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Bioorganic & Medicinal Chemistry 13 (2005) 4473–4484
Bioorganic & Medicinal Chemistry

Exploration of orally available calpain inhibitors: Peptidyl α-ketoamides containing an amphiphile at P3 site

Yoshihisa Shirasaki,* Hiroyuki Miyashita, Masazumi Yamaguchi, Jun Inoue and Masayuki Nakamura

Research Laboratory of Ocular Science, Senju Pharmaceutical Co., Ltd, 1-5-4 Murotani Nishi-ku Kobe, Hyogo 651-2241, Japan

Received 23 March 2005; revised 15 April 2005; accepted 15 April 2005
Available online 25 May 2005

Abstract—A novel series of dipeptidyl α-ketoamide derivatives with amphiphile was designed and synthesized as water-soluble calpain inhibitors. The introduction of amphiphiles at the P3 site increased water solubility without loss of membrane permeability and provided the oral available inhibitors. Extension of the ethylene glycol chain at the P3 site led to an improvement in persistence of plasma levels. In particular, introduction of a combination of a diethylene glycol methyl ether moiety at the P3 site, a phenylalanine residue at the P1 site and a cyclopropyl moiety at the P′ site was the most effective modification for an increase in plasma drug exposure.

1. Introduction

Calpains are nonlysosomal cysteine endoproteases and the family of these enzymes has grown rapidly in recent years.[1] Two well-known calpains, μ-calpain (calpain I) and m-calpain (calpain II), are ubiquitously found in mammalian cells and participate in a variety of biological processes and numerous diseases such as stroke, Alzheimar's disease, central nervous system (CNS) diseases, spinal cord injury, brain trauma, cardiac ischemia, muscular dystrophy, and cataracts.[2] Therefore, calpains are attractive targets for discovering novel therapeutic agents for diseases.

Many of the known calpain inhibitors are composed of a binding group and di- or tripeptide-based backbone, which is structurally related to the cleavage site of substrates of calpains. The binding groups are capable of binding to the catalytic center of the enzymes in irreversible or reversible manners.[3] Epoxide, haloketone, and vinyl sulfone work as irreversible binding groups. Aldehyde, α-ketoacid, α-ketoester, and α-ketoamide act as reversible binding groups, which form hemithioacetal or ketal with the SH group of the cysteine residue in the active site of calpains.

Several α-ketoamide inhibitors have demonstrated protective effects in in vivo experiments. For example, in an animal model of stroke, it has been shown that an original peptidyl α-ketoamide inhibitor, AK-275 (**1**), has a protective effect against ischemic brain damage (Fig. 1).[4] Furthermore, its water-soluble derivative, AK-295 (**2**), is also effective in an animal ischemia model via internal carotid artery administration.[5] Recently, benzoylalanine-derived ketoamides have been reported as non-peptidyl calpain inhibitors without an L-Leu or L-Val moiety at the P2 site. The representative compound showed neuroprotective efficacy in an experimental model of traumatic brain injury by intraperitoneal administration.[6]

Previously, our group has reported a dipeptidyl aldehyde inhibitor SJA6017 (**3**), which has efficacy as an anti-cataract agent in lens culture models.[7] However, in a rat retinal ischemia model,[8] the high oral dosage of **3** is needed for confirmation of the protective effect, because of its poor oral bioavailability. It appears that this poor availability is ascribed to the presence of an aldehyde group that is easily metabolized.[9] Furthermore, it is possible that the aldehyde group reacts with an amino or thiol moiety of various biological substances and proteins through a Schiff base and this may limit membrane permeation of the drugs.[10] Therefore, we examined whether introducing an α-ketoamide moiety instead of the aldehyde moiety could improve oral availability. The corresponding α-ketoamide

Keywords: Calpain inhibitor; Dipeptidyl α-ketoamides; Amphiphile; Water-solubility; Pharmacokinetics; Ethylene glycol.
* Corresponding author. Tel.: +81 78 997 1010; fax: +81 78 997 1016; e-mail: shirasaki@senju.co.jp

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doi:10.1016/j.bmc.2005.04.059

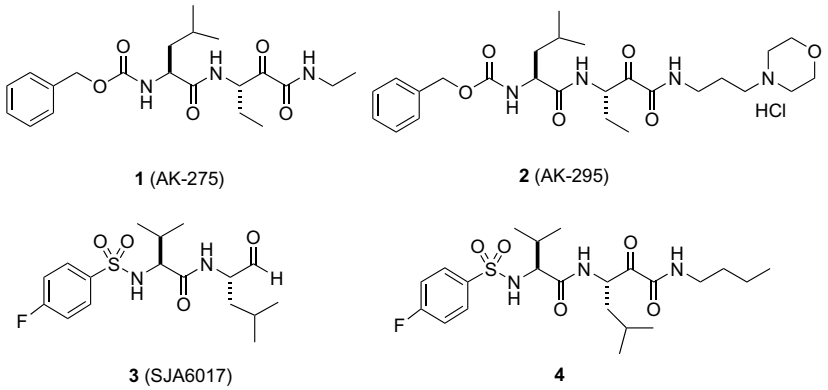


Figure 1. Structure of known calpain inhibitors.

analogue **4**[11] showed inhibitory activities equivalent to **3** and higher cellular permeability and higher metabolic stability than **3**. Unfortunately, it shows very low solubility that can be disadvantageous to the bioavailability. In fact, pharmacokinetic studies in monkey have demonstrated that **4** displayed poor oral availability.

To explore novel calpain inhibitors that have good oral availability, we designed water-soluble α-ketoamide derivatives based on known inhibitors such as **1**, **2**, and **4**. This study was focused on substitution of P3 *N*-capped moieties and P′ alkyl chain (Fig. 2). Although an introduction of ionic moieties such as sulfonate, carboxylate, and guanidino groups increase the water-solubility, the charged form of these molecules would show low cellular permeability.[9a,12] Thus, it is not easy to design the molecules with both suitable water solubility and membrane permeability. Therefore, we decided to introduce a non-ionic amphiphile as a water-soluble moiety to the P3 site of dipeptidyl α-ketoamide on the assumption that the introduction of amphiphile can increase water solubility without decreasing the cellular permeability. In this report, we describe the synthesis of peptidyl α-ketoamides containing amphiphile, their inhibitory activities, as well as the relationships between the chemical structures, in vitro pharmacokinetic data and in vivo pharmacokinetics in monkey.

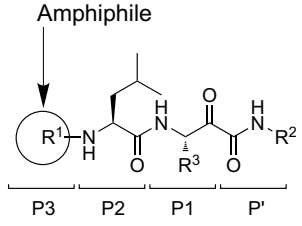


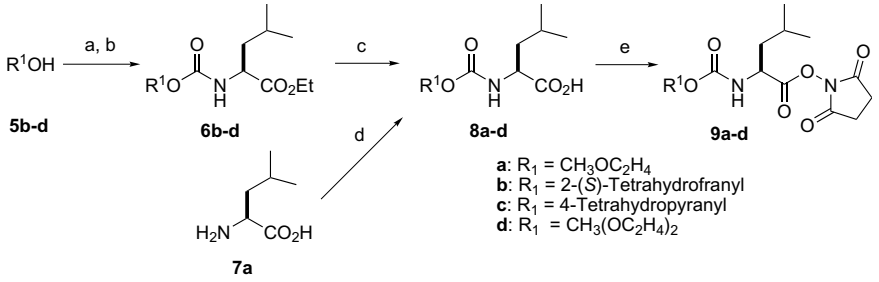
Figure 2. Strategy for oral available α-ketoamide.

2. Results and discussion

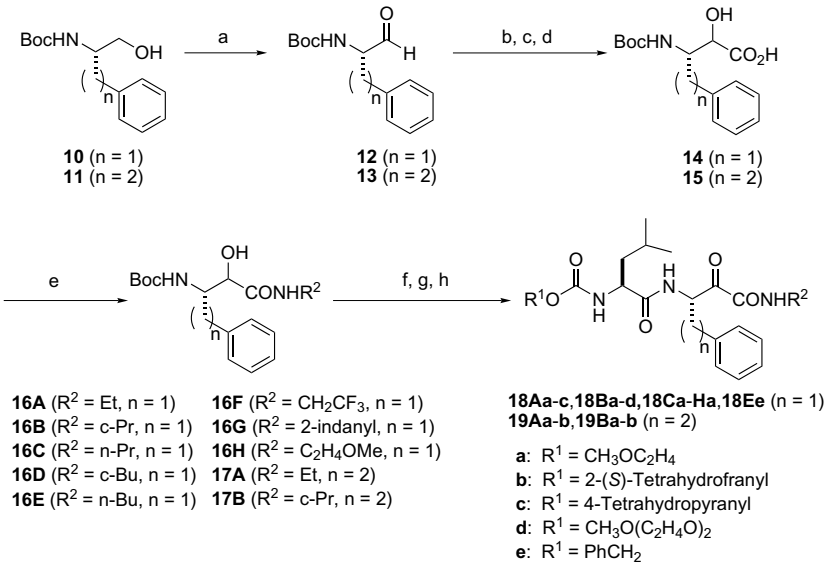
2.1. Chemistry

The new peptidyl α-ketoamides were synthesized as reported previously by Herbeson et al.[4b] The syntheses of the P2/P3 units are shown in Scheme 1. The alcohols **5b–d** as amphiphiles were coupled to L-leucine ethyl ester (L-Leu-OEt) through carbamate linkage using di-(*N*-succinimidyl) carbonate ($CO(OSu)_2$) to prepare the *N*-alkoxycarbonyl amino acid esters **6b–d**.[13] The esters were hydrolyzed with NaOH to yield the corresponding acids **8b–d**. The carboxylic acid **8a** was prepared by a direct condensation of L-Leu-OH (**7a**) and 2-methoxyethyl chloroformate. The carboxylic acids **8a–d** were converted to the corresponding *N*-succinimidyl esters **9a–d** as the P2/P3 units.

The P1/P′ units were prepared from Boc-protected amino alcohols **10** and **11** (Scheme 2). The alcohols **10** and **11** were oxidized by SO_3–pyridine complex in DMSO to afford the aldehydes **12** and **13**.[10] The aldehydes were converted to the cyanohydrins, which were directly hydrolyzed with HCl to give α-hydroxy-β-amino acids. The α-hydroxy-β-amino acids were protected with the



Scheme 1. Reagents: (a) $CO(OSu)_2$, Et_3N, MeCN; (b) L-leucine-OEt·HCl, Et_3N, CH_2Cl_2; (c) NaOH, aq EtOH; (d) $MeOC_2H_4OCOCl$, Et_3N, CH_2Cl_2; (e) *N*-hydroxysuccinimide, EDC, THF.



Scheme 2. Reagents and conditions: (a) SO_3/pyridine, DIPEA, DMSO/CH_2Cl_2; (b) $NaHSO_3$, aq MeOH; then NaCN, EtOAc; (c) concentrated HCl/dioxane (1:1), reflux; (d) Boc_2O, aq NaOH, dioxane; (e) R^2–NH_2, EDC, HOBt, DMF; (f) 4 M HCl/dioxane; (g) **9a**–**d** or Cbz-L-Leu-OSu, Et_3N, CH_2Cl_2; (h) Dess–Martin periodinane, CH_2Cl_2.

Boc group using di-*tert*-butyl dicarbonate (Boc_2O) and aqueous NaOH to afford the *N*-Boc-α-hydroxy-β-amino acids **14** and **15** as mixtures of diastereomers.[5b] The HOBt/EDC-mediated coupling between the acids and the various amines afforded the α-hydroxyamides **16A**–**H** and **17A**–**B**. The Boc group in **16** and **17** were removed and the resulting amines were coupled to *N*-succinimidyl esters **9a**–**d** or Cbz-L-Leu-OSu to afford the corresponding dipeptidyl α-hydroxyamide intermediates. Oxidation of the hydroxyl group of the intermediates with Dess–Martin periodinane gave the final products **18** and **19** (Table 1).[11]

2.2. Enzyme inhibition and water solubility

Table 1 shows the inhibitory activities against μ- and m-calpain and the water solubility of novel α-ketoamide derivatives containing an amphiphile at the P3 site. These compounds are less potent than the known inhibitor **18Ee**[14] that has a Cbz moiety at the P3 site, although they are sufficiently potent inhibitors (μ-calpain IC_{50} 0.086–0.45 μM). The conversion of alkyl chain at the P′ site did not affect the inhibitory activities toward calpains. For example, introduction of a 2-indanyl substituent, the most bulky group, did not alter the activities (**18Ga** vs **18Aa**). In the case of an (*S*)-3-tetrahydrofuranyl (THF) moiety at the P3 site, replacement of an Et group with a cyclopropyl (*c*-Pr) group resulted in a 2-fold increase in inhibitory activities (**18Bb** vs **18Ab**). Introduction of a trifluoroethyl (CH_2CF_3) moiety at the P′ site slightly reduced the activities compared to the isosteric Et analogue (**18Fa** vs **18Aa**). The reduction of activity may be due to an electrostatic repulsion between the CH_2CF_3 at the P′ site and the carboxylate group at the S′ site of the calpain enzyme.[15] Similar activity reduction has been reported by Donkor et al.[16] On the other hand, replacement of phenylalanine residue (Phe) with homophenylalanine (HomoPhe) at the P1 site also did not affect the activities (**19Aa** and **19Ba**). Thus, SAR at P′ and P1 region for the novel α-ketoamides were consistent with that of known α-ketoamides.[6,15–17]

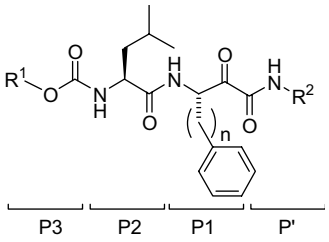
We recognized that the α-ketoamide derivatives containing amphiphile at the P3 site were water-soluble inhibitors. For instance, compound **18Ea**, which has an ethylene glycol methyl ether (mEG) moiety, is 20-fold more soluble in water than the corresponding Cbz derivative (**18Ee**). Decreasing of P′ alkyl chain length from *n*-Bu to Et gradually increases water solubility (**18Ea** vs **18Ca** vs **18Aa**). On the other hand, *c*-Pr compound (**18Ba**), which is more soluble than *n*-Pr compound (**18Ca**), is as soluble as Et compound (**18Aa**). A similar phenomenon was observed in other *c*-Pr derivatives (**18Ac** vs **18Bc** and **19Aa** vs **19Ba**). However, in the case of THF derivative, replacement of Et with *c*-Pr leads to 14-fold less water-solubility (**18Ab** vs **18Bb**). The extension of the ethylene glycol chain at the P3 site also resulted in an unexpected decline in water-solubility (**18Ba** vs **18Bd**). Thus, it is not easy to predict the water solubility from the combination of each building block in the modification of α-ketoamides.

Consequently, the introduction of amphiphiles enables us to provide α-ketoamide derivatives with acceptable water solubility, although it slightly decreased inhibitory activities. However, if the enhancement in water solubility leads to improvement in plasma exposure of the drug, it can compensate for the loss of activity.

2.3. Membrane permeability and metabolic stability

Membrane permeability and in vitro metabolic stability of the compounds are presented in Table 2. Most inhibitors were evaluated by Caco-2 permeability assay. These compounds showed generally acceptable permeability (P_{app} (apical → basolateral) > 10^{-6} cm/s).[18] Furthermore, immobilized artificial membranes (IAM)

Table 1. Enzyme inhibitory activity and solubility of α-ketoamide derivatives



Compd	Structure			IC_{50} (μM)		Solubility[c] (mg/mL)
	R^1	*n*	R^2	μ-Calpain[a]	m-Calpain[b]	
18Aa	$CH_3OC_2H_4$	1	Et	0.17	0.11	1.2
18Ab	THF[d]	1	Et	0.15	0.11	1.0
18Ac	THP[e]	1	Et	0.25	0.16	0.84
18Ba	$CH_3OC_2H_4$	1	*c*-Pr	0.11	0.10	1.3
18Bb	THF[d]	1	*c*-Pr	0.086	0.047	0.074
18Bc	THP[e]	1	*c*-Pr	0.12	0.13	0.76
18Bd	$CH_3(OC_2H_4)_2$	1	*c*-Pr	0.17	0.099	0.65
18Ca	$CH_3OC_2H_4$	1	*n*-Pr	0.099	0.070	0.33
18Da	$CH_3OC_2H_4$	1	c-Bu	0.17	0.080	0.066
18Ea	$CH_3OC_2H_4$	1	*n*-Bu	0.10	0.14	0.11
18Fa	$CH_3OC_2H_4$	1	$-CH_2CF_3$	0.45	0.34	0.071
18Ga	$CH_3OC_2H_4$	1	2-Indanyl	0.17	0.12	<0.005
18Ha	$CH_3OC_2H_4$	1	$-C_2H_4OCH_3$	0.18	0.11	0.74
19Aa	$CH_3OC_2H_4$	2	Et	0.30	0.20	0.22
19Ba	$CH_3OC_2H_4$	2	*c*-Pr	0.18	0.14	0.27
18Ee	$PhCH_2$	1	*n*-Bu	0.038	0.020	<0.005
3	—	—	—	0.021	0.021	0.0053
4	—	—	—	0.021	0.045	0.1

[a] Human erythrocyte μ-calpain.
[b] Porcine kidney m-calpain.
[c] Water-solubility in pH 7 buffer, at 25 °C.
[d] THF: 
[e] THP: 

chromatography analysis was performed to determine the capacity factors (k'_{IAM})[19] of inhibitors as a parameter of permeability. We confirmed that all compounds had acceptable membrane permeability that was deduced from the results of Caco-2 permeability assay and IAM chromatography analysis. Thus, introduction of amphiphile at the P3 site provided water-soluble inhibitors without loss of membrane permeability.

In vitro metabolic stability was determined by incubation of the inhibitors with human liver S9 fraction. The inhibitors showed low to moderate metabolic stability (17–64% of initial concentration). Increasing the alkyl chain length at the P′ and P1 sites reduced their metabolic stability. For instance, metabolic stability was reduced gradually by increasing the P′ alkyl chain from Et (**18Aa**) to *n*-Bu (**18Ea**). We confirmed that the increase of lipophilicity caused the reduction of metabolic stability in this series of inhibitors.

2.4. Pharmacokinetic analysis

The purpose of this study is to improve the pharmacokinetic (PK) properties of the peptidyl α-ketoamide derivatives. The α-ketoamide derivatives were evaluated for their PK properties by oral absorption experiments in monkeys. The C_{max} value and area under the plasma level concentration time curve ($AUC_{0\rightarrow4\,h}$) after 10 mg/kg oral administration are shown in Table 2. Compounds **18Ab**, **18Ba**, **18Bd**, and **18Ca** showed good PK properties. Compounds **18Bb**, **18Da**, **18Ea**, and **18Ee** displayed no or very low plasma drug levels. The most effective modification for the improvement in PK properties was introduction of a combination of mEG at the P3 site, Phe at the P1 site, and *c*-Pr at the P′ site (**18Ba** and **18Bd**). Thus, introduction of amphiphiles to dipeptidyl α-ketoamide led to enhancement of water-solubility without loss of adequate membrane permeability and improved the PK properties. In addition, extension of the P3 ethylene glycol chain, namely conversion of **18Ba** into **18Bd**, led to enhanced 2 and 4 h plasma levels with equal C_{max} value (Fig. 3).

The compounds with good PK properties possess high water-solubility and metabolic stability. Insoluble or metabolically unstable compounds displayed poor oral absorption. Therefore, in this series, the oral availability is mainly restricted water solubility and metabolic stability. However, since compound **18Ha**, having high solubility and metabolic stability, showed relatively low plasma $AUC_{0\rightarrow4\,h}$, some other minor factors such as carrier-mediated active transport and non-hepatic

Table 2. Solubility, permeability, and PK data of α-ketoamide derivatives

Compd	Solubility[a] (mg/mL)	Permeability		Metabolic stab[f] Human S9 (%)	Pharmacokinetics[g]	
		Caco-2 [b] P_{app}[c] (cm/s) × 10^{-6}	IAM[d] Log K'[e]		C_{max} (μM)	$AUC_{0\rightarrow4\ h}$[h] (μM h)
18Aa	1.2	3.1	1.13	64	0.30	0.31
18Ab	1.0	ND[i]	1.22	53	0.59	0.61
18Ba	1.3	2.7	1.20	64	1.2	1.9
18Bb	0.074	2.5	1.55	57	0.08	0.038
18Bc	0.76	3.0	1.36	27	0.26	0.27
18Bd	0.65	ND[i]	1.27	41	1.1	2.4
18Ca	0.33	6.3	1.28	42	0.65	0.87
18Da	0.066	ND[i]	1.53	17	BDL[j]	BDL[j]
18Ea	0.11	10.5	1.70	17	BDL[j]	BDL[j]
18Ha	0.74	2.0	1.13	60	0.21	0.16
19Ba	0.27	4.5	1.36	33	0.23	0.25
18Ee	<0.005	ND[i]	>2.50	ND[i]	BDL[j]	BDL[j]
3	0.0053	17.3	2.13	22	0.12	0.16
4	0.10	2.5	1.26	3	ND[i]	ND[i]

[a] Water-solubility in pH 7 buffer, at 25 °C.
[b] Caco-2 cell monolayer permeability assay.
[c] P_{app} is the apparent permeability coefficient[18] for apical to basolateral flux in 10^{-6} cm/s.
[d] Immobilized artificial membrane chromatography analysis.[19]
[e] Logarithm of the capacity factors k'_{IAM}.[19]
[f] Metabolic stability represented in residual percent after incubation with human S9 fraction for 0.5 h at 37 °C.
[g] Cynomolgus monkey, p.o. 10 mg/kg, dosed as a suspension in 0.5% carboxymethylcellulose.
[h] The area under the curves 0–4 h.
[i] Not determined.
[j] Below detection limit.

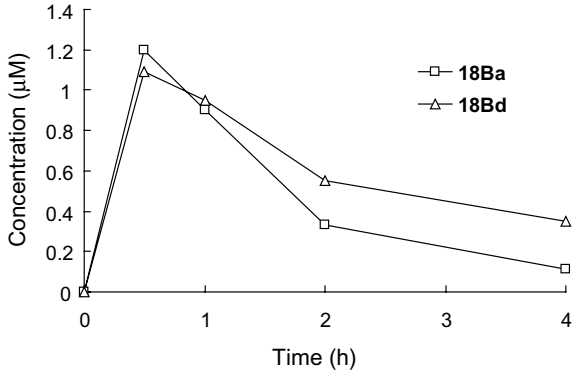


Figure 3. Mean plasma levels after oral administration (10 mg/kg) of **18Ba** and **18Bd** in cynomolgus monkeys ($n = 2$).

metabolism, in addition to water solubility and metabolic stability, would be partly attributed to the limitation of oral availability. We will hereafter need to investigate the involvement of these factors in pharmacokinetics.

3. Conclusion

Based on the assumption that the poor PK properties of dipeptidyl α-ketoamide **4** can be due to its insolubility, we modified the dipeptidyl α-ketoamides to improve their water solubility. Introduction of amphihiles at the P3 site led to increased water-solubility without a loss of membrane permeability, although potency was lower than with the starting α-ketoamide **4**. Since some of those inhibitors showed remarkable improvement of oral availability as compared with **4**, it is expected that the decrease in potency can be compensated by the enhancement in oral availability. The most effective modification for oral absorption is to introduce *c*-Pr at the P′ site and an mEG moiety at the P3 site in the series of α-ketoamides. Furthermore, the extension of the P3 ethylene glycol chain provided sustained plasma levels (compound **18Bd**). We will evaluate the efficacy of compound **18Bd** (SNJ-1945) in adequate in vivo pharmacological models to develop calpain inhibitors as orally available anti-retinopathy agents.

4. Experimental

4.1. General

Optical rotations were measured with a Horiba SEPA-200 polarimeter. Melting points were determined on a Yanaco micromelting point apparatus without correction. ^{1}H NMR spectra were recorded on a Varian Gemini-2000 spectrometer. Chemical shifts were reported in parts per million, and coupling constants (J) were reported in hertz. Matrix-assisted laser desorption ionization time-of-flight mass spectra (MALDI-TOF-MS) were obtained on a Perceptive Voyager DE mass spectrometer, and the mass numbers were corrected with an internal standard (α-cyano-4-hydroxycinnamic acid) and displayed accurately.

4.2. General procedure for the preparation of compounds 6b–d

4.2.1. *N*-(((*3S*)-Tetrahydrofuran-3-yloxy)carbonyl)-L-leucine ethyl ester (6b). To a stirred solution of (*S*)-3-hydroxytetrahydrofuran (1.0 g, 11 mmol) in acetonitrile

(50 mL) were added *N*,*N*′-disuccinimidyl carbonate (4.3 g, 17 mmol) and triethylamine (4.8 mL, 34 mmol) at room temperature. The resulting mixture was stirred at room temperature for 18 h and concentrated in vacuo. The residue was diluted into saturated $NaHCO_3$ (100 mL) and extracted with EtOAc (200 mL). The combined extracts were washed with saturated NaCl (100 mL), dried over $MgSO_4$, and concentrated in vacuo to give a brown oil. This mixed carbonate was used directly for the next reaction. To a solution of L-leucine ethyl ester hydrochloride (2.7 g, 14 mmol) and triethylamine (2.9 g, 28 mmol, 4.0 mL) in CH_2Cl_2 (50 mL) was added a solution of the resulting mixed carbonate (2.6 g, 11 mmol) in CH_2Cl_2 (20 mL). The mixture was stirred at room temperature for 18 h, and concentrated in vacuo. EtOAc (200 mL) was added to the residue, and the solution was washed with 1 M HCl (100 mL), saturated $NaHCO_3$ (100 mL), and saturated NaCl (100 mL), dried over $MgSO_4$, and concentrated in vacuo. The residue was washed with hexane to give **10b** (3.1 g, 98%) as a colorless solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.80–0.92 (m, 6H), 1.18 (t, 3H, J = 7.2), 1.37–1.71 (m, 3H), 1.85 (m, 1H), 2.12 (m, 1H), 3.61–3.84 (m, 4H), 3.95–4.15 (m, 3H), 5.10 (m, 1H), 7.64 (d, 1H, J = 8.1). MALDI-TOF-MS calcd for $C_{13}H_{23}NO_5$ $(M+Na)^+$, 296.1474, found, 296.1444.

4.2.2. *N*-((Tetrahydro-4*H*-pyran-4-yloxy)carbonyl)-L-leucine ethyl ester (6c). Brown oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.83–0.90 (m, 6H), 1.17 (t, 3H, J = 7.1), 1.38–1.71 (m, 5H), 1.83 (m, 2H), 3.41 (m, 2H), 3.81 (m, 2H), 3.93–4.16 (m, 3H), 4.68 (m, 1H), 7.55 (d, 1H, J = 8.1).

4.2.3. *N*-((5-Methoxy-3-oxapentyloxy)carbonyl)-L-leucine ethyl ester (6d). Colorless oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.80–0.90 (m, 6H), 1.18 (t, 3H, J = 7.1), 1.38–1.69 (m, 3H), 3.25 (s, 3H), 3.42–3.46 (m, 2H), 3.51–3.58 (m, 4H), 3.99 (m, 1H), 4.04–4.12 (m, 4H), 7.64 (d, 1H, J = 7.8). MALDI-TOF-MS calcd for $C_{14}H_{27}NO_6$ $(M+Na)^+$, 328.1736, found, 328.1775.

4.3. *N*-((2-Methoxyethoxy)carbonyl)-L-leucine (8a)

L-Leucine (**7a**) (25 g, 0.19 mol) was dissolved in 2 M NaOH (0.12 L). To this solution were slowly added chloroformic acid 2-methoxyethyl ester (30 g, 0.22 mol) and 1 M NaOH (0.22 L) at the same time under ice-cooled conditions. The mixture was stirred at room temperature for 18 h, diluted into water (600 mL), and washed with ethyl ether (2 × 200 mL). The aqueous phase was chilled in an ice bath and acidified to pH 3 with 6 M HCl. This mixture was extracted with EtOAc (5 × 150 mL). The organic phase was dried over $MgSO_4$ and concentrated in vacuo to yield **13a** (41 g, 92%) as a colorless oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.85 (d, 3H, J = 6.6), 0.88 (d, 3H, J = 6.6), 1.47 (m, 2H), 1.63 (m, 1H), 3.26 (s, 3H), 3.49 (t, 2H, J = 4.7), 3.92 (m, 1H), 4.06 (t, 2H, J = 4.5), 7.50 (d, 1H, J = 8.4), 12.50 (br s, 1H). MALDI-TOF-MS calcd for $C_{10}H_{19}NO_5$ $(M+Na)^+$, 256.1161, found, 256.1241.

4.4. General procedure for the preparation of compounds 8b–d

4.4.1. *N*-(((3*S*)-Tetrahydrofuran-3-yloxy)carbonyl)-L-leucine (8b). To a solution of **6b** (2.9 g, 11 mmol) in EtOH (100 mL) was added 1 M NaOH (33 mL). The mixture was stirred under ice-cooled conditions for 3 h made by the addition of HCl thereto (pH 3). The solution was concentrated in vacuo and extracted with EtOAc (100 mL). Then, the organic layer was separated, washed with 1 M HCl and saturated NaCl, dried over $MgSO_4$ and concentrated in vacuo. The residue was washed with hexane and EtOAc to give **8b** (2.6 g, 85%) as colorless crystals. Mp 94.9–96.0 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.85 (d, 3H, J = 6.3), 0.88 (d, 3H, J = 6.3), 1.38–1.73 (m, 3H), 1.85 (m, 1H), 2.10 (m, 1H), 3.61–3.85 (m, 4H), 3.94 (m, 1H), 5.10 (m, 1H), 7.48 (d, 1H, J = 7.8), 12.49 (br s, 1H). MALDI-TOF-MS calcd for $C_{11}H_{19}NO_5$ $(M+Na)^+$, 268.1161, found, 268.1181.

4.4.2. *N*-((Tetrahydro-4*H*-pyran-4-yloxy)carbonyl)-L-leucine (8c). Colorless oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.83–0.90 (m, 6H), 1.38–1.73 (m, 5H), 1.84 (m, 2H), 3.41 (m, 2H), 3.84 (m, 2H), 3.95 (m, 1H), 4.68 (m, 1H), 7.38 (d, 1H, J = 8.1), 12.49 (br s, 1H). MALDI-TOF-MS calcd for $C_{12}H_{21}NO_5$ $(M+Na)^+$, 282.1318, found, 282.1354.

4.4.3. *N*-((5-Methoxy-3-oxapentyloxy)carbonyl)-L-leucine (8d). Colorless oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.80–0.89 (m, 6H), 1.39–1.69 (m, 3H), 3.25 (s, 3H), 3.42–3.46 (m, 2H), 3.51–3.58 (m, 4H), 3.94 (m, 1H), 4.02–4.07 (m, 2H), 7.50 (d, 1H, J = 8.4), 12.45 (br s, 1H). MALDI-TOF-MS calcd for $C_{12}H_{23}NO_6$ $(M+Na)^+$, 300.1423, found, 300.1470.

4.5. General procedure for the preparation of compounds 9a–d

4.5.1. *N*-((2-Methoxyethoxy)carbonyl)-L-leucine *N*-hydroxysuccinimide ester (9a). Compound **8a** (20 g, 86 mmol) and *N*-hydroxysuccinimide (13 g, 0.11 mmol) were dissolved in THF (200 mL), and a suspension of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (21 g, 0.11 mol) in CH_2Cl_2 (200 mL) was added thereto. The mixture was stirred at room temperature for 18 h, and concentrated in vacuo. The residue was dissolved in EtOAc (300 mL), the solution was washed with 1 M HCl (150 mL), saturated $NaHCO_3$ (150 mL), and saturated NaCl (150 mL), dried over $MgSO_4$, and concentrated in vacuo to give **14a** (27 g, 95%) as a colorless oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.89 (d, 3H, J = 6.6), 0.93 (d, 3H, J = 6.6), 1.57–1.84 (m, 3H), 2.81 (s, 4H), 3.26 (s, 3H), 3.51 (t, 2H, J = 4.7), 4.10 (t, 2H, J = 4.7), 4.40 (m, 1H), 8.04 (d, 1H, J = 8.1). MALDI-TOF-MS calcd for $C_{14}H_{22}N_2O_7$ $(M+Na)^+$, 353.1325, found, 353.1301.

4.5.2. *N*-(((3*S*)-Tetrahydrofuran-3-yloxy)carbonyl)-L-leucine *N*-hydroxysuccinimide ester (9b). Colorless viscous oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.89 (d, 3H, J = 6.0), 0.92 (d, 3H, J = 6.3), 1.55–1.82 (m, 3H), 1.88

(m, 1H), 2.12 (m, 1H), 2.81 (s, 4H), 3.64–3.84 (m, 4H), 4.39 (m, 1H), 5.15 (m, 1H), 8.04 (d, 1H, $J = 7.8$).

4.5.3. *N*-((Tetrahydro-4*H*-pyran-4-yloxy)carbonyl)-L-leucine *N*-hydroxysuccinimide ester (9c). Colorless viscous oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.89 (d, 3H, $J = 6.0$), 0.92 (d, 3H, $J = 6.3$), 1.43–1.93 (m, 7H), 2.80 (s, 4H), 3.42 (m, 2H), 3.78–3.82 (m, 2H), 4.39 (m, 1H), 4.72 (m, 1H), 7.94 (d, 1H, $J = 7.8$). MALDI-TOF-MS calcd for $C_{16}H_{24}N_2O_7$ $(M+Na)^+$, 379.1428, found, 379.0930.

4.5.4. *N*-((5-Methoxy-3-oxapentyloxy)carbonyl)-L-leucine *N*-hydroxysuccinimide ester (9d). Colorless oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.90 (dd, 6H, $J =$ 9.5, 6.5), 1.56–1.80 (m, 3H), 2.80 (s, 4H), 3.24 (s, 3H), 3.41–3.46 (m, 2H), 3.50–3.54 (m, 2H), 3.56–3.60 (m, 2H), 4.08–4.11 (m, 2H), 4.39 (m, 1H), 8.05 (d, 1H, $J = 7.8$).

4.6. General procedure for the preparation of compounds 12 and 13

4.6.1. *N*-(*tert*-Butoxycarbonyl)-L-phenylalaninal (12). *N*-Boc-L-phenylalaninol (**10**) (69 g, 0.28 mol) was dissolved in DMSO (280 mL) and CH_2Cl_2 (140 mL), and cooled on an ice bath. *N*,*N*-Diisopropylethylamine (110 g, 0.82 mol) and a suspension of purified sulfur trioxide pyridine complex (130 g, 0.82 mol) in DMSO (100 mL) were added thereto. The mixture was stirred for 1 h under the same conditions. The reaction mixture was diluted with EtOAc (1.5 L), and the solution was washed with 1 M HCl (1 L), saturated $NaHCO_3$ (0.8 L), and saturated NaCl (0.8 L), dried over $MgSO_4$, and concentrated in vacuo. The residue was crystallized from a mixture of hexane and EtOAc to give **5a** (53 g, 77%) as colorless crystals. Mp 42.5–43.6 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.34 (s, 9H), 2.73 (m, 1H), 3.10 (dd, 1H, $J = 14.0$, 4.7), 4.08 (m, 1H), 7.15–7.34 (m, 6H), 9.53 (s, 1H). MALDI-TOF-MS calcd for $C_{14}H_{19}NO_3$ $(M+H)^+$, 250.1143, found, 250.1172.

4.6.2. *N*-(*tert*-Butoxycarbonyl)-L-homophenylalaninal (13). Colorless oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.40–1.42 (m, 9H), 1.72 (m, 1H), 1.96 (m, 1H), 2.58–2.73 (m, 2H), 3.77–3.84 (m, 1H), 7.16–7.31 (m, 5H), 7.43 (d, 1H, $J = 7.2$), 9.45 (s, 1H). MALDI-TOF-MS calcd for $C_{15}H_{21}NO_3$ $(M+H)^+$, 264.1599, found, 264.1686.

4.7. General procedure for the preparation of compounds 14 and 15

4.7.1. (3*S*)-3-(*tert*-Butoxycarbonylamino)-2-hydroxy-4-phenylbutanoic acid (14). Compound **12** (17 g, 67 mmol) was dissolved in MeOH (100 mL) and chilled to 5 °C. Sodium bisulfite (7.0 g, 67 mmol) was dissolved in water (150 mL) and chilled to 5 °C before addition to the aldehyde solution. The solution was added to the aldehyde solution and the mixture was stirred overnight at 5 °C. NaCN (4.0 g) was dissolved in water (150 mL) and added with EtOAc (300 mL) to the above mixture. The reaction was allowed to stir for 5 h at room temperature. The organic layer was separated, dried with $MgSO_4$, and concentrated in vacuo to yield crude cyanohydrin as a colorless viscous oil. This crude cyanohydrin was dissolved in 1,4-dioxane (250 mL), concentrated HCl (250 mL), and anisole (10 mL). The solution was gently refluxed and stirred overnight. The hydrolysis reaction was allowed to cool and then concentrated in vacuo to give a brown oil. The residue was dissolved in water (100 mL) and washed with ethyl ether (3×50 mL). The aqueous phase was then placed on a Dowex 50X8 (100–200 mesh) column (H^+ form; 25×2 cm). The column was washed with water until pH 5.5 and eluted with 2 M ammonium hydroxide (ca. 1.5 L). The eluent was evaporated in vacuo to yield crude α-hydroxy-β-amino acid as a white solid. The resulting α-hydroxy-β-amino acid was dissolved in 1 M NaOH (70 mL). To this solution was added a solution of di-*tert*-butyl dicarbonate (12 g, 56.9 mmol) in dioxane (70 mL). The reaction was stirred at room temperature while the pH was maintained at pH 11 with 1 M NaOH. The mixture was stirred at room temperature for 18 h, diluted into water (600 mL) and washed with ethyl ether (2×200 mL). The aqueous phase was chilled in an ice bath and acidified to pH 2 with 1 M HCl. This mixture was extracted with EtOAc (3×250 mL). The organic phase was dried with $MgSO_4$ and concentrated in vacuo to yield a mixture of diastereomers (ca. 55:45) of **14** (12 g, 72%) as a white solid. (Major diastereomer) ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.31 (s, 9 H), 2.75 (m, 2H), 3.87 (m, 1H), 4.00 (m, 1H), 6.39 (d, 1H, $J = 9.6$), 7.15–7.34 (m, 6H). (Minor diastereomer) ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.27 (s, 9H), 2.68 (m, 2H), 3.83–4.05 (m, 2H), 6.70 (d, 1H, $J = 9.0$), 7.12–7.33 (m, 6H), 12.55 (br s, 1H). MALDI-TOF-MS calcd for $C_{15}H_{21}NO_5$ $(M+Na)^+$, 318.1318, found, 318.1312.

4.7.2. (3*S*)-3-(*tert*-Butoxycarbonylamino)-2-hydroxy-5-phenylpentanoic acid (15). Colorless oil as a mixture of diastereomers. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.31–1.38 (m, 9H), 1.57–1.76 (m, 2H), 2.40–2.60 (m, 2H), 3.66–3.78 (m, 1H), 3.96 (m, 1H), 6.32 (d, 0.5H, $J = 9.6$), 6.68 (d, 0.5H, $J = 8.7$), 7.12–7.27 (m, 5H). MALDI-TOF-MS calcd for $C_{16}H_{23}NO_5$ $(M+Na)^+$, 332.1474, found, 332.1469.

4.8. General procedure for the preparation of compounds 16A–H, 17A–B

4.8.1. ((1*S*)-1-Benzyl-3-ethylamino-2-hydroxy-3-oxopropyl)carbamic acid 1,1-dimethylethyl ester (16A). Compound **14** (6.3 g, 21 mmol) and HOBt (3.0 g, 22.4 mmol) were dissolved in DMF (45 mL) and cooled on an ice bath. EDC (4.6 g, 24 mmol) was added followed by 70% aqueous ethylamine solution (3.0 mL). The solution was stirred for 18 h at room temperature. The solution was diluted into EtOAc (200 mL) and washed with 1 M HCl (150 mL), saturated $NaHCO_3$ (150 mL), and saturated NaCl (150 mL). The organic layer was dried over $MgSO_4$ and concentrated in vacuo to yield **16A** (5.8 g, 84%) as a white solid. Mp 122.7–123.8 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.02 (m, 3H), 1.28 (s, 9H), 2.54–2.67 (m, 2H), 3.11 (m, 2H), 3.94 (m, 1H), 4.01 (m, 1H), 5.65–5.85 (m, 1H), 6.55 (d, 1H, $J = 8.7$), 7.08–7.29 (m, 5H), 7.83 (m, 1H). MALDI-TOF-MS

calcd for $C_{17}H_{26}N_2O_4$ $(M+Na)^+$, 345.1791, found, 345.1762.

4.8.2. ((1*S*)-1-Benzyl-3-cyclopropylamino-2-hydroxy-3-oxopropyl)carbamic acid 1,1-dimethylethyl ester (16B). White solid. Mp 103.5–104.0 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.47 (m, 2H), 0.58 (m, 2H), 1.28 (s, 4.5H), 1.21 (s, 4.5H), 2.53–2.68 (m, 2.5H), 2.78 (m, 0.5H), 3.77 (dd, 0.5H, J = 6.6, 2.7), 3.97 (m, 1.5H), 5.50 (d, 0.5H, J = 6.3), 5.67 (d, 0.5H, J = 5.7), 6.14 (d, 0.5H, J = 9.3), 6.50 (d, 0.5H, J = 9.0), 7.14–7.32 (m, 5H), 7.75 (m, 1H). MALDI-TOF-MS calcd for $C_{18}H_{26}N_2O_4$ $(M+Na)^+$, 357.1791, found, 357.1803.

4.8.3. ((1*S*)-1-Benzyl-2-hydroxy-3-oxo-3-(propylamino)propyl)carbamic acid 1,1-dimethylethyl ester (16C). White solid. Mp 104.8–112.5 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.82 (m, 3H), 1.28 (s, 4.5H), 1.29 (s, 4.5H), 1.42 (m, 2H), 2.53–2.72 (m, 1.5H), 2.80 (m, 0.5H), 3.05 (m, 2H), 3.79 (m, 0.5H), 3.97 (m, 1.5H), 5.70 (d, 0.5H, J = 6.3), 5.76 (d, 0.5H, J = 5.7), 6.10 (d, 0.5H, J = 9.3), 6.54 (d, 0.5H, J = 8.7), 7.14–7.30 (m, 5H), 7.75–7.83 (m, 1H). MALDI-TOF-MS calcd for $C_{18}H_{28}N_2O_4$ $(M+Na)^+$, 359.1947, found, 359.2062.

4.8.4. ((1*S*)-1-Benzyl-3-cyclobutylamino-2-hydroxy-3-oxopropyl)carbamic acid 1,1-dimethylethyl ester (16D). White solid. Mp 94.7–96.5 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.28 (s, 4.5H), 1.29 (s, 4.5H), 1.60 (m, 2H), 1.99 (m, 2H), 2.09 (m, 2H), 2.54–2.67 (m, 1.5H), 2.79 (dd, 0.5H, J = 13.4, 6.8), 3.76 (dd, 0.5H, J = 6.3, 2.7), 3.89–4.04 (m, 1.5H), 4.20 (m, 1H), 5.57 (d, 0.5H, J = 6.3), 5.71 (d, 0.5H, J = 5.7), 6.15 (d, 0.5H, J = 9.3), 6.52 (d, 0.5H, J = 9.0), 7.13–7.30 (m, 5H), 7.92 (d, 1H, J = 8.4). MALDI-TOF-MS calcd for $C_{19}H_{28}N_2O_4$ $(M+Na)^+$, 371.1947, found, 371.1936.

4.8.5. ((1*S*)-1-Benzyl-3-butylamino-2-hydroxy-3-oxopropyl)carbamic acid 1,1-dimethylethyl ester (16E). White solid. Mp 114.9–116.5 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.85 (m, 3H), 1.15–1.44 (m, 4H), 1.28 (s, 4.5H), 1.29 (s, 4.5H), 2.48–2.68 (m, 1.5H), 2.80 (m, 0.5H), 3.07 (m, 2H), 3.79 (m, 0.5H), 3.97 (m, 1.5H), 5.68 (d, 0.5H, J = 6.3), 5.75 (d, 0.5H, J = 5.7), 6.10 (d, 0.5H, J = 9.3), 6.54 (d, 0.5H, J = 8.7), 7.11–7.30 (m, 5H), 7.77 (m, 1H). MALDI-TOF-MS calcd for $C_{19}H_{30}N_2O_4$ $(M+Na)^+$, 373.2104, found, 373.2164.

4.8.6. ((1*S*)-1-Benzyl-2-hydroxy-3-oxo-3-(2,2,2-trifluoroethylamino)propyl)carbamic acid 1,1-dimethylethyl ester (16F). White solid. Mp 103.1–104.8 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.15 (s, 2H), 1.28 (s, 7H), 2.60–2.70 (m, 1.5H), 2.80 (m, 0.5H), 3.81–4.09 (m, 4H), 5.90 (d, 0.5H, J = 6.6), 6.01 (d, 0.5H, J = 6.0), 6.14 (d, 0.5H, J = 9.3), 6.64 (d, 0.5H, J = 8.7), 7.13–7.30 (m, 5H), 8.38–8.49 (m, 1H). MALDI-TOF-MS calcd for $C_{17}H_{23}F_3N_2O_4$ $(M+Na)^+$, 399.1508, found, 399.1536.

4.8.7. ((1*S*)-1-Benzyl-3-(2-indanylamino)-2-hydroxy-3-oxopropyl)carbamic acid 1,1-dimethylethyl ester (16G). White solid. Mp 145.0–145.9 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.28 (s, 4.5H), 1.31 (s, 4.5H), 2.55–2.68 (m, 1.5H), 2.84 (m, 2.5H), 3.07–3.18 (m, 2H), 3.80–3.83 (m, 0.5H), 3.93–4.07 (m, 1.5H), 4.52 (m, 1H), 5.56 (d, 0.5H, J = 6.6), 5.75 (d, 0.5H, J = 5.4), 6.23 (d, 0.5H, J = 5.4), 6.56 (d, 0.5H, J = 9.3), 7.12–7.30 (m, 9H), 7.96 (m, 1H). MALDI-TOF-MS calcd for $C_{24}H_{30}N_2O_4$ $(M+Na)^+$, 433.2104, found, 433.2213.

4.8.8. ((1*S*)-1-Benzyl-2-hydroxy-3-(2-methoxyethylamino)-3-oxopropyl)carbamic acid 1,1-dimethylethyl ester (16H). White solid. Mp 113.5–116.5 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.28 (s, 4.5H), 1.29 (s, 4.5H), 2.53–2.67 (m, 1.5H), 2.79 (m, 0.5H), 3.23 (s, 1.5H), 3.23 (s, 1.5H), 3.29–3.39 (m, 4H), 3.81 (m, 0.5H), 3.91–4.08 (m, 1.5H), 5.76 (d, 0.5H, J = 6.0), 5.82 (d, 0.5H, J = 5.4), 6.11 (d, 0.5H, J = 9.6), 6.53 (d, 0.5H, J = 8.7), 7.14–7.30 (m, 5H), 7.74 (m, 1H). MALDI-TOF-MS calcd for $C_{18}H_{28}N_2O_5$ $(M+Na)^+$, 375.1896, found, 375.1937.

4.8.9. ((1*S*)-3-Ethylamino-2-hydroxy-3-oxo-1-(phenylethyl)propyl)carbamic acid 1,1-dimethylethyl ester (17A). White solid. Mp 134.4–134.9 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.95–1.03 (m, 3H), 1.38–1.41 (m, 9H), 1.59–1.81 (m, 2H), 2.50–2.66 (m, 2H), 3.03–3.13 (m, 2H), 3.70–3.80 (m, 1H), 3.88 (m, 1H), 5.59 (m, 1H), 6.14 (d, 0.5H, J = 9.3), 6.58 (d, 0.5H, J = 9.3), 7.13–7.30 (m, 5H), 7.77 (m, 1H). MALDI-TOF-MS calcd for $C_{18}H_{28}N_2O_4$ $(M+Na)^+$, 359.1947, found, 359.2041.

4.8.10. ((1*S*)-3-Cyclopropylamino-2-hydroxy-3-oxo-1-(phenylethyl)propyl)carbamic acid 1,1-dimethylethyl ester (17B). White solid. Mp 142.1–143.6 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.42–0.60 (m, 4H), 1.38–1.40 (m, 9H), 1.56–1.77 (m, 2H), 2.35–2.65 (m, 3H), 3.67–3.80 (m, 1H), 3.82–3.88 (m, 1H), 5.41 (d, 0.5H, J = 6.3), 5.54 (d, 0.5H, J = 6.0), 6.16 (d, 0.5H, J = 9.3), 6.56 (d, 0.5H, J = 9.0), 7.14–7.30 (m, 5H), 7.69 (d, 0.5H, J = 4.8), 7.74 (d, 0.5H, J = 4.2). MALDI-TOF-MS calcd for $C_{19}H_{28}N_2O_4$ $(M+Na)^+$, 371.1947, found, 371.1930.

4.9. General procedure for the preparation of compounds 18Aa–Ac, 18Ba–Bd, 18Ca–Ha, 18Ee, 19Aa–Ab, 19Ba–Bb

4.9.1. ((1*S*)-1-((((1*S*)-1-Benzyl-2,3-dioxo-3-(ethylamino)propyl)amino)carbonyl)-3-methylbutyl)carbamic acid 2-methoxyethyl ester (18Aa). Compound **16A** (5.5 g, 17 mmol) was dissolved in 4 N HCl/dioxane (65 mL) and stirred for 3 h at room temperature. The reaction mixture was evaporated to yield the deprotected amine hydrochloride (4.4 g, quant) as a white solid. To a solution of the deprotected amine hydrochloride (1.0 g, 4.0 mmol) and **9a** (1.2 g, 3.6 mmol) in DMF (10 mL) was added triethylamine (1.1 g, 11 mmol). The mixture was stirred at room temperature for 18 h and concentrated in vacuo. The residue was dissolved in EtOAc (50 mL) and the solution was washed with 1 M HCl (50 mL), saturated $NaHCO_3$ (50 mL), and saturated NaCl (50 mL), dried over $MgSO_4$, and concentrated in vacuo. The resulting white solid was washed with a mixture of hexane and EtOAc (9:1). To a solution of the resulting solid (0.7 g, 1.6 mmol) in CH_2Cl_2 (70 mL) was added Dess–Martin periodinane (1.0 g, 2.4 mmol).

The mixture was stirred at room temperature for 18 h. Aqueous 10% $Na_2S_2O_3$ (35 mL) and saturated aqueous $NaHCO_3$ (35 mL) were added, and the mixture was stirred for 30 min. The organic layer was separated, washed with 1 M HCl, saturated $NaHCO_3$, and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo. The residue was recrystallized from EtOAc/hexane to give **18Aa** (0.62 g, 88%) as colorless crystals. Mp 138.0–138.3 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.83 (d, 3H, J = 7.5), 0.85 (d, 3H, J = 7.2), 1.04 (t, 3H, J = 7.1), 1.35 (m, 2H), 1.56 (m, 1H), 2.82 (m, 1H), 3.14 (m, 3H), 3.25 (s, 3H), 3.47 (t, 2H, J = 4.5), 4.04 (m, 3H), 5.19 (m, 1H), 7.16–7.33 (m, 6H), 8.24 (d, 1H, J = 7.2), 8.70 (m, 1H). MALDI-TOF-MS calcd for $C_{22}H_{33}N_3O_6$ $(M+Na)^+$, 458.2267, found, 458.2361.

4.9.2. ((1*S*)-1-((((1*S*)-1-Benzyl-2,3-dioxo-3-(ethylamino)-propyl)amino)carbonyl)-3-methylbutyl)carbamic acid (3*S*)-tetrahydrofuran-3-yl ester (18Ab). Colorless crystal. Mp 158.9–160.7 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.83 (d, 3H, J = 6.6), 0.85 (d, 3H, J = 6.9), 1.04 (t, 3H, J = 7.1), 1.35 (m, 2H), 1.55 (m, 1H), 1.83 (m, 1H), 2.08 (m, 1H), 2.82 (m, 1H), 3.14 (m, 3H), 3.61–3.78 (m, 4H), 4.01 (m, 1H), 5.07 (m, 1H), 5.19 (m, 1H), 7.17–7.33 (m, 6H), 8.22 (d, 1H, J = 7.2), 8.69 (t, 1H, J = 5.7). MALDI-TOF-MS calcd for $C_{23}H_{33}N_3O_6$ $(M+H)^+$, 448.2447, found, 448.2509.

4.9.3. ((1*S*)-1-((((1*S*)-1-Benzyl-2,3-dioxo-3-(ethylamino)-propyl)amino)carbonyl)-3-methylbutyl)carbamic acid tetrahydro-4*H*-pyran-4-yl ester (18Ac). Colorless crystals. Mp 140.0–141.8 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.84 (m, 6H), 1.04 (t, 3H, J = 7.2), 1.35 (m, 2H), 1.49 (m, 3H), 1.79 (m, 2H), 2.82 (m, 1H), 3.14 (m, 3H), 3.41 (m, 2H), 3.78 (m, 2H), 4.02 (m, 1H), 4.66 (m, 1H), 5.19 (m, 1H), 7.15–7.33 (m, 6H), 8.22 (d, 1H, J = 7.2), 8.69 (t, 1H, J = 5.7). MALDI-TOF-MS calcd for $C_{24}H_{35}N_3O_6$ $(M+Na)^+$, 484.2424, found, 484.2486.

4.9.4. ((1*S*)-1-((((1*S*)-1-Benzyl-3-(cyclopropylamino)propyl)amino)carbonyl)-2,3-dioxo-3-methylbutyl)carbamic acid 2-methoxyethyl ester (18Ba). Colorless crystals. Mp 112.4–113.5 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.58 (m, 2H), 0.65 (m, 2H), 0.83 (d, 3H, J = 6.6), 0.85 (d, 3H, J = 6.6), 1.35 (m, 2H), 1.56 (m, 1H), 2.68–2.88 (m, 2H), 3.11 (m, 1H), 3.25 (s, 3H), 3.47 (t, 2H, J = 4.5), 4.04 (m, 3H), 5.17 (m, 1H), 7.17–7.34 (m, 6H), 8.25 (d, 1H, J = 7.2), 8.73 (d, 1H, J = 4.8). MALDI-TOF-MS calcd for $C_{23}H_{33}N_3O_6$ $(M+Na)^+$, 470.2267, found, 470.2441. $[\alpha]_D^{25}$ +6.3 (c 0.20, DMSO).

4.9.5. ((1*S*)-1-((((1*S*)-1-Benzyl-3-(cyclopropylamino)-2,3-dioxopropyl)amino)carbonyl)-3-methylbutyl)carbamic acid (3*S*)-tetrahydrofuran-3-yl ester (18Bb). Colorless crystals. Mp 169.2–170.5 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.58 (m, 2H), 0.65 (m, 2H), 0.83 (d, 3H, J = 8.1), 0.85 (d, 3H, J = 6.9), 1.34 (m, 2H), 1.55 (m, 1H), 1.83 (m, 1H), 2.08 (m, 1H), 2.79 (m, 2H), 3.12 (m, 1H), 3.61–3.80 (m, 4H), 4.02 (m, 1H), 5.08 (m, 1H), 5.17 (m, 1H), 7.22–7.35 (m, 6H), 8.24 (d, 1H, J = 6.6), 8.74 (d, 1H, J = 5.1). MALDI-TOF-MS calcd for $C_{24}H_{33}N_3O_6$ $(M+Na)^+$, 482.2267, found, 482.2586.

4.9.6. ((1*S*)-1-((((1*S*)-1-Benzyl-3-cyclopropylamino-2,3-dioxopropyl)amino)carbonyl)-3-methylbutyl)carbamic acid tetrahydro-4*H*-pyran-4-yl ester (18Bc). Colorless crystals. Mp 137.0–138.2 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.58 (m, 2H), 0.65 (m, 2H), 0.84 (m, 6H), 1.35 (m, 2H), 1.48 (m, 3H), 1.80 (m, 2H), 2.79 (m, 2H), 3.11 (m, 1H), 3.41 (m, 2H), 3.79 (m, 2H), 4.03 (m, 1H), 4.65 (m, 1H), 5.18 (m, 1H), 7.15–7.30 (m, 6H), 8.23 (d, 1H, J = 6.9), 8.73 (d, 1H, J = 5.4). MALDI-TOF-MS calcd for $C_{25}H_{35}N_3O_6$ $(M+H)^+$, 474.2604, found, 474.2643.

4.9.7. ((1*S*)-1-((((1*S*)-1-Benzyl-3-(cyclopropylamino)-2,3-dioxopropyl)amino)carbonyl)-3-methylbutyl)carbamic acid 5-methoxy-3-oxapentyl ester (18Bd). Colorless crystals. Mp 127.9–128.7 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.54–0.66 (m, 4H), 0.81–0.86 (m, 6H), 1.30–1.42 (m, 2H), 1.57 (m, 1H), 2.73 (m, 1H), 2.82 (dd, 1H, J = 14.3, 9.2), 3.11 (dd, 1H, J = 13.8, 4.2), 3.24 (s, 3H), 3.42–3.44 (m, 2H), 3.50–3.57 (m, 4H), 3.99–4.04 (m, 3H), 5.17 (m, 1H), 7.22–7.30 (m, 6H), 8.22 (d, 1H, J = 6.9), 8.71 (d, 1H, J = 4.8). MALDI-TOF-MS calcd for $C_{25}H_{37}N_3O_7$ $(M+Na)^+$, 514.2530, found, 514.2944. $[\alpha]_D^{25}$ +13.9 (c 0.20, DMSO).

4.9.8. ((1*S*)-1-((((1*S*)-1-Benzyl-2,3-dioxo-3-(propylamino)-propyl)amino)carbonyl)-3-methylbutyl)carbamic acid 2-methoxyethyl ester (18Ca). Colorless crystals. Mp 108.8–109.9 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.83 (m, 9H), 1.35 (m, 2H), 1.46 (m, 2H), 1.55 (m, 1H), 2.83 (dd, 1H, J = 14.0, 9.2), 3.08 (m, 3H), 3.25 (s, 3H), 3.48 (t, 2H, J = 4.4), 4.04 (m, 3H), 5.19 (m, 1H), 7.22–7.28 (m, 6H), 8.24 (d, 1H, J = 6.9), 8.68 (t, 1H, J = 5.6). MALDI-TOF-MS calcd for $C_{23}H_{35}N_3O_6$ (M+H)+, 450.2604, found, 450.2832.

4.9.9. ((1*S*)-1-((((1*S*)-1-Benzyl-3-(cyclobutylamino)propyl)amino)carbonyl)-2,3-dioxo-3-methylbutyl)carbamic acid 2-methoxyethyl ester (18Da). Colorless crystals. Mp 114.2–115.3 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.84 (m, 6H), 1.34 (m, 2H), 1.49–1.72 (m, 3H), 2.10 (m, 4H), 2.81 (dd, 1H, J = 13.8, 9.3), 3.10 (m, 1H), 3.25 (s, 3H), 3.47 (m, 2H), 4.03 (m, 3H), 4.22 (m, 1H), 5.15 (m, 1H), 7.24 (m, 6H), 8.24 (d, 1H, J = 7.2), 8.91 (d, 1H, J = 7.8). MALDI-TOF-MS calcd for $C_{24}H_{35}N_3O_6$ $(M+Na)^+$, 484.2424, found, 484.2400.

4.9.10. ((1*S*)-1-((((1*S*)-1-Benzyl-3-butylamino-2,3-dioxopropyl)amino)carbonyl)-3-methylbutyl)carbamic acid 2-methoxyethyl ester (18Ea). Colorless crystals. Mp 94.0–95.2 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.85 (m, 9H), 1.25 (m, 2H), 1.35 (m, 2H), 1.42 (m, 2H), 1.56 (m, 1H), 2.83 (dd, 1H, J = 13.8, 9.0), 3.10 (m, 3H), 3.25 (s, 3H), 3.47 (t, 2H, J = 4.5), 4.04 (m, 3H), 5.18 (m, 1H), 7.21–7.29 (m, 6H), 8.23 (d, 1H, J = 6.6), 8.67 (t, 1H, J = 6.0). MALDI-TOF-MS calcd for $C_{24}H_{37}N_3O_6$ $(M+H)^+$, 464.2760, found, 464.2870.

4.9.11. ((1*S*)-1-((((1*S*)-1-Benzyl-2,3-dioxo-3-(2,2,2-trifluoroethylamino)propyl)amino)carbonyl)-3-methylbutyl)carbamic acid 2-methoxyethyl ester (18Fa). Colorless crystals. Mp 152.5–153.9 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.84 (m, 6H), 1.34 (m, 2H), 1.55 (m, 1H), 2.86 (dd, 1H,

J = 14.0, 8.6), 3.10 (dd, 1H, J = 14.1, 4.8), 3.25 (s, 3H), 3.48 (t, 2H, J = 4.7), 3.90 (m, 2H), 4.04 (m, 3H), 5.14 (m, 1H), 7.21–7.31 (m, 6H), 8.34 (d, 1H, J = 6.9), 9.29 (m, 1H). MALDI-TOF-MS calcd for $C_{22}H_{30}F_3N_3O_6$ $(M+H)^+$, 490.2165, found, 490.2434.

4.9.12. ((1*S*)-1-((((1*S*)-1-Benzyl-2,3-dioxo-3-(2-indanylamino)propyl)amino)carbonyl)-3-methylbutyl)carbamic acid 2-methoxyethyl ester (18Ga). Colorless crystals. Mp 141.9–143.5 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.83 (d, 3H, J = 6.9), 0.86 (d, 3H, J = 6.9), 1.36 (m, 2H), 1.57 (m, 1H), 2.80–2.96 (m, 3H), 3.10–3.18 (m, 3H), 3.24 (s, 3H), 3.47 (t, 2H, J = 4.7), 4.04 (m, 3H), 4.50 (m, 1H), 5.19 (m, 1H), 7.13–7.30 (m, 10H), 8.29 (d, 1H, J = 6.9), 8.97 (d, 1H, J = 7.2). MALDI-TOF-MS calcd for $C_{29}H_{37}N_3O_6$ $(M+H)^+$, 524.2760, found, 524.2810.

4.9.13. ((1*S*)-1-((((1*S*)-1-Benzyl-2,3-dioxo-3-(2-methoxyethylamino)propyl)amino)carbonyl)-3-methylbutyl)carbamic acid 2-methoxyethyl ester (18Ha). Colorless crystals. Mp 127.0–127.9 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.83 (d, 3H, J = 6.9), 0.86 (d, 3H, J = 6.9), 1.35 (m, 2H), 1.56 (m, 1H), 2.83 (dd, 1H, J = 13.8, 9.0), 3.11 (dd, 1H, J = 14.0, 4.4), 3.24 (s, 3H), 3.25 (s, 3H), 3.16–3.34 (m, 2H), 3.39 (m, 2H), 3.48 (t, 2H, J = 4.5), 4.04 (m, 3H), 5.20 (m, 1H), 7.18–7.30 (m, 6H), 8.21 (d, 1H, J = 6.9), 8.66 (t, 1H, J = 5.4). MALDI-TOF-MS calcd for $C_{23}H_{35}N_3O_7$ $(M+Na)^+$, 488.2373, found, 488.2680.

4.9.14. ((1*S*)-1-((((1*S*)-2,3-Dioxo-3-ethylamino-1-(phenylethyl)propyl)amino)carbonyl)-3-methylbutyl)carbamic acid 2-methoxyethyl ester (19Aa). Colorless crystals. Mp 119.1–120.4 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.89 (t, 6H, J = 6.3), 1.03 (t, 3H, J = 7.2), 1.43 (t, 2H, J = 7.2), 1.61–1.85 (m, 2H), 2.07 (m, 1H), 2.56–2.74 (m, 2H), 3.07–3.17 (m, 2H), 3.25 (s, 3H), 3.49 (t, 2H, J = 4.7), 4.05–4.14 (m, 3H), 4.89 (m, 1H), 7.16–7.36 (m, 5H), 7.34 (d, 1H, J = 8.4), 8.33 (d, 1H, J = 6.9), 8.65 (t, 1H, J = 5.9). MALDI-TOF-MS calcd for $C_{23}H_{35}N_3O_6$ $(M+H)^+$, 450.2604, found, 450.2701.

4.9.15. ((1*S*)-1-((((1*S*)-2,3-Dioxo-3-ethylamino-1-(phenylethyl)propyl)amino)carbonyl)-3-methylbutyl)carbamic acid (3*S*)-tetrahydrofuran-3-yl ester (19Ab). Colorless crystals. Mp 111.9–114.5 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.89 (t, 6H, J = 6.3), 1.03 (t, 3H, J = 7.2), 1.43 (t, 2H, J = 7.4), 1.60–1.91 (m, 3H), 2.09 (m, 2H), 2.56–2.76 (m, 2H), 3.07–3.17 (m, 2H), 3.63–3.82 (m, 4H), 4.02–4.13 (m, 1H), 4.88 (m, 1H), 5.09–5.13 (m, 1H), 7.16–7.31 (m, 5H), 7.34 (d, 1H, J = 8.4), 8.34 (d, 1H, J = 6.9), 8.66 (t, 1H, J = 5.7). MALDI-TOF-MS calcd for $C_{24}H_{35}N_3O_6$ $(M+H)^+$, 462.2604, found, 462.2870.

4.9.16. ((1*S*)-1-((((1*S*)-2,3-Dioxo-3-cyclopropylamino-1-(phenylethyl)propyl)amino)carbonyl)-3-methylbutyl)carbamic acid 2-methoxyethyl ester (19Ba). Colorless crystals. Mp 109.7–111.1 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.53–0.68 (m, 4H), 0.87–0.91 (m, 6H), 1.43 (t, 3H, J = 7.2), 1.59–1.85 (m, 2H), 2.01–2.13 (m, 1H), 2.56–2.74 (m, 3H), 3.25 (s, 3H), 3.48–3.51 (m, 2H), 4.05–4.14 (m, 3H), 4.87 (m, 1H), 7.17–7.36 (m, 6H), 8.34 (d, 1H, J = 6.6), 8.69 (d, 1H, J = 5.1). MALDI-TOF-MS calcd for $C_{24}H_{35}N_3O_6$ $(M+H)^+$, 462.2604, found, 462.2742.

4.9.17. ((1*S*)-1-((((1*S*)-3-Cyclopropylamino-2,3-dioxo-1-(phenylethyl)propyl)amino)carbonyl)-3-methylbutyl)carbamic acid (3*S*)-tetrahydrofuran-3-yl ester (19Bb). Colorless crystals. Mp 115.8–116.2 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.56–0.59 (m, 4H), 0.88 (t, 6H, J = 6.3), 1.42 (t, 2H, J = 7.4), 1.60–1.91 (m, 3H), 2.09 (m, 2H), 2.56–2.76 (m, 3H), 3.63–3.81 (m, 4H), 4.05–4.13 (m, 1H), 4.87 (m, 1H), 5.09–5.13 (m, 1H), 7.20–7.35 (m, 6H), 8.34 (d, 1H, J = 6.9), 8.69 (d, 1H, J = 5.1). MALDI-TOF-MS calcd for $C_{25}H_{35}N_3O_6$ $(M+H)^+$, 474.2604, found, 474.2598.

4.9.18. ((1*S*)-1-((((1*S*)-1-Benzyl-3-butylamino-2,3-dioxopropyl)amino)carbonyl)-3-methylbutyl)carbamic acid benzyl ester (18Ee). Colorless crystals. Mp 150.1–153.8 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.85 (m, 9H), 1.24 (m, 2H), 1.33–1.49 (m, 4H), 1.58 (m, 1H), 2.83 (m, 1H), 3.11 (m, 3H), 4.07 (m, 1H), 5.01 (s, 2H), 5.19 (m, 1H), 7.23 (m, 5H), 7.35 (m, 6H), 8.28 (d, 1H, J = 7.2), 8.67 (t, 1H, J = 5.9). MALDI-TOF-MS calcd for $C_{28}H_{37}N_3O_5$ $(M+Na)^+$, 518.2631, found, 518.2847.

4.10. Determination of water-solubility

A suspension of inhibitor (1–5 mg) in pH 7 buffer solution (2.0 mL) was shaken at 25 °C for 5 h. The suspension was filtered through CHROMATODISK® (0.45 μm, GL Sciences), and the filtrate (1.0 mL) was quantified by the HPLC–UV method.

4.11. Inhibition assays for calpains

Inhibition assays were performed as described in the previous literature[10,11] using commercial μ-calpain (human erythrocyte, Calbiochem) and m-calpain (porcine kidney, Calbiochem). Assay solution including 0.5 mg/mL casein, 20 mM dithiothreitol, 50 mM Tris–HCl (pH 7.4), and 1.0 nmol of enzyme was used. The assay solution (200 μL) and DMSO (2.5 μL) including inhibitor of different concentrations were placed in each well. Reaction was started by addition of 20 mM $CaCl_2$ (50 μL) in a test well and 1 mM EDTA (50 μL) in a blank well. After incubation for 60 min at 30 °C, the mixture (100 μL) was transferred to another plate in which H_2O (100 μL) and Bio-Rad protein assay dye reagent (50 μL) were placed in each well. After incubation at room temperature for 15 min, the OD of the mixture was taken at 595 nm with a plate reader (Multiscan Multisoft, Labsystems). The percent inhibition was calculated from the difference of OD between the presence and absence of the compound. The IC_{50} was obtained from the graphical analysis of the concentration and the inhibition.

4.12. Metabolic stability

Human hepatic S9 fraction (S9) incubations were performed in the presence of an NADPH-generating system composed of 3 mM $MgCl_2$, 1 mM $NADP^+$, 5 mM glucose-6-phosphate, and 1 Unit/mL glucose-6-phosphate

dehydrogenase in a 50 mM potassium phosphate buffer (pH 7.4). All concentrations are relative to the final incubation volume. The compounds were added in acetonitrile to a final concentration of 5 μM. Incubations were conducted at 37 °C. After 30 min, aliquots of incubations (1 mL) were terminated by addition of 4 mL of acetonitrile. The precipitated proteins were removed by centrifugation, and supernatants were evaporated and the residue was dissolved in the mobile phase. The solution was analyzed by the HPLC–UV method.

4.13. Pharmacokinetic studies in monkey

The in vivo pharmacokinetic results were determined after oral (10 mg/kg, $n = 2$) single agent dosing to female cynomolgus monkeys. The compounds were administrated using a 0.5% carboxymethyl cellulose suspension formulation by nasogastric tube. Blood samples from each dosing were taken at pre-determined times (0.5, 1, 2, and 4 h) for analysis. All plasma samples were frozen and stored in −30 °C until analysis. The plasma concentration of each test compound was determined by the HPLC–UV method.

4.14. Caco-2 permeability studies[18]

Caco-2 cells were cultivated under aseptic conditions at 37 °C in an atmosphere of 90% relative humidity (5% CO_2). The culture medium consisted of 10% fetal bovine serum, antibiotic–antimicotic solution (GIBCO-BRL), non-essential amino acids solution (GIBCO-BRL), and 2 M L-glutamate. The culture medium was replaced every 2–3 days. The Caco-2 cells were seeded onto 24-well transwell plates (1×10^5 cells/cm^2) and grown for 22 days until monolayer was apparent.

The chamber was preincubated for 1 h at 37 °C in HBSS (apical, pH 6.5, 300 μL: basolateral, pH 7.4, 1000 μL). The test compounds and permeability reference markers (propranolol and ^{14}C-mannitol) were dissolved in HBSS with 0.1% DMSO (final concentration: 10 μM).

Independently, the test compounds and the reference markers were added to the apical side of the cell monolayer. Aliquots (500 μL) were removed from this compartment at 60 and 120 min. The concentration of the test samples were determined by LC/MS/MS or a liquid scintillation counter (^{14}C-mannitol) and P_{app} values were calculated as described in the literature. The P_{app} values of propranolol were determined to be 6.8×10^{-6} cm/s (at 60 min) and 8.5×10^{-6} cm/s (at 120 min) while those of ^{14}C-mannitol were found to be 0.8×10^{-6} cm/s (at 60 min) and 0.6×10^{-6} cm/s (at 120 min).

4.15. IAM Analysis[19]

The test compounds (5 mg) were dissolved with acetonitrile (0.1 mL). The solutions were diluted with the mobile phase (Dulbecco's Phosphate-Buffered Saline (pH 7.4)) to 100 mL. HPLC analysis (IAM Fast-Screen Mini column 4.6 × 30 mm from Regis Technologies Inc., 0.5 mL/min, at 27 °C column temperature) was performed on a Shimadzu LC-10A system equipped with a UV detector (monitoring at 250 nm). Aliquots (5–20 μL) of the sample solution were injected onto the HPLC column. The retention time (t_R) of each compound was determined and the k'_{IAM} values were calculated using the following equation:

$$k'_{IAM} = (t_R - t_0)/t_0,$$

where t_R is the retention times (min) of the test compound and t_0 corresponds to the void volume time. The $\mathrm{Log}\,k'_{IAM}$ ($\mathrm{Log}\,K'$) value of propranolol was determined to be 1.95.

Acknowledgments

The authors would like to thank Yuji Sakamoto, Atsuko Fujii, Misae Ohtaka, and Kazuko Nishiyama for their support of this work. Also, we would like to thank Drs. Yutaka Kawamatsu and Mitsuyoshi Azuma for useful discussions.

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