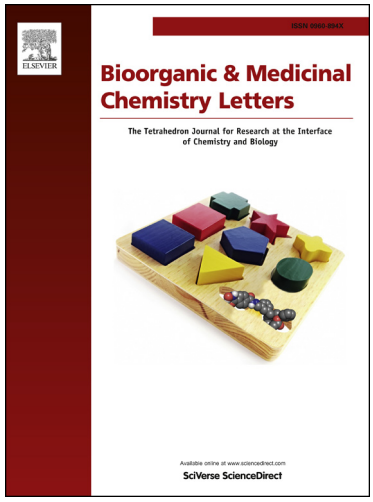


New β-phospholactam as a carbapenem transition state analog: synthesis of a broad-spectrum inhibitor of metallo-β-lactamases

Ke-Wu Yang, Lei Feng, Shao-Kang Yang, Mahesh Aitha, Alecander E. LaCuran, Peter Oelschlaeger, Michael W. Crowder

PII: S0960-894X(13)01047-0
DOI: http://dx.doi.org/10.1016/j.bmcl.2013.08.098
Reference: BMCL 20836

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 19 July 2013
Revised Date: 21 August 2013
Accepted Date: 26 August 2013

Please cite this article as: Yang, K-W., Feng, L., Yang, S-K., Aitha, M., LaCuran, A.E., Oelschlaeger, P., Crowder, M.W., New β-phospholactam as a carbapenem transition state analog: synthesis of a broad-spectrum inhibitor of metallo-β-lactamases, *Bioorganic & Medicinal Chemistry Letters* (2013), doi: http://dx.doi.org/10.1016/j.bmcl.2013.08.098

New β-phospholactam as a carbapenem transition state analog: synthesis of a broad-spectrum inhibitor of metallo-β-lactamases

Ke-Wu Yang[a,*], Lei Feng[a], Shao-Kang Yang[a], Mahesh Aitha[b], Alecander E. LaCuran[c], Peter Oelschlaeger[c], and Michael W. Crowder[b,*]

[a] *Key Laboratory of Synthetic and Natural Functional Molecule Chemistry of Ministry of Education, College of Chemistry and Materials Science, Northwest University, Xi'an 710069, P. R. China;* [b] *Department of Chemistry and Biochemistry, Miami University, 160 Hughes Hall, Oxford, OH 45056, USA;* [c] *Department of Pharmaceutical Sciences, College of Pharmacy, Western University of Health Sciences, 309 E. Second St., Pomona, CA 91766, USA.*

Corresponding authors: Tel/Fax: +86-29-8830-2429 (K.-W.Y.); +1-513-529-7274 (M.W.C.). E-mail address: kwyang@nwu.edu.cn (K.-W. Yang); crowdemw@MiamiOH.edu (M.W. Crowder)

Abstract

In an effort to test whether a transition state analog is an inhibitor of the metallo-β-lactamases, a phospholactam analog of carbapenem has been synthesized and characterized. The phospholactam **1** proved to be a weak, time-dependent inhibitor of IMP-1 (70%), CcrA (70%), L1 (70%), NDM-1 (53%), and Bla2 (94%) at an inhibitor concentration of 100 μM. The phospholactam **1** activated ImiS and BcII at the same concentration. Docking studies were used to explain binding and to offer suggestions for modifications to the phospholactam scaffold to improve binding affinities.

Since the antibiotic properties of penicillin were first discovered in the beginning of the last century, antibiotics have been well developed as miracle drugs in the treatment of bacterial infections in clinics.[1, 2] However, overuse of antibiotics has resulted in a large number of bacteria that produce β-lactamases, and the resulting bacteria are resistant to many commonly-used β-lactam antibiotics.[3-5] β-Lactamases catalyze the hydrolysis of the β-lactam ring of these antibiotics. They are divided into classes A, B, C, and D.[6] The B class enzymes, called metallo-β-lactamases (MβLs), are Zn(II)-dependent and hydrolyze nearly all known β-lactam-containing antibiotics, including penicillins, cephalosporins and carbapenems (Figure 1). There are no known clinical inhibitors of the MβLs.[4] The MβLs are further divided into B1, B2, and B3 subclasses.[6] Both B1 and B3 subclasses have a broad-spectrum substrate profile including penicillins, cephalosphorins and carbapenems. In contrast, the B2 subclass enzymes primarily hydrolyze carbapenems.

Given the enormous biomedical importance of MβLs, there has been a large amount of effort in identifying novel inhibitors of these enzymes.[4, 7] Succinic acid derivatives were shown to be inhibitors of IMP-1 with IC_{50} values in the nanomolar range.[8] Lienard *et al.* reported that *D*-captopril inhibits L1 and CcrA,[9] and thiomandelic acid was tested as an inhibitor for nine different MβLs.[10] Picolinic acid derivatives were shown to inhibit CphA with K_i values in the micromolar range,[11] while hydroxamic acid derivatives inhibit Fez-1 but not L1, IMP-1, BcII, or CphA.[12] A mechanism-based inhibitor of IMP-1 was reported that exhibited irreversible inhibition.[13] Penicillin-based inhibitors have been shown to be micromolar inhibitors of L1, BcII, and Bla2,[14] and some N-arylsulfonyl hydrazones are

inhibitors of IMP-1.[15] A series of pyrrole-based inhibitors of IMP-1 have recently been reported.[16] However, most of the inhibition reports have involved studies on one or two of the MβLs, and to the best of our knowledge, only three classes of inhibitors, thiomandelic acid,[10] thiols,[9, 17-19] and mercaptophosphonate compounds,[20] have been reported to be broad-spectrum inhibitors of the MβLs. Our goal is to develop broad-spectrum, transition state analog inhibitors of MβLs and to use these inhibitors as drug/inhibitor combinations to combat bacterial infections in which the bacteria produce a MβL.

Previous mechanistic studies have suggested a ring-opened intermediate, whose breakdown is rate-limiting, for L1, CcrA, and NDM-1 when using nitrocefin or chromacef as substrate.[21-23] When using other MβLs or substrates, nucleophilic attack or β-lactam ring cleavage appears to be rate-limiting.[24-26] Previous studies on peptidases, many of which exhibit rate-limiting bond cleavage, suggested that a tetrahedral transition state forms during the reaction.[27-29] The fact that several phosphinate, phosphonate, and phosphoramidate peptide analogs proved to be very tight binding inhibitors (one with a reported K_i of 10^{-15} M) strongly supported the existence of the tetrahedral transition state in these peptidases.[30-37] Since β-lactam-containing antibiotics are peptide mimics and since β-lactamases catalyze peptide bond cleavage, we hypothesize that a tetrahedral transition state may form during the reaction (Figure 2) and that a chemically-stable β-phospholactam may be a very tight binding inhibitor of the MβLs.

Toward this goal, a β-phospholactam analog of a carbapenem transition state (Figure 2) was synthesized by using a 12-step protocol, and the resulting compound **1** was characterized by NMR and MS. The inhibitory activities of the β-phospholactam **1** were

evaluated using MβLs from the three subclasses B1 (IMP-1, CcrA, Bla2, NDM-1), B2 (ImiS), and B3 (L1). In the absence of experimental structures of MβL-ligand complexes, docking studies can provide insights into possible binding modes of inhibitors[38] and β-lactam substrates.[39, 40] These studies have shown that substituents with high electron density, such as, thiols, carboxylates, and carbonyl groups interact electrostatically with the zinc ions and the positively-charged Lys224 conserved in many B1 and B2 MβLs.[41, 42] Here we assessed the binding mode of β-phospholactam **1** to the different MβLs using docking. Previously, non-cyclic phosphinates[43] and monocyclic β-phospholactams[44] were tested as inhibitors of MβLs; however, none of these compounds inhibited the tested enzymes, most likely due to the fact that these compounds did not have the correct structure to be recognized by the MβLs. Page and coworkers reported a number of studies using β-sultams and β-phospholactams as inhibitors of a serine β-lactamase, and these studies, along with a theoretical study,[45] explored the stability of these compounds at different pH's.[44, 46-48] None of these compounds tested were bicyclic. Rees and coworkers reported the synthesis of a 1,2-azaphosphetidine, which is a bicyclic β-phospholactam;[49] however, the compound was not tested as an inhibitor of any of the MβLs. Our efforts to synthesize this compound were unsuccessful using the published procedure; therefore, we developed a novel synthetic approach to obtain a bicyclic β-phospholactam **1** (Scheme 1). Dimethyl 2,5-dibromoadipate **3** as white solid was prepared by acylation, α-bromination, and esterification of adipic acid, using previously reported methods.[50] Adipate **3** was reacted with benzylamine in alkaline medium, and the resulting product was treated by acidification and alkalization to afford pure pyrrole dicarboxylate **4**. Compound **4** has a highly symmetric structure, which required harsh reduction conditions to

obtain the pyrrole monocarboxylate **5**. After attempting various reaction conditions, sodium borohydride was identified as the optimum reductant, and ethanol was identified as the optimum solvent. Alcohol **5** was converted to aldehyde **6** by Swern oxidation, and this product was used in the next step without further purification. Intermediate **6** was reacted with dimethyl phosphate in the presence of catalyst 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) at 0 °C to afford dimethyl phosphonate **7**. Dimethyl phosphonate was converted to monomethyl phosphonate **8** by treatment of **7** with NaI in acetone. The α-hydroxy was protected by treatment of **8** in pyridine with acetic anhydride in the presence of 4-dimethylaminopyridine (DMAP) to afford **9**. The removal of the benzyl protecting group was very challenging. A series of bases, including triethylamine, diisopropylethylamine, potassium acetate, sodium hydroxide, and NaH, and solvents were tested in an effort to remove the benzyl group. Fortunately, intermediate **9** was hydrogenated with 10% Pd/C in the presence of HCO_2NH_4 to afford **10**. The ring-closure step to yield **11** was carried out using NaH. Finally, the target compound β-phospholactam **1** was obtained by hydrolysis of **11** with LiOH to remove the methyl protecting groups. The overall yield was 0.68%. The product and intermediates were characterized and confirmed by NMR and MS.

The inhibitory activity of β-phospholactam **1** on MβLs was evaluated, and percent inhibition values are listed in Table 1. Without pre-incubation with enzymes, the β-phospholactam **1** activated CcrA and NDM-1 and showed 48, 22, and 37% inhibition against IMP-1, Bla2, and L1, respectively. After incubation with enzymes for 30 min, β-phospholactam **1** exhibited a 70% inhibition against IMP-1, CcrA, and L1, a 53% inhibition for NDM-1, and a 94% inhibition of Bla2, suggesting that β-phospholactam **1** is a

time-dependent inhibitor. At this point, it is unclear whether the time-dependent inhibition is caused by intact β-phospholactam **1**, a covalently-modified enzyme-inhibitor complex, or by a hydrolyzed product. Previous studies with a β-phospholactam and class C β-lactamase P99 revealed time-dependent inhibition was caused by a phosphonylated enzyme.[46] Nonetheless, stability studies revealed a relatively fast hydrolysis of monocyclic β-phospholactam in water.[44, 46] We believe that the different trend exhibited by the enzymes with and without pre-incubation is due to the enzymes behaving differently in DMSO, which is used as a solvent for the inhibitor. Unexpectedly, the inhibitor activates ImiS and BcII. The mechanism of this activation is unknown, but it could be due to an allosteric effect as a result of phosphonylation of solvent-exposed hydroxyl groups by β-phospholactam **1**.

In docking calculations (computational details can be found in the Supporting Information) the majority of conformations (between 42 and 50 out of 50 per complex) were found in clusters that correspond to the expected binding mode, which is the way that the hypothetical carbapenem transition state would bind. Since no crystal structure of any MβL-carbapenem transition state complex is available, the lowest-energy conformations docked to NDM-1 and L1 are compared to enzyme-hydrolyzed β-lactam complexes in Figure 3 and Table 2. In all complexes, one phosphinate oxygen, which corresponds to the oxygen derived from the β-lactam carbonyl, coordinates Zn_1, while the β-lactam nitrogen coordinates Zn_2. In some complexes (IMP-1 and CcrA), the phosphinate oxygen additionally coordinates Zn_2 (Figure S1), which may be due to the short Zn_1-Zn_2 distance of 3.6 Å and 3.5 Å, respectively, in these crystal structures. The carboxylate attached to C_7 of the five-membered ring forms a salt bridge with the Lys224 side chain, as is seen in the enzyme-hydrolyzed

β-lactam complexes, except in L1, which does not contain Lys224. Interestingly, in the L1-β-phospholactam **1** complex, which is compared to an L1-hydrolyzed moxalactam complex[51] in Table 2 and Figure S2, one phosphinate oxygen occupies the site occupied by the carboxylate oxygen coordinating Zn_1, while the other oxygen occupies the site of a water molecule that was localized between the two zinc ions, which are also very close (3.7 Å) in the crystal structure. As seen in Figure 3, the conformations of the β-phospholactam **1** docked to NDM-1 structures correspond very well to the co-crystallized hydrolyzed ampicillin and meropenem,[52] consistent with the similarity of the inhibitor with both the penicillin and carbapenem core. The more substrate-like binding mode in NDM-1 (Figure 3), where the Zn_1-Zn_2 distance is increased to 4.6 (PDB entry 4HL2) and 4.1 Å (PDB entry 4EYL), consistent with previous findings that the Zn_1-Zn_2 distance varies during the catalytic cycle[53, 54] as opposed to the binding mode with phosphinate oxygens coordinating both zinc ions when they are closer (Figures S1 and S2) could account for the slightly lower inhibition of NDM-1 versus that of IMP-1, CcrA, and L1 (Table 1). Based on the docking calculations, we cannot exclude the possibility that the inhibiting species is hydrolyzed β-phospholactam. When docking this product to NDM-1 (PDB entry 4EYL), the resulting phosphonate coordinated both zinc ions, but the carboxylate at C_7 did not form a salt bridge with Lys244 (data not shown). The most favorable binding energy was -12.4 kcal/mol, not significantly lower than -11.2 kcal/mol observed for the intact β-phospholactam **1**.

These structures also suggest how the binding affinity of this inhibitor scaffold could be improved. Currently, it only occupies the area close to the zinc ions. However, the active site of MβLs is rather a cleft and the inhibitor could be designed to better fill out that cleft by

extending the substituent at C_3 to something similar to R in penicillins (Figure 3A) and adding a substituent to C_7 that resembles R' of carbapenems (Figure 3C).

We have developed a prototype β-phospholactam analog of a carbapenem transition state, which exhibits broad-spectrum and time-dependent inhibitory activity against MβLs, with an inhibition percentage of 70% for IMP-1, CcrA, and L1, 53% for NDM-1, and 94% for Bla2. This study opened a way to develop β-phospholactam compounds as broad-spectrum inhibitors of MβLs.

Acknowledgements

This work was supported by grants (21272186 to K.W.Y) from the National Natural Science Fund of China and from the National Institutes of Health (GM093987 to M.W.C.) and National Science Foundation (CHE1151658 to M.W.C.). The Center for Macromolecular Modeling and Materials Design (CM_3D) at California State Polytechnic University, Pomona, where docking calculations were conducted, is supported by the W. M. Keck Foundation.

References

1. Mitcher, L. A.; Lemke, T. L.; Gentry, E. J. In *Foye's Principles of Medicinal Chemistry 6th ed.;* Lemke, T. L., Williams, D. A., Roche, V. F., Zito, S. W., Eds.; Wolters Kluwer Lippincott Williams & Wilkins, 2007.

2. Bush, K. *Curr. Opin. Pharmacol.* 2012, *12*, 527.

3. Bush, K.; Macielag, M. J. *Expert Opin. Thera. Patents* 2010, *20*, 1277.

4. Drawz, S. M.; Bonomo, R. A. *Clin. Microbiol. Rev.* 2010, *23*, 160.

5. Papp-Wallace, K. M.; Endimiani, A.; Taracila, M. A.; Bonomo, R. A. *Antimicro. Agents Chemo.* 2011, *55*, 4943.

6. Bush, K.; Jacoby, G. A. *Antimicro. Agents Chemo.* 2010, *54*, 969.

7. Fast, W.; Sutton, L. D. *Biochim. Biophys. Acta* 2013, *1834*, 1648.

8. Toney, J. H.; Hammond, G. G.; Fitzgerald, P. M.; Sharma, N.; Balkovec, J. M.; Rouen, G. P.; Olson, S. H.; Hammond, M. L.; Greenlee, M. L.; Gao, Y. D. *J. Biol. Chem.* 2001, *276*, 31913.

9. Lienard, B. M. R.; Garau, G.; Horsfall, L.; Karsisiotis, A. I.; Damblon, C.; Lassaux, P.; Papamicael, C.; Roberts, G. C. K.; Galleni, M.; Dideberg, O.; Frere, J. M.; Schofield, C. J. *Org. Biomol. Chem.* 2008, *6*, 2282.

10. Mollard, C.; Moali, C.; Papamicael, C.; Damblon, C.; Vessilier, S.; Amicosante, G.; Schofield, C. J.; Galleni, M.; Frere, J. M.; Roberts, G. C. K. *J. Biol. Chem.* 2001, *276*, 45015.

11. Horsfall, L. E.; Garau, G.; Lienard, B. M.; Dideberg, O.; Schofield, C. J.; Frere, J. M.; Galleni, M. *Antimicro. Agents Chemo.* 2007, *51*, 2136.

12. Lienard, B. M.; Horsfall, L. E.; Galleni, M.; Frere, J. M.; Schofield, C. J. *Bioorg. Med. Chem. Lett.* 2007, *17*, 964.

13. Hiromasa Kurosaki, Y. Y., Toshihiro Higashi, Kimitaka Soga, Satoshi Matsueda, Haruka Yumoto, Shogo Misumi, Yuriko Yamagata, Yoshichika Arakawa, Masafumi Goto,. *Angew.e Chem. Intern. Ed.* 2005, *44*, 3861.

14. Buynak, J. D.; Chen, H.; Vogeti, L.; Gadhachanda, V. R.; Buchanan, C. A.; Palzkill, T.; Shaw, R. W.; Spencer, J.; Walsh, T. R. *Bioorg. Med. Chem. Lett.* 2004, *14*, 1299.

15. Toney, J. H.; Moloughney, J. G. *Curr. Opin. Invest. Drugs* 2004, *5*, 823.

16. Mohamed, M. S.; Hussein, W. M.; McGeary, R. P.; Vella, P.; Schenk, G.; Abd El-Hameed, R. H. *Eur. J. Med. Chem.* 2011, *46*, 6075.

17. Bounaga, S.; Galleni, M.; Laws, A. P.; Page, M. I. *Bioorg. Med. Chem.* 2001, *9*, 503.

18. Bounaga, S.; Laws, A. P.; Galleni, M.; Page, M. I. *Biochem. J.* 1998, *331*, 703.

19. Yang, K. W. *Arch. Biochem. Biophys.* 1999, *368*, 1.

20. Lassaux, P.; Hamel, M.; Gulea, M.; Delbruck, H.; Mercuri, P. S.; Horsfall, L.; Dehareng, D.; Kupper, M.; Frere, J. M.; Hoffmann, K.; Galleni, M.; Bebrone, C. *J. Med. Chem.* 2010, *53*, 4862.

21. McManus-Munoz, S.; Crowder, M. W. *Biochemistry* 1999, *38*, 1547.

22. Wang, Z.; Benkovic, S. J. *J. Biol. Chem.* 1998, *273*, 22402.

23. Yang, H.; Aitha, M.; Hetrick, A. M.; Richmond, T. K.; Tierney, D. L.; Crowder, M. W. *Biochemistry* 2012, *51*, 3839.

24. Griffin, D. H.; Richmond, T. K.; Sanchez, C.; Moller, A. J.; Breece, R. M.; Tierney, D. L.; Bennett, B.; Crowder, M. W. *Biochemistry* 2011, *50*, 9125.

25. Sharma, N. P.; Hajdin, C.; Chandrasekar, S.; Bennett, B.; Yang, K. W.; Crowder, M. W. *Biochemistry* 2006, *45*, 10729.

26. Spencer, J.; Clark, A. R.; Walsh, T. R. *J. Biol. Chem.* 2001, *276*, 33638.

27. Grembecka, J.; Mucha, A.; Cierpicki, T.; Kafarski, P. *J. Med. Chem.* 2003, *46*, 2641.

28. Bradshaw, R. A. *Encyclopedia Biol. Chemistry* 2004, *1*, 96.

29. Copik, A. J.; Waterson, S.; Swierczek, S. I.; Bennett, B.; Holz, R. C. *Inorg. Chem.* 2005, *44*, 1160.

30. Wu, Z.; Walsh, C. T. *Proc. Nat. Acad. Sci.* 1995, *92*, 11603.

31. Wu, Z.; Wright, G. D.; Walsh, C. T. *Biochemistry* 1995, *34*, 2455.

32. Jacobsen, N. E.; Bartlett, P. A. *J. Am. Chem. Soc.* 1981, *103*, 654.

33. Bartlett, P. A.; Marlowe, C. K. *Biochemistry* 1983, *22*, 4618.

34. Bartlett, P. A.; Kezer, W. B. *J. Am. Chem. Soc.* 1984, *106*, 4282.

35. Hanson, J. E.; Kaplan, A. P.; Bartlett, P. A. *Biochemistry* 1989, *28*, 6294.

36. Bartlett, P. A.; Marlowe, C. K. *Biochemistry* 1991, *22*, 4618.

37. Kaplan, A. P.; Bartlett, P. A. *Biochemistry* 1991, *30*, 8165.

38. Irwin, J. J.; Raushel, F. M.; Shoichet, B. K. *Biochemistry* 2005, *44*, 12316.

39. Yuan, Q.; He, L.; Ke, H. *Antimicro. Agents Chemo.* 2012, *57*, 5157.

40. Pegg, K. M.; Liu, E. M.; Lacuran, A.; Oelschlaeger, P. *Antimicro. Agents Chemo.* 2013, *Epub*.

41. Garau, G.; Bebrone, C.; Anne, C.; Galleni, M.; Frere, J. M.; Dideberg, O. *J. Mol. Biol.* 2005, *345*, 785.

42. Widmann, M.; Pleiss, J.; Oelschlaeger, P. *Antimicro. Agents Chemo.* 2012, *56*, 3481.

43. Yang, K. W.; Brandt, J. J.; Chatwood, L. L.; Crowder, M. W. *Bioorg. Med. Chem. Lett.* 2000, *10*, 1087.

44. Page, M. I.; Laws, A. P. *Tetrahedron* 2000, *56*, 5631.

45. Yu, L.; Feng, D.; He, M.; Li, R.; Cai, Z. *J. Theor. Comput. Chem.* 2006, *5 Special Issue*, 421.

46. Page, M. I.; Laws, A. P.; Slater, M. J.; Stone, J. R. *Pure Appl. Chem.* 1995, *67*, 711.

47. Slater, M. J.; Laws, A. P.; Page, M. I. *Bioorg. Chem.* 2001, *29*, 77.

48. Wood, J. M.; Hinchliffe, P. S.; Laws, A. P.; Page, M. I. *J. Chem. Soc. Perkin Trans. I* 2002, *2*, 938.

49. Afarinkia, K.; Cadogan, J. I. G.; Rees, C. W. *J. Chem. Soc. Chem. Comm.* 1992, 285.

50. Daly, A. M.; Gilheany, D. G. *Tetrahedron: Asymmetry* 2003, *14*, 127.

51. Spencer, J.; Read, J.; Sessions, R. B.; Howell, S.; Blackburn, G. M.; Gamblin, S. J. *J. Am. Chem. Soc.* 2005, *127*, 14439.

52. Zhang, H.; Hao, Q. *FASEB J.* 2011, *25*, 2574.

53. Breece, R. M.; Hu, Z.; Bennett, B.; Crowder, M. W.; Tierney, D. L. *J. Am. Chem. Soc.* 2009, *131*, 11642.

54. Oelschlaeger, P.; Schmid, R. D.; Pleiss, J. *Protein Eng.* 2003, *16*, 341.

55. Humphrey, W.; Dalke, A.; Schulten, K. *J. Mol. Graphics* 1996, *14*, 33.

Table 1: Percent inhibition of MβLs by β-phospholactam **1**

Enzyme	**% inhibition**
L1	70 ± 7
IMP-1	70 ± 6
CcrA	69 ± 8
NDM-1	53 ± 6
Bla2	94 ± 4

The concentration of inhibitor was 100 μM.

Table 2: Summary of geometries of lowest-energy docked MβL-β-phospholactam **1** complexes (bold) in comparison to available MβL-hydrolyzed β-lactam complexes (italic).

Subclass	Complex	Binding Energy (kcal/mol)	$O(P_2)$-Zn1 *$O(C_2)$-Zn1* (Å)[e]	$O(P_2)$-Zn2 *$O(C_2)$-Zn2* (Å)[e]	N_1-Zn2 (Å)	Carboxylate(C_7) -Lys244 Nζ (Å)[f]
1	**IMP-1(1DD6)-βPL[a]**	**-10.8**	**1.9**	**1.8**	**3.1**	**2.9**
1	**CcrA(1A7T)-βPL**	**-11.9**	**1.8**	**1.9**	**3.1**	**2.7**
1	**NDM-1(4HL2)-βPL**	**-12.6**	**1.7/2.6**	**-**	**2.4**	**2.9**
1	*NDM-(4HL2)-hAMP[b]*		*2.4*	-	*2.2*	*2.9*
1	**NDM-1(4EYL)-βPL**	**-11.2**	**1.8/2.6**	**2.7**	**2.3**	**2.9**
1	*NDM-1(4EYL)-hMER[c]*		*2.3/2.7*	*2.5*	*2.2*	*3.2*
3	**L1(2AIO)_βPL**	**-11.8**	**2.0/2.1**	**1.9**	**2.8**	-
3	*L1(2AIO)-HOH_{600}*		*2.0*	*2.2*	-	**-**
3	*L1(2AIO)-hMOX[d]*		*2.4*	-	*2.4*	-

[a] βPL = β-phospholactam **1**

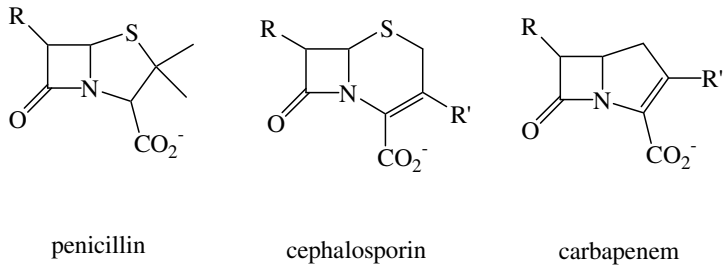
[b] hAMP = hydrolyzed ampicillin

[c] hMER = hydrolyzed meropenem

[d] hMOX = hydrolyzed moxalactam

[e] $O(P_2)$ and $O(C_2)$ designate the oxygen atoms bound to P_2 in the β-phospholactam **1** and C_2 in the β-lactams. Only atoms that are within a distance of 3.2 Å are reported. If both are within that distance, both are reported starting with the closer one.

[f] Average distance between the two carboxylate oxygens and Lys244 Nζ.



penicillin cephalosporin carbapenem

Figure 1. Structures of β-lactam-containing antibiotics.

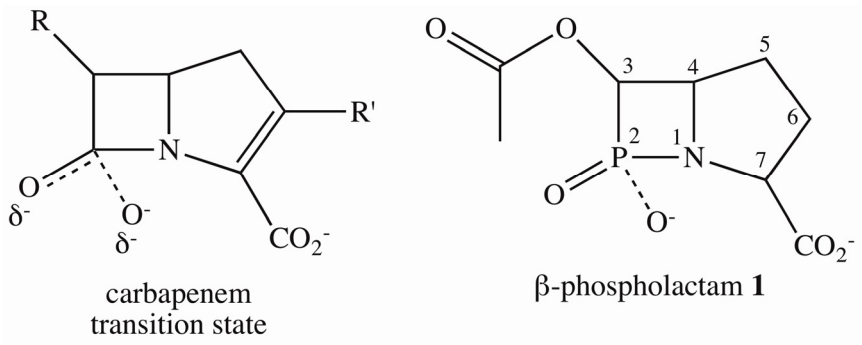


Figure 2. Structures of a hypothetical carbapenem transition state and β-phospholactam **1** synthesized in this study.

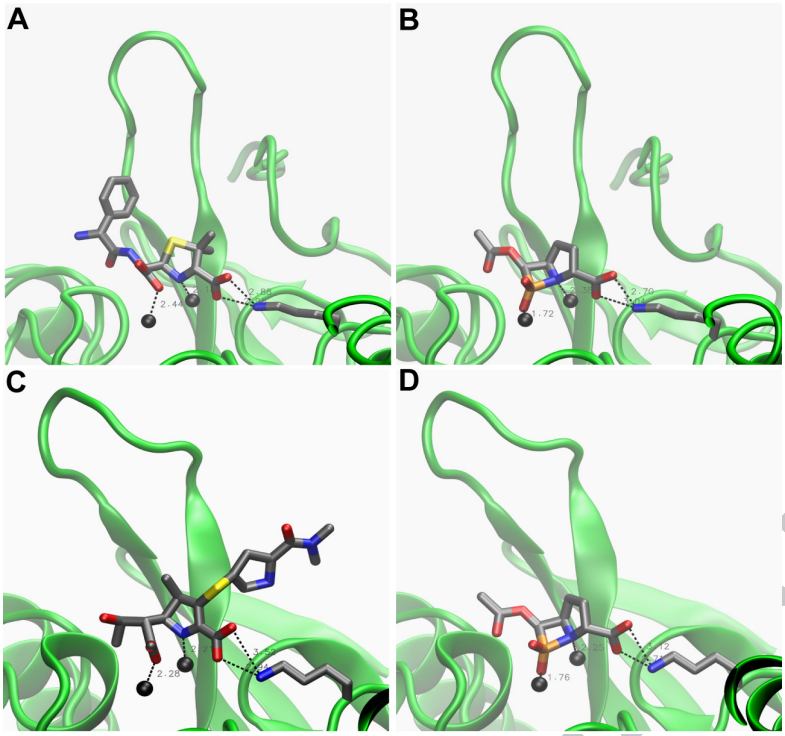
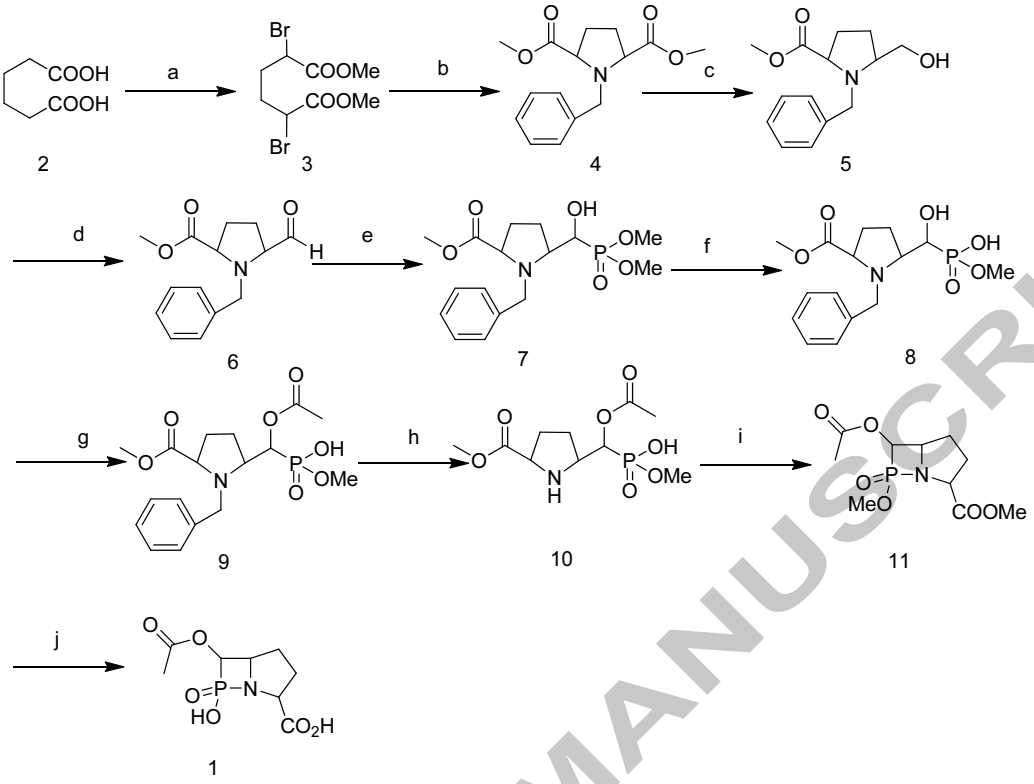


Figure 3. Complexes of NDM-1 co-crystalized with hydrolyzed β-lactams (A and C) and with the lowest-energy docked β-phospholactam **1** conformation (B and D). The coordinates of NDM-1 in panels A and B as well as hydrolyzed ampicillin in panel A are taken from PDB entry 4HL2. The coordinates of NDM-1 in panels C and D as well as hydrolyzed meropenem in panel C are taken from PDB entry 4EYL. The images were generated with VMD.[55] The protein backbone is shown as a green cartoon and zinc ions as black spheres (Zn_1 on the left and Zn_2 on the right). The β hairpin loop in the back is loop 3; loop 10 was removed for clarity. The hydrolyzed substrates as well as the β-phospholactam **1** inhibitor and the Lys224 side chain are shown as sticks colored by atom (C, gray; O, red, N, blue; S, yellow; P, orange). Key distances summarized in Table 2 are indicated by dashed lines.



Scheme 1. Synthetic route of β-phospholactam **1**: a: (1) acetyl chloride, (2) Br_2, (3) MeOH; b: benzylamine, K_2CO_3, CH_3CN; c: $NaBH_4$, EtOH; d: oxalyl chloride, DMSO, dichloromethane, -78 °C; e: dimethyl phosphonate, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), anhydrous THF; f: NaI, acetone; g: acetic anhydride, pyridine; h: Pd-C, H_2; i: NaH, 15-crown-5, dichloromethane; j: LiOH, H_2O, MeOH.

Supporting Information

New β-phospholactam as a carbapenem transition state analog: synthesis of a broad-spectrum inhibitor of metallo-β-lactamases

Ke-Wu Yang[a,*], Lei Feng[a], Shao-Kang Yang[a], Mahesh Aitha[b], Alecander E. LaCuran[c], Peter Oelschlaeger[c], and Michael W. Crowder[b,*]

[a]*Key Laboratory of Synthetic and Natural Functional Molecule Chemistry of Ministry of Education, College of Chemistry and Materials Science, Northwest University, Xi'an 710069, P. R. China;* [b] *Department of Chemistry and Biochemistry, Miami University, 160 Hughes Hall, Oxford, OH 45056, USA;* [c]*Department of Pharmaceutical Sciences, College of Pharmacy, Western University of Health Sciences, 309 E. Second St., Pomona, CA 91766, USA.*

Corresponding authors: Tel/Fax: +8629-8830-2429 (K.-W.Y.); +1-513-529-7274 (M.W.C.). E-mail address: kwyang@nwu.edu.cn (K.-W. Yang); crowdemw@MiamiOH.edu (M.W. Crowder)

Experiments

General methods

All starting materials were purchased from commercial sources and purified using standard methods. Analytical Thin Layer Chromatography (TLC) was carried out on silica gel F_{254} plates with visualization by ultraviolet radiation. ^{1}H, ^{31}P, and ^{13}C NMR spectra were recorded on a Varian INOVA400 MHz NMR spectrometer. Chemical shifts were given in part per million (ppm) on the delta scale. The peaks patterns are indicated as follows: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet doublet; m, multiplet. The spectra were recorded with TMS as internal standard or with 42.5% phosphoric acid as external reference. Coupling constants (*J*) were reported in hertz (Hz). Mass spectra were obtained on a micro TOF-Q (BRUKER) mass spectrometer. UV-visible spectra were recorded on an Agilent UV8453 spectrometer. Activity evaluation of inhibitors was performed on an Agilent 8453 UV-Vis spectrometer.

Dimethyl 2,5-dibromoadipate (3). Thionyl chloride (323 g, 2.71 mol) was added in 70 ml portions over 2 h to adipic acid (197 g, 1.35 mol) heated at 80 °C in a three-neck round bottom flask equipped with a reflux condenser and a constant pressure dropping funnel. The mixture was stirred until gas evolution ceased and partial solid adipic acid still remained. An additional 100 ml of thionyl chloride was added in 7 h, and heating was continued until gas evolution ceased. Bromine (473 g, 2.96 mol) was added dropwise to the pale yellow reaction mixture over an ice bath, and a white precipitate formed during the addition. The white precipitate was collected by filtration and recrystallized from MeOH to offer 253 g of **3** as

white power with a yield of 53%. ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 2.00~2.05 (m, 2H), 2.28~2.35 (m, 2H), 3.80 (s, 6H), 4.24~4.26 (t, 2H).

Dimethyl N-benzylpyrrolidine-2,5-dicarboxylate (4). A mixture of compound **3** (66.6 g, 0.2 mol), benzylamine (22.3 ml, 0.2 mol), potassium carbonate (33.4 g, 0.24 mol), toluene (140 ml), and H_2O (66 ml) was refluxed for 24 h. After cooling, the organic layer was separated, and the aqueous layer was extracted with hexane (2×100 mL). The organic layers were combined, washed with brine (2×100 ml), and dried over Na_2SO_4. The solvent was removed to give **4** as a colorless oil with a yield of 87%. ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): δ 2.02~2.07 (m, 4H), 3.41~3.45 (t, 2H), 3.56 (s, 6H), 3.91 (s, 2H).

Methyl N-benzyl-5-hydroxymethylproline ester (5). $NaBH_4$ (7.0 ml, 69.3 mmol) was added to a stirred solution of the dimethyl ester **4** (10.0 g, 66 mmol) in MeOH (150 ml). The reaction mixture was refluxed for 30 min, cooled to room temperature, and quenched by addition of cold water (50 ml). The mixture was extracted with dichloromethane (50 ml) and washed with a saturated solution of $NaHCO_3$ (3×20 ml) and brine (3×20ml), respectively. The organic phases were dried over Na_2SO_4, filtered, and evaporated *in vacuo* to give **5** as a pale-yellow oil in 45% yield. ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 1.73~2.08 (m, 1H), 3.01 (m, 1H), 3.32 (t, J = 10.8 Hz, 1H), 3.43 (s, 3H), 3.47 (d, 2H), 3.66~3.84 (dd, J = 36 Hz, 2H).

Methyl N-benzyl-5-(dimethoxyphosphoryl)(hydroxy)methylproline ester (7). To oxalyl chloride (2.7 ml, 27.6 mmol) in dry CH_2Cl_2 (20 ml) at -78 °C under argon was added

dropwise DMSO (4 ml, 55.2 mmol) in CH_2Cl_2 (5 ml). The mixture was stirred for 15 min, and compound **5** (5.0 g, 23 mmol) in CH_2Cl_2 (5 ml) was added over 30 min. After the reaction mixture was stirred at -78 °C for 30 min, triethylamine (9.0 ml, 59.8 mmol) was added, and the reaction was stirred for 30 min. The resulting mixture was warmed to room-temperature, poured into water, and extracted with CH_2Cl_2 (100 ml×3). The organic layer was washed sequentially with 1% HCl, water, 5% Na_2CO_3, water, and brine and dried over anhydrous Na_2SO_4. The solvents were removed *in vacuo* to afford aldehyde **6** as pale yellow oil, which was used directly in the next step reaction without further purification.

To dimethyl phosphite (1.5 ml, 16.5 mmol) in dry THF (20 ml) under argon was added 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (3 ml, 24 mmol). The reaction mixture was stirred for 1 h and cooled to 0 °C. Aldehyde **6** (4.7 g, 16 mmol) in THF (5 ml) was added, and the reaction was stirred at 0 °C for 48 h. The resulting mixture was poured into saturated NH_4Cl and extracted with ethyl acetate. The organic layer was washed twice with water and brine and dried over anhydrous $MgSO_4$. The solvents were removed *in vacuo*, and the residue was purified by column chromatography, eluting with ethyl acetate, to afford 5.2 g phosphonates **7** as a pale yellow oil, yield 91%. ^{31}P NMR (400 MHz, $CDCl_3$, δ ppm): 25.24, 25.78. ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 1.94~2.12 (m, 4H), 3.48 (s, 3H), 3.50~3.55 (m, 1H), 3.73~3.75 (dd, 2H), 3.78 (s, 3H), 3.83(s, 3H), 3.85~3.86 (t, 1H), 4.24~4.27 (d, *J*=12Hz, 1H), 7.22~7.36 (m, 5H). ^{13}C NMR (400 MHz, $CDCl_3$, δ ppm): 25.1, 30.2~30.9, 52.1, 53.3, 57.6, 60.1, 65.0~64.3, 66.4, 68.9, 70.5, 127.5, 128.8, 129.3, 128.4, 138.3, 176.1. HRMS(ESI^+) m/z: 380.1088 (Calcd for $[M+Na^+]^+$: 380.1341 m/z).

Methyl N-benzyl-5-(hydroxyl(hydroxy)(methoxy)phosphoryl)methylproline ester (8). A stirred mixture of compound **7** (0.43 g, 1.25 mmol), sodium iodide (0.19 g, 1.25 mmol), and acetone (3.0 g) was refluxed, and a clear solution was observed after 5 min. A white precipitate appeared after 45 min. The reaction mixture was cooled to 4 °C, and a white solid was filtered, washed with cooled acetone, and dried in the presence of P_2O_5 to offer phosphonic acid **8** as white powder, yield 60%. ^{31}P NMR (400 MHz, $CDCl_3$, δ ppm)：23.78. ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 1.82~1.92 (m, 4H), 3.35 (m, 1H), 3.40 (s, 3H), 3.48~3.49 (t, 1H), 3.61~3.67 (t, 3H), 3.77~3.78 (d, *J*=4Hz), 3.97~4.16 (dd, *J*=76Hz, 2H), 7.16~7.33 (m, 5H). ^{13}C NMR (400 MHz, $CDCl_3$, δ ppm): 24.9, 30.0, 30.8~30.0, 50.2, 51.8~52.0, 57.2, 58.5, 64.0, 64.8, 66.1, 69.7, 71.2, 127.3, 128.3, 129.5, 130.5, 137.4, 177.0. HRMS(ESI$^+$) m/z: 366.0955 (Calcd for [M+Na$^+$]$^+$: 366.1185)

Methyl N-benzyl-5-(acetoxy(hydroxy)(methoxy)phosphoryl)methylproline ester (9). Phosphonic acid **8** (300 mg, 0.44 mmol) in dry pyridine (5 ml) was treated with acetic anhydride (0.5 ml, 5.3 mmol) in the presence of 4-dimethylaminopyridine (DMAP) (50 mg), and the mixture was stirred for 24 h. The solvents were removed *in vacuo*, and the resulting residue was purified by column chromatography, eluting with ethyl acetate, to afford compound **9** as colorless waxy solid, 57% yield. ^{31}P NMR (400 MHz, $CDCl_3$, δ ppm)：16.91. ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 1.67~1.99 (m, 4H), 2.30 (s, 3H), 2.77 (t, 1H), 3.99 (s, 3H), 3.51~3.54 (d, *J*=12Hz, 6H), 3.77~3.80 (t, 1H), 3.85~3.86 (d, J=4Hz, 1H), 4.52~5.32 (dd, J=40Hz, 2H), 7.25~7.42 (m, 5H).

Methyl 5-(acetoxy(hydroxyl)(methoxy)phosphoryl)methylproline ester (10). A solution of compound **9** (0.44 g, 0.65 mmol) in MeOH (9 ml) was hydrogenated for 24 h in presence of H_2, 10% Pd/C (0.891 mg) and HCO_2NH_4 (2 × 0.286 g). The catalyst was removed by filtration through Celite, and the pad was washed with MeOH (5 × 10 mL). The solvent was removed *in vacuo* to afford crude product as a sodium salt. The crude product was purified by chromatographic separation on basic aluminum oxide and cation exchange resin and lyophilized to give the intermediate amine **10** as white powder, in 42% yield, which was hygroscopic. ^{31}P NMR (400 MHz, $CDCl_3$, δ ppm): 23.91. ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 1.94~2.43 (m, 4H), 2.23 (s, 3H), 3.62~3.64 (m, 1H), 3.67~3.70 (d, J=6Hz, 3H), 3.77 (s, 3H), 4.33~4.40 (m, 1H), 4.48~4.52 (t, 1H).

Methyl 3-acetoxy-2-methoxy-1-aza-2-phosphabicyclo [3.2.0] heptane-7-carboxylate 2-oxide (11). Phosphonic acid monoester **10** (5.0 mmol) was suspended in dry chloroform (50 ml), thionyl chloride was added (0.6 ml, 7.5 mmol) at 0 ^{o}C, and the mixture was stirred for 12 h at room temperature. The volatile components of the reaction mixture were evaporated under reduced pressure, and the resulting oily residue was redissolved in chloroform and evaporated again in order to remove hydrogen chloride and unreacted thionyl chloride. This step was repeated three times using dry chloroform. The resulting phosphonochloridate was dissolved in dry cooled CH_3CN (30 ml), and NaH was added. The mixture was stirred for two days at room temperature. The mixture was filtered to remove the undissolved salt, and the filtrate was evaporated *in vacuo* and purified by HPLC, eluting with 1:9 water/acetonitrile (flow rate 1 ml/min), to give β-phospholactam ester **11** as an oil, in

38% yield. ^{31}P NMR (400 MHz, D_2O, δ ppm): 15.48. ^{1}H NMR (400 MHz, D_2O, δ ppm): 1.95~2.45 (m, 4H), 2.21 (s, 3H), 3.59~3.62 (d, 3H), 3.69 (s, 3H), 3.91~3.96 (t, 1H), 4.35~4.40 (d, 1H), 4.45~4.50 (m, 1H). HRMS(ESI$^-$) m/z: 276.0625 (Calcd for [M-H$^+$]$^-$: 276.0715)

3-Acetoxy-2-hydroxy-1-aza-2-phosphabicyclo [3.2.0] heptane-7-carboxylic acid 2-oxide (1). Removal of the methyl groups of compound **11** was achieved by alkaline hydrolysis of the methyl ester in a 1.5 M LiOH/MeOH solution (2 equiv of LiOH was used). The solvent was removed by freeze-drying, and the mixture was treated with cation exchange resin to afford the β-phospholactam **1** as white solid, in 66% yield. ^{31}P NMR (400 MHz, D_2O, δ ppm): 17.10, 17.93. ^{1}H NMR (400 MHz, D_2O, δ ppm):1.94~2.45 (m, 4H), 2.13 (s, 3H), 3.85~3.95 (t, 1H), 4.08~4.11 (m, 1H), 4.27~4.29 (d, 1H). HRMS(ESI-) m/z: 248.0351 (Calcd for [M-H$^+$]$^-$: 248.0402 m/z)

Determination of inhibition percentage

Hydrolysis of nitrocefin was monitored at 482 nm using an Uvikon 8453 spectrophotometer. IMP-1, CcrA, L1, Bla2, and NDM-1 enzymes were used at fixed concentrations between 0.03 and 0.7 nM. β-Phospholactam **1** was dissolved in 50 mM cacodylate, pH 7.0, containing 50 μM $ZnCl_2$ and 15% DMSO; this was also the buffer used in the assays. In reactions that we did not pre-incubate the enzyme with inhibitor, we added the inhibitor (to a concentration of 100 μM) to a buffered solution of enzyme, then quickly added the substrate (at a concentration of 100 μM), and followed the hydrolysis of nitrocefin

at 482 nm. In the samples that we pre-incubated the enzyme with inhibitor, we added inhibitor to a stock of enzyme and allowed the enzyme/inhibitor mixture to incubate for 30 minutes at room temperature. We then took an aliquot of this mixture and added it to a buffered solution of nitrocefin and followed the hydrolysis of nitrocefin at 482 nm. All assays were conducted in triplicate, and data are reported as mean percentages of the rate without inhibitor present.

Docking calculations

The β-phospholactam **1** was docked into the active sites of the MβLs that it inhibited and for which high resolution structures are available: IMP-1 (PDB code 1DD6),[1] CcrA (PDB code 1A7T),[2] NDM-1 (PDB code 4HL2 (unpublished) and 4EYL,[3] and L1 (PDB code 2AIO).[4] The program AutoDock 4.2[5] and previously used charges (+1.4)[6] and van der Walls parameters (σ = 1.95 Å, ε = 0.25 kcal/mol)[7] for the zinc ions[8] were used and 50 conformations were generated for each complex. The flexible ligand was docked into each rigid monomeric receptor using a grid box with dimensions of 40 x 40 x 40 grid points equally spaced at 0.375 Å per grid and centered between the two active-site zinc ions. The maximum number of energy evaluations and generations were set to 2,500,000 and 27,000, respectively, and the mutation and crossover rates were set to 0.02 and 0.8, respectively. The rest of the parameters were set at their default values and all docking calculations were performed without constraints. Binding energies were calculated via the Lamarckian genetic algorithm and the conformations that constitute each cluster were defined by a root mean square deviation tolerance of 2.0 Å. The conformations with the lowest binding energy were

used for Figures 3, S1 and S2.

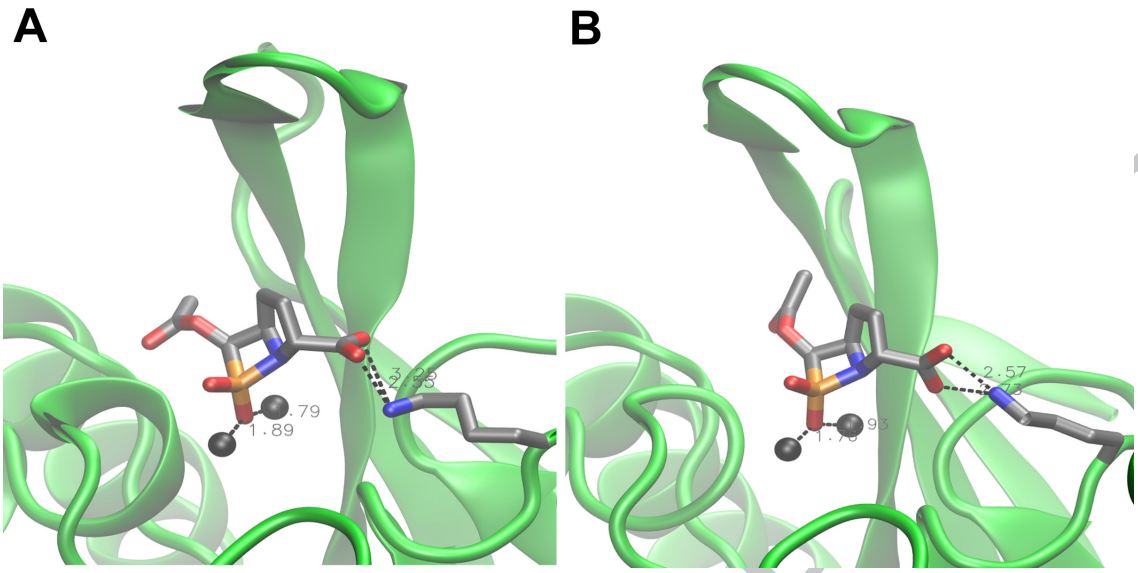


Figure S1. Complexes of IMP-1 (A) CcrA (B) with the lowest-energy docked β-phospholactam **1** conformation. The coordinates of IMP-1 and CcrA are taken from PDB entries 1DD6 and 1A7T, respectively. The images were generated with VMD.[9] The protein backbone is shown as a green cartoon and zinc ions as black spheres (Zn_1 on the left and Zn_2 on the right). The β hairpin loop in the back is loop 3; loop 10 was removed for clarity. The β-phospholactam **1** inhibitor and the Lys224 side chain are shown as sticks colored by atom (C, gray; O, red, N, blue; S, yellow; P, orange). Key distances below 3.0 Å summarized in Table 2 are indicated by dashed lines.

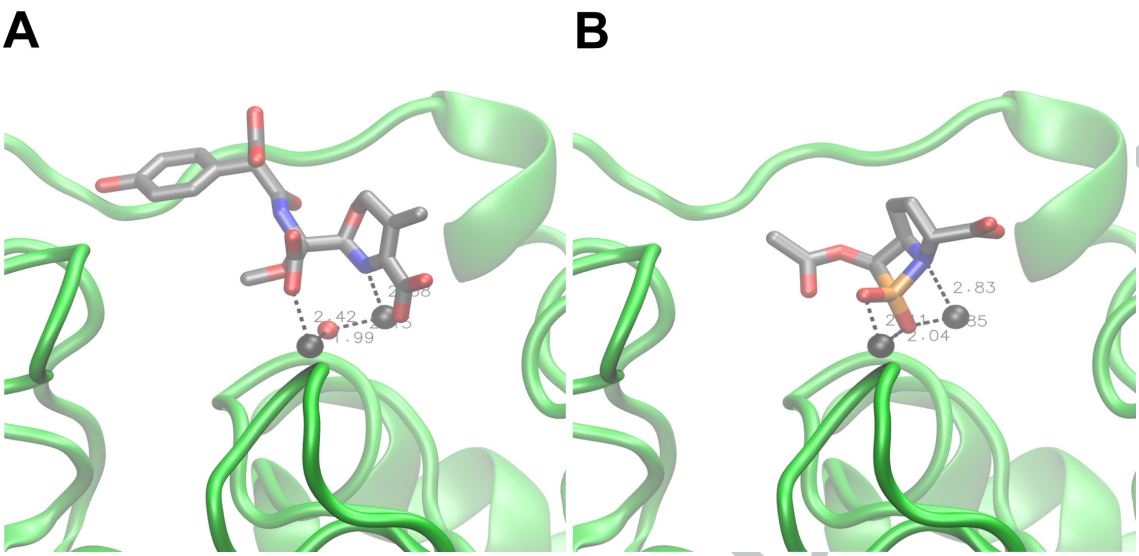


Figure S2. Complexes of L1 co-crystallized with hydrolyzed moxalactam (A, PDB entry 2AIO) and β-phospholactam **1** docked into the same structure (B). Orientation, rendering and color coding correspond to that in Figure S1. Loop 10, helix 4 and helix 5 were removed for clarity. The red sphere between Zn_1 and Zn_2 in A is a co-crystallized water molecule (HOH_{600}).

References

1. Concha, N. O.; Janson, C. A.; Rowling, P.; Pearson, S.; Cheever, C. A.; Clarke, B. P.; Lewis, C.; Galleni, M.; Frere, J. M.; Payne, D. J.; Bateson, J. H.; Abdel-Meguid, S. S. *Biochemistry* **2000,** *39*, 4288.

2. Fitzgerald, P. M. D.; Wu, J. K.; Toney, J. H. *Biochemistry* **1998,** *37*, 6791.

3. King, D. T.; Worrall, L. J.; Gruninger, R.; Strynadka, N. C. J. *J. Am. Chem. Soc.* **2012,** *134*, 11362.

4. Spencer, J.; Read, J.; Sessions, R. B.; Howell, S.; Blackburn, G. M.; Gamblin, S. J. *J. Am. Chem. Soc.* **2005,** *127*, 14439.

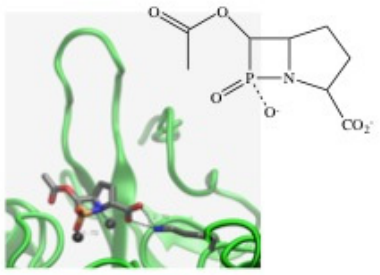
5. Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew, R. K.; Goodsell, D. S.; Olson, A. J. *J. Comput. Chem.* **2009,** *30*, 2785.

6. Irwin, J. J.; Raushel, F. M.; Shoichet, B. K. *Biochemistry* **2005,** *44*, 12316.

7. Stote, R. H.; Karplus, M. *Proteins-Struct. Func. Bioinformat.* **1995,** *23*, 12.

8. Pegg, K. M.; Liu, E. M.; Lacuran, A.; Oelschlaeger, P. *Antimicro. Agents Chemo.* **2013,** *Epub*.

9. Humphrey, W.; Dalke, A.; Schulten, K. *J. Mol. Graphics* **1996,** *14*, 33.



Graphical abstract