Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 1415–1418

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

COMPETITIVE INHIBITION OF A GLUTAMATE CARBOXYPEPTIDASE BY PHOSPHONAMIDOTHIONATE DERIVATIVES OF GLUTAMIC ACID

Chester E. Rodriguez, Haiyan Lu, Trang T. Dinh, Karyn L. Mlodnosky, Azar Dastgah, Vinh Q. Lam, C. Blake Nichols, and Clifford E. Berkman*

Department of Chemistry & Biochemistry, San Francisco State University
1600 Holloway Avenue, San Francisco, CA 94132, U.S.A.

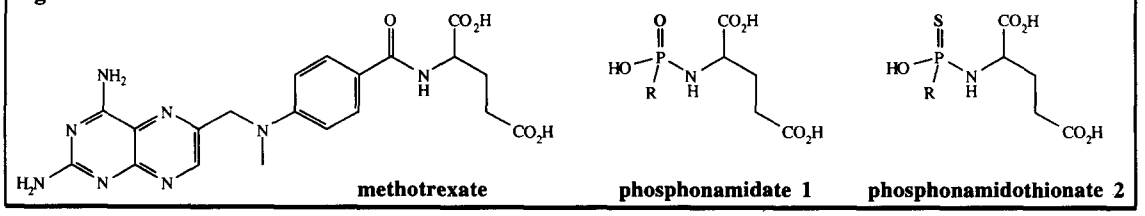
Received 9 February 1999; accepted 7 April 1999

Abstract: Several N-thiophosphonyl-glutamates were found to be potent competitive inhibitors of a zinc-dependent glutamyl hydrolase, carboxypeptidase G (CPG). Weak inhibition exhibited by an analogous *N*-phosphonyl-glutamate suggests that the enhanced potency of the phosphonamidothioates is due to the presence of their sulfur ligand and its favorable interactions with active site features, presumably zinc(II).

A number of relevant glutamate carboxypeptidases have been identified throughout the spectrum of living systems. A few such examples include rat brain *N*-acetylated-alpha-linked-acidic dipeptidase (NAALADase)[1] and prostate-specific membrane antigen (PSMA)[2] which are noted to hydrolyze the neuropeptide *N*-acetylaspartylglutamate while two forms of pteroylpoly-glutamate hydrolase (PPH) have been identified in human jejunum capable of hydrolytically cleaving C-terminal γ-glutamates from pteroylpoly-γ-glutamates.[3] In addition, various forms the carboxypeptidase G enzymes have been isolated from bacteria[4] which were noted to liberate glutamic acid from folates or folate derivatives, a representative of which (carboxypeptidase G2) has been employed in ADEPT[5] as well as in therapeutic rescue techniques following severe chemotherapeutic dosages of antifolates such as methotrexate (Figure 1).[6] All the above glutamate carboxypeptidases ultimately possess the ability to cleave C-terminal glutamates from respective substrates and are presumed to be metallocarboxypeptidases, although the specific metal ion cofactors required for optimal activity may differ.

Our focus in this study was to develop potent competitive inhibitors for glutamate carboxypeptidases. The acquisition of inhibitors for such enzymes may help to further our understanding of the biological role of these metallocarboxypeptidases as well as serve to elucidate germane active site features. For our inhibition studies, we chose the zinc(II)-dependent carboxypeptidase G (CPG) as a representative for this group of glutamate carboxypeptidases due not only to its ready commercial availability but its ability to catalytically liberate glutamic acid from a broad range of *N*-acyl-glutamates where the acyl group may be represented by a variety of organic acids including pteroates and amino acids.[4a] As for many metallopeptidases, phosphonamidates are presumed to be suitable competitive inhibitors by acting as transition-state or tetrahedral-intermediate mimics. However, we

Figure 1



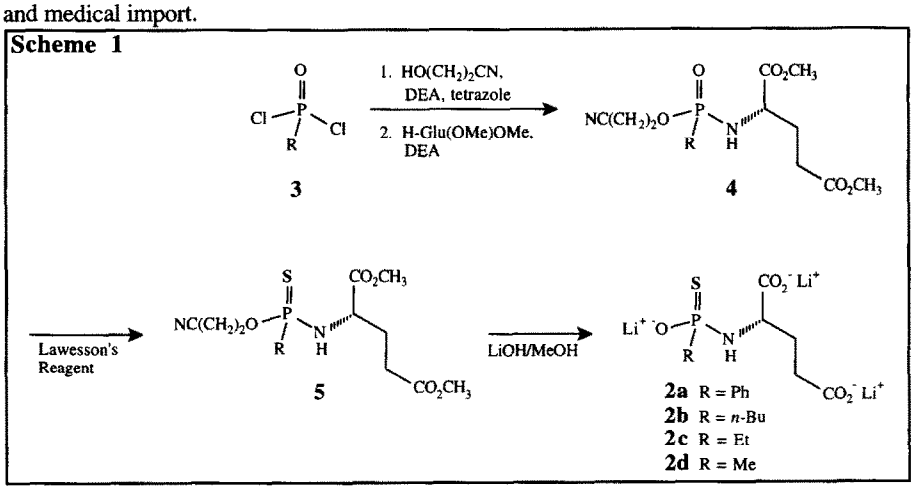
PII: S0960-894X(99)00198-5

found in an earlier study that such compounds (Figure 1; **1** R = Ph, Me) were not notably potent inhibitors of a semipurified preparation of CPG.[7]

The basis for the success of phosphonamidates as inhibitors of zinc metallopeptidases is presumably due to strong interactions of the phosphonyl oxygens with zinc(II). Therefore, replacement of a phosphonyl oxygen by ligands which may form more favorable interactions with zinc should enhance the inhibitory potency of such compounds. It is known that sulfur exhibits a high affinity for zinc(II) and that complexes with sulfur-containing ligands involve more covalent forces and as a consequence, the metal–ligand bonds in such zinc-sulfur complexes are correspondingly more stable.[8] Therefore, we anticipated that simple modifications to the design of phosphonamidates which incorporate a sulfur atom ligand on the central phosphorus could enhance the chelation of the active-site zinc(II), thus resulting in enhanced inhibitory potency.

The representative phosphonamidothionates **2** were thus targeted as putative inhibitors of CPG to exploit such stable zinc(II)-sulfur interactions and to mimic the hydrolytic tetrahedral intermediate (Figure 1). Although some progress towards such compounds have been pioneered recently, this design has been greatly overlooked in terms of generating potent transition-state or tetrahedral-intermediate analog protease inhibitors.[9] Furthermore, the preparation of phosphonamidothionates and demonstration that they are potent tetrahedral-intermediate analog inhibitors of metalloproteases may allow for an extrapolation of this design to other enzymatic systems of biological and medical import.

Scheme 1

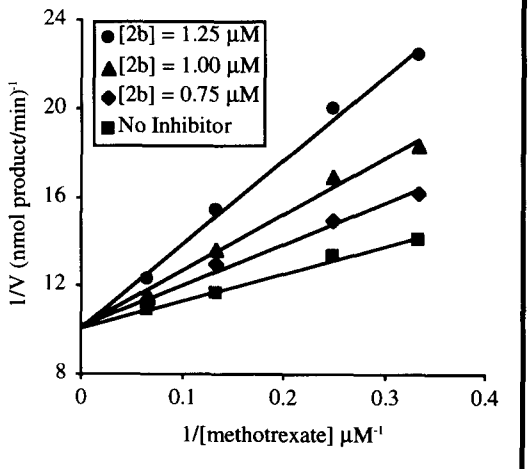


The synthesis of inhibitors **2a–2d** is outlined in Scheme 1 and exploits our two-step one-pot methodology to form the intermediate phosphonamidate esters **4** from phosphonyl dichlorides **3**.[10] Subsequent thionation with Lawesson's reagent[11] provided the precursors **5**. Mild basic hydrolysis of **5** with methanolic LiOH quantitatively removed the phosphorous-cyanoethyl and glutamyl-methyl esters simultaneously to give the desired phosphonamidothionates **2**.[12] It should be noted that the final products (**2a–d**) were prepared as a mixture of diastereomers (racemic at phosphorus) in an approximate ratio of 50:50 as determined by ^{31}P NMR. No attempts were made to separate the individual diastereomers during this investigation, however this will be addressed in future studies.

Once in hand, the phosphonamidothionates **2** were examined for inhibitory potency against CPG using a reversed phase HPLC-based method to monitor the hydrolysis of substrate (methotrexate).[13] Lineweaver–Burk analyses indicated that all four phosphonamidothionates (**2**) were competitive inhibitors of CPG (Figure 2;

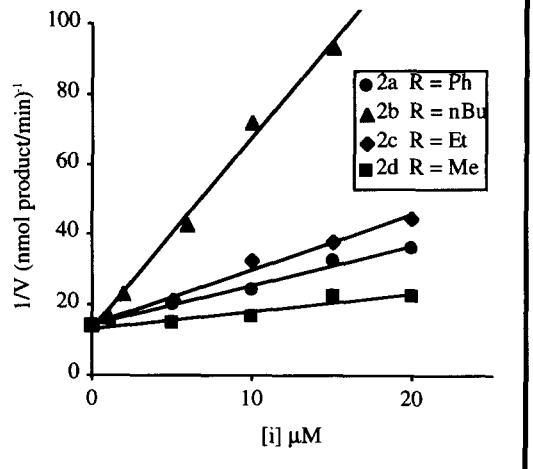
representative data shown for **2b**). For such experiments, methotrexate (K_M = 1.46 μM) concentrations were varied from 3 to 15 μM while inhibitor concentrations were varied from 0–20 μM. Dixon analyses (Figure 3) for each of the four inhibitors provided the K_i values shown in Table 1. These values were obtained using inhibitor concentrations described for the Lineweaver–Burk analysis in the presence of 10 μM methotrexate.

Figure 2. Lineweaver-Burk Plot of CPG Inhibition by 2b.*



*each point represents the average of triplicate determinations; correlation coefficients were all greater than 0.992.

Figure 3. Dixon Plot for Inhibitors 2.*



*[methotrexate] = 10 μM; each point represents the average of triplicate determinations; correlation coefficients = 0.993 **2a**, 0.982 **2b**, 0.994 **2c**, 0.943 **2d**.

Although the series of inhibitors is rather limited, cursory analysis of the data (Table 1) reveals the emergence of a structure–activity relationship dependent upon the length of the alkyl ligand on phosphorus. Hence, the greatest inhibition was observed when R = *n*Bu while shortening of the chain (R = Et or Me) significantly reduced inhibitory potency. More notable, however, were the results from the Dixon analysis performed for the phosphonamidate **1** (R = Ph; K_i = 46.7 μM) under the assay conditions described for the phosphonamidothionates **2**. Indeed, the aryl phosphonamidothionate **2a** exhibited much greater inhibitory potency against CPG than the corresponding phosphonamidate (**1** R = Ph). This result alone indicates that the sulfur ligand of the phosphonamidothionates imparts a significant influence on their interactions with the active site features of CPG, presumably with the active-site zinc(II) atom in particular.

In conclusion, the results from the inhibition data herein support the hypothesis that modifications to the design of traditional phosphonamidates by the replacement of an oxygen ligand with a sulfur atom on the central phosphorus can yield more potent metallopeptidase inhibitors. Although this phosphonamidothionate motif has been greatly overlooked in terms of generating potent tetrahedral-intermediate analog inhibitors, these results with phosphonamidothionates **2** may serve as precedence for the extrapolation of this design to other enzymatic systems.

Table 1. Inhibition of CPG[14] by Phosphonamidothionates 2

inhibitor		K_i* μM
2a	R = Ph	3.24
2b	R = *n*Bu	0.264
2c	R = Et	1.24
2d	R = Me	6.20

*K_i values were determined from the Dixon plots in Figure 3.

Acknowledgments: This investigation was supported in part through a grant from the National Institutes of Health, MBRS SCORE Program-NIGMS (Grant No. S06 GM52588-04) and a 'Research Infrastructure in Minority Institutions' award from the National Center for Research Resources with funding from the Office of Research on Minority Health, National Institutes of Health (Grant No. RR11805-02). The authors would also like to extend their gratitude to the Dept. of Education for the GAANN fellowship to C. E. Rodriguez (Grant No. P200A70233) and to the Department of Defense Science Scholars Program for support to A. Dastgah (Grant No. 6-93472).

References and Notes

1. Robinson, M. B.; Blakely, R. D.; Couto, R. Coyle, J. T. *J. Biol. Chem.* **1987**, *262*, 14498.

2. Carter, R. E.; Feldman, A. R.; Coyle, J. T. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 749.

3. Reisenauer, A. M.; Krumdieck, C. L.; Hlasted, C. H. *Science,* **1977**, *198*, 196.

4. a) Goldman, P. and Levy, C. C. Proc. Natl. Acad. Sci., **1967**, *58*, 1299. b) Levy, C. C. and Goldman, P. *J. Biol. Chem.* **1967**, *242*, 2933. c) McCullough, J. L.; Chabner, B. A.; Bertino, J. R. *J. Biol. Chem.* **1971**, *246*, 7207. c) Sherwood, R. F.; Melton, R. G.; Alwan, S. M.; Hughes, P. *Eur. J. Biochem.* **1985**, *148*, 447.

5. Bagshawe, K. D.; Springer, C. J.; Scarle, F.; Antoniw, P.; Sharma, S.K.; Melton, R. G.; Sherwood, R. F. *Br. J. Cancer.* **1988**, *58*, 700.

6. Adamson, P. C.; Balis, F. M.; McCully, C. L.; Godwin, K. S.; Poplack, D. G. *J. Clin. Oncol.* **1992**, *10*, 1359.

7. Rodriguez, C. E.; Holmes, H. M.; Mlodnosky, K. L.; Lam, V. Q.; Berkman, C. E. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1521.

8. Bell, C. F. in *Principles and Applications of Metal Chelation*; Atkins, P. W.; Holker, J. S. E.; Holliday, A. K., Eds.; Oxford University: Oxford, 1977. Prasad, A. S. in *Biochemistry of Zinc*; Frieden, E., Ed. Plenum: New York, 1993.

9. Suarez, A. I.; Lin, J.; Thompson, C. M. *Tetrahedron* **1985**, *41*, 5061. Thomspon, C. M.; Lin, J. *Tetrahedron Lett.* **1996**, *27*, 8979 .

10. Mlodnosky, K. L.; Holmes, H. M.; Lam, V. Q.; Berkman, C. E. *Tetrahedron Lett.* **1997**, *38*, 8803.

11. Jackson, J. A.; Berkman, C. E.; Thompson, C. M. *Tetrahedron Lett.* **1992**, *33*, 6061.

12. All compounds were fully characterized by ^{1}H and ^{31}P NMR (relative to H_3PO_4). **2a**, ^{1}H NMR (CD_3OD) δ 1.688–1.818 (m, 2H), 1.992–2.227 (m, 2H), 3.474–3.684 (m, 1H), 7.294–7.336 (m, 3H), 7.713–7.849 (m, 2H), ^{31}P NMR (CD_3OD) δ 49.93, 51.00; **2b**, ^{1}H NMR (CD_3OD) δ 0.777 and 0.785 (t, J = 7.3 Hz, 3H), 1.207–1.267 (m, 2H), 1.469–1.518 (m, 2H), 1.539–1.637 (m, 2H), 1.755–1.808 (m, 2H), 2.129–2.185 (m, 2H), 3.498–3.599 (m, 1H) ^{31}P NMR (CD_3OD) δ 68.30, 68.70; **2c**, ^{1}H NMR (D_2O) δ 0.987 and 1.056 (dt, J = 7.6 Hz, J = 4.8 Hz, 3H), 1.685–1.833 (m, 4H), 2.149–2.214 (m, 2H), 3.484 and 3.623 (dt, J = 6.4 Hz, J = 10.7 Hz, 1H) ^{31}P NMR (D_2O) δ 69.87, 71.42; **2d**, ^{1}H NMR (CD_3OD) δ 1.293 and 1.297 (d, J = 14.1 Hz, 3H), 1.541–1.645 (m, 2H), 1.943–2.108 (m, 2H), 3.309–3.422 (m, 1H) ^{31}P NMR (CD_3OD) δ 62.63, 63.35.

13. Methotrexate and its hydrolytic product (4-(N-[2,4-diamino-6-pteridinylmethyl]-N-methylamino)benzoic acid) were separated and quantified with an analytical reversed phase HPLC column (4.6 x 150 mm, Sphereclone 5u ODS(2)), Phenomenex, Torrence, CA, USA). The mobile phase for elution was CH_3OH/[potassium phosphate, 50 mM, pH 6.8] (22:78; v:v). At a flow rate of 1.2 mL/min, methotrexate and its hydrolytic product were detected at 304 nm with retention times of 8.8 and 13.9 min, respectively.

14. Careful time-course studies (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 min) were performed to define the linear portion of the initial rate (0–2 min) for the enzymatic hydrolysis of methotrexate. 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., approximately 5 % conversion to product was observed for incubations with the lowest substrate concentration, 3 μM, and in the absence of inhibitor). *Typical experimental procedures*: A typical incubation mixture (final volume 0.25 mL) was prepared by addition of 200μL TRIS buffer (50 mM, pH 7.3) to 25 μL of an enzyme solution (0.0375units/mL water). The enzymatic reaction was initiated by the addition of a 25 μL aqueous mixture containing both substrate and inhibitor. The reaction was allowed to proceed for one minute with constant shaking at 30 ^{0}C and was terminated by the addition of 100 μL methanolic TFA (1% trifluoroacetic acid by volume in methanol) followed by vortexing and centrifugation (7000*g*). A 100 μL aliquot of the resulting supernatant was then quantified by HPLC as described above.[13]