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Probing for a hydrophobic a binding register in prostate-specific membrane antigen with phenylalkylphosphonamidates

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Abstract—To explore for the existence of an auxiliary hydrophobic binding register remote from the active site of PSMA a series of phenylalkylphosphonamidate derivatives of glutamic acid were synthesized and evaluated for their inhibitory potencies against PSMA. Both the phenyl- and benzylphosphonamidates (**1a** and **1b)** exhibited only modest inhibitory potency against. The phenethyl analog **1c** was intermediate in inhibitory potency while inhibitors possessing a longer alkyl tether from the phenyl ring, resulted in markedly improved K_i values. The greatest inhibitory potency was obtained for the inhibitors in which the phenyl ring was extended furthest from the central phosphorus (**1f**, $n=5$ and **1g**, $n=6$). The slightly serrated pattern that emerged as the alkyl tether increased from three to six methylene units suggests that inhibitory potency is not simply correlated to increased hydrophobicity imparted by the phenylalkyl chain, but rather that one or more hydrophobic binding registers may exist remote from the substrate recognition architecture in the active site of PSMA.

1. Introduction

Prostate cancer remains the most common male malignancy and the second most common cause of cancer-related mortality in most Western societies.[1] Its incidence is associated with age, family history, and life style factors,[2] and varies strikingly among ethnic, racial, and national groups with noteworthy high rates of both incidence and mortality among African Americans.[3] As basic research provides an ever-growing understanding of this disease, the strategies for preventing, diagnosing, and treating prostate cancer are being reshaped. One notable discovery has been the identification of an over-expressed membrane-bound cell surface protein on prostate cancer cells, namely, prostate-specific membrane antigen (PSMA). PSMA, also known as folate hydrolase I (FOLH1) and glutamate carboxypeptidase II (GCP II), is a 750-amino acid type II membrane glycoprotein[4] and was discovered during the development of the LNCaP cell line; one which retains most of the known features of prostate cancer.[5]

Although PSMA is primarily expressed in normal human prostate epithelium, the importance of this enzyme lies with the fact that it is upregulated and strongly expressed in prostate cancer cells, including those of the metastatic disease state.[6] Recent studies have demonstrated PSMA expression in the endothelium of tumor-associated neovasculature of multiple nonprostatic solid malignancies,[7] including metastatic renal carcinoma.[8] It is not surprising that PSMA has attracted a great deal of attention as a target for immunotherapy.[9] In addition to its immunological importance, PSMA is also reported to possess three, yet poorly understood, enzymatic activities: the hydrolytic cleavage and liberation of glutamic acid from both γ-glutamyl derivatives of folic acid[10] and the neuropeptide NAAG[11] (*N*-acetylaspartylglutamate) as shown in Figure 1, and dipeptidyl peptidase IV activity.[12] PSMA is highly homologous to NAALADase (*N*-acetylated alpha-linked L-amino

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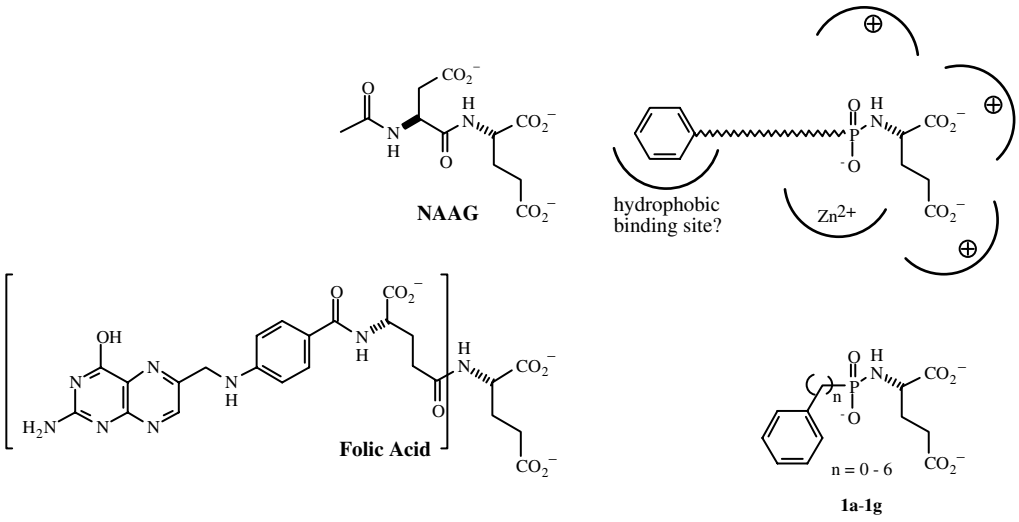


Figure 1. Substrates of PSMA and a binding model for a hydrophobic probing inhibitor.

dipeptidase) which is specifically characterized by its ability to hydrolyze the neuropeptide NAAG.[13] However, in contrast to NAALADase, which has been extensively studied due to its presumed regulatory role in glutamate neurotransmission, questions of medical interest remain to be answered for PSMA, especially with regard to its role in prostate cancer. It is our hypothesis that these enzymatic activities of PSMA can be exploited for chemotherapeutic strategies such as the selective inhibition by small molecule inhibitors.

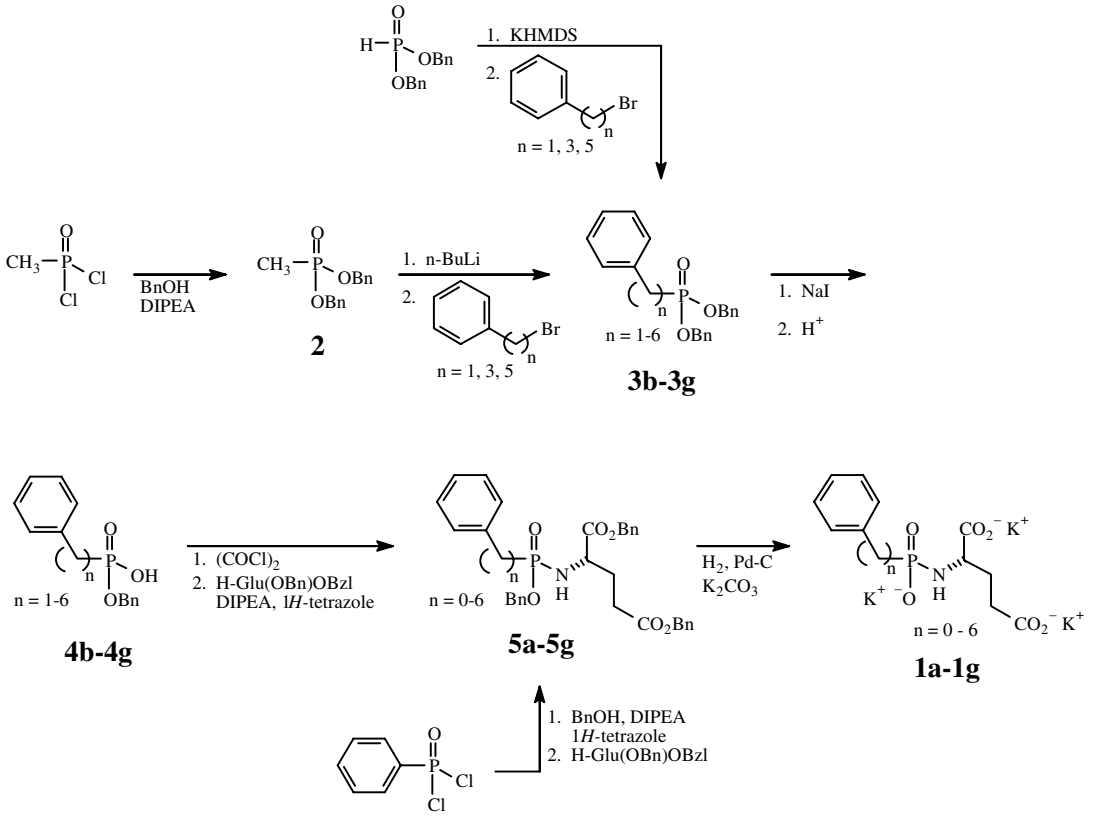
There is no crystal structure of PSMA as of yet, and thus the development of specific inhibitors must rely upon rational strategies to identify putative binding sites within or near the architecture of its active site. Although there have been a number of studies aimed at the acquisition of inhibitors of NAALADase,[14] there have been far fewer studies on the inhibition of PSMA by rationally designed, synthetic, small-molecule inhibitors.[15] The focus of this work was to identify the existence and relative location of hydrophobic binding site remote from the catalytic center of PSMA. To this end, we chose to prepare a series of phenylalkylphosphonamidate derivatives of glutamic acid (**1a**–**g**) Figure 1. The glutamic acid residue was incorporated to provide a sufficient substrate-recognition element to these inhibitors while the phosphonyl center would bind as a tetrahedral-intermediate analog to the active-site zinc (II) ions. A phenyl ring was selected as a probe for a putative remote hydrophobic binding site and as such, was tethered by an increasing number of (0–6) methylene units from the central phosphorus.

2. Results and discussion

The series of phenylalkylphosphonamidate inhibitors (**1a**–**g**) of PSMA were prepared as outlined in Scheme 1. Depending upon the length of the alkyl chain linking the phenyl probe to the central phosphorus, either dibenzyl methylphosphonate (**2**) or dibenzyl phosphite was selected as the nucleophile for alkylation to provide the phenylalkylphosphonate homologs **3**. As such, Michaelis–Becker alkylation of dibenzyl phosphite with the readily available phenylalkyl bromides ($Ph(CH_2)_n$ Br, $n = 1$, 3, or 5) provided the phenylalkylphosphonates **3b**,**d**, and **3f** in good yield. Not surprising, attempts at alkylating dibenzyl phosphite with 2-phenylethylbromide resulted in low yields with concomitant formation of styrene. Employing the same alkyl halides as above, the phenylalkylphosphonates **3c**,**e**, and **3g** were prepared by alkylation of dibenzyl methylphosphonate **2**. Care was taken to minimize the extent of dimerization of the methylphosphonate.[16]

Monodealkylation of the phenylalkylphosphonates was easily achieved with NaI[17] and following an acidic work-up provided the phosphonic acids **4b**–**g** in good yield. Formation of the phosphonochloridate with $(COCl)_2$[18] immediately followed by reaction with glutamic acid dibenzyl ester [H-Glu(OBn)OBn] afforded the phosphonamidate precursors **5b**–**g**. The phenylphosphonamidate **5a** was prepared directly from phenylphosphonic dichloride in a two-step, one-pot procedure we established previously.[19] Hydrogenolysis of the phosphonamidate precursors **5** in the presence of 1.5 M equiv of potassium carbonate provided the inhibitors **1a**–**g** in quantitative yield. We observed considerable degradation of the inhibitors during the deprotection step if the reaction mixture was not buffered with carbonate. Knowing that P–N bond is labile to acidic conditions, we presumed that as the acidic moieties were revealed during hydrogenolysis, their acidity compromised the chemical integrity of the the P–N bond.

With the acquisition of the hydrophobic inhibitor probes **1a**–**g**, we were interested in comparing their relative inhibitory potencies against purified PSMA rather than crude LNCaP cell lysates, which contains considerable amounts of PSMA′, a truncated, soluble cytosolic form of PSMA.[20] Since PSMA′ exhibits dual active-site kinetics as opposed to the single active-site kinetics of



Scheme 1. Synthesis of phenylalkylphosphonamidate derivatives of glutamic acid.

PSMA,[20] it was thought that employing the full-length membrane bound form of the enzyme would simplify the competitive inhibition kinetics. After obtaining the membrane-bound protein fraction of LNCaP cell lysates, active full-length PSMA was purified by antibody-affinity chromatography.

In order to monitor PSMA enzymatic activity, we chose to develop a convenient HPLC-based assay rather than use the conventional radioisotopic assay. The latter method involves incubating PSMA with radiolabeled NAAG, loading the enzymatic reaction mixture onto an ion-exchange column, selectively eluting and counting enzymatically liberated, radiolabeled glutamate. Cost and waste issues notwithstanding, this method is labor intensive and prone to various introductions of error. Our hypothesis was that we could develop a more convenient alternative assay for monitoring PSMA activity. The constraints we enforced upon our design of a novel substrate were that it must be based upon the structure of a known substrate for PSMA and bear a chromophore for convenient detection by HPLC. Knowing that folylpoly-γ-glutamates are substrates for PSMA, we prepared a simplified analog **7**, which bears a phenylazobenzoyl chromophore (Fig. 2). Using reversed-phase chromatography, the putative product **6** was cleanly separated from our synthetic substrate **7** with baseline resolution (Fig. 3). In terms of detection limits, we noted that this HPLC-based assay was sufficiently sensitive to conveniently detect concentrations of our synthetic substrate **7** and product **6** as low as 10 nM. Kinetic parameters were subsequently determined for diglutamate **7** with (Fig. 4) and found to be within the broad range reported for NAAG (2.6–540 nM).[21] Specifically, K_m and V_{max} for **7** were 280 nM and 622 nmol min^{-1} mg protein^{-1}, respectively. Indeed, the determined kinetic parameters for **7** with PSMA demonstrated the success of our design and development of a convenient alternative assay for monitoring PSMA assay. It should also be noted that,

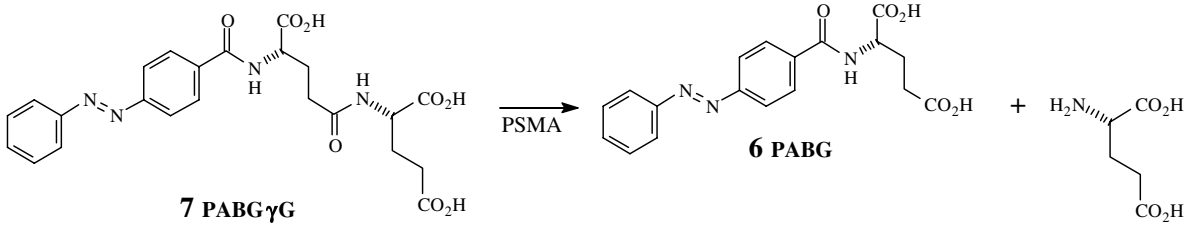


Figure 2. PSMA-mediated hydrolysis of PABGγG to PABG.

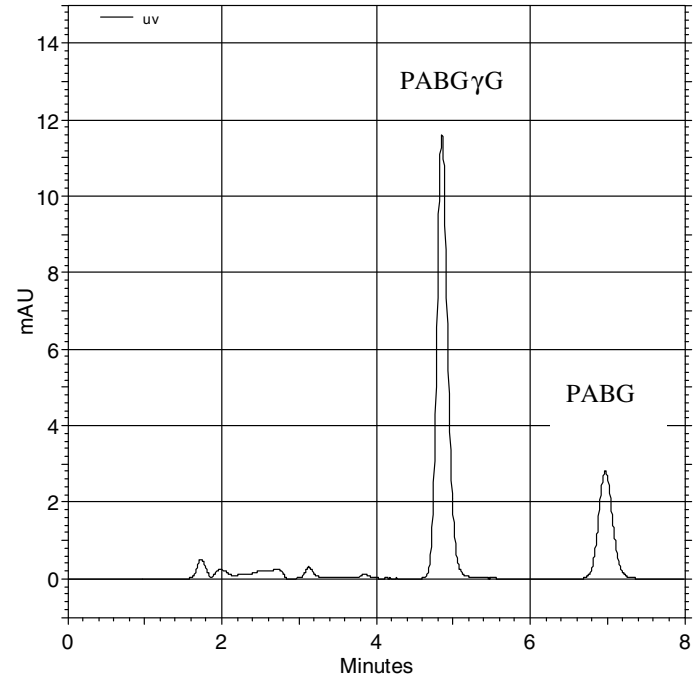


Figure 3. HPLC chromatogram of PABGγG and PABG. Lichrosphere C18 150×4.6 mm 5 μm; 40% ACN, 60% phosphate (pH 2.0, 25 mM) 1.0 mL/min; 325 nm.

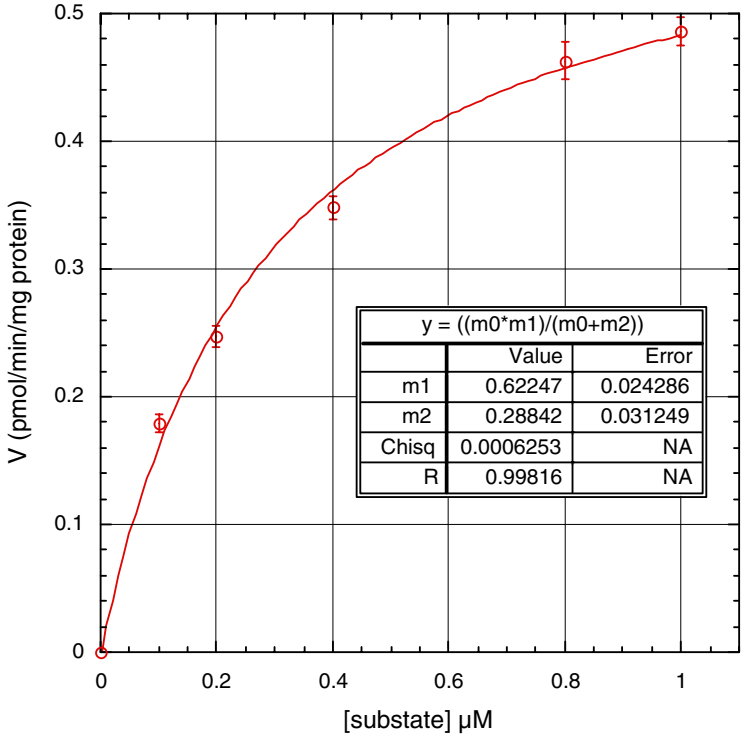


Figure 4. Kinetics of PSMA with PABGγG **7**.

purified PSMA maintained consistent enzymatic activity through numerous freeze-thaw cycles.

To ensure that the measured inhibition of PSMA by phosphonamidate inhibitors **1a**–**g** was physiologically relevant, enzyme activity assays were performed without the conventional addition of millimolar concentrations of $CoCl_2$. Although $CoCl_2$ is typically included in enzyme incubation systems to enhance measurable enzymatic activity, we found that the inclusion of 1.0 mM $CoCl_2$ in our assay resulted in a 27% decrease in enzymatic activity. Dixon analyses (Fig. 5) were conducted

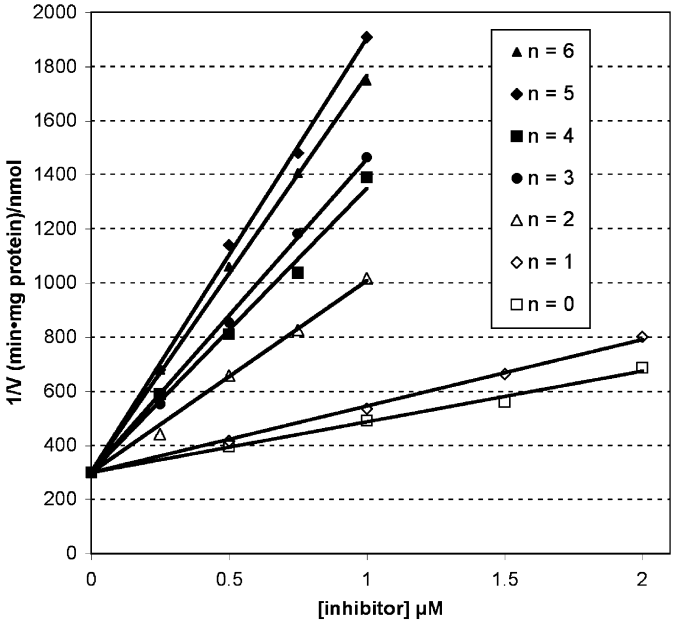


Figure 5. Dixon analysis of phosphonamidates **1a**–**g**.* (*Each point represents the average of triplicate determinations.)

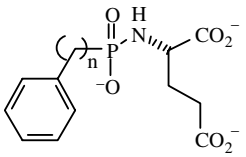
for inhibitors **1a**–**g** and the resulting K_i values are presented in Table 1. The initial substrate concentration for these assays was generally 1 μM while inhibitor concentrations were varied within the range of 0.25–2.0 μM depending upon the potency of the inhibitors.

Both the phenyl- and benzylphosphonamidates (**1a** and **1b)** exhibited only modest inhibitory potency against PSMA. It is likely that the phenyl ring in these compounds imparts steric congestion or unproductive interactions in the active site and precludes efficient binding of the inhibitors. The phenethyl analog **1c** was intermediate in inhibitory potency while inhibitors possessing a longer alkyl tether from the phenyl ring, resulted in markedly improved K_i values. The greatest inhibitory potency was obtained for the inhibitors in which the phenyl ring was extended furthest from the central phosphorus (**1f** and **1g**). These results are not inconsistent with the observations of Tenniswood and co-workers that phosphorus-containing inhibitors bearing large, yet remote fluorescent moieties were relatively potent inhibitors of PSMA/PSMA′ from crude LNCaP cell lysates.[15a]

The slightly serrated pattern that emerged as the alkyl tether increased from three to six methylene units suggests that inhibitory potency is not simply correlated to increased hydrophobicity imparted by the phenylalkyl chain nor does the pattern suggest that increasing the tether length allows for reduced unfavorable interactions by incrementally displacing the phenyl ring from an active site hosting a number of electrostatic interactions. Rather, the data suggests that one or more hydrophobic binding registers may exist remote from the substrate recognition architecture in the active site of PSMA as proposed by Wiest and co-workers.[22]

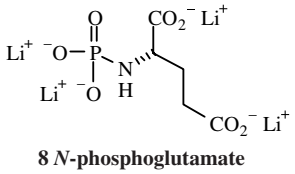
In order to better appreciate the significance of a potential hydrophobic binding site to inhibitory potency of PSMA, the K_i of *N*-phosphoglutamate[23] (**8**, Fig. 6) was determined. Not unlike the potent NAALADase inhibitor 2-PMPA, *N*-phosphoglutamate was identified as a very potent inhibitor of PSMA with a K_i value of 0.068 (±0.015) nM. Lacking the strong chelating interactions of the central dibasic phosphonamidate group with

Table 1. Inhibition of PSMA by phenylalkylphosphonamidates homologues



Inhibitor	*n*	K_i (nM)[a]
1a	0	603 (34)
1b	1	459 (12)
1c	2	159 (7.9)
1d	3	98 (5.1)
1e	4	107 (12)
1f	5	70 (5.3)
1g	6	77 (3.8)

[a] Standard deviation in parenthesis.



8 ***N*-phosphoglutamate**

Figure 6.

the catalytic zinc ions in PSMA, the phenylalkylphosphonamidates **1d–g** likely interact with a remote hydrophobic register site to elicit their enhanced inhibitory potency over the shorter homologs **1a–c**.

3. Conclusion

In summary, we have developed a convenient and sensitive HPLC-based assay for monitoring the enzymatic activity of PSMA using a novel synthetic γ-diglutamate derivative bearing a *N*-acyl chromophore. A comparison of the individual inhibitory potencies of phenylalkylphosphonamidate derivatives of glutamate supports the existence of a hydrophobic binding register remote from the catalytic cleft of PSMA. We expect that further elaboration of both the alkyl tether and the phenyl ring will improve the inhibitory potency and selectivity of these phosphonamidate derivatives of glutamic acid and such studies are currently underway.

4. Experimental

4.1. Synthesis

All solvents used in reactions and diisopropylethylamine (DIPEA) were both anhydrous and obtained as such from commercial sources. The HCl salt of glutamic acid dibenzyl ester [H-Glu(OBn)OBn] was neutralized by extraction with methylene chloride from a saturated aqueous solution of sodium bicarbonate. All other reagents were used as supplied unless otherwise stated. Liquid flash chromatography (silica or C18) was carried out using a Biotage 12i/40i system. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on a Bruker DRX 300 MHz NMR Spectrometer. ^{1}H NMR chemical shifts are relative to TMS ($\delta = 0.00$ ppm), $CDCl_3$ ($\delta = 7.26$ ppm), CD_3OD ($\delta = 4.87$ and 3.31 ppm), or D_2O ($\delta = 4.87$). ^{13}C NMR chemical shifts are relative to CD_3OD ($\delta = 49.15$ ppm) or $CDCl_3$ ($\delta = 77.23$ ppm). ^{31}P NMR chemical shifts in $CDCl_3$, CD_3OD, or D_2O were externally referenced to 85% H_3PO_4 ($\delta = 0.00$ ppm) in $CDCl_3$, CD_3OD, and D_2O, respectively. Combustion analyses were performed by Quantitative Technologies Inc., Whitehouse, NJ. High resolution mass spectra (FAB) were performed by the University of Notre Dame Mass Spectrometry Facility, Notre Dame, IN 46556-5670.

4.1.1. Dibenzyl methylphosphonate (2). Benzyl alcohol (4.55 mL, 44.0 mmol) and DIPEA (7.87 mL, 45.2 mmol) were sequentially added dropwise via syringe to a stirring solution of methyl phosphonic dichloride (3.00 g, 22.6 mmol) and 1*H*-tetrazole (0.16 g, 2.2 mmol) in dry benzene (30 mL) at 0 °C under an $Ar_{(g)}$ atmosphere. The reaction mixture was stirred for 0.5 h at 0 °C, then additional 3.5 h at room temperature. After filtering the salts, the solvent was removed under reduced pressure, and the crude oil was subjected to flash chromatography using 2:1 hexanes/ethyl acetate to afford the product as a light brown oil ($R_f \approx 0.08$). Yield: 89%. ^{1}H NMR ($CDCl_3$) δ: 1.46 (d, $J = 17.6$ Hz, 3H), 4.92–5.09 (m, 4H), 7.26–7.34 (m, 10H). ^{13}C NMR ($CDCl_3$) δ: 11.30, 13.20, 67.61, 67.67, 128.42, 128.93, 129.13, 136.90, 136.98. ^{31}P NMR ($CDCl_3$) δ: 31.93.

4.1.2. General procedure for phenylalkylphosphonates 3b,d, and 3f. A 0.5 M solution of potassium hexamethyldisilylzane (3.81 mL, 1.91 mmol) in toluene was added dropwise via syringe to a stirring solution of dibenzyl phosphite (421 μL, 1.91 mmol) in THF (5 mL) at 0 °C under an $Ar_{(g)}$ atmosphere. After 1 h, the appropriate phenylalkylbromide (2.29 mmol) was added via syringe and the reaction mixture was refluxed 2.5 h. The solvent was removed under reduced pressure and flash chromatography afforded the phenylalkylphosphonates as pale yellow oils.

4.1.3. Dibenzyl benzylphosphonate (3b). Chromatography conditions: hexanes/ethyl acetate 2:1, v:v, $R_f \approx 0.19$. Yield: 61.4%. ^{1}H NMR ($CDCl_3$) δ: 3.18 (d, $J = 21.7$ Hz, 2H), 4.91 (d, $J = 8.3$ Hz, 4H), 7.24–7.32 (m, 15H) ^{13}C NMR ($CDCl_3$) δ: 33.84, 35.55, 35.67, 68.26, 66.35, 127.62, 128.58, 129.00, 129.20, 130.50, 130.58, 131.81, 131.92, 136.97, 137.05 ^{31}P NMR ($CDCl_3$) δ: 27.72.

4.1.4. Dibenzyl 3-phenylpropylphosphonate (3d). Chromatography conditions: hexanes/ethyl acetate 3:1, v:v, $R_f \approx 0.11$. Yield: 65.2%. ^{1}H NMR ($CDCl_3$) δ: 1.67–1.93 (m, 4H), 2.62 (t, $J = 7.3$ Hz, 2H), 4.89–5.07 (m, 4H), 7.07–7.37 (m, 15H). ^{13}C NMR ($CDCl_3$) δ: 24.61, 25.21, 27.08, 33.84, 36.88, 37.10, 67.71, 67.78, 126.70, 128.55, 129.01, 129.12, 129.22, 130.48, 130.58, 137.12, 137.20, 141.54. ^{31}P NMR ($CDCl_3$) δ: 33.53.

4.1.5. Dibenzyl 5-phenylpentylphosphonate (3f). Chromatography conditions: hexanes/ethyl acetate 2:1 v:v, $R_f \approx 0.21$. Yield: 58.1%. ^{1}H NMR ($CDCl_3$) δ: 1.32–1.39 (m, 2H), 1.51–1.78 (m, 6H), 2.55 (t, $J = 7.5$ Hz, 2H), 4.90–5.08 (m, 4H), 7.11–7.34 (m, 15H). ^{13}C NMR ($CDCl_3$) δ: 22.85, 25.75, 27.62, 30.63, 30.87, 31.48, 36.26, 67.62, 67.76, 68.28, 126.37, 128.56, 128.95, 129.02, 129.24, 130.51, 130.61, 137.23, 137.29, 143.06. ^{31}P NMR ($CDCl_3$) δ: 33.83.

4.1.6. General procedure for dibenzyl phenylalkylphosphonates 3c,e and 3g. A 1.6 M solution of *n*-butyllithium (7.33 mL, 11.7 mmol) in hexanes was added dropwise via syringe to a stirring solution of dibenzyl methylphosphonate (2.70 g, 9.77 mmol) in dry THF (27 mL) at −78 °C under a N_2 atmosphere. After 15 min, the respective phenylalkylbromide (11.7 mmol) was added dropwise and the reaction stirred for 0.5 h at −78 °C, then additional 2 h at room temperature. The aqueous layer was extracted twice more with CH_2Cl_2, the organic layers were then combined, washed with brine, dried over $MgSO_4$, and the solvent was evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2, and washed with a half-saturated aqueous solution of NaCl. Flash chromatography afforded the dibenzyl phenylalkylphosphonates as light yellow oils.

4.1.7. Dibenzyl 2-phenylethylphosphonate (3c). Chromatography conditions: hexanes/ethyl acetate 1:1 v:v, $R_f \approx 0.45$. Yield: 47.2%. ^{1}H NMR ($CDCl_3$) δ: 2.02–2.14

(m, 2H), 2.84–2.93 (m, 2H), 4.96–5.11 (m, 4H), 7.11–7.41 (m, 15H). ^{13}C NMR ($CDCl_3$) δ: 27.77, 29.12, 29.18, 29.61, 67.83, 67.90, 127.00, 128.61, 128.70, 129.07, 129.27, 129.23, 137.08, 137.14, 141.35, 141.58. ^{31}P NMR ($CDCl_3$) δ: 32.18.

4.1.8. Dibenzyl 4-phenylbutylphosphonate (3e). Chromatography conditions: hexanes/ethyl acetate 2:1 v:v, $R_f \approx 0.20$. Yield: 42.9%. ^{1}H NMR ($CDCl_3$) δ: 1.58–1.83 (m, 6H), 2.52–2.57 (m, 2H), 4.91–5.08 (m, 4H), 7.08–7.33 (m, 15H). ^{13}C NMR ($CDCl_3$) δ: 22.59, 22.66, 25.67, 27.50, 32.66, 32.88, 35.95, 67.65, 67.71, 126.41, 128.52, 128.97, 129.19, 137.15, 137.21, 142.51. ^{31}P ($CDCl_3$) δ: 30.65.

4.1.9. Dibenzyl 6-phenylhexylphosphonate (3g). Chromatography conditions: hexanes/ethyl acetate 2:1 v:v, $R_f \approx 0.22$. Yield: 38.5%. ^{1}H NMR ($CDCl_3$) δ: 1.29–1.41 (m, 4H), 1.53–1.80 (m, 6H), 2.58 (t, J = 7.5 Hz, 2H), 4.94–5.10 (m, 4H), 7.11–7.36 (m, 15H). ^{13}C ($CDCl_3$) δ: 22.89, 22.96, 25.81, 27.66, 29.34, 30.89, 31.12, 31.77, 36.51, 67.67, 67.76, 126.31, 128.56, 128.62, 128.92, 128.99, 129.05, 129.25, 137.27, 137.34, 143.28. ^{31}P ($CDCl_3$) δ: 33.94.

4.1.10. General procedure for benzyl phenylalkylphosphonic acids 4b–g. A stirring solution of NaI (0.729 g, 4.86 mmol) and dibenzyl phenylalkylphosphonate **3** (4.05 mmol) in 2-butanone (15 mL) was refluxed 2.5 h under an $Ar_{(g)}$ atmosphere. The solvent was removed under reduced pressure and the resulting salt was collected and washed with ice-cold acetone to remove starting materials. Trace solvents were removed from the filtered solid under reduced pressure. The mono-sodium phosphonate salt was dissolved in a solution of 10% HCl (30 mL) and brine (30 mL) then extracted thrice with CH_2Cl_2 (40 mL). The organic layers were combined, dried over $MgSO_4$, and concentrated in vacuo to afford the benzyl phenylalkylphosphonic acids as white solids.

4.1.11. Benzyl benzylphosphonic acid (4b). Yield: 66.5%. ^{1}H NMR ($CDCl_3$) δ: 3.05 (d, J = 22.1 Hz, 2H), 4.80 (d, J = 7.6 Hz, 2H), 7.18–7.33 (m, 10H), 11.10 (s, 1H) ^{13}C NMR ($CDCl_3$) δ: 33.66, 33.52, 67.50, 127.57, 128.40, 128.88, 129.14, 130.59, 130.68, 131.89, 132.01, 136.90, 136.99. ^{31}P NMR ($CDCl_3$) δ: 29.86.

4.1.12. Benzyl 2-phenylethylphosphonic acid (4c). Yield: 52.5%. ^{1}H NMR (CD_3OD) δ: 1.86–1.98 (m, 2H), 2.69–2.78 (m, 2H), 4.92 (d, J = 8.4 Hz, 2H), 7.043–7.285 (m, 10H), 11.15 (s, 1H). ^{13}C NMR ($CDCl_3$) δ: 27.73, 29.04, 29.08, 29.59, 67.30, 67.38, 126.99, 128.43, 128.69, 129.00, 129.23, 136.92, 137.01, 141.36, 141.61. ^{31}P NMR (CD_3OD) δ: 30.52.

4.1.13. Benzyl 3-phenylpropylphosphonic acid (4d). Yield: 73.1%. ^{1}H NMR ($CDCl_3$) δ: 1.70–1.81 (m, 2H), 1.89–1.98 (m, 2H), 2.66 (t, J = 7.2 Hz, 2H), 5.00 (d, J = 7.8 Hz, 2H), 7.113–7.332 (m, 10H), 10.66 (s, 1H). ^{13}C NMR ($CDCl_3$) δ: 24.54, 24.59, 25.19, 27.09, 36.88, 37.11, 67.08, 67.16, 126.74, 128.31, 128.43, 128.97, 129.10, 129.17, 130.47, 136.96, 141.64. ^{31}P NMR ($CDCl_3$) δ: 33.20.

4.1.14. Benzyl 4-phenylbutylphosphonic acid (4e). Yield: 62.3%. ^{1}H NMR ($CDCl_3$) δ: 1.67–1.84 (m, 6H), 2.57 (t, J = 6.6 Hz, 2H), 5.02 (d, J = 7.8 Hz, 2H), 7.11–7.338 (m, 10H). ^{13}C NMR ($CDCl_3$) δ: 22.60, 22.65, 25.62, 27.51, 32.72, 32.94, 36.07, 67.07, 67.15, 26.48, 128.42, 128.94, 129.01, 129.21, 137.07, 137.30, 142.59. ^{31}P NMR ($CDCl_3$) δ: 35.88.

4.1.15. Benzyl 5-phenylpentylphosphonic acid (4f). Yield: 86.0%. ^{1}H NMR ($CDCl_3$) δ: 1.36–1.39 (m, 2H), 1.58–1.71 (m, 6H), 2.56 (t, J = 7.4 Hz, 2H), 4.97 (d, J = 7.8 Hz, 2H), 7.11–7.33 (m, 10H), 11.14 (s, 1H). ^{13}C NMR ($CDCl_3$) δ: 22.70, 22.76, 25.64, 27.55, 30.62, 30.85, 31.52, 33.75, 36.30, 66.95, 67.04, 67.71, 67.78, 126.37, 127.56, 128.41, 128.57, 128.95, 129.03, 129.19, 130.61, 130.69, 137.02, 137.12, 143.07. ^{31}P NMR ($CDCl_3$) δ: 36.20.

4.1.16. Benzyl 6-phenylhexylphosphonic acid (4g). Yield: 91.2%. ^{1}H NMR ($CDCl_3$) δ: 1.26–1.40 (m, 4H), 1.56–1.80 (m, 6H), 2.56 (t, J = 7.6 Hz, 2H), 4.98 (d, J = 8.1 Hz, 2H), 7.13–7.35 (m, 11H). ^{13}C NMR ($CDCl_3$) δ 22.78, 25.715, 27.60, 29.38, 30.90, 31.14, 31.83, 36.51, 66.96, 67.75, 126.21, 126.31, 128.40, 128.98, 129.14, 137.03, 137.15, 143.25. ^{31}P NMR ($CDCl_3$) δ: 36.32.

4.1.17. *N*-[Benzyloxy(phenyl)phosphinyl]-L-glutamic acid dibenzyl ester (5a). Benzyl alcohol (391 μL, 3.78 mmol) and DIPEA (724 μL, 4.16 mmol) were sequentially added dropwise via syringe to a stirring solution of phenylphosphonic dichloride (579 μL, 4.16 mmol) and 1-*H*-tetrazole (26.5 mg, 0.038 mmol) dissolved in dry benzene (8.0 mL) at 0 °C under an $Ar_{(g)}$ atmosphere. The reaction mixture was stirred for 0.5 h at 0 °C, then additional 2.5 h at room temperature. The reaction mixture was cooled to 0 °C and to it was sequentially added H-Glu(OBn)OBn (1.237 g, 3.78 mmol) and DIPEA (724 μL, 4.16 mmol) dropwise. The reaction mixture was stirred for 0.5 h at 0 °C and then 3.5 h at room temperature. The reaction mixture was filtered to remove salts and the solvent was removed under reduced pressure. Reversed-phase (C18) flash chromatography ($MeOH/H_2O$, 4:1, v:v) afforded the product as white solids ($R_f \approx 0.16$). Yield: 39.5%. ^{1}H NMR ($CDCl_3$) δ: 1.93–1.98 (m, 1H), 2.05–2.09 (m, 1H), 2.34–2.43 (m, 2H), 3.50–3.55 (m, 1H), 3.91–4.09 (m, 1H), 5.03–5.08 (m, 6H), 7.25–7.47 (m, 18H), 7.75–7.79 (m, 2H). ^{13}C NMR ($CDCl_3$) δ: 30.11, 30.20, 30.27, 30.43, 30.51, 53.55, 53.80, 66.93, 67.00, 67.85, 128.33, 128.42, 128.82, 128.87, 128.91, 129.01, 129.09, 129.17, 129.21, 131.96, 132.09, 132.14, 132.27, 132.73, 132.77, 132.81, 135.73, 136.39, 136.42, 137.01, 172.97, 173.29, 173.37, 173.43. ^{31}P NMR ($CDCl_3$) δ: 18.86, 19.52 (diastereomers). Anal. Calcd for $C_{32}H_{32}NO_6P$: C, 68.93; H, 5.78; N, 2.51. Found: C, 68.82; H, 5.91; N, 2.45.

4.1.18. General procedure for benzyl phenylalkylphosphonamidates 5b–g. $(COCl)_2$ (97.1 μL, 1.13 mmol) was added dropwise via syringe to a stirring solution of phenylalkylphosphonic acid **4** (0.942 mmol) and DMF (0.094 mmol) in CH_2Cl_2 (6 mL) at 0 °C under an $Ar_{(g)}$ atmosphere. After 0.5 h, the reaction mixture was warmed to room temperature and stirred for an additional 1.5 h. The solvent was removed under reduced

pressure, the residue was dissolved in dry toluene (2 mL), and the solvent was again removed under reduced pressure. The resulting crude phosphonochloridate was dissolved in benzene (2.54 mL) and added dropwise to a stirring solution of H-Glu(OBn)OBn (278 mg, 0.848 mmol), 1*H*-tetrazole (6.6 mg, 0.094 mmol), and DIPEA (181 μL, 1.04 mmol) in benzene (2.54 mL) at 0 °C under an $Ar_{(g)}$ atmosphere. After 0.5 h, the reaction mixture was warmed to room temperature and stirred for additional 3.5 h. The reaction mixture was filtered and the solvent of the filtrate was removed under reduced pressure. Reversed-phase (C18) flash chromatography ($MeOH/H_2O$, 4:1, v:v) afforded the products as white solids.

4.1.19. *N*-[Benzyloxy(benzyl)phosphinyl]-L-glutamic acid dibenzyl ester (5b). $R_f \approx 0.16$. Yield: 33.1%. ^1H NMR (CD_3OD) δ: 1.76–1.95 (m, 2H), 2.12–2.48 (m, 4H), 3.11–3.22 (m, 2H), 3.90–3.96 (m, 1H), 5.06–5.16 (m, 4H), 7.17–7.35 (m, 20H). ^{13}C NMR ($CDCl_3$) δ: 30.74, 30.79, 30.93, 31.02, 36.46, 36.55, 38.11, 38.27, 54.27, 54.55, 66.34, 66.67, 66.76, 66.96, 67.06, 67.49, 68.37, 127.93, 128.01, 128.51, 128.63, 128.80, 129.15, 129.21, 129.35, 129.39, 129.59, 129.63, 129.70, 129.78, 130.87, 130.95, 131.05, 132.97, 133.09, 136.28, 136.86, 137.66, 137.75, 173.43, 174.06. ^{31}P NMR ($CDCl_3$) δ: 29.25, 29.54 (diastereomers). Anal. Calcd for $C_{33}H_{34}NO_6P$: C, 69.34; H, 6.00; N, 2.45. found: C, 69.14; H, 6.00; N, 2.73.

4.1.20. *N*-[Benzyloxy(2-phenylethyl)phosphinyl]-L-glutamic acid dibenzyl ester (5c). $R_f \approx 15$. Yield: 31.3%. ^1H NMR ($CDCl_3$) δ: 1.93–2.10 (m, 4H), 2.37–2.43 (m, 2H), 2.86–2.89 (m, 2H), 3.03–3.12 (m, 1H), 4.02–4.14 (m, 1H), 4.87–5.14 (m, 6H), 7.11–7.33 (m, 20H). ^{13}C NMR ($CDCl_3$) δ: 29.21, 29.26, 29.92, 30.40, 30.68, 30.72, 31.60, 53.78, 53.89, 126.97, 128.28, 128.50, 128.76, 128.93, 128.97, 129.05, 129.24, 129.30, 135.78, 136.38, 137.23, 137.33, 141.52, 141.73, 173.00, 173.07, 173.71, 173.78. ^{31}P NMR ($CDCl_3$) δ: 33.23, 33.90 (diastereomers). Anal. Calcd for $C_{34}H_{36}NO_6P$: C, 69.73; H, 6.20; N, 2.39. Found: C, 69.48; H, 6.21; N, 2.37.

4.1.21. *N*-[Benzyloxy(3-phenylpropyl)phosphinyl]-L-glutamic acid dibenzyl ester (5d). $R_f \approx 0.14$. Yield: 54.5%. ^1H NMR ($CDCl_3$) δ: 1.62–2.13 (m, 6H), 2.40–2.47 (m, 2H), 2.52–2.58 (m, 2H), 3.93–3.96 (m, 1H), 4.97–5.10 (m, 6H), 7.06–7.29 (m, 20H). ^{13}C NMR (CD_3OD) δ: 24.67, 27.35, 27.41, 29.05, 29.16, 30.36, 30.45, 30.51, 30.69, 37.02, 37.23, 53.74, 53.87, 65.92, 66.00, 66.13, 66.20, 67.09, 67.96, 126.67, 128.25, 128.46, 128.81, 128.92, 129.02, 129.14, 129.21, 129.29, 135.85, 136.44, 137.34, 137.41, 141.68, 141.73, 173.00, 173.74. ^{31}P NMR ($CDCl_3$) δ: 33.66, 34.43 (diastereomers). Anal. Calcd for $C_{35}H_{38}NO_6P$: C, 70.10; H, 6.39; N, 2.34. Found: C, 70.05; H, 6.34; N, 2.13.

4.1.22. *N*-[Benzyloxy(4-phenylbutyl)phosphinyl]-L-glutamic acid dibenzyl ester (5e). $R_f \approx 0.14$. Yield: 47.6%. ^1H NMR ($CDCl_3$) δ: 1.61–1.75 (m, 3H), 1.85–2.16 (m, 3H), 2.34–2.56 (m, 4H), 2.98–3.13 (m, 1H), 3.98–4.09 (m, 1H), 4.82–5.12 (m, 6H), 7.11–7.33 (m, 20H). ^{13}C NMR ($CDCl_3$) δ: 22.82, 27.80, 27.93, 29.66, 30.42, 30.51, 30.58, 30.71, 30.77, 32.87, 33.10, 36.10, 53.80, 53.93, 65.98, 66.02, 66.12, 66.22, 67.19, 68.03, 128.27, 128.83, 128.93, 129.00, 129.06, 129.22, 129.35, 135.88, 136.47, 142.67, 173.05, 173.77. ^{31}P NMR ($CDCl_3$) δ: 34.62, 35.24 (diastereomers). Anal. Calcd for $C_{36}H_{40}NO_6P$: C, 70.46; H, 6.57; N, 2.28. Found: C, 68.15; H, 6.60; N, 2.36.

4.1.23. *N*-[Benzyloxy(5-phenylpentyl)phosphinyl]-L-glutamic acid dibenzyl ester (5f). $R_f \approx 0.12$. Yield: 49.1%. ^1H NMR ($CDCl_3$) δ: 1.33–1.36 (m, 2H), 1.52–1.69 (m, 6H), 1.85–1.94 (m, 1H), 2.05–2.14 (m, 1H), 2.39–2.43 (m, 2H), 2.56 (t, $J = 7.4$ Hz, 2H), 3.01–3.05 (m, 1H), 3.94–4.10 (m, 1H), 4.83–4.90 (m, 1H), 4.97–5.12 (m, 6H), 7.12–7.34 (m, 20H). ^{13}C NMR ($CDCl_3$) δ: 22.97, 30.46, 30.50, 30.68, 30.73, 31.55, 36.30, 53.83, 65.92, 66.05, 67.16, 68.00, 126.34, 128.23, 128.43, 128.82, 128.94, 129.04, 129.34, 129.31, 136.40, 143.08, 173.04, 173.80. ^{31}P NMR ($CDCl_3$) δ: 34.76, 35.40 (diastereomers). Anal. Calcd for $C_{37}H_{42}NO_6P$: C, 70.80; H, 6.74; N, 2.23. Found: C, 70.56; H, 6.60; N, 2.13.

4.1.24. *N*-[Benzyloxy(6-phenylhexyl)phosphinyl]-L-glutamic acid dibenzyl ester (5g). $R_f \approx 0.10$. Yield: 59.2%. ^1H NMR ($CDCl_3$) δ: 1.29–1.30 (m, 4H), 1.55–1.69 (m, 6H), 1.93–1.95 (m, 1H), 2.04–2.18 (m, 1H), 2.39–2.43 (m, 2H), 2.56 (t, $J = 7.6$ Hz, 2H), 3.02–3.06 (m, 1H), 3.95–4.05 (m, 1H), 4.83–4.87 (m, 1H), 4.98–5.12 (m, 6H), 7.14–7.31 (m, 20H). ^{13}C NMR ($CDCl_3$) δ: 22.96, 23.01, 27.91, 29.41, 29.59, 30.52, 30.68, 30.75, 31.04, 31.26, 31.83, 36.54, 53.77, 53.89, 65.87, 66.16, 67.13, 68.01, 126.29, 128.23, 128.43, 128.81, 128.92, 129.04, 129.21, 129.32, 136.44, 143.30, 173.09, 173.80. ^{31}P NMR ($CDCl_3$) δ: 34.85, 35.49 (diastereomers). Anal. Calcd for $C_{38}H_{44}NO_6P$: C, 71.12; H, 6.91; N, 2.18. Found: C, 71.08; H, 6.92; N, 2.16.

4.1.25. General procedure for phenyl- and phenylalkylphosphonamidates 1a–g. To a flask was added **5** (100 mg), THF (1 mL), 10% Pd/C (10 mg), K_2CO_3 (1.55 equiv), and water (1 mL). The mixture was then stirred vigorously under an $N_{2(g)}$ atmosphere for 1 min. The flask was then purged and charged with $H_{2(g)}$ (balloon pressure) and stirred for 4 h at room temperature. The reaction mixture was filtered using a Whatman 0.2 μm PTFE micropore filtration disk, the solvent was removed under reduced pressure, and the products were obtained as white solids in quantitative yield.

4.1.26. *N*-[Hydroxy(phenyl)phosphinyl]-L-glutamic acid tripotassium salt (1a). ^1H NMR (D_2O) δ: 1.64–1.70 (m, 2H), 2.02–2.08 (m, 2H), 3.29–3.32 (m, 1H), 7.35–7.38 (m, 3H), 7.55–7.63 (m, 2H). ^{13}C NMR (D_2O) δ: 32.98, 34.52, 57.26, 128.85, 129.01, 130.93, 131.28, 131.40, 136.71, 138.81, 161.62, 182.06, 183.83. ^{31}P NMR (D_2O) δ: 13.31. FAB-HRMS $[M+K]^+$ calcd 439.8872, found: 439.8872 for $C_{11}H_{11}K_4NO_6P$.

4.1.27. *N*-[Hydroxy(benzyl)phosphinyl]-L-glutamic acid tripotassium salt (1b). ^1H NMR (D_2O) δ: 1.79–1.85 (m, 2H), 2.15–2.23 (m, 2H), 2.99 (d, $J = 19.0$ Hz, 2H), 7.28–7.39 (m, 5H). ^{13}C NMR (D_2O) δ: 27.22, 32.82, 33.75, 33.92, 36.31, 36.82, 37.65, 38.36, 56.81, 125.83,

126.02, 128.63, 129.81, 129.87, 136.13, 136.23, 181.83, 183.40. ^{31}P NMR (D_2O) δ: 22.58. FAB-HRMS $[M+K]^+$ calcd 453.9029 found: 453.9029 for $C_{12}H_{13}K_4NO_6P$.

4.1.28. *N*-[Hydroxy(2-phenylethyl)phosphinyl]-L-glutamic acid tripotassium salt (1c). ^{1}H NMR (CD_3OD) δ: 1.75–1.92 (m, 4H), 2.21–2.28 (m, 2H), 2.74–2.82 (m, 2H), 3.56–3.69 (m, 1H), 7.24–7.40 (m, 5H). ^{13}C NMR ($CDCl_3$) δ: 30.16, 31.71, 32.92, 33.35, 34.08, 56.91, 125.09, 127.67, 127.86, 127.99, 143.71, 143.94, 181.04, 181.56. ^{31}P NMR ($CDCl_3$) δ: 25.29. FAB-HRMS $[M+H]^+$ calcd 429.9627 found: 429.9626 for $C_{13}H_{16}K_3NO_6P$.

4.1.29. *N*-[Hydroxy(3-phenylpropyl)phosphinyl]-L-glutamic acid tripotassium salt (1d). ^{1}H NMR (D_2O) δ: 1.48–1.59 (m, 2H), 1.79–1.88 (m, 4H), 2.69 (t, J = 7.2 Hz, 2H), 3.41–3.48 (m, 1H), 7.25–7.42 (m, 5H). ^{13}C NMR (D_2O) δ: 25.32, 28.20, 29.82, 32.70, 34.20, 36.56, 36.79, 56.80, 126.16, 128.83, 128.93, 142.97, 182.19, 183.31. ^{31}P NMR (D_2O) δ: 29.56. FAB-HRMS $[M+K]^+$ calcd 481.9342 found: 481.9342 for $C_{14}H_{17}K_4NO_6P$.

4.1.30. *N*-[Hydroxy(4-phenylbutyl)phosphinyl]-L-glutamic acid tripotassium salt (1e). ^{1}H NMR (D_2O) δ: 1.54–1.70 (m, 6H), 1.80–1.86 (m, 2H), 2.22 (t, J = 8.0 Hz, 2H), 2.66 (t, J = 7.2 Hz, 2H), 3.46–3.49 (m, 1H), 7.25–7.41 (m, 5H). ^{13}C NMR (D_2O) δ: 23.12, 28.42, 32.84, 32.69, 34.20, 34.99, 56.82, 126.00, 128.81, 143.68, 182.27, 183.34. ^{31}P NMR (D_2O) δ: 28.33. FAB-HRMS $[M+H]^+$ calcd 457.9939 found: 457.9939 for $C_{15}H_{20}K_3NO_6P$.

4.1.31. *N*-[Hydroxy(5-phenylpentyl)phosphinyl]-L-glutamic acid tripotassium salt (1f). ^{1}H NMR (D_2O) δ: 1.351.61 (m, 8H), 1.81–1.83 (m, 2H), 2.21 (t, J = 7.8 Hz, 2H), 2.62 (t, J = 6.8 Hz, 2H), 3.43–3.46 (m, 1H), 7.24–7.35 (m, 5H). ^{13}C NMR (D_2O) δ: 23.10, 28.49, 30.10, 30.22, 30.53, 32.77, 34.20, 35.10, 56.74, 56.85, 125.84, 126.06, 128.74, 128.84, 128.96, 143.82, 182.22, 183.34. ^{31}P NMR (D_2O) δ: 28.57. FAB-HRMS $[M-K]^-$ calcd 432.0381 found: 432.0390 for $C_{16}H_{21}K_2NO_6P$.

4.1.32. *N*-[Hydroxy(6-phenylhexyl)phosphinyl]-L-glutamic acid tripotassium salt (1g). ^{1}H NMR (D_2O) δ: 1.34–1.61 (m, 10H), 1.79–1.81 (m, 2H), 2.22 (t, J = 7.8 Hz, 2H), 2.63 (t, J = 7.5 Hz, 2H), 3.41–3.44 (m, 1H), 7.25–7.36 (m, 5H). ^{13}C NMR (D_2O) δ: 23.35, 23.39, 28.36, 28.73, 30.35, 30.51, 30.74, 31.02, 32.89, 32.94, 34.37, 35.34, 56.96, 126.06, 128.88, 128.96, 144.07, 182.30, 183.40. ^{31}P NMR (D_2O) δ: 28.71. FAB-HRMS $[M-K]^-$ calcd 446.0537 found: 446.0559 for $C_{17}H_{23}K_2NO_6P$.

4.1.33. *N*-[4-(Phenylazo)benzyol]-L-glutamic acid (6). *Step 1. (Deprotection of resin)*: *N*-α-Fmoc-L-glutamic acid-γ-*t*-butyl ester preloaded on Wang resin (0.5 g, 0.270 mmol; 0.54 mmol/g resin 100–200 mesh, Novabiochem San Diego, CA) was deprotected with 5 mL piperidine/DMF (20:80, v:v) for 30 min, vacuum filtered, washed, and vacuum filtered using the following solvent wash cycle: 3×5 mL DMF, 3×5 mL CH_2Cl_2, 2×5mL CH_3CN, 2×5mL diethyl ether. *Step 2 (Chromophore coupling)*: 4-(Phenylazo)benzoic acid (0.183 g, 0.81 mmol), benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP, 0.421 g, 0.81 mmol) and *N*-hydroxybenzotriazole (HOBt, 0.109 g, 0.81 mmol) were dissolved in DMF (5 mL) and added to the deprotected resin followed by the addition of DIPEA (0.282 mL, 1.62 mmol) via syringe. The reaction mixture was rocked 1 h. The resin was vacuum filtered then subjected to the solvent wash cycle described above. A standard Kaiser test was used to confirm complete loading. In the case that loading was incomplete, this step was repeated. *Step 3 (Cleavage of product from resin)*: A 5 mL solution of TFA/H_2O (4:1, v:v) was added to the resin and rocked 3 h. Resin was vacuum filtered then washed with CH_2Cl_2 (4×5 mL). The filtrate was collected and the solvent removed under reduced pressure to provide an orange solid (0.138 g). The product was purified by semi-prep HPLC (Alltech Semi-Prep Econosil C18 10μ, 250×22 mm) using a mobile phase composed of 65% A and 35% B (Solvent A = 0.1% aqueous TFA by volume; Solvent B = CH_3CN) to give a light yellow solid (0.032 g, 13% yield). ^{1}H NMR (D_2O with 1 equiv K_3PO_4 for solubility) δ: 1.98–2.18 (m, 2H), 2.22–2.32 (m, 2H), 4.25 (t, J = 5.90 Hz, 1H), 7.45–7.47 (m, 3H), 7.60–7.66 (m, 4H), 7.76 (d, J = 8.07 Hz, 2H). ^{13}C NMR (D_2O with 1 equiv K_3PO_4) δ: 30.32, 36.03, 57.88, 124.21, 124.37, 130.14, 131.22, 133.96, 137.33, 153.53, 155.42, 170.42, 180.30, 183.89. Mp 188–189 °C. λ_{max} = 325 nm, $\varepsilon = 3.00\times10^4$ $M^{-1}cm^{-1}$.

4.1.34. *N*-[4-(Phenylazo)benzyol]glutamyl-γ-glutamic acid (7). *Step 1 (Deprotection of resin)*: Deprotection of the resin was accomplished according to the procedure described above for **6**. *Step 2 (Amino acid coupling)*: A solution of *N*−α-Fmoc-L-glutamic acid-γ-*t*-butyl ester (0.345 g, 0.81 mmol), PyBOP (0.421 g, 0.81 mmol) and HOBt (0.109 g, 0.81 mmol) in DMF (5 mL) was added to the deprotected resin. DIPEA (0.282 mL, 1.62 mmol) was added via syringe and the reaction mixture was rocked 1 h. The resin was vacuum filtered then subjected to the solvent wash cycle described in *Step 3* for **6**. A standard Kaiser test was used to confirm complete loading. In the case that loading was incomplete, this step was repeated. *Step 4 (Chromophore coupling)*: The resin was first deprotected according to *Step 1*. 4-(Phenylazo)benzoic acid (0.183 g, 0.81 mmol), PyBOP (0.421 g, 0.81 mmol) and HOBt (0.109 g, 0.81 mmol) were dissolved in DMF (5 mL) and added to the deprotected resin followed by the addition of DIPEA (0.282 mL, 1.62 mmol) via syringe. The reaction mixture was rocked 1 h. The resin was vacuum filtered then subjected to the solvent wash cycle described in *Step 3* for **6**. A standard Kaiser test was used to confirm complete loading. In the case that loading was incomplete, this step was repeated. *Step 5 (Cleavage of product from resin)*: A 5 mL solution of TFA/H_2O (4:1, v:v) was added to the resin and rocked 3 h. Resin was vacuum filtered then washed with CH_2Cl_2 (4×5 mL). The filtrate was collected and the solvent removed under reduced pressure to provide a reddish

brown solid (0.145 g). The product was purified by semi-prep HPLC (Alltech Semi-Prep Econosil C18 10μ, 250×22 mm) using a mobile phase composed of 65% A and 35% B (Solvent A = 0.1% aqueous TFA by volume; Solvent B = CH_3CN) to give an orange solid (0.058 g, 23% yield). ^1H NMR (D_2O with 1 equiv K_3PO_4 for solubility) δ: 1.79–1.93 (m, 2H), 1.96–2.12 (m, 2H), 2.14–2.24 (m, 2H), 2.38–2.45 (m, 2H), 4.05–4.09 (m, 1H), 4.27–4.31 (m, 1H), 7.49 (d, J = 6.60 Hz, 2H), 7.64–7.68 (m, 5H), 7.80 (d, J = 8.40 Hz, 2H). ^{13}C NMR (D_2O) δ: 28.76, 29.52, 33.37, 35.04, 56.24, 56.46, 123.36, 123.49, 129.28, 130.36, 133.07, 136.46, 152.75, 154.66, 169.61, 175.82, 178.88, 179.65, 182.94. Mp 185–186 °C. λ_{max} = 325 nm, $\varepsilon = 2.58\times10^4$ $M^{-1}cm^{-1}$.

4.2. Purification of PSMA

4.2.1. Cells and media. LNCaP cells were obtained from ATCC (Manassus, VA). Cells were cultured in RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate, 90%; fetal bovine serum, 10% at 37 °C in a 5% CO_2 atmosphere.

4.2.2. Extraction of PSMA from Cell lysates. The media from the cell cultures were poured off and LNCaP cells were scraped from 200 75 cc flasks, washed in PBS and pelleted by centrifugation at 1000 rpm. The supernatant is discarded and the pellet was dounce-homogenized in 50 mM Tris, 150 mM NaCl pH 7.4. The membrane-containing fraction was isolated as a pellet by centrifugation at 100,000*g* for 15 min; the supernatant was discarded. This process of homogenization and centrifugation was repeated twice more. The pellet was then suspended in 50 mM Tris, 150 mM NaCl and 1.0% Triton X-100 to solubilize PSMA. After a final centrifugation at 100,000*g* the PSMA-containing supernatant was collected and quickly frozen in dry ice or $N_{2(l)}$.

4.2.3. PSMA purification. An antibody-affinity column was prepared by adding 500 μL Sepharose-3C6 Mab affinity resin (Northwest Biotherapeutics; Seattle, WA) into a Poly-Prep Chromatography Column (Bio-Rad Laboratories, Hercules, CA). The column was sequentially washed with 5 mL each: 50 mM Tris pH 7.5 containing 150 mM NaCl; 100 mM Glycine pH 2.5 containing 150 mM NaCl; and 50 mM Tris pH 7.5 containing 150 mM NaCl. Solubilized PSMA obtained from cell lysates above was loaded on the column and sequentially washed with 5 mL each of the following buffers: 50 mM Tris pH 7.5 containing 150 mM NaCl and 1.0% Triton-X 100; 1.0 M KCl containing 150 mM NaCl and 1.0% Triton-X 100; 50 mM Tris pH 7.5 containing 150 mM NaCl and 1.0% Triton-X 100, 50 mM Tris pH 9.0 containing 150 mM NaCl and 1.0% Triton-X 100. Purified active enzyme was finally eluted with 5 mL 100 mM Glycine pH 11.5 containing 150 mM NaCl directly into a Sephadex 10DG desalting column (Bio-Rad Laboratories, Hercules, CA) equilibrated with 50 mM Tris pH 7.5 containing 150 mM NaCl and 1.0% Triton-X 100. The eluate was collected in 0.5 mL aliquots, PSMA enzyme activity was determined by HPLC as described below, and active aliquots were pooled and the enzyme solution was quickly frozen in dry ice or $N_{2(l)}$ then stored at −20 °C.

4.3. HPLC Quantification of PABGγG and PABG

PABGγG and its hydrolytic product (PABG) were separated and quantified using an analytical reversed-phase HPLC column (Lichrosphere C18 5 μm, 150×4.6 mm; Phenomenex, Torrence, CA) with a mobile phase consisting of ACN/potassium phosphate [25 mM, pH 2.0 (adjusted with H_3PO_4)] at a respective volume ratio of 40:60. At a flow rate of 1.0 mL/min, PABGγG and its hydrolytic product (PABG) were detected at 325 nm with retention times of 4.8 and 6.9 min, respectively.

4.4. Determination of K_m and V_{max} for PABGγG/with purified PSMA

Working solutions of the substrate (PABGγG) were made in Tris buffer (50 mM, pH 7.5). A typical incubation mixture (final volume 250 μL) was prepared by the addition of 200 μL Tris buffer (50 mM, pH 7.4) to either 25 μL of a solution of purified PSMA (0.00214 μg) or 25 μL Tris buffer (50 mM, pH 7.4) as a negative control. The enzymatic reaction was initiated by the addition of 25 μL PABGγG (1–20 μM). The final concentration of PABGγG ranged from 0.1 to 2.0 μM. The reaction was allowed to proceed for 15 min with constant shaking at 37 °C and was terminated by the addition of 25 μL methanolic TFA (2% trifluoroacetic acid by volume in methanol) followed by vortexing and centrifugation (10 min at 7000*g*). An 85 μL aliquot of the resulting supernatant was subsequently quantified by HPLC as described above. Under the assay conditions described above, it was noted that the initial substrate concentration was not substantially depleted during the time course of the incubation (e.g., approximately 10% conversion to product was observed for incubations with the lowest substrate concentration, 0.1 μM).

4.5. PSMA inhibition assay

Working solutions of the substrate (PABGγG) and all inhibitors were made in Tris buffer (50 mM, pH 7.5). A typical incubation mixture (final volume 250 μL) was prepared by the addition of 175 μL Tris buffer (50 mM, pH 7.4) to either 25 μL of an inhibitor solution or 25 μL Tris buffer (50 mM, pH 7.4). 25 μL of a solution of purified PSMA (0.00855 μg) was added and the enzymatic reaction was initiated by the addition of 25 μL PABGγG (10 or 20 μM). In all cases, the final concentration of PABGγG was either 1 or 2 μM while the final inhibitor concentration varied: 0.5–2.0 μM for **1a** and **1b**; 0.25–1.0 μM for **1c**–**g**; and 0.0005–0.002 μM for **8**. The reaction was allowed to proceed for 15 min with constant shaking at 37 °C and was terminated by the addition of 25 μL methanolic TFA (2% trifluoroacetic acid by volume in methanol) followed by vortexing and centrifugation (10 min at 7000*g*). An 85 μL aliquot of the resulting supernatant was subsequently quantified by HPLC as described above. Under the assay conditions described above, it was noted that the initial sub-

strate concentration was not substantially depleted during the time course of the incubation (e.g., approximately 10% conversion to product was observed for incubations with the lowest substrate concentration (1 μM) in the absence of inhibitor.

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