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Synthesis and Biological Evaluation of Hydroxamate-Based Inhibitors of Glutamate Carboxypeptidase II

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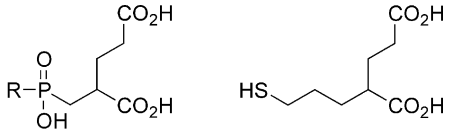
Abstract—A series of hydroxamic acids has been prepared as potential inhibitors of glutamate carboxypeptidase II (GCP II). Compounds based on a P1′ residue (primed-side inhibitors) were more potent than those based on a P1 group (unprimed-side inhibitors). Inhibitory potency of the primed-side GCP II inhibitors was found to be dependent on the number of methylene units between the hydroxamate group and pentanedioic acid. Succinyl hydroxamic acid derivative, 2-(hydroxycarbamoylmethyl)pentanedioic acid, is the most potent GCP II inhibitor with an IC_{50} value of 220 nM. The comparison of the results to those of other classes of GCP II inhibitors as well as hydroxamate-based MMP inhibitors provides further insight into the structure–activity relationships of GCP II inhibition.

Glutamate carboxypeptidase II (GCP II, EC 3.4.17.21), also known as *N*-acetylated α-linked acidic dipeptidase (NAALADase) and prostate-specific membrane antigen (PSMA), is a metallopeptidase which cleaves *N*-acetylaspartylglutamate (NAAG) into *N*-acetylaspartate and glutamate in the nervous system.[1] This enzyme belongs to the M28 peptidase family comprised of co-catalytic metallopeptidases.[2] Peptidases in this family contain two metal ions forming a co-catalytic active site with ligands consisting of five amino acid residues. Inhibition of GCP II has gained considerable attention as a strategy to suppress glutamate excitotoxicity leading to neurological disorders including stroke, spinal cord injury, amyotrophic lateral sclerosis (ALS), peripheral neuropathy, chronic pain, schizophrenia, and epilepsy.[3]

2-(Phosphonomethyl)pentanedioic acid (2-PMPA) **1a**[4] is one of the first potent inhibitors of GCP II with a K_i value of 0.2 nM.[5] 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 vigorously protects against injury in a neuronal culture model of ischemia and in rats after transient middle cerebral artery occlusion (MCAO).[6] We have extended our SAR studies to other zinc-binding groups and identified phosphinate and thiol-based GCP II inhibitors **1b**[7] and **2**,[8] which exhibited in vivo efficacy in animal models of various neurological disorders.



1a R = OH
1b R = pentafluorophenylmethyl

2

In order to further diversify the pharmacophore of GCP II inhibitors, we have examined hydroxamate group as an alternative zinc-binding group. The hydroxamate group of metalloprotease inhibitors is known to bind in a bidentate fashion to the active site zinc(II) ion and has proven to be one of the most effective zinc-binding

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groups particularly in the area of matrix metalloprotease (MMP) inhibitors.[9] As shown in Figure 1, our design of hydroxamate-based GCP II inhibitors is based on an endogenous substrate NAAG that can be divided into the three segments, 1-acetylamino-2-carboxyethyl group, scissile amide bond, and glutaric acid. Incorporation of a hydroxamic acid group into the first segment leads to a P1 group-containing inhibitor **3**, also conventionally referred to as an unprimed-side inhibitor. Attachment of a hydroxamic acid group to the glutaric acid segment through a methylene linker results in derivatives **4a–d**, which contain a P1′ group and thus are referred to as primed-side inhibitors. In this paper, we describe synthesis and biological evaluation of these hydroxamate-based inhibitors of GCP II.

Synthesis of (*S*)-**3** is illustrated in Scheme 1. *N*-Acetyl-L-aspartic acid β-benzyl ester (*S*)-**5**,[10] was coupled to *O*-benzylhydroxylamine to yield (*S*)-**6**. Subsequent catalytic hydrogenolysis afforded *N*-acetyl-L-aspartic acid α-hydroxamate (*S*)-**3**. Ready access to (*R*)-**5**[11] also allowed us to prepare the corresponding D-isomer (*R*)-**3** in a similar manner.

Synthesis of **4a–d** is outlined in Scheme 2. Conjugate addition of Meldrum's acid **7** to benzyl acrylate gave 5-substituted Meldrum's acid **8**.[12] Alcoholysis of **8** with benzyl alcohol[13] followed by coupling to *O*-benzylhydroxylamine afforded compound **9**. Removal of all benzyl groups from **9** by catalytic hydrogenolysis gave the malonyl hydroxamic acid derivative **4a**. The succinyl hydroxamic acid derivative **4b** was also synthesized from Meldrum's acid. One-pot tandem alkylation of Meldrum's acid with benzyl acrylate and allyl bromide gave disubstituted Meldrum's acid **10b**. The acetonide group of compound **10b** was selectively cleaved by the treatment with lithium hydroxide in THF/H_2O. Subsequent decarboxylation and coupling to benzyl alcohol afforded 2-allylglutaric acid dibenzyl ester **11b**. Oxidation of the terminal olefin into a carboxylic acid, followed by coupling to benzyloxyamine gave the fully protected precursor **12b**. The benzyl groups were removed by catalytic hydrogenolysis to yield the desired compound **4b**. The same method was successfully applied to the synthesis of two other analogues **4c** ($n=2$) and **4d** ($n=3$) by replacing allyl bromide with 4-bromo-1-butene and 5-bromo-1-pentene, respectively.

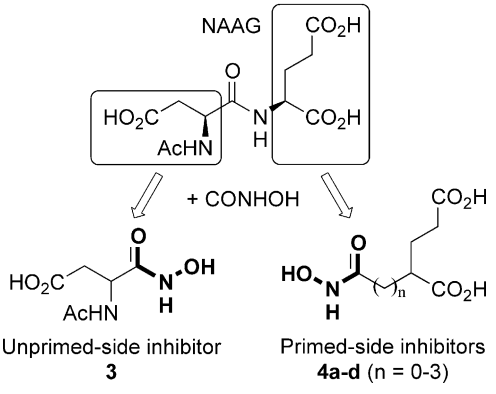
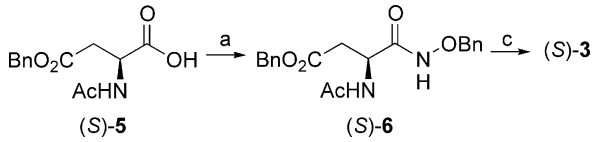


Figure 1. Design of hydroxamate-based GCP II Inhibitors.



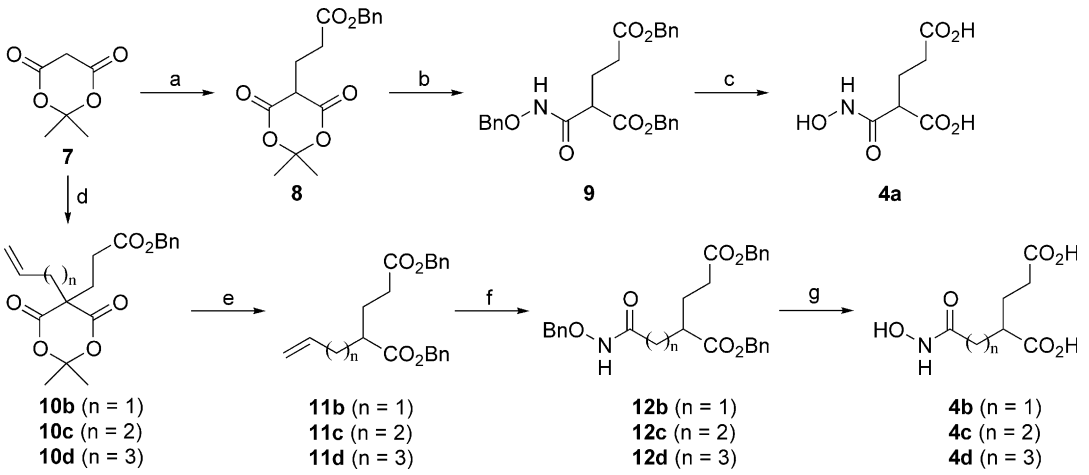
Scheme 1. (a) $BnONH_2$·HCl, EDC, DMAP, HOBt, Et_3N, CH_2Cl_2, rt, 51%; (b) H_2 (30 psi), Pd/C, MeOH, rt, 99%.

Inhibitory potencies of hydroxamic acids **3**, **4a–d** and other structurally relevant compounds (*S*)- and (*R*)-**13**,[14] (*S*)-**14**,[14] **15**,[15] and **16**[14] were evaluated using *N*-acetyl-L-aspartyl-[^{3}H]-L-glutamate as a substrate[5] and a purified recombinant GCP II.[16] For those compounds with an IC_{50} value less than 1 μM, a median effective concentration (EC_{50}) value in a cell culture model of cerebral ischemia[17] was also determined. The results are summarized in Table 1 along with previously reported representative GCP II inhibitors **1a–b** and **2** for comparison purposes.

The rationale for testing both enantiomers of **3** is based on the previously reported studies[18] on amino acid hydroxamate inhibitors of another metalloprotease from family M28, *Aeromonas proteolytica* aminopeptidase. Wilkes and Prescott found that this aminopeptidase was inhibited to a greater extent by D-leucine hydroxamic acid ($K_i=2.0$ nM) than by L-leucine hydroxamic acid ($K_i=350$ nM) despite its substrate specificity for L-amino acid residue in the N-terminal position. In the GCP II assay, neither (*S*)-**3** nor (*R*)-**3** exhibited inhibitory activity in concentration up to 100 μM. Similarly, no significant activity was observed with other commercially available amino acid hydroxamates (*S*)-**13**, (*R*)-**13**, and (*S*)-**14**, which potentially interact with S1 site of the enzyme.

The lack of GCP II inhibition by *N*-acetyl-L-aspartic acid α-hydroxamate **3** is in sharp contrast with the potent inhibition of *A. proteolytica* aminopeptidase by leucine hydroxamate.[19] The marked difference in inhibitory profile between the two metalloproteases can be explained by the substrate specificity of each enzyme. *A. proteolytica* aminopeptidase is specific for peptides possessing leucine as an NH_2-terminal residue[20] and thus potently inhibited by a P1 residue-containing inhibitors such as leucine hydroxamic acid. On the other hand, GCP II is known to hydrolyze not only NAAG but also γ-glutamyl linkage of folate and antifolate poly-γ-glutamates[21] in which case its S1 site is occupied by γ-glutamyl residue. The lower substrate specificity with respect to the P1 residue is presumably attributable to the lack of GCP II inhibition by the unprimed-side inhibitors.

In contrast to the unprimed-side inhibitors, all of the primed-side inhibitors **4a–d** showed IC_{50} values below 10 μM. The results are in a good agreement with the



Scheme 2. (a) Benzyl acrylate, K_2CO_3, $BnEt_3N^+Cl^-$, CH_3CN, 60 °C, 98%; (b) (i) BnOH, toluene, 90 °C; (ii) $BnONH_2$·HCl, EDC, DMAP, Et_3N, CH_2Cl_2, rt; 40%; (c) H_2 (30 psi), $Pd(OH)_2$/C, MeOH, rt, 90%; (d) (i) benzyl acrylate, K_2CO_3, $BnEt_3N^+Cl^-$, CH_3CN, 60 °C; (ii) $CH_2{=}CH(CH_2)_nBr$, 50 °C; 72% for **10b**, 41% for **10c**, 54% for **10d**; (e) (i) LiOH, THF/H_2O, rt; (ii) neat, 130 °C; (iii) BnOH, EDC, DMAP, CH_2Cl_2, rt; 71% for **11b**, 82% for **11c**, 39% for **11d**; (f) (i) RuO_2, $NaIO_4$, acetone/H_2O; (ii) $BnONH_2$·HCl, EDC, DMAP, Et_3N, CH_2Cl_2, rt; 78% for **12b**, 68% for **12c**, 45% for **12d**; (g) H_2 (30 psi), $Pd(OH)_2$/C, MeOH, rt, 98% for **4b**, 98% for **4c**, 73% for **4d**.

substrate specificity and inhibitory profile of the enzyme previously reported. GCP II only hydrolyzes peptides with glutamate as the COOH-terminal residue.[1] It suggests that the predominant binding determinants of peptide substrates for GCP II reside on the primed side of the scissile bond, and that the primed-side inhibitors based on a 2-substituted glutaric acid should exhibit more potency compared to the corresponding unprimed-side inhibitors. Indeed, recent SAR studies on GCP II inhibitors conducted by us and other groups demonstrated that a 2-substituted glutaric acid is an essential component for the potent inhibition of GCP II regardless of the choice of zinc-binding group.[4,7,8,22–25]

Inhibitory potency of **4a–d** against GCP II was found to be dependent on the number of methylene units between the hydroxamate group and pentanedioic acid. Succinyl hydroxamic acid derivative **4b** was found to be the most potent inhibitor of GCP II with an IC_{50} value of 220 nM. Increasing or decreasing the number of methylene units between the hydroxamate group and pentanedioic acid led to a decrease in inhibitory potency as observed in compounds **4a** and **4c–d**. The results are consistent with early SAR analysis by Johnson's group who demonstrated that the succinyl hydroxamate is the optimal backbone for the inhibition of MMP-1.[26]

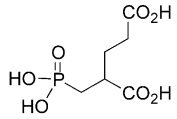
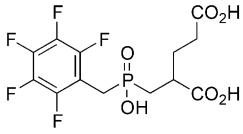
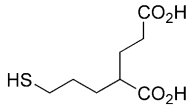
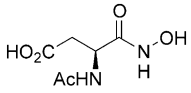
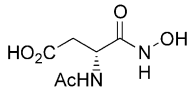
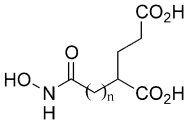
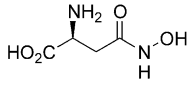
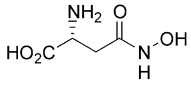
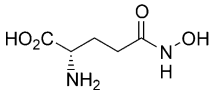
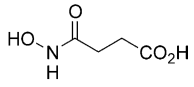
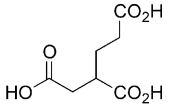
A truncated analogue of **4b**, *N*-hydroxy-succinamic acid **15**, was over 100-fold less potent than **4b** in inhibiting GCP II. The notable decline in potency upon removal of a propionate portion from **4b** suggests critical contribution of the P1′ side chain of **4b** to its strong affinity to GCP II. A similar trend was observed during our SAR studies on thiol and phosphonate-based GCP II inhibitors.[8,27] The poor inhibition by tricarboxylic acid derivative **16** can be attributed to its inability to effectively interact with an active site zinc ion and suggests a critical role for the hydroxamate group of **4b** as a zinc-binding group in interacting with GCP II.

The compound **4b** also provided dose-dependent protection in an established tissue culture model of cerebral ischemia with an EC_{50} value of 220 nM while the less potent GCP II inhibitor **4d** exhibited only weak neuroprotection. Despite their structural diversity, there is a good correlation for relative potency (IC_{50} value) of GCP II inhibitors (**1a–b**, **2**, **4b**, and **4d**) and their neuroprotective potency (EC_{50} value). This provides further evidence to support the hypothesis that the neuroprotective effect of these compounds is due to their ability to inhibit GCP II activity.

Comparison of different zinc-binding groups within 2-substituted glutarate derivatives arrived at the following preference in terms of GCP II inhibition: phosphonate (**1a**) ≫ phosphinate (**1b**) ≥ sulfhydryl (**2**) > hydroxamate (**4b**) ≫ carboxylate (**16**). Interestingly, this preference differs from that of MMPs, which are most effectively inhibited by hydroxamates but only weakly by the corresponding phosphonates.[9] While MMPs require one zinc ion for catalysis, GCP II utilizes two zinc ions that act cocatalytically. Therefore, the catalytic structure of MMPs is notably different from that of GCP II, which may explain the difference in the preference for zinc-binding group.

Crystallographic studies on a hydroxamate-based inhibitor bound to MMP revealed that the hydroxamate group acts as a bidentate ligand to the active site zinc ion with its two oxygen atoms.[28] The hydroxamate group provides additional interactions with the enzyme via the hydroxy group and nitrogen atom which form a hydrogen bond with the proximate Glu and Ala residues of the enzyme backbone, respectively. The relatively weak GCP II inhibition by the hydroxamate-based inhibitor **4b** may be attributed to its inability to guide the hydroxamate group in such way to achieve all

Table 1. Inhibition of GCP II by **1–4** and **13–16**

Compd	Structure	IC_{50} (nM)[a]	EC_{50} (nM)[a]
1a		0.30±0.05[b]	0.62[b]
1b		82±14[b]	9.5±2.1
2		90±26[b]	13±6
(*S*)-**3**		>100,000	—
(*R*)-**3**		>100,000	—
4a ($n=0$) **4b** ($n=1$) **4c** ($n=2$) **4d** ($n=3$)		4000±1400 220±40 1200±400 940±80	— 220[c] — 1100[c]
(*S*)-**13**		>100,000	—
(*R*)-**13**		>100,000	—
(*S*)-**14**		>100,000	—
15		43,000[c]	—
16		20,000±2000	—

[a]Values are the means±SD of at least two separate experiments unless otherwise noted.
[b]Values have been previously reported[6–8] and are included herein for reference purposes.
[c]Values are from a single experiment.

of these interactions with the enzyme. Further structural optimization with respect to other parts of the molecule may enhance the interaction of a hydroxamate group with the active site and lead to more potent hydroxamate-based inhibitors of GCP II.

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