HHS Public Access

Author manuscript

J Med Chem. Author manuscript; available in PMC 2018 February 02.

Published in final edited form as:

J Med Chem. 2017 September 28; 60(18): 7799–7809. doi:10.1021/acs.jmedchem.7b00825.

Discovery of a *para*-Acetoxy-benzyl Ester Prodrug of a Hydroxamate-Based Glutamate Carboxypeptidase II Inhibitor as Oral Therapy for Neuropathic Pain

Rana Rais[†,‡], **Jan Vávra**[▽], **Tomáš Tichý**[▽], **Ranjeet P. Dash**[†,‡], **Alexandra J. Gadiano**[†], **Lukáš Tenora**[▽], **Lenka Monincová**[▽], **Cyril Bařinka**[○], **Jesse Alt**[†], **Sarah C. Zimmermann**[†,‡], **C. Ethan Slusher**[†], **Ying Wu**[†], **Krystyna Wozniak**[†], **Pavel Majer**[*,▽], **Takashi Tsukamoto**[*,†,‡], and **Barbara S. Slusher**[*,†,‡,§,∥,⊥,#]

[†]Johns Hopkins Drug Discovery, Johns Hopkins School of Medicine, Baltimore, Maryland 21205, United States

[‡]Departments of Neurology, Johns Hopkins School of Medicine, Baltimore, Maryland 21205, United States

[§]Psychiatry and Behavioral Sciences, Johns Hopkins School of Medicine, Baltimore, Maryland 21205, United States

[∥]Neuroscience, Johns Hopkins School of Medicine, Baltimore, Maryland 21205, United States

[⊥]Medicine, Johns Hopkins School of Medicine, Baltimore, Maryland 21205, United States

[#]Oncology, Johns Hopkins School of Medicine, Baltimore, Maryland 21205, United States

[▽]Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, vvi, 166 10 Prague, Czech Republic

[○]Institute of Biotechnology, Academy of Sciences of the Czech Republic, vvi, 166 10 Prague, Czech Republic

Abstract

[*]**Corresponding Authors:** For B.S.S.: phone, 410-614-0662; fax, 410-614-0659; bslusher@jhmi.edu; address, Johns Hopkins Drug Discovery, 855 North Wolfe Street, Baltimore, Maryland 21205, United States.; T.T.: phone, 410-614-0982; fax, 410-614-0659; ttsukamoto@jhmi.edu; address, Johns Hopkins Drug Discovery, 855 North Wolfe Street, Baltimore, Maryland 21205, United States.; P.M.: phone, +420-220-183-125; majer@uochb.cas.cz; address, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic vvi, Flemingovo nam 2, 166 10 Prague, Czech Republic.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmed-chem.7b00825. $^{1}H–^{13}C$ NMR and HMBC spectra for **26**; $^{1}H–^{13}C$ NMR and high resolution mass spectra for all prodrugs of **1**; in vivo data for the positive control gabapentin (PDF) Molecular formula strings (CSV)

ORCID

Rana Rais: 0000-0003-4059-2453

Barbara S. Slusher: 0000-0001-9814-4157

Author Contributions

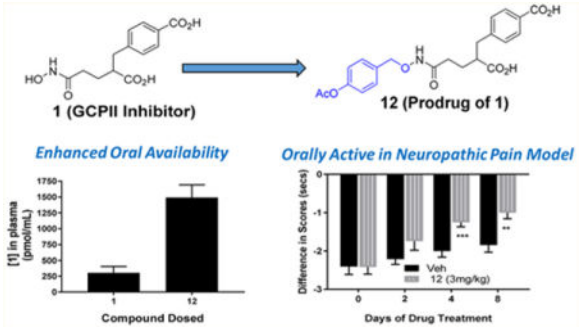
The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

4-Carboxy-α-[3-(hydroxyamino)-3-oxopropyl]-benzenepropanoic acid **1** is a potent hydroxamate-based inhibitor of glutamate carboxypeptidase II. In an attempt to improve its poor oral pharmacokinetics, we synthesized a series of prodrugs by masking its hydrophilic hydroxamate group. Prodrugs were evaluated for oral availability in mice and showed varying degree of plasma exposure to **1**. Of these, *para*-acetoxybenzyl-based, 4-(5-(((4-acetoxybenzyl)oxy)amino)-2-carboxy-5-oxopentyl)benzoic acid, **12**, provided 5-fold higher plasma levels of **1** compared to oral administration of **1** itself. Subsequently, *para*-acetoxybenzyl-based prodrugs with additional ester promoiety(ies) on carboxylate(s) were examined for their ability to deliver **1** to plasma. Isopropyloxycarbonyloxymethyl (POC) ester **30** was the only prodrug that achieved substantial plasma levels of **1**. In vitro metabolite identification studies confirmed stability of the ethyl ester of benzoate while the POC group was rapidly hydrolyzed. At oral daily dose-equivalent of 3 mg/kg, **12** exhibited analgesic efficacy comparable to dose of 10 mg/kg of **1** in the rat chronic constrictive injury model of neuropathic pain.

Graphical abstract



INTRODUCTION

In the nervous system, a membrane-bound metalloprotease termed glutamate carboxypeptidase II (GCPII) catalyzes the hydrolysis of the abundant neuropeptide *N*-acetylaspartylglutamate (NAAG), generating glutamate, a major excitatory neurotransmitter, in the extracellular compartment.[1] Inhibition of GCPII has been extensively explored as a therapeutic approach to treatment of neurological disorders associated with glutamate excitotoxicity. To date, a wide variety of small molecule GCPII inhibitors have been identified[2] and many have shown in vivo efficacy in preclinical models of neurological disorders including neuropathic pain.[3–5] Nearly all potent GCPII inhibitors possess a zinc-binding group, such as phosphonate,[6] phosphinate,[7] urea,[8] thiol,[9] and hydroxamate,[10] that interacts with the catalytic zinc atom(s) in the enzyme's active site. Among them, the hydroxamate-based inhibitors represented by **1** (Figure 1) exhibit an unprecedented mode of binding, in which its two carboxylate groups occupy the nonprime side of the carboxypeptidase while the glutamate-binding S1′ pocket remains unoccupied.[10] In addition, despite its high polarity, compound **1** exhibited good distribution to sciatic nerves,[10] which is believed to be the site of action for GCPII inhibitors for attenuating neuropathic pain.[4,11] Compound **1**, however, suffers from poor oral bioavailability, limiting its therapeutic utility as a treatment for chronic pain. We encountered a similar challenge with

2-phosphonomethyl pentanedioic acid (2-PMPA),[6] a potent GCPII inhibitor with negligible oral availability due to its highly polar molecular characteristics. Recently, we reported that oral administration of tris-isopropyloxycarbonyloxymethyl (POC) ester derivative of 2-PMPA resulted in nearly 20-fold enhancement in the plasma exposure of 2-PMPA in mice compared to oral administration of 2-PMPA itself.[12] This approach represents the first successful demonstration of an orally active GCPII prodrug. We hypothesized that this strategy could be extended to other GCPII inhibitors of high polarity.

To this end, we have explored a series of prodrugs of **1**, in which the hydroxamate and/or carboxylate group(s) was (were) masked to form more lipophilic species in an attempt to improve oral absorption. Here we present the design, synthesis, and pharmacological evaluation of prodrugs derived from **1** (Figure 1). In addition to acetyl derivative, we introduced *O*-4-acetoxy benzyl (PAB) and 2-oxo-1,3-dioxol-4-yl methyl (ODOL) groups for the first time as promoieties for hydroxamate-based drugs. The prodrug with the most desirable pharmacokinetics properties was evaluated in an animal model of neuropathic pain following oral administration to assess its in vivo efficacy.

RESULTS

Chemistry

In all of the prodrugs reported herein, a promoiety was incorporated into the hydroxamate group of **1**. This is based on our previous findings from thiol-based GCPII inhibitors, which have been shown to exhibit good oral bioavailability despite the presence of two carboxylate groups.[13] We also explored prodrugs in which carboxylate group(s) of **1** were modified in addition to the hydroxamate group in order to assess the effects of the added promoiety(ies) on the oral pharmacokinetic profile.

Synthesis of three prodrugs of **1** bearing a single promoiety at its hydroxamate group is illustrated in Scheme 1. Dicarboxylate **2** was converted to the corresponding di-*t*-butyl ester **3**. The terminal olefin of **3** was oxidized to a carboxylate group, providing compound **4**. Coupling with hydroxylamine followed by acetylation and subsequent ester hydrolysis afforded acetylated prodrug **7**. A similar approach but involving coupling of **4** with 4-((aminooxy)methyl)phenyl acetate **10** or 4-((aminooxy)methyl)-5-methyl-1,3-dioxol-2-one **15** provided PAB and ODOL-based prodrugs **12** and **17**, respectively.

Scheme 2 illustrates the synthesis of prodrugs **23**, **25**, and **30** containing multiple promoieties. Prodrug **23** was synthesized by first converting dicarboxylate **2** into the diethyl ester **18**. Two-step conversion of the aliphatic carboxylate ethyl ester into *t*-butyl ester **20** followed by oxidation of the terminal olefin afforded **21**. Coupling of **21** to **10** and subsequent hydrolysis of the *t*-butyl ester afforded the desired prodrug **23**. We also synthesized triple-promoiety prodrug **25** by oxidizing diethyl ester **18** followed by coupling with **10**. Preparation of prodrug **30** bearing a benzoate isopropyloxycarbonylmethyl (POC) was initiated by conversion of dicarboxylate **2** into mono-POC ester **26**. The selective incorporation of the POC group into the benzoate moiety of **26** was confirmed by $^{1}H–^{13}C$ HMBC spectrum (Supporting Information). The methylene protons (5.99 ppm) of the POC group showed three-bond coupling with the carbonate carbonyl (153.4 ppm) of the POC

group and another carbonyl peak at 164.9 ppm. This peak (164.9 ppm) was assigned to the benzoate carbonyl because of its three-bond coupling with the aromatic protons at 8.01 ppm. Compound **26** was subsequently condensed with 2-(trimethylsilyl)ethan-1-ol to give diester **27**. Oxidation of **27** into **28** followed by coupling with **10** and selective hydrolysis of trimethylsilylethyl ester by TFA afforded the desired product **30**.

GCPII Activity of Prodrugs

Compound **1** and its six prodrugs **7**, **12**, **17**, **23**, **25**, and **30** were tested for their inhibition potency against GCPII. While **1** exhibited nM potency as previously described (IC_{50} = 44 nM),[14] prodrugs **7**, **12**, **23**, **25**, and **30** had IC_{50} values >5 μM (Figure 2). Considering structural data on the compound **1**/GCPII complex reported previously,[14] the lack of inhibitory potency of the prodrugs is likely due to the inability of the modified hydroxamate function to chelate active-site zinc ions. The only exception was **17**, which exhibited GCPII inhibition, albeit with 12-fold less potency compared to **1** (IC_{50} = 540 nM; Figure 2). Subsequent stability testing of **17** under identical GCPII enzyme assay conditions (30 m in aqueous buffer; pH 8.0), however, revealed partial hydrolysis to **1**, likely accounting for its GCPII inhibitory activity.

Drug Metabolism and Pharmacokinetics

The six prodrugs **7**, **12**, **17**, **23**, **25**, and **30** were given to mice orally at a dose equivalent of 10 mg/kg of compound **1**, and plasma levels of **1** were measured 30 min after the administration (Figure 2). For comparison, compound **1** was also administered orally at 10 mg/kg to assess the ability of the prodrugs to achieve enhanced plasma levels of **1**. Plasma levels of **1** following oral administration of the prodrugs containing a single promoiety at the hydroxamate group such as **7**, **12**, and **17** were found to be equal or higher than those following oral administration of compound **1** itself. Compound **12** masked with a *para*-acetoxybenzyl moiety at the hydroxamate group was a particularly effective prodrug, displaying 5-fold increase in plasma exposure to compound **1**. Oral administration of *para*-acetoxybenzyl-based prodrugs with additional ester promoiety-(ies) **23** and **25**, however, resulted in negligible plasma levels of **1**. In contrast, *para*-acetoxybenzyl-based prodrug **30** with a benzoate isopropyloxycarbonyloxymethyl (POC) ester achieved 4-fold increase in plasma levels of **1** as compared to those of oral administration of compound **1** itself.

To understand the structure–activity relationship in terms of bioconversion of prodrugs into compound **1**, we assessed metabolism of prodrugs **12**, **23**, and **30** in plasma (Figure 1A). Because compound **1** (Figure 3B) was found to be completely stable in mouse plasma (Figure 3C), we assumed that the plasma samples treated with a prodrug contain the remaining prodrug, partially hydrolyzed intermediate(s), and/or compound **1**. Figure 3D shows an extracted-ion (428.142 for **12**) chromatogram of **12** as reference. An extracted mass (280.080 for **1** and 428.080 for **12**) chromatogram of plasma sample treated with **12** for 60 min (Figure 3E) lost the peak corresponding to **12** and displayed a new peak corresponding to **1**, suggesting complete conversion of prodrug **12** to **1** in plasma. In contrast, prodrug **23** (Figure 3F) was not converted into **1** in plasma despite the complete loss of **23** after 60 min incubation. An extracted mass (280.080 for **1**, 308.120 for **31**, and 428.080 for **12**) chromatogram shows a new peak matching the mass of **31**, ethyl ester of **1**

(Figure 3G). The results suggest that only *para*-acetoxybenzyl moiety was removed from **23** in plasma. This explains the negligible plasma levels of **1** following oral administration of **23** (Figure 2). Although speculative, this could be partially attributable to the inability of plasma-rich hydrolases to recognize benzoate esters as a substrate. The inability to hydrolyze the benzoate ester likely prevented formation of **12** from **23** as well. This is apparent as formation of **12** would be immediately followed by the generation of **1** as is evident from the plasma stability data of **12** (Figure 3D). Interestingly, prodrug **30** (Figure 3H) containing a POC ester was completely hydrolyzed in plasma along with its *para*-acetoxybenzyl moiety to produce **1** as a sole metabolite as shown in an extracted mass (280.090 for **1** and 428.142 for **30**) chromatogram (Figure 3I) of plasma treated with prodrug **30**. This can be attributed to the ability of hydrolases to act on the carbonate portion of the POC ester away from the benzoate carbonyl moiety.[15] This is in a good agreement with the enhanced plasma levels of **1** following oral administration of **30** as compared to **23** (Figure 2).

Chronic Constrictive Injury Model of Neuropathic Pain

On the basis of the highest exposure to compound **1** achieved by oral administration, prodrug **12** was subsequently tested for its antinociceptive effects following oral administration using the rat chronic constriction injury model of neuropathic pain.[16] We have previously shown analgesic effects of compound **1** in the same model following 10 mg/kg/day oral dosing. Given the improved plasma exposure achieved by oral administration of **12**, we chose to test **12** at 4.6 mg/kg, an equivalent of 3 mg/kg of compound **1**. Gabapentin (50 and 100 mg/kg po daily) was used as a positive control. Hyperalgesia testing was initiated 10 days postsurgery by determining withdrawal latencies in response to a constant thermal stimulus. As shown in Figure 4A, prodrug **12** significantly reduced thermal hyperalgesia relative to the vehicle-treated control on days 4 and 8. In contrast, as shown in Figure 4B, prodrug **12** had no significant effect on absolute latency of the sham operated side, suggesting a selective antihyperalgesic effect on the injured nerve. Similarly, gabapentin (100 mg/kg po) significantly reversed allodynia that was maintained throughout the dosing period (Supporting Information, Figure S4).

When compared directly to **1**, despite the higher doses (10 mg/kg/day), oral administration of **1** itself first displayed significantly reduced thermal hyperalgesia on day 8[10] as opposed to day 4 for the lower dose of **12**. These findings clearly demonstrate the pharmacological advantages of prodrug **12** over the parent compound **1**, presumably due to the improved oral pharmacokinetics of **1** achieved by prodrug **12**.

CONCLUSIONS

The concept of hydroxamate prodrugs has been only recently reported in the literature, including carbamates,[17,18] *O*-acyl derivatives,[19] and 1,4,2-dioxazol-5-one.[20] However, little has been investigated on the oral pharmacokinetics of these prodrugs to assess their pharmacological advantages over the parent compounds. In our work, systematic assessment of prodrugs derived from hydroxamate-based GCPII inhibitor **1** provided key insights into the design strategy for creating an improved oral pharmacokinetic profile. As far as the

hydroxamate group is concerned, incorporation of a *para*-acetoxybenzyl group as represented by prodrug **12** resulted in substantial improvement in pharmacokinetics of **1** as compared to acetyl and (5-methyl-2-oxo-1,3-dioxol-4-yl)methyl groups. Prodrugs **23** and **25** containing additional pro-moiety(ies), however, failed to deliver **1** in plasma following oral administration. The inability of **23** and **25** to generate **1** in plasma can be attributed to the resistance of the benzoate ethyl ester to enzymatic hydrolysis reaction. Indeed, *para*-acetoxybenzyl-based prodrug **30** containing a POC ester was able to release **1** in plasma, presumably because of its carbonate site prone to enzymatic hydrolysis reaction. Consistent with the improved oral pharmacokinetics demonstrated by prodrug **12**, at oral daily doses equivalent to 3 mg/kg of **1**, prodrug **12** exhibited analgesic efficacy superior to that of oral daily doses of 10 mg/kg of **1**[10] and similar to standard of care gabapentin in the rat chronic constrictive injury model (CCI) of neuropathic pain. These findings collectively suggest that the prodrug approach is an effective strategy for improving oral activity of hydroxamate-based GCPII inhibitors. To the best of our knowledge, compound **12** represents the first example of hydroxamate-derived prodrugs with improved in vivo oral pharmacokinetics. This approach has potential application to other hydroxamate-based drugs such as HDAC and MMP inhibitors.

EXPERIMENTAL SECTION

General

The commercially available HPLC grade methanol, catalysts, and reagent grade materials were used without further purification. TLC was performed on silica gel 60 F254-coated aluminum sheets (Merck), and spots were detected by a solution of $Ce(SO_4)_2{\cdot}4H_2O$ (1%) and $H_3P(Mo_3O_{10})_4$ (2%) in sulfuric acid (10%). Flash chromatography was performed on silica gel 60 (0.040–0.063 mm, Fluka) or on Biotage KP-C18-HS or KP-Sil SNAP cartridges using the Isolera One HPFC system (Biotage, Inc.). All chemicals were purchased from Sigma-Aldrich and were used without further purification. Preparative HPLC was performed on a JASCO system equipped with Jasco PU-986 pump and UV-975 detector using an YMC C18 column (5 μm, 20 mm × 250 mm) with a linear gradient of 2–60% acetonitrile/water (0.1% TFA) over 50 min at a flow rate 10 mL/min. NMR spectra were measured on a Bruker Avance III 400 or 500 spectrometer operating at 400.1 MHz (^{1}H) and 100.8 MHz (^{13}C) or at 500.0 MHz (^{1}H) and 125.7 MHz (^{13}C). Chemical shift assignment and confirmation of the structure of compound **26** was achieved by a combination of ^{1}H, ^{13}C, and ^{1}H–^{13}C HMBC NMR experiments. The ESI mass spectra were recorded using Waters Micromass ZQ mass spectrometer equipped with an ESCi multimode ion source and operated by MassLynx software. The low resolution ESI mass spectra were recorded using a quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-Tof micro, Waters) and high-resolution ESI mass spectra using a hybrid FT mass spectrometer combining a linear ion trap MS and the Orbitrap mass analyzer (LTQ Orbitrap XL, Thermo Fisher Scientific). The conditions were optimized for suitable ionization in the ESI Orbitrap source (sheath gas flow rate 35 au, aux gas flow rate 10 au of nitrogen, source voltage 4.3 kV, capillary voltage 40 V, capillary temperature 275 °C, tube lens voltage 155 V). Purity for each compound was established using HPLC (Jasco Inc.) equipped with a Reprosil 100 C18, 5 μm, 250 mm × 4 mm column. The analysis was performed using a gradient of 2% CH_3CN/98% H_2O with

0.1% TFA to 100% CH_3CN, with UV detection at λ = 210 nm. All final compounds tested were confirmed to be of ≥95% purity.

tert-Butyl 4-(2-(tert-Butoxycarbonyl)hex-5-en-1-yl)benzoate (3)—A solution of 4-(2-carboxyhex-5-en-1-yl)benzoic acid **2** (7.07 g, 28.5 mmol) in anhydrous dichloromethane (120 mL) was cooled to −65 °C, and an excess of isobutylene (23.7 g, 422 mmol) was condensed into the mixture followed by addition of conc H_2SO_4 (1.6 mL, 28.5 mmol). The reaction vessel was sealed and allowed to reach room temperature. The mixture was stirred overnight, then cooled to −65 °C and poured into saturated aqueous solution of $NaHCO_3$. The reaction mixture was extracted with ether (100 mL × 2), and combined organic extracts were dried over $MgSO_4$ and concentrated in vacuo. The residual oil was chromatographed on silica gel (acetone/hexane, 1:6) to yield 8.49 g of **3** as a syrupy oil (83% yield). ^{1}H NMR (DMSO-d_6) δ 1.28 (s, 9H), 1.53 (s, 9H), 1.52 (m, 1H), 1.61 (m, 1H), 1.96–2.08 (m, 2H), 2.55 (m, 1H), 2.80 (m, 2H), 4.96 (dm, 1H, J = 10.2), 5.00 (dm, J = 17.1 Hz, 1H), 5.77 (ddt, J = 10.3, 17.1, 6.6 Hz, 1H), 7.29 (m, 2H), 7.80 (m, 2H). ^{13}C NMR (DMSO-d_6) δ 27.8, 28.0, 31.0, 31.3, 37.9, 46.9, 80.0, 80.7, 115.6, 129.1, 129.3, 129.5, 138.0, 144.8, 165.1, 173.8. HRMS (ESI): $[M + Na]^+$ m/z 383.21938 (calcd 383.21928 for $C_{22}H_{32}O_4Na^+$).

5-(tert-Butoxy)-4-(4-(tert-butoxycarbonyl)benzyl)-5-oxopentanoic Acid (4)—To a solution of **3** (8.48 g, 23.5 mmol) in acetonitrile (450 mL) and water (450 mL) was added RuO_2 hydrate (356 mg, 2.35 mmol) followed by addition of $NaIO_4$ (50.4 g, 235 mmol) portionwise over the course of 80 min at 0 °C. The mixture was slowly warmed up to 20 °C and stirred until the starting material disappeared on TLC (~2 h). The reaction mixture was then filtered through a pad of Celite and washed with additional acetonitrile. The solvents were then removed on rotavap. and the residual material was extracted with EtOAc (250 mL × 3). The combined organic extracts were dried over $MgSO_4$ and concentrated. The residual material was chromatographed on silica gel (acetone/hexane, 3:7) to give 7.78 g of **4** as an oil (87% yield). ^{1}H NMR (DMSO-d_6) δ 1.29 (s, 9H), 1.53 (s, 9H), 1.66–1.71 (m, 2H), 2.16–2.28 (m, 2H), 2.58 (m, 1H), 2.77–2.84 (m, 2H), 7.30 (m, 2H), 7.80 (m, 2H). ^{13}C NMR (DMSO-d_6) δ 27.2, 27.8, 28.0, 31.4, 37.7, 46.7, 80.2, 80.7, 129.1, 129.3, 129.6, 144.7, 165.1, 173.5, 174.0. HRMS (ESI): $[M – H]^-$ m/z 377.19682 (calcd 377.19696 for $C_{21}H_{29}O_6$).

tert-Butyl 4-(2-(tert-Butoxycarbonyl)-5-(hydroxyamino)-5-oxopentyl)benzoate (5)—Hydroxylamine hydrochloride (5.94 g, 84.6 mmol, 8 equiv) and NaOH (3.45 g, 84.55 mmol, 8 equiv) were placed in a Schlenk flask and put under an argon atmosphere. After adding methanol (60 mL), the mixture was cooled to 0 °C while stirring. After 15 min, the cooling bath was removed and the mixture was stirred for additional 1 h. The mixture was then filtered through a pad of Celite, yielding the filtrate containing NH_2OH for the later use. Meanwhile, to a solution of **4** (4.00 g, 10.57 mmol) in THF (100 mL) in a Schlenk flask were added triethylamine (2.2 mL, 15.85 mmol, 1.5 equiv) and ethyl chloroformate (1.6 mL, 15.86 mmol, 1.5 equiv) under an argon atmosphere, and the mixture was stirred at rt for 1 h. A white precipitate formed almost immediately. The filtrate containing NH_2OH prepared earlier was added, and the mixture was stirred at rt overnight. Volatiles were then removed on a rotavap, and water (100 mL) was added. The mixture was extracted with EtOAc (250

mL × 3). The combined extracts were dried over $MgSO_4$ and concentrated. The residual material was chromatographed on silica gel (hexanes/EtOAc, 2:3 containing 1% AcOH) to afford 2.63 g of **5** as an orange oil (63% yield, 6.69 mmol). ^{1}H NMR (DMSO-d_6) δ 1.29 (s, 9H), 1.53 (s, 9H), 1.66–1.71 (m, 2H), 1.92–2.03 (m, 2H), 2.53 (m, 1H), 2.76–2.84 (m, 2H), 7.29 (m, 2H), 7.80 (m, 2H), 8.71 (bs, 1H), 10.40 (bs, 1H). ^{13}C NMR (DMSO-d_6) δ 27.8, 28.0, 27.9, 29.9, 37.7, 47.0, 80.1, 80.7, 129.1, 129.3, 129.6, 144.7, 165.1, 168.5, 173.5. HRMS (ESI): $[M - H]^-$ m/z 392.20771 (calcd 392.20786 for $C_{21}H_{30}O_6N$).

tert-Butyl 4-(5-(Acetoxyamino)-2-(tert-butoxycarbonyl)-5-oxopentyl)benzoate (6)—To a solution of **5** (6.78 g, 17.2 mmol) in anhydrous pyridine (100 mL) was dropwise (over 15 min) added acetic anhydride (1.6 mL, 17.2 mmol) at 0 °C. The mixture was kept in cooling bath overnight. The mixture was evaporated and coevaporated with toluene. The residual material was then chromatographed on a silica gel column in EtOAc/hexanes, (1:1 to 3:2) to afford 5.74 g of **6** as a colorless oil (76% yield, 13.2 mmol). ^{1}H NMR (DMSO-d_6) δ 1.29 (s, 9H), 1.53 (s, 9H), 1.68–1.77 (m, 2H), 2.14 (s, 3H), 2.12–2.20 (m, 2H), 2.59 (m, 1H), 2.77–2.85 (m, 2H), 7.30 (m, 2H), 7.80 (m, 2H), 11.64 (bs, 1H). ^{13}C NMR (DMSO-d_6) δ 18.2, 27.4, 27.8, 28.0, 29.6, 37.6, 46.7, 80.2, 80.7, 129.1, 129.3, 129.6, 144.6, 165.1, 168.7, 169.2, 173.4. HRMS (ESI): $[M - H]^-$ m/z 434.21759 (calcd 434.21843 for $C_{23}H_{32}O_7N$).

4-(5-(Acetoxyamino)-2-carboxy-5-oxopentyl)benzoic Acid (7)—To a solution of **6** (0.90 g, 2.07 mmol) in dichloromethane (10 mL) were added TFA (5 mL) and *i*-Pr_3SiH (428 μL, 2.07 mmol) under an argon atmosphere. The mixture was stirred at rt for 1 h, when disappearance of the starting material was confirmed on TLC. Volatiles were removed on a rotavap, and EtOAc (5 mL) was added to the residual material. A short sonication caused a precipitate formation. After adding hexanes (10 mL) followed by a short sonication, the mixture was filtered to give 0.57 g of **7** as a white solid (86% yield, 1.78 mmol). ^{1}H NMR (DMSO-d_6) δ 1.66–1.78 (m, 2H), 2.13 (s, 3H), 2.10–2.23 (m, 2H), 2.10–2.23 (m, 2H), 2.63 (m, 1H), 2.81 (dd, J = 13,7, 6.2 Hz, 1H), 2.88 (dd, J = 13.7, 8.6 Hz, 1H), 7.31 (m, 2H), 7.85 (m, 2H), 11.63 (bs, 1H), 12.57 (vbs, 2H). ^{13}C NMR (DMSO-d_6) δ 18.3, 27.1, 29.7, 37.5, 45.8, 129.0, 129.3, 129.5, 144.9, 167.5, 168.8, 169.3, 175.8. HRMS (ESI): $[M - H]^-$ m/z 322.09274 (calcd 322.09323 for $C_{15}H_{16}O_7N$).

4-((1,3-Dioxoisoindolin-2-yloxy)methyl)phenyl Acetate (9)—To a suspension of NaH (768 mg, 19.2 mmol, 1.1 equiv) in DMF (40 mL) was slowly (over 10 min) added a solution of *N*-hydroxyphthalimide (3.23 g, 19.2 mmol, 1.1 equiv) in DMF (40 mL) at 0 °C under an argon atmosphere. After 10 min of stirring, a solution of 4-(bromomethyl)phenyl acetate **8** (4.00 g, 17.5 mmol) in DMF (40 mL) was added. The mixture was stirred at rt for 2 h, then water (100 mL) was added and the mixture was extracted with EtOAc (200 mL × 3). The combined organic extracts were washed with water (200 mL × 3), dried over $MgSO_4$, and concentrated to give a yellowish solid, which was sonicated in EtOAc (10 mL). After adding hexanes (40 mL), the mixture was sonicated for additional 5 min. The resulting mixture was filtered to afford 4.23 g of **9** as an off-white solid (78% yield, 13.6 mmol). ^{1}H NMR ($CDCl_3$) δ 2.29 (s, 3H), 5.20 (s, 2H), 7.12 (d, J = 8.5 Hz, 2H), 7.57 (d, J = 8.5 Hz, 2H), 7.71–7.78 (m, 2H), 7.79–7.86 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 21.3, 79.3, 121.9, 123.7,

129.0, 131.2, 131.5, 134.6, 151.5, 163.6, 189.4. HRMS (ESI): $[M + Na]^+$ *m*/*z* 334.06863 (calcd 334.06859 for $C_{17}H_{13}O_5NNa$).

4-(Aminooxymethyl)phenyl Acetate (10)—To a suspension of **9** (3.80 g, 12.2 mmol) in THF (30 mL) and ethanol (3 mL) was added 1 M N_2H_4 in THF (12.22 mL, 12.22 mmol, 1 equiv) at rt under an argon atmosphere. The mixture turned slightly yellowish, and the solid dissolved. In a minute, a precipitate appeared again. The mixture was stirred at rt for 1 h, and then it was filtered under an argon atmosphere. The filtrate was used immediately in the subsequent reaction.

tert-Butyl 4-(5-(((4-Acetoxybenzyl)oxy)amino)-2-(tert-butoxycarbonyl)-5-oxopentyl)benzoate (11)—To a mixture of 5-(*tert*-butoxy)-4-(4-(*tert*-butoxycarbonyl)benzyl)-5-oxopentanoic acid **4** (3.08 g, 8.15 mmol), EDC·HCl (2.34 g, 12.2 mmol, 1.5 equiv), and DMAP (50 mg, 0.41 mmol, 0.05 equiv) in DMF (60 mL) were added *i*-Pr_2NEt (4.28 mL, 24.4 mmol, 3 equiv) and the filtrate containing 4-(aminooxymethyl)phenyl acetate **10** prepared as above (theoretically 2.21 g, 12.2 mmol, 1.5 equiv) at once at rt under an argon atmosphere. The mixture was stirred at rt for 3 h. Then water (100 mL) was added and the mixture was extracted with EtOAc (120 mL × 3). The combined organic extracts were washed water (100 mL × 3), dried over $MgSO_4$, and concentrated. The residue was chromatographed on a silica gel column (hexanes/EtOAc, 1:1) to afford a yellowish oil containing a solid. The mixture was sonicated in EtOAc (5 mL × 5), and then with hexanes (25 mL). The suspension was kept still for 10 min for the precipitate to settle, and the supernatant was separated. The combined supernatants were concentrated to give 881 mg of **11** as a yellowish oil (20% yield, 1.63 mmol). ^{1}H NMR ($CDCl_3$) δ 1.32 (s, 9H), 1.58 (s, 9H), 1.81–1.93 (m, 2H), 1.96–2.21 (m, 2H), 2.30 (s, 3H), 2.54–2.66 (m, 1H), 2.70–2.80 (m, 1H), 2.88–2-.98 (m, 1H), 4.73–4.94 (m, 2H), 7.08 (d, *J* = 8.1 Hz, 2H), 7.20 (d, *J* = 7.9 Hz, 2H), 7.39 (d, *J* = 7.7 Hz, 2H), 7.88 (d, *J* = 8.3 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 21.2, 27.8, 28.0, 28.2, 30.8, 38.4, 47.2, 77.5, 80.9, 81.2, 121.8, 128.8, 129.5, 130.2, 130.4, 133.0, 143.8, 150.9, 165.8, 169.5, 170.2, 173.9. HRMS (ESI): $[M + Na]^+$ *m*/*z* 564.25690 (calcd 564.25679 for $C_{30}H_{39}O_8NNa$).

4-(5-(((4-Acetoxybenzyl)oxy)amino)-2-carboxy-5-oxopentyl)-benzoic Acid (12)—To a solution of **11** (365 mg, 0.67 mmol) in dichloromethane (10 mL) were added TFA (5 mL) and *i*-Pr_3SiH (276 μL, 1.35 mmol, 2 equiv) under an argon atmosphere. The mixture was stirred at rt for 1 h, after which time no starting material was detected by TLC. Volatiles were removed on a rotavap, and residual TFA was coevaporated with toluene (5 mL × 3). EtOAc (1 mL) was then added to the residual material and the mixture was sonicated for 5 min, resulting in white precipitate formation. The mixture was centrifuged and the supernatant was removed. The precipitate was sonicated in chloroform (2 mL), filtered, and washed with chloroform (5 mL) to give 165 mg of **12** as a white solid (57% yield, 0.38 mmol). ^{1}H NMR (DMSO-d_6) δ 1.61–1.79 (m, 2H), 1.92–2.11 (m, 2H), 2.27 (s, 3H), 2.53–2.64 (m, 1H), 2.73–2.92 (m, 2H), 4.75 (s, 2H), 7.12 (d, *J* = 8.5 Hz, 2H), 7.30 (d, *J* = 8.3 Hz, 2H), 7.41 (d, *J* = 8.5 Hz, 2H), 7.85 (d, *J* = 8.3 Hz, 2H). ^{13}C NMR (DMSO-d_6) δ 20.9, 27.1, 29.9, 37.3, 45.8, 76.1, 121.7, 128.8, 129.0, 129.3, 130.0, 133.6, 144.7, 150.4, 167.2, 168.7, 169.1, 175.6. HRMS (ESI): $[M - H]^-$ *m*/*z* 428.13480 (calcd 428.13509 for $C_{22}H_{22}O_8N$).

2-((5-Methyl-2-oxo-1,3-dioxol-4-yl)methoxy)isoindoline-1,3-dione (14)—To a suspension of NaH (287 mg, 7.18 mmol, 1.1 equiv) in DMF (10 mL) was slowly added (over 5 min) a solution of *N*-hydroxyphthalimide (1.21 g, 7.18 mmol, 1.1 equiv) in DMF (10 mL) at 0 °C under an argon atmosphere. After 5 min of stirring, a solution of **13** (1.00 g, 6.53 mmol) in DMF (5 mL) was added. The mixture was warmed up to rt and then heated at 70 °C for 1 h, during which time the dark-red mixture turned medium beige and the starting material disappeared on TLC. Water (50 mL) was then added to the mixture, and the resulting mixture was extracted with EtOAc (120 mL × 3). The combined organic extracts were washed with water (100 mL × 3) to remove DMF, dried over $MgSO_4$, and concentrated. EtOAc (4 mL) was then added to the residual material, and it was sonicated for 10 min. Hexanes (40 mL) were added, and after a short sonication and filtration 1.64 g of **14** was obtained as an off-white solid (92% yield, 5.98 mmol). ^{1}H NMR ($CDCl_3$) δ 2.14 (s, 3H), 4.95 (s, 2H), 7.76–7.81 (m, 2H), 7.83–7.88 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 9.5, 66.7, 124.0, 128.7, 132.8, 135.0, 142.2, 151.9, 163.3. HRMS (ESI): $[M + Na]^+$ m/z 298.03227 (calcd 298.03221 for $C_{13}H_9O_6NNa$).

4-((Aminooxy)methyl)-5-methyl-1,3-dioxol-2-one (15)—To a solution of **14** (218 mg, 0.79 mmol) in THF (5 mL) was added N_2H_4 (1.0 M solution in THF, 793 μL, 0.79 mmol) under an argon atmosphere. A precipitate formed almost immediately, which slowly (5 min) disappeared, leaving a homogeneous mixture and then rapidly a precipitate formed again. The mixture was stirred at rt for 1 h, then it was filtered through a microfilter syringe, and the resulting filtrate containing **15** was used immediately in the subsequent reaction.

tert-Butyl 4-(2-(tert-Butoxycarbonyl)-5-(((5-methyl-2-oxo-1,3-dioxol-4-yl)methoxy)amino)-5-oxopentyl)benzoate (16)—To a solution of **4** (200 mg, 0.53 mmol) in THF (10 mL) were added triethylamine (89 μL, 0.63 mmol, 1.2 equiv) and ethyl chloroformate (63 μL, 0.63 mmol, 1.2 equiv). The mixture was stirred at rt for 1.5 h, then the filtrate containing **15** prepared above was added at once. The mixture was stirred at rt for 16 h. Water (30 mL) was then added, and the mixture was extracted with EtOAc (100 mL × 3), dried over $MgSO_4$, and concentrated. The residual material was chromatographed on a silica gel column (hexanes/EtOAc, 1:1) to afford 104 mg of **16** as a yellowish oil (39% yield, 0.21 mmol). ^{1}H NMR ($CDCl_3$) δ 1.35 (s, 9H), 1.58 (s, 9H), 1.81–1.94 (m, 2H), 2.05–2.26 (m, 2H), 2.13 (s, 3H), 2.56–2.66 (m, 1H), 2.76 (dd, J = 6.3, 13.8, Hz, 1H), 2.95 (dd, J = 8.8, 13.8, Hz, 1H), 4.67 (s, 2H), 7.20 (d, J = 8.2 Hz, 2H), 7.88 (d, J = 8.1 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 9.5, 27.7, 28.1, 28.3, 30.7, 38.5, 47.3, 65.4, 81.1, 81.4, 128.9, 129.7, 130.4, 133.7, 141.1, 143.8, 152.3, 165.8, 168.8, 174.0. HRMS (ESI): $[M + Na]^+$ m/z 528.22055 (calcd 528.22040 for $C_{26}H_{35}O_9NNa$).

4-(2-Carboxy-5-(((5-methyl-2-oxo-1,3-dioxol-4-yl)methoxy)-amino)-5-oxopentyl)benzoic Acid (17)—To a solution of **16** (74 mg, 0.15 mmol) in dichloromethane (5 mL) were added TFA (1 mL) and *i*-Pr_3SiH (60 μL, 0.29 mmol, 2 equiv) under an argon atmosphere. The mixture was stirred at rt for 30 min. Volatiles were removed on a rotavap, and residual TFA was coevaporated with toluene (2 mL × 2). The residual material was sonicated in chloroform (1 mL), filtered, and washed with chloroform (3 mL) to give 35 mg of **17** as a white solid (61% yield, 0.09 mmol). ^{1}H NMR (DMSO-d_6) δ 1.62–

1.74 (m, 2H), 1.91–2.10 (m, 2H), 2.05 (s, 3H), 2.54–2.63 (m, 1H), 2.74–2.93 (m, 2H), 4.59 (s, 2H), 7.31 (d, J = 7.8 Hz, 2H), 7.85 (d, J = 7.9 Hz, 2H). ^{13}C NMR (DMSO-d_6) δ 8.7, 27.0, 27.6, 29.9, 37.3, 45.8, 63.9, 128.8, 129.0, 129.3, 133.5, 140.9, 144.6, 152.1, 167.2, 168.8, 175.5. HRMS (ESI): $[M - H]^-$ m/z 392.09845 (calcd 392.09870 for $C_{18}H_{18}O_9N$).

Ethyl 4-(2-(Ethoxycarbonyl)hex-5-en-1-yl)benzoate (18)—To a solution of **2** (1.00 g, 4.03 mmol) in ethanol (20 mL) was dropwise added $SOCl_2$ (1.77 mL, 24.4 mmol) at 0 °C over 10 min. The mixture was stirred at rt for 24 h. Volatiles were then removed on a rotavap, and the residual material was chromatographed on a silica gel column (hexanes/EtOAc, 6:1) to afford 1.12 g of **18** as a yellow oil (91% yield, 3.68 mmol). ^{1}H NMR ($CDCl_3$) δ 1.14 (t, J = 7.1 Hz, 3H), 1.38 (t, J = 7.1 Hz, 3H), 1.52–1.62 (m, 1H), 1.72–1.83 (m, 1H), 1.98–2.15 (m, 2H), 2.64–2.73 (m, 2H), 2.80 (dd, J = 13.6, 6.4 Hz, 1H), 2.97 (dd, J = 13.5, 8.8 Hz, 1H), 3.98–4.11 (m, 2H), 4.36 (q, J = 7.1 Hz, 2H), 4.94–5.04 (m, 2H), 5.68–5.80 (m, 1H), 7.22 (d, J = 8.3 Hz, 2H), 7.94 (d, J = 8.3 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 14.3, 14.5, 31.4, 31.5, 38.5, 46.8, 60.5, 61.0, 115.5, 128.8, 129.0, 129.8, 137.6, 144.8, 166.7, 175.2. HRMS (ESI): $[M + Na]^+$ m/z 327.15670 (calcd 327.15668 for $C_{18}H_{24}O_4Na$).

2-(4-(Ethoxycarbonyl)benzyl)hex-5-enoic Acid (19)—To a solution of **18** (500 mg, 1.64 mmol) in EtOH (20 mL) was dropwise added a solution of NaOH (74 mg, 1.81 mmol, 1.1 equiv) in water (10 mL) over 10 min at 0 °C. The mixture was stirred at 0 °C for 3 h and at rt for 68 h. Volatiles were removed on a rotavap, and the residue was acidified with concentrated HCl to pH ~ 1 at 0 °C. The mixture was extracted with EtOAc (100 mL × 3), dried over $MgSO_4$, and concentrated. The residual material was chromatographed on a silica gel column (hexanes/EtOAc, 1:1) to afford 243 mg of **19** as a yellowish solid (54% yield, 0.75 mmol). ^{1}H NMR ($CDCl_3$) δ 1.14 (t, J = 7.2 Hz, 1H), 1.53–1.64 (m, 1H), 1.74–1.85 (m, 1H), 1.99–2.17 (m, 2H), 2.65–2.75 (m, 1H), 2.83 (dd, J = 13.6, 6.2 Hz, 1H), 3.00 (dd, J = 13.6, 8.9 Hz, 1H), 3.99–4.10 (m, 2H), 4.95–5.06 (m, 2H), 5.69–5.82 (m, 1H), 7.27 (d, J = 8.3 Hz, 2H), 8.02 (d, J = 8.2 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 14.3, 31.5, 31.5, 38.6, 46.8, 60.5, 115.8, 127.6, 129.2, 130.5, 137.6, 146.0, 172.0, 175.2. HRMS (ESI): $[M - H]^-$ m/z 275.12885 (calcd 275.12888 for $C_{16}H_{19}O_4$).

Ethyl 4-(2-(tert-Butoxycarbonyl)hex-5-en-1-yl)benzoate (20)—To a solution of **19** (243 mg, 0.88 mmol) in dichloromethane (30 mL) were added isobutylene (10 mL) and H_2SO_4 (49 μL, 0.88 mmol) at −78 °C. The flask was sealed, and the mixture was stirred at rt for 40 h. The mixture was cooled to −78 °C again and poured into a saturated solution of $NaHCO_3$. After warming up to rt, dichloro-methane was removed on a rotavap and the mixture was extracted with EtOAc (80 mL × 3). The combined organic extracts were dried over $MgSO_4$ and concentrated. The residual material was chromatographed on a silica gel column (hexanes/EtOAc, 6:1) to afford 225 mg of **20** as a colorless oil (77% yield, 0.68 mmol). ^{1}H NMR ($CDCl_3$) δ 1.16 (t, J = 7.1 Hz, 3H), 1.51–1.57 (m, 1H), 1.58 (s, 9H), 1.71–1.82 (m, 1H), 1.98–2.15 (m, 2H), 2.62–2.72 (m, 1H), 2.79 (dd, J = 13.5, 6.5 Hz, 1H), 2.97 (dd, J = 13.6, 8.6 Hz, 1H), 3.99–4.13 (m, 2H), 4.93–5.04 (m, 2H), 5.68–5.80 (m, 1H), 7.20 (d, J = 8.3 Hz, 2H), 7.89 (d, J = 8.2 Hz, 1H). ^{13}C NMR ($CDCl_3$): δ 14.3, 28.3, 31.4, 31.5, 38.5, 46.8, 60.5, 81.0, 115.5, 128.9, 129.7, 130.3, 137.7, 144.3, 165.9, 175.2. HRMS (ESI): $[M + Na]^+$ m/z 355.18804 (calcd 355.18798 for $C_{20}H_{28}O_4Na$).

5-(tert-Butoxy)-4-(4-(ethoxycarbonyl)benzyl)-5-oxopentanoic Acid (21)—To a solution of **20** (225 mg, 0.68 mmol) in acetonitrile (15 mL) and water (15 mL) was added RuO_2 hydrate (14 mg, 0.10 mmol) followed by addition of $NaIO_4$ (1.45 g, 6.77 mmol) at 0 °C. The mixture was stirred at 0 °C for 15 min and at rt for 6 h, after which no starting material was seen on TLC. The reaction mixture was then filtered through a pad of Celite and washed with additional acetonitrile. The solvents were then removed on a rotavap, and the residual material was extracted with EtOAc (100 mL × 3). The combined organic extracts were dried over $MgSO_4$ and concentrated. The residual material was chromatographed on a silica gel column (hexanes/EtOAc, 1:1 containing 1% AcOH) to give 132 mg of **21** as a colorless oil (56% yield, 0.38 mmol). ^{1}H NMR ($CDCl_3$) δ 1.16 (t, J = 7.1 Hz, 1H), 1.58 (s, 9H), 1.80–2.00 (m, 2H), 2.30–2.49 (m, 2H), 2.69–2.77 (m, 1H), 2.81 (dd, J = 13.5, 6.6 Hz, 1H), 3.01 (dd, J = 13.5, 8.2 Hz, 1H), 4.01–4.13 (m, 2H), 7.21 (d, J = 8.3 Hz, 2H), 7.90 (d, J = 8.3 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 14.3, 26.7, 28.3, 31.6, 38.4, 46.4, 60.8, 81.1, 128.9, 129.7, 130.5, 143.7, 165.8, 174.5, 178.3. HRMS (ESI): $[M + Na]^+$ m/z 373.16211 (calcd 373.16216 for $C_{19}H_{26}O_6Na$.

Ethyl 4-(5-(((4-Acetoxybenzyl)oxy)amino)-2-(tert-butoxycarbonyl)-5-oxopentyl)benzoate (22)—To a solution of **21** (65 mg, 0.19 mmol) in THF (10 mL) were added triethylamine (31 μL, 0.22 mmol, 1.2 equiv) and ethyl chloroformate (22 μL, 0.22 mmol, 1.2 equiv). After stirring at rt for 1.5 h, a freshly prepared THF solution of **10** (theoretically 50 mg, 0.28 mmol) was added to the mixture. The mixture was stirred at rt for 14 h, quenched with water (20 mL), and extracted with EtOAc (70 mL × 3). The combined organic extracts were dried over $MgSO_4$ and concentrated. The residual material was chromatographed on a silica gel column (hexanes/EtOAc, 1:1) to afford 78 mg of **22** as a colorless oil (82% yield, 0.15 mmol). ^{1}H NMR ($CDCl_3$) δ 1.13 (t, J = 7.1 Hz, 1H), 1.57 (s, 9H), 1.82–1.95 (m, 2H), 2.03–2.19 (m, 2H), 2.30 (s, 3H), 2.64–2.75 (m, 1H), 2.79 (dd, J = 13.6, 6.3 Hz, 1H), 2.97 (dd, J = 13.6, 8.6 Hz, 1H), 4.03 (q, J = 7.1 Hz, 2H), 4.62–4.94 (m, 2H), 7.0d, J = 8.1 Hz, 2H), 7.18 (d, J = 8.1 Hz, 2H), 7.37 (d, J = 8.1 Hz, 2H), 7.88 (d, J = 8.4 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 14.3, 21.2, 27.6, 28.3, 30.9, 38.4, 46.6, 60.7, 77.4, 81.0, 121.9, 128.9, 129.1, 129.7, 130.5, 133.1, 143.7, 151.1, 165.8, 169.5, 170.2, 174.6. HRMS (ESI): $[M + Na]^+$ m/z 536.22475 (calcd 536.22549 for $C_{28}H_{35}O_8NNa$).

5-(((4-Acetoxybenzyl)oxy)amino)-2-(4-(ethoxycarbonyl)benzyl)-5-oxopentanoic Acid (23)—To a solution of **22** (88 mg, 0.15 mmol) in dichloromethane (1 mL) were added i-Pr_3SiH (31 μL, 0.15 mmol) and TFA (0.5 mL) under an argon atmosphere. The mixture was stirred at rt for 1 h. Then volatiles were removed on rotavap, and TFA was removed by coevaporating with toluene (1 mL × 3). The residual material was purified by preparative HPLC to give 19 mg of **23** as a white hygroscopic solid (27%, 0.04 mmol). ^{1}H NMR (DMSO-d_6) δ 1.05 (t, J = 7.1 Hz, 3H), 1.66–1.79 (m, 2H), 1.92–2.05 (m, 2H), 2.27 (s, 3H), 2.62–2.71 (m, 1H), 2.84 (d, J = 7.6 Hz, 2H), 3.97 (q, J = 7.1 Hz, 2H), 4.76 (bs, 2H), 7.12 (d, J = 8.5 Hz, 2H), 7.27 (d, J = 8.3 Hz, 2H), 7.41 (d, J = 8.5 Hz, 2H), 7.84 (d, J = 8.2 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 14.0, 20.9, 27.2, 29.8, 37.4, 45.8, 59.9, 76.1, 121.7, 129.0, 129.1, 129.3, 130.0, 133.6, 144.1, 150.4, 167.3, 168.6, 169.2, 174.0. HRMS (ESI): $[M + Na]^+$ m/z 458.18099 (calcd 458.18094 for $C_{24}H_{28}O_8N$).

5-Ethoxy-4-(4-(ethoxycarbonyl)benzyl)-5-oxopentanoic Acid (24)—To a solution of **18** (1.00 g, 3.29 mmol) in acetonitrile (50 mL) and water (50 mL) was added RuO_2 hydrate (66 mg, 0.49 mmol), followed by addition of $NaIO_4$ (7.04 g, 32.85 mmol) at 0 °C. The mixture was stirred at 0 °C for 10 min and at rt for 18 h. The reaction mixture was then filtered through a pad of Celite and washed with additional acetonitrile. The volatile solvents were then removed on a rotavap, and the mixture was extracted with EtOAc (150 mL × 3). The combined organic extracts were dried over $MgSO_4$ and concentrated. The residual material was chromatographed on a silica gel column (hexanes/EtOAc, 1:1 containing 1% AcOH) to give 579 mg of **24** as a colorless oil (55%, 1.80 mmol). ^{1}H NMR ($CDCl_3$) δ 1.15 (t, J = 7.1 Hz, 3H), 1.38 (t, J = 7.1 Hz, 3H), 1.81–2.02 (m, 2H), 2.30–2.51 (m, 2H), 2.69–2.78 (m, 1H), 2.79–3.05 (m, 2H), 4.06 (qd, J = 7.1, 1.4 Hz, 2H), 4.36 (q, J = 7.1 Hz, 2H), 7.23 (d, J = 8.3 Hz, 2H), 7.95 (d, J = 8.4 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 14.3, 14.5, 26.8, 31.7, 38.5, 46.4, 60.8, 61.0, 129.0, 129.0, 129.9, 144.2, 166.7, 174.5, 178.6. HRMS (ESI): $[M + Na]^+$ m/z 345.13093 (calcd 345.13086 for $C_{17}H_{22}O_6Na$).

Ethyl 4-(5-(((4-Acetoxybenzyl)oxy)amino)-2-(ethoxycarbonyl)-5-oxopentyl)benzoate (25)—To a solution of **24** (100 mg, 0.31 mmol) in THF (5 mL) were added triethylamine (57 μL, 0.40 mmol, 1.3 equiv) and ethyl chloroformate (40 μL, 0.40 mmol, 1.3 equiv). After stirring at rt for 1.5 h, a freshly prepared THF solution of **10** (theoretically 84 mg, 0.46 mmol) was added to the mixture. The mixture was stirred at rt for 14 h, quenched with water (50 mL), and extracted EtOAc (100 mL × 3). The combined organic extracts were dried over $MgSO_4$ and concentrated. The residual material was chromatographed on a silica gel column (hexanes/EtOAc, 1:1) to afford 55 mg, which was further purified by preparative HPLC to give 31 mg of **25** as a colorless oil (21%, 0.06 mmol). ^{1}H NMR ($CDCl_3$) δ 1.12 (t, J = 7.1 Hz, 3H), 1.38 (t, J = 7.1 Hz, 3H), 1.84–1.97 (m, 2H), 2.05–2.19 (m, 2H), 2.30 (s, 3H), 2.64–2.76 (m, 1H), 2.81 (dd, J = 13.6, 6.1 Hz, 1H), 2.99 (dd, J = 13.6, 8.7 Hz, 1H), 4.04 (q, J = 7.2 Hz, 2H), 4.36 (q, J = 7.1 Hz, 2H), 4.71–4.94 (m, 2H), 7.09 (d, J = 8.1 Hz, 2H), 7.21 (d, J = 8.0 Hz, 2H), 7.38 (d, J = 8.1 Hz, 2H), 7.94 (d, J = 8.4 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 14.3, 14.5, 21.3, 30.9, 38.5, 60.8, 61.0, 77.4, 122.0, 128.9, 129.0, 129.9, 130.5, 134.4, 144.2, 151.2, 166.7, 169.6, 174.6. HRMS (ESI): $[M + H]^+$ m/z 486.21229 (calcd 486.21224 for $C_{26}H_{32}O_8N$).

2-(4-((((Isopropoxycarbonyl)oxy)methoxy)carbonyl)benzyl)hex-5-enoic Acid (26)—To a solution of **2** (4.90 g, 19.74 mmol) in DMF (200 mL) was added triethylamine (8.3 mL, 59.21 mmol), and the mixture was stirred at rt for 10 min. Chloromethyl isopropyl carbonate (8.1 mL, 59.2 mmol) was added dropwise at 0 °C over 10 min, and the mixture was stirred at rt for 24 h. Volatiles were removed on a rotavap, and the residual material was chromatographed on a silica gel column (hexanes/acetone, 3:1) to afford 1.59 g of **26** as a yellow oil (22% yield, 4.35 mmol). ^{1}H NMR ($CDCl_3$) δ 1.33 (d, J = 6.3 Hz, 6H), 1.62 (m, 1H), 1.80 (m, 1H), 2.07–2.21 (m, 2H), 2.75 (m, 1H), 2.86 (dd, J = 13.7, 6.6 Hz, 1H), 3.05 (dd, J = 13.7, 8.2 Hz, 1H), 4.94 (sept, J = 6.3 Hz, 1H), 4.97–5.05 (m, 2H), 5.75 (m, 1H), 5.99 (s, 2H), 7.28 (m, 2H), 8.01 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 21.6, 30.8, 31.2, 37.9, 46.1, 73.1, 82.2, 115.7, 127.2, 129.1, 130.3, 137.2, 145.3, 153.4, 164.9, 180.3. HRMS (ESI): $[M - H]^-$ m/z 363.14462 (calcd 363.14493 for $C_{19}H_{23}O_7$).

(Isopropoxycarbonyloxy)methyl 4-(2-((2-(Trimethylsilyl)ethoxy)-carbonyl)hex-5-enyl)benzoate (27)—To a solution of **26** (1.59 g, 4.35 mmol) in dichloromethane (15 mL) were added DCC (1.38 g, 6.55 mmol, 1.5 equiv) and DMAP (54 mg, 0.44 mmol, 0.1 equiv) under an argon atmosphere. After 15 min of stirring, 2-(trimethylsilyl)-ethan-1-ol (718 μL, 4.79 mmol, 1.1 equiv) was added and the mixture was stirred at rt for 16 h. Volatiles were removed on a rotavap, and the residual material was chromatographed on silica gel column (hexanes/EtOAc, 20:1) to afford 1.34 g of **27** as a yellow oil (66% yield, 2.89 mmol). ^{1}H NMR ($CDCl_3$) δ 0.01 (s, 9H), 0.89 (m, 2H), 1.32 (d, J = 6.3 Hz, 6H), 1.57 (m, 1H), 1.79 (m, 1H), 2.02–2.15 (m, 2H), 2.68 (m, 1H), 2.81 (dd, J = 13.7, 6.3 Hz, 1H), 2.99 (dd, J = 13.7, 8.8 Hz, 1H), 4.03–4.13 (m, 2H), 4.94 (sept, J = 6.3, 1H), 4.97–5.04 (m, 2H), 5.75 (m, 1H), 5.99 (s, 2H), 7.25 (m, 2H), 7.99 (m, 2H). ^{13}C NMR ($CDCl_3$) δ –1.6, 17.3, 21.6, 31.3, 31.4, 38.4, 46.7, 62.6, 73.1, 82.1, 115.4, 127.0, 129.1, 130.2, 137.5, 145.8, 153.4, 164.9, 175.1. HRMS (ESI): $[M + Na]^+$ m/z 487.21217 (calcd 487.21225 for $C_{24}H_{36}O_7NaSi$).

4-(4-((((Isopropoxycarbonyl)oxy)methoxy)carbonyl)benzyl)-5-oxo-5-(2-(trimethylsilyl)ethoxy)pentanoic Acid (28)—To a solution of **27** (8.48 g, 23.5 mmol) in acetonitrile (450 mL) and water (450 mL) was added RuO_2 hydrate (350 mg, 2.63 mmol) at once at 0 °C, followed by addition of $NaIO_4$ (50.4 g, 230 mmol) portionwise (over 80 min) at 0 °C. The mixture was stirred at 0 °C for 1 h and at rt for 2 h. The reaction mixture was then filtered through a pad of Celite and washed with additional acetonitrile. The volatile solvents were then removed on a rotavap, and brine (70 mL) was added. The mixture was extracted with EtOAc (120 mL × 3). The combined organic extracts were dried over $MgSO_4$ and concentrated. The residual material was chromatographed on a silica gel column (hexanes/acetone, 7:3) to afford 7.78 g of **28** as a syrup (87% yield, 20.4 mmol). ^{1}H NMR ($CDCl_3$) δ 0.01 (s, 9H), 0.88 (m, 2H), 1.33 (d, J = 6.3 Hz, 6H), 1.87 (m, 1H), 1.96 (m, 1H), 2.34–2.48 (m, 2H), 2.74 (m, 1H), 2.83 (dd, J = 13.7, 6.4 Hz, 1H), 3.03 (dd, J = 13.7, 8.5 Hz, 1H), 4.03–4.15 (m, 2H), 4.94 (sept, J = 6.3, 1H), 5.99 (s, 2H), 7.27 (m, 2H), 8.00 (m, 2H). ^{13}C NMR ($CDCl_3$) δ –1.6, 17.3, 21.6, 26.7, 31.3, 38.4, 46.3, 63.0, 73.0, 82.1, 127.2, 129.1, 130.3, 145.2, 153.4, 164.9, 174.4, 177.3. HRMS (ESI): $[M - H]^-$ m/z 481.18949 (calcd 481.18993 for $C_{23}H_{33}O_9Si$).

((Isopropoxycarbonyl)oxy)methyl 4-(5-(((4-Acetoxybenzyl)oxy)-amino)-5-oxo-2-((2-(trimethylsilyl)ethoxy)carbonyl)pentyl)benzoate (29)—To a solution of **28** (583 mg, 1.21 mmol) in THF (10 mL) were added triethylamine (187 μL, 1.33 mmol) and ethyl chloroformate (131 μL, 1.33 mmol). After stirring at rt for 2 h, a freshly prepared THF solution of **10** (theoretically 241 mg, 1.33 mmol) was added to the mixture. The mixture was stirred at rt for 16 h. Volatiles were removed on rotavap and the residual material was chromatographed on a silica gel column (hexanes/EtOAc, 1:1) to afford 646 mg of **29** as a colorless oil (83%, 1.00 mmol). ^{1}H NMR ($CDCl_3$) δ 0.01 (s, 9H), 0.89 (m, 2H), 1.33 (d, J = 6.3 Hz, 6H), 1.93 (m, 2H), 2.03, 2.13 (2 × bs, 2H), 2.70 (m, 1H), 2.82 (dd, J = 13.7, 6.1 Hz, 1H), 3.00 (dd, J = 13.7, 8.9 Hz, 1H), 4.07 (m, 2H), 4.79, 4.88 (2 × bs, 2H), 4.94 (sept, J = 6.3, 1H), 5.99 (s, 2H), 7.09 (m, 2H), 7.25 (m, 2H), 7.40 (m, 2H), 7.99 (m, 2H), 8.37 (bs, 1H). ^{13}C NMR ($CDCl_3$) δ –1.6, 17.3, 21.1, 21.6, 27.5, 30.7, 38.4, 46.5, 63.0, 73.0, 77.4,

82.1, 121.8, 127.1, 129.1, 130.2, 130.4, 133.0, 145.2, 150.9, 153.4, 164.8, 169.5, 170.0, 174.6. HRMS (ESI): $[M - H]^-$ m/z 644.25244 (calcd 644.25326 for $C_{32}H_{42}O_{11}NSi$).

5-(((4-Acetoxybenzyl)oxy)amino)-2-(4-((((isopropoxycarbonyl)-oxy)methoxy)carbonyl)benzyl)-5-oxopentanoic Acid (30)—To a solution of **29** (646 mg, 1.00 mmol) in dichloromethane (5 mL) was added TFA (1.5 mL) at 0 °C under an argon atmosphere. The mixture was stirred at 0 °C for 15 min and at rt for 4 h. Volatiles were removed on a rotavap, and the residual TFA was removed by coevaporating with toluene. The residual material was purified on HPLC and lyophilized to give 267 mg of **30** as a white fluffy solid (49% yield, 0.49 mmol). ^{1}H NMR ($CDCl_3$) δ 1.31 (d, J = 6.3 Hz, 6H), 1.78–1.92 (m, 2H), 2.00–2.14 (m, 2H), 2.31 (s, 3H), 2.69 (m, 1H), 2.77 (m, 1H), 3.02 (dd, J = 13.8, 8.2 Hz, 1H), 4.73, 4.80 (2 × bs, 2H), 4.93 (m, 1H), 5.97 (s, 2H), 7.04 (m, 2H), 7.25 (m, 2H), 7.36 (m, 2H), 7.98 (m, 2H), 8.86 (bs, 1H). ^{13}C NMR ($CDCl_3$) δ 21.1, 21.6, 27.3, 30.4, 38.1, 46.0, 73.1, 77.9, 82.1, 121.7, 127.2, 129.1, 130.5, 130.9, 133.0, 145.0, 150.8, 153.4, 164.9, 170.0, 170.2, 178.3. HRMS (ESI): $[M - H]^-$ m/z 544.18144 (calcd 544.18243 for $C_{27}H_{30}O_{11}N$).

IC_{50} Determination of Prodrugs

Inhibition constants of prodrugs were determined using the radioenzymatic assay with ^{3}H-NAAG (radiolabeled at the terminal glutamate) as we have previously described.[14] Briefly, rhGCPII (30 ng/mL) was preincubated in the presence of inhibitors at varying concentrations, in 20 mM Tris, 150 mM NaCl, pH 8.0, for 15 min at 37 °C in the total volume of 80 μL. The reaction was initiated by the addition of 40 μL of the mixture of 0.31 μM NAAG (Sigma) and 15 nM ^{3}H-NAAG (50 Ci/mmol in Tris buffer, PerkinElmer) to the total reaction volume of 120 μL. Following 20 min incubation, the reaction was terminated by addition of 120 μL of potassium phosphate (200 mM), EDTA (50 mM), and β-mercaptoethanol (2mM), pH 7.4. The released glutamate was separated by ion-exchange chromatography and quantified by liquid scintillation counting. GraphPad Prism software (GraphPad Software, San Diego, CA, USA) was used for data fitting, and IC_{50} values were calculated from the inhibition curves where applicable.

In Vitro Plasma Metabolite Identification Studies

Plasma stability studies were conducted in mouse plasma as previously described.[12] Compound **1** and prodrugs (**12**, **23** and **30**) (10 μM) were spiked in plasma and incubated in an orbital shaker at 37 °C. At zero and 60 min post incubation, 100 μL aliquots of the mixture in triplicate were removed and the reaction quenched by addition of two times the volume of ice-cold acetonitrile spiked with the internal standard (losartan 5 μM). The samples were vortexed for 30 s and centrifuged at 12000g for 10 min. Supernatant (50 μL) was diluted with 50 μL of water and transferred to a 250 μL of polypropylene vial sealed with a Teflon cap. Prodrug disappearence and metabolite identification were analyzed over time using an Agilent 1260 HPLC coupled to a ESI-TOF Agilent 6530 with Agilent Jet Stream Technology (Agilent Technologies, Santa Clara, CA). Samples were separated on a Waters 1.7 μm, C18, 100 mm × 2.1 mm column at a flow rate 0.3 mL/min. The concentration of mobile phase B (0.1% FA in acetonitrile) was gradually increased from 10 to 100% in mobile phase A (0.1% FA in water) over 7 min. The mass spectrometry

instrument was operated in a negative or positive (for JV-2946) ion mode with a voltage of +3.00 kV applied to the capillary. The temperature, the flow rate of the nitrogen drying gas, the pressure of the nitrogen nebulizing gas, the temperature, and the flow rate of the sheath gas were set at 325 °C, 10 L/min, 40 psi, 390 °C, and 11/min, respectively.

Plasma Pharmacokinetic Studies in Mice

All pharmacokinetic studies in mice were conducted according to protocols approved by the Animal Care and Use Committee at Johns Hopkins University (Baltimore, MD). Briefly, male CD 1 mice between 25 and 30 g were obtained from Harlan Laboratories (Indianapolis, Indiana) and maintained on a 12 h light–dark cycle with ad libitum access to food and water. Compound **1** and its prodrugs were administered to male mice as a single (po) dose of 10 mg/kg equivalent. Dosing solutions were prepared on the day of the experiment in 50 mM HEPES buffered saline and pH adjusted to 7.4. The mice were sacrificed by pentobarbital injection at 30 min post drug administration, and blood collected via cardiac puncture was placed into iced heparin coated BD microtainers. Blood samples were spun at 200*g* for 15 min, and plasma was removed and stored at −80 °C until LC-MS analysis as previously described.[10] Briefly, chromatographic analysis was performed using an Accela ultra high-performance system consisting of an analytical pump an autosampler coupled with TSQ Vantage mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA). Separation of the analyte was achieved using Agilent Eclipse Plus column (100 mm × 2.1 mm i.d.) packed with a 1.8 μm C18 stationary phase. The mobile phase used was composed of 0.1% formic acid in acetonitrile and 0.1% formic acid in H_2O with gradient elution, starting with 5% (organic) and linearly increasing to 99% up to 2.5 min, maintaining at 99% (2.5–3.5 min), and reequlibrating to5% by 4.5 min. The total run time for each analyte was 6 min. The mass transitions $(M - H)^-$ used for (**1**) were 279.986 > 203.066, 247.173, and for losartan were 421.131 > 156.996, 179.112.

Chronic Constrictive Injury Model of Neuropathic Pain

All experimental protocols were approved by the Institutional Animal Care and Use Committee at Johns Hopkins University (Baltimore, MD) and adhered to all of the applicable institutional and governmental guidelines for the humane treatment of laboratory animals. Male Sprague–Dawley rats (200–250 g; n = 10–15 per group) were subjected to peripheral neuropathy injury by constriction of the sciatic nerve performed according to methods described previously.[10,16,21] In brief, the common sciatic nerve was exposed and four ligatures (4.0 chromic gut) tied loosely around it with 1 mm spacing. Wound clips were used to close wounds, and animals were returned to home cages for recovery. Hyperalgesia testing was initiated 10 days postsurgery. Pain sensitivity was assessed by determining withdrawal latencies in response to a constant thermal stimulus directed to the plantar surface of the hind paw using a Basile Plantar apparatus (Ugo Basile, Vaarese, Italy) according to the method described by Hargreaves et al.[22] Apparatus was calibrated so that normal rats respond to the stimulus within 15 s of application. Withdrawal latency (i.e., the time taken for the rat to withdraw its paw from the heat source) was measured to the nearest 0.1 s. The “difference in score” was calculated by subtracting the average latency of the paw on the nonligated versus ligated side. Each animal was tested during two experimental sessions per week, with 3 days allowed to elapse between test sessions. Test agents, prodrug

12 (3 mg/kg equivalent of **1**), or gabapentin (50 mg/kg and 100 mg/kg po) as a positive control were dosed daily throughout the study via oral gavage and 1 h before testing on experimental days. Statistical analysis with students *t*-test was used to determine differences between control and treatment group.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by NIH grant RO1CA161056 (to B.S.S.) and the Institute of Organic Chemistry and Biochemistry of the Academy of Sciences of the Czech Republic, vvi (RVO 61388963), and in part supported by the CAS (RVO: 86652036), MEYS CR (LM2015043 CIISB), and project BIOCEV (CZ.1.05/1.1.00/02.0109) from the ERDF to C.B.

ABBREVIATIONS

2-PMPA	2-phosphonomethyl pentanedioic acid
CCI	chronic constriction injury
ESI-TOF	electrospray ionization-time-of-flight mass spectrometer
GCPII	glutamate carboxypeptidase II
IC_{50}	half-maximal inhibitory concentration
NAAG	*N*-acetylaspartylglutamate
ODOL	2-oxo-1,3-dioxol-4-yl methyl
po	per oral
PAB	*O*-4-acetoxy benzyl
POC	isopropyloxycarbonyloxymethyl
rhGCPII	recombinant human GCPII

References

1. Slusher, BS., Rojas, C., Coyle, JT. Glutamate Carboxypeptidase II A2. In: Rawlings, ND., Salvesen, G., editors. Handbook of Proteolytic Enzymes. Academic Press; New York: 2013. p. 1620-1627.
2. Vornov JJ, Hollinger KR, Jackson PF, Wozniak KM, Farah MH, Majer P, Rais R, Slusher BS. Still NAAG′ing after all these years: The continuing pursuit of GCPII inhibitors. Adv Pharmacol. 2016; 76:215–255. [PubMed: 27288079]
3. Barinka C, Rojas C, Slusher B, Pomper M. Glutamate carboxypeptidase II in diagnosis treatment of neurologic disorders prostate cancer. Curr Med Chem. 2012; 19:856–870. [PubMed: 22214450]
4. Yamamoto T, Nozaki-Taguchi N, Sakashita Y, Inagaki T. Inhibition of spinal N-acetylated-alpha-linked acidic dipeptidase produces an antinociceptive effect in the rat formalin test. Neuroscience. 2001; 102:473–479. [PubMed: 11166133]

5. Yamamoto T, Nozaki-Taguchi N, Sakashita Y. Spinal N-acetyl-alpha-linked acidic dipeptidase (NAALADase) inhibition attenuates mechanical allodynia induced by paw carrageenan injection in the rat. Brain Res. 2001; 909:138–144. [PubMed: 11478930]
6. Jackson PF, Cole DC, Slusher BS, Stetz SL, Ross LE, Donzanti BA, Trainor DA. Design synthesis and biological activity of a potent inhibitor of the neuropeptidase N-acetylated alpha-linked acidic dipeptidase. J Med Chem. 1996; 39:619–622. [PubMed: 8558536]
7. Jackson PF, Tays KL, Maclin KM, Ko YS, Li W, Vitharana D, Tsukamoto T, Stoermer D, Lu XC, Wozniak K, Slusher BS. Design pharmacological activity of phosphinic acid based NAALADase inhibitors. J Med Chem. 2001; 44:4170–4175. [PubMed: 11708918]
8. Kozikowski AP, Nan F, Conti P, Zhang J, Ramadan E, Bzdega T, Wroblewska B, Neale JH, Pshenichkin S, Wroblewski JT. Design of remarkably simple yet potent urea-based inhibitors of glutamate carboxypeptidase II (NAALADase). J Med Chem. 2001; 44:298–301. [PubMed: 11462970]
9. Majer P, Jackson PF, Delahanty G, Grella BS, Ko YS, Li W, Liu Q, Maclin KM, Polakova J, Shaffer KA, Stoermer D, Vitharana D, Wang EY, Zakrzewski A, Rojas C, Slusher BS, Wozniak KM, Burak E, Limsakun T, Tsukamoto T. Synthesis biological evaluation of thiol-based inhibitors of glutamate carboxypeptidase II: discovery of an orally active GCP II inhibitor. J Med Chem. 2003; 46:1989–1996. [PubMed: 12723961]
10. Novakova Z, Wozniak K, Jancarik A, Rais R, Wu Y, Pavlicek J, Ferraris D, Havlinova B, Ptacek J, Vavra J, Hin N, Rojas C, Majer P, Slusher BS, Tsukamoto T, Barinka C. Unprecedented binding mode of hydroxamate-based inhibitors of Glutamate Carboxypeptidase II: Structural characterization biological activity. J Med Chem. 2016; 59:4539–4550. [PubMed: 27074627]
11. Vornov JJ, Wozniak KM, Wu Y, Rojas C, Rais R, Slusher BS. Pharmacokinetics pharmacodynamics of the glutamate carboxypeptidase II inhibitor 2-MPPA show prolonged alleviation of neuropathic pain through an indirect mechanism. J Pharmacol Exp Ther. 2013; 346:406–413. [PubMed: 23776202]
12. Majer P, Jancarik A, Krecmerova M, Tichy T, Tenora L, Wozniak K, Wu Y, Pommier E, Ferraris D, Rais R, Slusher BS. Discovery of orally available prodrugs of the Glutamate Carboxypeptidase II (GCPII) inhibitor 2-Phosphonomethylpentanedioic Acid (2-PMPA). J Med Chem. 2016; 59:2810–2819. [PubMed: 26930119]
13. Majer P, Jackson PF, Delahanty G, Grella BS, Ko Y-S, Li W, Liu Q, Maclin KM, Poláková J, Shaffer KA, Stoermer D, Vitharana D, Wang EY, Zakrzewski A, Rojas C, Slusher BS, Wozniak KM, Burak E, Limsakun T, Tsukamoto T. Synthesis biological evaluation of thiol-based inhibitors of Glutamate Carboxypeptidase II: Discovery of an orally active GCP II inhibitor. J Med Chem. 2003; 46:1989–1996. [PubMed: 12723961]
14. Novakova Z, Wozniak K, Jancarik A, Rais R, Wu Y, Pavlicek J, Ferraris D, Havlinova B, Ptacek J, Vavra J, Hin N, Rojas C, Majer P, Slusher BS, Tsukamoto T, Barinka C. Unprecedented binding mode of hydroxamate-based inhibitors of Glutamate Carboxypeptidase II: Structural characterization biological activity. J Med Chem. 2016; 59:4539–4550. [PubMed: 27074627]
15. Wiemer AJ, Wiemer DF. Prodrugs of phosphonates phosphates: crossing the membrane barrier. Top Curr Chem. 2014; 360:115–160.
16. Bennett GJ, Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain. 1988; 33:87–107. [PubMed: 2837713]
17. Schlimme S, Hauser AT, Carafa V, Heinke R, Kannan S, Stolfa DA, Cellamare S, Carotti A, Altucci L, Jung M, Sippl W. Carbamate prodrug concept for hydroxamate HDAC inhibitors. ChemMedChem. 2011; 6:1193–1198. [PubMed: 21416613]
18. Silhar P, Eubanks LM, Seki H, Pellett S, Javor S, Tepp WH, Johnson EA, Janda KD. Targeting botulinum A cellular toxicity: a prodrug approach. J Med Chem. 2013; 56:7870–7879. [PubMed: 24127873]
19. Raji I, Ahluwalia K, Oyelere AK. Design synthesis and evaluation of antiproliferative activity of melanoma-targeted histone deacetylase inhibitors. Bioorg Med Chem Lett. 2017; 27:744–749. [PubMed: 28131715]
20. Flipo M, Charton J, Hocine A, Dassonneville S, Deprez B, Deprez-Poulain R. Hydroxamates: relationships between structure plasma stability. J Med Chem. 2009; 52:6790–802. [PubMed: 19821586]

21. Attal N, Jazat F, Kayser V, Guilbaud G. Further evidence for ‘pain-related’ behaviours in a model of unilateral peripheral mononeuropathy. Pain. 1990; 41:235–251. [PubMed: 2164179]

22. Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain. 1988; 32:77–88. [PubMed: 3340425]

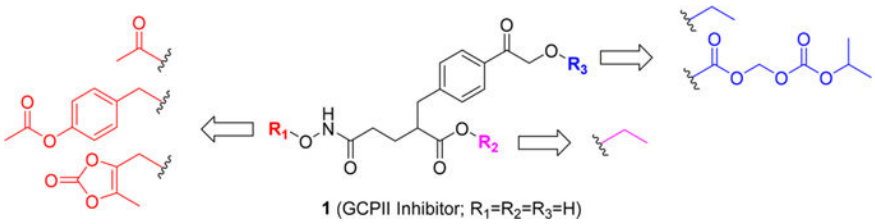


Figure 1.
Prodrug approach for hydroxamate-based GCPII inhibitor **1**.

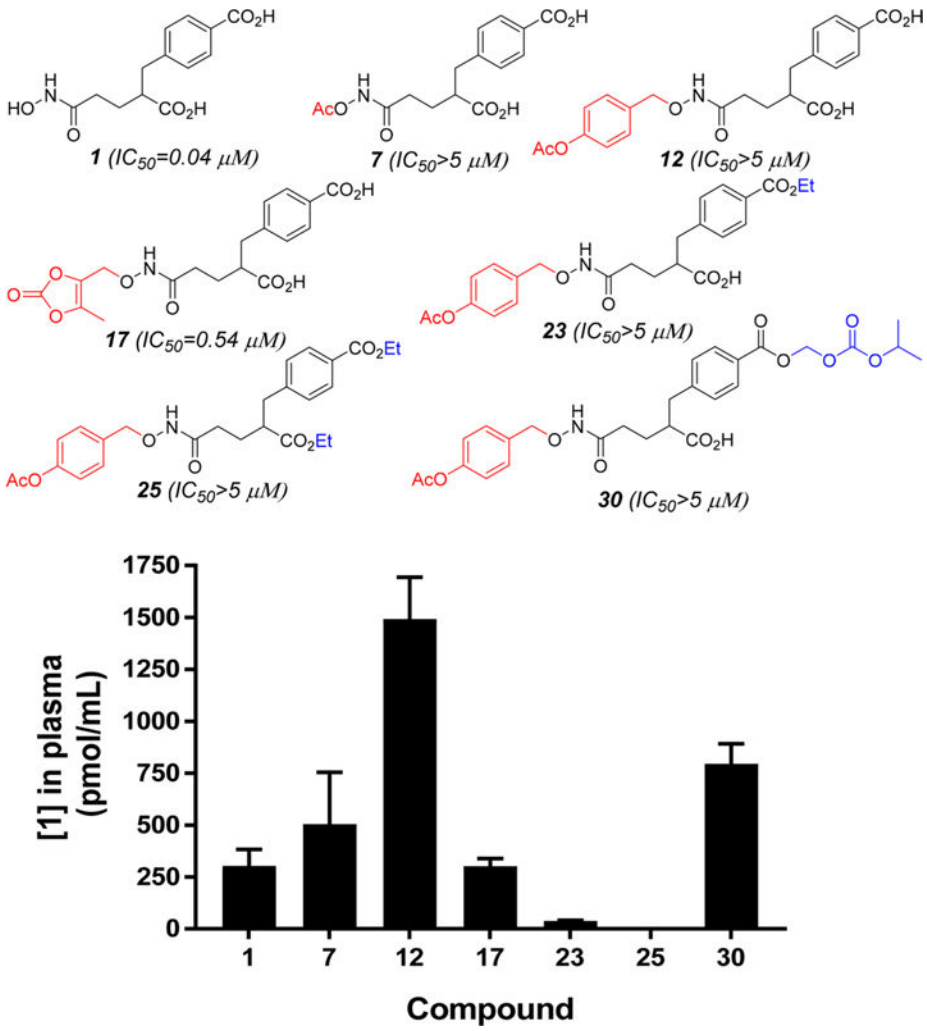


Figure 2.

Structures, IC_{50}, and plasma concentrations of compound **1** following oral administration of prodrugs in mice. All prodrug except **17** were >10-fold less potent versus **1** in inhibiting GCP II activity. Six prodrugs, **7**, **12**, **17**, **23**, **25**, and **30**, given po to mice at a dose equivalent of 10 mg/kg of compound **1**, and plasma levels of **1** were measured 30 min after administration. Compound **12** showed the highest improvement, delivering concentrations of **1** at greater than 5-fold in plasma vs **1**. Data expressed as mean ± SEM, (n = 3).

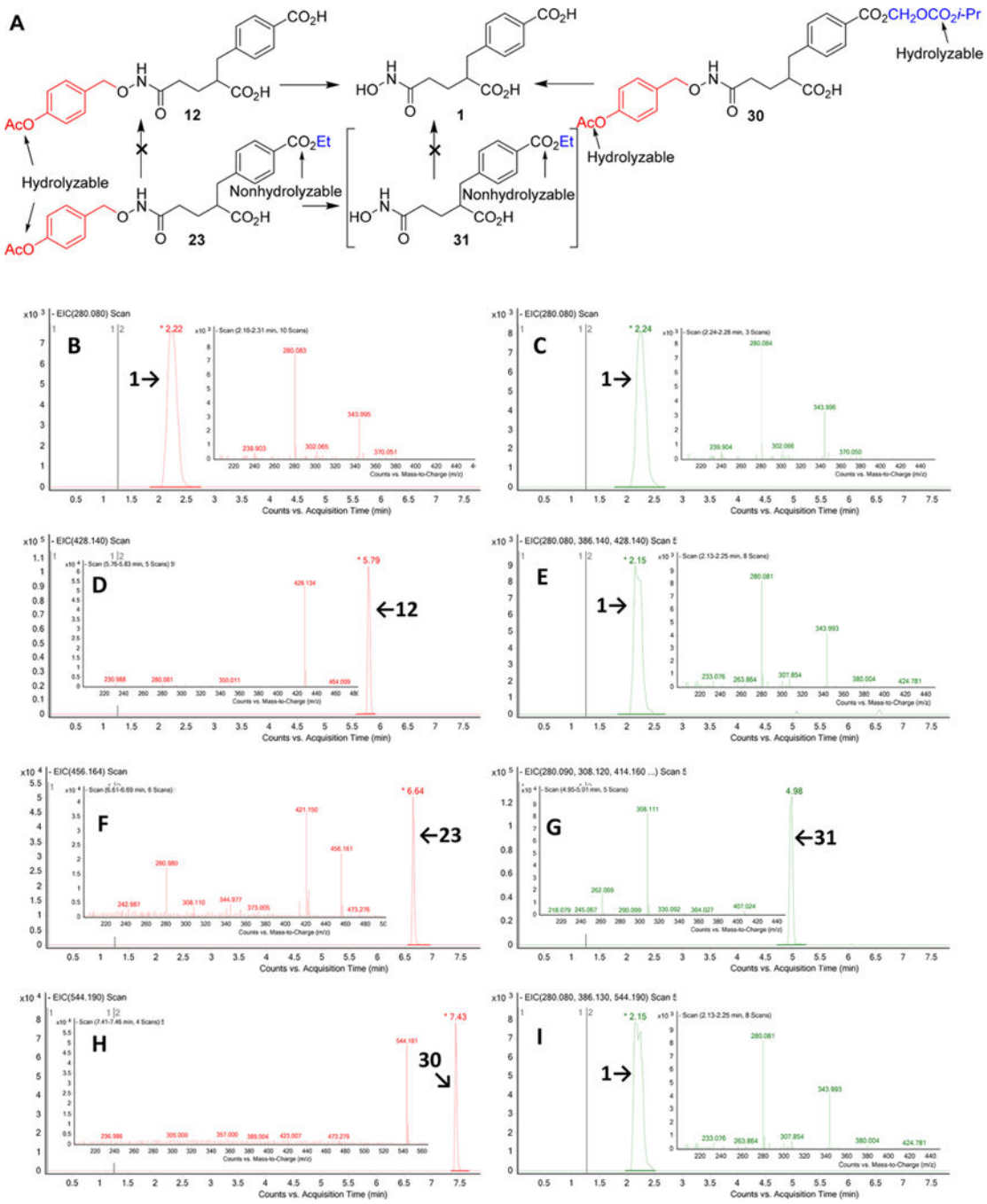


Figure 3.

Metabolism of prodrugs **12**, **23**, and **30** in mouse plasma. (A) Putative metabolic pathways for **12**, **23**, and **30** in plasma. Mass extracted chromatograms of **1** and its prodrugs **12**, **23**, and **30** in mouse plasma at 0 min (B, D, F, and H) and after 60 min incubation (C, E, G, and H).

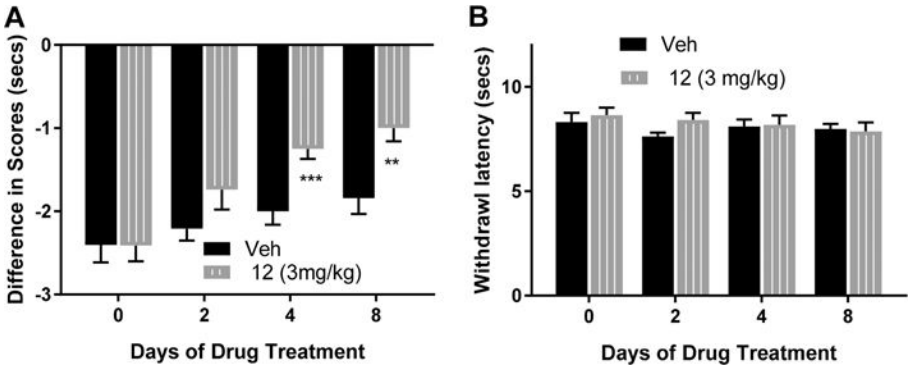
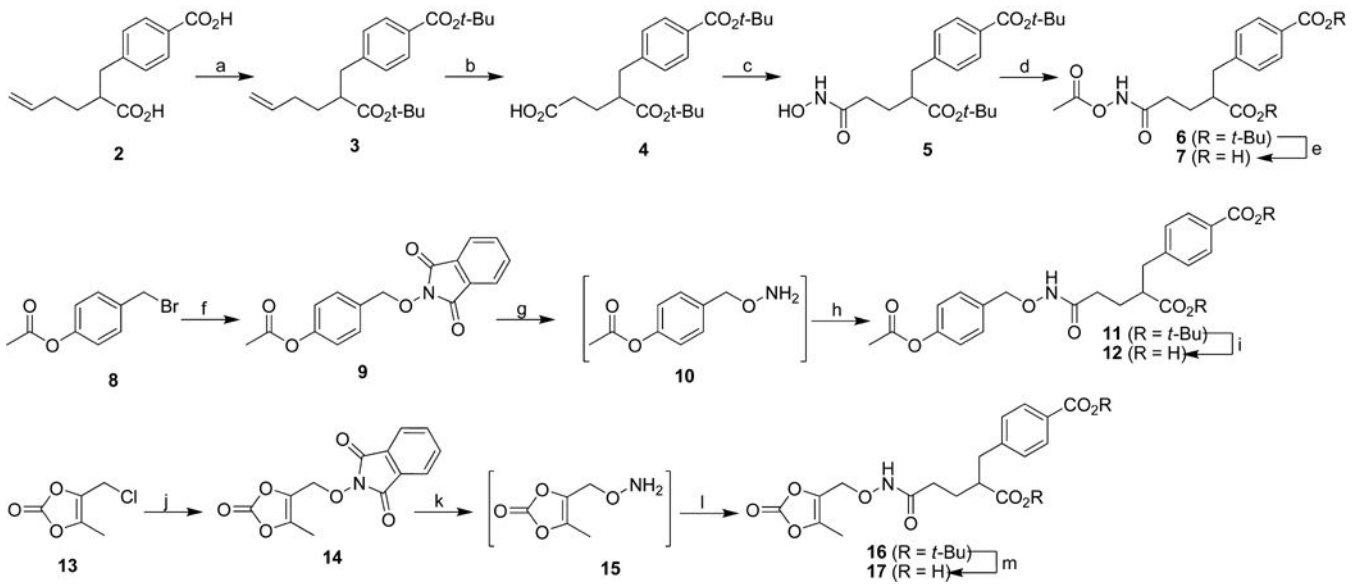


Figure 4.

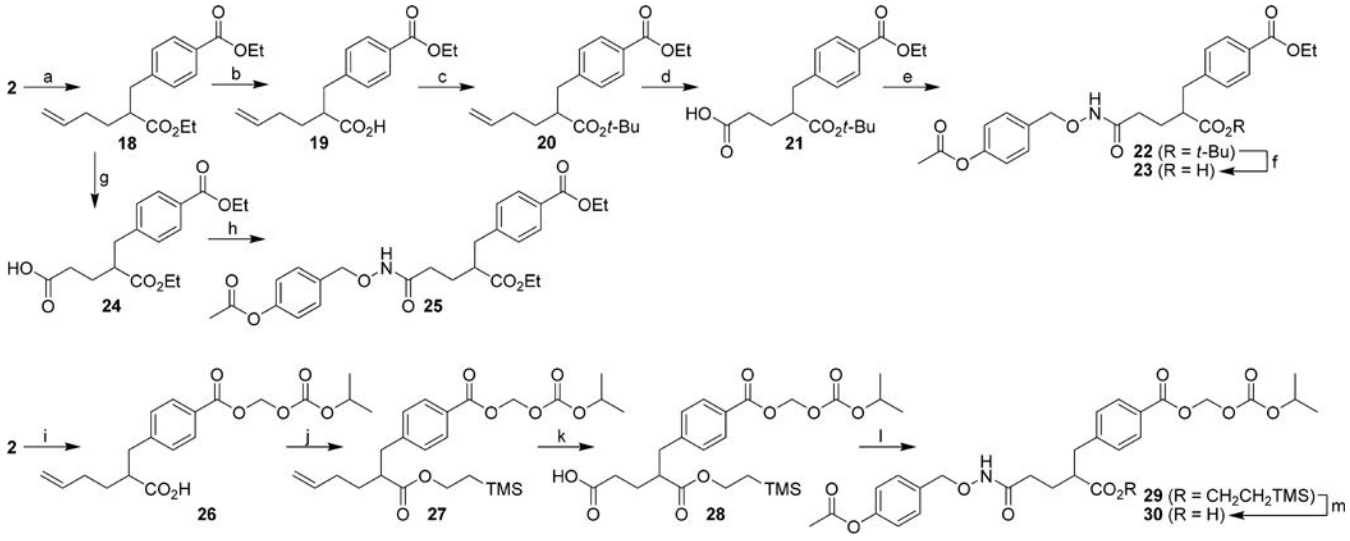
Antinociceptive effects of prodrug **12** in the rat chronic constriction injury (CCI) model of neuropathic pain. (A) Oral administration of **12** at 4.6 mg/kg/day (equivalent of 3 mg/kg/day of compound **1**) significantly attenuated CCI-induced hyperalgesic state on the ligated side relative to the vehicle-treated control (** denotes $p < 0.01$, *** denotes $p < 0.001$). (B) Compound **12** did not affect the response latency to the thermal stimulus applied to the sham-operated side.



Scheme 1.

Synthesis of 7, 12, and 17[a]

[a]Reagents and conditions: (a) isobutylene, H_2SO_4, CH_2Cl_2, rt; (b) $RuO_2{\cdot}H_2O$, $NaIO_4$, $CH_3CN–H_2O$, rt; (c) triethylamine, ethyl chloroformate, NH_2OH, THF–methanol, rt; (d) Ac_2O, pyridine, 0 °C; (e) TFA, *i*-Pr_3SiH, CH_2Cl_2, rt; (f) *N*-hydroxyphthalimide, NaH, DMF, rt; (g) N_2H_4, THF, rt; (h) **4**, EDC·HCl, DMAP, DIEA, DMF, rt; (i) TFA, *i*-Pr_3SiH, CH_2Cl_2, rt; (j) *N*-hydroxyphthalimide, NaH, DMF, 60 °C; (k) N_2H_4, THF, rt (l) **4**, triethylamine, ethyl chloroformate, THF, rt; (m) TFA, *i*-Pr_3SiH, CH_2Cl_2, rt.



Scheme 2.

Synthesis of 23, 25, and 30[a]

[a]Reagents and conditions: (a) $SOCl_2$, EtOH, rt; (b) NaOH, EtOH–H_2O; rt; (c) isobutylene, H_2SO_4, CH_2Cl_2, rt; (d) $RuO_2{\cdot}H_2O$, $NaIO_4$, CH_3CN–H_2O, rt; (e) triethylamine, ethyl chloroformate, **10**, THF, rt; (f) TFA, *i*-Pr_3SiH, CH_2Cl_2, rt; (g) $RuO_2{\cdot}H_2O$, $NaIO_4$, CH_3CN-H_2O, rt; (h) triethylamine, ethyl chloroformate, **10**, THF, rt; (i) triethylamine, chloromethyl isopropyl carbonate, DMF, rt; (j) 2-(trimethylsilyl)ethan-1-ol, DCC, DMAP, CH_2Cl_2, rt; (k) $RuO_2{\cdot}H_2O$, $NaIO_4$, CH_3CN–H_2O, rt; (l) triethylamine, ethyl chloroformate, **10**, THF, rt; (m) TFA, CH_2Cl_2, rt.