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TITLE: Synthesis of PSA Inhibitors as SPECT- and PET-Based Imaging Agents for Prostate Cancer

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14. ABSTRACT Prostate-Specific Antigen (PSA) is a well known serine protease used extensively as a biomarker for prostate cancer. PSA is produced by the normal epithelial cells of the prostate. High levels of enzymatically active PSA (high µg to mg/ml concentrations, in the extracellular fluid) are produced by androgen dependent as well as androgen independent prostate cancers. Even though the expression of PSA is closely related to the cancer progression its role in the cancer pathobiology is not well established. To aid the better understanding of PSA role in prostate cancer development and to expand the number of possible therapeutic agents we prepared a library of novel peptidyl boronic acid based inhibitors. Protease inhibitors commonly bind to their targets through intermolecular hydrogen bonds and/or disulfide bonds. To prepare more selective inhibitors we modified the S1 binding site of the inhibitors to incorporated bromopropylglycine group. This allowed the inhibitors to reach deeper in the specificity pocket and participate in halogen bond formation. The new candidates for targeted inhibition were screened for their ability to inhibit PSA and chymotrypsin.					
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Introduction

Prostate cancer is the most common cancer among men of all races and the second leading cause of cancer death, according to the CDC. Nearly 1 in 6 men will develop prostate cancer in their lifetime and as a result about 30,000 of them will die this year in US alone (1, 2). Generally, inflammatory processes in the prostate are related to higher PSA production. While a small percentage of poorly differentiated prostate cancers produce low levels of PSA, the majority (>95%) continue to produce high levels that can be readily measured in the blood of patients as the disease progresses from hormone sensitive to castration resistant to chemotherapy refractory (3-8). There are two major clinical problems associated with the treatment of metastatic prostate cancer. First, all men undergoing androgen ablation, eventually relapse and no longer respond to hormone treatment. Therefore, there is an urgent need for more effective therapies for patients with metastatic disease. In addition, the clinical development of such novel therapies is limited by the inability to adequately measure the response of prostate cancer metastases in the majority of patients. The lack of sensitive imaging techniques limits not only the follow-up of patients' response to therapy but also the detection of prostate cancer metastases. The development of new therapeutics is also highly dependent on the existence of selective biomarkers and proven imaging techniques. Therefore, intervention at this level and efforts resulting in decrease of the active PSA may subsequently slow down the advancement of the disease and may become an efficient way to control the cancer progression. Based on the PSA specific cleavage map and earlier identified substrates we developed potent and selective PSA inhibitors with inhibition constant in the nanomolar range.

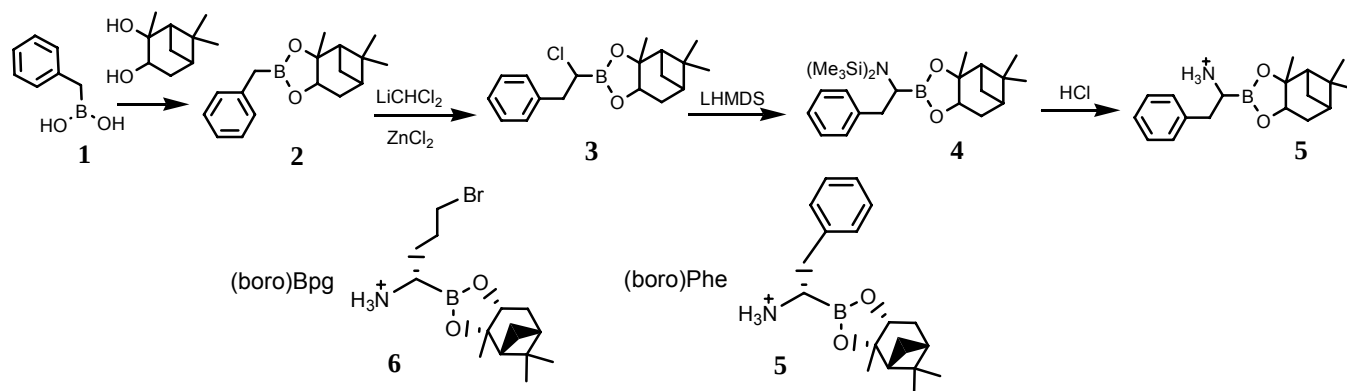
Body

In partial fulfillment of the goals of this proposal the following tasks outlined in the approved Statement of Work are completed.

Task 1: Synthesize a series of boronic acid - peptide conjugates as PSA inhibitors and characterize them for their ability to selectively bind to and inhibit the enzymatic activity of PSA vs. other extracellular proteases. (Completed)

1. Synthesis, purification and characterization of peptidyl boronic acids:

Using the synthetic schemes suggested in the proposal we were able to prepare phenylalanine boronic acid. The final product was achieved using commercially available starting materials and well studied protocols developed by Matteson and Winssinger (9, 10).



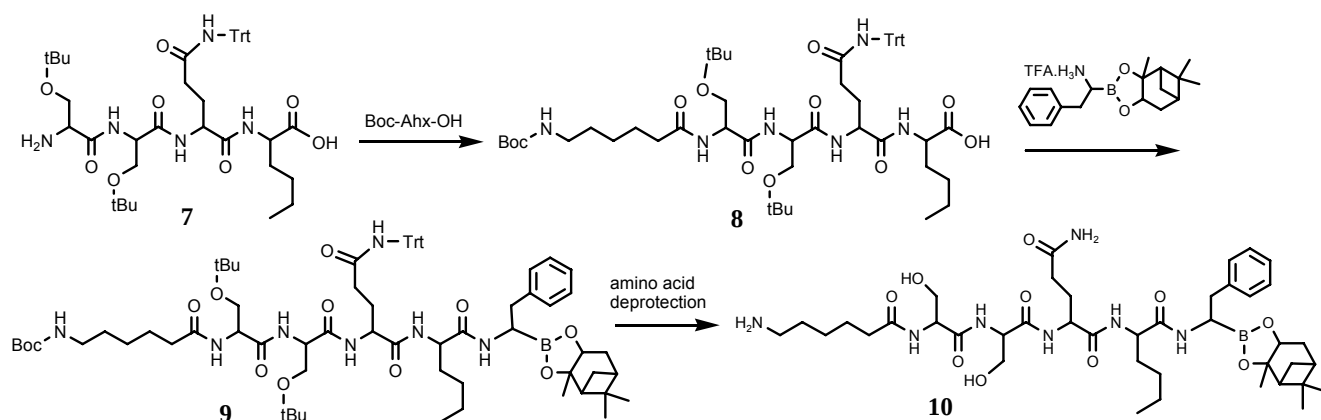
Scheme 1. Synthesis of phenylalanine boronic acid and structure of bromopropylglycine boronic acid

We were unable to prepare methionine and glutamine derivatives of boronic acids due to synthetic difficulties. However, we wanted to compare aromatic vs. aliphatic residue for a better fit for the active site. Thus, bromopropylglycine boronic acid was selected because it somewhat mimics hydrophobic residues like Leu and Nle, which have shown optimal binding due to their medium length hydrocarbon chains. The polar character of the bottom of the pocket prompted us to select polar ending for the amino acid residue such as bromine.

All final products were purified using reverse phase semi-preparative HPLC. The products were characterized by ^1H and ^{13}C NMR on a 400 MHz Bruker Advance NMR Spectrometer. Mass spectra were measured on a Bruker 3000 Esquire Mass Spectrometer equipped with ESI. All synthetic protocols and structural characterizations for the boronic acids and their precursors are reported in the Appendices section.

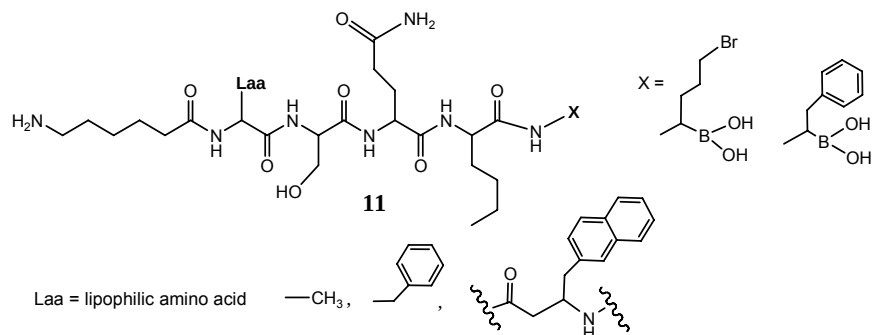
2. Synthesis, purification and characterization of inhibitors containing other natural and unnatural amino acids and/or peptidomimetics in P4:

Eleven peptidyl boronic acids were successfully prepared according to the suggested procedure described in the grant proposal. The peptide moieties were prepared on a solid phase support using Apex 396 peptide synthesizer. The products were obtained using standard Fmoc protocol, starting with preloaded Fmoc-Nle-OH onto a 2-ClTrt resin. The exact protocol is described in the Appendices section.



Scheme 2. Synthesis of peptidyl boronic acid

Inhibitors containing Alanine, Phenylalanine and Naphthylalanine at P4 were also prepared and tested. Replacing the second serine with a hydrophobic amino acid allowed us to manipulate the lipophilicity of the peptides with expectation of delaying the clearance.



Scheme 3. General structure of peptide boronic acid and lipophilic amino acid substituents at P4

The final products were purified on a semi-prep RP-HPLC column from Phenomenex: Luna C18, particle size 10 μ , 250 x 10 mm. The HPLC used for the analysis and purification was equipped with photodiode array detector and purchased from Waters (Milford, MA).

Of the HPLC methods developed for the purification of the peptides the following two had the best purification conditions:

^a- HPLC method: gradient of 15-90% buffer B over 25 min at 5 mL/min

^b- HPLC method: gradient of 10-95% buffer B over 25 min at 5 mL/min

Buffer A = 0.1% TFA in 5% MeOH and 95% water

Buffer B = 0.1% TFA in MeOH

The retention times (R_t , min) of all products are reported in Table 1.

3. K_i determination for PSA inhibition:

Candidate inhibitors were incubated at different concentrations with enzymatically active PSA (Calbiochem) in the presence of the PSA substrate Mu-SRKSQQY-AMC (K_m = 140 μ M) (California Peptides). Change in fluorescence due to proteolytic release of AMC was followed using a 96 well fluorometric plate reader. The velocity of each reaction (change in RFU/time) was determined and plotted vs. concentration of the inhibitors.

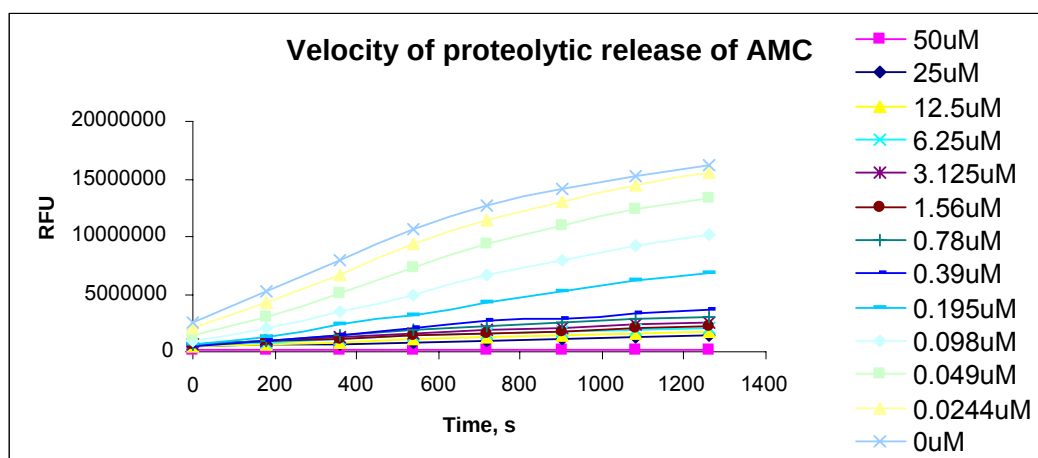


Figure 1. Time progression curves for the inhibition of PSA by Z-SSKn(boro)Br over a range of concentrations

The inhibition constant (K_i) of each compound was determined by solving the equation: $K_i = 1/\text{slope} \times 1/(1+[S]/K_m)$, where the slope is obtained from a linear plot of $V_o/V_i - 1$ vs. $[I]$. The reciprocal of the slope is also equal to the $K_{i(\text{app})}$, apparent inhibition constant. $[S]$ = substrate concentration, $[I]$ = inhibitor concentration, K_m = Michaelis constant, V_o = rate of substrate hydrolysis in the absence of inhibitor, and V_i = rate in the presence of inhibitor. The K_i values for PSA inhibition are reported in Table 1.

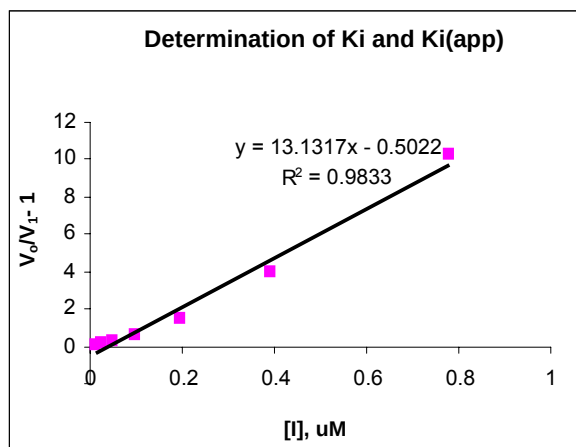


Figure 2. Linear plot of $V_0/V_i - 1$ vs. $[I]$ for Z-SSKn(boro)Br used for calculation of K_i

4. Selectivity evaluation of individual inhibitors with K_i for PSA of <200 nM against extracellular proteases:

The inhibitors were assayed for their specificity for PSA relative to chymotrypsin using the method described above. Suc-AAPF-AMC (Sigma, USA) was used as a substrate for Chymotrypsin (Sigma, USA). The determined inhibitory constants for Chymotrypsin are reported in Table 1. All assays were performed in triplicate and the average value at each point was plotted and reported.

Inhibitor	m/z, [M+H-18] ⁺ , [M+H] ⁺	R _t , min	K _i (nM) PSA inhibition	K _i (nM) Chymotrypsin inhibition
Z-SSKn(boro)F	697.0	25.6 ^a	164.503	18.108
Z-SSKn(boro)Br	729.0	24.2 ^a	24.228	196.882
Ahx-SSQn(boro)L	642.1, 660.0	21.9 ^b	43.053	137.837
Ahx-SQn(boro)F	589.4, 607.4	20.9 ^a	22.01	3.765
Ahx-SQn(boro)Br	620.9, 638.9	19.0 ^a	302.096	250.575
Ahx-ASQn(boro)F	660.0, 678.0	23.1 ^b	310.286	36.22
Ahx-ASQn(boro)Br	690.1, 708.3	20.7 ^a	52.11	165.921
Ahx-FSQn(boro)F	736.6, 754.5	24.5 ^a	61.484	0.135
Ahx-FSQn(boro)Br	768.5, 786.5	22.9 ^a	72.288	580.724
Ahx-NaphSQn(boro)F	800.0, 818.0	24.6 ^a	228.782	7.214
Ahx-NaphSQn(boro)Br	831.9, 849.8	24.9 ^a	102.463	505.676

^a- HPLC method: gradient of 15-90%, 0.1% TFA in MeOH over 25 min at 5 mL/min

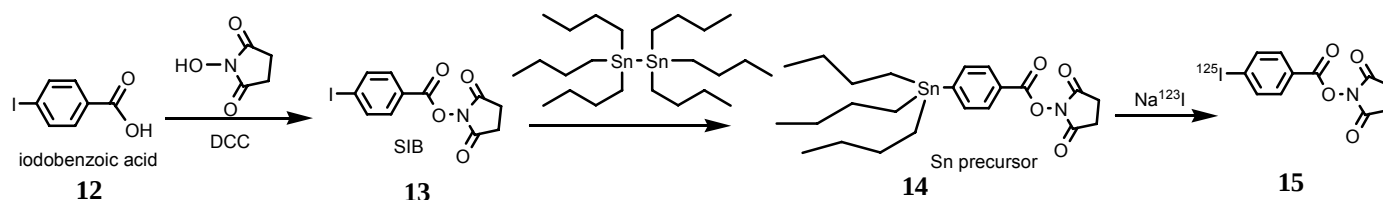
^b- HPLC method: gradient of 10-95%, 0.1% TFA in MeOH over 25 min at 5 mL/min

Table 1. Chemical structures, exact masses detected by ESI/MS, HPLC retention times and inhibition constants of PSA inhibitors

Task 2: Selected PSA inhibitors will be labeled with I-125 at the amino terminus. The pharmacokinetics of these inhibitors will be evaluated *in vivo* in animals bearing isogenic PSA positive and negative prostate cancer xenografts. (Experiment in progress)

1. Optimization of HPLC purification method for each labeled inhibitor:

An optimal solvent condition for analytical RP-HPLC purification is being determined based on the polarity of the products. For this task non-radioactive iodine labeled products are being prepared. For the cold runs N-hydroxysuccinimidyl-4-iodobenzoate (SIB) was prepared in one step according to procedure reported by Vaidyanathan (11). The following synthetic scheme is being used to prepare the precursor for radiolabeling.



Scheme 4. Synthesis of ¹²⁵I-SIB for radioiodination of peptidyl boronic acids

The products were purified on analytical RP-HPLC column from Phenomenex: Luna C18, particle size 5 μ , 150 x 4.60 mm.

HPLC method: gradient of 30-100% buffer B over 16 min at 1.25 mL/min, then gradient of 0-70% buffer A over 12 min at the same flow rate.

Buffer A = 0.1% TFA in 5% acetonitrile and 95% water

Buffer B = 0.1% TFA in acetonitrile

The following retention times were determined: iodobenzoic acid - 10.57 min, SIB - 12.54 min, and Sn-precursor - 24.40 min.

Key Research Accomplishments

- Synthesized, purified and characterized a library of eleven peptidyl boronic acids.
- Explored the inhibition potential of (boro)Bpg vs. (boro)Phe and compared aromatic vs. aliphatic substituent of the boronic acid.
- Examined the importance of serine at P4 and the possibility of replacing it with hydrophilic amino acids, such as alanine, phenylalanine and naphthylalanine.
- Evaluated the products as inhibitors of PSA by following the enzyme kinetics.
- Evaluated the selectivity of PSA inhibitors by determining the inhibitory constants for chymotrypsin.
- Prepared the precursors for radiolabeling and the non-radioactive iodine carrier and established their HPLC purification conditions.

Reportable Outcomes

Based on the conducted research and results from the study the PI had submitted two abstracts and attended two conferences:

- “Novel peptidyl boronic acid based inhibitors of PSA”, M. B. Kostova, D. M. Rosen, S. R. Denmeade at 240th ACS National Meeting, Boston, MA, August 2010
- “Synthesis and biological evaluation of selective inhibitors of prostate-specific antigen”, M. B. Kostova, D. M. Rosen, S. R. Denmeade at CDMRP IMPaCT Conference, Orlando, FL, March 2011

The PI also co-authored the following article:

- LeBeau AM, Kostova M, Craik CS, Denmeade SR. Prostate-specific antigen: an overlooked candidate for the targeted treatment and selective imaging of prostate cancer. *Biol Chem.* 2010; 391:333-43.

The PI has also presented the findings at our local Chemical Therapeutics program meetings at Johns Hopkins University.

Conclusion

Base on previously published data of peptidyl aldehydes we were able to create a new library of drug candidates with improved inhibitory properties. The presence of bromine at the end of the side chain of the active site residue allows for halogen bond formation. The formation of such halogen bonds provides a unique base for inhibitor - enzyme recognition, therefore we were able to prepare more specific and selective inhibitors of PSA. Also, the results of this study revealed additional information about the structure of the active site of PSA and the structural requirements of a nanomolar inhibitor.

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Appendices

(+)-Pinanediol Phenylmethyl-1-boronate (2). To a solution of 1 g (7.36 mmol) benzyl boronic acid 1 in 80 mL of ether were added 1 eqv. of (+)-pinanediol (1.25 g) and 3 g of MgSO_4 . The solution was stirred for 2-3 days and the product formation was followed by NMR. The reaction mixture was filtered out and the organic layer was washed with satd. NaHCO_3 (20 mL x 2) and brine (20 mL x 2). After drying the organic layer was concentrated under low pressure and kept under high vacuum overnight to yield 85% colorless oil. The product was used for the next step without purification. ^1H NMR (CDCl_3): δ 0.85 (s, 3H), 1.07 (d, J = 11.0, 1H), 1.28 (s, 3H), 1.39 (s, 3H), 1.9 (m, 2H), 2.05 (m, 1H), 2.2 (m, 1H), 2.28 (m, 1H), 2.35 (s, 2H), 4.29 (dd, J = 8.9, 2.1, 1H), 7.16-7.34 (m, 5H); ^{13}C NMR (CDCl_3): δ 23.95, 26.50, 28.31, 28.68, 36.22, 38.36, 39.48, 51.31, 78.34, 85.89, 110.64, 124.84, 128.47, 129.13, 138.86; MS (ESI) m/z 288.0 $[\text{M}+\text{H}_2\text{O}]^+$, 293.1 $[\text{M}+\text{Na}]^+$.

(+)-Pinanediol (S)-1-chloro-2-phenylethaneboronate (3).(12, 13) In a three-neck flask equipped with thermometer, a solution of 10 mL of freshly distilled THF and 1.3 mL of DCM was cooled to -100°C (in ethanol/liquid nitrogen bath) under argon atmosphere. Chilled at -78°C $n\text{-BuLi}$ (8.6 mmol; 2.5 M in hexane) was added via syringe by running it down the cold wall of the reaction flask over a period of 15 min. White precipitate of LiCHCl_2 was formed and after 10 min of stirring, cold solution of 0.38 g (1.4 mmol) of (+)-pinanediol phenylmethyl-1-boronate 2 in 2 mL of dry THF was added. After 10 min of stirring at -100°C , 8 mL of ZnCl_2 solution (0.5 M in THF) were added. The solution was stirred overnight and the temperature was allowed to rise to room temperature. The reaction mixture was evaporated and re-dissolved in hexane. The organic phase was washed with satd. NH_4Cl , water, and brine, then dried and concentrated to give about 80% crude product as colorless oil. The product was used for the next step without purification. ^1H NMR (CDCl_3): δ 0.84 (s, 3H), 1.08 (d, J = 11.2, 1H), 1.28 (s, 3H), 1.36 (s, 3H), 1.90 (m, 2H), 2.08 (m, 1H), 2.19 (m, 1H), 2.35 (m, 1H), 3.11 (dd, J = 8.6, 13.7, 1H), 3.23 (dd, J = 7.9, 13.9, 1H), 3.68 (dd, J = 8.5, 7.8, 1H), 4.46 (dd, J = 2.0, 11.3, 1H), 7.20-7.35 (m, 5H); ^{13}C NMR (CDCl_3): δ 23.95, 26.22, 27.02, 28.33, 29.71, 35.15, 38.24, 39.35, 40.45, 51.22, 78.56, 86.79, 126.72, 126.75, 128.35, 129.20, 129.24, 138.42. MS (ESI) m/z 283.0, $[\text{M}-\text{Cl}+\text{H}]^+$.

(+)-Pinanediol (R)-1-(N-Bis(trimethylsilyl)amino)-2-phenylethane-1-boronate (4). An oven dried flask containing 5 mL of freshly distilled THF was cooled to -78°C and flushed with argon. After addition of 7.2 mL (1.25 eqv.) of lithium bis(trimethylsilyl)amide, $\text{LiN}(\text{SiMe}_3)_2$ (1 M solution in THF) the contents of the flask were stirred for 10 min. Crude boronic ester 3 (1.8 g, 5.6 mmol) was dissolved in 10 mL of THF and added dropwise. The reaction mixture was stirred overnight and allowed to warm to room temperature. The resulting solution was concentrated under reduced pressure, reconstituted in 25 mL of DCM and filtered through Celite. The organic layer was dried with MgSO_4 and evaporated to give 2.5g of crude product as light brown oil with m/z 444, corresponding to $[\text{M}+\text{H}]^+$. ^1H NMR (CDCl_3): δ 0.09 (s, 18H), 0.83 (s, 3H), 0.97 (d, J = 10.6, 1H), 1.27 (s, 3H), 1.38 (s, 3H), 1.82 (m, 2H), 2.01 (m, 1H), 2.12 (m, 1H), 2.30 (m, 1H), 2.67 (m, 1H), 2.74 (dd, J = 13.2, 7.2, 1H), 3.05 (m, 1H), 4.30 (dd, J = 8.6, 2.1, 1H), 7.20-7.32 (m, 5H).

(+)-Pinanediol (*R*)-1-Amino-2-phenylethane-1-boronate trifluoroacetate salt (5). 0.5 g of the crude compound 4 (1.1 mmol) were dissolved in 10 mL of ethyl ether and cooled down to 0°C. To the mixture was added TFA (0.25 mL, 3.3 mmol) and the reaction was allowed to stir for 3h. The TFA salt crashed out of solution and was filtered. The final product was washed three times with cold ethyl ether resulting in white powder with 73% yield (0.34 g). ¹H NMR (methanol-d₄): δ 0.89 (s, 3H), 1.11 (d, J = 10.8, 1H), 1.34 (s, 3H), 1.42 (s, 3H), 1.93 (m, 2H), 2.06 (m, 1H), 2.26 (m, 1H), 2.43 (m, 1H), 3.03 (dd, J = 8.1, 14.0, 1H), 3.11 (dd, J = 6.4, 14.2, 1H), 3.25 (dd, J = 6.5, 8.0, 1H), 4.50 (d, J = 8.2, 1H), 7.30-7.37 (m, 5H); ¹³C NMR (methanol-d₄): δ 24.25, 27.37, 27.49, 28.81, 35.92, 36.46, 39.38, 40.77, 52.40, 80.33, 89.14, 128.58, 130.00, 130.35, 137.88; MS (ESI) *m/z* 300.1, [M]⁺.

General Method for Synthesis of Peptidyl Boronic Acids. The desired N-terminus protected peptide Boc-Ahx- or Z- (1 mmol) was dissolved in 50 mL of CH₂Cl₂ and the solution was cooled in ice-bath. HOBt (1.5 mmol, 0.229 g) and EDC (1.5 mmol, 0.288 g) were added to the mixture and stirred for 15 min. Boronic acid pinanediol ester (1 eqv.) was added and after 10 min of stirring at 0°C *N*-methylmorpholine (2 mmol, 0.23 mL) was added. The solution was stirred overnight and the temperature was allowed to warm slowly to room temperature. The reaction mixture was washed with water x 2, and brine x 2. The organic layer was dried with MgSO₄ and evaporated to dryness to yield crude protected peptide in 95-97% yield. The resulting white powder was then stirred with 95% TFA in water to remove all of the peptide side chain protecting groups. After 1h of stirring at room temperature the reaction was concentrated in vacuo and resulting residue was resuspended in a 1:1 mixture of ethyl ether/water (40 mL). Phenyl boronic acid (0.305 g, 2.5 mmol) was then added to remove the (+)-pinanediol protecting group. The deprotection was allowed to go overnight at room temperature. The aqueous layer containing the fully deprotected peptidyl boronic acid was washed with ethyl ether (30 mL, 3 x) and lyophilized. The product was purified by HPLC and the resulting white powder was characterized by MS.

Enzyme assays. Peptidyl boronic acids were dissolved in DMSO at 20 mM. Stock solutions could be stored at -20°C and were stable for one year. More diluted solutions were prepared as necessary. The activity of the inhibitors was determined by monitoring the change of fluorescence of AMC substrate (ex. 370 nm; em. 465 nm) due to hydrolysis by PSA. Product formation was followed over 30 min. The K_m for the substrate Mu-SRKSQQY-AMC was previously determined (14) to be 140 μM. Kinetic measurements were done in triplicate and run at 37°C in PSA buffer (50 mM TRIS, 100 mM NaCl, pH 7.8). Reactions were performed on 96-well plate where the final concentration of PSA was 2.5 μg/mL (2 nM active PSA) and the substrate was 300 μM. The assay mixture contained 100 μL of buffer, peptidyl boronic acid in DMSO (20 μL), PSA solution (20 μL) and Mu-SRKSQQY-AMC in buffer (60 μL). The following concentrations of inhibitors were employed for the assay: 50, 25, 12.5, 6.25, 3.1, 1.56, 0.78, 0.39, 0.19, 0.098, 0.049 and 0.0244 μM.

Selectivity of the compounds was determined by screening the products against chymotrypsin. Chymotrypsin assays were performed by monitoring the fluorescence of Suc-AAPF-AMC substrate at the above described conditions. The K_m value of chymotrypsin for the substrate Suc-AAPF-AMC was determined to be 14 μM. Reactions were performed on 96-well plate where the final concentration of chymotrypsin was 2 nM and the substrate was 25 μM. The assay mixture contained peptidyl boronic acid in DMSO (20 μL), Suc-AAPF-AMC in PSA buffer (90 μL) and chymotrypsin solution (90 μL). The chymotrypsin substrate solution was added last to each assay solution and the kinetic data was collected immediately.