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Inhibition of Glutamate Carboxypeptidase II by Phosphonamidothionate Derivatives of Glutamic Acid

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A limited series of *N*-thiophosphonyl-glutamates were found to be inhibitors of the prostate-specific membrane antigen (PSMA) form of glutamate carboxypeptidase II. Comparative inhibitory profiles of an analogous *O*-thiophosphonyl-2-hydroxyglutarate revealed that the amido-linkage of the *N*-thiophosphonyl-glutamate provides a significant enhancement of inhibitory potency presumably due to significant hydrogen-bonding interactions with acceptor groups in the active-site of PSMA resulting in tighter binding. An analogous *N*-phosphonyl-glutamate exhibited significantly greater inhibitory potency than the parent *N*-thiophosphonyl-glutamate indicating that the sulfur ligand of the *N*-thiophosphonyl-glutamates is responsible for less favorable active-site interactions than oxygen, potentially due to steric crowding from the longer P–S bond or as a result of active-site metal substitution of Co(II) for Zn(II) arising from assay conditions.

Keywords: Prostate-specific membrane antigen inhibition; Glutamate carboxypeptidase II inhibition; Phosphonamidothionate; Phosphonamidoic acid; Phosphonothionate; Phosphonothioic acid

INTRODUCTION

One of the glutamate carboxypeptidase II (GCP2) enzymes of recent medical interest is the membrane-bound prostate-specific membrane antigen (PSMA) which has been reported as possessing a specific, yet not-well understood, folate hydrolase activity.[1] The importance of this enzyme is due to its strong expression in prostate cancer cells and was discovered during the development of the LNCaP cell line which retains most of the known features of prostate cancer.[2] It has been noted that a cytosolic form of PSMA is predominant in normal cells while a membrane bound form predominates in prostate cancer cells. Moreover, a 100-fold difference in expression of the ratios of the mRNA messages

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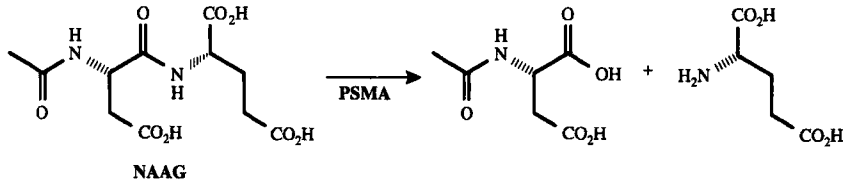


FIGURE 1 PSMA-mediated hydrolysis of NAAG.

encoding the two forms of PSMA has been observed, which may be indicative of a disease state.[1] In a recent study, the soluble form of PSMA found in normal cells, was observed to display two catalytic site kinetics, while the membrane-bound form exhibited a much lower activity characteristic of single site kinetics.[3] As to what this difference means in terms of folic acid metabolism remains unclear.

PSMA possesses a very high sequence homology to another GCP2 enzyme, the membrane-bound NAALADase (*N*-acetylated-alpha-linked acidic dipeptidase). NAALADase is characterized by its ability to hydrolyze the neuropeptide *N*-acetylaspartylglutamate (NAAG; Fig. 1).[1] PSMA also exhibits such activity which is commonly assayed by monitoring the hydrolysis of NAAG.[2] In contrast to NAALADase which has been extensively studied due to its presumed regulatory role in glutamate neurotransmission,[4] questions of medical interest remain to be answered for PSMA including its possible role in folic acid metabolism as well as its poorly understood NAALADase-type activity. The acquisition of inhibitors for PSMA may help to further the current understanding of the biological role of this metallocarboxypeptidases as well as serve to elucidate germane active site features. Furthermore, it is expected that inhibitors of PSMA will similarly inhibit NAALADase due to their high degree of homology.

The previously reported inhibitory potency of structures such as 2-(phosphonomethyl) pentanedioic acid (2-PMPA; Fig. 2) against NAALADase was presumably due to characteristic chelating interactions of the phosphonyl oxygen ligands with active site metal ions.[5] The phosphonamidothionate-derivatives of glutamic acid (1), which differ from the traditional phosphonamidate tetrahedral-intermediate inhibitors by the substitution of sulfur for one of the phosphonyl oxygens, were recently found to be potent competitive inhibitors of a bacterial glutamate carboxypeptidase, carboxypeptidase G (CPG).[6] Because it is known that sulfur exhibits a high affinity for zinc(II) and that zinc(II) complexes with sulfur-containing ligands involve more covalent forces and are as a result more stable[7,8] replacement of a phosphonyl oxygen with sulfur was anticipated to lead to more favorable inhibitor-enzyme interactions. Although additional progress towards such compounds has been pioneered recently, this design has been greatly overlooked in terms of generating potent

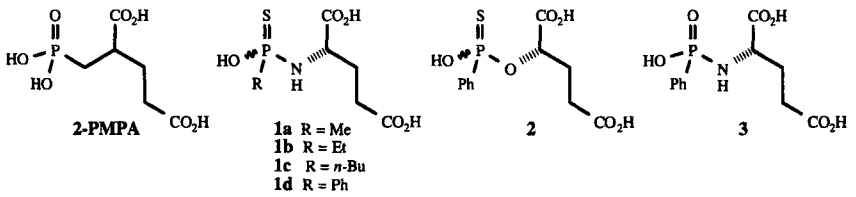


FIGURE 2 Phosphorus-containing inhibitiors of GCP2.

transition-state or tetrahedral-intermediate analog protease inhibitors.[9,10]

Our focus in this study was to examine several phosphonamidothionate-derivatives of glutamic acid (**1**) as potent competitive inhibitors for PSMA. In order to provide greater insight into the nature of the putative interactions of phosphonamidothionates (**1**) with PSMA, the representative phosphonothionate (**2**) and phosphonamidate (**3**) were also considered for comparison.

MATERIALS AND METHODS

THF, 3-hydroxypropionitrile, and triethylamine (TEA) were freshly distilled prior to use. All other reagents were of the highest grade available and were used as supplied unless otherwise stated. Liquid (flash) chromatography was carried out using silica gel 60 (230–400 mesh).[11] ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on a Bruker DRX 300 MHz NMR Spectrometer. ^{1}H NMR and ^{13}C NMR chemical shifts are relative to CD_3OD ($\delta = 4.87$, 3.31 ppm and $\delta = 49.15$ ppm, respectively). ^{31}P NMR chemical shifts in CD_3OD are relative to 85% H_3PO_4 ($\delta = 0.00$ ppm). High resolution mass spectra (FABHRMS) analyses were performed on a JEOL JMS-AX505HA Mass Spectrometer by the University of Notre Dame Mass Spectrometry Facility, Notre Dame, IN 46556-5670. Liquid scintillation was performed using a Beckman LS6500 with Ecoscint (National Diagnostics; Altanta, GA) as a scintillator.

Synthesis

2-(S)-[Hydroxy(phenyl)phosphinothioyloxy]-pentanedioic Acid Trilithium Salt (2)

A solution of 3-hydroxypropanenitrile (0.21 g, 3 mmol) in THF (5.0 mL) was added into a stirring solution of dichlorophenylphosphine (0.59 g, 3.3 mmol) and TEA (0.5 mL, 3.3 mmol) in THF (15 mL) at −40°C. The resulting solution was stirred for 0.5 h and then warmed to ambient temperature. A solution of (*S*)-dimethyl 2-hydroxyglutarate[12] (0.7 g, 3.9 mmol) and TEA (1.0 mL, 6.6 mmol) in 5 mL THF was added to the stirred reaction mixture for 1.5 h, followed by the addition of sulfur (0.15 g, 4.8 mmol). The solution was stirred for 15 h, filtered and concentrated under reduced pressure. Chromatography (CH_2Cl_2:CH_3OH, 100:1, v:v) gave (**4**) as a pale yellow oil. Phosphonothionate ester (**4**) (0.5 mmol) was dissolved in methanol (2 mL) to which was added aqueous lithium hydroxide (2 mL, 1.0 M). The resulting solution was stirred at room temperature for 18 h then filtered. The solvent was evaporated *in vacuo* to give (**2**) as a white residue. The residue was resuspended in anhydrous methanol, filtered (0.2 μm Teflon membrane), and concentrated *in vacuo* to give the trilithium salt of the desired product (**2**) as a white solid. ^{1}H NMR (CD_3OD) δ 1.76–1.97 (m, 2H), 2.14–2.23 (m, 2H), 3.82–3.86 (m, 1H), 7.17–7.21 (m, 3H), 7.78–7.83 (m, 2H). ^{13}C NMR (CD_3OD) δ 32.39 (d, $J = 6.9$ Hz), 35.40, 77.60 (d, $J = 6.8$ Hz), 128.46 (d, $J = 3.8$ Hz), 128.65(d, $J = 4.1$ Hz), 130.89, 131.64 (d, $J = 10.6$ Hz), 132.23 (d, $J = 10.7$ Hz), 142.20 (d, $J = 138.3$ Hz), 142.94 (d, $J = 144.8$ Hz), 180.33, 182.94. ^{31}P NMR (CD_3OD) δ 68.70, 69.44. FABHRMS $(M-Li)^-$ for $C_{11}H_{10}Li_2O_6PS$: found 315.0225; requires 315.0256.

2-(S)-[Hydroxy(phenyl)phosphinyl]-L-glutamic Acid Trilithium Salt (3)

N-[2-Cyanoethoxy(phenyl)phosphinyl]-L-glutamic acid dimethyl ester[13] (0.5 mmol) was dissolved in methanol (2 mL) to which was added aqueous lithium hydroxide (2 mL, 1.0 M). The resulting solution was stirred at room temperature for 18 h then filtered. The solvent was evaporated *in vacuo* to give (**3**) as a white residue. The residue was resuspended in anhydrous methanol, filtered (0.2 μm Teflon membrane), and concentrated *in vacuo* to give

the trilithium salt of the desired product (**3**) as a white solid. ^{1}H NMR (D_2O) δ 1.84–1.91 (m, 2H), 2.23–2.28 (m, 2H), 3.47–3.62 (m, 1H), 7.54–7.59 (m, 3H), 7.76–7.83 (m, 2H). ^{13}C NMR (D_2O) δ 32.41, 33.92, 72.27 128.23, 128.41, 130.34, 130.74, 135.95, 138.09, 181.49 (d, $J = 4.8$ Hz), 183.31. ^{31}P NMR (D_2O) δ 16.64. FABHRMS $(M-Li)^-$ for $C_{11}H_{11}NLi_2O_6P$: found 298.0649; requires 298.0644.

Enzyme Preparation

The culture medium was poured off 3 LNCaP cultures, grown on 25×150 mm plates containing RPMI 1640 medium with 5% fetal bovine serum, 1% glutamine, and 1% pyruvate. The remaining cells were scraped into phosphate buffer saline solution (PBS) and centrifuged at 1400 rpm for 10 min. The resulting cell pellets were transferred to 1.5 mL centrifuge tubes, redissolved in PBS, and centrifuged a second time. After discarding the supernatant, the pellets were sonicated in 1 mL of lysis buffer [150 mM NaCl, 25 mM Tris (pH 7.4), 1% Nonidet P-40], centrifuged (10,000*g*), and the resulting supernatant was stored at −80°C until use.

Enzyme Inhibition

The assay for the hydrolysis of NAAG was similar to that described previously with only slight modfications.[4] A typical incubation mixture (final volume 1.0 mL) was prepared by addition of 50 μL of Tris buffer (50 mM, pH 7.4, 1 mM $CoCl_2$) to 820 μL of water. This was followed by the addition of 100 μL of inhibitor and 20 μL of membrane protein solution, obtained as mentioned above. The reaction was initiated by the addition of 10 μL of the radiolabeled substrate *N*-acetyl-L-aspartyl-L-[3,4-^{3}H]glutamate (1 mM, 99.77 μCi/μmol). The reaction was allowed to proceed for 15 min with constant shaking at 37°C, and then terminated with 1.0 mL of ice cold sodium phosphate (0.1 M, pH 7.4). The cleaved glutamate product was subsequently separated from unreacted substrate by ion-exchange chromatography as follows: aliquots of the assay mixture was applied to water-prewashed minicolumns (2 cm) of defined AG 1-X8 anion exchange resin (200–400 mesh; formate form) prepared in 5 in. Pasteur capillary pipettes. Elution of the radiolabeled glutamate residue was achieved with 1.8 mL of 0.4 M formate solution. The product eluate was then diluted with 15 mL scintillator and its radioactivity was determined by liquid scintillation counting. Under the assay conditions described below for a typical incubation, it was noted that the initial substrate concentration was not substantially depleted during the time course of the incubation (e.g. < 2% conversion to product was observed for incubations in the absence of inhibitor) thus allowing for adequate steady-state kinetic analysis.

RESULTS AND DISCUSSION

Phosphonamidothionates (**1**) were available from a previous study,[13] the synthesis of (**2**) is outlined in Scheme 1, and the preparation of compound (**3**) required only mild basic deprotection of a previously available precursor.[13] It should be noted that the phosphonamidothionates (**1**) and the phosphonothionate (**2**) were prepared as a mixture of diastereomers (unresolved at phosphorus) in an approximate ratio of 50 : 50 as determined by ^{31}P NMR. Although the diastereomers of (**2**) were well resolved by ^{31}P NMR, ^{1}H NMR afforded no resolution of these diastereomers and only the ipso-carbon of the phenyl ligand to phosphorus were resolved as dual sets of doublets in the ^{13}C NMR spectrum. No attempts were made to separate the individual diastereomers during this investigation, however this topic will be explored in future studies. It is anticipated that the acquisition of the individual diasteromers of (**1**) and (**2**) may indeed aid in mapping the active-site architecture of

PSMA and similar enzymes of interest (e.g. NAALADase).

In order to confirm that phosphonamidothionates (**1**), (**2**) and (**3**) were suitably stable under the assay conditions, 10 mM samples of (**1c**), (**2**) or (**3**) in Tris buffer (100 mM, pH 7.0) were monitored by ^{31}P NMR spectroscopy. Although under these conditions, first-order desulfurization of representative compound (**1c**) to (**3**) was noted to occur ($t_{1/2} = 134$ min) it was determined to be suitably stable during the course of the assays. Unlike the phosphonamidothionate (**1c**), no detectable desulfurization of phosphonothionate (**2**) was detected after 18 h. This enhanced stability was presumably due to the decreased electron density contribution of the phosphonothionate oxygen ligand compared to that of the nitrogen ligand in phosphonamidothionate (**1c**). Furthermore, no evidence for the hydrolysis of the P–N linkage of phosphonamidate (**3**) was observed under these conditions.

The phosphonamidothionates (**1**) were examined for inhibitory potency against PSMA using a previously described radioenzymatic assay to monitor the liberation of glutamic acid from NAAG.[4] For this study, PSMA was extracted from the LNCaP human prostate cancer cell line because it contains high levels of NAAG-hydrolyzing activity and can be grown with ease when compared to other cell lines such as Du145 or PC-3.[3] Dixon analyses for phosphonamidothionates (**1**) were conducted by varying the inhibitor concentration from 10 to 90 μM in the presence of 10 μM NAAG ($K_M = 0.2$ μM)[14] and the results obtained are shown in Table I. For purposes of direct comparison, K_i values obtained previously for these same compounds with the glutamate carboxypeptidase CPG are also presented in Table I.[15]

Remarkably, the most potent of the four phosphonamidothionates (**1**) against either PSMA or CPG was the *n*-butyl analog (**1c**). However, unlike that observed for CPG, no clear structure-activity relationship (SAR) for the alkyl series was evident for PSMA. It can be postulated that as the alkyl ligand is extended from methyl to ethyl, increased steric bulk congests the active-site of PSMA and as a result, inhibitory potency decreases. Although a further decrease in inhibitory potency would then be expected for the *n*-butyl analog, a secondary and more favorable hydrophobic interaction with the butyl chain may predominate. The observation that the aryl phosphonamidothionate (**1d**) exhibited the weakest inhibitory potency may be due to both steric crowding and insufficient hydrophobic interactions.

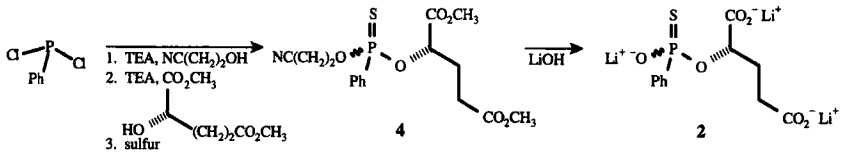
TABLE I Inhibition of PSMA and CPG*

Inhibitor	PSMA K_i μM	CPG[6] K_i μM
1a	0.719	6.20
1b	1.09	1.24
1c	0.104	0.264
1d	6.39	3.24
2	14.6	3.43†
3	1.11	22.7†

*Corr. coeff. for all Dixon analyses > 0.94.
†Determined as previously described[6].

As noted above, the inhibitors (**1a–d**) were prepared as a mixture of two diastereomers (unresolved at phosphorus) in an approximate ratio of 50:50 as determined by ^{31}P NMR. It is quite possible that the inhibition of PSMA and CPG by these compounds is stereoselective; one diastereomer being more potent and primarily responsible for the inhibition of the mixture. As such, any conclusions regarding SAR should remain tentative until the diastereomers are resolved and stereoselective inhibition studies are executed.

In order to elucidate specific interactions of the phosphonamidothionate inhibitors (**1**) with PSMA and to contrast those with CPG, K_i values for both (**2**) and (**3**) (direct analogs of (**1d**)) were obtained (Table I). It was proposed that (**2**) could address the importance of the sulfur ligand while (**3**) could address the potential hydrogen bonding to the active site through the amido-hydrogen. The observed weaker inhibitory potency of (**2**) against PSMA compared with (**1d**) suggests that



SCHEME 1

the amido-hydrogens of phosphonamidothionates (**1**) participate as donors in significant hydrogen-bonding interactions with acceptor groups in the active-site of the enzyme resulting in tighter binding; a trend similar to one observed previously for phosphonamidate and phosphonate inhibitors of thermolysin.[16]

In contrast to CPG, replacement of the oxygen ligand of the phosphonamidate (**3**) with sulfur in the analogous phosphonamidothionate (**1d**) resulted in a dramatic loss of inhibitory activity against PSMA. One possible explanation for this result, postulated previously for inhibitors of carboxypeptidase A, may be attributed to the considerably greater length of the P–S bond (1.85 Å) compared to that of the P–O bond (1.39 Å).[17] Hence, the relatively significant difference in bond length between P–S and P–O could cause steric congestion around the zinc ion of a metalloprotease thus leading to weaker enzyme inhibitor interactions. Alternatively, an artifact of the assay conditions for PSMA may provide the source for additional explanation. High concentrations of Co(II) are traditionally employed in assays for PSMA-mediated NAAG-hydrolyzing activity for the reason that it stimulates enzymatic activity.[18] As a result, a possibility exists for active site metal substitution in the presence of excess Co^{2+} (100 μM) although no chelating agents were employed to promote such substitution. The addition of significant amounts of an extrinsic metal ion to an enzyme reaction mixture has resulted in a type of site-directed mutagenesis by which the naturally occurring metals in metalloproteases become substituted.[19] Such was the case for angiotensin-I-converting enzyme in which cadmium and cobalt ions were substituted for zinc ions to characterize the binding of inhibitors to this enzyme.[20] Active site metal substitution could thus explain the reduced relative inhibitory potency of (**1d**) compared with (**3**) as sulfur ligands have been observed to form less stable complexes with cobalt than oxygen.[21]

In conclusion, the results from the inhibition data derived here reveal that the phosphonamidothionates (**1**) were relatively potent inhibitors of PSMA. Bioisosteric replacement of the amido moiety in such compounds with oxygen reduces inhibitory potency revealing the importance of putative hydrogen-bonding interactions through the amido-hydrogen. Somewhat unexpectedly, replacement of the phosphonamidothionate sulfur ligand with oxygen enhanced inhibitory potency, potentially due to active-site metal substitution or simply reduced steric crowding. Regardless, the results further support the hypothesis that phosphonamidothionate are indeed laudable candidates as potent tetrahedral-intermediate analog inhibitors of metallopeptidases and may serve as precedence for the extrapolation of this design to other enzymatic systems of medical import.

Acknowledgements

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