Supporting Information

Structure-Activity Relationship of ^{18}F-labeled phosphoramidate peptidomimetic Prostate-Specific Membrane Antigen (PSMA)-targeted inhibitor analogues for PET imaging of prostate cancer

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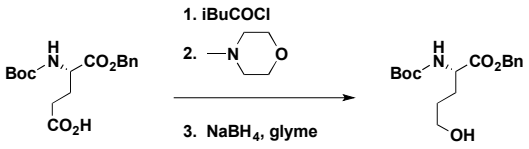
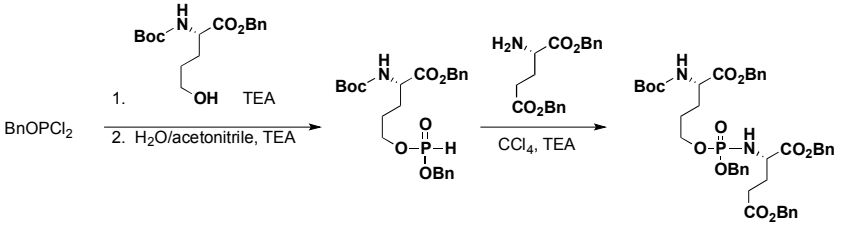
Section 1: Synthesis of 7 and precursors.



A

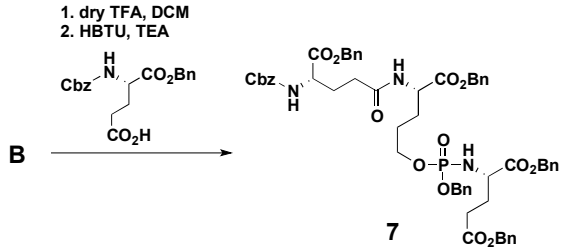
Benzyl 2-((tert-butoxycarbonyl)amino)-5-hydroxypentanoate, A. Boc-Glu(OBn) (1g, 1 equiv) and *N*-Methylmorpholine (3.55 mmol, 1.2 equiv) were dissolved in 3 mL glyme and stirred at -15C. iso-Butyloxychloride (2.96 mmol, 1 equiv) was then added and stirred for an additional 15 min. The resulting white precipitate was filtered off and $NaBH_4$ (4.44 mmol, 1.5 equiv) was added to the filterate along with 4 mL of water and stirred for 15 min. Th eraction mixture was dissolved in ethylacetate (EtoAc) and extracted with brine thrice . The organic layer was dried over Mg_2SO_4 and rotary-evaporated at 40°C. Pure product was obtained on drying (0.726 g, 76%). Characterization confirmed formation of the product.



B

Dibenzyl 2-(((R)-(benzyloxy)(((R)-5-(benzyloxy)-4-((tert-butoxycarbonyl) amino)-5-oxopentyl)oxy)phosphoryl)amino)pentanedioate, B. In a flame dried 100 mL flask, 10 mL dry dichloromethane (DCM) was taken, argon flushed and cooled over dried ice. PCl_2OBn (2.31 mM, 1.5 equiv) and triethyamine (1.855 mM, 1.2 equiv) was added and stirred. **A** (1.56 mM, 1 equiv) was dissolved in 10 mL of DCM and added to the reaction mixture in parts. After complete addition, dry ice was replaced with ice bath and stirred for 5 h. 1:1 mixture of water:acetonitrile (ACN) was added and stirred for additional 1h. The reaction mixture was rotary-evaporated to remove the organic solvent, dissolved in EtOAc, and washed successively with 10% HCl, 10% $NaHCO_3$, and brine. Organic layer was dried, concentrated to remove solvent, and dried overnight. Crude phosphite was dissolved in 10 mL dry ACN, flushed with $argon_{(g)}$, cooled on ice and 5 mL of CCl_4 was added. NH_2-Glu$(OBn)_2$ (1.546 mM, 1 equiv) and TEA (4.638 mM, 3.2 equiv) together were dissolved in 10 mL ACN and added to the phosphite in parts and stirred for 5 h. The reaction mixture was concentrated and purified by C-18 column chromatography using 80:20 MeOH:water as the mobile phase. Product **B** was obtained as pale yellow oil (36.7% yield). ^{1}HNMR (300 MHz, $CDCl_3$): δ 2.04 (s, 9H), 2.05–2.06 (m, 3H), 2.07 (m, 4h), 2.09 (m, 4h), 3.48–3.52 (m, 1H), 3.91 (t, 4h), 4.94 (m, 4h), 5.07 (m, 4h) , 7.30-7.31 (m, 20H). ^{13}C NMR (300 MHz, $CDCl_3$): δ 28.5, 28.9, 30.0, 53.8, 53.9, 66.6, 66.7, 67.2, 67.5, 76.9, 77.3, 77.7, 135.5, 154.8, 172.61, 172.65. ^{31}P NMR (300

MHz, $CDCl_3$): δ 8.41, 8.44. ESI mass spectroscopy (M+Na): Calculated 802.3, found 825.3 for $C_{43}H_{51}N_2O_{11}P^+$.



(2S)-dibenzyl 2-(((benzyloxy)(((S)-5-(benzyloxy)-4-((S)-5-(benzyloxy)-4-((tert-butoxycarbonyl)amino)-5-oxopentanamido)-5-oxopentyl)oxy)phosphoryl)amino) pentanedioate, 7. Cbz-Glu(OBn) (0.15g, 1equiv) was dissolved in 3mL of dry DMF in a flame dried flask and argon flushed. HBTU (0.44mmol, 1.1 equiv.) and triethylamine (0.44mmol, 1.1 equiv.) was added and stirred for 30 minutes for pre-activation of the carboxylic acid. In a separate flask, **B** was dissolved in 2mL dry DCM, argon flushed and cooled over ice bath. 1mL of dry TFA was added and stirred for 15 min. DCM was then evaporated off, reaction mixture dissolved in ethylacetate and washed with 10% $NaHCO_3$ (till pH neutralized), brine and organic layer dried on anhy. Na_2SO_4. It was then redissolved in 2mL dry DMF added to the flask with the pre-activated acid and stirred overnight under $Argon_{(g)}$. The reaction mixture was dissolved in ethyleacetate, and washed with 10% $NaHCO_3$ and brine. Organic layer dried over Na_2SO_4 and dried under vaccum. Purification was carried out using reversed phase C18 chromatography with 80% MeOH-water as the mobile phase. Pure **7** was isolated in 40% yield. ^{1}HNMR (300 MHz, $CDCl_3$): δ 1.45-1.6 (m, 3H), 1.80-1.98 (m, 2H), 2.04-2.18 (m, 2H), 2.20–2.34 (m, 3H), 2.38–2.48 (m, 2H), 3.45 (m, 1H), 3.82 (t, 2h), 4.37 (m, 1H), 4.56 (m, 1H), 4.81-5.00 (m, 2h), 5.04-5.2 (m, 10H) , 5.89 (d, 1H, -NH), 6.56 (d, 1H, -NH), 6.70 (d, 1H, -NH), 7.22-7.40 (m, 25H). ^{31}P NMR (300 MHz, $CDCl_3$): δ 8.46. ESI mass spectroscopy (M+Na): calculated 1055.40 (M), found 1078.5 for $C_{58}H_{62}N_3O_{14}P^+$.

Section 2: IC_{50} and Mode of inhibition determinations.

General method of IC_{50}. Inhibition studies were performed as previously described with minor modifications.[1, 2] Description is provided in Supplementary material (Section-2). Briefly, working solutions of the substrate N-[4-phenylazo-benzoyl]-glutamyl-γ-glutamic acid, (PABGgG) and inhibitor were prepared in Tris buffer (50 mM, pH 7.4). Working solutions of purified PSMA were diluted in Tris buffer (50 mM, pH 7.4 containing 1% Triton X-100) to provide 15-20% conversion of substrate to product in the absence of inhibitor. A typical incubation mixture (final volume of 250 µL) was prepared by the addition of either 25 µL of an inhibitor solution or 25 µL TRIS buffer (50 mM, pH 7.4) to 175 µL TRIS buffer (50 mM, pH 7.4). PABGgG (25 µL, 10 µM) was added to the

above solution. The enzymatic reaction was initiated by the addition of 25 µL of the PSMA working solution. In all cases, the final concentration of PABGgG was 1 µM while the enzyme was incubated with five serially diluted inhibitor concentrations providing a range of inhibition from 10% to 90%. The reaction was allowed to proceed for 15 min with constant shaking at 37 ^{o}C and was terminated by the addition of 25 µL methanolic TFA (2% v/v trifluoroacetic acid in methanol) followed by vortex. The quenched incubation mixture was quickly buffered by the addition of 25 µL K_2HPO_4 (0.1 M), vortexed, and centrifuged (10 min at 7,000 g). An 85 µL aliquot of the resulting supernatant was subsequently quantified by HPLC as previously described.[3, 4] IC_{50} values were calculated using KaleidaGraph 3.6 (Synergy Software, Reading, PA).

Mode of inhibition Study. The mode of inhibition studies followed the procedure described in our previous work.[5] Description is provided in Supplementary material (Section-2). The concentration of PSMA (2.5µg/mL) was 100-fold greater than used in the typical ezyme activity assays. The enzyme was pre-incubated for 10 minutes with 0.1 µM of inhibitor (40 µL), at approximately 10-fold greater than the IC_{50} value. The solution was diluted with 1 mM of substrate in 50 mM tris + 1% triton buffer (100-fold, total volume 3960 µL). The formation of product was monitored every 5 minutes for 1 hour. A control sample was defined as incubation described here without inhibitor.

Section 3: Crystallographic studies - Structure determination and refinement.

Table S1. Data collection and refinement statistics

Data collection statistics		
Inhibitor	**4**	**6**
PDB code	4LQG	---
Wavelength (Å)	0.918	0.918
Space group	I222	I222
Unit-cell parameters *a, b, c* (Å)	101.9, 130.3, 158.3	100.4, 130.5, 157.6
Resolution limits (Å)	50-1.77 (1.87-1.77)	50-1.71 (1.81-1.71)
Number of unique reflections	102,407 (15967)	111,302 (17797)
Redundancy	4.15 (4.14)	5.82 (5.80)
Completeness (%)	99.4% (97.4%)	99.7% (99.4%)
$I/\sigma I$	13.47 (2.36)	23.42 (3.60)
R_{merge}	0.093 (0.693)	0.052 (0.551)
Refinement Statistics		
Resolution limits (Å)	19.51-1.77 (1.811-1.766)	29.25-1.71 (1.754-1.710)
Total number of reflections	97,284 (7,086)	105,653 (7,750)
Number of reflections in working set	92,164 (6,731)	100,093 (7,342)
Number of reflections in test set	5,120 (355)	5,560 (408)
R/R_{free} (%)	15.9/18.2	16.3/18.0
Total number of non-H atoms	6791	6438
Number of non-H protein atoms	5923	5791
Number Inhibitor molecules	1	1
Number of water molecules	591	409
Average B-factor ($Å^2$)	13.7	16.8
Protein atoms	12.2	15.6
Waters	20.8	22.2
Inhibitor	31.8	63.7
[&]Ramachandran Plot (%)		
Most favored	97.4	97.8
Additionally allowed	2.5	2.1
Disallowed	0.1	0.1
R.m.s. deviations: bond lengths (Å)	0.019	0.016
bond angles (°)	1.7	1.5
planarity (Å)	0.009	0.009
chiral centers ($Å^3$)	0.1	0.1
Missing residues	545-546, 654- 655	654-655

* Values in parenthesis are for the highest resolution shells.

[&] Structures were analyzed using the MolProbity package

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