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INHIBITION OF GLUTAMATE CARBOXYPEPTIDASE BY PHOSPHORYL AND THIOPHOSPHORYL DERIVATIVES OF GLUTAMIC AND 2-HYDROXYGLUTARIC ACID

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Representative phosphoryl and thiophosphoryl derivatives of (S)-glutamic or (S)-2-hydroxyglutaric acid were synthesized and evaluated for their inhibitory potency against the glutamate carboxypeptidase, carboxypeptidase G (CPG). It was observed that the inhibition of CPG was highly sensitive to the individual phosphorus ligands. The most potent inhibitors were the dibasic phosphoryl and thiophosphoryl derivatives of glutamic acid and the monobasic thiophosphoryl derivatives of 2-hydroxyglutaric acid.

Keywords: Inhibition; metallopeptidase; phosphate; phosphoramidate; phosphoramidothionate; phosphorothionate

INTRODUCTION

Our research efforts recently have been aimed at developing potent competitive inhibitors for glutamate carboxypeptidases such as N-acetylated- α -linked-acidic dipeptidase (NAALADase),¹ prostate-specific membrane antigen (PSMA),² pteroylpoly-glutamate hydrolase (PPH),³ and carboxypeptidase G (CPG).⁴ The acquisition of inhibitors

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for such enzymes is expected to further the understanding of the biological role of these metallocarboxypeptidases as well as to serve in the elucidation of germane active site features. In addition, inhibitors of CPG₂ (closely related to CPG) recently have been sought for the use in inhibiting non-tumor-localized enzyme in ADEPT strategies.⁵

Based on preliminary evidence, alkylphosphonyl and alkylthiophosphonyl derivatives of glutamic and 2-hydroxyglutaric acid (**1–4**) have exhibited promise as potent tetrahedral-intermediate analog inhibitors of glutamate-liberating metallopeptidases known as glutamate carboxypeptidases. Although the phosphonyl motif often provides suitable inhibitory potency toward metallopeptidases, thiophosphonyl analogs maintain the unique property of probing enzyme active-site architecture with complementary chiral phosphorus centers.⁶ We observed that, in some cases, one alkylthiophosphonyl stereoisomer possesses inhibitory potency notably greater than either a stereoisomer of antipodal phosphorus stereochemistry or its respective alkylphosphonyl analog.⁷ The basis for the enhanced inhibitory potency of such compounds, especially against zinc-metallopeptidases, presumably is due to favorable zinc-sulfur interactions within enzyme active sites.⁸

Although simple N-phosphonyl and N-thiophosphonyl derivatives of amino acids can be readily obtained from C-protected amino acids, the corresponding derivatives of 2-hydroxyacids are more challenging due to the lack of readily available oxygen analogs of amino acids. However, the resulting isosteric analogs of phosphonamidothionates and phosphonamidates, such as phosphonothionates and phosphonates, are expected not only to be similarly promising (as are metallopeptidase inhibitors), but possess the additional benefit of being less prone to desulfurization and hydrolysis under acidic conditions.⁹

The focus of the current study was to examine the effect of isosteric replacement of the proximal alkyl ligand carbon atom in previous phosphonyl and thiophosphonyl inhibitors with an oxygen atom upon glutamate carboxypeptidase inhibition. To this end, representative

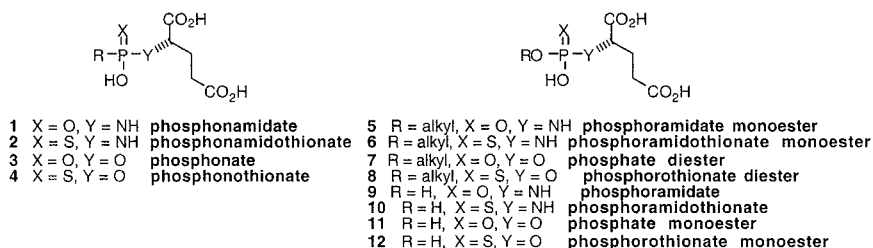


FIGURE 1

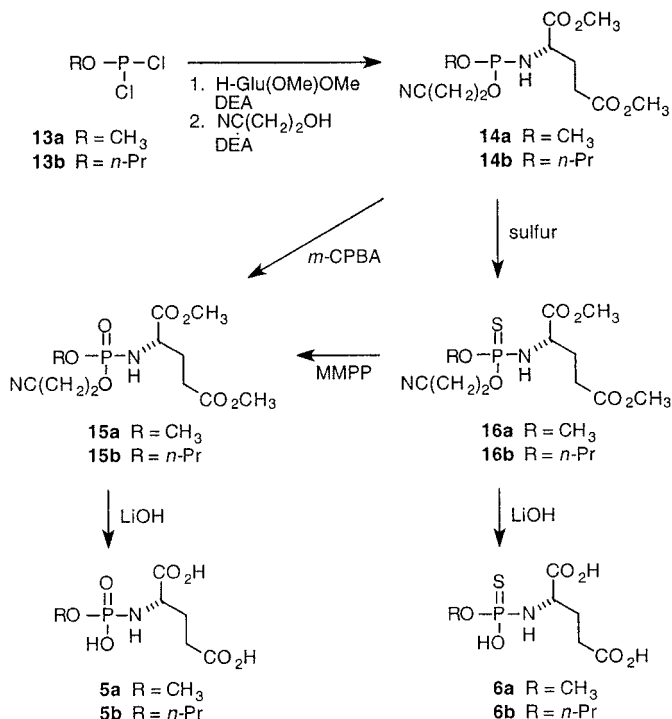
compounds **5–12** were synthesized and evaluated for inhibitory potency against glutamate carboxypeptidase. In line with our laboratory's interest in the inhibition of glutamate carboxypeptidases, CPG was selected as a model enzyme for its versatility and ready availability.

Results and Discussion

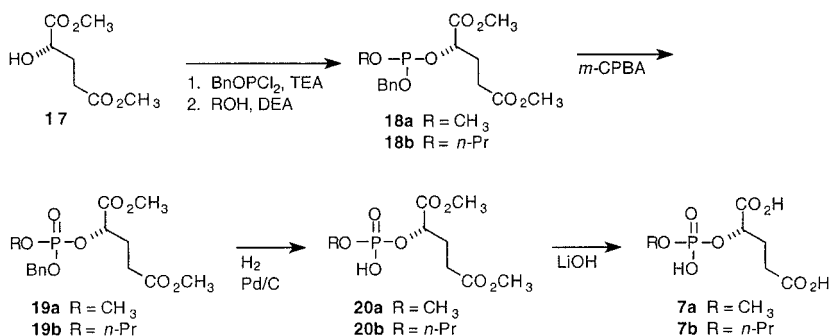
Due to the significant structural similarities of the target inhibitors **5–12**, the synthetic strategies for many of these compounds or their synthetic intermediates were sustained when possible. In a few cases, however, a different approach was necessary and therefore pursued. Beginning with dichlorophosphite **13**,* phosphoroamidite intermediates **14** were obtained by a sequential one-pot reaction with glutamic acid dimethyl ester and 2-cyanoethanol. Subsequent oxidation with *m*CPBA or thionation with sulfur gave the phosphoramidates **15** or phosphoramidothioates **16** respectively (Scheme 1). The yield for preparing **15a** was notably improved by subjecting phosphoramidothioate **16a** to oxidative desulfurization conditions using MMPP. Subsequent deprotection of intermediates **15** and **16** with LiOH, provided **5** and **6** respectively.

The synthesis of compounds **7a**, **7b** was achieved through the strategy outlined in Scheme 2. Reaction of benzyl dichlorophosphite with (*S*)-2-hydroxyglutarate **17**¹⁰ and the corresponding alcohol in a two-step, one-pot reaction gave phosphites **18**, which were summarily subjected to oxidation with *m*CPBA to give phosphate precursors **19**. Deprotection of the benzyl group by catalytic hydrogenolysis with 5% Pd/C in MeOH afforded the phosphate monoesters **20** which were further deprotected with LiOH, finally yielding the fully deprotected targets **7**. It should be noted that the use of dibenzyl ester of glutaric acid instead of the dimethyl ester did not provide the phosphoric acids **7** by a one-step deprotection through catalytic hydrogenolysis as expected. It was hypothesized that the initial deprotection at the alpha-carboxylate could react with the phosphorus center to form a cyclic pentacoordinated intermediate similar to that proposed for the N-phosphoryl glutamic acid.⁹

*Methyl dichlorophosphite **13a** was available from Aldrich Chemical Company (Milwaukee, WI, U.S.A.). Propyl dichlorophosphite **13b** was prepared as follows. A solution of 1-propanol (7.5 mL, 100 mmol) in diethyl ether (50 mL) was added to a stirring solution of phosphorus trichloride (9.0 mL, 100 mmol) in diethyl ether (50 mL) at -78°C . The resulting solution was stirred for 15 min while being allowed to warm to room temperature. The solution was concentrated in vacuo and purified by vacuum distillation (20 mmHg, 109°C) to give propyl dichlorophosphite as a colorless liquid in 85.7% yield. ^1H NMR: (CDCl_3) δ 1.91–1.45 (t, 3H, $J = 7.4$ Hz), 1.66–1.83 (m, 2H), 4.14–4.25 (dt, 2H, $J = 6.4$ and 12.9 Hz). ^{31}P NMR: (CDCl_3) δ 179.09.

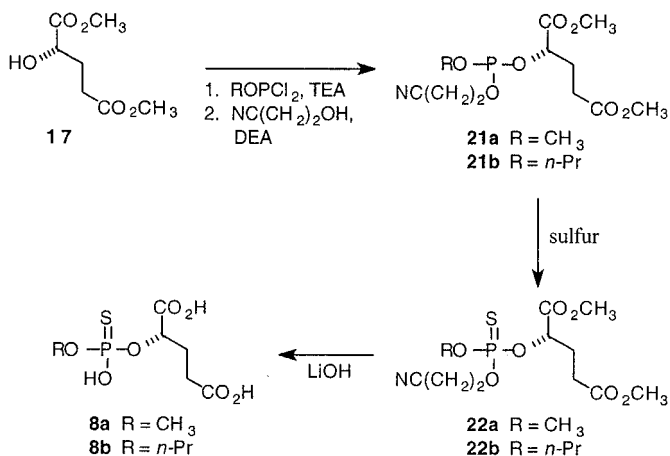


SCHEME 1



SCHEME 2

Due to the poisoning effect of sulfur on palladium catalysts, thio-phosphoric acid derivatives **8** were prepared by an alternative approach shown in Scheme 3 in which 2-cyanoethoxy was incorporated as the phosphorus protective group rather than benzyloxy. Thus, the reaction of alkyl dichlorophosphites with (*S*)-2-hydroxyglutarate **17** and

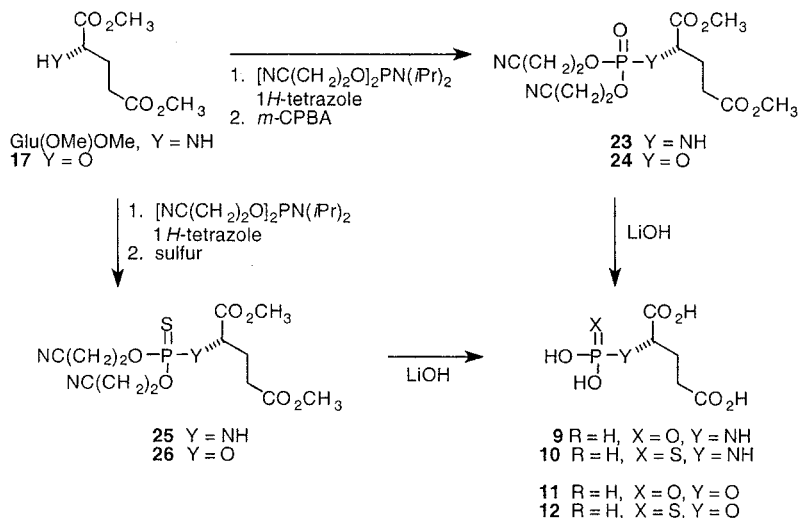


SCHEME 3

2-cyanoethanol provided phosphites **21**, which were summarily thionated with sulfur to give phosphorothionates **22**. Subsequent mild basic hydrolysis with LiOH provided the phosphorothionate diesters **8**.

Employing our recently developed amine-exchange reaction on phosphoramidites,¹¹ the respective phosphoramidate and phosphoramidothionate precursors **23** and **25** were prepared by the reaction of bis(2-cyanoethyl)-N,N-diisopropylphosphoramidite with Glu(OMe)₂, followed respectively by oxidation or thionation. Deprotection of **23** or **25** with LiOH provided **9** and **10** respectively. Phosphitylation of (S)-2-hydroxyglutarate **17** with bis(2-cyanoethyl)-N,N-diisopropylphosphoramidite followed by oxidation or thionation gave the corresponding phosphate **24** or thiophosphate **26** respectively. Mild hydrolysis with LiOH afforded the fully deprotected targets, **11** and **12**.

In our ongoing studies with alkyl- and arylphosphonamidothionates, we have noticed only moderate stability ($t_{1/2} = 35\text{--}130$ min) when subjected to the conditions of the enzyme incubations. Knowing that these phosphonamidothionates are prone to desulfurization under such conditions, it was hypothesized that a carbon-to-oxygen isosteric replacement would impart greater stability of compounds **6** over their alkylphosphonamidothionate analogs. Indeed, we observed that for over 16 h, the phosphoramidothionates **6** were completely stable, exhibiting no desulfurization under the conditions of the inhibition assay (pH 7.3). Presumably, this enhanced stability is due to increased stabilization of the phosphorus monoacid anion along with a correspondingly lower pKa of the monoacid itself. Incidentally, it also was noted that inhibitors **5** and **7–12** were similarly stable under the assay conditions.



SCHEME 4

For the inhibitors that possessed a sulfur ligand (**X**) on phosphorus (Table I; **6**, **8**, **10**, **12**) notable differences were observed in their ^{31}P NMR chemical shifts. While the relatively upfield chemical shifts for inhibitors **10** and **12** at approximately 46 ppm suggest that they

TABLE I Inhibition of CPG by Phosphoryl and Thiophosphoryl Derivatives of Glutamic and 2-Hydroxyglutaric Acid

Inhibitor	R	X	Y	^{31}P NMR ^a	K_i (μM) ^b
9	H	O	NH	8.66	2.1 (0.9730)
5a	CH ₃	O	NH	10.11	65 (0.9079)
5b	CH ₃ CH ₂ CH ₂	O	NH	8.49	31 (0.9589)
10	H	S	NH	46.38	0.53 (0.9960)
6a	CH ₃	S	NH	58.61, 59.24 ^c	424 (0.9950)
6b	CH ₃ CH ₂ CH ₂	S	NH	56.36, 57.12 ^c	27 (0.9695)
11	H	O	O	3.93	170 (0.9800)
7a	CH ₃	O	O	1.80	24 (0.9932)
7b	CH ₃ CH ₂ CH ₂	O	O	0.82	26 (0.9990)
12	H	S	O	46.52	44 (0.9980)
8a	CH ₃	S	O	59.55, 60.72	4.7 (0.9823)
8b	CH ₃ CH ₂ CH ₂	S	O	56.91, 58.32	5.7 (0.9995)

^aChemical shifts in CD₃OD referenced to 85% H₃PO₄.

^bCorrelation coefficient in parenthesis.

^cChemical shifts in D₂O referenced to 85% H₃PO₄.

exist as the thiol tautomer (P-SH), the chemical shifts near 60 ppm for inhibitors **6** and **8** suggests a preference for thiono tautomer (P=S). The latter assertion is in agreement with a similar observation reported previously for a series of phosphonamidothionates.¹²

Once obtained in sufficient quantity, the individual inhibitors **5–12** were examined for inhibitory potency against CPG. It should be noted that **6a, 6b** and **8a, 8b** were individually prepared as a mixture of two diastereomers differing only by their phosphorus configuration and their diastereomeric ratios ranged from 1:1.1 to 1:1.3. Resolution of their respective precursors (**16a,b** and **22a,b**) by flash silica gel chromatography were unsuccessful and no further attempts were made to resolve these diastereomeric inhibitors. As such, **6a, 6b** and **8a, 8b** were evaluated for inhibitory potency as individual mixtures of diastereomers. Employing the hydrolysis of methotrexate⁴ as the conventional measure of enzymatic activity, both K_m and V_{max} were initially determined ($1.3 \mu M$ and $15 \mu mol \min^{-1} mg\text{-protein}^{-1}$ respectively). Dixon analyses were subsequently performed to obtain K_i values for **5–12** (Table I). In all Dixon analyses, the initial substrate (methotrexate) concentration was $10 \mu M$ and triplicate determinations were made and averaged for each inhibitor concentration, which generally varied within a range from 5 to $120 \mu M$ depending on the potency of the individual inhibitors.

Of the series of compounds examined, the most potent inhibitor was the phosphonamidothionate **10** ($K_i = 0.53 \mu M$). Although less potent than **10**, inhibitors **8a, 8b**, and **9** exhibited strong and similar potency ($K_i = 4.7, 5.7$, and $2.1 \mu M$ respectively) against CPG when compared to the remaining compounds. The trend observed for inhibition results of the phosphorus diacids **9–12** did not completely coincide with the trends observed for the phosphoryl monoacids **5–8**. The thiono analogs **10** and **12** were respectively more potent inhibitors than **9** and **11**. Thus, for these phosphoryl diacids (**9–12**), it was observed that substitution of one of the oxygen ligands to phosphorus with sulfur generally enhanced the inhibitory potency. This observation also was in agreement with the trend observed for the phosphoryl monoacids derivatives of 2-hydroxyglutaric acid, **7a, 7b** and **8a, 8b** ($Y=O$) but contrary to that observed for the phosphoryl monoacids derivatives of glutamic acid, **5a, 5b** and **6a, 6b** ($Y=NH$).

Although the respective isosteric replacement of the P–N linkage in the diacids **9** and **10** ($Y=NH$) with that of the P–O linkage found in **11** and **12** ($Y=O$) resulted in dramatically weaker inhibitors of CPG, the converse was generally true for the phosphoryl monoacids **5–8**. In addition, the isosteric replacement of the P–N linkage in the monoacids **5a, 5b** and **6a, 6b** with a P–O linkage (**7a, 7b** and **8a, 8b**) appeared to significantly reduce the importance of the alkoxy ligand. Therefore, the

NH ligand may influence the orientation of the phosphorus group in the enzyme active site, potentially through hydrogen bonding interactions through the amido hydrogen.

The apparent reduction in the significance of the alkoxy ligands in **7a**, **7b** and **8a**, **8b** suggests that these ligands may not be involved in binding interactions with complimentary features within the active site, thus allowing both the $\text{O}=\text{P}-\text{O}^-$ or the $\text{S}=\text{P}-\text{O}^-$ moieties to fully participate in coordinating with the active site zinc ions. For these compounds as well as for the diacids **9–12** which possess no alkoxy ligand, the $\text{S}=\text{P}-\text{O}^-$ moiety appears to provide enhanced inhibitory potency over $\text{O}=\text{P}-\text{O}^-$, presumably through greater chelation of the active site zinc ions. In contrast to the trend observed for the **7a**, **7b** and **8a**, **8b** ($\text{Y}=\text{O}$), the inhibitory potency of the monoacids **5a**, **5b** and **6a**, **6b** ($\text{Y}=\text{NH}$) was indeed dependent upon the length of the alkoxy ligand.

Conclusion

In summary, the procurement of a limited series of phosphoryl and thiophosphoryl derivatives of (*S*)-glutamic or (*S*)-2-hydroxyglutaric acid was achieved using slight modifications of previously established methodologies. The significance of such compounds lies with their potential to serve as potent inhibitors of glutamate carboxypeptidases. Examination of the inhibitory data for these compounds with CPG revealed that the inhibition was sensitive to the individual phosphorus ligands. The most potent inhibitors were the dibasic phosphoryl (**9**) and thiophosphoryl (**10**) derivatives of glutamic acid and the monobasic thiophosphoryl derivatives of 2-hydroxyglutaric acid (**8a**, **8b**). eventual resolution of the individual phosphorus stereoisomers of **6a**, **6b** and **8a**, **8b** may provide additional information on the effects of phosphorus stereochemistry upon the interactions with active site features. Future studies involving the examination of this or an extended series of phosphonothioic acids against more medically relevant glutamate carboxypeptidase such as NAALADase or PSMA are anticipated to provide greater insight into the characteristics of those enzymes and allow for the development of potentially therapeutic agents.

EXPERIMENTAL

Synthesis

General

All solvents used in reactions (benzene, CH_2Cl_2 , THF), 3-hydroxypropionitrile, diisopropylethylamine (DEA), and triethylamine

(TEA) were freshly distilled prior to use. All other reagents were used as supplied unless otherwise stated. Liquid (flash) chromatography was carried out using silica gel 60 (230–400 mesh). ^1H , ^{13}C , and ^{31}P NMR spectra were recorded on a Bruker DRX 300 MHz NMR Spectrometer. ^1H NMR chemical shifts are relative to TMS ($\delta = 0.00$ ppm), CDCl_3 ($\delta = 7.26$ ppm), or CD_3OD ($\delta = 4.87$ and 3.31 ppm). ^{13}C NMR chemical shifts are relative to CD_3OD ($\delta = 49.15$ ppm or CDCl_3 ($\delta = 77.23$)). ^{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).

General procedure for 2-cyanoethyl alkyl [methyl (1-carbomethoxybutanoate)] phosphoramidothionates (16). A solution of H-Glu(OMe)-OMe (0.58 g, 3.29 mmol) in THF (25 mL) was added dropwise to a solution of alkylidichlorophosphite (3.31 mmol) and diisopropylethylamine (0.69 mL, 3.96 mmol) in THF (25 mL) at -78°C . After removal of external cooling, stirring was continued for 20 min. The solution was filtered and concentrated in vacuo. The reaction mixture was dissolved in THF (25 mL) at -78°C . To this was added a solution of 3-hydroxypropionitrile (0.25 mL, 3.29 mmol) and diisopropylethylamine (0.69 mL, 3.96 mmol) in THF (25 mL). The resulting solution was stirred for 20 min while being allowed to warm to room temperature. The solution was filtered and sulfur (0.22 g, 6.96 mmol) was added. The solution was stirred at room temperature for 2 h, filtered, concentrated in vacuo, and purified by silica gel column chromatography to give a pale yellow oil **16a** (hexane:ethyl acetate:dichloromethane 1:1:1, $v:v:v$, $R_f = 0.28$) or **16b** (hexane:ethyl acetate:dichloromethane 2:1:1, $v:v:v$, $R_f = 0.22$). **16a**: ^1H NMR (CDCl_3) δ 1.89–2.02 (m, 1H), 2.12–2.25 (m, 1H), 2.37–2.54 (m, 2H), 2.70–2.82 (m, 2H), 3.63–3.84 (m, 9H), 4.02–4.29 (m, 3H); ^{31}P NMR (CDCl_3) δ 74.10, 74.59 (1.3:1 diastereomers); Anal. Calcd. for $\text{C}_{11}\text{H}_{19}\text{N}_2\text{O}_6\text{PS}$: C, 39.05; H, 5.66; N, 8.28. Found: C, 38.94; H, 5.54; N, 8.12. **16b**: ^1H NMR (CDCl_3) δ 0.94–1.01 (m, 3H), 1.67–1.75 (m, 2H), 1.91–2.02 (m, 1H), 2.10–2.20 (m, 1H), 2.43–2.52 (m, 2H), 2.74–2.81 (m, 2H), 3.72 (s, 3H), 3.78 and 3.80 (s, 3H), 3.97–4.02 (m, 2H), 4.03–4.16 (m, 1H), 4.17–4.24 (m, 2H); ^{31}P NMR (CDCl_3) δ 72.17, 72.62 (1.3:1 diastereomers); Anal. Calcd. for $\text{C}_{13}\text{H}_{23}\text{N}_2\text{O}_6\text{PS}$: C, 42.62; H, 6.33; N, 7.65. Found: C, 42.54; H, 6.18; N, 7.46.

2-Cyanoethyl methyl [methyl (1-carbomethoxybutanoate)] phosphoramidate (15a). Dichloromethane was neutralized with basic aluminum oxide and dried over warm molecular sieves prior to use. MMPP

(0.31 g, 5.05 mmol) was added to a solution of the phosphoramidothionate **16a** (0.15 g, 4.55 mmol) in CH_2Cl_2 (10 mL). The solution was refluxed 4 h, filtered, concentrated in vacuo, and purified by silica gel column chromatography (dichloromethane:acetone 2:1, *v:v*, $R_f = 0.20$) to give a pale yellow oil **15a**. ^1H NMR (CDCl_3) δ 1.99–2.05 (m, 1H), 2.11–2.24 (m, 1H), 2.38–2.57 (m, 2H), 2.73–2.85 (m, 2H), 3.41–3.54 (m, 1H), 3.71–3.83 (m, 9H), 3.88–4.02 (m, 1H), 4.16–4.28 (m, 2H); ^{31}P NMR (CDCl_3) δ 9.54, 9.62 (1.2:1 diastereomers); Anal. Calcd. for $\text{C}_{11}\text{H}_{19}\text{N}_2\text{O}_7\text{P}$: C, 41.00; H, 5.94; N, 8.69. Found: C, 40.97; H, 5.78; N, 8.48.

2-Cyanoethyl propyl [methyl (1-carbomethoxybutanoate)] phosphoramidate (15b). The procedure to prepare **15b** was identical to those described for the synthesis of compound **16b** with the exception that the intermediate phosphoramidite **14b** was intercepted with *m*CBPA rather than sulfur. The resulting reaction mixture was stirred at room temperature for 15 min, concentrated in vacuo, and redissolved in CH_2Cl_2 (20 mL). The solution was extracted twice with 10% wt. sodium carbonate, dried over MgSO_4 , filtered, concentrated in vacuo, and purified by silica gel column chromatography (dichloromethane:acetone 3:1, *v:v*, $R_f = 0.28$) to give a clear yellowish oil **15b**. ^1H NMR (CDCl_3) δ 0.95–1.02 (m, 3H), 1.65–1.76 (m, 2H), 1.89–2.01 (m, 1H), 2.09–2.19 (m, 1H), 2.37–2.54 (m, 2H), 2.70–2.83 (m, 2H), 3.29–3.39 (t, 1H), 3.70 (s, 3H), 3.78 (s, 3H), 3.91–4.06 (m, 3H), 4.14–4.31 (m, 2H); ^{31}P NMR (CDCl_3) δ 8.25, 8.35 (1.3:1 diastereomers); Anal. Calcd. for $\text{C}_{13}\text{H}_{23}\text{N}_2\text{O}_7\text{P}$: C, 44.57; H, 6.62; N, 8.00. Found: C, 45.17; H, 6.61; N, 7.51.

General procedure for benzyl alkyl [methyl (1-carbomethoxybutanoate)] phosphates (19). Into a flask charged with benzyl dichlorophosphite* (645 mg, 3.09 mmol) in anhydrous ether (40.0 mL) was added a solution of (*S*)-dimethyl 2-hydroxyglutarate (436 mg, 2.47 mmol) and triethylamine (0.43 mL, 3.09 mmol) in anhydrous ether (15.0 mL) via syringe at -70°C under an argon atmosphere. The resulting solution was stirred 0.5 h. A solution of the corresponding alcohol (6.18 mmol) and TEA (1.2 mL, 6.18 mmol) in anhydrous ether (15.0 mL) was added dropwise to the reaction mixture (-70°C), which was then allowed to warm to ambient temperature. The intermediate phosphite **18** was oxidized by the addition of *m*CPBA (832 mg, 4.94 mmol). The solution was stirred an additional 1 h, filtered, concentrated in vacuo, and

*Benzyl dichlorophosphite was prepared as follows: A solution of benzyl alcohol (100 mmol) in diethyl ether (50 mL) was added to a stirring solution of phosphorous trichloride (9.0 mL, 100 mmol) in diethyl ether (50 mL) at -78°C . The resulting solution was stirred for 15 min while being allowed to warm to room temperature. The solution was concentrated in vacuo and purified by vacuum distillation to give benzyl dichlorophosphite as a colorless liquid.

purified by flash chromatography to give **19a** (59%) or **19b** (62%) [ether: methanol 100:1 v/v (**19a**: Rf = 0.32. **19b**: Rf = 0.28)]. **19a**: ^1H NMR (CDCl_3) δ 2.04–2.35 (m, 2H), 2.36–2.66 (m, 2H), 3.67 (d, J = 5.4 Hz, 3H), 3.73 and 3.75 (s, 3H), 3.76 and 3.78 (s, 3H), 4.84–4.93 (m, 1H), 5.11 (d, J = 17.7 Hz, 1H), 5.14 (dd, J = 17.1 Hz, J = 0.8 Hz, 1H), 7.27–7.66 (m, 5H); ^{31}P NMR (CDCl_3) δ 0.29; Anal. Calcd. for $\text{C}_{15}\text{H}_{21}\text{O}_8\text{P}$: C, 50.00; H, 5.83; Found: C, 49.68; H, 5.65. **19b**: ^1H NMR (CDCl_3) δ 0.93 and 0.92 (t, J = 7.5 Hz, 3H), 1.60–1.74 (m, 2H), 2.10–2.31 (m, 2H), 2.38–2.51 (m, 2H), 3.65 and 3.67 (s, 3H), 3.73 and 3.74 (s, 3H), 3.96–4.09 (m, 2H), 4.81–4.92 (m, 1H), 5.10 (d, J = 16.2 Hz, 1H), 5.13 (dd, J = 15.3 Hz, J = 0.9 Hz, 1H), 7.26–7.55 (m, 5H); ^{31}P NMR (CDCl_3) δ -0.63; Anal. Calcd. for $\text{C}_{17}\text{H}_{25}\text{O}_8\text{P}$: C, 52.51; H, 6.44; Found: C, 50.86; H, 6.36.

General procedure for 2-cyanoethyl alkyl [methyl (1-carbomethoxybutanoate)] phosphorothionates (22). A solution of (S)-dimethyl 2-hydroxyglutarate (0.53 g, 3.0 mmol) and triethylamine (0.40 g, 4.0 mmol) in anhydrous ether (20.0 mL) was added via syringe to a stirring solution of alkyl dichlorophosphite (3.9 mmol) in anhydrous ether (60 mL) at -70°C under an argon atmosphere. The resulting solution was stirred for 20 min at -70°C , followed by the addition of a solution of 3-hydroxypropionitrile (0.34 g, 4.8 mmol) and triethylamine (0.51 g, 5.0 mmol) in anhydrous ether (20.0 mL). The solution was stirred an additional 20 min and warmed to the room temperature. Sulfur (0.16 g, 4.78 mmol) was added and the reaction mixture was stirred overnight. Subsequent filtration and concentration in vacuo afforded a yellow oil, which was purified by flash chromatography to give the ester **22a** (49%, ether:hexane 2.5:1, v/v, Rf = 0.19) and **22b** (52%, ethyl acetate:hexane 3:1, v/v, Rf = 0.41). **22a**: ^1H NMR (CDCl_3) δ 2.14–2.30 (dm, 2H), 2.44–2.52 (m, 2H), 2.74–2.84 (m, 2H), 3.69 (s, 3H), 3.79 (s, 3H), 3.79 and 3.84 (d, J = 12.0 Hz, 3H), 4.23–4.38 (m, 2H), 4.96–5.04 (m, 1H); ^{31}P NMR (CDCl_3) δ 70.34, 70.76 (1.3:1 diastereomers); Anal. Calcd. for $\text{C}_{11}\text{H}_{18}\text{NO}_7\text{PS}$: C, 38.94; H, 5.31; N, 4.13; Found: C, 39.23; H, 5.19; N, 4.12. **22b**: ^1H NMR (CDCl_3) δ 0.97 (t, J = 7.2 Hz, 3H), 1.57–1.78 (m, 2H), 2.08–2.34 (dm, 2H), 2.41–2.58 (m, 2H), 2.72–2.88 (m, 2H), 3.69 (s, 3H), 3.78 (s, 3H), 4.02–4.15 (m, 2H), 4.21–4.38 (m, 2H), 4.95–5.05 (m, 1H); ^{31}P NMR (CDCl_3) δ 68.65, 69.16 (1.1:1 diastereomers); Anal. Calcd. for $\text{C}_{13}\text{H}_{22}\text{NO}_7\text{PS}$: C, 42.51; H, 5.99; N, 3.81; Found: C, 43.07; H, 5.94; N, 3.70.

O,O-Bis(2-cyanoethyl) methyl 1-carbomethoxybutanoate phosphoramidate (**23**) and phosphate (**24**). 1H-tetrazole (0.08 g, 1.2 mmol) dissolved in 6 mL acetonitrile was added to a solution of bis(2-cyanoethyl)-N',N'-diisopropylphosphoramidite (0.27 g, 1.0 mmol), L-glutamic acid

dimethyl ester hydrochloride (0.25 g, 1.2 mmol) or (*S*)-dimethyl 2-hydroxyglutarate (0.21 g, 1.2 mmol), and diisopropylethylamine (0.26 g, 2.0 mmol) in THF (1.5 mL) under an argon atmosphere at room temperature. The mixture was stirred for 0.5 h, followed by the addition of tert-butyl hydroperoxide (0.18 g, 2.0 mmol), and stirred another 3 h. After filtration of the resulting reaction mixture, the solvent was removed under reduced pressure. Purification by flash chromatography provided **23** (ethyl acetate, *R_f* = 0.16) and **24** (ethyl acetate, *R_f* = 0.36). **23**: ^1H NMR (CDCl_3) δ 1.90–2.20 (dm, 2H), 2.36–2.49 (m, 2H), 2.70–2.82 (m, 4H), 3.40–3.62 (m, 1H), 3.70 (s, 3H), 3.78 (s, 3H), 3.86–4.02 (m, 1H), 4.17–4.31 (m, 4H); ^{31}P NMR (CDCl_3) δ 7.54; Anal. Calcd. for $\text{C}_{13}\text{H}_{20}\text{N}_3\text{O}_7\text{P}$: C, 43.21; H, 5.54; N: 11.63; Found: C, 45.18; H, 5.56; N: 11.52. **24**: ^1H NMR (CDCl_3) δ 2.12–2.39 (dm, 2H), 2.41–2.59 (m, 2H), 2.73–2.88 (m, 4H), 3.71 (s, 3H), 3.79 (s, 3H), 4.32 (dt, J = 8.1 Hz, J = 6.2 Hz, 2H), 4.41 (dt, J = 8.4 Hz, J = 6.2 Hz, 2H), 4.96 (dt, J = 3.3 Hz, J = 4.5 Hz, 1H); ^{31}P NMR (CDCl_3) δ –2.35; Anal. Calcd. for $\text{C}_{13}\text{H}_{19}\text{N}_2\text{O}_8\text{P}$: C, 43.09; H, 5.25; N: 6.63; Found: C, 42.84; H, 5.08; N: 7.94.

O,O-Bis(2-cyanoethyl) methyl 1-carbomethoxybutanoate phosphoramidothionate (**25**) and phosphorothionate (**26**). To a solution of bis(2-cyanoethyl)-*N,N'*-diisopropylphosphoramidite (0.27 g, 1.0 mmol), L-glutamic acid dimethyl ester hydrochloride (0.25 g, 1.2 mmol) with diisopropylethylamine (0.26 g, 2.0 mmol) in THF or (*S*)-dimethyl 2-hydroxyglutarate (0.21 g, 1.2 mmol) in THF (1.5 mL) was added a solution of 1H-tetrazole (0.08 g, 1.2 mmol) in acetonitrile (6 mL) dropwise under argon at room temperature for 30 min. Sulfur (0.06 g, 2 mmol) was then added and the reaction mixture was stirred overnight. Evaporation of solvent under reduced pressure followed by purification by flash chromatography gave **25** (ether, *R_f* = 0.21) or **26** (hexane:ether 1:9, *R_f* = 0.42). **25**: ^1H NMR (CDCl_3) δ 1.72–1.85 (m, 1H), 1.95–2.11 (m, 1H), 2.31–2.38 (m, 2H), 2.61–2.70 (m, 4H), 3.59 (s, 3H), 3.67 (s, 3H), 3.80–4.05 (m, 1H), 4.07–4.16 (m, 4H); ^{31}P NMR (CDCl_3) δ 71.88; Anal. Calcd. for $\text{C}_{13}\text{H}_{20}\text{N}_3\text{O}_6\text{PS}$: C, 41.38; H, 5.31; N: 11.14; Found: C, 41.41; H, 5.21; N: 11.01. **26**: ^1H NMR (CDCl_3) δ 2.06–2.15 (m, 1H), 2.17–2.29 (m, 1H), 2.33–2.48 (m, 2H), 2.72–2.84 (m, 4H), 3.63 (s, 3H), 3.73 (s, 3H), 4.21–4.34 (m, 4H), 4.91–4.99 (m, 1H); ^{31}P NMR (CDCl_3) δ 67.66; Anal. Calcd. for $\text{C}_{13}\text{H}_{19}\text{N}_2\text{O}_7\text{PS}$: C, 41.27; H, 5.03; N: 7.41; Found: C, 41.18; H, 4.94; N: 7.56.

General Procedure for **5**, **6**, **8**, **9**, **10**, **11**, **12**

Phosphoramidate **15** or **23**, phosphoramidothionate **16** or **25**, phosphorothionates **22** or **26**, or phosphate **24** (0.5 mmol) was dissolved in

methanol (2.5 mL) to which was added aqueous LiOH (2.5 mL, 1.0 M). The resulting solution was stirred at room temperature for 16 h and then filtered. The solvent was evaporated in vacuo to give the corresponding crude products. The residue was resuspended in anhydrous methanol, filtered (0.45 μ M Teflon membrane), and concentrated in vacuo to give the trilithium salt of the desired product as a white solid.

Methyl hydrogen (1-carboxybutanoic acid) phosphoramidate (5a). ^1H NMR (CD_3OD) δ 1.72–1.98 (m, 2H), 2.10–2.29 (m, 2H), 3.42 (d, J = 10.8 Hz, 3H), 3.40–3.61 (m, 1H); ^{13}C NMR (CD_3OD) δ 33.86, 35.52, 52.28 (d, J = 4.6 Hz), 58.26, 182.37 (d, J = 3.4 Hz), 183.40; ^{31}P NMR (CD_3OD) δ 10.11; FABHRMS (M-Li) $^-$ calcd. 252.0437, found 252.0460 for $\text{C}_6\text{H}_9\text{Li}_2\text{NO}_7\text{P}$.

Propyl hydrogen (1-carboxybutanoic acid) phosphoramidate (5b). ^1H NMR (CD_3OD) δ 0.88 (t, J = 7.5 Hz, 3H), 1.46–1.58 (m, 2H), 1.81–2.02 (m, 2H), 2.13–2.41 (m, 2H), 3.48–3.60 (m, 1H), 3.61–3.72 (m, 2H); ^{13}C NMR (CD_3OD) δ 10.98, 25.37, 33.91, 35.52, 58.22, 67.28, 182.48, 183.51; ^{31}P NMR (CD_3OD) δ 8.49; FABHRMS (M-Li) $^-$ calcd. 280.0750, found 280.0759 for $\text{C}_8\text{H}_{13}\text{Li}_2\text{NO}_7\text{P}$.

Methyl hydrogen (1-carboxybutanoic acid) phosphoramidothionate (6a). ^1H NMR (CD_3OD) δ 1.58–1.82 (m, 2H), 1.98–2.12 (m, 2H), 3.31 and 3.34 (d, J = 13.2 Hz, 3H), 3.42–3.58 (m, 1H); ^{13}C NMR (CD_3OD) δ 33.65 (d, J = 11.1 Hz), 35.46 (d, J = 6.2 Hz), 52.68 (dd, J = 4.2 Hz, J = 17.1 Hz), 58.24, 181.90 (dd, J = 4.9 Hz, J = 11.8 Hz), 183.43 (d, J = 14.0 Hz); ^{31}P NMR (CD_3OD) δ 60.90, (D_2O) δ 58.61, 59.24 (1:1.3 diastereomers); FABHRMS (M-Li) $^-$ calcd. 268.0209, found 268.0214 for $\text{C}_6\text{H}_9\text{Li}_2\text{NO}_6\text{PS}$.

Propyl hydrogen (1-carboxybutanoic acid) phosphoramidothionate (6b). ^1H NMR (CD_3OD) δ 0.87 and 0.88 (t, J = 7.2 Hz, 3H), 1.42–1.60 (m, 2H), 1.72–1.93 (m, 2H), 2.08–2.30 (m, 2H), 3.58–3.80 (m, 3H); ^{13}C NMR (CD_3OD) δ 11.05, 25.08 (d, J = 8.8 Hz), 33.78 (d, J = 4.7 Hz), 35.44 (d, J = 11.5 Hz), 58.22, 67.87 (dd, J = 6.1 Hz, J = 12.3 Hz), 181.90 (dd, J = 5.9 Hz, J = 20.1 Hz), 183.46 (d, J = 19.3 Hz); ^{31}P NMR (CD_3OD) δ 58.88, 58.92, (D_2O) δ 56.36, 57.12 (1:1.3 diastereomers); FABHRMS (M-Li) $^-$ calcd. 296.0522, found 296.0535 for $\text{C}_8\text{H}_{13}\text{Li}_2\text{NO}_6\text{PS}$.

Methyl hydrogen (1-carboxybutanoic acid) phosphorothionate (8a). ^1H NMR (CD_3OD) δ 1.92–2.03 (m, 2H), 2.16–2.26 (m, 2H), 3.45 and 3.46 (d, J = 13.6 Hz, 3H), 4.52–4.63 (m, 1H); ^{13}C NMR (CD_3OD) δ 32.29 (d, J = 35.4 Hz), 35.03 (d, J = 53.1 Hz), 53.50 (d, J = 22.8 Hz), 78.62 (d, J = 112.5 Hz), 179.73 and 179.86, 182.95 and 183.05; ^{31}P NMR (CD_3OD)

δ 59.55, 60.72 (1.3:1 diastereomers); FABHRMS (M-Li)⁻ calcd. 269.0049, found 269.0035 for C₆H₈Li₂O₇PS.

Propyl hydrogen (1-carboxybutanoic acid) phosphorothionate (8b). ¹H NMR (CD₃OD) δ 0.90 and 0.91 (t, J = 7.4 Hz, 3H), 1.53–1.66 (m, 2H), 2.02–2.17 (m, 2H), 2.22–2.38 (m, 2H), 3.78–3.89 (m, 2H), 4.60–4.77 (m, 1H); ¹³C NMR (CD₃OD) δ 10.87, 24.98 (d, J = 34.5 Hz), 32.24 and 32.43 (d, J = 25.8 Hz), 34.92 and 35.16, 68.70 and 68.87 (d, J = 24.9 Hz), 78.21 and 78.80 (d, J = 23.4 Hz), 179.67 and 179.86, 182.89 and 183.01; ³¹P NMR (CD₃OD) δ 56.91, 58.32 (1:1.1 diastereomers); FABHRMS (M-Li)⁻ calcd. 297.0362, found 297.0356 for C₈H₁₂Li₂O₇PS.

N-(1-Carboxybutanoic acid) phosphoramidic acid (9). ¹H NMR (CD₃OD) δ 1.66–1.74 (m, 2H), 1.94–2.05 (m, 2H), 3.35–3.40 (m, 1H); ¹³C NMR (CD₃OD) δ 29.87, 34.07, 57.25, 181.97, 183.21; ³¹P NMR (CD₃OD) δ 8.66; FABHRMS (M-Li)⁻ calcd. 244.0362, found 244.0364 for C₅H₆Li₃NO₇P.

N-(1-Carboxybutanoic acid) phosphoramidothionic acid (10). ¹H NMR (CD₃OD) δ 1.83–1.99 (m, 2H), 2.14–2.36 (m, 2H), 3.65–3.73 (m, 1H); ¹³C NMR (CD₃OD) δ 30.29, 33.80, 57.53, 182.14, 183.66; ³¹P NMR (CD₃OD) δ 46.38; FABHRMS (M-Li)⁻ calcd. 260.0134, found 260.0107 for C₅H₆Li₃NO₆PS.

O-(1-Carboxybutanoic acid) phosphoric acid (11). ¹H NMR (CD₃OD) δ 1.72–1.93 (m, 2H), 1.96–2.08 (m, 1H), 2.12–2.24 (m, 1H), 4.19–4.30 (m, 1H); ¹³C NMR (CD₃OD) δ 28.44, 30.61, 71.71, 178.28, 180.91; ³¹P NMR (CD₃OD) δ 3.93; FABHRMS (M-Li)⁻ calcd. 245.0202, found 245.0221 for C₅H₅Li₃O₈P.

O-(1-Carboxybutanoic acid) phosphorothionic acid (12). ¹H NMR (CD₃OD) δ 1.95–2.19 (dm, 2H), 2.29–2.33 (m, 2H), 4.50–4.56 (m, 1H); ¹³C NMR (CD₃OD) δ 31.43 (d, J = 4.0 Hz), 33.90, 76.06 (d, J = 5.7 Hz), 180.70 (d, J = 4.9 Hz), 182.72; ³¹P NMR (300 MHz, CD₃OD) δ 46.52; FABHRMS (M-Li)⁻ calcd. 260.9974, found 260.9967 for C₅H₅Li₃O₇PS.

General Procedure for Alkyl Hydrogen (1-Carboxybutanoic Acid) Phosphates (7)

A mixture of **19** (0.33 mmol) and palladium on carbon (5%, 26 mg) in methanol (12 ml) was stirred for 3 h under a hydrogen atmosphere. The resulting reaction mixture was filtered and concentrated in vacuo to give **20** as a yellow oil. Intermediate **20** 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 16 h, filtered, and the solvent evaporated in vacuo to give 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 **7** as a white solid. **7a**: ^1H NMR (CD_3OD) δ 1.95–2.06 (m, 2H), 2.10–2.28 (m, 2H), 3.50 (d, $J = 10.9$ Hz, 3H), 4.36–4.42 (m, 1H); ^{13}C NMR (CD_3OD) δ 32.39 (d, $J = 16.5$ Hz), 34.97, 53.33 (d, $J = 21.0$ Hz), 77.86 (d, $J = 22.2$ Hz), 179.81, 182.78; ^{31}P NMR (CD_3OD) δ 1.80; FABHRMS (M-Li) $^-$ calcd. 253.0277, found 253.0294 for $\text{C}_6\text{H}_8\text{Li}_2\text{O}_8\text{P}$. **7b**: ^1H NMR (CD_3OD) δ 0.85 (t, $J = 7.2$ Hz, 3H), 1.47–1.59 (m, 2H), 1.98–2.03 (m, 2H), 2.04–2.27 (m, 2H), 3.70–3.80 (m, 2H), 4.37–4.43 (m, 1H); ^{13}C NMR (CD_3OD) δ 10.83, 25.22 (d, $J = 32.4$ Hz), 32.48, 34.96, 68.33 (d, $J = 19.2$ Hz), 77.75 (d, $J = 21.9$ Hz), 179.81, 182.82; ^{31}P NMR (CD_3OD) δ 0.82; FABHRMS (M-Li) $^-$ calcd. 281.0590, found 281.0582 for $\text{C}_8\text{H}_{12}\text{Li}_2\text{O}_8\text{P}$.

Enzyme Inhibition

General

Glutamate carboxypeptidase (carboxypeptidase G from *Pseudomonas* sp. strain ATCC 25301, C 4053) and methotrexate were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of the highest purity and purchased from commercial sources.

Determination of K_m and V_{max} . Each incubation mixture (final volume 0.25 mL) was prepared by the addition of 200 μL TRIS buffer (50 mM, pH 7.3) to 25 μL methotrexate (concentration varied from 10 to 100 μM) dissolved in this same buffer. The enzymatic reaction was initiated by the addition of 25 μL of an enzyme solution (0.17–0.21 μg protein/mL buffer). The reaction was allowed to proceed for one minute with constant shaking at 30°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 subsequently quantified by HPLC. 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 \times 150 mm, Spherclone 5u ODS(2), Phenomenex, Torrance, CA) with a mobile phase of CH_3OH /[potassium phosphate, 50 mM, pH 6.8] (22:78, v:v). At a flow rate of 0.9 mL/min, methotrexate and its hydrolytic product were detected at 304 nm with retention times of 3.8 and 7.7 min respectively. For the determination of K_m and V_{max} 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 reaction. 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, 1 μ M).

Inhibition assay procedures. A typical incubation mixture (final volume 0.25 mL) was prepared by the addition of 200 μ L TRIS buffer (50 mM, pH 7.3) to either a 25 μ L mixture of both methotrexate and inhibitor in buffer or 25 μ L of a buffered solution of methotrexate alone. The enzymatic reaction was initiated by the addition of 25 μ L of an enzyme solution (0.17–0.21 μ g protein/mL buffer). In all cases, the final concentration of methotrexate was 10 μ M while the inhibitor concentration varied from 7.5 to 120 μ M. The reaction was allowed to proceed for one minute with constant shaking at 30°C and was terminated by the addition of 100 μ L methanolic TFA (1% trifluoroacetic acid by volume in methanol) followed by vortexing and centrifugation (7000 g). A 100 μ L aliquot of the resulting supernatant was then quantified by HPLC as described above.

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