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Article

Dipeptides as Effective Prodrugs of the Unnatural Amino Acid (+)-2-Aminobicyclo[3.1.0]hexane-2,6-dicarboxylic Acid (LY354740), a Selective Group II Metabotropic Glutamate Receptor Agonist

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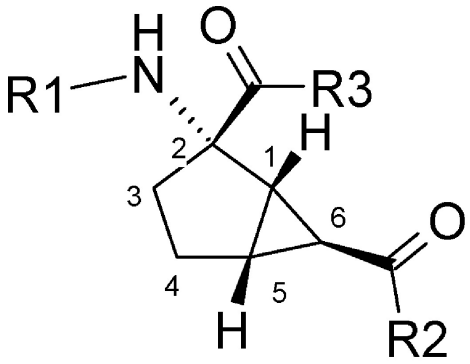

R1=H, R2=R3=OH	LY354740
R1= aa, R2=R3=OH	N-peptides
R1=H, R2=aa, R3=OH	C-6 peptides
R1=H, R2=OH, R3=aa	C-2 peptides
R1=R3=aa, R2=OH	N,C-2 peptides

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Dipeptides as Effective Prodrugs of the Unnatural Amino Acid (+)-2-Aminobicyclo[3.1.0]hexane-2,6-dicarboxylic Acid (LY354740), a Selective Group II Metabotropic Glutamate Receptor Agonist

Ana Belén Bueno,*,† Iván Collado,† Alfonso de Dios,† Carmen Domínguez,† José Alfredo Martín,† Luisa M. Martín,† María Angeles Martínez-Grau,† Carlos Montero,† Concepción Pedregal,† John Catlow,‡ D. Scott Coffey,‡ Michael P. Clay,‡ Anne H. Dantzig,‡ Terry Lindstrom,‡ James A. Monn,‡ Haiyan Jiang,‡ Darryle D. Schoepp,‡ Robert E. Stratford,‡ Linda B. Tabas,‡ Joseph P. Tizzano,‡,§ Rebecca A. Wright,‡ and Marc F. Herin#

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(+)-2-Aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid (**1**), also known as LY354740, is a highly potent and selective agonist for group II metabotropic glutamate receptors (mGlu receptors 2 and 3) tested in clinical trials. It has been shown to block anxiety in the fear-potentiated startle model. Its relatively low bioavailability in different animal species drove the need for an effective prodrug form that would produce a therapeutic response at lower doses for the treatment of anxiety disorders. We have investigated the increase of intestinal absorption of this compound by targeting the human peptide transporter hPepT1 for active transport of di- and tripeptides derived from **1**. We have found that oral administration of an N dipeptide derivative of **1** (**12a**) in rats shows up to an 8-fold increase in drug absorption and a 300-fold increase in potency in the fear-potentiated startle model in rats when compared with the parent drug **1**.

Introduction

The treatment of neurological or psychiatric disorders, such as anxiety, has been linked to selective activation of metabotropic excitatory amino acid receptors. Glutamate receptors are classified into ionotropic glutamate receptors (iGlu receptors),[1] which are ligand-gated ion channels, and metabotropic glutamate receptors (mGlu receptors),[2] which are G-protein-coupled receptors involved in the production of second messengers. There are currently eight different subtypes of mGlu receptors that have been cloned from human and rat species, which are designated mGlu1−mGlu8. These have been subdivided into three subgroups (I, II, and III) based on higher structural homology and shared pharmacology within each mGlu receptor group. Group II mGlu receptors include mGlu2 and mGlu3 receptors, which are negatively coupled to cyclic adenosine monophosphate (cAMP) formation.

(+)-2-Aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid (**1**), also known as LY354740,[3] is a potent group II mGlu receptor agonist tested in clinical trials. It has been shown to suppress enhanced glutamatergic excitations in brain synapses (e.g., hippocampus, prefrontal cortex, amygdala, locus coeruleus) involved in anxiety/stress disorders. Oral or parenteral administration of **1** also demonstrated anxiolytic activity in the fear-potentiated startle model in rats.[4] More recently, systemic administration of **1** was shown to prevent lactate-induced panic-like responses in panic-prone rats.[5]

In vivo studies in rats and dogs[6] show that **1** is a safe drug that is not metabolized by either species but excreted unchanged in the urine. Brain penetration following systemic administration[7] has been reported. Pharmacokinetic studies demonstrate that the oral bioavailability is relatively low in rats (10%) and moderate in dogs (40%) apparently because of low transfer across the intestinal epithelial membrane. This fact made it advisable to search for a prodrug that would increase the absorption of this amino acid in the intestinal tract.

Most orally administered drugs rely on passive diffusion to cross cell membranes. However, developments in biotechnology and peptide synthesis as well as new screening strategies in the past years have led to the exploitation of the unique pharmacological activities of peptides and peptidomimetics as potential drugs. These developments have spurred great interest in the human intestinal peptide transporter, hPepT1, as being appealing for oral drug delivery.[8] Although intestinal peptide transport is not fully understood, it is known that, unlike the structurally restrictive amino acid mediated transport systems,[9] the peptide transport carrier hPepT1 has broad substrate specificity and recognizes not only di- or tripeptides[10,11] but also peptidomimetics such as β-lactam antibiotics,[12] cephalosporins,[13] angiotensin-converting enzyme (ACE) inhibitors,[14] renin inhibitors[15] and even compounds without an obvious peptide bond or equivalent, such as δ-aminolevulinic acid[16] and ω-amino fatty acids.[17] The peptide carrier has also been targeted for increasing the availability of drugs with low permeability by designing

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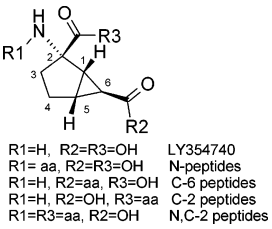


Figure 1.

peptide or peptidomimetic prodrugs that are transported through the intestine by the hPepT1 and then hydrolyzed prior to or after the delivery of the drug to the systemic circulation. This strategy has been used to increase the availability of L-acyclovir and azidothymidine (AZT) by making 5′-amino acid ester prodrugs[18] and the availability of L-methyldopa by making L-methyldopa-L-Phe[19] and D-phenylglycine-L-methyldopa.[20] Interestingly, the studies made on L-methyldopa revealed that both approaches, N dipeptides and C dipeptides of this amino acid, succeeded in increasing the uptake of the drug. In addition to the increased bioavailability compared to the parent drug, this approach is also attractive because of the low potential for side effects following release of the drug, because these promoieties would be natural amino acids.

The goal of this work was to increase the oral bioavailability of **1** by making oligopeptides that would be substrates for the intestinal peptide transporter hPepT1. Since there are three points for attachment of the amino acids to **1**, we have synthesized C-2, C-6, and N peptides with a variety of amino acids and dipeptides as potential prodrugs of **1** (see Figure 1).

Chemistry

The starting material for the synthesis of all the prodrugs was the parent drug **1**, which was synthesized as described in the literature.[3] Regio- and chemoselective protections of the parent provided four key intermediates (**2**−**5**) in which only one functionality remains unprotected (Scheme 1). Coupling of these intermediates with the conveniently protected amino acids or dipeptides followed by full deprotection provided all the prodrugs.

Synthesis of C-2 Peptides through Intermediates 2 and 3. We developed two different routes to access to C-2 peptides. Both were based on the protection of the free amine and the acid on C-6 prior to the coupling with amino acids. First, the free amine of **1** was protected as an allyl carbamate by standard methods. The resulting diacid was regioselectively[21] esterified with 1 equiv of allyl alcohol in the presence of EDCI, providing pure compound **2** after column chromatography (Scheme 2).

Alternatively, **1** was reacted with HCl in MeOH[22] to provide the C-6 methylated derivative as the only regioisomer. Protection of the free amine with Boc_2O led to **3** in 75% yield (Scheme 2).

The free acid on intermediates **2** and **3** was coupled with acid-protected amino acids to provide fully protected C-2 dipeptides **6** and **7**. When commercially available, the amino acids were selected as allyl esters for reacting with **2** or as methyl esters for reacting with **3** so that both esters in **6** and **7** (R_2 and R_3; see Scheme 2) could be hydrolyzed in a single step. Full deprotection of the amino acids, using different reaction conditions depending on the nature of the protecting groups (see Experimental Section), provided the free peptides or the HCl salts. The C-2 dipeptides synthesized using either route, along with the amino acids utilized, and the yields in each step are collected in Table 1.

Synthesis of C-6 Peptides through Intermediate 4. The *N*-Alloc of **1** in the presence of paraformaldehyde under acid catalysis[23] provided intermediate **4**, where the α-amino acid functionality has been protected as an oxazolidinone. Coupling of the free acid on C-6 under standard conditions with the L-phenylalanine allyl ester provided **9**. Reaction of this compound with Pd(0) in the presence of dimethylbarbituric acid (DMBA) removed the two allyl groups and the oxazolidinone in a single step. The zwitterion thus formed was transformed into the hydrochloride (**10**·HCl) with HCl in EtOAc. Compound **10** was the only C-6 peptide synthesized for this study (Scheme 3).

Synthesis of N Peptides through Intermediate 5. The reaction of **1** with thionyl chloride in methanol[24] led to the dimethyl ester **5** in quantitative yield. Coupling of **5** with *N*-Boc protected amino acids or dipeptides provided fully protected di- and tripeptides **11a**−**o** that, after basic treatment to remove the methyl esters followed by acid deprotection of the *N*-Boc, yielded the final peptides **12a**−**o** as hydrochlorides (Scheme 4 and Table 1).

Synthesis of N,C-2 Peptides through Intermediate 3. Compound **3** was coupled with L-Leu-OMe following standard conditions to provide the protected dipeptide **6k**. Treatment of the dipeptide with HCl to remove the Boc protecting group followed by the coupling of the free amine with Boc-L-Ala afforded the fully protected tripeptide **13**. Hydrolysis of the esters with LiOH and removal of the Boc group with HCl gave rise to the hydrochloride **14**·HCl (Scheme 5).

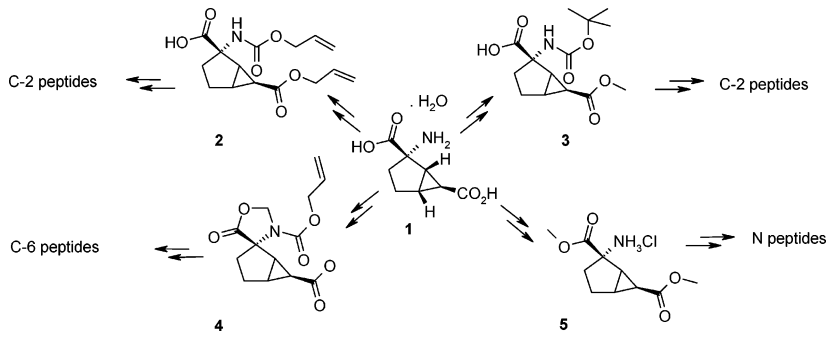
All the peptides synthesized (collected in Table 1 and Schemes 3 and 5) are white solids as the free zwitterion or the HCl salt.

In Vivo Exposure

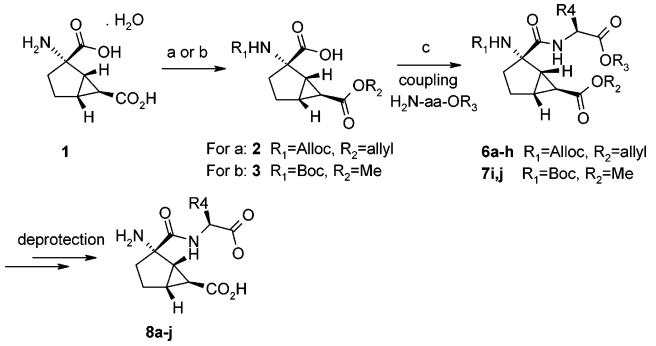
Rat Screen. Studies measuring the plasma concentrations of the parent in rats were performed by collecting blood samples 0.5, 1, and 3 h after oral administration of a single dose of the peptide. This assay provided a rank order of the relative efficacy of the different peptides in delivering **1** to the plasma. The results are summarized in Table 2. These data suggest a high specificity in the transport and/or hydrolysis of the different families of peptides in the rat model. While a high plasma concentration of **1** was obtained when N dipeptides were orally administered to the animals, the compound was almost undetected when the C-6 dipeptide **10** or the N,C-2 tripeptide **14** was administered. It is unknown whether the low plasma exposure of **1** after oral administration of the N,C dipeptide or the C-6 dipeptide is due to poor absorption or poor bioconversion of the prodrugs because we did not pursue further studies with these two peptides. With respect to C-2 dipeptides, only two C-2 dipeptides (**8b** and **8f**) provided plasma concentrations of the parent drug in a range similar to those obtained with the N dipeptides.

Rat Bioavailabilities. The absolute oral bioavailability in rats was determined for those dipeptides that

Scheme 1



Scheme 2[a]



[a] (a) (i) AllocCl, $NaHCO_3$; (ii) EDCI, HOBT, Et_3N, DMAP, AllylOH, 30%; (b) (i) HCl(g), MeOH; (ii) Boc_2O, K_2CO_3, 75%; (c) (coupling) EDCI, DMAP, HOBT, Et_3N, H_2N-(amino acid)-OR_3.

had shown high plasma concentration of parent in the three-time-point rat screen. The results are collected in Table 2. The concentration of prodrug circulating was also measured.

The only three C-2 dipeptides evaluated in this study, **8b**, **8c**, and **8f**, did not show a complete in vivo bioconversion to the parent **1**, with high concentrations of the prodrug detected at all time points. However, for two of them (**8b** and **8f**, Table 2), the systemic availability of **1** was high after oral administration. This indicates that in some cases at least, C-2 peptides are well-absorbed, presumably via the PepT1 transporter. The evaluated N dipeptides (**12a**, **12c**, **12e**, **12f**, and **12j**), on the other hand, were completely bioconverted in rats to the parent **1** with high bioavailability (71−105%).

The curves of two representative dipeptides, **8b** as an example of a C-linked dipeptide and **12a** as an example of an N-linked dipeptide, are shown for comparison (Figure 2). When **8b** was orally administered to Fischer rats at a dose of 8 mg/kg (27 μM), it was only partially converted into **1**. High concentrations of the prodrug remained in the rat plasma. These data were compared to equimolar intravenous administrations of **8b** or **1**. This allowed us to determine that the oral bioavailability of the prodrug was 42% while the systemic availability of **1** was 74%. The intravenous data also demonstrated that 29% of the iv dose of **8b** was recovered as **1** in rat plasma. This indicates that **1** formed from **8b** after oral administration was partly formed during the first pass and partly from **8b** available systemically. The intravenous data suggest that the bioconversion is the rate-limiting step of the elimination of **8b** because **8b** was eliminated at the same rate as **1** formed from **8b**. These data also indicate that the plasma clearance of **8b** was 3-fold lower than that after intravenous administration of **1**, explaining the higher plasma concentrations of **8b** observed in Figure 2.

In comparison, the N-linked prodrug **12a** was rapidly absorbed and completely biotransformed when administered to Fischer rats. Indeed the maximum plasma concentration of **1** appeared 1 h after oral administration and only a very low amount of the prodrug was detectable 30 min after administration (Figure 2). A comparison of the area under the curve (AUC) for **1** with the AUC determined after intravenous administration of **1** indicated that the oral bioavailability of **1** was 84% following oral administration of the N-linked prodrug.

In Vitro Bioconversion

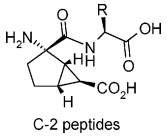
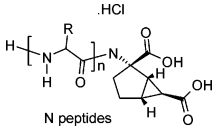
Two selected peptides (**8b** and **12a**) were incubated with rat liver homogenate and human jejunum in order to assess the stability of the prodrugs to enzymatic hydrolysis. The results are presented in Figure 3. Only the N dipeptide prodrug **12a** was unstable when exposed to enzymes located in the brush-border and citosol of the enterocytes (jejunum homogenate) or to the liver enzymes. These data are in agreement with the exposure studies in which it was shown that only N dipeptide prodrugs were completely bioconverted in vivo to the parent drug in rats. Compound **8b** is transported to the blood stream, but it is not fully hydrolyzed by peptidases to the parent drug.

In Vitro Transport Results

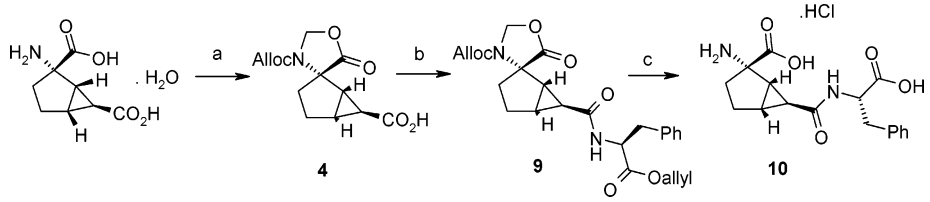
The in vivo results presented in the previous section demonstrated the enhanced exposure after oral administration to rats of dipeptides of **1** when compared to the parent drug itself. We wondered if an active transport mechanism such as the intestinal peptide transporter, PepT1, might be responsible for the greater oral absorption that was observed. With this aim we examined the uptake of a known substrate of the intestinal peptide transporter, radiolabeled glycylsarcosine (Gly-Sar), into Caco-2 cells and into Chinese hamster ovary cells transfected with human PepT1 (CHO/hPepT1) in the presence of selected peptides.

Transport into Caco-2 Cell Line. Initially, uptake of 100 μM Gly-Sar into Caco-2 cells was measured in the absence and presence of a selected peptide at a final concentration of 1 mM. This assay provided a rank order of the relative affinity of the transporter for the various peptides. As shown in Figure 4, **1** and the C-2 peptides showed weaker inhibition of Gly-Sar uptake than the corresponding N peptides ($p < 0.05$).[25]

Table 1. Synthesis of C-2 Peptides and N Peptides

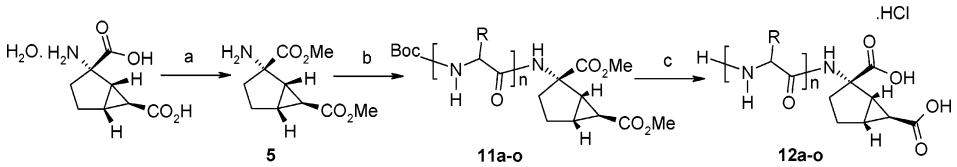
series	protected amino acid	intermediate	protected peptide (% yield)	peptide (% yield)
 C-2 peptides	L-Ala-OEt	**2**	**6a** (92%)	**8a** (38%)
	L-Leu-OAllyl	**2**	**6b** (98%)	**8b** (50%)
	L-Phe-OAllyl	**2**	**6c** (79%)	**8c** (48%)
	L-Val-OAllyl	**2**	**6d** (82%)	**8d** (56%)
	Gly-OEt	**2**	**6e** (76%)	**8e** (70%)
	L-Met-OMe	**2**	**6f** (90%)	**8f** (50%)
	L-Pro-OMe	**2**	**6g** (69%)	**8g** (89%)
	L-Ile-OAllyl	**2**	**6h** (72%)	**8h** (31%)
	L-Tyr-OMe	**3**	**7i** (95%)	**8i** (87%)
	L-Ser(OTBDMS)-OMe	**3**	**7j** (85%)	**8j**·HCl (20%)
 N peptides	Boc-L-Ala	**5**	**11a** (50%)	**12a**·HCl (80%)
	Boc-D-Ala	**5**	**11b** (93%)	**12b** (80%)
	Boc-L-Leu	**5**	**11c** (95%)	**12c**·HCl (48%)
	Boc-D-Leu	**5**	**11d** (88%)	**12d**·HCl (71%)
	Boc-L-Phe	**5**	**11e** (87%)	**12e**·HCl (44%)
	Boc-L-Val	**5**	**11f** (87%)	**12f**·HCl (64%)
	Boc-Gly	**5**	**11 g** (88%)	**12g** (44%)
	Boc-L-Met	**5**	**11h** (89%)	**12h** (78%)
	Boc-L-Pro	**5**	**11i** (78%)	**12i**·HCl (90%)
	Boc-L-Ile	**5**	**11j** (79%)	**12j**·HCl (64%)
	Boc-L-Tyr	**5**	**11k** (73%)	**12k** (35%)
	Boc-L-Ser(OTBDMS)-OH	**5**	**11l** (82%)	**12l** (11%)
	α-Boc-ϵ-Boc-L-Lys	**5**	**11m** (94%)	**12m**·2HCl (82%)
	Boc-L-Ala-L-Ala	**5**	**11n** (90%)	**12n** (45%)
	Boc-L-Leu-L-Ala	**5**	**11o** (95%)	**12o** (30%)

Scheme 3[a]



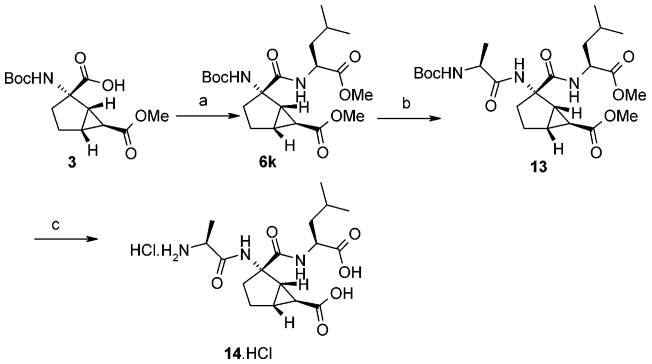
[a] (a) (i) AllocCl, $NaHCO_3$; (ii) CH_2O, *p*-TsOH, 65% two steps; (b) EDCI, DMAP, HOBT, Et_3N, H_2N-Phe-Oallyl, 47%; (c) (i) DMBA, $(Ph_3P)_4Pd$ cat.; (ii) saturated HCl in EtOAc, 91% two steps.

Scheme 4[a]



[a] (a) MeOH, $SOCl_2$; (b) EDCI, DMAP, HOBT, Et_3N, BocHN-(amino acid)$_n$-OH; (c) (i) LiOH; (ii) saturated HCl in EtOAc.

Scheme 5[a]

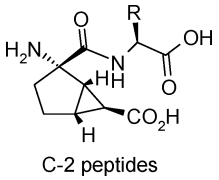
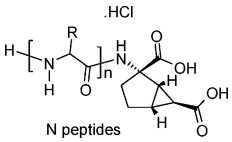
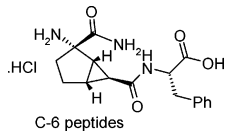
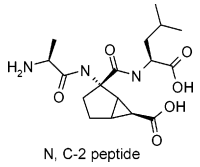


[a] (a) EDCI, HOBT, Et_3N, DMAP, L-Leu-OMe, 40%; (b) (i) HCl(g), MeOH; (ii) EDCI, HOBT, Et_3N, DMAP, BocHN-L-Ala, 48%; (c) (i) LiOH; (ii) saturated HCl in EtOAc, 81%.

To further determine the affinity of the peptide transporter, dose−response curves of selected compounds were examined for their effect on the uptake of Gly-Sar into Caco-2 cells or CHO/hPepT1 cells. The results are summarized in Table 3.

Some interesting observations can be made from these results: (a) Gly-Sar uptake was inhibited by the prodrugs (di- and tripeptides) but not by the amino acid analogue **1**. (b) Compounds that show a high concentration of parent in the three-point rat screen show high affinity for the transporter, such as **12a** (0.12 mM), **12c** (1.43 mM), and **12e** (0.52 mM). Compounds with lower affinities (**12b** and **12o**) show lower concentrations of parent in the same assay (entries 12 and 25 of Table 2), although further studies would be necessary to determine whether these compounds are poorly transported or hydrolyzed or both. (c) The uptake assay by CHO/hPepT1 is more predictive of the absorption of C-2 dipeptides than the Caco-2 assay because compounds **8b** and **8c** are absorbed, as demonstrated by the high concentrations of prodrug detected in plasma (see entries 2 and 3, Table 2). These in vivo data correlate with a good affinity for the transporter shown in the

Table 2. Plasma Concentrations (ng/mL) and AUC Values (ng·h/mL) of Prodrug and **1** in Rats after an Oral Dose of 5 mg equiv **1**/kg

series	entry		aa[a]	**1** at 0.5 h (ng/mL)[b]	**1** at 1 h (ng/mL)[b]	**1** at 3 h (ng/mL)[b]	oral *F*,[c] %	AUC of prodrug
 C-2 peptides	1	**8a**	Ala	0	0	0		
	2	**8b**	Leu	450	798	919	74	21264
	3	**8c**	Phe	20	45	10	0.3	11367
	4	**8d**	Val	10	17	10		
	5	**8e**	Gly	19	0	0		
	6	**8f**	Met	602	808	889	66	16648
	7	**8g**	Pro	0	0	0		
	8	**8h**	Ile	18	34	20		
	9	**8i**	Tyr	30	44	124		
	10	**8j**	Ser	36	0	53		
 N peptides	11	**12a**	Ala	3732	3381	1770	85	0
	12	**12b**	D-Ala	81	191	525		
	13	**12c**	Leu	4488	4323	1152	94	0
	14	**12d**	D-Leu	1478	894	877		
	15	**12e**	Phe	1710	2763	1274	71	194
	16	**12f**	Val	2387	2124	1136	94	ND[d]
	17	**12g**	Gly	1707	1992	ND[d]		
	18	**12h**	Met	3720	2107	ND[d]		
	19	**12i**	Pro	319	455	670		
	20	**12j**	Ile	2009	1457	2032	105	ND[d]
	21	**12k**	Tyr	1915	2163	ND[d]		
	22	**12l**	Ser	1497	1365	ND[d]		
	23	**12m**	Lys	74	147	246		
	24	**12n**	Ala-Ala	3620	1554	734		
	25	**12o**	Leu-Ala	2315	1337	660		
 C-6 peptides	26	**10**	Phe	0	0	0		
 N, C-2 peptide	27	**14**	Ala-Leu	44	26	55		

[a] The configuration of the amino acid is L unless otherwise indicated. [b] Plasma concentration of **1** after oral dosing of corresponding prodrug. [c] Bioavailability of parent from prodrug. [d] ND: not determined.

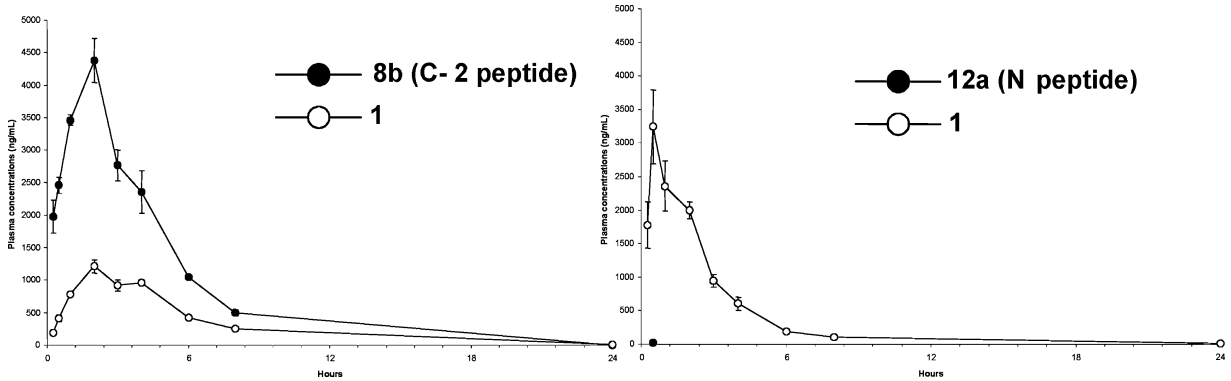


Figure 2. Comparison of plasma concentrations vs time profiles for **8b** and **12a** in rats following an oral dose of 5 mg equiv **1**/kg.

CHO/hPepT1 assay. (d) N Dipeptides gave better inhibition of uptake by the human intestinal PepT1 transporter than N tripeptides (compare IC_{50} for dipeptides **12a** (0.12 mM) and **12c** (1.43 mM) with IC_{50} for tripeptides **12n** (2.88 mM) and **12o** (5.26 mM) and their correlation with entries 11, 13, 24, and 25 in Table 2). (e) Although only two examples of unnatural D-peptides are available (compare **12a** with **12b** and **12c** with **12d**, Table 3), dipeptides with the L-configuration on the amino acid seem to have better affinity for the carrier than the corresponding dipeptides with the unnatural D-configuration.

These in vitro data and their correlation with in vivo results suggest that these dipeptides are actively transported into the intestinal enterocyte by the peptide carrier hPepT1 prior to exiting the enterocyte into the blood stream.

In Vitro Binding to mGlu Receptors 2/3

To test the affinity for mGlu receptors 2/3, compound **12a** (tested as the HCl salt) was examined for displacement of mGlu receptors 2/3 ligand binding. For these studies, **12a**·HCl displacement of high-affinity mGlu receptors 2/3 antagonist ligand [^{3}H]LY341495 binding

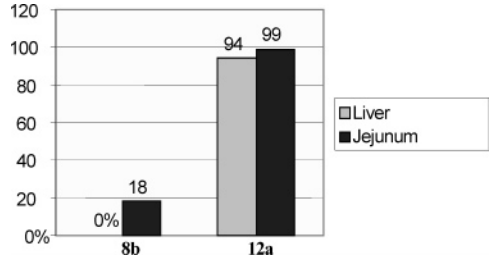


Figure 3. In vitro bioconversion of **8b** and **12a** incubated for 1 h at 10 μM in liver or jejunum homogenates.

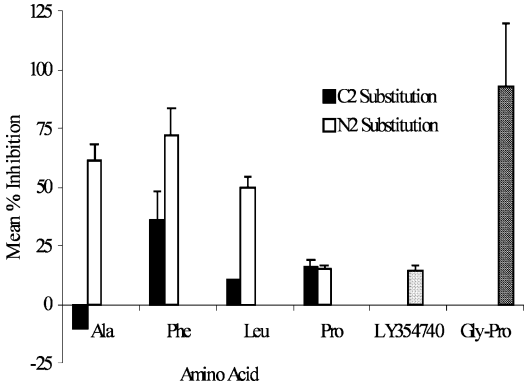


Figure 4. Comparison of the influence of C-2 and N dipeptide prodrugs on 100 μM ^{14}C Gly-Sar uptake in Caco-2 cells. **1** and Gly-Pro controls are also shown. Results are presented as percentage inhibition (mean ± SC of three to six filters) of ^{14}C Gly-Sar uptake relative to control filters that were exposed only to ^{14}C Gly-Sar.

Table 3. Affinity of the Transport Carrier for Selected Di- and Tripeptides and **1**

series		IC_{50} (mM) Caco-2	IC_{50} (mM) hPepT1
1	**1**	>10	>19
C-2 dipeptides	**8b**	>10	0.62
	8c	2.68	1.54
N peptides	**12a**	0.30	0.12
	12b	>10	6.66
	12c	5.01	1.43
	12d	6.31	1.7
	12e	0.42	0.52
	12n	7.29	2.88
	12o	>10	5.26
N,C-2 tripeptides	**14**	3.05	1.34

to cell membranes from human mGlu2, human mGlu3, and native rat brain tissues was determined.[26] **1** was used as a positive control/comparator in these studies.

As shown in Figure 5 (left and middle panels) and Table 4, **1** potently displaced [^{3}H]LY341495 binding to human mGlu2 and human mGlu3 expressing cell membranes with nanomolar potencies. In contrast, **12a**·HCl did not appreciably displace [^{3}H]LY341495 binding at up to 10 000 nM, which is 80- to 120-fold higher than the K_i values for the parent molecule in these tissues.

Table 4. Affinity of **1** versus Prodrug **12a**·HCl for Recombinant Human and Native Rat Brain mGluR2 Receptors

	displacement of [^{3}H]LY341495 binding ($N = 3$)	
receptor preparation	mean K_i ± SE (nM) for **1**	K_i (nM) for **12a**·HCl
human mGlu2	84.7 ± 11.1	>10 000
human mGlu3	125.6 ± 4.8	>10 000
rat forebrain	80.0 ± 7.3	>10 000

[^{3}H]LY341495 binding to rat forebrain membranes represents mGlu receptors 2/3 binding to native tissue. Rat brain tissue also represents the species used for in vivo efficacy data with **12a**·HCl (see below). As shown in Figure 5 (right panel) and Table 4, **1** displaced [^{3}H]-LY341495 binding to rat forebrain membranes with a potency similar to that observed in human recombinant receptors. In contrast, **12a**·HCl did not appreciably displace [^{3}H]LY341495 binding to rat forebrain membranes at up to 10 000 nM.

In summary, ligand binding data demonstrate that **12a**·HCl, unlike **1**, had no intrinsic affinity at human recombinant or native rat mGlu receptors 2/3.

In Vivo Actions in Rat Fear-Potentiated Startle Anxiety Model

To study the oral potencies of **12a** (tested as the HCl salt) in comparison to **1** in an mGlu receptors 2/3 linked therapeutic animal model, studies in the rat fear-potentiated startle assay were performed. This model was specifically chosen because it is highly sensitive to mGlu receptors 2/3 agonists such as **1**[27] and served as the basis for the development of **1** for anxiety disorders in humans. To verify that the actions of compound **12a**·HCl in this model were mGlu receptors 2/3 mediated, as we have shown previously for the parent,[27b] the ability of LY341495 (an mGlu receptors 2/3 antagonist)[28] to block compound-mediated suppression of fear-potentiated startle was also determined. As a positive control in each experiment, diazepam (0.6 mg/kg ip) was used. All experiments were performed in fed rats.

As shown in Figure 6, the parent compound **1** significantly suppresses rat fear-potentiated startle with a minimally effective dose (MED or lowest dose where $p < 0.05$ compared to control) of 3.0 mg/kg (Table 5). This is compared to 0.3 mg/kg **1** reported by Helton et al.[27a] However, in this more recent experiment (Figure 6, left panel), 0.3 mg/kg **1** suppressed fear-potentiated

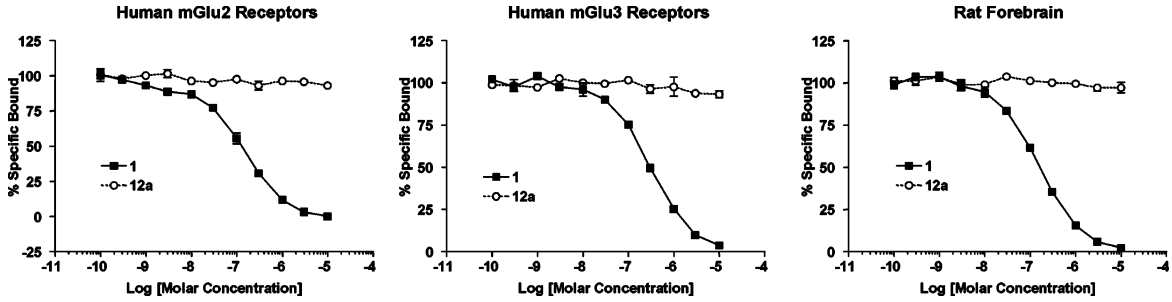


Figure 5. Unlike **1**, **12a**·HCl does not displace [^{3}H]LY341495 binding to human recombinant or rat brain mGluR2 receptors.

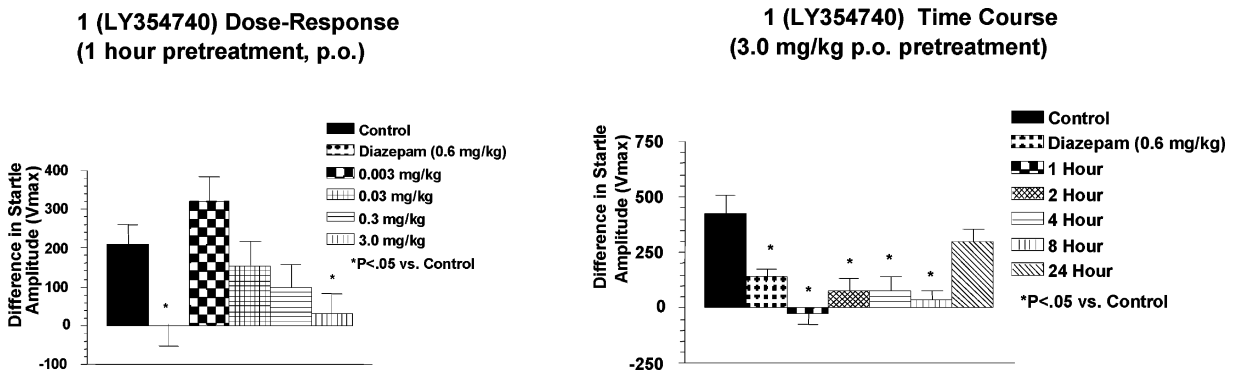


Figure 6. Potency, onset, and duration of **1** in rat fear-potentiated startle. **1** was dosed orally in fed rats. The positive control in each experiment, diazepam (0.6 mg/kg) was given ip 30 min prior to testing.

Table 5. Comparison **1** and **12a**·HCl in the Rat Fear-Potentiated Startle Assay

parameter measured	**1**	**12a**·HCl
MED (1 h pretreatment)	0.3[a]−3.0 mg/kg po	0.01 mg/kg po
onset of effect	≤1 h @ 3.0 mg/kg po	≤1 h @ 0.01 and 0.1 mg/kg po
duration of effect	8−24 h @ 3.0 mg/kg po	4−8 h @ 0.01 mg/kg po ≥24 h @ 0.1 mg/kg po

[a] Data from ref 27a.

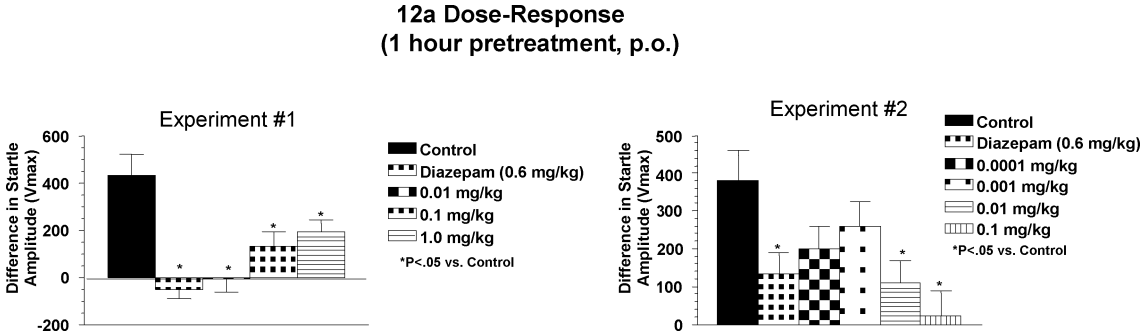


Figure 7. Oral potency of **12a**·HCl in rat fear-potentiated startle. **12a**·HCl was dosed orally (po) in fed rats. Two separate experiments with overlapping doses of **12a**·HCl were performed to determine the MED for oral activity. The positive control in each experiment, diazepam (0.6 mg/kg), was given ip 30 min prior to testing.

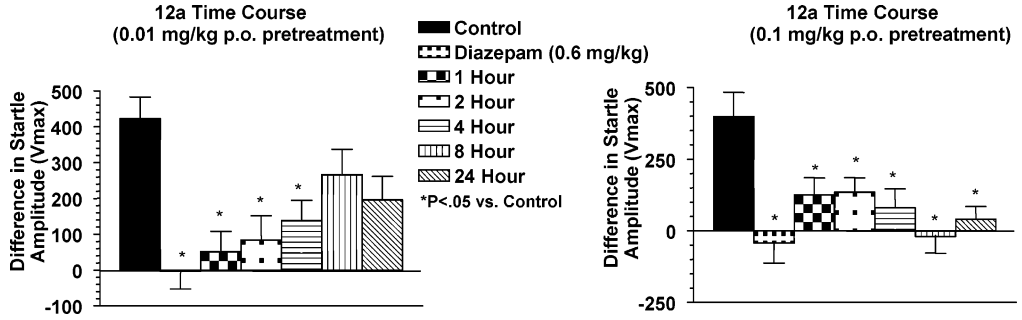


Figure 8. Time course for **12a**·HCl suppression of rat fear-potentiated startle. **12a**·HCl (0.01 mg/kg po (left panel), 0.1 mg/kg po (right panel)) was administered to fed rats at the designated pretreatment times. The positive control in the experiment, diazepam (0.6 mg/kg), was given ip 30 min prior to testing.

startle by ∼50%, but this was not statistically significant ($p = 0.08$). At 3.0 mg/kg **1** had an onset of 1 h and was active between 8 and 24 h following administration (Figure 6, right panel).

As shown in Figure 7, in two separate experiments with overlapping doses, **12a**·HCl significantly blocked fear-potentiated startle with an MED of 0.01 mg/kg po. As shown in Figure 8, at an MED dose of 0.01 mg/kg po, **12a**·HCl had an onset of 1 h and was still active at up to 4 h in this test. At a dose of 0.1 mg/kg po **12a**·HCl, a significant effect was observed within 1 h, and **12a**·HCl was still active at up to 24 h postadministration. Thus, in this test, **12a**·HCl was observed to be ∼300-fold more potent than the parent compound **1**. Furthermore, a dose of 0.1 mg/kg po **12a**·HCl produced a longer-lasting effect when compared to a 30-fold higher dose (3.0 mg/kg) of the parent. Figure 9 shows that the suppression of fear-potentiated startle by **12a**·HCl was reversed by LY341495, an mGlu receptors 2/3 antagonist.

Summary of Efficacy Data

In vitro studies showed that the prodrug of **1**, **12a**·HCl, had no appreciable affinity for mGlu receptors 2/3.

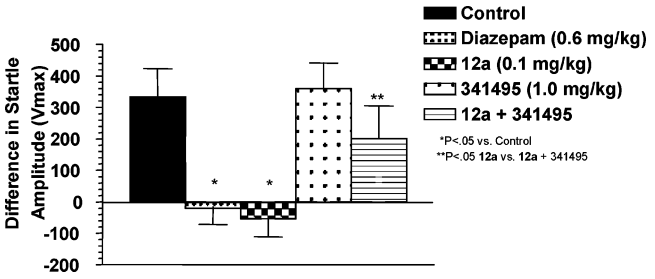


Figure 9. Reversal **12a**·HCl suppression of fear-potentiated startle by the mGluR2 antagonist LY341495. **12a**·HCl (0.1 mg/kg) was dosed orally (po) in fed rats 1 h prior to testing. LY341495 was given sc at 1 mg/kg 1 h prior to testing. The positive control, diazepam (0.6 mg/kg), was given ip 30 min prior to testing.

This indicates that the in vivo pharmacology of this compound in rats and humans likely reflects the conversion of the prodrug to the parent molecule **1**, which then acts at mGlu receptors 2/3 to produce a therapeutic effect.

When given orally to fed rats, **12a**·HCl was active in the rat fear-potentiated startle test at ~30−300 times lower doses when compared to **1**. Importantly, antagonist studies with LY341495 also demonstrated that the actions of **12a**·HCl in the anxiety test in vivo were group II mGlu receptors mediated. These animal data suggest that anxiolytic doses of **12a**·HCl in humans would be considerably lower than that of the parent compound. If this in vivo animal model data directly predict human anxiety responses, **12a**·HCl would produce anxiolytic effects in humans at 30- to 300-fold lower doses than the parent compound.

Conclusions

A series of di- and tripeptides of **1** have been investigated in this study as prodrugs of the parent amino acid **1** in order to increase the relatively low oral bioavailability of this compound in rats (10%). In vitro transport studies have shown the broad specificity of the human PepT1 transporter that recognizes a variety of peptides (N dipeptides, N tripeptides, and C-2 dipeptides of **1**) suggesting that these peptides are actively transported by the peptide carrier. In vitro bioconversion studies of two model dipeptides, **8b** and **12a**, show complete hydrolysis of **12a** in the presence of rat liver or human jejunum, while **8b** is more stable to enzymatic hydrolysis. In vivo studies performed with rats correlate with the in vitro results. Thus, while N dipeptides are rapidly absorbed and bioconverted in vivo to the parent drug, C-2 dipeptides fail to be transported or to completely biotransform.[29] As a result, N dipeptides are more suitable prodrugs of **1**, improving significantly the intestinal uptake of the parent drug. *N*-Ala dipeptide (**12a**) shows a 84% oral bioavailability in rats, with no prodrug circulating 30 min after oral administration. The released promoiety is the safe L-Ala. In vivo efficacy experiments performed with **12a** have shown this compound to be 300-fold more active in the fear-potentiated startle model of anxiety in rats than the parent compound **1**.

In vitro results obtained with human cells (human PepT1 for absorption model and human jejunum for bioconversion model) suggest that a dipeptide prodrug-like **12a** could also be a suitable prodrug for **1** in humans.

Experimental Section

Biology Methods. In Vivo Results. Male Fisher 344 rats weighing 190−250 g were obtained from Harlan (Indianapolis, IN) and housed in polycarbonate cages in a light-controlled environment (12 h of light, 12 h of dark) with free access to food and water. The animals were acclimated to the housing environment for 3 days before drug administration. Water was provided ad libitum throughout the study, and the animals were fasted overnight prior to dosing, with the food being returned approximately 4 h postdose. The rats received either an oral dose of the prodrug that was the molar equivalent to a 5 mg/kg dose of compound **1** dissolved in pH 2.5 20 mM $KHPO_4$ or an intravenous dose of compound **1** at 2.5 mg/kg dissolved in water. Blood was collected from the orbital sinus for the first two time points, and the last time point blood was collected from carbon dioxide anesthetized animals by cardiac puncture. The blood samples were combined with 50 mL of a 100 mM phenylmethylsulfonyl fluoride solution to inhibit hydrolysis of the prodrug. The sample was then centrifuged, and the resulting plasma was stored at −20 °C or below until analysis. There were three plasma samples per time point, and each rat was collected from three time points.

In Vitro Bioconversion. 1 prodrugs were incubated for 1 h with liver and jejunum homogenates. The liver homogenate was prepared by probe-sonicating a fresh liver sample diluted 3-fold with pH 7.4 100 mM phosphate buffer with 5 mM $MgCl_2$ while being kept on ice. The jejunum homogenate was prepared by scraping an approximate 2 in. section (human) with a razor blade, diluting the preparation in 2 mL of 5 mM Tris and 50 mM manitol, probe-sonicating, and diluting with 2 mL of 100 mM phosphate pH 7.4 with 5 mM $MgCl_2$ while being kept on ice. The incubation was then performed in a 96-well plate by gently shaking 0.8 mL of homogenate with 40 mL of a 200 mM prodrug solution at 37 °C for 1 h. The reaction was stopped with 50 mL of a 100 mM phenylmethylsulfonyl fluoride solution, and the samples were stored at −20 °C or below until analysis.

Samples were extracted utilizing solid-phase extraction and analyzed by LC/MS/MS. One hundred microliters of a sample was combined with 20 mL of internal standard (LY459477) and 500 mL of water. The sample was then slowly extracted at approximately 1 drop/3 s on a SAX 100 mg cartridge (IST) that had been preconditioned with 0.5 mL of methanol and 0.5 mL of water. The cartridge was then washed with 0.5 mL of water and 1 mL of methanol. To elute of the prodrug, the cartridge was washed with 0.5 mL of ethyl acetate (1% trifluoroacetic acid and 1% water), dried for 10 min under full vacuum, and eluted with 0.25 mL of 0.2% acetic acid. To extract compound **1**, the cartridge was eluted with 0.8 mL of 3% trifluroracetic acid, 5% water, and 93% methanol.

Uptake by the Intestinal Human PepT1 Transporter. Caco-2 Cells. For cells grown on filters, inhibition of 100 mM [^{14}C]Gly-Sar was measured in the absence or presence of 1 mM of prodrugs, dipeptide control, or **1**. The flux buffer was 25 mM MES, pH 6.0, which also contained 125 mM NaCl, 5.2 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 10 mM glucose. Prior to an experiment, cells were washed once in pH 7.4 buffer (25 mM HEPES used as the buffering agent) and starved for 30−60 min. This buffer was removed, and 0.3 mL of the pH 6 buffer containing Gly-Sar, 100 mM mannitol, and 1 mM of a competing substrate was added to the cells and incubated for 15 min on an orbital shaker at 37 °C. Cells and filters were washed twice in cold pH 7.4 buffer, 0.3 mL on top and 1 mL on the bottom. Individual filters were then removed from the plastic filter cup housing using a scalpel and placed in scintillation vials with 10 mL of ReadySolv HP (Beckman Coulter, Fullerton, CA). Samples were then counted for 5 min. With mannitol as a marker, extracellular fluid remaining on the cells was <0.1% of the initial amount applied. Alternatively for cells grown in 96-well plates, inhibition of 40 mM [3H]Gly-

Sar was measured over a concentration range of 0.125−2.0 mM for the prodrugs or 0.5−10 mM for **1**. On the day of an experiment, the medium was removed and replaced with 0.1 mL of the 25 mM HEPES pH 7.4 buffer used in the 24-well plate filter experiments. Sixty minutes later, this buffer was replaced with 40 mL of the 25 mM MES pH 6 buffer also described above. After 15 min at 37 °C in a humidified orbital shaker, cells were washed three times with 0.2 mL of ice-cold pH 7.4 buffer. Preliminary studies demonstrated linear uptake of 40 mM Gly-Sar between 5 and 15 min. Cells were then lysed by adding 50 mL of 1% Triton-X 100, and the plates were allowed to sit overnight at ambient temperature. Subsequently, an aliquot of 10 mL was removed for analysis of protein content using the Pierce BCA protein assay reagent. A volume of 200 mL of Packard Microscint 40 was added to the remaining 40 mL, sealed using Packard Top Seal and mixed, and tritium was counted for 5 min on a Perkin-Elmer Top Count NXT microplate scintillation and luminescence counter (Perkin-Elmer Life Sciences, Boston, MA). Uptake rate was calculated on the basis of total protein content. Residual extracellular fluid remaining on the cells was estimated using [^{3}H]inulin as the marker. IC_{50} values were calculated using a computer-generated curve-fitting program, BRAVO/SAS, and represent the concentration that gave 50% inhibition of the maximum inhibition observed in the experiment. For a given experiment, each inhibitor concentration was measured in duplicate and the average percent inhibition at each concentration was used in the curve-fitting. For most inhibitors, at least two experiments (on different days) were performed.

CHO/PepT1 Cells. Inhibition of 40 mM [^{3}H]Gly-Sar was measured over a concentration range of 0.01−3.15 mM for the prodrugs or 0.59−19 mM for **1**. The flux buffer was sodium-free modified EBSS buffer at pH 6.0 as previously published.[25] Cells were washed once in EBSS pH 7.4 and starved for 30−45 min. Buffer was removed, and an aliquot of 40 mL of flux buffer was added to cells and incubated for 5−20 min on an orbital shaker at 37 °C. Cells were washed three times with ice-cold EBSS pH 7.4 using a Perkin-Elmer Filtermate 196 with the air and vacuum off. Excess fluid was removed, and cells were lysed as described above except with 40 mL of Triton-X 100. After the plates were allowed to sit overnight, 30 mL was transferred to an opaque bottom Packard culture plate with the addition of 200 mL of Microscint 40 per well. The methods for analysis of protein content and extracellular volume, as well as IC_{50} determinations, were the same as described for the Caco-2 96-well experiments. For each inhibitor, at least two independent determinations of IC_{50} (from separate experiment days) were made.

In Vitro Receptor Binding. Membranes from cells expressing recombinant human mGlu receptors were prepared by scraping attached cells from T-150 flasks, centrifuging at approximately 1000*g* for 5 min, and freezing resultant pellets at −20 °C until the day of assay. Rat brain tissue was obtained by decapitation from adult male Sprague-Dawley rats (150−250 g, Harlan/Sprague-Dawley, Indianapolis, IN). Forebrain tissue (cortex, hippocampus, and striatum) was dissected and homogenized in 30 mM Tris-HCl + 2.5 mM $CaCl_2$ buffer (pH 7.6 at 5 °C). The homogenized tissue was washed three times by centrifugation, then incubated for 30 min at 37 °C followed by three more washes, then resuspended in 10 volumes of buffer and frozen at −20 °C.

Frozen tissue preparations were thawed on the day of assay, suspended in ice-cold assay buffer (10 mM potassium phosphate pH 7.6 + 100 mM potassium bromide), homogenized, and washed 3× by centrifugation at 50000*g* for 10 min. To start the binding reaction, washed tissue (0.015−0.020 mg of protein) was added to deep-well polypropylene microtiter plates containing [^{3}H]LY341495 (1 nM) and appropriate concentrations of test compounds in assay buffer. The final assay volume was 0.5 mL. Nonspecific binding was defined with 1 mM L-glutamate. Assay plates were incubated on ice for 30−45 min, and the reaction was terminated by rapid filtration. Filters were placed in minivials with scintillation cocktail and counted on a Beckman LS6000 counter. Protein concentration was determined using the Pierce Coomassie microassay. Affinity constants (K_i) were calculated using the GraphPad Prism program.

Fear-Potentiated Startle in Rats. Apparatus and Procedures. SR-LAB (San Diego Instruments, San Diego, CA) chambers were used for conditioning sessions and for producing and recording startle responses. A respondent conditioning procedure was used to produce potentiation of startle responses. The fear-potentiated startle paradigm was conducted over 3 consecutive days. All 3 days began with a 5 min adaptation period before the trial started. On day 1 (baseline startle) after the adaptation period, the animals received 30 trials of 120 dB auditory noise. Startle responding was measured through transducers located under the startle platforms. Recorded values represent a maximum change in voltage (V_{max}). The mean startle amplitude (V_{max}) was used to assign animals to groups ($n = 8$) with similar mean values before conditioning began. Day 2 consisted of conditioning the animals. Each animal received 0.5 mA of shock for 500 ms preceded by a 5 s presentation of light (15 W) that remained on for the duration of the shock. Ten presentations of the light and shock were administered. Animals tested in the preconditioning paradigm were administered compound prior to conditioning. On day 3, 24 h after conditioning, startle testing sessions were conducted. Ten trials of acoustic startle (120 dB), non-light-paired, were presented at the beginning of the session to minimize the influence of the initial rapid phase of habituation to the stimulus. This was followed by 20 random trials of the noise alone and 20 random trials of noise preceded by light. Animals tested in the postconditioning paradigm were administered compounds prior to testing on day 3.

Statistical Analysis. With exclusion of the first 10 trials, the startle response amplitudes for each trial type were averaged for each animal. Data were presented as the difference between light + noise and noise alone. Differences in startle response amplitudes were analyzed by JMP statistical software using a one-way ANOVA (analysis of variance), followed by a Dunnett's post hoc analysis to determine differences between control and treatment groups. Interaction experiments between agonists and antagonists were analyzed using the Student's *T* test. Group differences were considered to be significant at $p < 0.05$.

Chemistry Methods. (1*S*,2*S*,5*R*,6*S*)-2-Allyloxycarbonylaminobicyclo[3.1.0]hexane-2,6-dicarboxylic Acid 6-Allyl Ester 2. (1*S*,2*S*,5*R*,6*S*)-2-Aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid monohydrate **1** (15.0 g, 73.9 mmol) was slowly dissolved in 250 mL of saturated $NaHCO_3$ (250 mL) in a round-bottom flask provided with a stopper. After complete solution, dioxane (100 mL) and allyl chloroformate (15.7 mL, 147.8 mmol) were added at room temperature, and the mixture was stirred overnight. The reaction mixture was diluted with water (100 mL) and washed with ethyl acetate (3×). The organic layer was extracted once with saturated $NaHCO_3$. The combined aqueous layers were acidified to pH 1 with 4 N HCl and extracted with ethyl acetate (2×). The organic layer was dried over magnesium sulfate, filtered, and concentrated to provide an oil (13.4 g, 67% yield) that was used without further purification. ^{1}H NMR (CD_3OD) δ: 6.01−5.82 (m, 2 H), 5.35−5.13 (m, 4 H), 4.51 (d, $J = 5.1$ Hz, 4 H), 2.48−1.78 (m, 5 H), 1.69−1.62 (m, 1 H), 1.45−1.29 (m, 1 H). ^{13}C NMR (CD_3OD) δ: 176.7, 176.6, 158.3, 134.2, 117.4, 67.3, 66.3, 35.8, 33.1, 29.9, 27.0, 22.0.

To a suspension of the compound obtained in the previous step (the Alloc derivative of **1**) (13.4 g, 49.8 mmol) in dichloromethane (400 mL), *N*-ethyl-*N*′-dimethylaminopropylcarbodiimide (9.55 g, 49.8 mmol) and (dimethylamino)pyridine (0.61 g, 5.0 mmol) were added at room temperature under nitrogen. Allyl alcohol (3.4 mL, 49.8 mmol) was added, and the mixture was stirred overnight at room temperature. The reaction mixture was diluted with dichloromethane and washed with water (2×). The organic layer was dried over magnesium sulfate, filtered, and concentrated to provide **2** (6.8 g, 44% yield) as an oil. ^{1}H NMR (CD_3OD) δ: 6.01−5.82 (m, 1 H), 5.35−

5.13 (m, 2 H), 4.51 (d, J = 5.1 Hz, 2 H), 2.48−1.78 (m, 5 H), 1.69−1.62 (m, 1 H), 1.45−1.29 (m, 1 H).

(1*S*,2*S*,5*R*,6*S*)-2-*tert*-Butoxycarbonylaminobicyclo[3.1.0]-hexane-2,6-dicarboxylic Acid 6-Methyl Ester (3). A 250 mL flask provided with a stopper was charged with (1*S*,2*S*,5*R*,6*S*)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid monohydrate **1** (18.08 g, 89.0 mmol), and 90 mL of a 2.5 M HCl(g) in methanol was added. The suspension was vigorously stirred, and methanol (50 mL) was added after 10 min. The solution was stirred for 5 h. Diethyl ether was added, and the solid was filtered and rinsed with diethyl ether. After the sample was dried under high vacuum, the 6-methyl ester derivative of **1** was obtained as a fine powder (5.5 g, 87% yield): mp 245 °C (dec). $[\alpha]^{25}_{D}$ +2° (*c* 1.25, MeOH). ^{1}H NMR (D_2O) δ: 3.65 (s, 3H), 2.30−2.00 (m, 6H), 1.56 (m, 1H). ^{13}C NMR (D_2O) δ: 174.4, 172.9, 65.9, 52.6, 32.6, 30.0, 29.2, 26.2, 21.2.

The 6-methyl ester derivative (8.6 g, 43.2 mmol), di-*tert*-butyl dicarbonate (18.8 g, 86.3 mmol), and potassium carbonate (11.9 g, 86.3 mmol) were dissolved in dioxane (200 mL) and water (100 mL). The solution was stirred at room temperature for 2 days. Hydrochloric acid (1 N) was added dropwise to give pH 1, and it was extracted with ethyl acetate. The organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated to provide **3** as a white solid (9.7 g, 75% yield): mp 164−165 °C. $[\alpha]^{25}_{D}$ −30° (*c* 1.6, MeOH). ^{1}H NMR ($CDCl_3$) δ: 3.65 (s, 3 H), 2.45−1.87 (m, 5 H), 1.71 (t, J = 2.9 Hz, 1 H), 1.41 (s, 9 H), 1.30−1.09 (m, 1 H). ^{13}C NMR ($CDCl_3$) δ: 177.8, 172.9, 156.2, 81.6, 66.4, 51.7, 34.7, 32.5, 28.4, 28.1 (3C), 26.4, 21.0.

(1*S*,2*S*,5*R*,6*S*)-3-Allyloxycarbonyl-5-oxooxazolidine-4-spiro-2′-bicyclo[3,1,0]-hexane-6′-carboxylic Acid (4). The Alloc derivative of **1** (see synthesis of **2**) (19.2 g, 64.3 mmol), paraformaldehyde (7.7 g, 257.3 mmol), and *p*-toluenesulfonic acid (0.61 g, 3.2 mmol) were refluxed in 200 mL of benzene with azeotropic removal of water for 2 h. The mixture was cooled to room temperature, diluted with 200 mL of ethyl acetate, washed with brine, dried over $MgSO_4$, filtered, and concentrated to afford a slightly hygroscopic solid (17.6 g, 97%). ^{1}H NMR ($CDCl_3$) δ: 6.10−5.90 (m, 1 H), 5.41−5.21 (m, 4 H), 4.65 (dt, J = 5.6, 1.3 Hz, 2 H), 2.51−2.49 (m, 1 H), 2.33−2.18 (m, 2 H), 2.04−1.92 (m, 3 H), 1.75−1.70 (m, 1 H). ^{13}C NMR (CD_3OD) δ: 176.1, 175.4, 153.0, 133.5, 118.9, 78.4, 67.8, 67.4, 33.1, 27.2 (×2), 26.0, 23.9.

(1*S*,2*S*,5*R*,6*S*)-2-Aminobicyclo[3.1.0]hexane-2,6-dicarboxylic Acid Dimethyl Ester Hydrochloride (5). Thionyl chloride (807 mL, 11.1 mol) was added to methanol (9.5 L) over a period of 1 h while maintaining the temperature between 2 and 20 °C. The solution was maintained for 30 min, then (1*S*,2*S*,5*R*,6*S*)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid monohydrate **1** (1.61 kg, 7.92 mol) was added. The resulting solution was heated to 47 °C and maintained between 47 and 50 °C for 17 h. An amount of approximately 7.3 L of methanol was then removed by vacuum distillation (47−50 °C, 240−275 mmHg). The remaining methanol was removed by azeotropic distillation with *tert*-butyl methyl ether (MTBE) at atmospheric pressure (added MTBE (10 L), removed 8.5 L; added MTBE (10 L), removed 8.5 L; added MTBE (8 L), removed 5.1 L). During the course of the distillations a white solid began to precipitate from the solution. After completion of the distillations, MTBE (2 L) was added to the resulting slurry, which was cooled to 22 °C. The solid was filtered, rinsed with MTBE (2 L), and dried under vacuum to afford 1.94 kg (98%) of **5** as a white solid: mp 193−194 °C. $[\alpha]^{25}_{D}$ +22° (*c* 1.0, MeOH). ^{1}H NMR (D_2O) δ: 3.86 (s, 3H), 3.67 (s, 3H), 2.31−2.04 (m, 6H), 1.57 (m, 1H). ^{13}C NMR (methanol-d_4) δ: 171.9, 170.2, 65.6, 52.8, 51.2, 32.4, 29.9, 28.5, 26.2, 20.7. Anal. ($C_{10}H_{16}NO_4Cl$) C, H, N, Cl.

General Procedure A: Coupling 2−5 with Protected Amino Acids. In a round-bottom flask intermediates **2**−**5** (1.0 equiv), *N*-ethyl-*N*′-dimethylaminopropylcarbodiimide (1.4 equiv), (dimethylamino)pyridine (0.1 equiv), *N*-hydroxybenzotriazole (1.2 equiv), the protected amino acid (1.2 equiv), and triethylamine (2 equiv) were dissolved in dichloromethane (0.1 M solution) under nitrogen. The resulting solution was stirred at room temperature overnight. The solvent was removed under vacuum, the residue was diluted with ethyl acetate (50 mL) and washed with saturated $KHSO_4$ (2×) and with $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated under vacuum. The residue was purified by column chromatography on silica gel (eluent hexane/ethyl acetate).

General Procedure B: Deprotection of Allyl Derivatives. The corresponding allylated compound (1.0 equiv) was dissolved in dry dichloromethane (0.1 M solution) under nitrogen. 1,3-Dimethylbarbituric acid (0.5 equiv for each allyl group to be removed) and tetrakis(triphenylphosphine)-palladium(0) (0.02 equiv) were added, and the resulting solution was heated at 35 °C for 2 h. After the mixture was cooled to room temperature, if a solid was formed, it was filtered, washed with dichloromethane, ethyl acetate, and diethyl ether, and dried under vacuum to provide the product. In case the solid did not appear, the solvent was removed under vacuum and a large amount of diethyl ether was added. After the mixture was stirred for 30 min, the solid was filtered, washed with ether and ethyl acetate, and dried to provide the product.

General Procedure C: Deprotection of *N*-Boc, 6-Methyl Derivatives. The corresponding 2-*N*-Boc-6-methyl ester dipeptide (1.0 equiv) was dissolved in THF and an equal volume of 2.5 N aqueous LiOH (10−20 equiv) was added. The reaction mixture was stirred at room temperature for 1−3 h. After dilution with water, the mixture was washed with ethyl acetate. The aqueous layer was acidified to pH 2 with 1 N HCl and extracted with ethyl acetate. The combined organic extracts were washed with brine, dried over sodium sulfate, filtered, and concentrated. The foamy solid was dissolved in a solution of ethyl acetate saturated with hydrogen chloride (g) (5−10 mL/mmol), and the resulting mixture was stirred overnight. The solid was filtered, rinsed with diethyl ether, and dried under high vacuum to provide the product.

General Procedure D: Deprotection of 2,6-Dimethyl Esters. The corresponding 2′-*N*-Boc-2,6-dimethyl ester dipeptide derivative (1.0 equiv) was dissolved in THF, and an equal volume of 2.5 N aqueous LiOH (10−20 equiv) was added. The reaction mixture was stirred at room temperature for 1−3 h. The solution was diluted with water and washed with EtOAc (2×). The aqueous layer was acidified to pH 1−2 with 1 N hydrochloric acid and extracted with ethyl acetate (5×). The combined organic layer was washed with brine, dried over sodium sulfate, filtered, and evaporated under vacuum to dryness. The crude *N*-Boc-dicarboxylic acid was dissolved in a saturated solution of hydrogen chloride gas in ethyl acetate (5−10 mL/mmol), and the mixture was stirred for 16 h. The resulting white precipitate was filtered, rinsed with ethyl ether, and dried under high vacuum to afford the hydrochloride salt as a fine white powder.

If further purification was needed, the crude amino diacid was chromatographed over a C8 reverse-phase support, eluting with acetonitrile in water with 0.05% of trifluoroacetic acid to provide, after drying, the amino diacid as a zwitterion.

(1*S*,2*S*,5*R*,6*S*)-2-Amino-2-[(1′*S*)-carboxyethyl]carbamoylbicyclo[3.1.0]hexane-6-carboxylic Acid (8a). (1*S*,2*S*,5*R*,6*S*)-2-Allyloxycarbonylamino-2-[(1′*S*)-ethoxycarbonylethyl]-carbamoylbicyclo[3.1.0]hexane-6-carboxylic Acid Allyl Ester (6a). The reaction of L-alanine ethyl ester and **2** as described in general procedure A provided compound **6a** as a white solid that was used for the next step without further purification (92% yield).

The allyl groups of compound **6a** were removed following general procedure B. The residue was suspended in 2 mL of THF, and 4 mL of 2.5 N LiOH was added. The reaction mixture was stirred for 2 h, acidified to pH 3 with 6 N HCl, and evaporated to dryness. Purification by ion exchange chromatography provided **8a** as a white solid (38% yield): mp >300 °C (changed color at 290 °C). $[\alpha]^{25}_{D}$ −55° (*c* 0.5, 1 N HCl). ^{1}H NMR (D_2O) δ: 4.12 (q, J = 7.3 Hz, 1 H), 2.14−2.13 (m, 2 H), 2.00−1.90 (m, 3 H), 1.79 (t, J = 3.0 Hz, 1 H), 1.59−1.41 (m, 1

H), 1.25 (d, J = 7.3 Hz, 3 H). ^{13}C NMR (D_2O) δ: 174.8, 177.2, 170.1, 66.1, 50.8, 30.9, 29.7, 28.4, 24.9, 22.8, 16.8.

(1*S*,2*S*,5*R*,6*S*)-2-Amino-2-[(1′*S*)-carboxy-3′-methylbutyl]-carbamoylbicyclo[3.1.0]hexane-6-carboxylic Acid (8b). (1*S*,2*S*,5*R*,6*S*)-2-Allyloxycarbonylamino-2-[(1*S*)-allyloxycarbonyl-3-methylbutyl]carbamoylbicyclo[3.1.0]hexane-6-carboxylic Acid Allyl Ester (6b). L-Leucine allyl ester hydrochloride (1.3 equiv), (dimethylamino)pyridine (0.1 equiv), a slurry of 5-([1,4′]bipiperidinyl-1′-sulfonyl)benzotriazol-1-ol in 20 mL of dimethylformamide previously heated at 60 °C (1.2 equiv), a solution of *N*-ethyl-*N*′-dimethylaminopropylcarbodiimide (1.4 equiv) and **2** (1.0 equiv) in 20 mL of dichloromethane and triethylamine (1.2 equiv) were mixed in a screw-capped tube and stirred in an orbital agitator overnight. Dichloromethane was removed under vacuum, and the DMF solution was diluted with 150 mL of ethyl acetate and washed 1 N HCl and brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated to provide **6b** (98% yield) as an oil that was used for the next step without further purification. ^{1}H NMR ($CDCl_3$) δ: 7.13 (bs, 1 H), 5.95−5.79 (m, 2 H), 5.60 (s, 1 H), 5.34−5.07 (m, 7 H), 4.63−4.50 (m, 6 H), 2.49−2.34 (m, 3 H), 2.10−1.85 (m, 4 H), 1.71 (t, J = 2.7 Hz, 1 H), 1.41 (d, J = 7.3 Hz, 3 H), 1.28−1.12 (m, 1 H).

Compound **6b** (1.0 equiv) was dissolved in dry dichloromethane (0.1 M solution) under nitrogen. 1,3-Dimethylbarbituric acid (6.0 equiv) and tetrakis(triphenylphosphine)-palladium(0) (0.03 equiv) were added, and the solution was heated at 35 °C for 2 h. After the mixture was cooled to room temperature, the solvent was removed under vacuum and the resulting residue was dissolved in a solution of ethyl acetate saturated with hydrogen chloride gas and stirred for 2 h. The reaction mixture was filtered, and the filtrate was washed with ethyl acetate and ether and dried to provide **8b** as a white solid (50% yield): mp 130.5−131.7 °C. ^{1}H NMR (DMSO-d_6/TFA-d) δ: 4.45−4.38 (m, 1 H), 2.27−2.21 (m, 2 H), 2.11−1.90 (m, 4 H), 1.77−1.54 (m, 4 H), 0.95 (dd, J = 6.18, 5.10 Hz, 6 H). ^{13}C NMR (DMSO-d_6/TFA-d) δ: 173.7, 173.2, 170.1, 65.9, 51.2, 31.7, 31.1, 29.5, 25.7, 24.9, 23.4, 21.6, 21.2. MS (electrospray), *m/z*: 299.16 (M^+ + H).

(1*S*,1′*S*,2*S*,5*R*,6*S*)-2-Amino-2-[(1′*S*)-carboxy-2-phenylethyl]carbamoylbicyclo[3.1.0]hexane-6-carboxylic Acid (8c). (1*S*,2*S*,5*R*,6*S*)-2-Allyloxycarbonylamino-2-[(1′*S*)-allyloxycarbonyl-2′-phenylethyl]carbamoylbicyclo[3.1.0]-hexane-6-carboxylic Acid Allyl Ester (6c). The reaction of L-phenylalanine allyl ester and **2** as described in general procedure A provided compound **6c** as a white solid that was used for the next step without further purification (79% yield): mp 220 °C (with decomposition). ^{1}H NMR ($CDCl_3$) δ: 7.27−7.08 (m, 5 H), 7.01−6.97 (bd, 1 H), 5.96−5.75 (m, 3 H), 5.64 (bs, 1 H), 5.32−5.17 (m, 6 H), 4.83 (c, J = 6.4 Hz, 1 H), 4.59−4.49 (m, 6 H), 3.17−3.09 (m, 2 H), 2.42 (bs, 1 H), 2.30 (dd, J = 13.2, 7.5 Hz, 1 H), 2.03−1.84 (m, 3 H), 1.66 (t, J = 3.0 Hz, 1H), 1.24−1.08 (m, 1H).

Compound **6c** was deprotected as described in the general procedure B. The solid was stirred in MeOH for 15 min and filtered to provide **8c** as a white solid (48% yield): mp >300 °C. ^{1}H NMR (D_2O) δ: 7.07−7.04 (m, 5 H), 4.35−4.32 (m, 1 H), 3.15−3.08 (m, 1 H), 2.85−2.74 (m, 1 H), 1.88 (m, 1 H), 1.74−1.74 (m, 1 H), 1.52−1.10 (m, 5 H). ^{13}C NMR (DMSO-d_6) δ: 174.0, 173.3, 171.6, 138.5, 129.7 (2C), 128.3 (2C), 126.5, 65.6, 54.7, 37.0, 33.8, 32.8, 28.6, 26.1, 21.7.

(1*S*,2*S*,5*R*,6*S*)-2-Amino-2-[(1′*S*)-carboxy-2′-methylpropyl]-carbamoylbicyclo[3.1.0]hexane-6-carboxylic Acid (8d). (1*S*,2*S*,5*R*,6*S*)-2-Allyloxycarbonylamino-2-[(1′*S*)-allyloxycarbonyl-2′-methylpropyl]carbamoylbicyclo[3.1.0]hexane-6-carboxylic Acid Allyl Ester (6d). The reaction of L-valine allyl ester and **2** as described in general procedure A provided compound **6d** as a white solid that was used for the next step without further purification (82% yield). ^{1}H NMR ($CDCl_3$) δ: 5.88 (m, 3H), 5.49−5.17 (m, 7H), 4.64−4.51 (m, 7H), 2.55 (bs, 1H), 2.43 (dd, J = 13.2, 8.3 Hz, 1H), 2.30−1.87 (m, 4H), 1.71 (t, J = 2.7 Hz, 1H), 1.29−1.13 (m, 1H), 0.96 (d, J = 6.7 Hz, 3H), 0.90 (d, J = 7.0 Hz, 3H).

Compound **6d** was deprotected as described in the general procedure B. The solid was stirred in MeOH for 15 min and filtered to provide **8d** as a white solid (56% yield): mp >300 °C. $[\alpha]^{25}_D$ −48° (*c* 1, 1 N HCl). ^{1}H NMR (DMSO-d_6) δ: 4.05 (m, 1 H), 2.18−1.85 (m, 7 H), 1.28−1.17 (m, 1 H), 0.86 (d, J = 6.4 Hz, 3 H), 0.84 (d, J = 6.5 Hz, 3 H). ^{13}C NMR (DMSO-d_6) δ: 174.2, 173.4, 173.2, 65.7, 58.1, 35.4, 33.3, 30.9, 28.7, 26.8, 21.4, 19.7, 18.3.

(1*S*,2*S*,5*R*,6*S*)-2-Amino-2-[(1′*S*)-carboxymethyl]carbamoylbicyclo[3.1.0]hexane-6-carboxylic Acid (8e). (1*S*,1′*S*,2*S*,5*R*,6*S*)-2-Allyloxycarbonylamino-2-(ethoxycarbonylmethylcarbamoyl)bicyclo[3.1.0]hexane-6-carboxylic Acid Allyl Ester (6e). The reaction of glycine ethyl ester and **3** as described in general procedure A provided compound **6e** as a white solid that was used for the next step without further purification (76% yield). ^{1}H NMR ($CDCl_3$) δ: 7.02 (m, 1 H), 5.99−5.94 (m, 2 H), 5.64 (bs, 1 H), 5.34−5.16 (m, 4 H), 4.54 (dt, J = 5.6, 1.1 Hz, 4 H), 4.19 (q, J = 7.3 Hz, 2 H), 4.07−3.99 (m, 2 H), 2.52 (b, J = 3.5 Hz, 1 H), 2.42 (dd, J = 13.2, 8.3 Hz, 1 H), 2.21−2.07 (m, 2 H), 1.94 (dd, J = 12.9, 7.8 Hz, 1 H), 1.74 (t, J = 3.0 Hz, 1 H), 1.27 (t, J = 7.0 Hz, 3 H), 1.30−1.15 (m, 1H).

Compound **6e** was deprotected as described in the general procedure B. The residue was suspended in 2 mL of THF, and an amount of 4 mL of 2.5 N aqueous lithium hydroxide was added. The reaction mixture was stirred for 2 h, acidified to pH 3 with 6 N HCl, and concentrated to dryness. Purification by ion exchange chromatography provided **8e** as a white solid (70% yield): mp 240 °C (with decomposition). $[\alpha]^{25}_D$ −40° (*c* 0.5, 1 N HCl). ^{1}H NMR (D_2O + pyridine-d_5) δ: 3.75 (s, 2 H), 2.10−2.00 (m, 5 H), 1.76 (t, J = 2.7 Hz, 1 H), 1.68−1.55 (m, 1 H). ^{13}C NMR (D_2O + pyridine-d_5) δ: 179.0, 175.9, 171.2, 66.5, 43.6, 31.1, 29.5, 27.9, 25.0, 24.3.

(1*S*,2*S*,5*R*,6*S*)-2-Amino-2-[(1′*S*)-carboxy-3′-methylthiopropyl]carbamoylbicyclo[3.1.0]hexane-6-carboxylic Acid (8f). (1*S*,2*S*,5*R*,6*S*)-2-Allyloxycarbonylamino-2-[(1′*S*)-methoxycarbonyl-3′-methylthiopropyl]carbamoylbicyclo[3.1.0]-hexane-6-carboxylic Acid Allyl Ester (6f). The reaction of L-methionine methyl ester hydrochloride and **2** as described in general procedure A provided compound **6f** as a white foamy solid that was used for the next step without further purification (90% yield). ^{1}H NMR ($CDCl_3$) δ: 7.21 (brs, 1 H), 5.89 (m, 2 H), 5.40−5.20 (m, 4 H), 4.67 (m, 1 H), 4.52 (d, 4 H, J = 7.0 Hz), 3.74 (s, 3 H), 2.55−2.37 (m, 4 H), 2.08 (s, 3 H), 2.30−1.89 (m, 5 H), 1.71 (t, 1 H, J = 2.4 Hz), 1.21 (m, 1 H). ^{13}C NMR ($CDCl_3$) δ: 172.5, 172.2, 171.9, 155.7, 132.4, 131.9, 118.3, 117.9, 67.0, 65.9, 65.3, 52.4, 51.5, 34.3, 32.7, 31.2, 28.9, 26.4, 21.2, 15.3.

Compound **6f** was deprotected as described in the general procedure B. The residue obtained was dissolved in 10 mL of 2.5 N lithium hydroxide and stirred overnight. The solution was acidified to pH 2 with 1 N aqueous HCl and extracted with ethyl acetate (4×). The combined organic extracts were dried over $MgSO_4$ and concentrated to dryness. The resulting material was purified by anion exchange chromatography to provide **8f** as a white solid (50% yield): mp 72−74 °C. $[\alpha]^{25}_D$ −62° (*c* 0.95, 1 N HCl). ^{1}H NMR (D_2O) δ: 4.26 (dd, 1 H, J = 8.0, 4.6 Hz), 2.54−2.31 (m, 2 H), 2.00 (s, 3 H), 2.10−1.85 (m, 7 H), 1.77 (t, 1 H, J = 2.7 Hz), 1.58 (m, 1 H). ^{13}C NMR (D_2O) δ: 178.4, 177.3, 170.6, 66.4, 54.6, 31.2, 30.2, 29.7, 29.5, 28.0, 25.3, 23.9, 14.2. MS (electrospray), *m/z*: 317 (M^+ + 1).

(1′*S*,2′*S*,5′*R*,6′*S*)-2′-Amino-6′-carboxybicyclo[3.1.0]-hexane-2′-carbonylpyrrolidine-2*S*-carboxylic Acid (8g). (1*S*′,2*S*′,5*R*′,6*S*′)-1-(6′-Allyloxycarbonyl-2′-allyloxycarbonylamino-2′-bicyclo[3.1.0]hexane-2′-carbonylpyrrolidine-2*S* carboxylic Acid Methyl Ester (6g). The reaction of L-proline methyl ester hydrochloride and **2** as described in general procedure A provided compound **6g** as a white foamy solid that was used for the next step without further purification (69% yield). ^{1}H NMR ($CDCl_3$) δ: 5.87 (m, 2 H), 5.25 (m, 4 H), 4.54 (d, 4 H, J = 5.9 Hz), 3.70 (s, 3 H), 3.69 (m, 1 H), 2.54 (m, 2 H), 2.13−1.85 (m, 7 H), 1.64 (m, 1 H), 1.19 (m, 1 H). ^{13}C NMR ($CDCl_3$) δ: 172.8, 172.2, 170.0, 155.8, 154.7, 132.5, 132.0,

118.0, 117.7, 66.8, 65.6, 65.1, 60.4, 51.9, 47.5, 34.6, 32.5, 31.5, 28.9, 27.8, 25.4, 20.8.

Compound **6g** was deprotected as described in the general procedure B, providing **8g** as a white solid (89% yield): mp 274 °C. $[\alpha]^{25}_D$ −103° (*c* 0.92, 1 N HCl). ^{1}H NMR (D_2O) δ: 4.25 (m, 1 H), 2.08 (m, 2 H), 1.82−2.30 (m, 10 H), 1.70 (m, 1 H), 1.44 (m, 1 H). ^{13}C NMR (D_2O) δ: 172.1, 169.5, 125.7, 67.1, 58.6, 45.8, 32.7, 32.3, 27.9, 27.1, 25.7, 22.3, 21.8. MS (electrospray), *m/z*: 303, 265.

(1*S*,2*S*,5*R*,6*S*)-2-Amino-2-[(1′*S*)-carboxy-(2′*R*)-methylbutyl]carbamoylbicyclo[3.1.0]hexane-6-carboxylic Acid (8h). (1*S*,2*S*,5*R*,6*S*)-2-Allyloxycarbonylamino-2-[(1′*S*)-allyloxycarbonyl-(2′*R*)-methylbutyl]carbamoylbicyclo[3.1.0]-hexane-6-carboxylic Acid Allyl Ester (6h). The reaction of L-isoleucine allyl ester and **2** as described in general procedure A provided compound **6h** as a white solid that was used for the next step without further purification (72% yield). ^{1}H NMR ($CDCl_3$) δ: 5.90−5.87 (m, 3 H), 5.53 (bs, 1 H), 5.37−5.16 (m, 6 H), 4.63−4.52 (m, 7 H), 2.53 (bs, 1 H), 2.40 (dd, *J* = 13.4, 8.3 Hz, 1 H), 2.12−1.87 (m, 4 H), 1.70 (t, *J* = 3.0 Hz, 1 H), 1.48−1.05 (m, 3 H), 0.90 (t, *J* = 7.3 Hz, 3 H), 0.90 (d, *J* = 7.0 Hz, 3 H).

Compound **6h** was deprotected as described in the general procedure B. The residue was purified by cation exchange chromatography, eluting with 2 N ammonia in methanol. The yellow solid obtained was stirred with methanol for 15 min, filtered, and dried to provide **8h** as a white solid (31% yield): mp >300 °C (changed color at 291 °C). $[\alpha]^{25}_D$ −40° (*c* 0.5, 1 N HCl). ^{1}H NMR (DMSO-d_6) δ: 4.03 (bs, 1 H), 1.82 (m, 7 H), 1.54−1.38 (m, 1 H), 1.29−1.02 (m, 2 H), 0.87−0.80 (m, 6 H). ^{13}C NMR (DMSO-d_6) δ: 174.4, 173.5, 173.2, 65.7, 57.6, 37.7, 35.7, 33.7, 28.5, 27.0, 25.2, 21.6, 16.1, 12.0.

(1*S*,2*S*,5*R*,6*S*)-2-Amino-2-[(1′*S*)-carboxy-2′-(4″-hydroxyphenyl)ethyl]carbamoylbicyclo[3.1.0]hexane-6-carboxylic Acid (8i). (1*S*,2*S*,5*R*,6*S*)-2-*tert*-Butoxycarbonylamino-2-[2-(4-hydroxyphenyl)-1′*S*-methoxycarbonylethyl]carbamoylbicyclo[3.1.0]hexane-6-carboxylic Acid Methyl Ester (7i). The reaction of l-tyrosine methyl ester hydrochloride and **3** as described in general procedure A provided compound **7i** as a white solid that was used for the next step without further purification (95% yield). ^{1}H NMR ($CDCl_3$) δ: 6.98 (d, 2 H, *J* = 8.2 Hz), 6.73 (d, 2 H, *J* = 8.3 Hz), 5.11 (brs, 1 H), 4.81 (dd, 1 H, *J* = 7.5, 6.1 Hz), 3.71 (s, 3 H), 3.67 (s, 3 H), 3.15 (dd, 1 H, *J* = 13.7, 6.2 Hz), 2.99 (dd, 1 H, *J* = 13.9, 6.2 Hz), 2.45 (dd, 1 H, *J* = 12.6, 7.5 Hz), 2.25−1.82 (m, 4 H), 1.64 (t, 1 H, *J* = 2.9 Hz), 1.40 (s, 9 H), 1.12 (m, 1 H). ^{13}C NMR ($CDCl_3$) δ: 173.0, 172.0, 155.5, 155.5, 155.2, 130.2 (2 C), 127.0, 115.6 (2 C), 80.7, 67.0, 53.4, 52.2, 51.8, 37.1, 35.2, 32.8, 28.7, 28.0 (3 C), 26.8, 21.3.

Compound **7i** was deprotected as described in the general procedure C to provide **8i** as a white solid (87% yield): mp 174−176 °C. $[\alpha]^{25}_D$ −47° (*c* 1.1, 1 N HCl). ^{1}H NMR (D_2O) δ: 7.13 (d, 2 H, *J* = 8.0 Hz), 6.80 (d, 2 H, *J* = 6.8 Hz), 4.79 (m, 1 H), 3.32 (dd, 1 H, *J* = 14.2, 5.4 Hz), 2.96 (dd, 1 H, *J* = 14.2, 10.7), 2.25−2.10 (m, 2 H), 1.92−1.79 (m, 2 H), 1.59−1.41 (m, 3 H). ^{13}C NMR (CD_3OD) δ: 173.1, 173.0, 169.5, 156.0, 129.7, 114.9, 65.9, 53.7, 35.2, 31.1, 30.4, 28.9, 24.9, 21.4. MS (electrospray), *m/z*: 349 ($M^+ + 1$), 332.

(1*S*,2*S*,5*R*,6*S*)-2-Amino-2-[(1′*S*)-carboxy-(2′*R*)-hydroxypropyl]carbamoylbicyclo[3.1.0]hexane-6-carboxylic Acid Hydrochloride (8j). (1*S*,2*S*,5*R*,6*S*)-Methyl 2-[(2′*S*)2-*tert*-Butoxycarbonylamino-3-(*tert*-butyldimethylsilanyloxy)-propionylamino]bicyclo[3.1.0]hexane-2,6-dicarboxylate (7j). The reaction of (2*S*)-2-amino-3-(*tert*-butyldimethylsilanyloxy)propionic acid methyl ester[30] and **3** as described in general procedure A provided compound **7j** as a white foamy solid (82% yield). ^{1}H NMR ($CDCl_3$) δ: 7.44 (bs, 1 H), 5.38 (bs, 1 H), 4.16−4.05 (m, 1 H), 3.94 (dd, *J* = 9.7, 4.3 Hz, 1 H), 3.72 (s, 3 H), 3.65 (s, 3 H), 3.60−3.51 (m, 1 H), 2.64 (dd, *J* = 14.0, 8.6 Hz, 1 H), 2.30−2.11 (m, 2 H), 2.03−1.97 (m, 2 H), 1.72 (m, 1 H), 1.43 (s, 9 H), 1.26−1.09 (m, 1 H), 0.88 (s, 9 H), 0.11 (s, 3 H), 0.10 (s, 3 H).

Compound **7j** was dissolved in dry THF (10 mL) under nitrogen, and 1 M tetrabutylammonium fluoride was added. The reaction mixture was stirred at room temperature for 1 h, the solvent was evaporated, and the residue was purified by chromatography (eluent EtOAc). The solid was subjected to the general procedure of deprotection C. The final hydrochloride was purified by HPLC to provide **8j** as a very hygroscopic white solid (11% yield). $[\alpha]^{25}_D$ −14° [*c* 1, 1 N HCl]. ^{1}H NMR ($CDCl_3$) δ: 4.01−3.71 (m, 3 H), 2.39 (dd, *J* = 6.4, 3.0 Hz, 1 H), 2.19−1.86 (m, 4 H), 1.59 (t, *J* = 2.7 Hz, 1 H), 1.41−1.25 (m, 1 H). ^{13}C NMR (D_2O) δ: 177.0, 175.9, 167.8, 66.4, 60.2, 54.4, 34.2, 31.4, 29.1, 25.5, 20.7. MS (electrospray) *m/z*: 273 ($M^+ + 1$).

Because of the hygroscopicity of the compound, it was dissolved in 10 mL of a solution of saturated HCl(g) in EtOAc and stirred for 2 h. The white precipitate was filtered and washed with EtOAc. The **8j**·HCl salt was more stable to moisture than the corresponding zwitterion: mp 197−200 °C. IR (KBr): 3436, 3213, 1711, 1684 cm^{-1}.

(1*S*,2*S*,5*R*,6*S*)-2-Amino-6-[(1′*S*)-carboxy-2′-phenylethyl]-carbamoylbicyclo[3.1.0]hexane-2-carboxylic Acid Hydrochloride (10·HCl). (1*S*,2*S*,5*R*,6*S*)-3-Allyloxycarbonyl-5-oxo-4-spiro-6′-[(1″*S*)-carboxy-2″-phenylethyl]carbamoyl-2′(bicyclo[3,1,0]hexane)oxazolidine (9). The reaction of L-phenylalanine allyl ester and **4** as described in general procedure A provided compound **9** as a white foamy solid (47%). ^{1}H NMR (CD_3OD) δ: 7.33−7.24 (m, 3 H), 7.15−7.10 (m, 2 H), 6.17 (d, *J* = 7.8 Hz, 1 H), 6.00−5.75 (m, 2 H), 5.34−5.18 (m, 6 H), 4.87 (dt, *J* = 8.1, 5.9 Hz, 1 H), 4.61−4.57 (m, 4 H), 3.12 (d, *J* = 6.2 Hz, 2 H), 2.43−2.15 (m, 3 H), 2.07−1.86 (m, 3 H), 1.77−1.70 (m, 1 H).

Compound **9** was deprotected as described in the general procedure B. The residue was dissolved in a solution of ethyl acetate saturated with hydrogen chloride gas and stirred for 2 h. The reaction mixture was filtered, and the filtrate was washed with ethyl acetate and ether providing **10**·HCl as a white solid (91%). ^{1}H NMR (CD_3OD) δ: 7.33−7.23 (m, 5 H), 4.71 (dd, *J* = 8.41, 5.40 Hz, 1 H), 3.22 (dd, *J* = 13.81, 5.20 Hz, 1 H), 3.00 (dd, *J* = 14.01, 8.41 Hz, 1 H), 2.21−1.86 (m, 6 H), 1.58−1.43 (m, 1 H). ^{13}C NMR (CD_3OD) δ: 173.8, 171.5, 171.3, 128.9 128.0, 126.4, 65.4, 53.6, 37.1, 31.4, 30.1, 27.4, 26.3, 22.2.

(1*S*,2*S*,5*R*,6*S*)-2-[(2′*S*)-(2′-Amino)propionyl]aminobicyclo[3.1.0]hexane-2,6-dicarboxylic Acid (12a). (1*S*,2*S*,5*R*,6*S*)-2-[(2′*S*)-(2′-*tert*-Butoxycarbonylamino)propionyl]-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic Acid Dimethyl Ester (11a). The reaction of **5** with *N*-Boc-L-alanine following general procedure A provided **11a** as a foamy white solid (50% yield): mp 51−52 °C. $[\alpha]^{25}_D$ −28° (*c* 0.52, $CHCl_3$). ^{1}H NMR ($CDCl_3$) δ: 7.28 (bs, 1H), 5.04 (brd, 1H, *J* = 7.6 Hz), 4.16 (m, 1H), 3.74 (s, 3H), 3.66 (s, 3H), 2.49 (dd, 1H, *J* = 13.9, 8.3 Hz), 2.42 (dd, 1H, *J* = 6.3, 2.8 Hz), 2.18−1.89 (m, 3H), 1.70 (t, 1H, *J* = 2.9 Hz), 1.45 (s, 9H), 1.33 (d, 3H, *J* = 7.0 Hz), 1.19 (m, 1H). ^{13}C NMR ($CDCl_3$) δ: 172.8, 172.6, 172.6, 155.7, 80.2, 66.3, 52.6, 51.8, 49.5, 34.4, 32.0, 28.2, 28.1, 26.6, 21.1, 17.6.

Compound **11a** was deprotected following general procedure D to provide **12a**·HCl as a white solid (80% yield): mp >250 °C, dec. $[\alpha]^{25}_D$ −7.8° (*c* 1.0, MeOH). ^{1}H NMR (CD_3OD) δ: 3.96 (q, 1H, *J* = 7.0 Hz), 2.47 (dd, 1H, *J* = 6.3, 2.7 Hz), 2.37 (dd, 1H, *J* = 13.6, 8.2 Hz), 2.18−1.92 (m, 3H), 1.66 (t, 1H, *J* = 3.0 Hz), 1.53 (d, 3H, *J* = 7.0 Hz), 1.46−1.34 (m, 1H). ^{13}C NMR (CD_3OD) δ: 175.2, 174.7, 170.2, 66.4, 49.0, 36.6, 32.0, 28.5, 26.3, 21.2, 16.6.

For complete characterization it was transformed into the crystalline sulfonate salt (**12a**·HSO_3Me). (1*S*,2*S*,5*R*,6*S*)-2-[(2′*S*)-(2′-Amino)propionyl]aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid hydrochloride (**12a**·HCl) (1.0 g, 3.42 mmol) was dissolved in water (1 mL), and 1.0 N NaOH (3.42 mL, 3.42 mmol) was added. The solution was maintained in the refrigerator for 24 h. The solution remained clear. Acetone (2 mL) was added, and the solution was stored in the refrigerator for 16 h. A white solid precipitated out of solution, and the mixture could not be stirred. Acetone (4 mL) was added, and the mixture was stirred at room temperature, then filtered and dried to afford 630 mg of the title compound as a white crystalline solid corresponding to the zwitterion (72% yield), which contained 2−4% NaCl. ^{1}H NMR (CD_3OD) δ: 3.93 (q, *J*

= 7.1 Hz, 1 H), 2.48 (dd, *J* = 6.6, 2.9 Hz, 1 H), 2.32 (dd,, *J* = 13.5, 8.4 Hz, 1 H), 2.20−2.08 (m, 1 H), 2.01−1.90 (m, 2 H), 1.61 (t, *J* = 2.9 Hz, 1 H), 1.51 (d, *J* = 7.0 Hz, 3 H), 1.48−1.33 (m, 1 H). ^{13}C NMR (CD_3OD) *δ*: 176.9 (2 C), 171.1, 68.0, 50.1, 35.9, 33.2, 29.7, 27.3, 22.5, 17.6.

A solution of **12a** (1.07 g, 3.00 mmol), methanesulfonic acid (0.584 mL, 9.00 mmol), and dioxane (10 mL) was stirred for 48 h. The mixture was filtered and dried to afford **12a**·HSO_3-Me as a crude, white, amorphous solid (1.05 g). A sample of this solid (1.0 g) was dissolved in MeOH (10 mL). The solution was concentrated to 3.3 g total weight, and seed crystals were added. Ethyl acetate (10 mL) was then added to the mixture over a period of 15 min. The mixture was stirred for 30 min, filtered, and dried under vacuum to afford 830 mg of the title compound as a white, crystalline solid (78% yield). ^{1}H NMR (CD_3OD) *δ*: 3.96 (q, *J* = 7.1 Hz, 1 H), 2.71 (s, 3 H), 2.45 (dd, *J* = 6.4, 2.7 Hz, 1 H), 2.38 (dd, *J* = 13.9, 8.4 Hz, 1 H), 2.20−2.08 (m, 1 H), 2.01−1.93 (m, 2 H), 1.67 (t, *J* = 2.9 Hz, 1 H), 1.52 (d, *J* = 7.0 Hz, 3 H), 1.46−1.35 (m, 1 H). ^{13}C NMR (CD_3OD) *δ*: 176.3, 175.7, 171.2, 67.4, 50.0, 39.5, 35.7, 33.1, 29.5, 27.4, 22.2, 17.6. Anal. ($C_{12}H_{20}N_2O_8S$) C, H, N.

(1*S*,2*S*,5*R*,6*S*)-2-[(2′*R*)-(2′-Amino)propionyl]aminobicyclo[3.1.0]hexane-2,6-dicarboxylic Acid Hydrochloride (12b·HCl). (1*S*,2*S*,5*R*,6*S*)-2-[(2′*R*)-*tert*-Butoxycarbonylamino)propionyl]aminobicyclo[3.1.0]hexane-2,6-dicarboxylic Acid Dimethyl Ester (11b). The reaction of **5** with *N*-Boc-D-alanine following general procedure A provided **11b** as a foamy white solid (93% yield). ^{1}H NMR ($CDCl_3$) *δ*: 7.03 (s, 1 H), 5.00 (s, 1 H), 4.12 (br m, 1 H), 3.72 (s, 3 H), 3.64 (s, 3 H), 2.52 (dd, *J* = 14.0, 8.5 Hz, 1 H), 2.39 (dd, *J* = 6.5, 3.0 Hz, 1 H), 2.18−2.10 (m, 1 H), 2.01 (app. quintet, *J* = 3.5 Hz, 1 H), 1.94 (dd, *J* = 13.5, 8.0 Hz, 1 H), 1.67 (t, *J* = 3.0 Hz, 1 H), 1.44 (s, 9 H), 1.31 (d, *J* = 7.0 Hz, 3 H), 1.24−1.17 (m, 1 H). ^{13}C NMR ($CDCl_3$) *δ*: 172.8, 172.7, 172.6, 155.7, 80.2, 66.1, 52.7, 51.8, 49.6, 34.4, 32.0, 28.2, 28.1, 26.6, 21.0, 17.4.

Compound **11b** was deprotected following general procedure D to provide **12b**·HCl as a white solid (80% yield). ^{1}H NMR (CD_3OD) *δ*: 3.92 (q, *J* = 7.1 Hz, 1 H), 2.56 (dd, *J* = 6.4, 2.7 Hz, 1 H), 2.25 (*J* = 14.0, 8.5 Hz, 1 H), 2.13−2.06 (m, 1 H), 2.01 (m, 1 H), 1.95 (dd, *J* = 12.8, 8.0 Hz, 1 H), 1.62 (t, *J* = 3.0 Hz, 1 H), 1.51 (d, *J* = 6.9 Hz, 3 H), 1.43−1.37 (m, 1 H). ^{13}C NMR (CD_3OD) *δ*: 176.4, 175.7, 171.2, 67.3, 50.0, 35.5, 32.8, 30.0, 26.7, 21.9, 17.7.

(1*S*,2*S*,5*R*,6*S*)-2-[(2′*S*)-(2′-Amino-4′-methyl)pentanoyl]aminobicyclo[3.1.0]hexane-2,6-dicarboxylic Acid Hydrochloride (12c·HCl). (1*S*,2*S*,5*R*,6*S*)-2-[(2′*S*)-(2′-*tert*-Butoxycarbonylamino-4′-methyl)pentanoyl]aminobicyclo[3.1.0]hexane-2,6-dicarboxylic Acid Dimethyl Ester (11c). The reaction of **5** with *N*-Boc-L-leucine following general procedure A provided **11c** as a foamy white solid (95% yield): mp 62−63 °C. $[\alpha]^{25}_D$ −27° (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$) *δ*: 7.00 (bs, 1H), 4.97 (bd, 1H, *J* = 8.4), 4.05 (m, 1H), 3.69 (s, 3H), 3.62 (s, 3H), 2.40 (m, 2H), 2.08−1.84 (m, 3H), 1.67−1.58 (m, 3H), 1.41 (m, 1H), 1.41 (s, 9H), 1.22 (m, 1H), 0.90 (d, 3H, *J* = 6.2 Hz), 0.89 (d, 3H, *J* = 6.1 Hz). ^{13}C NMR ($CDCl_3$) *δ*: 172.8, 172.6, 172.5, 155.8, 80.0, 66.2, 52.5, 51.7, 40.7, 34.3, 31.9, 28.2, 28.1, 26.4, 24.6, 22.7, 22.0, 21.0.

Compound **11c** was deprotected following general procedure D to provide **12c**·HCl as a white solid (48% yield): mp 135−137 °C. $[\alpha]^{25}_D$ +0.3° (*c* 1.0, MeOH). ^{1}H NMR (methanol-d_4) *δ*: 3.84 (bt, 1H, *J* = 6.6 Hz), 2.52 (dd, 1H, *J* = 6.2, 2.8 Hz), 2.31 (dd, 1H, *J* = 13.5, 8.6 Hz), 2.21−1.89 (m, 3H), 1.74−1.59 (m, 4H), 1.44 (m, 1H), 1.02 (d, 3H, *J* = 6.4 Hz), 0.99 (d, 3H, *J* = 6.2 Hz). ^{13}C NMR (methanol-d_4) *δ*: 175.3, 174.9, 169.7, 66.6, 51.8, 40.6, 34.4, 32.0, 28.7, 26.1, 24.4, 21.8, 21.5, 21.3.

(1*S*,2*S*,5*R*,6*S*)-2-[(2′*R*)-Amino-4′-methyl)pentanoyl]aminobicyclo[3.1.0]hexane-2,6-dicarboxylic Acid Hydrochloride (12d·HCl). (1*S*,2*S*,5*R*,6*S*)-2-[2′*R*-*tert*-Butoxycarbonylamino-4′-methyl)pentanoyl]aminobicyclo[3.1.0]hexane-2,6-dicarboxylic Acid Dimethyl Ester (11d). The reaction of **5** with *N*-Boc-D-leucine following general procedure A provided **11d** as a foamy white solid (88% yield). ^{1}H NMR (CD_3OD) *δ*: 7.92 (1H, bs), 4.57−4.48 (1H, m), 3.75 (3H, s), 3.70 (3H, s), 2.60 (1H, bs), 2.30−1.70 (8H, m), 1.43 (9H, s), 0.99−0.90 (6H, m). MS *m/z*: 327 [M + H − CO_2′Bu].

Compound **11d** was deprotected following general procedure D to provide **12d**·HCl as a white solid (71% yield). ^{1}H NMR (CD_3OD) *δ*: 3.62 (1H, t, 7.2 Hz), 3.02 (1H, t), 2.18 (1H, dd, 2.3 Hz, 6.4 Hz), 2.04−1.91 (1H, m), 1.90−0.80 (7H, m), 0.68 (6H, 2 × t, 6.7 Hz, 6.8 Hz). MS *m/z*: 299 [M + H].

(1*S*,2*S*,5*R*,6*S*)-2-[(2′*S*)-(2′-Amino)-3′-phenylpropionyl]aminobicyclo[3.1.0]hexane-2,6-dicarboxylic Acid Hydrochloride (12e·HCl). (1*S*,2*S*,5*R*,6*S*)-2-[(2′*S*)-(2′-*tert*-Butoxycarbonylamino)-3′-phenylpropionyl]aminobicyclo[3.1.0]hexane-2,6-dicarboxylic Acid Dimethyl Ester (11e). The reaction of **5** with *N*-Boc-L-phenylalanine following general procedure A provided **11e** as a foamy white solid (87% yield): mp 60−61 °C. $[\alpha]^{25}_D$ −2° (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$) *δ*: 7.33−7.21 (m, 5H), 6.64 (bs, 1H), 5.13 (brd, 1H, *J* = 6.9 Hz), 4.32 (q, 1H, *J* = 7.0 Hz), 3.72 (s, 3H), 3.66 (s, 3H), 3.03 (d, 2H, *J* = 7.1 Hz), 2.48−2.38 (m, 2H), 2.08−1.84 (m, 3H), 1.60 (t, 1H, *J* = 2.9 Hz), 1.41 (s, 9H), 1.14−1.02 (m, 1H). ^{13}C NMR ($CDCl_3$) *δ*: 172.5, 172.4, 171.3, 155.4, 136.8, 129.4, 128.5, 126.7, 80.1, 66.1, 55.3, 52.5, 51.7, 38.1, 34.1, 31.7, 28.2, 28.0, 26.3, 20.9.

Compound **11e** was deprotected following general procedure D to provide **12e**·HCl as a white solid (44% yield): mp 122−123 °C. $[\alpha]^{25}_D$ −2° (*c* 1.0, MeOH). ^{1}H NMR (methanol-d_4) *δ*: 7.41−7.26 (m, 5H), 4.13 (dd, 1H, *J* = 7.6, 6.1 Hz), 3.28 (dd, 1H, *J* = 14.2, 6.2 Hz), 3.07 (dd, 1H, *J* = 14.2, 7.6 Hz), 2.55 (dd, 1H, *J* = 6.4, 2.8 Hz), 2.31−1.89 (m, 4H), 1.64 (t, 1H, *J* = 2.8 Hz), 1.35 (m, 1H). ^{13}C NMR (methanol-d_4) *δ*: 178.8, 178.3, 172.2, 137.9, 133.2, 132.6, 131.3, 70.0, 57.9, 41.0, 37.7, 35.4, 32.3, 29.4, 24.7.

(1*S*,2*S*,5*R*,6*S*)-2-[(2′*S*)-(2′-Amino)-3′-methylbutyryl]aminobicyclo[3.1.0]hexane-2,6-dicarboxylic Acid Hydrochloride (12f·HCl). (1*S*,2*S*,5*R*,6*S*)-2-[(2′*S*)-(2′-*tert*-Butoxycarbonylamino)-3′-methylbutyryl]aminobicyclo[3.1.0]hexane-2,6-dicarboxylic Acid Dimethyl Ester (11f). The reaction of **5** with *N*-Boc-L-valine following general procedure A provided **11f** as a foamy white solid (87% yield): mp 63−65 °C. $[\alpha]^{25}_D$ −1° (*c* 1.16, $CHCl_3$). ^{1}H NMR ($CDCl_3$) *δ*: 6.75 (bs, 1H), 5.06 (bd, 1H, *J* = 8.8 Hz), 3.90 (dd, 1H, *J* = 8.9, 8.8 Hz), 3.73 (s, 3H), 3.66 (s, 3H), 2.54 (dd, 1H, *J* = 13.8, 8.3 Hz), 2.41 (dd, 1H, *J* = 5.9, 2.4 Hz), 2.23−1.88 (m, 4H), 1.71 (t, 1H, *J* = 2.9 Hz), 1.44 (s, 9H), 1.30−1.19 (m, 1H), 0.97 (d, 3H, *J* = 6.8 Hz), 0.93 (d, 3H, *J* = 6.8 Hz). ^{13}C NMR ($CDCl_3$) *δ*: 172.5, 172.4, 171.8, 155.8, 79.8, 66.2, 59.3, 52.4, 51.7, 34.3, 31.9, 30.9, 28.2, 27.9, 26.5, 21.1, 18.9, 17.6.

Compound **11f** was deprotected following general procedure E to provide **12f**·HCl as a white solid (64% yield): mp 217−219 °C. $[\alpha]^{25}_D$ + 6° (*c* 0.86, MeOH). ^{1}H NMR (D_2O) *δ*: 3.78 (d, 1H, *J* = 5.8 Hz), 2.56 (dd, 1H, *J* = 6.9, 2.9 Hz), 2.19−1.95 (m, 5H), 1.66 (t, 1H, *J* = 3.0 Hz), 1.50 (m, 1H), 0.99 (d, 3H, *J* = 6.9 Hz), 0.98 (d, 3H, *J* = 6.9 Hz). ^{13}C NMR (D_2O + methanol-d_4) *δ*: 177.0, 176.0, 169.3, 66.3, 58.1, 34.0, 31.3, 30.0, 29.5, 25.1, 20.9, 17.3, 16.9.

(1*S*,2*S*,5*R*,6*S*)-2-(2-Aminoacetylamino)bicyclo[3.1.0]hexane-2,6-dicarboxylic Acid (12 g). (1*S*,2*S*,5*R*,6*S*)-2-[(2-*tert*-Butoxycarbonylaminoacetylamino)bicyclo[3.1.0]hexane-2,6-dicarboxylic Acid Dimethyl Ester (11g). The reaction of **5** with *N*-Boc-glycine following general procedure A provided **11g** as a foamy white solid (88% yield). ^{1}H NMR ($CDCl_3$) *δ*: 7.01 (bs, 1 H), 5.28 (bs, 1 H), 3.75 (d, *J* = 5.6 Hz, 2 H), 3.72 (s, 3 H), 3.63 (s, 3 H), 2.54−2.38 (m, 2 H), 2.15−1.87 (m, 3 H), 1.68 (t, *J* = 2.9 Hz, 1 H), 1.43 (s, 9 H), 1.31−1.11 (m, 1 H).

Compound **11g** was deprotected following general procedure D to provide **12g**·HCl as a white solid (64% yield). Further purification of the chlorohydrate with a C8 reverse-phase support, eluting with MeOH/H_2O, provided, after drying, the zwitterion **12g** as a white solid (44% yield): mp 149−156 °C. $[\alpha]^{25}_D$ −6° (*c* 1, MeOH). ^{1}H NMR (MeOH-d_4) *δ*: 3.68 (s, 2 H), 2.37 (dd, *J* = 6.2, 3.0 Hz, 1 H), 2.19−2.08 (m, 1 H), 2.01−1.85 (m, 3 H), 1.59 (t, *J* = 2.7 Hz, 1 H), 1.37−1.21 (m, 1 H).

(1*S*,2*S*,5*R*,6*S*)-2-[(2′*S*)-(2′-Amino)-(4′-methylthio)butyryl]aminobicyclo[3.1.0]hexane-2,6-dicarboxylic Acid (12h).

(1*S*,2*S*,5*R*,6*S*)-2-[(2′*S*)-(2′-*tert*-Butoxycarbonylamino)-(4′-methylthio)butyryl]aminobicyclo[3.1.0]hexane-2,6-dicarboxylic Acid Dimethyl Ester (11h). The reaction of **5** with *N*-Boc-L-methionine following general procedure A provided **11h** as a foamy white solid (89% yield). ^{1}H NMR ($CDCl_3$) δ: 7.04 (brs, 1 H), 5.15 (d, 1 H, J = 8.3 Hz), 4.13 (m, 1 H), 3.61 (s, 3 H), 3.53 (s, 3 H), 2.48−2.25 (m, 4 H), 1.97 (s, 3 H), 2.15−1.71 (m, 5 H), 1.57 (t, 1 H, J = 3.0 Hz), 1.31 (s, 9 H), 1.13 (m, 1 H). ^{13}C NMR ($CDCl_3$) δ: 172.4, 172.3, 171.5, 155.4, 79.9, 66.1, 52.7, 52.4, 51.6, 34.2, 31.8, 31.4, 29.7, 28.1 (3 C), 27.9, 26.4, 20.9, 15.0.

Compound **11h** was deprotected following general procedure D to provide **12h**·HCl as a white solid (64% yield). Further purification of the chlorohydrate with a C8 reverse-phase support, eluting with acetonitrile in water with 0.05% of trifluoroacetic acid, provided, after drying, **12h** as a zwitterion (78% yield): mp 164 °C. $[\alpha]^{25}_D$ +13° (*c* 1.1, MeOH). ^{1}H NMR (methanol-d_4) δ: 3.97 (t, 1 H, J = 6.4 Hz), 2.64−2.56 (m, 2 H), 2.48−2.38 (m, 2 H), 2.11 (s, 3 H), 2.19−1.91 (m, 5 H), 1.66 (t, 1 H, J = 3.0 Hz), 1.38 (m, 1 H). ^{13}C NMR (methanol-d_4) δ: 174.7, 174.1, 168.3, 66.1, 52.0, 34.1, 31.5, 30.8, 28.1, 28.0, 25.9, 20.8, 13.6. MS (electrospray), *m/z*: 317 (M^+ + 1), 229.

(1*S*,2*S*,5*R*,6*S*)-2-[(2′*S*)-(Pyrrolidine-2-carbonyl)]aminobicyclo[3.1.0]hexane-2,6-dicarboxylic Acid Hydrochloride (12i·HCl). (1*S*,2*S*,5*R*,6*S*)-2-[(2′*S*)-(1′-*tert*-Butoxycarbonylpyrrolidine-2′-carbonyl)amino]bicyclo[3.1.0]hexane-2,6-dicarboxylic Acid Dimethyl Ester (11i). The reaction of **5** with *N*-Boc-L-proline following general procedure A provided **11i** as a foamy white solid (78% yield). ^{1}H NMR ($CDCl_3$) δ: 4.2 (bs, 1H), 3.71 (s, 3H), 3.63 (s, 3H), 3.30 (bs, 2H), 2.47−2.45 (m, 2H), 2.03−1.77 (m, 8H), 1.65 (t, 1H, J = 3 Hz), 1.47 (s, 9H), 1.20−1.00 (m, 1H).

Compound **11i** was deprotected following general procedure D to provide **12i**·HCl as a white solid (90% yield): mp 237−240 °C. $[\alpha]^{25}_D$ −49° (*c* 0.5, 1 N HCl). ^{1}H NMR (methanol-d_4) δ: 4.31−4.24 (m, 1H), 3.37−3.28 (m, 2H), 2.45−2.41 (m, 3H), 2.14−1.98 (m, 6H), 1.65 (t, 1H, J = 2 Hz), 1.44−1.32 (m, 1H). ^{13}C NMR (methanol-d_4) δ:174.7, 174.1, 168.4, 66.2, 59.5, 46.1, 34.3, 31.6, 29.6, 28.0, 26.0, 23.4, 20.8.

(1*S*,2*S*,5*R*,6*S*)-2-[(2′*S*,3′*S*)-(2′-Amino-3′-methylpentanoylamino)bicyclo[3.1.0]hexane-2,6-dicarboxylic Acid Hydrochloride (12j·HCl). (1*S*,2*S*,5*R*,6*S*)-2-[(2′*S*,3′*S*)-(2′-(2-*tert*-Butoxycarbonylamino-3-methylpentanoylamino)-bicyclo[3.1.0]hexane-2,6-dicarboxylic Acid Dimethyl Ester (11j). The reaction of **5** with *N*-Boc-L-isoleucine following general procedure A provided **11j** as a foamy white solid (79% yield). ^{1}H NMR ($CDCl_3$) δ: 6.75 (bs, 1 H), 5.06 (bd, J = 8.3 Hz, 1 H), 3.90 (dd, J = 8.9, 6.7 Hz, 1 H), 3.70 (s, 3H), 3.63 (s, 3 H), 2.54−2.39 (m, 2 H), 2.21−1.72 (m, 4 H), 1.67 (t, J = 3.2 Hz, 1 H), 1.60−1.40 (m, 1H), 1.41 (s, 9H), 1.22−1.0 (m, 2H), 0.91 (d, J = 6.7 Hz,3 H), 0.87 (t, J = 7.3 Hz, 3 H).

Compound **11j** was deprotected following general procedure D to provide **12j**·HCl as a white solid (64% yield): mp >300 °C. $[\alpha]^{25}_D$ −5° (*c* 1, 1 N HCl). ^{1}H NMR (MeOH-d_4) δ: 8.93 (s, 1 H), 3.73 (d, J = 5.4 Hz, 1 H), 2.54 (dd, J = 6.4, 3.0 Hz, 1 H), 2.35−1.91 (m, 5 H), 1.67−1.15 (m, 4 H), 1.06 (d, J = 7.0 Hz, 3 H), 0.98 (t, J = 7.3 Hz, 3 H). ^{13}C NMR (MeOH-d_4) δ: 176.2, 175.7, 169.6, 67.4, 58.7, 38.1, 35.3, 32.9, 29.8, 26.9, 25.2, 22.2, 15.1, 11.7.

(1*S*,2*S*,5*R*,6*S*)-2-[(2′*S*)-(2′-Amino)-(3′-*p*-hydroxyphenyl)-propionyl]aminobicyclo[3.1.0]hexane-2,6-dicarboxylic Acid (12k). (1*S*,2*S*,5*R*,6*S*)-2-[(2′*S*)-(2′-*tert*-Butoxycarbonylamino)-(3′-*p*-hydroxyphenyl)propionyl]aminobicyclo-[3.1.0]hexane-2,6-dicarboxylic Acid Dimethyl Ester (11k). The reaction of **5** with *N*-Boc-L-tyrosine following general procedure A provided **11k** as a foamy white solid (73% yield). ^{1}H NMR (methanol-d_4) δ: 7.03 (d, 2 H, J = 8.0 Hz), 6.72 (d, 2 H, J = 8.0 Hz), 5.21 (bs, 1 H), 4.28 (m, 1 H), 3.75 (s, 3 H), 3.63 (s, 3 H), 2.91 (m, 2 H), 2.39 (m, 2 H), 2.15−1.80 (m, 3 H), 1.59 (t, 1 H, J = 4.0 Hz), 1.39 (s, 9 H). ^{13}C NMR (methanol-d_4) δ: 173.6, 171.7, 155.3, 130.4 (2 C), 127.8, 121.2, 115.4 (2 C), 80.3, 66.2, 52.6, 51.8, 37.4, 34.1, 31.7, 28.1 (3 C), 26.3, 22.5, 21.0.

Compound **11k** was deprotected following general procedure D to provide **12k**·HCl as a white solid. Further purification of the chlorohydrate with a C8 reverse-phase support, eluting with acetonitrile in water with 0.05% of trifluoroacetic acid, provided, after drying, **12k** as a zwitterion (35% yield): mp 169 °C. $[\alpha]^{25}_D$ −2° (*c* 0.95, MeOH). ^{1}H NMR (methanol-d_4) δ: 7.13 (d, 2 H, J = 8.6 Hz), 6.79 (d, 2 H, J = 8.6 Hz), 4.02 (dd, 1 H, J = 7.5, 5.9 Hz), 3.18 (dd,1 H, J = 14.2, 5.9 Hz), 2.94 (dd, 1 H, J = 14.4, 7.5 Hz), 2.54 (dd, 1 H, J = 6.3, 2.8 Hz), 2.34−1.89 (m, 4 H), 1.66 (t, 1 H, J = 3.0 Hz), 1.35 (m, 1 H). ^{13}C NMR (methanol-d_4) δ: 174.9, 174.3, 168.5, 156.7, 130.3, 124.5, 115.4, 66.0, 54.2, 36.3, 33.9, 31.5, 28.3, 25.5, 20.8. MS (electrospray), *m/z*: 349 (M^+ + 1), 331.

(1*S*,2*S*,5*R*,6*S*)-2-[(2′*S*)-2′-Amino-3′-hydroxypropionyl-amino)bicyclo[3.1.0]hexane-2,6-dicarboxylic Acid (12l). (1*S*,2*S*,5*R*,6*S*)-2-[(2′*S*)-2-*tert*-Butoxycarbonylamino-3-(*tert*-butyldimethylsilanyloxy)propionylamino]bicyclo[3.1.0]-hexane-2,6-dicarboxylic Acid Dimethyl Ester (11l). The reaction of **5** with (2*S*)-2-*tert*-butoxycarbonylamino-3-(*tert*-butyldimethylsilanyloxy)propionic acid methyl ester[31] following general procedure A provided **11l** as a foamy white solid (82% yield). ^{1}H NMR ($CDCl_3$) δ: 7.44 (bs, 1 H), 5.38 (bs, 1 H), 4.16−4.05 (m, 1 H), 3.94 (dd, J = 9.7, 4.3 Hz, 1 H), 3.72 (s, 3 H), 3.65 (s, 3 H), 3.60−3.51 (m, 1 H), 2.64 (dd, J = 14.0, 8.6 Hz, 1 H), 2.30−2.11 (m, 2 H), 2.03−1.97 (m, 2 H), 1.72 (m, 1 H), 1.43 (s, 9 H), 1.26−1.09 (m, 1 H), 0.88 (s, 9 H), 0.11 (s, 3 H), 0.10 (s, 3 H).

Compound **11l** was dissolved in dry THF (10 mL) under nitrogen, and 1 M tetrabutylammonium fluoride in THF was added. The reaction mixture was stirred at room temperature for 1 h, the solvent was evaporated, and the residue was purified by silica gel chromatography, eluting with ethyl acetate. The desilylated material was subjected to general procedure D. The resulting chlorohydrate was purified by HPLC to provide **12l** as a very hygroscopic white solid (11% yield): mp (HCl salt) 197−200 °C. $[\alpha]^{25}_D$ −14° [*c* 1, 1 N HCl]. ^{1}H NMR ($CDCl_3$) δ: 4.01−3.71 (m, 3 H), 2.39 (dd, J = 6.4, 3.0 Hz, 1 H), 2.19−1.86 (m, 4 H), 1.59 (t, J = 2.7 Hz, 1 H), 1.41−1.25 (m, 1 H). ^{13}C NMR (D_2O) δ: 177.0, 175.9, 167.8, 66.4, 60.2, 54.4, 34.2, 31.4, 29.1, 25.5, 20.7.

(1*S*,2*S*,5*R*,6*S*)-2-[(2′*S*)-(2′,6′-Diamino)hexanoyl]aminobicyclo[3.1.0]hexane-2,6-dicarboxylic Acid Dihydrochloride (12m·2HCl). (1*S*,2*S*,5*R*,6*S*)-2-[(2′*S*)-(2′,6′-Bis-*tert*-butoxycarbonylamino)hexanoyl]aminobicyclo[3.1.0]hexane-2,6-dicarboxylic Acid Dimethyl Ester (11m). The reaction of **5** with *N*α-Boc-*N*ϵ-Boc-L-lysine following general procedure A provided **11m** as a foamy white solid (94% yield): mp 65−67 °C. $[\alpha]^{25}_D$ −11° (*c* 1.05, $CHCl_3$). ^{1}H NMR ($CDCl_3$) δ: 6.97 (brs, 1H), 5.12 (brd, 1H, J = 7.0 Hz), 4.76 (brs, 1H), 4.06 (brq, 1H, J = 7.4 Hz), 3.75 (s, 3H), 3.66 (s, 3H), 3.11 (AB system, 2H), 2.50 (dd, 1H, J = 13.8, 8.3 Hz), 2.42 (dd, 1H, J = 6.5, 2.7 Hz), 2.23−1.76 (m, 5H), 1.69 (t, 1H, J = 2.9 Hz), 1.63−1.11 (m, 5H), 1.43 (s, 9H), 1.42 (s, 9H). ^{13}C NMR ($CDCl_3$) δ: 172.6, 172.5, 172.3, 156.1, 155.8, 80.1, 66.3, 53.7, 52.7, 51.9, 39.8, 34.4, 32.0, 31.7, 29.5, 28.4, 28.3, 28.1, 26.6, 22.3, 21.1.

Compound **11m** was deprotected following general procedure D to provide **12m**·2HCl as a white solid (82% yield): mp 75−77 °C. $[\alpha]^{25}_D$ −1° (*c* 1.0, MeOH). ^{1}H NMR (D_2O) δ: 3.90 (t, 1H, J = 6.3 Hz), 2.84 (bt, 2H, J = 7.6 Hz), 4.42 (dd, 1H, J = 6.3, 2.8 Hz), 2.09−1.73 (m, 6H), 1.59−1.26 (m, 6H). ^{13}C NMR (D_2O + methanol-d_4) δ: 177.1, 176.3, 169.4, 66.1, 52.4, 38.8, 33.8, 31.2, 30.2, 29.3, 26.3, 25.1, 20.8, 20.6.

(1*S*,2*S*,5*R*,6*S*)-2-[2′(*S*)-(2″(*S*)-Aminopropionylamino)-propionylamino]bicyclo[3.1.0]hexane-2,6-dicarboxylic Acid (12n). 2(*S*)-(2′(*S*)-*tert*-Butoxycarbonylaminopropionylamino)propionic Acid (*N*-Boc-L-alanyl-L-alanine). In a flask containing L-alanyl-L-alanine (5 mmol) were added di-*tert*-butyl dicarbonate (10 mmol) dissolved in 15 mL of dioxane and 15 mL of saturated aqueous sodium bicarbonate. The reaction mixture was stirred at room temperature overnight. It was diluted with water (100 mL) and washed with EtOAc (9 × 50 mL). The aqueous layer was acidified to pH 1 with 6 N hydrochloric acid and extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, filtered, and

evaporated to provide the dipeptide as an oil (88% yield). ^{1}H NMR ($CDCl_3$) δ: 9.90(bs, 1H), 7.04 (bs, 1 H), 5.46 (bs, 1 H), 4.54 (m, 1 H), 4.20 (m, 1 H), 1.50−1.30 (m, 15 H).

(1*S*,2*S*,5*R*,6*S*)-2-[2′(*S*)-(2″(*S*)-*tert*-butoxycarbonylamino-propionylamino)propionylamino]bicyclo[3.1.0]hexane-2,6-dicarboxylic Acid Dimethyl Ester (11n). The reaction of **5** with *N*-Boc-L-alanyl-L-alanine following general procedure A provided **11n** as a foamy white solid (90% yield). ^{1}H NMR ($CDCl_3$) δ: 7.26 (bs, 1 H), 6.66 (bd, J = 7.5 Hz, 1 H), 4.98 (bd, J = 7.0 Hz, 1 H), 4.46 (quint, J = 7.2 Hz, 1 H), 4.13 (m, J = 7.2 Hz, 1 H), 3.74 (s, 3H), 3.65 (s, 3H), 2.48−2.36 (m, 2H), 2.14−1.87 (m, 3H), 1.69 (t, J = 2.4 Hz, 1H), 1.43 (s, 9 H), 1.36 (d, J = 7.0 Hz, 3 H), 1.35 (d, J = 7.0 Hz, 3 H).

Compound **11n** was deprotected following general procedure D to provide **12n**·HCl as a white solid. Purification by C8 reverse-phase chromatography, eluting with MeOH/H_2O, provided the zwitterion **12n** as a white solid (45% yield): mp 193 °C. $[\alpha]^{25}_D$ −25° (*c* 1.05, MeOH). IR (KBr): 3380, 3285, 1664 cm^{-1}. ^{1}H NMR (MeOH-d_4) δ: 8.67 (bs, 1 H), 4.43 (q, J = 7.3 Hz, 1 H), 3.93 (q, J = 7.0 Hz, 1 H), 2.45−2.34 (m, 2 H), 2.16−1.88 (m, 3 H), 1.66 (t, J = 3.2 Hz, 1 H), 1.50 (d, J = 7.3 Hz, 3 H), 1.35 (d, J = 7.3 Hz, 3 H), 1.33 (m, 1 H). ^{13}C NMR (MeOH-d_4) δ: 176.8, 176.3, 175.0, 171.0, 67.7, 50.6, 50.4, 36.1, 33.3, 29.8, 27.8, 22.7, 18.6, 18.0.

(1*S*,2*S*,5*R*,6*S*)-2-[(2′*S*)-((2″*S*)-Amino-4-methylpentanoylamino)propionylamino]bicyclo[3.1.0]hexane-2,6-dicarboxylic Acid (12o). 2(*S*)-(2′(*S*)-*tert*-Butoxycarbonylamino-4-methylpentanoylamino)propionic Acid (*N*-Boc-L-leucinyl-L-alanine). In a flask containing L-leucinyl-L-alanine (5 mmol) were added di-*tert*-butyl dicarbonate (10 mmol) dissolved in 15 mL of dioxane and 15 mL of saturated aqueous sodium bicarbonate. The reaction mixture was stirred at room temperature overnight. It was diluted with water (100 mL) and washed with EtOAc (2 × 50 mL). The aqueous layer was acidified to pH 1 with 6 N hydrochloric acid and extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, filtered, and evaporated to provide acid (*N*-Boc-L-leucinyl-L-alanine as a foamy white solid (78% yield). ^{1}H NMR ($CDCl_3$) δ: 6.90 (bs, 1 H), 5.15 (bs, 1 H), 4.63−4.46 (m, 1 H), 4.17−4.06 (m, 2 H), 1.75−1.43 (m, 15 H), 0.95−0.91 (m, 6 H).

(1*S*,2*S*,5*R*,6*S*)-2-[(2′*S*)-(2″*S*)-*tert*-Butoxycarbonylamino-4-methylpentanoylamino)propionylamino]bicyclo[3.1.0]-hexane-2,6-dicarboxylic Acid Dimethyl Ester (11o). The reaction of **5** with *N*-Boc-L-leucinyl-L-alanine following general procedure A provided **11o** as a foamy white solid (95% yield). ^{1}H NMR ($CDCl_3$) δ: 7.09 (s, 1 H), 6.62 (d, J = 7.3 Hz, 1 H), 4.90 (d, J = 7.3 Hz, 1 H), 4.45 (m, 1 H), 4.08 (m, 1 H), 3.72 (s, 3 H), 3.65 (s, 3 H), 2.47−2.37 (m, 2 H), 2.07−1.53 (m, 7 H), 1.43 (s, 9 H), 1.34 (d, J = 7.0 Hz, 3 H), 1.40−1.20 (m, 1H), 0.95−0.92 (m, 6 H).

Compound **11o** was deprotected following general procedure D to provide **12o**·HCl as a white solid. Purification by C8 reverse-phase chromatography, eluting with MeOH/H_2O, provided the zwitterion **12o** as a white solid (30% yield): mp 195−201 °C. $[\alpha]^{25}_D$ −10° (*c* 1.01, MeOH). IR (KBr): 3414, 1669 cm^{-1}. ^{1}H NMR (MeOH-d_4) δ: 4.46 (q, J = 7.0 Hz, 1 H), 3.87 (m, 1 H), 2.45−2.35 (m, 2 H), 2.16−1.60 (m, 7 H), 1.37 (d, J = 7.3 Hz, 3 H), 1.39−1.20 (m, 1H), 0.99 (m, 6 H). ^{13}C NMR (DMSO-d_6) δ: 174.9, 174.7, 172.8, 169.3, 66.2, 51.8, 48.8, 41.0, 34.8, 32.5, 28.3, 27.0, 24.6, 23.7, 23.2, 21.8, 19.6.

(1*S*,2*S*,5*R*,6*S*)-2-{(2*S*)-[(2*S*)-((2*S*)-Aminopropionylamino)propionylamino]propionylamino}-2-[(1′*S*)-carboxy-3′-methylbutyl]carbamoylbicyclo[3.1.0]hexane-2,6-dicarboxylic Acid Hydrochloride (14·HCl). (1*S*,2*S*,5*R*,6*S*)-2-*tert*-Butoxycarbonylamino-2-[(1*S*)-3-methyl-1-methoxycarbonylbutyl]carbamoylbicyclo[3.1.0]hexane-6-carboxylic Acid Methyl Ester (6k). The reaction of L-leucine methyl ester and **3** as described in general procedure A followed by flash column chromatography of the crude dipeptide (heptane/EtOAc, 7:3 to 4:6) afforded **6k** as a white solid (0.921 g, 40% yield). ^{1}H NMR ($CDCl_3$) δ: 4.98 (1H, bs), 4.54−4.46 (1H, m), 3.63 (3H, s), 3.60 (3H, s), 2.36 (2H, bs), 2.02−1.94 (2H, m), 1.78 (1H, dd, 7.7 Hz, 5.0 Hz), 1.58−1.52 (4H, m), 1.42 (9H, s), 1.08−1.02 (1H, m), 0.82 (6H, 2 × t, 6.5 Hz). LCMS *m/z*: 327 [M + H − CO2^{t}Bu]$^+$.

(1*S*,2*S*,5*R*,6*S*)-2-[(2*S*)-*tert*-Butoxycarbonylamino)propionyl]amino-2-[(1*S*)-3-methyl-1-methoxycarbonylbutyl]-carbamoylbicyclo[3.1.0]hexane-6-carboxylic Acid Methyl Ester (13). Compound **6k** was dissolved in 1.0 N HCl in EtOAc and stirred at room temperature for 3 h. The solvent was removed under vacuum, and the residue was reacted with *N*-Boc-L-alanine following general procedure A, providing **13** as a foamy white solid (48% yield). ^{1}H NMR ($CDCl_3$) δ: 7.30 (1H, d, 6.0 Hz), 6.79 (1H, bs), 4.95 (1H, d, 6.3 Hz), 4.54−4.46 (2H, m), 3.63 (3H, s), 3.60 (3H, s), 2.45−2.39 (2H, m), 2.04−2.00 (2H, m), 1.89−1.83 (2H, m), 1.59−1.53 (4H, m), 1.42 (9H, s), 1.23 (6H, 2 × t, 6.7 Hz), 1.18−1.14 (1H, m), 0.80 (3H, d, 7.0 Hz). LCMS *m/z*: 498 [M + H]$^+$ and 398 [M + H − $CO_2{}^t$Bu]$^+$.

Compound **13** was deprotected following general procedure D to provide **14**·HCl as a white solid (81% yield). ^{1}H NMR (D_2O) δ: 4.34−4.29 (1H, m), 3.95 (1H, q, 6.8 Hz), 2.41 (1H, dd, 6.6 Hz, 2.7 Hz), 2.09−2.01 (2H, m), 1.9−1.3 (7H, m), 1.40 (3H, d, 6.8 Hz), 0.76 (6H, 2 × t, 5.9 Hz). ^{13}C NMR (D_2O) δ: 173.7, 173.4, 173.1, 172.6, 52.5, 52.2, 51.4, 41.6, 33.1, 29.1, 28.7, 27.0, 25.4, 23.2, 22.3, 21.7. LCMS *m/z*: 369 M$^+$.

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Supporting Information Available: Additional information on the uptake of the intestinal human PepT1 transporter and the fear-potentiated startle model and analytical instrumentation and methods for HPLC purity determination. This material is available free of charge via the Internet at http://pubs.acs.org.

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