Synthesis and Biological Evaluation of Thiol-Based Inhibitors of Glutamate Carboxypeptidase II: Discovery of an Orally Active GCP II Inhibitor

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A series of 2-(thioalkyl)pentanedioic acids were synthesized and evaluated as inhibitors of glutamate carboxypeptidase II (GCP II, EC 3.4.17.21). The inhibitory potency of these thiol-based compounds against GCP II was found to be dependent on the number of methylene units between the thiol group and pentanedioic acid. A comparison of the SAR of the thiol-based inhibitors to that of the phosphonate-based inhibitors provides insight into the role of each of the two zinc-binding groups in GCP II inhibition. The most potent thiol-based inhibitor, 2-(3-mercaptopropyl)pentanedioic acid (IC_{50} = 90 nM), was found to be orally bioavailable in rats and exhibited efficacy in an animal model of neuropathic pain following oral administration.

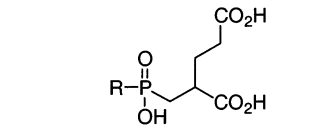
Introduction

Glutamate plays a key role as an excitatory neurotransmitter in both the central and peripheral nervous systems.[1,2] Excess glutamate, however, is implicated in a number of neurological disorders, including stroke, spinal cord injury, amyotrophic lateral sclerosis (ALS), peripheral neuropathy, chronic pain, schizophrenia, and epilepsy. Conventional therapeutic approaches for treating these diseases have been focused on blocking postsynaptic glutamate receptors with small molecules. To this end, a wide variety of glutamate receptors have been evaluated as therapeutic targets for neurological disorders associated with excess glutamate toxicity. Of these glutamate receptors, the *N*-methyl-D-aspartate (NMDA) receptor has been the most extensively studied over the past decade and several NMDA receptor antagonists are currently in clinical trials.[3]

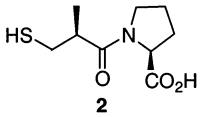
An alternative therapeutic strategy to blocking glutamate receptors would be the upstream reduction of presynaptic glutamate. One of the major sources of glutamate in the nervous system is believed to be hydrolysis of *N*-acetylaspartyl glutamate catalyzed by a metalloprotease glutamate carboxypeptidase II (GCP II).[4] Inhibition of GCP II, therefore, has gained considerable attention as a strategy to suppress glutamate excitotoxicity leading to neurological disorders.[5] Thus, there has been a substantial interest in the discovery of potent and selective GCP II inhibitors.

Earlier studies on GCP II inhibitors by us and other groups suggest that effective GCP II inhibitors are capable of interacting with both an active site zinc ion and the glutamate recognition site (P1′ site) of the enzyme. Indeed, most of the published potent GCP II inhibitors contain a glutarate moiety (including a glutamate) in part of the molecules in addition to a zinc-binding group.[6−12] 2-(Phosphonomethyl)pentanedioic acid (2-PMPA, **1a**; Chart 1)[13] is one of the earliest potent

Chart 1



1a R = OH
1b R = pentafluorophenylmethyl



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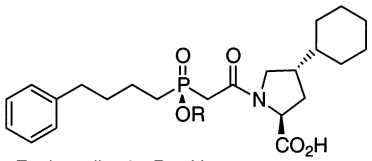
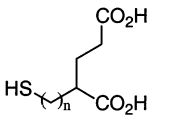


Fosinoprilat **3a** R = H
Fosinopril **3b** R = CH(*i*-C_3H_7)$OCOCH_2CH_3$



4a (n = 0) **4b** (n = 1)
4c (n = 2) **4d** (n = 3)
4e (n = 4) **4f** (n = 5)

inhibitors of GCP II with a K_i value of 0.2 nM.[14] The high potency of compound **1a** can be attributed to the strong chelation of the phosphonate group to an active site zinc atom as well as the interaction of the glutarate (pentanedioic acid) portion of the inhibitor with the glutamate recognition site of GCP II. 2-PMPA has been extensively utilized to study the mechanism and physiological role of GCP II as well as the potential therapeutic effects of GCP II inhibition. For example, we have demonstrated that 2-PMPA considerably reduces the ischemia-induced rise in extracellular glutamate and

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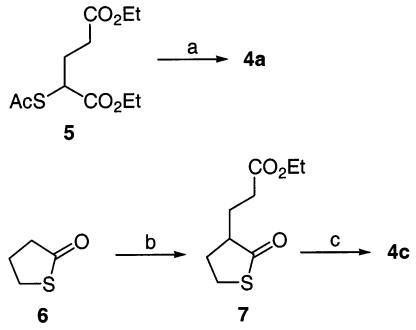
Scheme 1. Synthesis of Compounds **4a** and **4c**[a]



[a] Reagents and conditions: (a) THF, 2 M NaOH, 40%; (b) LDA, ethyl 3-bromopropionate, THF, −78 °C, 16%; (c) THF, 2 M NaOH, 66%.

vigorously protects against injury in a neuronal culture model of ischemia and in rats after transient middle cerebral artery occlusion (MCAO).[15]

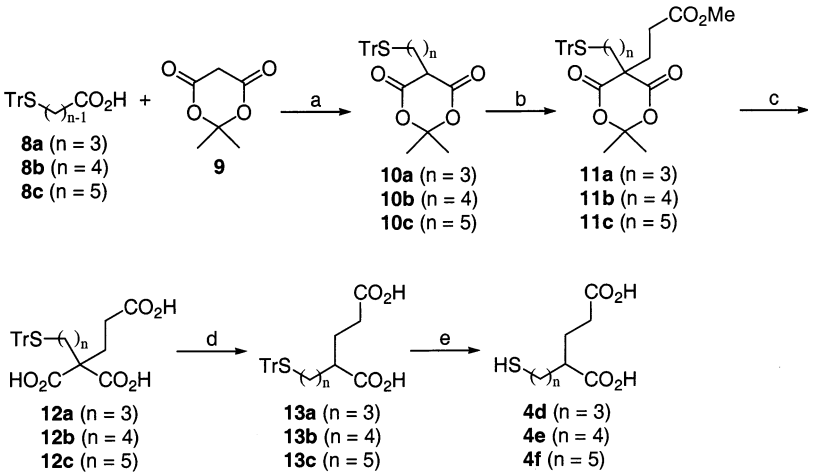
Subsequently, we carried out extensive SAR studies using compound **1a** as a template and identified a series of potent phosphinate-based inhibitors of GCP II.[6] Among these compounds, 2-(hydroxypentafluorophenylmethylphosphinoylmethyl)pentanedioic acid **1b** exhibited efficacy not only in the MCAO model of ischemia (30 mg/kg iv bolus followed by 6 mg/kg/h iv infusion for 4 h) but also in the chronic constriction injury (CCI) model of neuropathic pain (10 mg/kg ip daily). Additionally, compound **1b** prevents long-term diabetic neuropathy in type 1 diabetic BB/Wor rats.[16] However, a relatively poor pharmacokinetic profile of compound **1b**, due to its highly polar zinc-binding group phosphinate,[17] limited its potential as a therapeutic agent, particularly in the area of chronic neurological disorders such as diabetic neuropathy.

Assuming that the phosphonate and phosphinate functional groups are the primary cause of poor oral bioavailability of **1a** and **1b,** respectively, our focus has shifted to a different class of molecules in pursuit of the discovery of orally active GCP II inhibitors. As represented by a potent inhibitor of angiotensin converting enzyme **(**ACE), captopril **2**,[18] the thiols are one of the most extensively studied classes of metalloprotease inhibitors. More importantly, many thiol-based metalloprotease inhibitors exhibit pharmacokinetic profiles superior to phosphorus-based compounds. For example, while captopril **2** is orally active, fosinoprilat **3a**, a phosphinate-based ACE inhibitor, suffers from poor oral bioavailability and is provided as a prodrug, fosinopril **3b**. The present study was driven by the idea that replacements of the phosphonomethyl group of **1a** with thioalkyl groups could result in significant improvement in oral bioavailability without considerable loss of inhibitory potency against GCP II. One of the key objectives of this study is to locate an optimal position for the thiol group to achieve maximum affinity to GCP II by inserting a different number of methylene units between the thiol group and the α-position of glutarate moiety. In this paper, we describe the design, synthesis, and biological evaluation of 2-(thioalkyl)pentanedioic acids **4a**−**f** and their analogues, leading to the discovery of a potent thiol-based GCP II inhibitor that is orally active in an animal model of neuropathic pain.

Chemistry

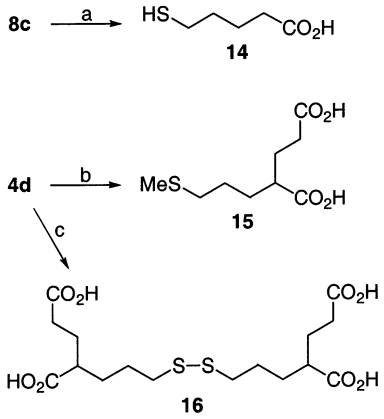
The synthesis of 2-(thioalkyl)pentanedioic acids **4a**−**f** is outlined in Schemes 1 and 2. The shortest analogue **4a** ($n = 0$) was obtained by base-mediated hydrolysis of diethyl 2-(acethylthio)glutarate **5**.[19] Compound **4b** ($n = 1$) was prepared according to the previously described procedure.[20] Compound **4c** ($n = 2$) was synthesized by alkylation of γ-thiobutyrolactone **6** with ethyl 3-bromopropionate followed by base-mediated hydrolysis of the alkylated thiolactone **7**. In the final step of the syntheses of these target compounds, it is essential to remove residual oxygen from the solvent prior to the base-mediated hydrolysis reaction in order to prevent the formation of disulfide derivatives. An attempt to synthesize **4d** ($n = 3$) by a similar manner using δ-thiovalerolactone was unsuccessful because of the formation of multiple unidentified byproducts. Alternatively, **4d** was synthesized using Meldrum's acid as a starting material as outlined in Scheme 2. Reductive coupling of *S*-trityl-3-mercaptopropionic acid **8a** to Mel-

Scheme 2. Synthesis of Compounds **4d**−**f**[a]



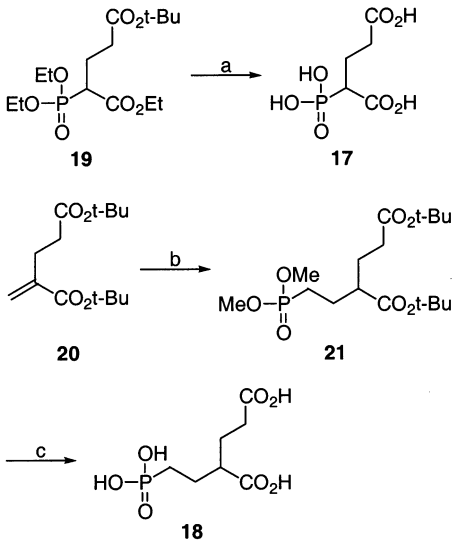
[a] Reagents and conditions: (a) (i) DCC, DMAP, CH_2Cl_2, −5 °C; (ii) $NaBH_4$, AcOH, CH_2Cl_2, −5 °C, 75% for **10a**, 77% for **10b**, 74% for **10c**; (b) methyl acrylate, NaOAc, *t*-BuOH, 60 °C, 81% for **11a**, 86% for **11b**, 79% for **11c**; (c) 1.75 M NaOH, 100 °C, >100% crude; (d) DMSO, 130 °C, 96% for **13a**, quantitative yields for **13b** and **13c**; (e) triisopropylsilane, TFA, CH_2Cl_2, room temp, 75% for **4d**, 72% for **4e**, 77% for **4f**.

Scheme 3. Synthesis of Compounds **14**−**16**[a]



[a] Reagents and conditions: (a) triisopropylsilane, TFA, CH_2Cl_2, room temp, 67%; (b) dimethyl sulfate, 2.75 M NaOH, 50 °C, 57%; (c) oxygen, 1.75 N NaOH, room temp, 94%.

Scheme 4. Synthesis of Compounds **17** and **18**[a]



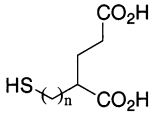
[a] Reagents and conditions: (a) 6 M HCl, reflux, 73%; (b) dimethyl methylphosphonate, CuI, *n*-BuLi, −78 °C, 15%; (c) 6 M HCl, reflux, 46%.

drum's acid **9** gave 5-monosubstituted Meldrum's acid derivative **10a**.[21] Michael addition of **10a** to methyl acrylate followed by simultaneous hydrolysis of both acetonide and methyl ester of **11a** afforded triacid **12a**. Subsequent decarboxylation in DMSO and the removal of the trityl group from **13a** by TFA/triisopropylsilane afforded **4d**. The same method was successfully applied to the synthesis of two other analogues **4e** ($n = 4$) and **4f** ($n = 5$) from *S*-trityl-4-mercaptobutanoic acid **8b** and *S*-trityl-5-mercaptopentanoic acid **8c**, respectively.

The syntheses of several analogues of **4d** are outlined in Scheme 3. The truncated analogue of **4d**, 5-mercaptopentanoic acid **14**, was obtained by removing a trityl group from *S*-trityl-5-mercaptopentanoic acid **8c**. Two S-substituted analogues of **4d**, the *S*-methyl derivative **15** and the disulfide derivative **16**, were prepared by treating **4d** with dimethyl sulfate and oxygen, respectively, under the basic conditions.

As shown in Scheme 4, two homologues of 2-PMPA **1a**, 2-phosphorylpentanedioic acid **17** and 2-(3-phosphonoethyl)pentanedioic acid **18**, were also synthesized in order to extend our SAR studies to phosphonate-based compounds. The shorter analogue **17** was synthesized by hydrolysis of the fully protected precursor **19**.[22] The synthesis of the longer analogue **18** involves 1,4-addition of dimethyl methylphosphonate to 2-substituted acrylate ester **20**[23] followed by hydrolysis of all the ester groups.

Table 1. Inhibition of GCP II by Thiol-Based Inhibitors



compd	*n*	IC_{50} (nM)
4a	0	2600 ± 800
4b	1	6000 ± 2800
4c	2	580 ± 190
4d	3	90 ± 26
4e	4	1100 ± 200
4f	5	2600 ± 500

Results and Discussion

In Vitro GCP II Inhibition. The ability of compounds **4a**−**f** to inhibit GCP II was evaluated using *N*-acetyl-L-aspartyl-[^{3}H]-L-glutamate as a substrate.[14] As summarized in Table 1, the inhibitory potency of these compounds against GCP II was found to be dependent on the number of methylene units between the thiol group and pentanedioic acid. Compound **4d** was found to be the most potent inhibitor of GCP II with an IC_{50} value of 90 nM. Increasing or decreasing the number of methylene units between the thiol group and pentanedioic acid led to a gradual loss of inhibitory potencies of the resulting compounds **4a**−**c** and **4e**,**f**. This observation was unexpected because prior SAR studies on two other zinc metalloproteases, ACE[24] and carboxypeptidase A (CPA),[25] suggest that a 3-mercaptopropanoyl moiety is the most effective backbone for the P1′ position in inhibiting these two enzymes. For example, the optimal acyl chain length for mercaptoalkanoyl derivatives of proline for ACE inhibition is that of 3-mercaptopropanoylproline, which led to discovery of captopril **2**.[24] In the area of CPA, 2-benzyl-3-mercaptopropanoic acid is the most potent thiol-based inhibitor with a K_i value of 11 nM.[25] In contrast to these inhibitory profiles for ACE and CPA, the 3-mercaptopropionic acid analogue **4b** was a poor GCP II inhibitor with an IC_{50} value of 6 μM, nearly 70-fold less potent than the most potent inhibitor **4d**.

The shift of optimal chain length for the mercaptoalkanoyl moiety from mercaptopropanoyl (for ACE and CPA) to mercaptopentanoyl (for GCP II) may be largely due to a structural difference in the active site between ACE/CPA and GCP II. Both ACE and CPA are known to have one zinc ion at their active site. On the other hand, the catalytic domain of GCP II is believed to contain two zinc ions forming a cocatalytic active site with metal ligands.[26] Obviously, the structure of an optimal pharmacophore would largely depend on which

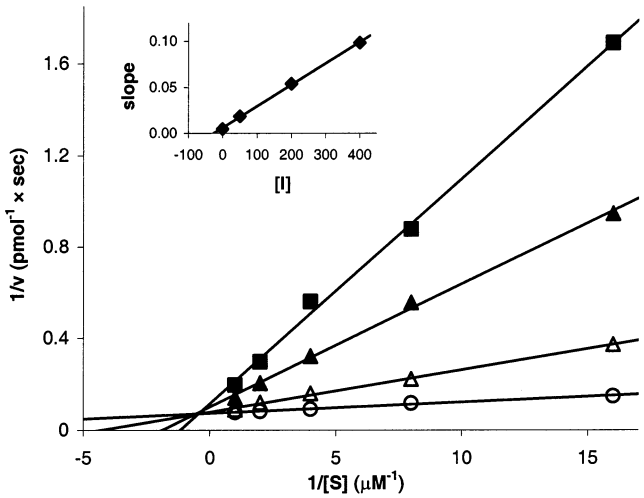
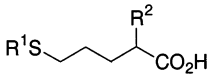


Figure 1. Double-reciprocal plot of the hydrolysis of NAAG by GCP II in the presence of compound **4d**. The units of substrate concentration are μM, while the units of velocity are pM/s. Concentrations of **4d** were 0 (○), 50 (△), 200 (▲), and 400 nM (■). Inset: Replot of the double-reciprocal plot K_{mapp}/V_{max} vs [I].

Table 2. Inhibition of GCP II by S-Substituted Analogues of **4d**



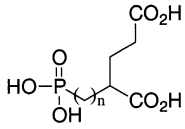
compd	R^1	R^2	IC_{50} (nM)
4d	H	$(CH_2)_2CO_2H$	90 ± 26
14	H	H	1400 ± 500
13a	Ph_3C	$(CH_2)_2CO_2H$	>10000
15	CH_3	$(CH_2)_2CO_2H$	6500 ± 1500
16	4,6-dicarboxyhexylsulfanyl	$(CH_2)_2CO_2H$	3100 ± 1500

zinc ion the sulfhydryl group of GCP II inhibitor interacts with. Further discussion on this issue, however, would best await an X-ray crystal structure of a GCP II inhibitor complex.

We conducted further kinetic studies on **4d** to determine its mode of GCP II inhibition. The double-reciprocal plot of velocity (v) vs substrate concentration ([S]) for inhibitor **4d** shows a pattern characteristic of competitive inhibition ($K_i = 30$ nM), suggesting that the compound **4d** occupies the substrate-binding pocket of GCP II and takes advantage of bindings to both the active site zinc and the glutamate recognition site (Figure 1). The results demonstrate that attaching a thiol group to the α-position of glutarate using a methylene linker is a valid strategy to identify potent thiol-based GCP II inhibitors.

Inhibition of GCP II by several analogues of **4d** is summarized in Table 2. A truncated analogue of **4d**, 5-mercaptopantanoic acid **14**, was nearly 16-fold less potent than **4d** in inhibiting GCP II. The notable decline in potency upon removal of a propionate portion from **4d** suggests critical contribution of the P1′ side chain of **4d** to its strong affinity to GCP II. A similar trend was observed during our SAR studies on phosphonate-based GCP II inhibitors. A significant loss of inhibitory potency was observed with 3-phosphonopropionic acid, a truncated analogue that lacks the propionate P1′ side chain of **1a**.[27] Lack of inhibitory potency observed with *S*-trityl analogue **13a**, a synthetic precursor for **4d**, can be attributed to the increased size of the molecule and/or inability to bind to an active site zinc ion. Significant loss of potency was observed even with the *S*-methyl analogue **15**. Kozikowski's group recently discovered a thiol-containing urea-based GCP II inhibitor, (*R*)-Cys-C(O)-(*S*)-Glu.[10] Interestingly, neither *S*-methyl[28] nor *S*-*tert*-butyl[10] analogues exhibited significant loss of inhibitory potency, indicating a distinct role for the free sulfhydryl group of **4d** as a zinc-binding group in the interaction with GCP II. One of the potential metabolites, the disulfide analogue **16**, also showed significantly lower potency than **4d** against GCP II.

Table 3. Inhibition of GCP II by Phosphonate-Based Inhibitors



compd	n	IC_{50} (nM)
17	0	1400 ± 500
1	1	0.30 ± 0.05
18	2	1200 ± 600

Comparison of the GCP II inhibitory profile of the thiol-based inhibitors to that of the phophonate-based inhibitors revealed fundamental differences between the two classes of compounds. Table 3 summarizes the effect of the phosphonoalkyl chain length of phosphonate-based compounds on GCP II inhibition. In striking contrast to thiol-based GCP II inhibitors whose potency changes gradually upon deletion or insertion of one methylene unit from/to **4d**, both truncation (compound **17**) and extension (compound **18**) of 2-PMPA by one methylene group increased IC_{50} value by over 3 orders of magnitude. This difference in inhibitory profiles can be explained by a distinct mode of interaction with the catalytic zinc by each of the two zinc-binding groups. Maximum interaction of the phosphonate group with a catalytic zinc ion can be attained when the phosphonate group bivalently coordinates with the two active site zinc ions. Using molecular modeling studies, Kozikowski's group demonstrated that the phosphonate group of compound **1a** is finely positioned to achieve this interaction while its glutarate group optimally interacts with the glutamate recognition site of the enzyme, resulting in extremely potent GCP II inhibition.[29] The considerable loss of potency by its homologues **17** and **18** is presumably due to their inability to take advantage of both of the two key interactions simultaneously. None of the thiol-based GCP II inhibitors are nearly as potent as 2-PMPA because of the inability of monodentate thiolate to coordinate with the zinc ions. The effect of chain length on potency is relatively modest for this class of compounds because contribution of the thiol−zinc interaction to the overall affinity is less significant compared to contribution of the phosphonate−zinc interaction of **1a**.

Selectivity of Compound 4d. Since compound **4d** contains a zinc-binding group as well as a portion that mimics glutamate, it is important to assess the selectivity of this compound for GCP II over other potentially relevant proteins such as metalloproteases and glutamate receptors. Thus, compound **4d** was evaluated in a panel of assays listed in Table 4 at 10 μM, over 100-fold higher

Table 4. Selectivity of Compound **4d**[a]

glutamate receptor	zinc metalloprotease
AMPA	angiotensin converting enzyme (ACE)
kainate	matrix metalloproteinase 1 (MMP-1)
NMDA (agonist)	matrix metalloproteinase 2 (MMP-2)
NMDA (glycine)	matrix metalloproteinase 3 (MMP-3)
NMDA (phencyclidine)	matrix metalloproteinase 7 (MMP-7)
NMDA (polyamine)	matrix metalloproteinase 9 (MMP-9)
	neutral endopeptidase (NEP)

[a] Compound **4d** showed significant responses (≥50% stimulation or inhibition) in none of the assays listed in this table.

than its IC_{50} value for GCP II (90 nM). At this concentration, compound **4d** exhibited significant activity (≥50% stimulation or inhibition) against neither glutamate receptors nor various metalloproteases. The results confirm that compound **4d** is a selective inhibitor of GCP II and useful in evaluating pharmacological benefit of GCP II inhibition.

Pharmacokinetic Studies. The phamacokinetic data for **4d** in fasted male Sprague−Dawley rats are summarized in Table 5. Following intravenous or oral administration (10 mg/kg) of **4d** to rats, blood samples were analyzed for plasma concentration of both **4d** and **16**. Compound **4d** is rapidly absorbed after oral administration as indicated by a short T_{max} (less than 30 min) with a mean peak plasma concentration value of 2.23 μg/mL. As expected from pharmacokinetic studies on captopril,[30] the disulfide derivative **16** was also detected in plasma samples after both intravenous (AUC = 0.19 μg·h/mL) and oral (AUC = 0.053 μg·h/mL) administrations of **4d**. These AUC values, however, are significantly lower than those of **4d**, 2.55 and 1.82 μg·h/mL for intravenous and oral administrations, respectively. The oral bioavailability (F) of **4d** in rats was 71%. The pharmacokinetic evaluation in rats clearly demonstrates that compound **4d** is capable of achieving high blood levels following oral administration. Compound **4d** represents, to the best of our knowledge, the first orally bioavailable inhibitor of GCP II.

Chronic Constriction Injury Model of Neuropathic Pain. We have subsequently tested **4d** for its antinociceptive effects using the rat chronic constriction injury (CCI) model[31] of neuropathic pain by oral administration. As shown in Figure 2, compound **4d** (10 mg kg^{-1} day^{-1}) significantly reduced thermal hyperalgesia relative to the vehicle-treated control. The antinociceptive effect of the compound was significant starting from 8 days of drug treatment and persisted to the end of the study. Although a similar level of potency was achieved by intraperitoneal administration of the phosphinate-based GCP II inhibitor **1b**,[6] compound **4d** offers a major advantage over **1b** with regard to its route of administration, since an orally active drug is much preferred in the treatment of chronic pain.

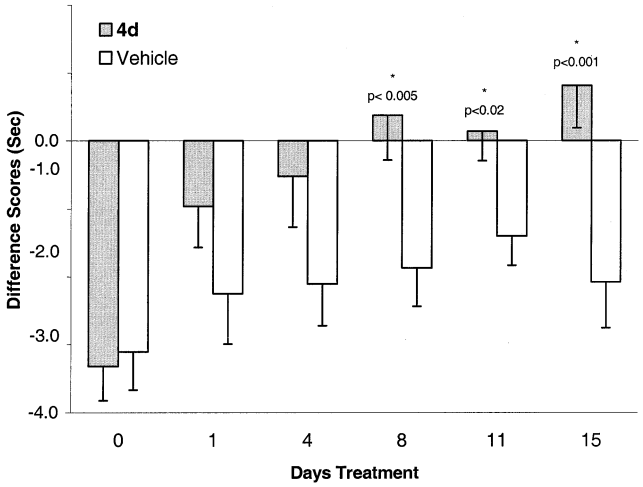


Figure 2. Chronic constriction injury (CCI) model of neuropathic pain. Compound **4d** (10 mg $kg^{-1}day^{-1}$, po) significantly attenuated CCI-induced hyperalgesic state relative to the vehicle-treated control.

Conclusion

A series of 2-(thioalkyl)glutaric acids were synthesized and evaluated as inhibitors of GCP II. The inhibitory potency of these compounds against GCP II was found to be dependent on the number of methylene units between the thiol group and pentanedioic acid. Our SAR analysis clearly indicates that both a 5-mercaptopentanoic acid backbone and a propionic acid P1′ side chain are essential for the potent inhibition of GCP II. Although none of the thiol-based compounds are as nearly potent as 2-PMPA, **1a**, we have shown that **4d** is orally bioavailable and efficacious in an animal model of peripheral neuropathy by oral administration, offering ideal approaches to the management of neuropathic pain. Further pharmacological evaluations of **4d** are currently underway in various animal models of the neurological disorders associated with glutamate excitotoxicity.

Experimental Section

General. All reactions were performed under nitrogen. Unless otherwise noted, all materials were obtained from commercial suppliers and used without further purification. Melting points were obtained on a Mel-Temp apparatus and are uncorrected. ^{1}H NMR spectra were recorded at 300 or 400 MHz. ^{13}C NMR spectra were recorded at 75 or 100 MHz. ^{31}P NMR spectra were recorded at 162 MHz. Elemental analyses were obtained from Atlantic Microlabs, Norcross, GA. *S*-Trityl-3-mercaptopropionic acid **8a** was purchased from Bachem, King of Prussia, PA. Preparation of compound **10a** from **8a** was previously described.[21] Bioassays listed in Table 4 were performed by MDS Pharma Services, Bothell, WA.

2-Mercaptopentanedioic Acid (4a). To a solution of diethyl 2-acetysulfanylpentanedioic acid **5**[19] (0.20 g, 0.76 mmol) in THF (4 mL) was added 2 M NaOH (1.5 mL) at 0 °C, and the resulting mixture was stirred under nitrogen at room

Table 5. Pharmacokinetic Profile of **4d** in Rats

compd	C_{max} (μg/mL)	T_{max} (h)	AUC_{inf} (μg·h/mL)	CL/F ((L/h)/kg)	$T_{1/2}$ (h)	F (%)
			Intravenous Administration of **4d** (10 mg/kg, n = 8)			
4d			2.55 ± 0.51	1.02 ± 0.23	1.05 ± 0.38	
16			0.19 ± 0.36		0.37 ± 0.06	
			Oral Administration of **4d** (10 mg/kg, n = 10)			
4d	2.23 ± 1.06	0.16 ± 0.19	1.82 ± 0.22	1.39 ± 0.18	1.18 ± 0.30	71
16	0.044 ± 0.054	0.90 ± 0.22	0.053 ± 0.034		1.94 ± 0.77	

temperature overnight. After addition of water (8 mL), the reaction mixture was washed with EtOAc (5 mL). The aqueous layer was acidified to pH 1 with 6 M HCl and extracted with EtOAc (20 mL × 3). The combined extracts were washed with brine, dried over $MgSO_4$, and concentrated. The residual solid was recrystallized from EtOAc/hexanes to give 0.050 g of **4a** as a white solid (40% yield): mp 88.6−89.6 °C. ^{1}H NMR (THF-d_8) δ 2.08−1.95 (m, 2H), 2.29−2.17 (m, 1H), 2.50−2.46 (m, 2H), 3.48−3.40 (m, 1H); ^{13}C NMR (THF-d_8) δ 30.2, 30.3, 39.3, 172.4, 172.9. Anal. ($C_5H_8O_4S$) C, H, S.

3-(2-Oxotetrahydrothiophen-3-yl)propionic Acid Ethyl Ester (7). To a solution of lithium diisopropylamide (2.0 M solution, 100 mL, 200 mmol) in THF (100 mL) was added dropwise a solution of γ-thiobutyrolactone **6** (20.0 g, 196 mmol) in THF (20 mL) at −75 °C, and the resulting mixture was stirred at −75 °C for 30 min. To the mixture was added dropwise a solution of ethyl 3-bromopropionate (39.0 g, 216 mmol) in THF (20 mL) at −75 °C. The reaction mixture was allowed to gradually warm to room temperature overnight. The mixture was then poured into H_2O (200 mL) and extracted with EtOAc (100 mL × 3). The combined organic layers were dried over $NaSO_4$ and concentrated under reduced pressure. The residue was purified by column chromatography (10% EtOAc/hexanes) to give 6.50 g of **7** as a colorless oil (16% yield): ^{1}H NMR ($CDCl_3$) δ 1.26 (t, J = 7.3 Hz, 3H), 1.68−1.74 (m, 1H), 1.85−1.98 (m, 1H), 2.07−2.18 (m, 1H), 2.45 (t, J = 7.8 Hz, 2H), 2.43−2.50 (m, 2H), 3.26−3.32 (m, 2H), 4.13 (q, J = 7.0 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 14.6, 25.4, 30.6, 32.2, 32.2, 51.1, 60.9, 173.2, 210.1.

2-(2-Mercaptoethyl)pentanedioic Acid (4c). To a solution of **7** (2.10 g, 10.4 mmol) in THF (15 mL) was added 2 M NaOH (35 mL), and the resulting mixture was stirred under an inert atmosphere at room temperature overnight. The reaction mixture was washed with Et_2O (20 mL × 2), acidified with 2 M HCl to pH 2, and extracted with EtOAc (20 mL × 3). The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure to give 1.33 g of **4c** as a white solid (66% yield): ^{1}H NMR ($CDCl_3$) δ 1.43 (t, J = 8.3 Hz, 1H), 1.65−1.75 (m, 1H), 1.85−2.15 (m, 3H), 2.48 (t, J = 7.3 Hz, 2H), 2.40−2.80 (m, 3H), 11.0−11.7 (br, 2H); ^{13}C NMR ($CDCl_3$) δ 22.6, 26.8, 32.0, 36.4, 43.6, 179.9, 181.8. Anal. ($C_7H_{12}SO_4$) C, H, S.

4-Tritylsulfanylbutyric Acid (8b). To a solution of tritylmercaptan (15.18 g, 55 mmol) in toluene (50 mL) was added sodium methoxide (4.37 M solution in methanol, 27.5 mL, 120 mmol). To the mixture was slowly added a solution of 4-bromobutyric acid (10.02 g, 60 mmol) in methanol (22.5 mL) at 5−10 °C. The mixture was allowed to warm to 50 °C and was stirred for 2 h. The solvent was removed under reduced pressure, and the residue was taken up with water (200 mL). The aqueous solution was acidified with 1 M H_2SO_4 and extracted with EtOAc (200 mL × 3). The combined extracts were dried over $MgSO_4$ and concentrated under reduced pressure. The crude material was recrystallized from EtOAc/hexanes to afford 15.20 g of **8b** as a white solid (76% yield): ^{1}H NMR ($CDCl_3$) δ 1.67 (quint, J = 7.1 Hz, 2H), 2.22 (t, J = 7.1 Hz, 2H), 2.31 (t, J = 7.1 Hz, 2H), 7.18−7.44 (m, 15H); ^{13}C NMR ($CDCl_3$) δ 24.0, 31.5, 33.2, 67.1, 127.0, 128.3, 130.0, 145.2, 178.9.

5-Tritylsulfanylpentanoic Acid (8c). By the same methods described above and by use of 5-bromovaleric acid, compound **8c** was produced as a white solid (71% yield): mp 124−125 °C; ^{1}H NMR ($CDCl_3$) δ 1.41 (quint, J = 7.3 Hz, 2H), 1.58 (quint, J = 7.4 Hz, 2H), 2.16 (t, J = 7.3 Hz, 2H), 2.20 (t, J = 7.3 Hz, 2H), 7.1−7.3 (m, 9H), 7.3−7.5 (m, 6H); ^{13}C NMR ($CDCl_3$) δ 23.9, 27.9, 31.4, 33.4, 66.52, 126.6, 127.8, 129.6, 144.9, 179.3.

2,2-Dimethyl-5-(4-tritylsulfanylbutyl)[1,3]dioxane-4,6-dione (10b). Compound **10b** was prepared as previously described for the preparation of **10a**,[21] except **8b** was used in place of **8a**: white solid (77% yield); ^{1}H NMR ($CDCl_3$) δ 1.34−1.45 (m, 4H), 1.74 (s, 3H), 1.75 (s, 3H), 1.92−2.02 (m, 2H), 2.10−2.19 (m, 2H), 3.41 (t, J = 5.2 Hz, 1H), 7.15−7.45 (m, 15H); ^{13}C NMR ($CDCl_3$) δ 26.0, 26.1, 26.8, 28.4, 28.5, 31.6, 46.0, 66.5, 104.8, 126.5, 127.8, 129.6, 144.9, 165.4.

2,2-Dimethyl-5-(5-tritylsulfanylpentyl)[1,3]dioxane-4,6-dione (10c). Compound **10b** was prepared as previously described for the preparation of **10a**,[21] except **8c** was used in place of **8a**: white solid (74% yield); ^{1}H NMR ($CDCl_3$) δ 1.25−1.45 (m, 6H), 1.74 (s, 3H), 1.76 (s, 3H), 1.98−2.05 (m, 2H), 2.12−2.18 (m, 2H), 3.44 (t, J = 5.5 Hz, 2H), 7.17−7.42 (m, 15H); ^{13}C NMR ($CDCl_3$) δ 26.1, 26.3, 26.9, 28.2, 28.4, 28.7, 31.8, 46.0, 66.4, 104.8, 126.5, 127.8, 129.6, 145.0, 165.5.

3-[2,2-Dimethyl-4,6-dioxo-5-(3-tritylsulfanylpropyl)-[1,3]dioxan-5-yl]propionic Acid Methyl Ester (11a). A solution of **10a** (4.6 g, 10 mmol), methyl acrylate (4.5 mL, 50 mmol), and sodium acetate (1.64 g, 20 mmol) in *tert*-butyl alcohol (15 mL) was stirred at 60 °C overnight. The solvent was removed under reduced pressure, and the residue was taken up with EtOAc (50 mL). The solution was washed with aqueous 5% $KHSO_4$ (50 mL × 3) and brine (50 mL), dried over $MgSO_4$, and concentrated. The solid residue was recrystallized from EtOAc/hexanes to provide 4.42 g of **11a** as a white solid (81% yield): ^{1}H NMR ($CDCl_3$) δ 1.23−1.33 (m, 2H), 1.67 (s, 3H), 1.74 (s, 3H), 1.84−1.92 (m, 2H), 2.10−2.16 (m, 2H), 2.21−2.28 (m, 2H), 2.34−2.40 (m, 2H), 3.65 (s, 3H), 7.17−7.43 (m, 15H); ^{13}C NMR ($CDCl_3$) δ 24.5, 29.4, 29.5, 29.6, 31.4, 32.6, 37.8, 51.9, 52.8, 66.7, 105.6, 126.7, 127.9, 129.5, 144.7, 168.4, 172.0.

3-[2,2-Dimethyl-4,6-dioxo-5-(4-tritylsulfanylbutyl)[1,3]-dioxan-5-yl]propionic Acid Methyl Ester (11b). Compound **11b** was prepared as described for the preparation of **11a**, except **10b** was used in place of **10a**: white solid (86% yield); ^{1}H NMR ($CDCl_3$) δ 1.16−1.40 (m, 4H), 1.71 (s, 3H), 1.75 (s, 3H), 1.81−1.87 (m, 2H), 2.07−2.15 (m, 2H), 2.26−2.34 (m, 2H), 2.36−2.42 (m, 2H), 3.66 (s, 3H), 7.16−7.44 (m, 15H); ^{13}C NMR ($CDCl_3$) δ 24.7, 28.4, 29.4, 29.5, 29.6, 31.3, 32.6, 38.3, 51.9, 53.0, 66.5, 105.7, 126.6, 127.8, 129.5, 144.8, 168.6, 172.1.

3-[2,2-Dimethyl-4,6-dioxo-5-(5-tritylsulfanylpentyl)-[1,3]dioxan-5-yl]propionic Acid Methyl Ester (11c). Compound **11c** was prepared as described for the preparation of **11a**, except **10c** was used in place of **10a**: white solid (79% yield); ^{1}H NMR ($CDCl_3$) δ 1.11−1.38 (m, 6H), 1.69 (s, 3H), 1.76 (s, 3H), 1.88−1.94 (m, 2H), 2.08−2.13 (m, 2H), 2.28−2.34 (m, 2H), 2.38−2.44 (m, 2H), 3.66 (s, 3H), 7.17−7.42 (m, 15H); ^{13}C NMR ($CDCl_3$) δ 25.0, 28.1, 28.7, 29.4, 29.5, 29.7, 31.7, 32.6, 38.6, 51.9, 53.1, 66.4, 105.6, 126.5, 127.8, 129.5, 144.9, 168.7, 172.1.

2-(3-Tritylsulfanylpropyl)pentanedioic Acid (13a). A suspension of **11a** (5.47 g, 10 mmol) in 1.75 N NaOH (40 mL) was stirred at 100 °C for 3 h. The resulting homogeneous solution was cooled to room temperature, acidified to pH 1 by 1 M H_2SO_4, and extracted with EtOAc. The extract was washed with brine (100 mL × 3), dried over $MgSO_4$, and concentrated to give **12a** as a crude material (>100% crude yield). This material was dissolved in DMSO (30 mL), and the solution was stirred at 130 °C for 3 h. The solvent was removed under reduced pressure, and the residue was taken up in EtOAc (50 mL). The organic solution was washed with water (50 mL × 3) and brine (50 mL), dried over $MgSO_4$, and concentrated. The residual material was recrystallized from EtOAc/hexanes to give 4.30 g of **13a** as a white solid (96% yield for two steps): ^{1}H NMR (CD_3OD) δ 1.26−1.43 (m, 3H), 1.44−1.54 (m, 1H), 1.61−1.80 (m, 2H), 2.08−2.32 (m, 5H), 7.16−7.41 (m, 15H); ^{13}C NMR (CD_3OD) δ 27.5, 28.2, 32.5, 32.6, 32.7, 45.5, 67.7, 127.7, 128.8, 130.7, 146.3, 176.7, 178.9. Anal. ($C_{27}H_{28}O_4S$) C, H, S.

2-(4-Tritylsulfanylbutyl)pentanedioic acid (13b). Compound **13b** was prepared as described for the preparation of **13a**, except **11b** was used in place of **11a**: white solid (quantitative yield); ^{1}H NMR (CD_3OD) δ 1.18−1.48 (m, 6H), 1.66−1.83 (m, 2H), 2.08−2.18 (m, 2H), 2.21−2.32 (m, 3H), 7.15−7.42 (m, 15H); ^{13}C NMR (CD_3OD) δ 27.6, 28.4, 29.5, 32.6, 32.7, 32.8, 45.8, 67.7, 127.7, 128.8, 130.8, 146.5, 176.8, 179.3.

2-(5-Tritylsulfanylpentyl)pentanedioic Acid (13c). Compound **13c** was prepared as described for the preparation of **13a**, except **11c** was used in place of **11a**: white solid (quantitative yield); ^{1}H NMR (CD_3OD) δ 1.08−1.56 (m, 8H),

1.70−1.83 (m, 2H), 2.07−2.15 (m, 2H), 2.22−2.34 (m, 3H), 7.15−7.42 (m, 15H); ^{13}C NMR (CD_3OD) δ 27.8, 28.4, 29.6, 29.8, 32.7, 32.8, 33.1, 45.9, 67.7, 127.7, 128.8, 130.8, 146.5, 176.8, 179.4.

2-(3-Mercaptopropyl)pentanedioic Acid (4d). To a solution of **13a** (4.49 g, 10 mmol) in dichloromethane (15 mL) was added trifluoroacetic acid (5 mL). The reaction mixture turned yellowish brown. To this mixture was slowly added triisopropylsilane (1.74 g, 11.0 mmol). The brown color gradually turned light-yellow, and the temperature of the reaction spontaneously rose because of the exothermic reaction. After the mixture was stirred for 30 min, the solvent was removed under reduced pressure and the residual material was partitioned into water and hexanes (1:1 by volume, 100 mL). The aqueous layer was washed with hexanes (50 mL × 2), basified to pH 13 with 40% aqueous NaOH, and allowed to stand for 3 h under an atmosphere of nitrogen to convert the cyclized byproduct (thiolactone) back to **4d**. The reaction mixture was then acidified to pH 1 with 1 M H_2SO_4, saturated with sodium chloride, and extracted with EtOAc (100 mL × 3). The combined extracts were dried over $MgSO_4$, concentrated, and purified by silica gel chromatography (EtOAc/hexanes) to give 1.55 g of **4d** as a white solid (75% yield): mp 51−53 °C; ^{1}H NMR (CD_3OD) δ 1.55−1.73 (m, 4H), 1.74−1.88 (m, 2H), 2.25−2.42 (m, 3H), 2.44−2.54 (m, 2H); ^{13}C NMR (CD_3OD) δ 24.7, 28.3, 31.9, 32.6, 32.8, 45.5, 176.8, 179.2. Anal. ($C_8H_{14}O_4S$) C, H, S.

Although compound **4d** is stable at 25 °C in the solid state, its aqueous solution stability is largely dependent on pH and oxygen level of the media. To avoid formation of disulfide **16**, it is essential to degas the media by bubbling argon prior to the preparation of an aqueous solution of **4d** for biological evaluation.

2-(4-Mercaptobutyl)pentanedioic Acid (4e). Compound **4e** was prepared as described for the preparation of **4d**, except **13b** was used in place of **13a**: white solid (72% yield); ^{1}H NMR (CD_3OD) δ 1.38−1.55 (m, 3H), 1.56−1.66 (m, 3H), 1.74−1.88 (m, 2H), 2.24−2.40 (m, 3H), 2.46−2.53 (m, 2H); ^{13}C NMR (CD_3OD) δ 24.7, 27.0, 28.4, 32.6, 32.8, 35.0, 45.9, 176.8, 179.3. Anal. ($C_9H_{16}O_4S\cdot 0.1H_2O$) C, H, S.

2-(5-Mercaptopentyl)pentanedioic Acid (4f). Compound **4f** was prepared as described for the preparation of **4d**, except **13c** was used in place of **13a**: white solid (77% yield); ^{1}H NMR (CD_3OD) δ 1.26−1.68 (m, 8H), 1.72−1.88(m, 2H), 2.24−2.41 (m, 3H), 2.43−2.52 (m, 2H); ^{13}C NMR (CD_3OD) δ 24.8, 27.8, 28.4, 29.2, 32.7, 33.2, 35.0, 45.9, 176.8, 179.4. Anal. ($C_{10}H_{18}O_4S$) C, H, S.

5-Mercaptopentanoic Acid (14). To a solution of **8c** (0.753 g, 2.0 mmol) in dichloromethane (5 mL) were added trifluoroacetic acid (5 mL) and trisopropylsilane (0.348 g, 2.2 mmol) at 0 °C, and the mixture was stirred at room temperature for 2 h. The solvent was removed under reduced pressure, and the resulting light-yellow solid was dissolved in dichloromethane and passed through a column of silica gel (dichloromethane) to give 0.179 g of a colorless oil (67% yield): ^{1}H NMR (CD_3OD) δ 1.37 (t, J = 7.8 Hz, 1H), 1.6−1.8 (m, 4H), 2.39 (t, J = 7.1 Hz, 2H), 2.56 (q, J = 7.2 Hz, 2H), 7.0−8.0 (br, 1H); ^{13}C NMR (CD_3OD) δ 23.3, 24.2, 33.2, 33.3, 179.2. Anal. ($C_5H_{10}O_2S$) C, H. S: calcd, 23.89; found, 23.20.

2-(3-Methylsulfanylpropyl)pentanedioic Acid (15). To a solution of **4d** (1.00 g, 4.8 mmol) in 2.75 M NaOH (8 mL) was added dimethyl sulfate (0.61 g, 4.8 mmol) at 0 °C, and the mixture was stirred at 50 °C for 1 h. The reaction mixture was acidified to pH 1 with 1 M HCl and extracted with EtOAc (50 mL × 2). The combined extracts were dried over sodium sulfate and concentrated to give 0.60 g of **15** as a white solid: 57% yield; mp 48−49 °C; ^{1}H NMR ($CDCl_3$) δ 1.57−2.04 (m, 6H), 2.09(s, 3H), 2.42−2.52 (m, 5H); ^{13}C NMR (CDCl3) δ 15.5, 26.6, 26.9, 30.8, 32.0, 33.9, 44.5, 179.7, 182.0. Anal. ($C_9H_{16}O_4S$) C, H, S.

2-[3-(4,6-Dicarboxyhexyldisulfanyl)propyl]pentanedioic Acid (16). A solution of **4d** (2.06 g, 10.0 mmol) in 1.75 M NaOH (20 mL) was stirred vigorously in an oxygen atmosphere for 1−2 days. Progress of the reaction was followed by TLC (hexane/EtOAc = 2:1, containing 2% AcOH). After completion of the reaction, the mixture was acidified by 1 M H_2SO_4 and extracted with EtOAc. The extract was dried over $MgSO_4$ and concentrated to give 1.95 g of **16** as a colorless oil (94% yield): ^{1}H NMR (CD_3OD) δ 1.55−1.94 (m, 12H), 2.25−2.47 (m, 6H), 2.62−2.78 (m, 4H); ^{13}C NMR (CD_3OD) δ 27.8, 28.4, 32.0, 32.6, 39.3, 45.6, 176.8, 179.1. Anal. ($C_{16}H_{26}O_8S_2\cdot 0.2H_2O$) C, H, S.

2-Phosphonopentanedioic Acid (17). A solution of 2-(diethoxyphosphoryl)pentanedioic acid 5-*tert*-butyl ester 1-ethyl ester **19**[22] (0.10 g, 0.28 mmol) in 6 M HCl (20 mL) was refluxed overnight. The solvent was removed under reduced pressure and dried by concentrating from toluene (50 mL × 3). The residual material was lyophilized to give 0.05 g (73% yield) of **17** as a semisolid (76% yield): ^{1}H NMR (D_2O) δ 1.8−1.7 (m, 2H), 2.1−2.0 (m, 2H), 2.6−2.5 (m, 1H); ^{31}P NMR (D_2O) δ 19.4. Anal. ($C_5H_9O_7P\cdot 1.7H_2O$) C, H.

2-[2-(Dimethoxyphosphoryl)ethyl]pentanedioic Acid Di-*tert*-butyl Ester (21). To a solution of dimethyl methylphosphonate (2.50 g, 20.0 mmol) in THF (100 mL) were added CuI (0.380 g, 2.0 mmol) and *n*-BuLi (1.6 M solution, 12.5 mL, 20.0 mmol) at −78 °C, and the mixture was stirred at that temperature for 10 min. To the mixture was slowly added 2-methylenepentanedioic acid di-*tert*-butyl ester **20**[23] (5.13 g, 20.0 mmol) over 20 min, and the reaction mixture was stirred at −78 °C for an additional 3 h. The mixture was poured into ice-cold 1 M HCl (100 mL) and extracted with EtOAc (100 mL × 3). The combined extracts were washed with water, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by column chromatography (EtOAc/hexanes, 1:1) to give 1.10 g of **21** as a colorless oil (15% yield): ^{1}H NMR ($CDCl_3$) δ 1.50 (m, 18H), 1.70 (m, 6H), 2.33 (m, 3H), 3.79 (m, 6H); ^{31}P NMR ($CDCl_3$) δ 32.6.

2-(2-Phosphonoethyl)pentanedioic Acid (18). A solution of **21** (0.60 g, 1.6 mmol) in 6 M HCl (40 mL) was refluxed overnight. The solvent was removed under reduced pressure and dried by concentrating from toluene (50 mL × 3). The residue was then purified by ion-exchange resin to give 0.20 g of **18** as a colorless oil (46% yield): ^{1}H NMR (D_2O) δ 1.51−1.63 (m, 6H), 2.14−2.27 (m, 3H); ^{31}P NMR (D_2O) δ 31.6. Anal. ($C_7H_{13}O_7P\cdot 1.7H_2O$) C, H.

GCP II Assay. The GCP II assay was carried out as outlined previously.[14] Briefly, radiolabeled NAA[^{3}H]G was incubated with purified recombinant GCP II[32] (20 pM) in Tris buffer (pH 7.6, 50 mM) containing $CoCl_2$ (1 mM) in a total volume of 50 μL for 15 min at 37 °C. The reaction was terminated with phosphate buffer (0.1 M, pH 7.4, 50 μL), and the material was applied to a strong anion exchange minicolumn (AG 1-X8 anion exchange resin, 200−400 mesh, formate form). The [^{3}H]glutamate was eluted with 180 μL of formate (1.0 M) while unreacted NAA[^{3}H]G remained bound to the column. The eluate was transferred to a lumaplate and dried. The radioactivity was measured with a top scintillation counter. IC_{50} values were determined at the NAAG concentration of 30 nM. The values are the mean ± SD of two or more independent experiments. The K_i value of compound **4d** was determined from the initial velocity of the hydrolysis at NAAG concentrations ranging from 60 nM to 1 μM. An apparent value of K_m ($K_{m\ app}$) was obtained from the reciprocal plot ($1/v$ vs $1/[S]$) for each set of data. The K_i value was obtained directly from a replot of the slopes of the reciprocal plots (K_{mapp}/V_{max}) vs inhibitor concentration.[33]

CCI Studies. The chronic constriction injury model of neuropathic pain was performed as previously reported.[31] In brief, sciatic nerve ligation, consisting of four ligatures being tied loosely around the sciatic nerve at 1 mm intervals proximal to the nerve trifurcation, was performed on rats. Following this treatment, rats exhibited a unilateral thermal hyperalgesia and allodynia. Animals received compound **4d** (10 mg/kg po daily) or vehicle, starting 10 days after surgery. Animals were habituated to the Hargreaves apparatus. The infrared heat source was directed onto the dorsal surface of the hindpaw, and the time taken for the animal to withdraw its paw was noted. The difference score (between the latency

of the response for the paw on the operated side versus the control side) was determined. Treatment with compound **4d** normalized the difference scores between the two paws compared to the continued hyperalgesic vehicle-treated controls.

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