Supporting Information

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

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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.

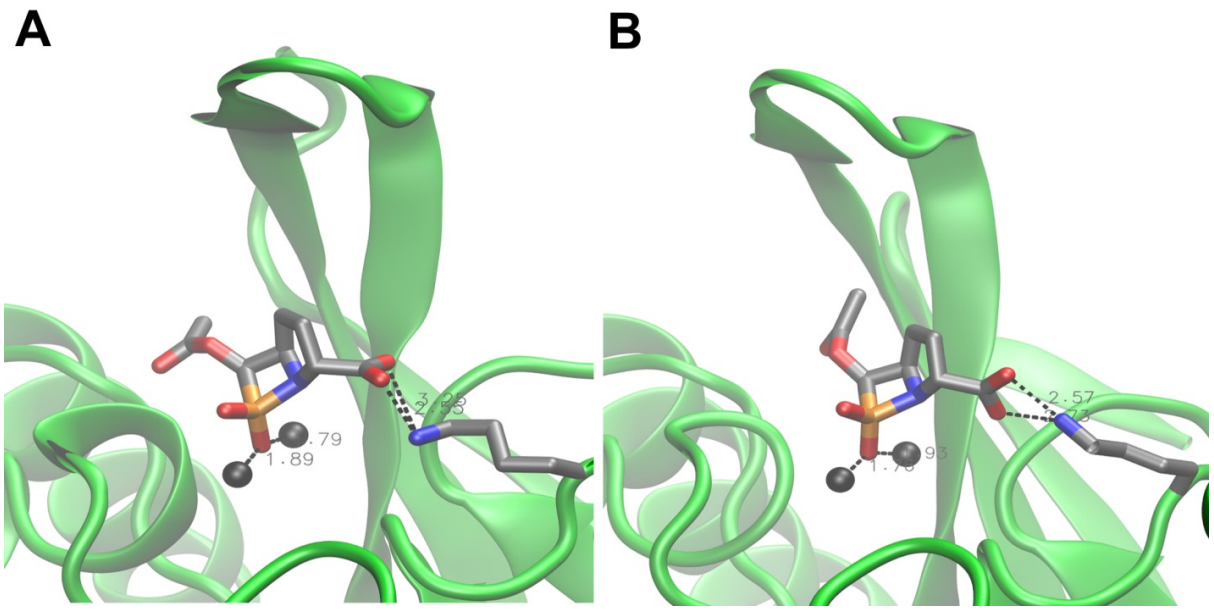

Figure S1. Complexes of IMP-1 (A) CcrA (B) with the lowest-energy docked β-phospholactam **1** conformation. The coordinates of IMP-1 and CcrA are taken from PDB entries 1DD6 and 1A7T, respectively. The images were generated with VMD.[9] The protein backbone is shown as a green cartoon and zinc ions 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.

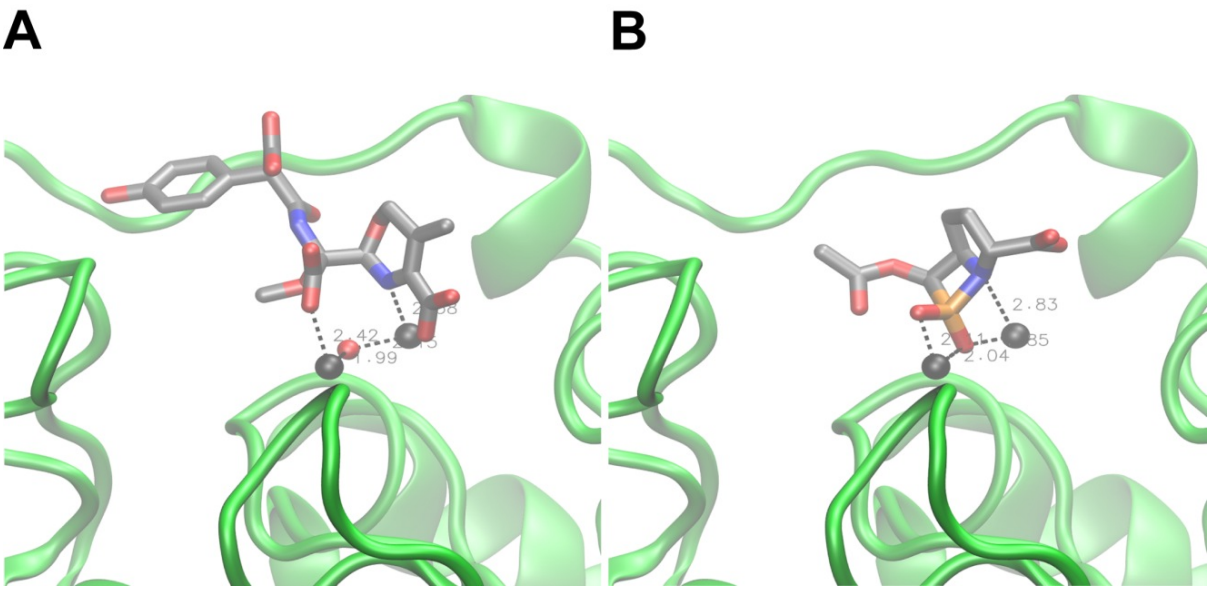

Figure S2. Complexes of L1 co-crystallized with hydrolyzed moxalactam (A, PDB entry 2AIO) and β-phospholactam **1** docked into the same structure (B). Orientation, rendering and color coding correspond to that in Figure S1. Loop 10, helix 4 and helix 5 were removed for clarity. The red sphere between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

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