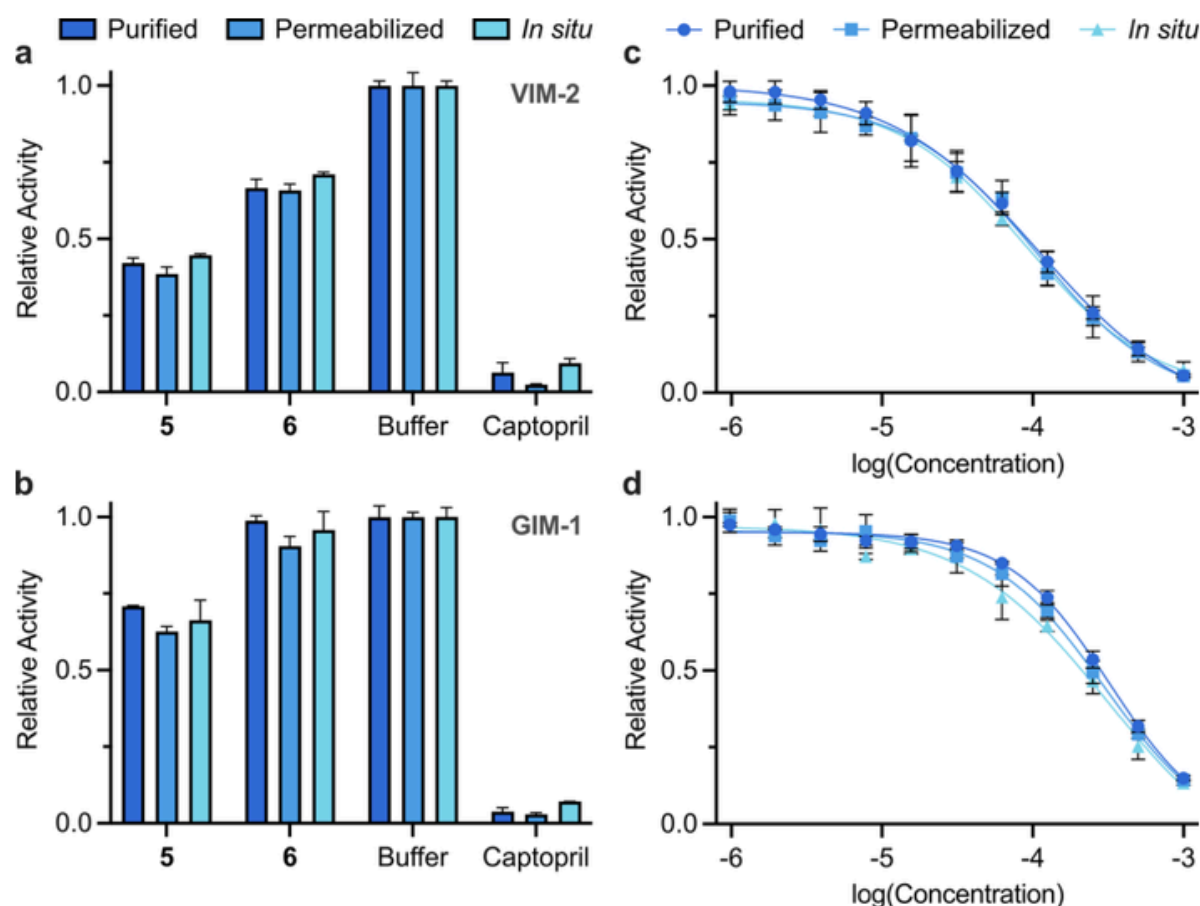


**Figure 2**

**Membrane permeability of compounds 5 and 6.** Relative activity of VIM-2 (a) and GIM-1 (b) encapsulated in outer membrane vesicles (*in situ*), after permeabilization, and as purified enzymes, measured in the absence (buffer) and presence of inhibitors. Error bars represent the standard deviation of three independent measurements. Compounds were tested at a final concentration of 100  $\mu$ M. Dose-response curves for compound **5** (c) and compound **6** (d) obtained using VIM-2 in outer membrane vesicles (*in situ*), permeabilized vesicles, and as purified enzyme, showing relative activity as a function of inhibitor concentration.

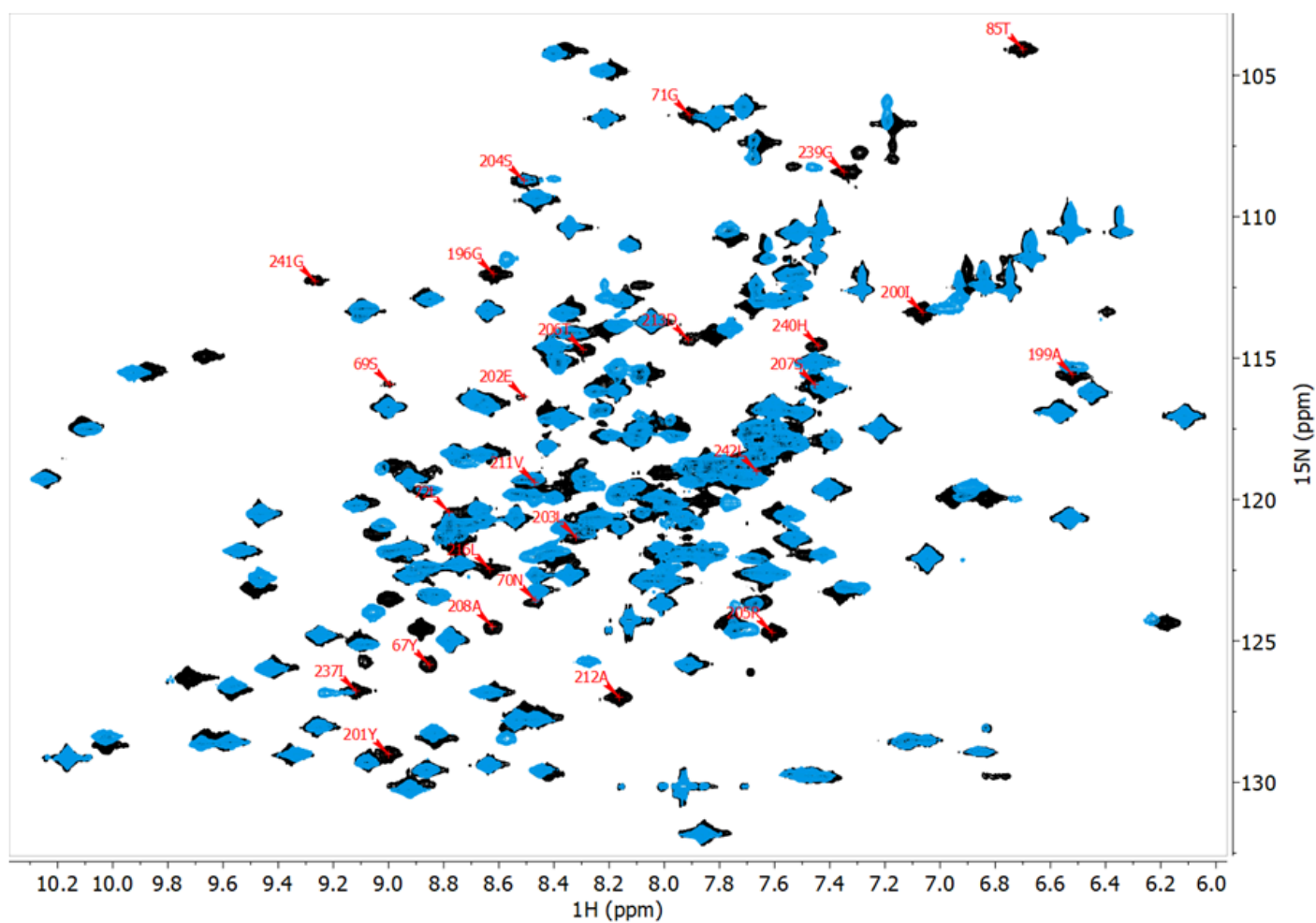


**Figure 3**

**Superimposition of the  $^1\text{H}$ ,  $^{15}\text{N}$  HSQC spectra (600 MHz, pH 7.0, 25 °C) of VIM-2 in the absence (black) and in the presence of 10 molar equivalent of compound 5 (blue). Amino acid residues showing significant chemical shift perturbations are labelled with red.**

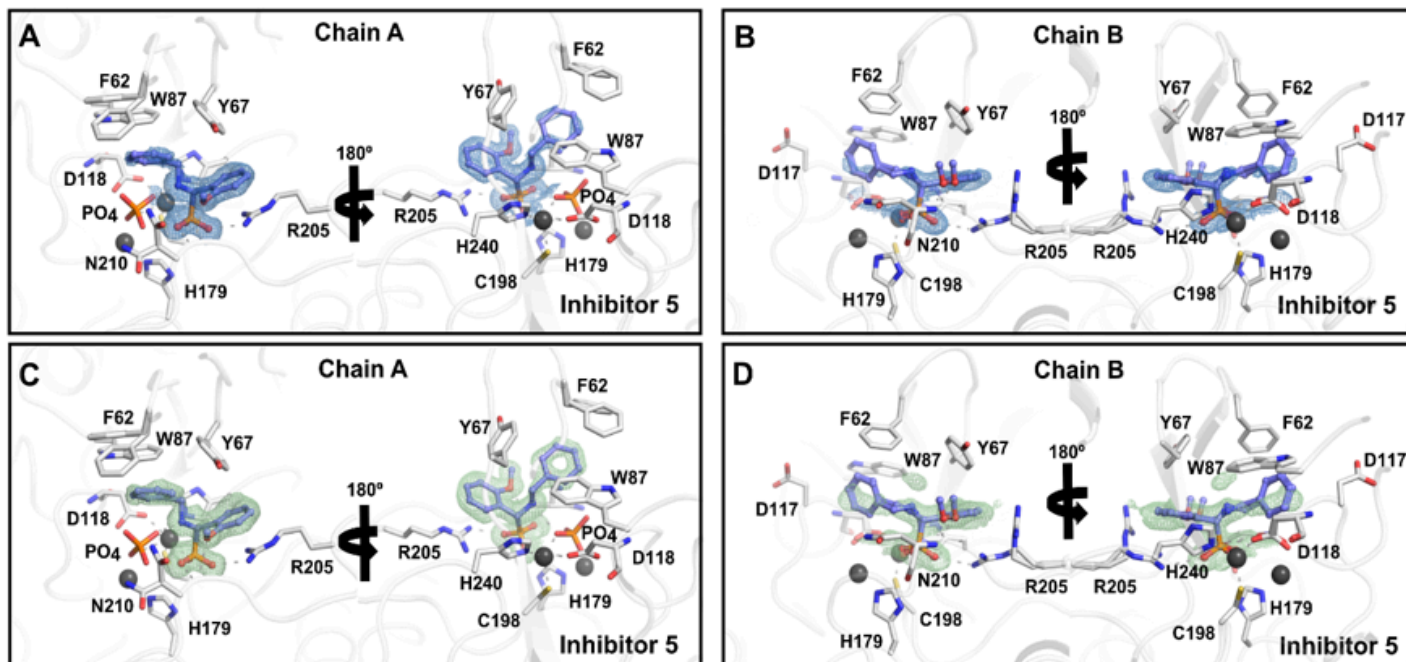


**Figure 4**

Electron density of inhibitor 5 in complex with VIM-2. 2Fo-Fc map of inhibitor 5 in (a) chain A and (b) chain B. Difference electron density polder-OMIT maps of inhibitor 5 in (c) chain A and (d) chain B. Each panel is shown with a 180 ° rotation, for better visibility of the inhibitor. VIM-2 is shown as grey cartoon and the inhibitor molecule as a ball-and-stick model. The 2Fo-Fc map is shown as blue mesh at an RMSD-level of 1, and the polder-OMIT map is shown as green mesh at a RMSD-level of 3.

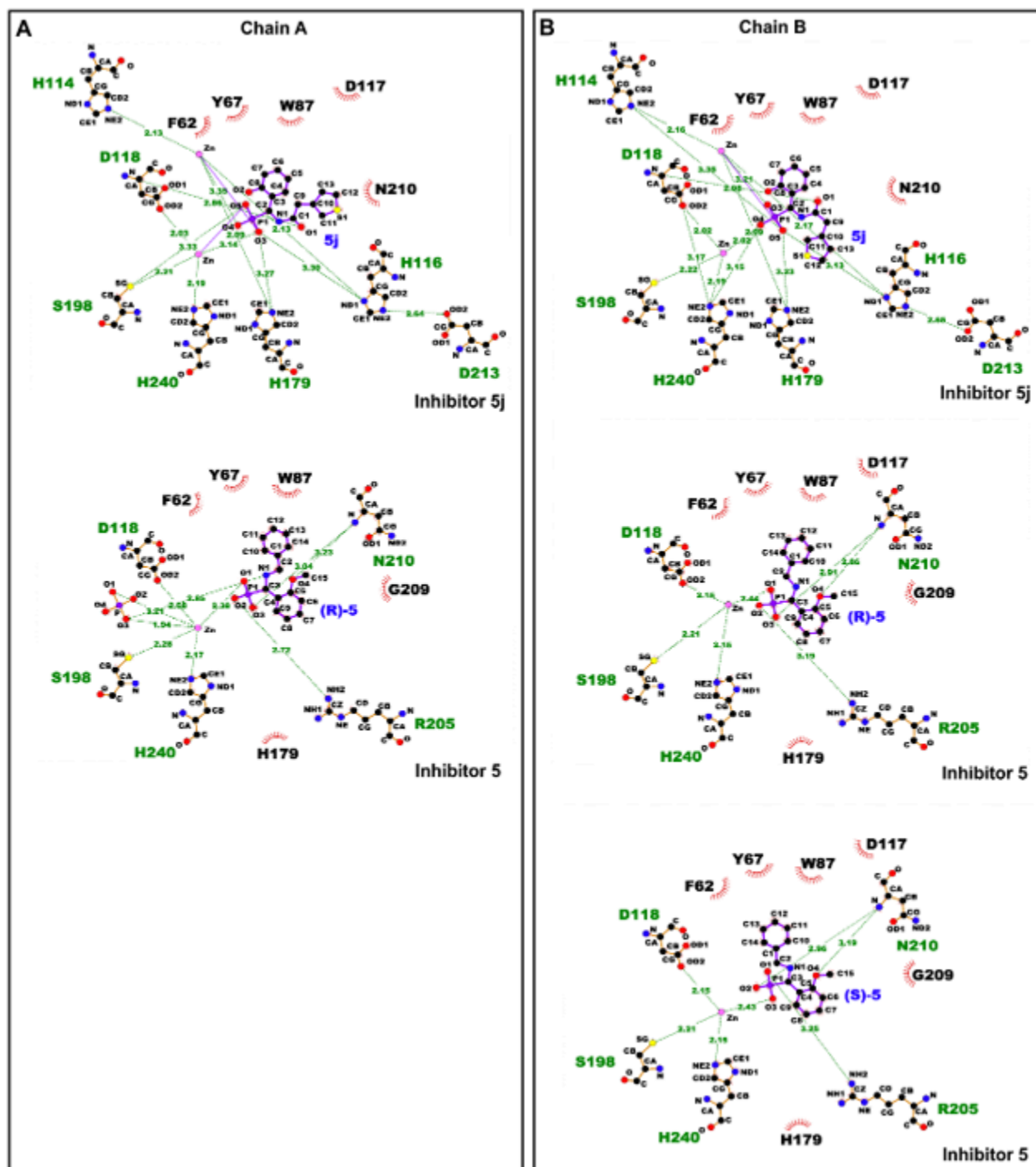


Figure 5

LigPlot+ VIM-2 in complex with inhibitors 5 and 5j. Interactions of inhibitors 5j and 5 with VIM-2 in (a) chain A and (b) chain B. Protein residues are shown in brown, the ligand in purple. Both the inhibitor and the protein are shown with atom specific colour coding, namely carbon – black, oxygen – red, nitrogen – blue, sulphur – yellow, and zinc ions – pink.

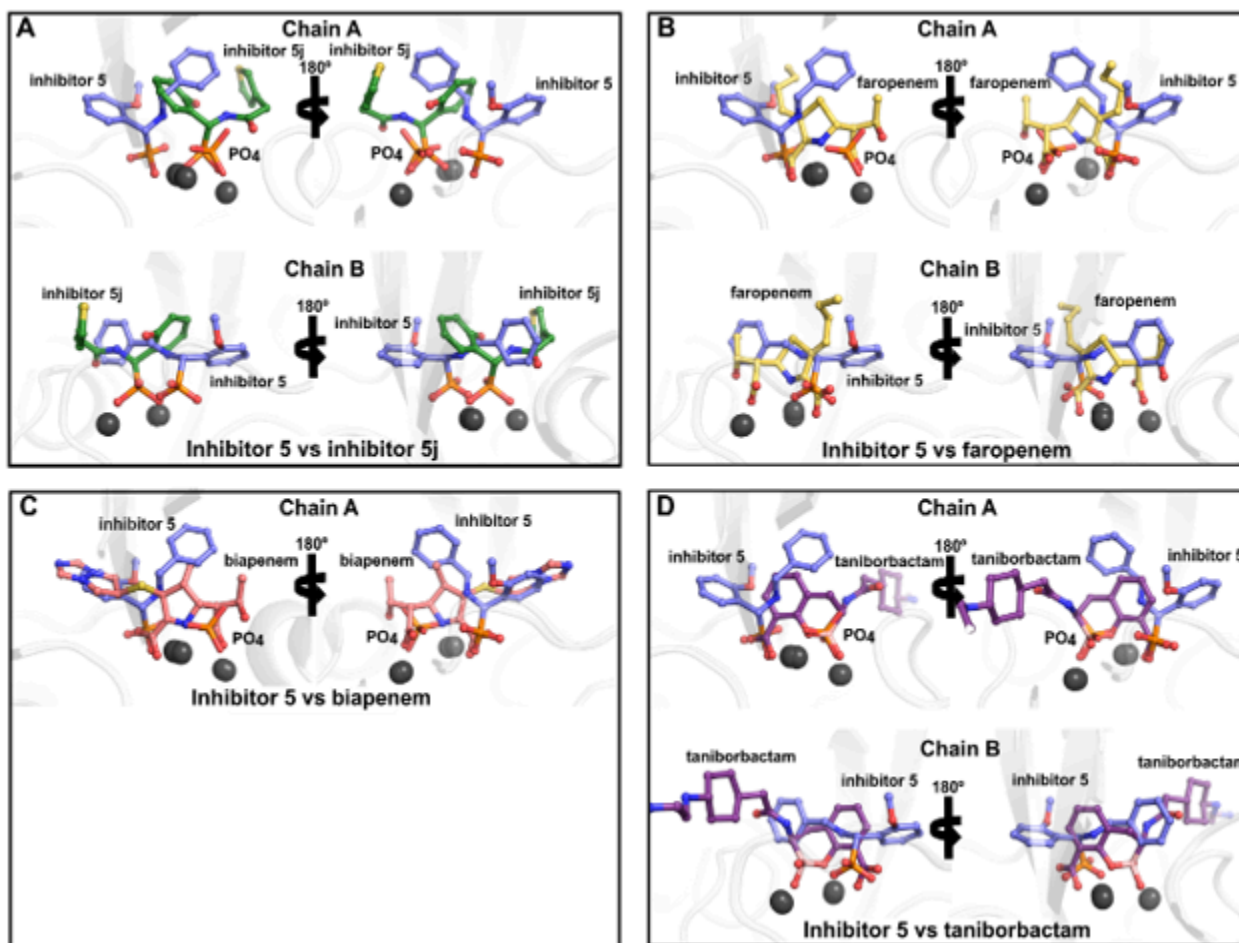


**Figure 6**

Comparison of the binding modes of inhibitors and hydrolysed antibiotics in complex with VIM-2. Binding modes in chain A and chain B of VIM-2 complexes (a) inhibitor 5 in blue compared to inhibitor 5j in green (PDB ID 9F0S), (b) inhibitor 5 in blue compared to faropenem in yellow (PDB ID 7A60), (c) inhibitor 5 in blue compared to biapenem in red (PDB ID 6Y6J), and (d) inhibitor 5 in blue compared to taniborbactam in purple (PDB ID 6SP7). Each panel with a 180° rotation for clarity. VIM-2 in complex with biapenem crystallized in a different space group (I222, one molecule per ASU) compared to all other complexes (C2, two molecules per ASU) presented here.

## Supplementary Files

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