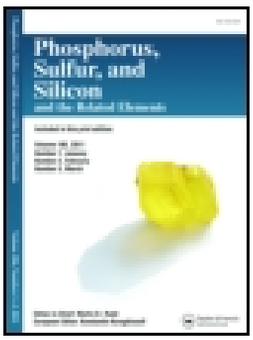

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SHORT COMMUNICATION

Synthesis and inhibitory activity of acetamidophosphonic acids against metallo-β-lactamases

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ABSTRACT

Metallo-β-lactamases (MβLs) are the target enzymes of antibiotic resistance and the phosphonic drugs make great influence to the development of contemporary medicine. Eleven acetamidophosphonic compounds were prepared and evaluated as inhibitors of the MβLs. Compounds **4, 5, 7, 9**, and **10** exhibited specific inhibitory activity against the MβL NDM-1 and CcrA with an IC_{50} value range of 17 to 354 μM. Analysis of the structure–activity relationship showed that both the acetamido linker and the position of the substituent on the phenyl ring played an important role in the inhibitory abilities of the inhibitors against MβLs.

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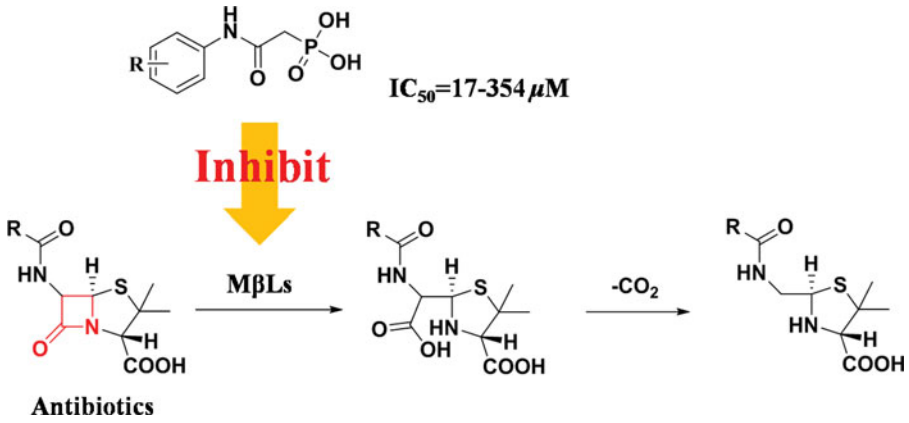
Metallo-β-lactamase; acetamidophosphonic acid; inhibitor; synthesis

GRAPHICAL ABSTRACT



Introduction

β-lactam antibiotics, as one of the three largest antibiotic classes (the others being the macrolides and fluoroquinolones), have been widely used in clinical applications for a long time. There are no words to describe the important role that these antibiotics play in human health.[1,2] However, during the past 70 years, β-lactam antibiotic resistance of gram-negative bacteria caused by the overuse of these drugs has caused worldwide attention. The resistance threatens the lives of hundreds of thousands of people every year.[2–4]

β-lactamases are the primary cause of β-lactam antibiotic resistance because they catalyze the hydrolysis of β-lactam ring of the drugs.[5] β-lactamases are subdivided into four classes: A, B, C, and D.[6–8] The classes A, C, and D enzymes are also called serine β-lactamases (SβLs) for an essential serine residue in their active site. However, the class B enzymes, also called metallo-β-lactamases (MβLs), require one or two Zn ions in the active sites to exhibit their activity. MβLs are further divided into three subclasses: B1, B2, and B3.[9–10] New Delhi metallo-β-lactamase-1 (NDM-1), a B1 subclass enzyme and discovered in 2008, has become a formidable threat to human health, prompting the World Health Organization to issue a global warning.[11] MβLs inactivate almost all β-lactam antibiotics, but there is no inhibitor of MβLs available for clinical purpose to date.[9]

We are interested in MβLs inhibitor templates that can be developed into broad-spectrum inhibitors of the MβLs. The phosphorus compounds are important substrates in the study of biochemical processes, and tetracoordinate pentavalent phosphorus compounds are widely used as biologically active compounds.[12,13] By nature, the phosphonic group [R-PO(OH)$_2$] is

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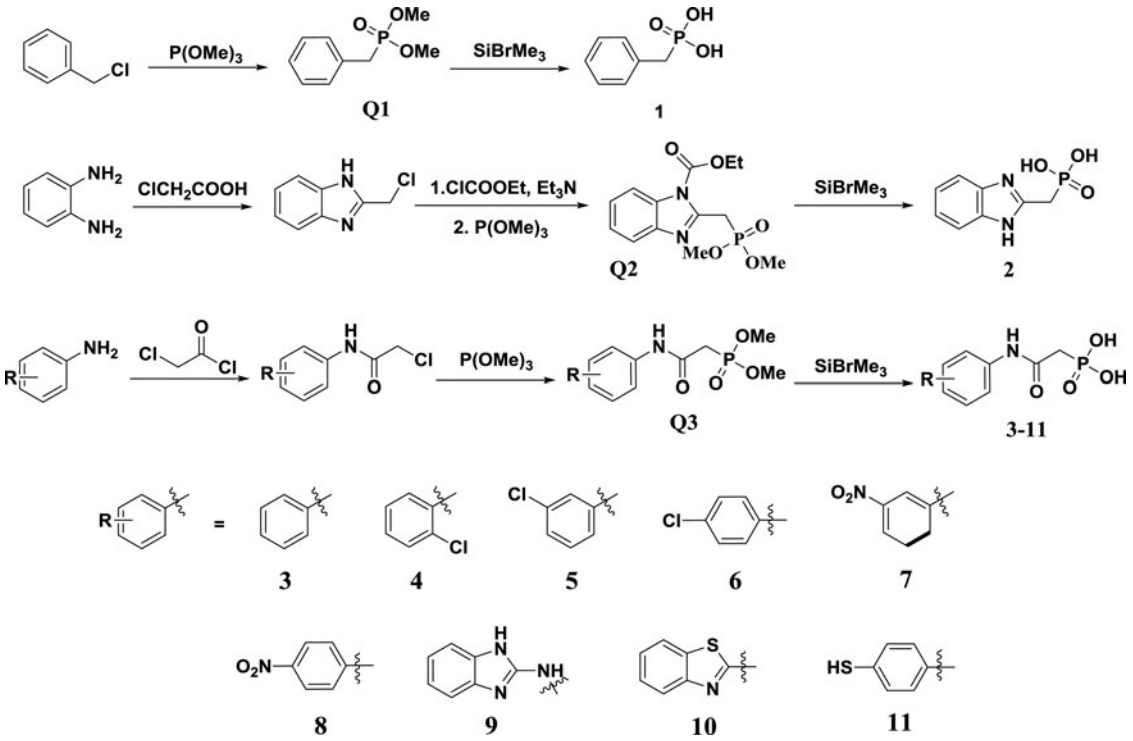

Scheme 1. Synthetic route to acetamidophosphonic acids.

introduced into a vast number of structural diverse molecules, which are involved in numerous biological functions. Several clinical drugs also comprise a phosphonic acid with prominent examples being anticancer, antibacterial, and anti-HIV agents.[14–16] In this study, the acetamidophosphonic acids were synthesized and evaluated as potential inhibitors of MβLs.

Results and discussion

Eleven acetamidophosphonic acids were synthesized by a synthetic route shown in **Scheme 1** and characterized by NMR and MS. The compounds **9, 10**, and **11** have not been reported. The acetamidophosphonic acids were prepared by the Arbuzov reaction, and the phosphonic acids were obtained by conversion of the phosphate esters in the presence of bromotrimethylsilane. The structures of the synthesized phosphonic acids are shown in **Figure 1**.

In vitro, the synthesized acetamidophosphonic acids were tested as inhibitors of MβL NDM-1 (subclass B1b), CcrA (subclass B1a), ImiS (subclass B2), and L1 (subclass B3) by employing the spectrometric method as described by Bush et al. using cefazolin (imipenem for ImiS) as the substrate.[17] The determined IC_{50} values of the phosphonic acids against MβLs are listed in **Table 1**. The results reveal most of these compounds to be potential inhibitors of the B1 subclass MβL NDM-1 and CcrA.

Compounds **2, 4, 5, 7, 9, 10**, and **11** showed a certain inhibitory activity on NDM-1 with an IC_{50} value range from 17 to 354 μM. Compounds **3, 4, 5, 7, 9**, and **10** can inhibit CcrA with an IC_{50} value range from 30 to 140 μM. We can see compounds **4, 5, 7, 9**, and **10** showed inhibitory activity to defense NDM-1 and CcrA. Among these phosphonic acids, **2** and **9**

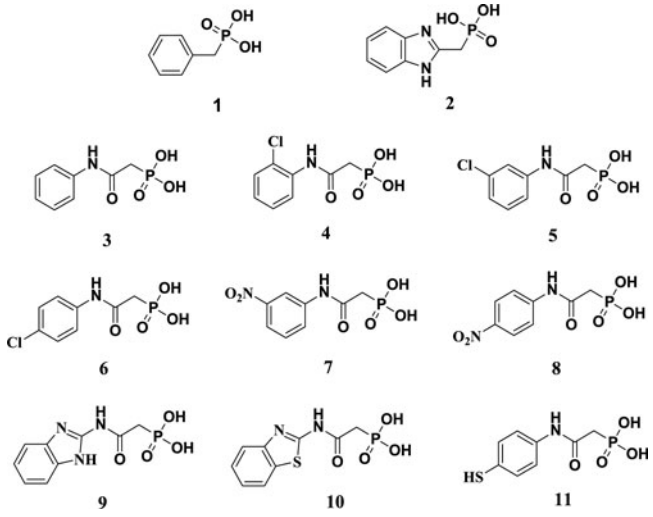

Figure 1. Structures of acetamidophosphonic acids.

Table 1. IC_{50} values of acetamidophosphonic acids against MβLs (μM).

Compounds	NDM-1*	CcrA*
1	—	—
2	30	—
3	—	52
4	55	30
5	354	140
6	—	—
7	60	63
8	—	—
9	17	99
10	158	103
11	137	—

*The antibiotic used was cefazolin;"-": no inhibitory activity.

exhibited high inhibitory activities against NDM-1 with the IC_{50} value of 30 and 17 μM, and **4** showed a slightly higher inhibitory activity for CcrA with IC_{50} value of 30 μM.

The acetamidophosphonic acids **2, 4, 7**, and **9** showed an outstanding inhibitory activity on NDM-1, with **2** and **9** being even more active than **4** and **7**. This is probably due to the structural difference. Both **2** and **9** contain a benzimidazole group, whereas **4** and **7** exhibit an acetamido linker and halogenophenyl or nitrophenyl ring. Moreover, **2** and **11** do not inhibit CcrA like **1, 6**, and **8** although **2** has a benzimidazole group but no acetamido linker, and **11** has an acetamido linker and, in addition, a sulfhydrylphenyl ring. Furthermore, compounds **3, 4**, and **7** showed better inhibitory activity against CcrA, with **4** exhibiting the best inhibitory activity. All three compounds contain an acetamido linker without a heterocyclic group.

The acetamidophosphonic acids **1, 6**, and **8** do not exhibit inhibitory activity against all tested MβLs, in which **1** contains benzyl without an acetamido linker or heterocyclic group, while **6** and **8** contain an acetamido linker and halogeno or nitro substituent. Compound **3** has a similar structure as **6** and **8** but without a substituent at the phenyl ring and showed inhibitory activity on CcrA.

In conclusion, the aromatic phosphonic acids with acetamido and heterocyclic substituents exhibit inhibitory activity against NDM-1 with the benzimidazole derivatives showing the best inhibitory activity. Regarding CcrA, the acetamido linker is essential for the inhibitory activities of the compounds, and a substituent in the *ortho*- or *meta*-position of the phenyl ring improves the inhibitory activity of the compounds compared with the *para*-substituted ones.

Conclusion

Eleven acetamidophosphonic acids have been synthesized, characterized, and tested as inhibitors of MβLs. The results indicated that partial phosphonic acids displayed specific inhibitory ability on the B1 subclass of MβLs NDM-1 and CcrA. The analysis of the structure–activity relationship reveals that the acetamido linker plays an important role in the inhibitory activity of the enzymes, and substitution in *ortho*-position of the phenyl ring improves the inhibitory activity of the compounds.

Experimental

Materials and methods

Chemicals were purchased from Aladin and were used without further purification. The reaction progress was monitored by thin-layer chromatography (TLC) using Merck silica gel 60 aluminum sheets (F_{254}). NMR spectra were recorded with a Bruker DRX 400 MHz spectrometer. ^{1}H (400 MHz), ^{13}C (101 MHz), and ^{31}P NMR (162 MHz) spectra were recorded with TMS as internal standard. The peak patterns are indicated as follows: s, singlet; d, doublet; t, triplet; sept, septuplet; m, multiplet. HRMS spectra were recorded on a BrukerMicrOTOF-Q II mass spectrometer. Inhibition studies were performed on an Agilent-8453 UV-visible spectrometer. All antibiotics used were purchased from Sigma-Aldrich.

General procedure for the preparation of phosphate esters

Under the protection of N_2, triethyl phosphate (0.012 mol) was added to the corresponding chloride (0.01 mol) at room temperature; the resulting mixture was refluxed at 120°C for 12 h, during which time the reaction was monitored by TLC (R_f = 0.5, AcOEt/petroleumether 2:1). The cooled mixture was purified by flash chromatography ($CHCl_3$/AcOEt 4:1) to yield the phosphonates **Q1, Q2**, and **Q3**.

General procedure for the preparation of phosphate acids

To a stirred solution of the phosphonates **Q1, Q2**, and **Q3** (14.0 mmol) in DCM (30 mL) NaI (0.03 mol) was added within 10 min. Then, Me_3SiBr (0.03 mol) was added dropwise to the reaction mixture and the mixture was heated to 60°C for 5 h. The solid target products **1**–**11** are obtained after cooling.

Spectroscopic data of products

Dimethyl benzylphosphonate (Q1): Colorless oil, 93%. ^{1}H NMR ($CDCl_3$): δ 9.39 (s, 1H), 7.54–7.47 (m, 2H), 7.21 (t, J = 7.6 Hz, 2H), 7.02 (s, 1H), 3.83 (d, J = 11.2 Hz, 6H), 3.13 (d, J = 8.4 Hz, 2H). ^{31}P NMR ($CDCl_3$): δ 25.64.

Ethyl 2-[(dimethoxyphosphoryl)methyl]-1H-benzo[d]imidazole-1-carboxylate (Q2): Yellow oil, 79%. ^{1}H NMR (MHz, $CDCl_3$): δ 7.79–7.54 (m, 2H), 7.19 (s, 2H), 4.46–4.36 (m, 2H), 3.98–3.85 (m, 2H), 3.76–3.62 (m, 6H), 1.46–1.27 (m, 3H). ^{13}C NMR ($CDCl_3$): δ 149.92, 146.97, 141.78, 132.54, 124.49, 124.07, 119.38, 114.65, 64.13, 52.81, 29.18, 27.80, 13.70. ^{31}P NMR ($CDCl_3$): δ 24.42.

Dimethyl [2-oxo-2-(phenylamino)ethyl]phosphonate (Q3–3): Yellow oil, 85%.^{1}H NMR ($CDCl_3$): δ 9.39 (s, 1H), 7.54–7.47 (m, 2H), 7.21 (t, J = 7.7 Hz, 2H), 7.02 (t, J = 7.4 Hz, 1H), 3.83 (d, J = 11.2 Hz, 6H), 3.13 (d, J = 8.4 Hz, 2H). ^{13}C NMR ($CDCl_3$): δ 162.18, 137.98, 128.78, 124.27, 119.80, 53.40, 36.07, 34.77. ^{31}P NMR ($CDCl_3$): δ 25.61.

Dimethyl 2-[(2-chlorophenyl)amino]-2-oxoethylphosphonate (Q3–4): Yellow oil, 88%. ^{1}H NMR ($CDCl_3$): δ 8.93 (s, 1H), 8.23 (dd, J = 8.2, 1.4 Hz, 1H), 7.36 (dd, J = 8.0, 1.4 Hz, 1H), 7.24 (td, J = 8.2, 1.4 Hz, 1H), 7.09–7.03 (m, 1H), 3.83 (d, J = 11.2 Hz, 6H), 3.15 (d, J = 9.0 Hz, 2H). ^{13}C NMR ($CDCl_3$): δ 162.32, 134.44, 129.19, 127.46, 125.18, 123.67, 122.38, 53.33, 36.11, 34.81. ^{31}P NMR ($CDCl_3$): δ 25.64.

Dimethyl {2-[(3-chlorophenyl)amino]-2-oxoethyl}phosphonate (Q3–5): Yellow oil, 83%. ^{1}H NMR ($CDCl_3$): δ 9.80 (s, 1H), 7.60 (t, J = 2.0 Hz, 1H), 7.32–7.25 (m, 1H), 7.06 (t, J = 8.1 Hz, 1H), 6.98–6.92 (m, 1H), 3.86 (d, J = 11.2 Hz, 6H), 3.14 (d, J = 21.8 Hz, 2H).^{31}P NMR ($CDCl_3$): δ 25.57.

Dimethyl {2-[(4-chlorophenyl)amino]-2-oxoethyl}phosphonate (Q3–6): Yellow oil, 90%. ^{1}H NMR ($CDCl_3$): δ 9.67 (s, 1H), 7.38 (d, J = 8.8 Hz, 2H), 7.10 (d, J = 8.8 Hz, 2H), 3.85 (d, J = 11.2 Hz, 6H), 3.13 (d, J = 8.6 Hz, 2H). ^{13}C NMR ($CDCl_3$): δ 162.29, 136.79, 129.14, 128.74, 120.79, 53.58, 36.21, 34.91. ^{31}P NMR ($CDCl_3$): δ 25.54.

Dimethyl {2-[(3-nitrophenyl)amino]-2-oxoethyl}phosphonate (Q3–7): Yellow oil, 86%. ^{1}H NMR ($CDCl_3$): δ 10.14 (s, 1H), 8.25 (d, J = 2.0 Hz, 1H), 7.79 (d, J = 8.0 Hz, 1H), 7.76–7.70 (m,

1H), 7.24 (s, 1H), 3.93 (d, $J = 11.2$ Hz, 6H), 3.22–3.13 (m, 2H). ^{31}P NMR ($CDCl_3$): δ 25.30.

Dimethyl {2-[(4-nitrophenyl)amino]-2-oxoethyl}phosphonate (Q3–8): Yellow oil, 86%. ^{1}H NMR ($CDCl_3$): δ 9.92 (s, 1H), 8.04 (d, $J = 9.1$ Hz, 2H), 7.61 (d, $J = 9.1$ Hz, 2H), 3.89 (d, $J = 11.3$ Hz, 6H), 3.15 (d, $J = 8.4$ Hz, 2H). ^{13}C NMR ($CDCl_3$): δ 162.52, 148.16, 139.23, 129.41, 124.61, 118.34, 113.94, 53.68, 36.34, 35.05. ^{31}P NMR ($CDCl_3$): δ 25.09.

Dimethyl {2-[(1H-benzo[d]imidazol-2-yl)amino]-2-oxoethyl}phosphonate (Q3–9): Yellow oil, 77%. ^{1}H NMR ($CDCl_3$): δ 7.33 (s, 2H), 7.31–7.28 (m, 2H), 7.26 (dd, $J = 4.4$, 2.4 Hz, 1H), 3.64 (s, 6H), 3.21–3.14 (m, 2H). ^{31}P NMR ($CDCl_3$): δ 23.26.

Dimethyl [2-(benzo[d]thiazol-2-ylamino)-2-oxoethyl]phosphonate (Q3–10): yellow oil, 83%. ^{1}H NMR ($CDCl_3$): δ 7.68 (d, $J = 7.8$ Hz, 1H), 7.49 (t, $J = 7.8$ Hz, 1H), 7.39–7.31 (m, 2H), 3.84 (d, $J = 11.2$ Hz, 6H), 3.72–3.69 (m, 3H), 3.31 (d, $J = 21.6$ Hz, 2H). ^{31}P NMR ($CDCl_3$): δ 26.04.

Dimethyl {2-[(4-mercaptophenyl)amino]-2-oxoethyl}phosphonate (Q3–11): yellow oil, 81%. ^{1}H NMR ($CDCl_3$): δ 9.60 (s, 1H), 7.46 (d, $J = 8.7$ Hz, 2H), 7.15 (d, $J = 8.7$ Hz, 2H), 3.91 (d, $J = 11.2$ Hz, 6H), 3.20 (d, $J = 8.6$ Hz, 2H). ^{13}C NMR ($CDCl_3$): δ 162.03, 135.67, 133.30, 127.57, 120.18, 53.42, 36.05, 34.76, 16.55. ^{31}P NMR ($CDCl_3$): δ 25.73.

Benzylmethylphosphonic acid (1): White solid, yield 92%. ^{1}H NMR (DMSO-d_6): δ 8.13 (s, 2H), 7.30–7.23 (m, 4H), 7.20 (dd, $J = 5.6$, 2.8 Hz, 1H), 2.97 (d, $J = 6.0$ Hz, 2H). ^{13}C NMR (DMSO-d_6): δ 134.19, 134.10, 129.94, 129.88, 128.20, 128.17, 126.15, 126.12, 35.99, 34.67. ^{31}P NMR (DMSO-d_6): δ 22.10. HRMS $[M-H]^-$ m/z for $C_7H_9O_3P$: calcd. 171.0189, obsd. 171.0027.

Benzimidazolyl-2-methylphosphonic acid (2): White solid, yield 87%. ^{1}H NMR (CD_3OD-d_4): δ 8.23–8.20 (m, 1H), 7.85–7.82 (m, 1H), 7.68 (t, $J = 6.3$ Hz, 2H), 4.74 (q, $J = 7.1$ Hz, 2H), 1.59 (t, $J = 7.1$ Hz, 3H). ^{13}C NMR (CD_3OD-d_4): δ148.24, 127.57, 127.28, 126.17, 116.23, 114.51, 113.37, 66.88, 12.90. ^{31}P NMR (CD_3OD-d_4): δ13.49.

[N-(Phenylcarbamoyl)methyl]phosphonicacid (3): White solid, yield 87%. ^{1}H NMR (DMSO-d_6): δ 9.97 (d, $J = 9.1$ Hz, 1H), 7.61–7.53 (m, 2H), 7.28 (t, $J = 7.9$ Hz, 2H), 7.02 (s, 1H), 2.83 (dd, $J = 6.3$, 3.5 Hz, 2H).^{13}C NMR (DMSO-d_6): δ 160.80, 130.57, 120.15, 115.39, 111.61, 31.52, 30.34. ^{31}P NMR(DMSO-d_6):δ17.34. HRMS $[M-H]^-$ m/z for $C_8H_{10}NO_4P$: calcd. 214.0247, obsd. 214.0049.

[N-(2-Chlorophenyl)carbamoyl]methylphosphonic acid (4): White solid, yield 82%. ^{1}H NMR (DMSO-d_6): δ 9.57 (s, 1H), 8.01 (d, $J = 7.1$ Hz, 1H), 7.49 (dd, $J = 8.0$, 1.4 Hz, 1H), 7.35–7.29 (m, 1H), 7.14 (td, $J = 7.8$, 1.5 Hz, 1H), 2.95–2.93 (m, 2H). ^{13}C NMR (DMSO-d_6): δ 164.85, 135.13, 129.46, 127.56, 125.46, 124.20, 123.70, 37.62. ^{31}P NMR (DMSO-d_6): δ 17.35.HRMS $[M-H]^-$ m/z for $C_7H_9ClNO_4P$: calcd. 247.9858, obsd. 247.9904.

[N-(3-Chlorophenyl)carbamoyl]methylphosphonic acid (5): White solid, yield 71%. ^{1}H NMR (DMSO-d_6): δ 9.81 (s, 1H), 7.76 (d, $J = 8.7$ Hz, 2H), 7.40 (s, 2H), 2.89 (d, $J = 6.8$ Hz, 2H). ^{13}C NMR (DMSO-d_6): δ 166.97, 138.22, 128.50, 126.69, 120.78, 51.60. ^{31}P NMR (DMSO-d_6): δ 18.15. HRMS $[M-H]^-$ m/z for $C_7H_9ClNO_4P$: calcd. 247.9858, obsd. 247.9911.

[N-(4-Chlorophenyl)carbamoyl]methylphosphonic acid (6): White solid, yield 82%.^{1}HNMR (DMSO-d_6): δ 10.47 (s, 1H), 7.64 (d, $J = 8.7$ Hz, 2H), 7.34–7.29 (m, 2H), 2.82 (dd, $J = 8.7$, 12.2 Hz, 2H). ^{13}C NMR (DMSO-d_6): δ 166.97, 138.22, 128.50, 126.69, 120.78, 51.60. ^{31}P NMR (DMSO-d_6): δ 18.15. HRMS $[M-H]^-$ m/z for $C_7H_9ClNO_4P$: calcd. 247.9858, obsd. 247.9912.

[N-(3-Nitrophenyl)carbamoyl]methylphosphonic acid (7): White solid, yield 81%.^{1}H NMR (CD_3OD-d_4): δ 8.74 (s, 1H), 7.95 (dd, $J = 13.4$, 7.9 Hz, 2H), 7.59 (t, $J = 8.2$ Hz, 1H), 3.02–2.91 (m, 2H). ^{13}C NMR (CD_3OD-d_4): δ 175.36, 157.53, 150.51, 139.81, 134.72, 127.38, 122.74, 47.97. ^{31}P NMR (CD_3OD-d_4): δ 15.80. HRMS $[M-H]^-$ m/z for $C_8H_9N_2O_6P$: calcd. 259.0098, obsd. 259.0165.

[N-(4-Nitrophenyl)carbamoyl]methylphosphonic acid (8): White solid, yield 80%.^{1}H NMR (CD_3OD-d_4): δ 8.35–8.25 (m, 2H), 7.93 (dd, $J = 9.1$, 2.6 Hz, 2H), 4.93 (s, 2H), 3.20–3.04 (m, 2H).^{31}P NMR (CD_3OD-d_4): δ 18.22. HRMS $[M-H]^-$ m/z for $C_8H_9N_2O_6P$: calcd. 259.0098, obsd. 259.0169.

[N-Benzimidazolylcarbamoyl]methylphosphonic acid (9): White solid, yield 65%.^{1}H NMR (DMSO-d_6): δ 7.96 (dd, $J = 6.2$, 3.1 Hz, 2H), 7.67 (dd, $J = 6.2$, 3.1 Hz, 2H), 3.89 (s, 1H), 3.11 (d, $J = 6.3$ Hz, 1H). ^{31}P NMR (DMSO-d_6): δ 15.30. HRMS $[M-H]^-$ (m/z) for $C_9H_{10}N_3O_4P$: calcd. 255.0414, obsd. 255.2366.

[N-Benzothiazolylphenylcarbamoyl]methylphosphonic acid (10): White solid, yield 69%. ^{1}H NMR (DMSO-d_6): δ 7.98 (d, $J = 7.9$ Hz, 1H), 7.75 (d, $J = 8.0$ Hz, 1H), 7.43 (d, $J = 8.1$ Hz, 1H), 7.32 (d, $J = 7.3$ Hz, 1H), 3.72 (s, 2H), 3.15 (d, $J = 8.5$ Hz, 2H). ^{31}P NMR (DMSO-d_6): δ 19.04.

[N-(4-Sulfhydrylphenyl)carbamoyl]methylphosphonic acid (11): White solid, yield 55%.^{1}H NMR (DMSO-d_6): δ 10.21 (s, 1H), 7.63–7.56 (m, 2H), 7.42 (d, $J = 8.5$ Hz, 1H), 7.28 (d, $J = 8.7$ Hz, 1H), 3.81 (s, 3H), 2.86 (d, $J = 7.8$ Hz, 1H), 2.57 (s, 3H). ^{31}P NMR (DMSO-d_6): δ 16.80.

Enzyme preparations

All MβLs used were purified as described in the references for L1,[17] ImiS,[18] CcrA,[19] and NDM-1.[20]

Over-expression and purification of L1

L1 was over-expressed and purified as previously described. The gene that encodes L1 was ligated into pET26b, and *E. coli* DH5R cells were transformed with the resulting plasmid pET26b(+)L1. Presence of the insert was confirmed with DNA sequencing. BL21(DE3) *E. coli* cells were then transformed with the pET26b(+)L1 plasmid, and the resulting cells were used for over-expression of protein. L1 was determined quantitatively by monitoring the absorbance at 280 nm and using an extinction coefficient of 54,614 M^{-1} cm^{-1}.

Over-expression and purification of ImiS

ImiS was over-expressed and purified as previously described. BL21(DE3) *E. coli* cells were transformed with a plasmid containing the gene for ImiS, pET-26b-ImiS. A culture of these cells in LB medium was used for over-expression of protein. The concentration of ImiS was determined using Beer's law and an extinction coefficient of 37,250 M^{-1} cm^{-1} at 280 nm.

Over-expression and purification of CcrA

CcrA was over-expressed and purified as previously described. BL21(DE3) *E. coli* cells were transformed with plasmid pMSZ02-CcrA and a culture of these cells in LB medium was used for protein over-expression. Protein concentration was determined using Beer's law and an extinction coefficient of 39,000 M^{-1} cm^{-1} at 280 nm.

Over-expression and purification of NDM-1

The over-expression plasmid, pET26b-NDM-1, was used for expression of NDM-1 as previously described. The over-expression plasmid, pET26b-NDM-1, was used to transform BL21(DE3) *E. coli* cells. The crude protein NDM-1 was further purified by running through a G75 column. Protein purity was ascertained by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. The protein concentration was determined using Beer's law and an extinction coefficient of 27,960 M^{-1} cm^{-1} at 280 nm.

Determination of IC_{50} values

The inhibition studies were conducted at 25°C using imipenem as substrate of ImiS and cefazolin V as substrate of CcrA, NDM-1, and L1.[21] Compounds were dissolved in a small volume of DMSO and diluted with 50 mM Tris, pH 7.0. The final DMSO concentration in the reaction mixture was less than 0.5%, which did not alter the enzyme activity. The substrate concentrations were varied between 20 and 140 μM, inhibitor concentrations were varied between 0 and 10 μM, and the enzyme concentrations were varied between 3 and 60 nM. The enzyme and inhibitor were preincubated for 30 min before starting the kinetic experiments.

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