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Stereoselective Inhibition of Glutamate Carboxypeptidase by Chiral Phosphonothioic Acids

Haiyan Lu and Clifford E. Berkman*

Department of Chemistry & Biochemistry, San Francisco State University, 1600 Holloway Avenue, San Francisco, CA 94132, USA

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Abstract—A series of phosphonothioic acid derivatives of (*S*)-2-hydroxyglutarate with various alkyl or aryl ligands to the central phosphorus atom was examined for stereoselective inhibition of the glutamate carboxypeptidase, carboxypeptidase G. The inhibitory potencies of these stereoisomers were compared to corresponding synthetic phosphonic acid analogues in order to reveal the significance of the sulfur ligand of the phosphonothioic acid motif upon the inhibition of this metallopeptidase. The acquisition of the individual phosphonothioic acid diastereomers was achieved through the resolution of the respective phosphonate ester precursors. In all cases, the $(+)_P$-diastereomers of these phosphonothioic acid derivatives of (*S*)-2-hydroxyglutarate were found to be more potent inhibitors of glutamate carboxypeptidase than the corresponding $(-)_P$ antipodes with the most dramatic difference observed for the butyl isomers (13.6-fold). Based upon K_i values obtained, the most potent inhibitor of the series by nearly an order of magnitude was the $(+)_P$-*n*-butylphosphonothioic acid derivative, revealing specific structural and stereochemical requirements by this glutamate carboxypeptidase. With the exception of the $(+)_P$-*n*-butyl analogue, the isosteric replacement of oxygen with sulfur of the phosphonic acid moiety did not enhance inhibitory potency.

Introduction

Based upon our preliminary evidence, compounds containing the phosphonamidothionate motif show strong promise as potent tetrahedral-intermediate analogue inhibitors of metallopeptidases with the unique value of probing enzyme active-site architecture with complementary chiral phosphorus centers.[1] The basis for the enhanced inhibitory potency of such compounds, especially against zinc-metallopeptidases, is presumably due to favorable and covalent-like zinc–sulfur interactions within enzyme active sites.[2] Isosteric analogues of phosphonamidothionates, such as phosphonothionates, are also expected to be similarly promising with the additional benefit of being less prone to desulfurization and hydrolysis under acidic conditions.[3]

Our research efforts have been aimed at developing potent competitive inhibitors for glutamate carboxypeptidases such as *N*-acetylated-alpha-linked-acidic dipeptidase (NAALADase),[4] prostate-specific membrane antigen (PSMA),[5] pteroylpoly-glutamate hydrolase (PPH),[6] and carboxypeptidase G (CPG).[7] The acquisition of inhibitors 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 CPG_2 (closely related to CPG) have recently been sought for the use in inhibiting non-tumor-localized enzyme in ADEPT strategies.[8]

The focus of the present study was to explore the significance of phosphorus stereochemistry of chiral phosphonothioic acids upon the inhibition of metallocarboxypeptidases. 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. The individual stereoisomers of a series of phosphonothioic acid derivatives of (*S*)-2-hydroxyglutarate (**10**–**13**) were thus targeted as synthetic and putative stereoselective inhibitors of CPG. The rationale for the selection of the hydrocarbon ligands to phosphorus was based upon this particular enzyme's capability of hydrolyzing a variety of alkyl and aryl amides of glutamic acid.[7] Indeed, we recently noted that for this glutamate carboxypeptidase, both methotrexate and *N*-[4-(4-nitrophenyl)butanoyl] glutamic acid exhibited very similar kinetic profiles as substrates, the later substrate having a slightly lower K_m and higher V_{max}.[9] For comparison, the phosphonic acid analogues **14**–**17** were targeted in order to determine the significance of the sulfur ligand upon the inhibition by the phosphonothioic acids **10**–**13**.

*Corresponding author. Tel: +1-415-338-6495; fax: +1-415-338-2384; e-mail: cberkman@sfsu.edu

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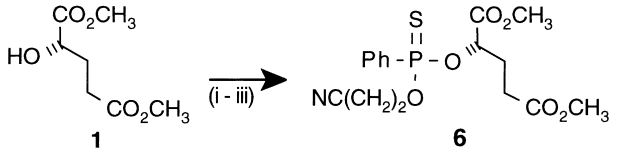
Results and Discussion

It was envisioned that the immediate precursor **6** to the representative target phosphonothioic acid **10** could be easily prepared from (*S*)-dimethyl 2-hydroxyglutarate[10] and dichloro phenylphosphine sulfide as shown in Scheme 1. Although **6** was readily obtained in this way, exhaustive attempts at flash chromatography[11] failed to resolve the individual diastereomers of **6** prior to deprotection. Consequently, attention was redirected at first separating the more polar P-chiral diastereomers of the oxon (P=O) analogues **2**–**5** of phosphonothionates **6**–**9** as outlined in Scheme 2.

The phosphonates **2**–**5** were prepared from the corresponding alkyl or aryl phosphonic dichlorides in a two-step, one-pot, 1*H*-tetrazole-catalyzed reaction.[12] Mild basic hydrolysis of these phosphonates with LiOH quantitatively yielded the phosphonic acid derivatives **14**–**17**. Flash chromatography conveniently resolved each of the diastereomeric pairs (**2a**–**5a** and **2b**–**5b**) and subsequent thionation with Lawesson's reagent stereospecifically generated the individual respective phosphonothionate diastereomers **6a**–**9a** and **6b**–**9b**, an observation consistent with one noted previously.[13] Mild basic hydrolysis of the individual diastereomeric phosphonothionates **6**–**9** with LiOH quantitatively and stereospecifically provided the desired individual diastereomeric phosphonothioic acid derivatives **10**–**13**, respectively.



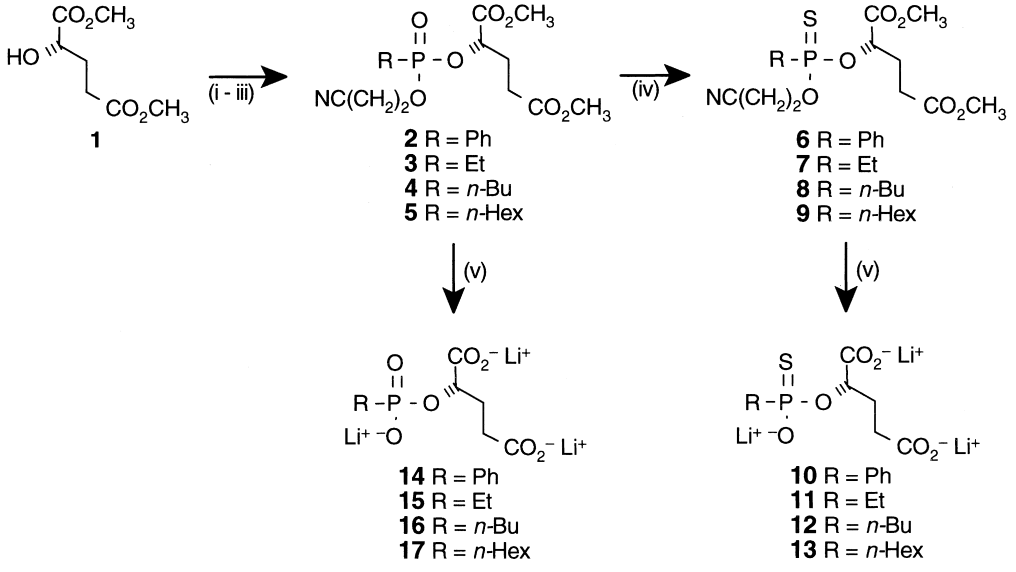
Scheme 1. (i) $PhPCl_2$, DEA, THF, 0 °C, rt; (ii) $NC(CH_2)_2OH$; (iii) sulfur.

It was noted that the ^{31}P NMR chemical shifts of all synthetic intermediates were characteristic of their phosphorus chemical motifs (Table 1). In all cases, the ^{31}P NMR resonances of **6**–**9** shifted upfield approximately 20 ppm upon hydrolysis, consistent with the loss of an alkoxy ligand to a tetracoordinated phosphorus atom.[14] Nearly all the specific rotations determined were levorotary, presumably due to the influence of the (*S*)-configuration of the glutamate alpha carbon. In general, the greatest difference in the specific rotation between diastereomeric pairs was that observed for the phosphonothionates **6**–**9**.

Table 1. Specific rotation[a] and ^{31}P NMR chemical shift data

Compound	R	^{31}P NMR (δ)		$[\alpha]_D^{25}$
2a $(+)_P$	Ph	20.45	−24.18	(1.9) $CHCl_3$
2b $(-)_P$		20.82	−26.67	(0.60) $CHCl_3$
6a $(-)_P$	Ph	89.63	−35.73	(2.8) $CHCl_3$
6b $(+)_P$		90.02	−6.72	(2.1) $CHCl_3$
10a $(-)_P$	Ph	69.45[b]	−5.41	(0.54) H_2O
10b $(+)_P$		68.72[b]	−2.16	(0.55) H_2O
3a $(-)_P$	Et	35.72	−27.31	(0.50) $CHCl_3$
3b $(+)_P$		36.91	−21.36	(0.50) $CHCl_3$
7a $(-)_P$	Et	104.9	−36.07	(0.58) $CHCl_3$
7b $(+)_P$		106.5	−11.47	(0.60) $CHCl_3$
11a $(-)_P$	Et	84.16[b]	−10.62	(0.55) H_2O
11b $(+)_P$		83.09[b]	−1.18	(0.54) H_2O
4a $(-)_P$	*n*-Bu	34.86	−20.69	(1.7) $CHCl_3$
4b $(+)_P$		36.01	−16.44	(0.90) $CHCl_3$
8a $(-)_P$	*n*-Bu	103.40	−34.56	(0.80) $CHCl_3$
8b $(+)_P$		105.20	−7.17	(1.7) $CHCl_3$
12a $(-)_P$	*n*-Bu	80.82[b]	−4.22	(0.54) H_2O
12b $(+)_P$		81.49[b]	3.00	(0.57) H_2O
5a $(-)_P$	*n*-Hex	34.83	−29.14	(0.58) $CHCl_3$
5b $(+)_P$		35.96	−12.75	(0.56) $CHCl_3$
9a $(-)_P$	*n*-Hex	103.4	−30.92	(0.54) $CHCl_3$
9b $(+)_P$		105.2	−5.41	(0.52) $CHCl_3$
13a $(-)_P$	*n*-Hex	82.47[b]	−8.96	(0.51) H_2O
13b $(+)_P$		81.50[b]	−1.12	(0.59) H_2O

[a]Concentrations (g/100 mL) in parentheses.
[b]Chemical shifts were externally referenced to H_3PO_4 (85%) in CD_3OD, all others externally referenced to H_3PO_4 (85%) in $CDCl_3$.



Scheme 2. (i) $RP(O)Cl_2$, DEA, tetrazole, benzene, 4 °C, rt; (ii) $NC(CH_2)_2OH$; (iii) chromatographic separation of diastereomers; (iv) Lawesson's reagent, toluene, reflux; (v) aqueous LiOH/MeOH.

No complete correlation of the relative ^{31}P NMR chemical shifts to specific rotations was observed for compounds **2a**–**13b**. Coincidentally, the faster migrating phosphonothionate diastereomers (**6a**–**9a**) by TLC and their corresponding phosphonothioic acids (**10a**–**13a**) were also identified as the $(-)_P$-isomers, while the $(+)_P$-isomers were identified as **6b**–**13b**. Although it could be assumed that the $(-)_P$-isomers **10a**–**13a** maintained the same stereochemical configuration at phosphorus and opposite to that of the $(+)_P$-isomers **6b**–**13b,** it should be noted that the absolute phosphorus stereochemistry for these compounds or their intermediates could not be conclusively determined. It is anticipated that the future use of alternative protecting ligands on phosphorus may provide crystalline intermediates, thus allowing for the ultimate determination of the phosphorus stereochemistry. The identification and development of such ligands is currently underway.

Once obtained in sufficient quantity, the individual phosphonothioic acid diastereomers **10**–**13** as well as the phosphonic acid analogues **14**–**17** were examined for inhibitory potency against the glutamate carboxypeptidase CPG. Employing the hydrolysis of methotrexate[1] as the conventional measure of enzymatic activity, both K_m and V_{max} were initially determined (1.6 μM and 25 μmol min^{-1} mg $protein^{-1}$). Dixon analyses were subsequently performed to obtain K_i values for **14**–**17** as well as the individual diastereomers **10**–**13** (Table 2). In all Dixon analyses, triplicate determinations were made and averaged for each inhibitor concentration, which generally varied from 8.5 to 65 μM in the presence of 10 μM methotrexate depending upon the potency of the individual inhibitors.

For both series of the phosphonothioic and phosphonic acid derivatives, the greatest inhibitory potency was exhibited by the phenyl and *n*-butyl congeners (**10**, **12**, **14** and **16**). Based upon the K_i values obtained, the greatest stereoselectivity of inhibition (13.6-fold) was observed for the butylphosphonothionates **12**, while the remaining diastereomeric pairs exhibited only mild stereoselectivity (≤2-fold). Although a 2-fold difference in K_i was observed for the ethyl isomers **11**, their abbreviated chain length compared to that of the butyl isomers resulted in decreased inhibitory potency, presumably due to diminished hydrophobic interactions within the active site. On the other hand, the weakly inhibitory hexyl isomers **13** exhibited negligible stereoselectivity, apparently due to a diminished significance of the chiral phosphorus center in enzyme–inhibitor interactions by the large hexyl ligand.

Table 2. Stereoselective inhibition of CPG

Compound	R	K_i (μM)	r[a]
10a $(-)_P$	Ph	4.31	0.992
10b $(+)_P$	Ph	2.74	0.999
14	Ph	2.42	0.988
11a $(-)_P$	Et	20.0	0.989
11b $(+)_P$	Et	10.0	0.998
15	Et	8.80	0.982
12a $(-)_P$	*n*-Bu	6.24	0.992
12b $(+)_P$	*n*-Bu	0.46	0.995
16	*n*-Bu	2.98	0.999
13a $(-)_P$	*n*-Hex	19.2	0.976
13b $(+)_P$	*n*-Hex	18.5	0.976
17	*n*-Hex	5.00	0.945

[a]Corr. coeff. of Dixon analysis.

For each of the phosphonothioic acid diastereomeric pairs, the $(+)_P$-isomers exhibited greater inhibitory potency than the corresponding $(-)_P$-isomers. The stereoselective inhibition of this glutamate carboxypeptidase suggests that $(+)_P$ configuration of the phosphonothioic acids allows for additional or enhanced active-site interactions through the alkyl or aryl ligand. However, the relatively unique inhibitory potency of **12b** suggests that butyl ligand of this isomer presents the greatest complementarity to the enzyme, presumably through hydrophobic interactions which are not as complete nor as accessible for the phenyl, ethyl, or hexyl ligands of **10b**, **11b** and **13b**, respectively. Regardless, the significance of phosphonothioic acid stereochemistry with regard to metallopeptidase inhibition has been revealed through course of this study.

In most cases, the presence of a sulfur ligand on the phosphonothioic acid derivatives **10**–**13** did not exhibit a marked enhancement of inhibitory potency over the respective phosphonic acid derivatives **14**–**17**, an observation in contrast to the results obtained with analogous phosphonamidothioic and phosphonamidoic acid derivatives of glutamic acid.[1] Only in the case of **12b** was a significant enhancement of inhibitory potency observed over the respective phosphonic acid **16**. The rather unique inhibitory potency of **12b** may be due to an altogether greater complementarity to the enzyme active site rather than a single zinc–sulfur interaction as initially proposed.

Conclusion

In summary, a convenient sequence for the procurement of the individual phosphorus diastereomers of chiral phosphonothioic acid derivatives of (*S*)-2-hydroxyglutarate from phosphonic dichlorides has been identified. The significance of such compounds, like their phosphonamidothioic acid analogues,[1] lies with their potential to serve as potent inhibitors and chiral probes of glutamate carboxypeptidases. Of the series of phosphonothioic acids examined, the most notable difference in stereoselective inhibitory potency was observed for the butyl diastereomers **12**, with the $(+)_P$-isomer possessing unique potency against the glutamate carboxypeptidase, CPG. Although in most cases the phosphonamidothioic acid derivatives did not show enhanced inhibitory potency over the corresponding phosphonic acid analogues, the results do suggest that with the appropriate ancillary structural features of a phosphonic acid metallopeptidase inhibitor, the isosteric replacement of an oxygen ligand with sulfur may allow for improved inhibitory potency over oxon analogues along with significant stereoselective inhibition, presumably through greater

enzyme complementarity. Future studies involving the examination of this or a similar series of phosphonothioic acids against other relevant glutamate carboxypeptidases 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. It is also expected that results described herein will encourage the extrapolation of this design to other metallocarboxypeptidases of medical import.

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)[11] 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). ^{31}P NMR chemical shifts in $CDCl_3$ or CD_3OD are relative to 85% H_3PO_4 ($\delta = 0.00$ ppm). 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.

General procedure for phosphononates 2–5. A solution of an alkyl or aryl phosphonic dichloride (1.80 mmol) was added to a stirring solution of 1H-tetrazole (12.6 mg, 0.18 mmol) and diisopropylethylamine (0.68 mL, 3.90 mmol) and (*S*)-dimethyl 2-hydroxyglutarate[10] (317.2 mg, 1.8 mmol) in benzene (18 mL) at 4 °C. The resulting solution was stirred overnight followed by the addition of 3-hydroxypropanenitrile (142 mg, 2.0 mmol) with continued stirring for an additional 3 h. The reaction mixture was filtered and concentrated under reduced pressure. Column chromatography (ethyl ether:methanol, 100:3, v:v) gave **2**–**5** as colorless oils (67.01%, 57.76%, 62.26%, and 51.98% yield, respectively) and afforded the separation of the individual stereoisomers (ethyl ether:methanol, 100:3, v:v: **2a**, $R_f = 0.17$; **2b**, $R_f = 0.12$; **3a**, $R_f = 0.11$; **3b**, $R_f = 0.08$; **4a**, $R_f = 0.18$; **4b**, $R_f = 0.12$; **5a**, $R_f = 0.20$; **5b**, $R_f = 0.12$).

(+)$_P$-2-(*S*)-[2-Cyanoethoxy(phenyl)phosphinoyloxy]-pentanedioic acid dimethyl ester (2a). ^1H NMR (300 MHz, $CDCl_3$) δ 2.05–2.24 (m, 2H), 2.31–2.42 (m, 2H), 2.73–2.93 (m, 2H), 3.60 and 3.81 (s, 3H), 4.28–4.54 (dm, 2H), 5.00 (dt, $J = 7.8$ Hz, $J = 4.5$ Hz, 1H), 7.47–7.65 (dm, 3H), 7.81–7.90 (m, 2H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 20.51 (d, $J = 6.4$ Hz), 28.65 (d, $J = 6.5$ Hz), 29.59, 52.45, 53.40, 61.25 (d, $J = 5.8$ Hz), 73.79 (d, $J = 5.9$ Hz), 117.32, 129.30, 129.51, 132.26, 132.40, 133.92 (d, $J = 3.0$ Hz), 171.22, 173.16.

(−)$_P$-2-(*S*)-[2-Cyanoethoxy(phenyl)phosphinoyloxy]-pentanedioic acid dimethyl ester (2b). ^1H NMR (300 MHz, $CDCl_3$) δ 2.17–2.39 (m, 2H), 2.43–2.62 (m, 2H), 2.78 (dt, $J = 6.4$ Hz, $J = 3.6$ Hz, 2H), 3.65 and 3.69 (s, 3H), 4.23–4.34 (m, 2H), 4.99 (ddd, $J = 9.0$ Hz, $J = 7.1$ Hz, $J = 4.8$ Hz, 1H), 7.47–7.64 (dm, 3H), 7.82–7.90 (m, 2H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 20.51 (d, $J = 7.1$ Hz), 28.86 (d, $J = 5.6$ Hz), 29.65, 52.54, 53.16, 61.00 (d, $J = 4.9$ Hz), 74.00 (d, $J = 5.7$ Hz), 117.11, 127.08 (d, $J = 192.2$ Hz), 129.19, 129.40, 132.46, 132.60, 133.86 (d, $J = 3.2$ Hz), 170.36 (d, $J = 3.3$ Hz), 173.35.

(−)$_P$-2-(*S*)-[2-Cyanoethoxy(ethyl)phosphinoyloxy]-pentanedioic acid dimethyl ester (3a). ^1H NMR (300 MHz, $CDCl_3$) δ 1.21 (dt, $J = 20.87$ Hz, $J = 7.7$ Hz, 3H), 1.78–1.92 (m, 2H), 2.08–2.32 (m, 2H), 2.37–2.55 (m, 2H), 2.71–2.88 (m, 2H), 3.70 and 3.79 (s, 3H), 4.28–4.45 (m, 2H), 4.93 (dt, $J = 7.5$ Hz, $J = 4.6$ Hz, 1H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 6.99 (d, $J = 3.7$ Hz), 20.09 (d, $J = 144.0$ Hz), 20.52 (d, $J = 7.2$ Hz), 28.77 (d, $J = 6.3$ Hz), 29.80, 52.51, 53.29, 60.67 (d, $J = 5.8$ Hz), 73.60 (d, $J = 6.6$ Hz), 117.44, 171.41, 173.21.

(+)$_P$-2-(*S*)-[2-Cyanoethoxy(ethyl)phosphinoyloxy]-pentanedioic acid dimethyl ester (3b). ^1H NMR (300 MHz, $CDCl_3$) δ 1.23 (dt, $J = 21.0$ Hz, $J = 7.7$ Hz, 3H), 1.89–2.03 (m, 2H), 2.11–2.34 (m, 2H), 2.39–2.58 (m, 2H), 2.76 (t, $J = 6.2$ Hz, 2H), 3.70 and 3.78 (s, 3H), 4.16–4.29 (m, 2H), 4.95–5.02 (m, 1H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 7.06, 20.16 (d, $J = 141.6$ Hz), 20.60 (d, $J = 6.4$ Hz), 28.83 (d, $J = 3.9$ Hz), 29.76, 52.55, 53.28 (d, $J = 2.6$ Hz), 60.07, 73.64, 117.19, 171.00, 173.36.

(−)$_P$-2-(*S*)-[2-Cyanoethoxy(*n*-butyl)phosphinoyloxy]-pentanedioic acid dimethyl ester (4a). ^1H NMR (300 MHz, $CDCl_3$) δ 0.93 (t, $J = 7.3$ Hz, 3H), 1.37–1.48 (m, 2H), 1.54–1.69 (m, 2H), 1.77–1.88 (m, 2H), 2.08–2.31 (m, 2H), 2.69 (ddd, $J = 24.0$ Hz, $J = 16.8$ Hz, $J = 6.5$ Hz, 2H), 2.70–2.88 (m, 2H), 3.70 and 3.78 (s, 3H), 4.27–4.44 (m, 2H), 4.92 (dt, $J = 7.5$ Hz, $J = 4.6$ Hz, 1H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 14.16, 20.54 (d, $J = 6.0$ Hz), 24.27 (d, $J = 18.0$ Hz), 24.82 (d, $J = 4.5$ Hz), 26.54 (d, $J = 141.9$ Hz), 28.79 (d, $J = 6.8$ Hz), 29.80, 52.56, 53.33, 60.64 (d, $J = 6.9$ Hz), 73.53 (d, $J = 6.9$ Hz), 117.51, 171.50, 173.26.

(+)$_P$-2-(*S*)-[2-Cyanoethoxy(*n*-butyl)phosphinoyloxy]-pentanedioic acid dimethyl ester (4b). ^1H NMR (300 MHz, $CDCl_3$) δ 0.93 (t, $J = 7.3$ Hz, 3H), 1.36–1.49 (m, 2H), 1.57–1.71 (m, 2H), 1.88–1.98 (m, 2H), 2.11–2.34 (m, 2H), 2.34–2.58 (m, 2H), 2.76 (t, $J = 6.2$ Hz, 2H), 3.70 and 3.78 (s, 3H), 4.15–4.32 (m, 2H), 4.95–5.01 (m, 1H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 14.14, 20.56 (d, $J = 6.0$ Hz), 24.12 (d, $J = 18.3$ Hz), 24.80 (d, $J = 5.8$ Hz), 26.48 (d, $J = 139.7$ Hz), 28.77 (d, $J = 5.8$ Hz), 29.70, 52.51 53.25, 60.03 (d, $J = 6.4$ Hz), 73.57 (d, $J = 6.6$ Hz), 117.21, 170.98 (d, $J = 2.4$ Hz), 173.34.

(−)$_P$-2-(*S*)-[2-Cyanoethoxy(*n*-hexyl)phosphinoyloxy]-pentanedioic acid dimethyl ester (5a). ^1H NMR (300 MHz, $CDCl_3$) δ 0.89 (t, $J = 6.7$ Hz, 3H), 1.28–1.42 (dm, 6H), 1.63–1.68 (m, 2H), 1.77–1.86 (m, 2H), 2.11–2.18 (m, 2H), 2.36–2.55 (m, 2H), 2.70–2.87 (m, 2H), 3.70 and

3.78 (s, 3H), 4.30–4.45 (m, 2H), 4.93 (dt, $J=7.5$ Hz, $J=4.7$ Hz, 1H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 14.19, 20.06 (d, $J=6.3$ Hz), 22.29 (d, $J=5.4$ Hz), 22.57, 26.36 (d, $J=141.6$ Hz), 28.30 (d, $J=7.0$ Hz), 29.32, 30.35 (d, $J=17.6$ Hz), 31.37, 52.08, 52.85, 60.18 (d, $J=7.0$ Hz), 73.06 (d, $J=7.0$ Hz), 117.02, 171.02, 172.78.

(+)$_P$-2-(*S*)-[2-Cyanoethoxy(*n*-hexyl)phosphinoyloxy]-pentanedioic acid dimethyl ester (5b). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.89 (t, $J=7.0$ Hz, 3H), 1.27–1.42 (dm, 6H), 1.63–1.67 (m, 2H), 1.87–1.93 (m, 2H), 2.10–2.34 (m, 2H), 2.38–2.57 (m, 2H), 2.75 (t, $J=6.2$ Hz, 2H), 3.70 and 3.78 (s, 3H), 4.15–4.32 (m, 2H), 4.98 (ddd, $J=8.3$ Hz, $J=3.5$ Hz, $J=1.1$ Hz, 1H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 14.14, 20.07 (d, $J=6.7$ Hz), 22.26 (d, $J=5.6$ Hz), 22.53, 26.28 (d, $J=139.4$ Hz), 28.29 (d, $J=5.8$ Hz), 29.21, 30.31 (d, $J=17.8$ Hz), 31.35, 52.01, 52.75, 59.54 (d, $J=6.4$ Hz), 73.07 (d, $J=6.6$ Hz), 116.74, 170.49 (d, $J=2.4$ Hz), 172.85.

General procedure for phosphonothionates 6–9. Refluxing a solution of **2**–**5** (0.11 g, 0.34 mmol) and Lawesson's reagent (0.076 g, 0.19 mmol) in toluene (2 mL) for 6 h followed by concentration in vacuo gave a yellow oil that was purified by reversed phase (C-18) preparative TLC [methanol:water, 70:30, v:v (**6a**: $R_f=0.43$. **6b**: $R_f=0.40$. **7a**: $R_f=0.54$. **7b**: $R_f=0.51$. **8a**: $R_f=0.37$. **8b**: $R_f=0.33$) and methanol:water, 80:20, v:v (**9a**: $R_f=0.42$. **9b**: $R_f=0.40$)].

(−)$_P$-2-(*S*)-[2-Cyanoethoxy(phenyl)phosphinothioyloxy]-pentanedioic acid dimethyl ester (6a). 71.28% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.11–2.17 (m, 2H), 2.27–2.33 (m, 2H), 2.77–2.86 (m, 2H), 3.59 and 3.82 (s, 3H), 4.34–4.44 (dm, 2H), 5.12–5.15 (m, 1H), 7.49–7.53 (dm, 3H), 7.90–7.98 (m, 2H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 20.24 (d, $J=8.3$ Hz), 28.46 (d, $J=6.8$ Hz), 29.73, 52.42, 53.35, 61.54 (d, $J=5.6$ Hz), 74.64 (d, $J=6.2$ Hz), 117.43, 129.09, 129.29, 131.43, 131.59, 132.82 (d, $J=158.3$ Hz), 133.55 (d, $J=3.3$ Hz), 171.33 (d, $J=1.8$ Hz), 173.20. Anal. calcd for $C_{16}H_{20}NO_6PS$: C, 49.86; H, 5.19; N, 3.63. Found: C, 49.49; H, 4.89; N, 3.67.

(+)$_P$-2-(*S*)-[2-Cyanoethoxy(phenyl)phosphinothioyloxy]-pentanedioic acid dimethyl ester (6b). 76.92% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.18–2.35 (m, 2H), 2.42–2.59 (m, 2H), 2.76 (dt, $J=6.2$ Hz, $J=2.3$ Hz, 2H), 3.61 and 3.70 (s, 3H), 4.29 (dt, $J=9.5$ Hz, $J=6.2$ Hz, 2H), 5.12 (ddd, $J=12$ Hz, $J=7.0$ Hz, $J=4.9$ Hz, 1H), 7.45–7.61 (dm, 3H), 7.91–7.98 (m, 2H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 20.30 (d, $J=8.2$ Hz), 28.80 (d, $J=5.9$ Hz), 29.79, 52.56, 53.03, 61.33 (d, $J=4.7$ Hz), 74.64 (d, $J=5.7$ Hz), 117.21, 128.96, 129.17, 131.67, 131.84, 132.60 (d, $J=153.9$ Hz), 133.51 (d, $J=3.3$ Hz), 170.49 (d, $J=3.5$ Hz), 173.39. Anal. calcd for $C_{16}H_{20}NO_6PS$: C, 49.86; H, 5.19; N, 3.63. Found: C, 49.55; H, 5.02; N, 3.48.

(−)$_P$-2-(*S*)-[2-Cyanoethoxy(ethyl)phosphinothioyloxy]-pentanedioic acid dimethyl ester (7a). 73.26% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.32 (dt, $J=23.3$ Hz, $J=7.6$ Hz, 3H), 2.11–2.41 (m, 4H), 2.47–2.63 (m, 2H), 2.78–2.95 (m, 2H), 3.69 and 3.77 (s, 3H), 4.36–4.50 (m, 2H), 5.17 (ddd, $J=11.8$ Hz, $J=7.3$ Hz, $J=4.4$ Hz, 1H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 6.90 (d, $J=7.1$ Hz), 19.68 (d, $J=7.5$ Hz), 28.24 (d, $J=110.5$ Hz), 27.98 (d, $J=6.4$ Hz), 29.39, 52.00 (d, $J=6.6$ Hz), 52.71 (d, $J=6.0$ Hz), 60.64 (d, $J=6.0$ Hz), 74.07 (d, $J=6.9$ Hz), 116.90, 170.88, 172.66. Anal. calcd for $C_{12}H_{20}NO_6PS$: C, 42.72; H, 5.93; N, 4.15. Found: C, 43.29; H, 5.58; N, 3.68.

(+)$_P$-2-(*S*)-[2-Cyanoethoxy(ethyl)phosphinothioyloxy]-pentanedioic acid dimethyl ester (7b). 66.74% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.22 (dt, $J=23.3$ Hz, $J=7.6$ Hz, 3H), 2.07–2.28 (m, 4H), 2.35–2.54 (m, 2H), 2.73 (t, $J=6.3$ Hz, 2H), 3.68 and 3.75 (s, 3H), 4.07–4.37 (dm, 2H), 5.09 (ddd, $J=11.8$ Hz, $J=7.0$ Hz, $J=4.8$ Hz, 1H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 7.37 (d, $J=5.0$ Hz), 20.25 (d, $J=9.0$ Hz), 28.23, 28.62 (d, $J=3.6$ Hz), 29.75, 52.43 (d, $J=5.2$ Hz), 53.07 (d, $J=5.2$ Hz), 60.59 (d, $J=6.0$ Hz), 74.45 (d, $J=5.7$ Hz), 117.24, 170.96, 173.26. Anal. calcd for $C_{12}H_{20}NO_6PS$: C, 42.72; H, 5.93; N, 4.15. Found: C, 43.22; H, 5.87; N, 3.94.

(−)$_P$-2-(*S*)-[2-Cyanoethoxy(*n*-butyl)phosphinothioyloxy]-pentanedioic acid dimethyl ester (8a). 68.46% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.94 (t, $J=7.2$ Hz, 3H), 1.42–1.47 (m, 2H), 1.61–1.68 (m, 2H), 2.00–2.17 (dm, 4H), 2.40–2.47 (m, 2H), 2.75–2.79 (m, 2H), 3.70 and 3.78 (s, 3H), 4.28–4.35 (m, 2H), 5.07–5.11 (m, 1H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 14.23, 20.25 (d, $J=7.6$ Hz), 24.00 (d, $J=19.8$ Hz), 25.34 (d, $J=4.1$ Hz), 28.53 (d, $J=7.7$ Hz), 29.93, 35.16 (d, $J=113.8$ Hz), 52.55, 53.31, 61.15 (d, $J=6.9$ Hz), 74.52 (d, $J=7.5$ Hz), 117.56, 171.48 (d, $J=3.7$ Hz), 173.29. Anal. calcd for $C_{14}H_{24}NO_6PS$: C, 46.00; H, 6.57; N, 3.83. Found: C,46.22; H, 6.66; N, 3.69.

(+)$_P$-2-(*S*)-[2-Cyanoethoxy(*n*-butyl)phosphinothioyloxy]-pentanedioic acid dimethyl ester (8b). 67.24% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.94 (t, $J=7.3$ Hz, 3H), 1.39–1.49 (m, 2H), 1.60–1.73 (m, 2H), 2.09–2.29 (m, 4H), 2.47–2.57 (m, 2H), 2.74 (t, $J=6.2$ Hz, 2H), 3.70 and 3.77 (s, 3H), 4.09–4.38 (dm, 2H), 5.11 (ddd, $J=11.7$ Hz, $J=6.9$ Hz, $J=4.9$ Hz, 1H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 13.60, 19.68 (d, $J=7.9$ Hz), 23.36 (d, $J=19.8$ Hz), 24.60 (d, $J=4.8$ Hz), 28.05 (d, $J=6.0$ Hz), 29.19, 34.73 (d, $J=110.3$ Hz), 51.90, 52.54, 59.93 (d, $J=6.3$ Hz), 73.85 (d, $J=6.5$ Hz), 116.63, 170.41 (d, $J=3.3$ Hz), 172.73. Anal. calcd for $C_{14}H_{24}NO_6PS$: C, 46.00; H, 6.57; N, 3.83. Found: C,46.11; H, 6.15; N, 3.92.

(−)$_P$-2-(*S*)-[2-Cyanoethoxy(*n*-hexyl)phosphinothioyloxy]-pentanedioic acid dimethyl ester (9a). 74.63% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.89 (t, $J=7.0$ Hz, 3H), 1.25–1.39 (m, 6H), 1.62–1.74 (m, 2H), 2.02–2.31 (m, 4H), 2.40–2.52 (m, 2H), 2.73–2.79 (m, 2H), 3.69 and 3.78 (s, 3H), 4.27–4.35 (m, 2H), 5.04–5.10 (m, 1H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 14.21, 19.78 (d, $J=7.7$ Hz), 22.60, 22.80 (d, $J=4.1$ Hz), 28.08 (d, $J=7.7$ Hz), 29.48, 30.07 (d, $J=19.3$ Hz), 31.42, 34.96 (d, $J=113.5$ Hz), 52.08, 52.84, 60.69 (d, $J=6.8$ Hz), 74.05 (d, $J=7.4$ Hz), 117.09, 171.04, 172.81. Anal. calcd for $C_{16}H_{28}NO_6PS$: C, 48.85; H, 7.12; N, 3.56. Found: C, 48.64; H, 7.18; N, 3.41.

(+)$_P$-2-(*S*)-[2-Cyanoethoxy(*n*-hexyl)phosphinothioyloxy]-pentanedioic acid dimethyl ester (9b). 70.26% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.89 (t, $J=6.9$ Hz, 3H),

1.25–1.44 (m, 6H), 1.61–1.75 (m, 2H), 2.09–2.31 (m, 4H), 2.37–2.56 (m, 2H), 2.74 (t, $J=6.2$ Hz, 2H), 3.70 and 3.77 (s, 3H), 4.09–4.19 (m, 1H), 4.27–4.38 (m, 1H), 5.11 (ddd, $J=11.8$ Hz, $J=6.9$ Hz, $J=4.9$ Hz, 1H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 14.18, 19.84 (d, $J=7.8$ Hz), 22.59, 22.68 (d, $J=4.8$ Hz), 28.22 (d, $J=6.0$ Hz), 29.35, 30.06 (d, $J=19.4$ Hz), 31.43, 35.15 (d, $J=110.0$ Hz), 52.05, 52.69, 60.09 (d, $J=6.5$ Hz), 74.00 (d, $J=6.5$ Hz), 116.76, 170.58, 172.89. Anal. calcd for $C_{16}H_{28}NO_6PS$: C, 48.85; H, 7.12; N, 3.56. Found: C, 48.68; H, 7.09; N, 3.38.

General procedure for phosphonothioic acids 10–13. Phosphonthionates **6–9** (0.5 mmol) were each 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 a white residue which was resuspended in anhydrous methanol, filtered (0.2 μm Teflon membrane), and concentrated in vacuo to give the trilithium salt of the desired phosphonothioic acids **10–13**, respectively, as white solids.

(−)$_P$-2-(*S*)-[Hydroxy(phenyl)phosphinothioyloxy]-pentanedioic acid trilithium salt (10a). 96.33% yield. ^{1}H NMR (300 MHz, CD_3OD) δ 1.93–1.95 (m, 2H), 2.06–2.13 (m, 2H), 4.78–4.83 (m, 1H), 7.10–7.25 (m, 3H), 7.76–7.83 (m, 2H). ^{13}C NMR (300 MHz, CD_3OD) δ 32.39 (d, $J=6.9$ Hz), 35.40, 77.70 (d, $J=6.8$ Hz), 128.48, 128.67, 130.82 (d, $J=2.9$ Hz), 131.64, 131.78, 142.94 (d, $J=145.9$ Hz), 180.35 (d, $J=2.0$ Hz), 182.96. FABHRMS $(M-Li)^-$ calcd 315.0256, found 315.0225 for $C_{11}H_{10}Li_3O_6PS$.

(+)$_P$-2-(*S*)-[Hydroxy(phenyl)phosphinothioyloxy]-pentanedioic acid trilithium salt (10b). 95.99% yield. ^{1}H NMR (300 MHz, CD_3OD) δ 1.96–2.07 (m, 2H), 2.16–2.24 (m, 2H), 4.46–4.53 (m, 1H), 7.21–7.24 (m, 3H), 7.82–7.89 (m, 2H). ^{13}C NMR (300 MHz, CD_3OD) δ 32.73 (d, $J=6.8$ Hz), 34.92, 77.66 (d, $J=6.5$ Hz), 128.42, 128.60, 130.87 (d, $J=3.0$ Hz), 132.16, 132.30, 142.20 (d, $J=138.3$ Hz), 179.60 (d, $J=4.7$ Hz), 183.05. FABHRMS $(M-Li)^-$ calcd 315.0256, found 315.0250 for $C_{11}H_{10}Li_3O_6PS$.

(−)$_P$-2-(*S*)-[Hydroxy(ethyl)phosphinothioyloxy]-pentanedioic acid trilithium salt (11a). 98.61% yield. ^{1}H NMR (300 MHz, CD_3OD) δ 1.01 (dt, $J=20.5$ Hz, $J=7.7$ Hz, 3H), 1.56–1.75 (m, 2H), 1.87–1.98 (m, 2H), 2.07–2.18 (m, 2H), 4.68–4.82 (m, 1H). ^{13}C NMR (300 MHz, CD_3OD) δ 8.86 (d, $J=3.5$ Hz), 31.74 (d, $J=116.9$ Hz), 32.42, 35.46, 77.33 (d, $J=7.5$ Hz), 180.58, 183.02. FAB HRMS $(M-Li)^-$ calcd 267.0256, found 267.0251 for $C_7H_{10}Li_3O_6PS$.

(+)$_P$-2-(*S*)-[Hydroxy(ethyl)phosphinothioyloxy]-pentanedioic acid trilithium salt (11b). 98.20% yield. ^{1}H NMR (300 MHz, CD_3OD) δ 1.10 (dt, $J=20.4$ Hz, $J=7.7$ Hz, 3H), 1.69–1.82 (m, 2H), 2.03–2.11 (m, 2H), 2.27–2.41 (m, 2H), 4.69–4.74 (m, 1H). ^{13}C NMR (300 MHz, CD_3OD) δ 8.66 (d, $J=4.8$ Hz), 31.27 (d, $J=104.0$ Hz), 32.97 (d, $J=3.8$ Hz), 35.09, 77.64 (d, $J=6.5$ Hz), 180.12 (d, $J=4.4$ Hz), 183.15. FABHRMS $(M-Li)^-$ calcd 267.0256, found 267.0276 for $C_7H_{10}Li_2O_6PS$.

(−)$_P$-2-(*S*)-[Hydroxy(*n*-butyl)phosphinothioyloxy]-pentanedioic acid trilithium salt (12a). 97.28% yield. ^{1}H NMR (300 MHz, CD_3OD) δ 0.80 (t, $J=7.4$ Hz, 3H), 1.16–1.30 (m, 2H), 1.49–1.56 (m, 2H), 1.57–1.73 (m, 2H), 1.90–2.01 (m, 2H), 2.16–2.19 (m, 2H), 4.74–4.82 (m, 1H). ^{13}C NMR (300 MHz, CD_3OD) δ 14.37, 25.21 (d, $J=19.0$ Hz), 27.54 (d, $J=3.6$ Hz), 32.46 (d, $J=7.5$ Hz), 35.42, 38.77 (d, $J=107.6$ Hz), 77.29, 180.50, 182.99. FABHRMS $(M-Li)^-$ calcd 295.0569, found 295.0532 for $C_9H_{14}Li_3O_6PS$.

(+)$_P$-2-(*S*)-[Hydroxy(*n*-butyl)phosphinothioyloxy]-pentanedioic acid trilithium salt (12b). 98.03% yield. ^{1}H NMR (300 MHz, CD_3OD) δ 0.81 (t, $J=7.4$ Hz, 3H), 1.23–1.30 (m, 2H), 1.53–1.59 (m, 2H), 1.66–1.74 (m, 2H), 1.98–2.04 (m, 2H), 2.22–2.28 (m, 2H), 4.62–4.70 (m, 1H). ^{13}C NMR (300 MHz, CD_3OD) δ 14.39, 25.31 (d, $J=19.1$ Hz), 27.35 (d, $J=3.3$ Hz), 32.98 (d, $J=4.2$ Hz), 35.08, 38.39 (d, $J=102.5$ Hz), 77.56 (d, $J=6.4$ Hz), 180.09 (d, $J=4.2$ Hz), 183.14. FABHRMS $(M-Li)^-$ calcd 295.0569, found 295.0569 for $C_9H_{14}Li_3O_6PS$.

(−)$_P$-2-(*S*)-[Hydroxy(*n*-hexyl)phosphinothioyloxy]-pentanedioic acid trilithium salt (13a). 96.62% yield. ^{1}H NMR (300 MHz, CD_3OD) δ 0.79 (t, $J=6.9$ Hz, 3H), 1.21–1.27 (m, 6H), 1.54–1.74 (dm, 4H), 1.96–2.01 (m, 2H), 2.17–2.22 (m, 2H), 4.73–4.80 (m, 1H). ^{13}C NMR (300 MHz, CD_3OD) δ 14.62, 23.84, 25.37 (d, $J=3.5$ Hz), 31.98 (d, $J=18.7$ Hz), 32.48 (d, $J=7.2$ Hz), 32.97, 35.45, 39.07 (d, $J=107.5$ Hz), 77.24 (d, $J=7.5$ Hz), 180.61, 183.00. FABHRMS $(M-Li)^-$ calcd 323.0882, found 323.0876 for $C_{11}H_{18}Li_3O_6PS$.

(+)$_P$-2-(*S*)-[Hydroxy(*n*-hexyl)phosphinothioyloxy]-pentanedioic acid trilithium salt (13b). 94.98% yield. ^{1}H NMR (300 MHz, CD_3OD) δ 0.78 (t, $J=6.6$ Hz, 3H), 1.10–1.31 (m, 6H), 1.51–1.71 (dm, 4H), 1.96–2.01 (m, 2H), 2.19–2.26 (m, 2H), 4.60–4.68 (m, 1H). ^{13}C NMR (300 MHz, CD_3OD) δ 14.61, 23.85, 25.16 (d, $J=3.3$ Hz), 32.07 (d, $J=18.8$ Hz), 32.93, 32.97, 35.05, 38.67 (d, $J=102.3$ Hz), 77.51 (d, $J=6.5$ Hz), 180.10 (d, $J=4.4$ Hz), 183.15. FABHRMS $(M-Li)^-$ calcd 323.0882, found 323.0880 for $C_{11}H_{18}Li_3O_6PS$.

General procedure for phosphonic acids 14–17. Phosphonates **2–5** (0.5 mmol) were each 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 a white residue which was resuspended in anhydrous methanol, filtered (0.2 μm Teflon membrane), and concentrated in vacuo to give the trilithium salt of the desired phosphonic acids **14–17**, respectively, as white solids.

2-[Hydroxy(phenyl)phosphinoyloxy]-pentanedioic acid trilithium salt (14). 97.32% yield. ^{1}H NMR (300 MHz, CD_3OD) δ 1.78–1.85 (m, 2H), 2.00–2.18 (m, 2H), 4.21–4.28(m, 1H), 7.36–7.46 (m, 3H), 7.61–7.68 (m, 2H). ^{13}C NMR (300 MHz, CD_3OD) δ 30.85 (d, $J=4.15$ Hz), 33.10, 75.10 (d, $J=5.1$ Hz), 128.12, 128.30, 130.94 (d, $J=2.7$ Hz), 131.05, 131.18, 133.37 (d, $J=176.9$ Hz), 178.59 (d, $J=3.6$ Hz), 182.96. ^{31}P NMR (CD_3OD) δ

15.24. FABHRMS $(M-Li)^-$ calcd 299.0467, found 299.0468 for $C_{11}H_{10}Li_3O_7P$.

2-[Hydroxy(ethyl)phosphinoyloxy]-pentanedioic acid trilithium salt (15). 96.51% yield. ^1H NMR (300 MHz, CD_3OD) δ 1.02 (dt, $J=18.0$ Hz, $J=7.5$ Hz, 3H), 1.39–1.53(m, 2H), 1.87–2.07 (m, 2H), 2.18–2.25 (m, 2H), 4.37–4.44 (m, 1H). ^{13}C NMR (300 MHz, CD_3OD) δ 8.30 (d, $J=3.5$ Hz), 21.85 (d, $J=139.2$ Hz), 32.72 (d, $J=5.3$ Hz), 35.18, 76.53 (d, $J=6.1$ Hz), 180.39 (d, $J=2.5$ Hz), 182.89. ^{31}P NMR (CD_3OD) δ 26.93. FABHRMS $(M-Li)^-$ calcd 251.0485, found 251.0488 for $C_7H_{10}Li_3O_7P$.

2-[Hydroxy(*n*-butyl)phosphinoyloxy]-pentanedioic acid trilithium salt (16). 96.22% yield. ^1H NMR (300 MHz, CD_3OD) δ 0.51 (t, $J=7.3$ Hz, 3H), 0.92–1.02(m, 2H), 1.09–1.18 (m, 4H), 1.62–1.71 (m, 2H), 1.87–1.94 (m, 2H), 4.06–4.12 (m, 1H). ^{13}C NMR (300 MHz, CD_3OD) δ 14.32, 25.61 (d, $J=18.0$ Hz), 27.02 (d, $J=4.6$ Hz), 29.06 (d, $J=137.4$ Hz), 32.76 (d, $J=5.7$ Hz), 35.19, 76.51 (d, $J=6.3$ Hz), 180.35 (d, $J=2.6$ Hz), 182.89. ^{31}P NMR (CD_3OD) δ 15.24. ^{31}P NMR (CD_3OD) δ 27.52. FABHRMS $(M-Li)^-$ calcd 279.0798, found 279.0788 for $C_9H_{14}Li_3O_7P$.

2-[Hydroxy(*n*-hexyl)phosphinoyloxy]-pentanedioic acid trilithium salt (17). 95.78% yield. ^1H NMR (300 MHz, CD_3OD) δ 0.81 (t, $J=6.3$ Hz, 3H), 1.20–1.29 (m, 6H), 1.42–1.54 (m, 4H), 1.95–2.26 (m, 2H), 2.20–2.26 (m, 2H), 4.38–4.42 (m, 1H). ^{13}C NMR (300 MHz, CD_3OD) δ 14.86, 24.12, 25.13, 28.75, 30.58, 32.67(d, $J=18.38$ Hz), 33.12(d, $J=12.90$ Hz), 35.50, 76.80, 180.69, 183.18. ^{31}P NMR (CD_3OD) δ 25.75. FABHRMS $(M-Li)^-$ calcd 307.1111, found 307.1107 for $C_{11}H_{18}Li_3O_7P$.

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 1 min 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 (4.6×150 mm, Spherclone 5 μ ODS(2), Phenomenex, Torrence, 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 μM).

Inhibition assay procedure. A typical incubation mixture (final volume 0.25 mL) was prepared by the addition of 200 μL TRIS buffer (50 mM, pH 7.3) to either a 25 μL mixture of both methotrexate and inhibitor in buffer or 25 μL of a buffered solution of methotrexate alone. The enzymatic reaction was initiated by the addition of 25 μL of an enzyme solution (0.17–0.21 μg protein/mL buffer). In all cases, the final concentration of methotrexate was 10 μM while the inhibitor concentration varied from 7.5 to 120 μM. The reaction was allowed to proceed for 1 min 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 then quantified by HPLC as described above.

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