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Exploration of orally available calpain inhibitors. Part 3: Dipeptidyl α-ketoamide derivatives containing pyridine moiety

Yoshihisa Shirasaki,* Hiroyuki Miyashita† and Masazumi Yamaguchi

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

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Abstract—Calpain-mediated proteolysis has been implicated as a major process in neuronal cell death including retinal neurological degeneration. The previously reported calpain inhibitor SJA6017 (**1**) showed oral efficacy in a retinal pharmacological model, but its oral bioavailability was low due to the metabolic lability and low water-solubility. The purpose of present study was to identify good orally bioavailable calpain inhibitors. A series of water-soluble dipeptidyl α-ketoamides containing a pyridine moiety at P3 were designed, synthesized, and evaluated for their oral bioavailability and retinal penetration. Introduction of a pyridineethanol moiety provided the potent α-ketoamide inhibitor **8** with good oral bioavailability. Compound **8** showed about 12-fold higher retinal AUC than **1**.

1. Introduction

Calpains are Ca^{2+}-activated cytoplasmic cysteine endoproteases and at least 15 genes of these enzymes were identified.[1–3] Two major isoforms, μ-calpain (calpain I) and m-calpain (calpain II), are ubiquitously found in mammalian cells. Calpains have been implicated in numerous diseases such as muscular dystrophy, cardiac ischemia, cataract, and various neurological disorders including stroke, Alzheimer's disease, central nervous system diseases, spinal cord injury, and traumatic brain injury (TBI).[4–7] Previously, our researchers have shown that calpains play important roles in retinal neuronal cell death induced by retinal ischemia-reperfusion[8] and ocular hypertension.[9] It has also been reported that an activation of calpains is found in *rd1* mice, which are an accepted animal model of retinitis pigmentosa.[10,11] These indicate that calpain is an attractive target for discovering a novel therapeutic agent of retinal neurological disorders such as glaucoma and retinitis pigmentosa.

A number of irreversible and reversible calpain inhibitors have so far been identified.[12–30] Reversible inhibitors are favored for the treatment of chronic diseases because irreversible inhibitors may react with various proteins and cause unexpected adverse effects. Since most of retinal diseases are chronic diseases, intravenous administration of a therapeutic agent is inconvenient. Topical instillation to the eyes cannot readily deliver drugs into the retina because several pathological and anatomical barriers exist.[31] Therefore, oral drugs are desirable for the treatment of retinal diseases. Our group has reported a highly potent reversible inhibitor, dipeptidyl aldehyde **1** (SJA6017),[32–34] which showed neuroprotective efficacy in the rat retinal ischemia model via intravenous administration.[8] However, **1** required the relatively high dose (500 mg/kg) in oral administration for the efficacy and showed poor bioavailability due to a metabolically labile aldehyde moiety.[35] Consequently, we examined the introduction of more chemically stable warheads such as α-ketoamide,[29] and cyclic hemiacetal,[29] hydrazone moiety[30] instead of the aldehyde moiety (Fig. 1). The resulting α-ketoamide analog **2**[29] showed potent inhibitory activities, higher cellular permeability, and metabolic stability than **1**, but displayed poor plasma exposure in monkeys after oral administration due to its extremely low aqueous solubility.[35]

Our aim was to explore potent and orally bioavailable calpain inhibitors. We attempted to enhance the water-solubility of ketoamide **2** to improve oral bioavailability. Introduction of a strong ionic moiety such as sulfonic acid, carboxylic acid or guanidine moieties increases

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* Corresponding author. Tel.: +81 78 997 1010; fax: +81 78 997 1016; e-mail: shirasaki@senju.co.jp
† Present address: Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan.

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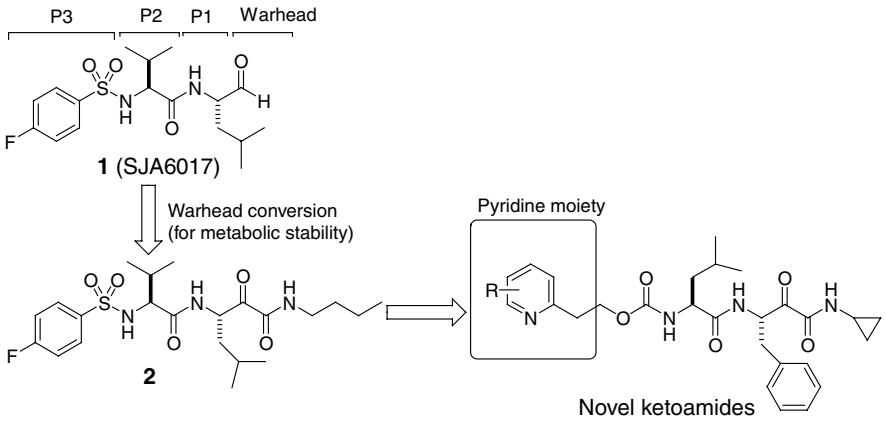


Figure 1. Drug design concept of novel calpain inhibitors.

aqueous solubility but would lead to low cellular permeability.[36–39] Therefore, we decided to introduce pyridine moiety, which was a weak basic moiety, to P3 site of dipeptidyl α-ketoamide. Phe-Leu residues and cyclopropyl group were placed at P1–P2 site and P′ site, respectively, because these moieties were optimal substituents in our previous report.[35] In this report, we describe the synthesis of pyridineethanol α-ketoamides, their inhibitory activities, metabolic stability data, and in vivo pharmacokinetics (PK) in rat plasma and retinas.

2. Results and discussion

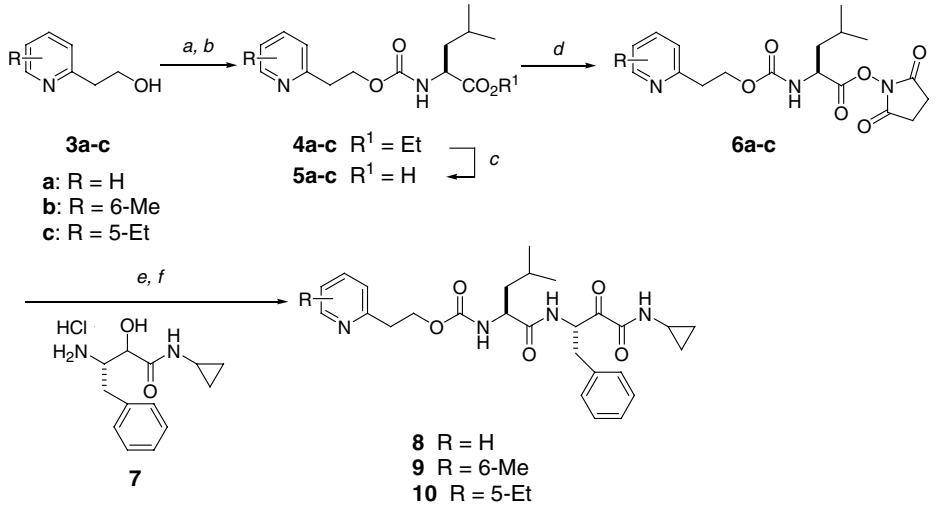
2.1. Chemistry

As shown in Scheme 1, the ketoamides **8**–**10** were synthesized by using a previously reported method.[27,35] 2-Pyridineethanols **3a**–**c** were coupled to a leucine ethyl ester by using *N*,*N*′-disuccinimidyl carbonate to afford ether esters **4a**–**c**. These esters were hydrolyzed to corresponding carboxylic acids **5a**–**c** and then they were converted to succinimide esters **6a**–**c**. Condensation of **6a**–**c** and β-hydroxyaminoacid **7**[27] in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 1-hydroxybenzotriazole (HOBt) followed by DMSO oxidation with sulfurtrioxide/pyridine complex (SO_3/pyridine) provided the target ketoamides **8**–**10**. Compounds **8**–**10** were obtained as single diastereomers on the basis of the ^{1}H NMR spectra.

2.2. Enzyme inhibitory activity, aqueous solubility, and metabolic stability

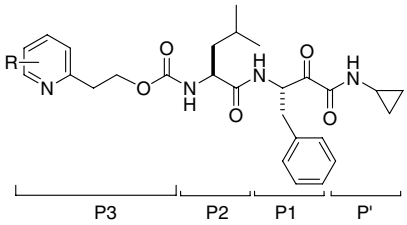
The structures, inhibitory activities, and water-solubility of the novel pyridineethanol α-ketoamides **8**–**10** are summarized in Table 1. The potencies of **8**–**10** were higher than that of starting ketoamide **2** and approximately equal to that of aldehyde **1**. Increases in potencies may result from an enhancement of lipophilic interaction between the pyridine moiety and the S3 subsite of calpains. The introduction of a methyl or ethyl group to the pyridine ring did not substantially affect the activities (**8** vs **9** or **10**).

Compounds **8**–**10** were 2- to 18-fold in a neutral condition (phosphate buffer, pH 7.0) and 3700-fold in acidic conditions (aqueous HCl, pH 1.2) more soluble than



Scheme 1. Reagents: (a) $CO(OSu)_2$, TEA, MeCN; (b) L-leucine-OEt HCl, TEA, CH_2Cl_2; (c) NaOH, aq EtOH; (d) HOSu, EDC, CH_2Cl_2; (e) TEA, CH_2Cl_2; (f) SO_3/pyridine, DIPEA, CH_2Cl_2.

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



Compound	R	IC_{50}[a] (μM)		Solubility[b] (mg/mL)	Metab[c] (%)	PK[d]	
		μ-Calpain	m-Calpain			C_{max} (μM)	AUC[e] (μM h)
1	—	0.028	0.023	0.1	3	—	(0.90)[f]
2	—	0.084	0.048	0.0053	21	—	—
8	H	0.038	0.019	0.087 (>20)[g]	23	8.6	6.8
9	6-Me	0.031	0.018	0.022 (>20)[g]	1	4.7	1.1
10	5-Et	0.041	0.017	0.013 (>20)[g]	1	2.1	0.86

[a] The IC_{50} values were determined by the fluorescence assay method.
[b] Aqueous-solubility in pH 7 buffer, at 25 °C.
[c] Metabolic stability represented in residual percent after incubation with human S9 for 0.5 h at 37 °C.
[d] Pharmacokinetics in rats (n = 4–5), po 10 mg/kg, dosed as a suspension in 0.5% CMC–Na.
[e] The area under the curves for 0–4 h.
[f] Dose normalized AUC value (normalized to 10 mg/kg) when **1** was dosed at po 500 mg/kg.
[g] Aqueous-solubility in aqueous HCl (pH 1.2), at 25 °C.

ketoamide **2**. Incorporation of pyridineethanol moiety significantly improved the aqueous solubility in acidic conditions.

Non-substituted analog **8** exhibited the highest metabolic stability (23%) in this series. Introduction of an alkyl substituent on the pyridine ring resulted in poor metabolic stability (**8** vs **9** or **10**). LC/MS analysis of the S9 incubations indicated that **8**–**10** were oxidized at the pyridineethanol moiety. However, the positions of oxidation were not identified by the data. It has been previously reported that the structurally related pyridine derivatives were oxidized mainly at the methylene/methyl moieties of the benzylic position or the nitrogen atom of the pyridine ring by CYP enzymes. This suggests that the positions of oxidation of **8**–**10** are similar to these pyridine derivatives.[40–42] The proposed metabolic pathways of these compounds are shown in Figure 2. We postulated that the increase in metabolic turnover via an introduction of an alkyl group was caused by an increase in both the number of benzylic methylene/methyl moieties, which are easily oxidized by CYP enzymes, and the affinities toward CYP enzymes through an enhancement of lipophilicity.[40–42] The change of steric environment also may be involved in the metabolic turnover. A similar increase in metabolic turnover was reported previously by Chiba et al.[43]

2.3. PK evaluation

Preliminary PK properties of these ketoamides **8**–**10** after single oral administration (10 mg/kg) were evaluated in rats and the data are summarized in Table 1. Non-substituted pyridine derivative **8** showed the highest plasma concentration among these compounds. Alkyl substituted analogs **9** and **10** exhibited a relatively low plasma exposure, which is presumably due to their low aqueous solubility in neutral conditions and poor in vitro metabolic stability. Therefore, we selected compound **8** for further PK evaluation. Thus, introduction of both

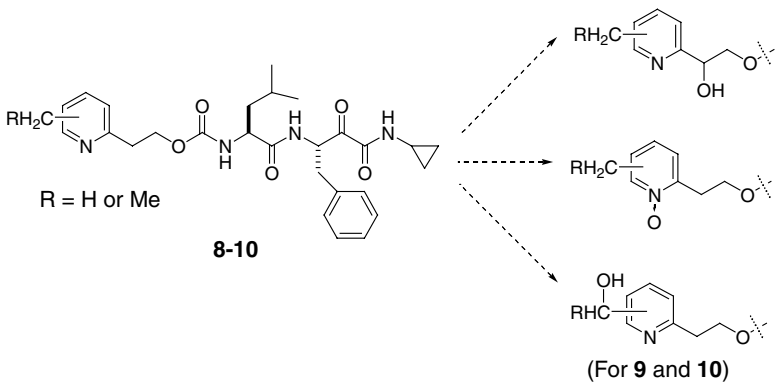


Figure 2. Proposed metabolic pathways of ketoamides **8**–**10**.

Table 2. Plasma PK parameters of ketoamide **8** and aldehyde **1** in rats (n = 4–5)

Compound	Route	Dose (mg/kg)	AUC[a] (μM h)	C_{max} (μM)	T_{max} (h)	$T_{1/2}$[b] (h)	V_{ss}[c] (L/kg)	Cl[d] (mL/min/kg)	F[e] (%)
1	po	500	45	9.4	1.0	—	—	—	18
	iv	3	1.5	—	—	0.7	6.1	89	
8	po	10	6.8	8.6	0.25	—	—	—	45
	iv	3	4.5	—	—	2.1	0.98	22	

[a] The area under the curves for 0 h to infinity.
[b] The terminal half-life.
[c] The steady-state volume of distribution.
[d] The total clearance.
[e] Oral bioavailability.

the pyridineethanol and the α-ketoamide moieties remarkably improved oral plasma exposure of the inhibitor.

Encouraged by the preliminary PK results of **8**, we decided to determine the oral bioavailability and retinal PK properties and compare them to those of potent aldehyde **1**, which showed retinal neuroprotective efficacy at an oral dose of 500 mg/kg. The oral and intravenous plasma PK data in rats are shown in Table 2. Ketoamide **8** demonstrated rapid absorption (T_{max} = 0.25 h) and high oral bioavailability (F = 45%) (Fig. 2). The total plasma clearance of **8** following bolus iv administration was moderate (Cl = 22 mL/min/kg) and lower than that of **1** (Cl = 89 mL/min/kg). Terminal half-life ($T_{1/2}$) of **8** was greater than that of **1**, in spite of a smaller steady-state volume of distribution (V_{ss}) of **8**. Higher in vitro metabolic stability of **8** may contribute to the lower plasma clearance in rats. We also confirmed that α-ketoamide moiety provided a more metabolically stable inhibitor in vivo compared to aldehyde moiety.

The retinal PK data after oral administration and physicochemical properties of inhibitors **8** and **1** are summarized in Table 3. Ketoamide **8** was able to penetrate into the retina, and showed about a 12-fold higher dose-normalized retinal AUC value ($nAUC_r$) and higher retina-to-plasma AUC ratio (R/P ratio) than **1**. The terminal half-life ($T_{1/2}$) of **8** in the retina was approximately equal to **1**. We confirmed that introduction of a pyridineethanol moiety provided not only the good oral bioavailability but also the higher retinal penetration. The results indicate that a 12-fold less dose of **8** can show the equal retinal efficacy to that of **1**. Although the lipophilicity of **8** ($c\log D_7$ 3.31) is similar to that of **1** ($c\log D_7$ 3.21), compound **8** showed higher R/P ratio. Penetration of drugs into the retina is restricted by the inner and outer blood–retinal barrier (BRB). The outer BRB is composed of the retinal-pigmented epithelium (RPE) and choroid. The RPE-choroid permeability by the passive transcellular transport depends on lipophilicity of drugs.[44] This suggests that **1** may be excreted from the retina by efflux transporters such as P-gp and MRP in BRB.

Table 3. Retinal PK parameters after single oral administration in rats

Compound	R/P ratio[a]	$nAUC_r$ (μM h)	$T_{1/2}$[b] (h)	$c\log D_7$[c]
1	0.062	0.060[d]	2.8	3.21
8	0.10	0.71	2.9	3.31

[a] Retina-to-plasma AUC ratio.
[b] The terminal half-life in the retina.
[c] The dissociative partition coefficient ACD $c\log D_7$ was calculated using Solaris v 4.76 (ACD/Labs).
[d] The dose normalized area under the curves for 0 h to infinity in retina (normalized to 10 mg/kg).

3. Conclusion

We synthesized a series of ketoamide-based inhibitors containing a pyridine moiety based on the premise that the introduction of a basic moiety may lead to an increase in oral bioavailability through the enhancement of aqueous solubility. We have identified ketoamide **8** as a potent calpain inhibitor, which showed good oral bioavailability and a higher R/P ratio than the potent calpain inhibitor **1**. Ketoamide **8** exhibited about a 12-fold higher retinal AUC and would show retinal efficacy at a lower dose than aldehyde lead **1**. The compound **8** (SNJ-2008) may become a candidate for further development as an orally available drug for the treatment of retinal disorders.

4. Experimental

4.1. General

All reagents were of commercial grade and used without further purification. Melting points were obtained using a Yanaco micromelting point apparatus without correction. ^{1}H NMR and ^{13}C NMR spectra were measured using a Varian Gemini-2000 Spectrometer. Chemical shifts were reported in parts per million (δ value), and coupling constants (J) were reported in hertz. Tetramethylsilane (δ 0) or DMSO (δ 39.7) was used as an internal standard (^{1}H NMR and ^{13}C NMR, respectively). Splitting patterns were indicated as follows: s, singlet; d, doublet; t, triplet; m, multiplet. IR spectra were recorded on a ThermoNicolet Avatar 380 FTIR spectrophotometer, and the representative absorption bands were reported. Specific rotations were measured with Horiba SEPA-2000 model. Analytical HPLC was performed with a Shimadzu Class LC-10A system (LC-10AD, SPD-10A, SIL-10A, CTO-10A, CBM-10A). Electron ionization mass spectra (EIMS)

and high-resolution electron ionization mass spectra (HREIMS) were recorded on JEOL JMS-AX505W (EI, 70 eV). Elemental analyses were performed on an Elementar Vario EL analyzer. LC–MS/MS spectra were obtained on an MDS-Sciex API 4000 triple–quadrupole mass spectrometer equipped with a turbo ion spray source.

4.2. General procedure for the preparation of ethyl esters 4a–c

4.2.1. *N*-((2-(Pyridin-2-yl)ethyloxy)carbonyl)-L-leucine ethyl ester (4a). To a stirred solution of 2-pyridineethanol (**3a**) (5.0 g, 41 mmol) in acetonitrile (100 mL) were added *N*,*N*′-disuccinimidyl carbonate ($CO(OSu)_2$) (15 g, 57 mmol) and triethylamine (12 g, 122 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 saturated NaCl (100 mL), dried over $MgSO_4$, and concentrated in vacuo to give a mixed carbonate. To a solution of L-leucine ethyl ester hydrochloride (5.8 g, 30 mmol) and triethylamine (7.6 g, 75 mmol) in CH_2Cl_2 (100 mL) was added a solution of the resulting mixed carbonate 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 water (100 mL), saturated $NaHCO_3$ (100 mL), and saturated NaCl (100 mL), dried over $MgSO_4$, and concentrated in vacuo to give **4a** (8.5 g, 69%) as a colorless viscous oil. $[\alpha]_D^{25}$ −13.6 (*c* 0.10, DMSO). ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.82–0.88 (m, 6H), 1.17 (t, 3H, *J* = 7.1 Hz), 1.36–1.63 (m, 3H), 3.03 (t, 2H, *J* = 6.8 Hz), 3.96–4.13 (m, 3H), 4.33 (t, 2H, *J* = 6.6 Hz), 7.23 (dd, 1H, *J* = 6.8, 5.0 Hz), 7.30 (d, 1H, *J* = 7.8 Hz), 7.52 (d, 1H, *J* = 8.1 Hz), 7.71 (m, 1H), 8.50 (m, 1H).

4.2.2. *N*-((2-(6-Methylpyridin-2-yl)ethyloxy)carbonyl)-L-leucine ethyl ester (4b). Colorless viscous oil. $[\alpha]_D^{25}$ −207.58° (*c* 0.13, DMSO). ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.83–0.88 (m, 6H), 1.15–1.20 (m, 3H), 1.37–1.65 (m, 3H), 2.43–2.48 (m, 3H), 2.95–3.02 (m, 2H), 3.97–4.12 (m, 3H), 4.31 (t, 2H, *J* = 6.8 Hz), 7.01–7.17 (m, 2H), 7.51 (d, 1H, *J* = 7.8 Hz), 7.59 (t, 1H, *J* = 7.7 Hz).

4.2.3. *N*-((2-(5-Ethylpyridin-2-yl)ethyloxy)carbonyl)-L-leucine ethyl ester (4c). Colorless viscous oil. $[\alpha]_D^{25}$ −62.8° (*c* 0.13, DMSO). ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.83–0.90 (m, 6H), 1.15–1.22 (m, 6H), 1.39–1.68 (m, 3H), 2.55–2.63 (m, 2H), 2.97–3.03 (m, 2H), 3.98–4.11 (m, 3H), 4.29–4.34 (m, 2H), 7.21 (d, 1H, *J* = 7.5 Hz), 7.50–7.57 (m, 2H), 8.36 (m, 1H).

4.3. General procedure for the preparation of acids 5a–c

4.3.1. *N*-((2-(Pyridin-2-yl)ethyloxy)carbonyl)-L-leucine (5a). To a solution of **4a** (8.5 g, 28 mmol) in EtOH (150 mL) was added 1 M NaOH (60 mL). The solution was concentrated in vacuo and washed with Et_2O (2× 100 mL). Then, aqueous layer was separated, made pH 4 by addition of HCl. The solution was extracted with EtOAc (2× 100 mL) and the organic layer was dried over $MgSO_4$ and concentrated in vacuo. The residue was crystallized with hexane and EtOAc to give **5a** (5.5 g, 71%) as colorless crystals. Mp 101.9–102.5 °C. $[\alpha]_D^{25}$ −11.8° (*c* 0.14, DMSO). ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.82–0.88 (m, 6H), 1.38–1.70 (m, 3H), 3.02 (t, 2H, *J* = 6.8 Hz), 3.93 (m, 1H), 4.32 (t, 2H, *J* = 6.8 Hz), 7.23 (m, 1H), 7.30 (d, 1H, *J* = 8.1 Hz), 7.37 (d, 1H, *J* = 8.1 Hz), 7.71 (m, 1H), 8.50 (d, 1H, *J* = 4.2 Hz).

4.3.2. *N*-((2-(6-Methylpyridin-2-yl)ethyloxy)carbonyl)-L-leucine (5b). Colorless viscous oil. $[\alpha]_D^{25}$ 4294.3° (*c* 0.12, DMSO). ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.82–0.87 (m, 6H), 1.38–1.65 (m, 3H), 2.42–2.50 (m, 3H), 2.97 (t, 2H, *J* = 6.8 Hz), 3.93 (m, 1H), 4.29 (t, 2H, *J* = 6.8 Hz), 7.03–7.09 (m, 2H), 7.37 (d, 1H, *J* = 8.1 Hz), 7.58 (t, 1H, *J* = 7.7 Hz).

4.3.3. *N*-((2-(5-Ethylpyridin-2-yl)ethyloxy)carbonyl)-L-leucine (5c). Colorless viscous oil. $[\alpha]_D^{25}$ −7.1° (*c* 0.16, DMSO). ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.82–0.87 (m, 6H), 1.14–1.20 (m, 3H), 1.39–1.65 (m, 3H), 2.53–2.62 (m, 2H), 2.98 (t, 2H, *J* = 6.8 Hz), 3.93 (m, 1H), 4.29 (t, 2H, *J* = 6.6 Hz), 7.19 (m, 1H), 7.36 (d, 1H, *J* = 8.4 Hz), 7.55 (m, 1H), 8.34 (m, 1H).

4.4. General procedure for the preparation of succinimide esters 6a–c

4.4.1. *N*-(((Pyridine-2-yl)ethyloxy)carbonyl)-L-leucine *N*-hydroxysuccinimide ester (6a). Compound **5a** (3.0 g, 11 mmol) and *N*-hydroxysuccinimide (1.6 g, 14 mmol) were dissolved in THF (30 mL), and a suspension of EDC (2.7 g, 14 mmol) in CH_2Cl_2 (30 mL) was added thereto. The mixture was stirred at room temperature for 18 h and concentrated in vacuo. The residue was dissolved in EtOAc (100 mL), the solution was washed with water (50 mL), saturated $NaHCO_3$ (50 mL) and saturated NaCl (50 mL), dried over $MgSO_4$, and concentrated in vacuo to give **6a** (3.2 g, 79%) as a colorless viscous oil. $[\alpha]_D^{25}$ −7.7° (*c* 0.24, DMSO). ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.82–0.91 (m, 6H), 1.42–1.76 (m, 3H), 2.76–2.81 (m, 4H), 3.00–3.06 (m, 2H), 4.30–4.40 (m, 3H), 7.23 (dd, 1H, *J* = 7.1 Hz, 5.3 Hz), 7.30 (d, 1H, *J* = 7.8 Hz), 7.71 (m, 1H), 7.90 (d, 1H, *J* = 8.1 Hz), 8.50 (d, 1H, *J* = 4.5 Hz).

4.4.2. *N*-((2-(6-Methylpyridin-2-yl)ethyloxy)carbonyl)-L-leucine *N*-hydroxysuccinimide ester (6b). Colorless viscous oil. $[\alpha]_D^{25}$ 1814.9° (*c* 0.10, DMSO). ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.83–0.92 (m, 6H), 1.49–1.77 (m, 3H), 2.43 (s, 3H), 2.81 (s, 4H), 2.99 (t, 2H, *J* = 6.5 Hz), 4.29–4.42 (m, 3H), 7.07–7.09 (m, 2H), 7.58 (t, 1H, *J* = 7.7 Hz), 7.91 (d, 1H, *J* = 8.4 Hz).

4.4.3. *N*-((2-(5-Ethylpyridin-2-yl)ethyloxy)carbonyl)-L-leucine *N*-hydroxysuccinimide ester (6c). Colorless viscous oil. $[\alpha]_D^{25}$ 124.5° (*c* 0.13, DMSO). ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.75–0.92 (m, 6H), 1.12–1.25 (m, 3H), 1.36–1.72 (m, 3H), 2.54–2.63 (m, 2H), 2.81–2.83 (m, 4H), 2.96–3.02 (m, 2H), 4.04 (m, 1H), 4.29–4.37 (m, 2H), 7.21 (d, 1H, *J* = 7.8 Hz), 7.53 (m, 1H), 7.90 (d, 1H, *J* = 7.8 Hz), 8.34 (m, 1H).

4.5. General procedure for the preparation of compounds 8–10

4.5.1. ((1*S*)-1-((((1*S*)-1-Benzyl-3-(cyclopropylamino)-2,3-dioxopropyl)amino)carbonyl)-3-methylbutyl)carbamic acid 2-(pyridin-2-yl)ethyl ester (8). To a solution of **6a** (2.0 g, 5.3 mmol) and **7** (1.4 g, 5.3 mmol) in CH_2Cl_2 (50 mL) was added triethylamine (1.6 g, 15.9 mmol). After stirring for 18 h at room temperature, the mixture was concentrated in vacuo. The residue was dissolved in EtOAc, and the solution was washed with water, saturated $NaHCO_3$, and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo to give crude hydroxylamide (1.6 g, 61%) as a white solid. The resulting crude hydroxylamide was used for the next step without further purification. To a solution of the crude hydroxyl amide (1.1 g, ca. 2.2 mmol) in DMSO (10 mL) and CH_2Cl_2 (7 mL) was added *N*,*N*-diisopropylethylamine (0.86 g, 6.6 mmol) under the ice-cooling condition. After stirring for 10 min, a suspension of purified sulfur trioxide pyridine complex (1.1 g, 6.6 mmol) in DMSO (10 mL) was added to the above mixture thereto. The mixture was stirred for 1 h under the same condition. The reaction mixture was diluted with EtOAc, and the solution was washed with water, saturated $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo. The residue was crystallized from EtOAc/hexane to yield **8** (0.70 g, 39%) as colorless crystals. Mp 138.0–138.3 °C. $[\alpha]_D^{25}$ −108.9° (*c* 0.1, DMSO). ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.58–0.66 (m, 4H), 0.83 (t, 6H, J = 7.1 Hz), 1.31–1.35 (m, 2H), 1.53 (m, 1H), 2.74 (m, 1H), 2.81 (dd, 1H, J = 14.1 Hz, 9.3 Hz), 3.02 (t, 2H, J = 6.3), 3.11 (dd, 1H, J = 14.0 Hz, 4.1 Hz), 4.01 (m, 1H), 4.28–4.32 (m, 2H), 5.17 (m, 1H), 7.14–7.34 (m, 8H), 7.75 (t, 1H, J = 6.8 Hz), 8.23 (d, 1H, J = 7.2 Hz), 8.51 (d, 1H, J = 4.2 Hz), 8.71 (d, 1H, J = 4.5 Hz). ^{13}C NMR (75 MHz, DMSO-d_6) δ 5.5, 5.6, 21.7, 22.7, 23.1, 24.2, 35.3, 37.3, 40.9, 52.8, 55.3, 63.3, 121.8, 123.6, 126.7, 128.4 (2C), 129.2 (2C), 136.6, 137.5, 149.2, 156.0, 158.2, 162.1, 172.8, 196.2. IR (KBr) ν_{max} 3282, 2955, 1714, 1649, 1536, 1361, 1259, 1120, 1069, 751, 696 (cm^{-1}). EIMS *m*/*z* 494 (M^+). HREIMS *m*/*z* calcd for $C_{27}H_{34}N_4O_5$ (M^+) 494.2529, found 494.2524. Anal. Calcd for $C_{27}H_{34}N_4O_5{\cdot}0.2H_2O$: C, 65.09; H, 6.96; N, 11.25. Found: C, 65.15; H, 7.01; N, 11.31.

4.5.2. ((1*S*)-1-((((1*S*)-1-Benzyl-3-(cyclopropylamino)-2,3-dioxopropyl)amino)carbonyl)-3-methylbutyl)carbamic acid 2-(6-methylpyridin-2-yl)ethyl ester (9). Colorless crystals. Mp 151.7–152.4 °C. $[\alpha]_D^{25}$ −80.0° (*c* 0.1, DMSO). ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.54–0.66 (m, 4H), 0.76–0.86 (m, 6H), 1.54 (m, 1H), 2.43 (s, 3H), 2.73–2.86 (m, 2H), 2.96 (t, 2H, J = 6.5 Hz), 3.11 (m, 1H), 4.03 (m, 1H), 4.21–4.34 (m, 2H), 5.17 (m, 1H), 7.07–7.30 (m, 8H), 7.58 (t, 1H, J = 7.7 Hz), 8.23 (d, 1H, J = 6.9 Hz), 8.72 (d, 1H, J = 4.8 Hz). ^{13}C NMR (75 MHz, DMSO-d_6) δ 5.5, 5.6, 21.7, 22.6, 23.1, 24.2, 35.3, 37.3, 40.9, 52.8, 55.3, 63.4, 120.5, 121.0, 126.6, 128.4 (2C), 129.2 (2C), 136.9, 137.5, 156.0, 157.4, 157.5, 162.1, 172.8, 196.2. IR (KBr) ν_{max} 3300, 2961, 1720, 1655, 1536, 1456, 1239, 1120, 1072, 748, 697 (cm^{-1}). EIMS *m*/*z* 508 (M^+). HREIMS *m*/*z* calcd for $C_{28}H_{36}N_4O_5$ (M^+) 508.2686, found 508.2673. Anal. Calcd for $C_{28}H_{36}N_4O_5$: C, 66.12; H, 7.13; N, 11.02. Found: C, 65.91; H, 7.13; N, 11.10.

4.5.3. ((1*S*)-1-((((1*S*)-1-Benzyl-3-(cyclopropylamino)-2,3-dioxopropyl)amino)carbonyl)-3-methylbutyl)carbamic acid 2-(5-Ethylpyridin-2-yl)ethyl ester (10). Colorless crystals. Mp 104.8–106.0 °C. $[\alpha]_D^{25}$ −190.0° (*c* 0.1, DMSO). ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.58–0.66 (m, 4H), 0.75–0.85 (m, 6H), 1.17 (t, 3H, J = 7.7 Hz), 1.33–1.36 (m, 2H), 1.53 (m, 1H), 2.58 (dd, 2H, J = 15.5, 8.3 Hz), 2.74–2.85 (m, 2H), 2.94–2.98 (m, 2H), 3.12 (m, 1H), 4.04 (m, 1H), 4.28–4.29 (m, 2H), 5.17 (m, 1H), 7.13–7.26 (m, 7H), 7.55 (d, 1H, J = 8.1 Hz), 8.22–8.35 (m, 2H), 8.75 (m, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 5.6 (2C), 15.5, 21.7, 22.6, 23.1, 24.2, 25.1, 35.3, 36.8, 40.9, 52.8, 55.3, 63.4, 123.1, 126.6, 128.4 (2C), 129.2 (2C), 135.9, 136.8, 137.5, 148.7, 155.5, 156.0, 162.1, 172.8, 196.2. IR (KBr) ν_{max} 3300, 2961, 1720, 1658, 1539, 1453, 1236, 1123, 1072, 742, 697 (cm^{-1}). EIMS *m*/*z* 522 (M^+). HREIMS *m*/*z* calcd for $C_{29}H_{38}N_4O_5$ (M^+) 522.2842, found 522.2836. Anal. Calcd for $C_{29}H_{38}N_4O_5{\cdot}0.2H_2O$: C, 66.19; H, 7.36; N, 10.65. Found: C, 65.98; H, 7.37; N, 10.82.

4.6. Inhibition assays for calpains

Calpain inhibition assays were performed as described in the previous literature[45] using commercial μ-calpain (human erythrocyte, Calbiochem) and m-calpain (porcine kidney, Calbiochem). Assay solution including 80 mM Tris–HCl (pH 7.4), 15 mM β-mercaptoethanol, and 15 nmol of enzyme was used. The assay solution (150 μL), 0.5 mM Boc-Val-Leu-Lys-AMC (50 μL, Bachem), and DMSO (2.5 μL) including inhibitor were placed in each well of 96-well plates. Reaction was started by the addition of 25 mM $CaCl_2$ (50 μL) to a test well and 1 mM EDTA (50 μL) in a blank well. Enzyme activity was determined by increase of fluorescence (λ_{ex} = 360 nm, λ_{em} = 440 nm) monitored at 37 °C using a CYTO FLUOR multi-well plate reader (Perseptive Biosystems). Percent enzyme inhibition was determined by comparison of this activity to that of a solution without inhibitor.

4.7. Metabolic stability

Metabolic stability studies in 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 U/mL glucose-6-phosphate dehydrogenase in a 50 mM potassium phosphate buffer (pH 7.4). The human S9 prepared by XenoTech LLC (Lenexa, CS) was purchased from Nosan Corporation (Yokohama, Japan). All concentrations were relative to the final incubation volume (5 mL). The compounds were added in acetonitrile to a final concentration of 5 μM. Incubations were conducted at 37 °C. After 30 min, 1 mL of incubations was terminated by the addition of 4 mL of acetonitrile. Precipitated proteins were removed by centrifugation, and supernatants were evaporated and the residue was dissolved in mobile phase. The solution was analyzed by LC–MS/MS.

4.8. Pharmacokinetic studies in rats

The in vivo pharmacokinetic study was determined after oral or intravenous single agent dosing (iv 3 mg/kg for **1** and **8**, po 10 mg/kg for **8**–**10** or 500 mg/kg for **1**) to male Sprague–Dawley rats (n = 4–5/time point, weight 200–250 g). The compounds were formulated as a suspension in 0.5% carboxymethyl cellulose solution (CMC) for oral administration. The compounds were administered in a vehicle consisting of 60% polyethylene glycol and 40% water at a concentration of 10 mg/mL for iv administration. The animals were anesthetized with isoflurane at a pre-determined time (0.1 (iv only), 0.25, 0.5, 1, 2, 4 and 8 h) and blood was sampled from the abdominal aorta into heparinized syringes. The blood was centrifuged to obtain plasma. Then, eyeballs were excised and isolated the retinas. All plasma samples were frozen and stored at −30 °C until analysis. The frozen plasma and retinas were fused at room temperature and plasma samples were prepared by solid-phase extraction procedure using OASIS® HLB (60 mg) cartridges by Waters. The retinas were extracted with MeOH, evaporated in vacuo, and reconstituted with mobile phase. The plasma and retinal concentrations of each test compound were determined by turbo ion spray on an MDS-Sciex API 4000 triple–quadrupole mass spectrometer equipped with a turbo ion spray source using multiple reaction monitoring (MRM). Samples were chromatographed using a NANOSPACE SI-2 (Shiseido) HPLC system with Shiseido Capcell pak® C18 MG-II column (75 × 1.5 mm, 5 μm) at 40 °C using a mobile phase ($MeOH/H_2O/HCO_2H$ = 40:60:0.1) at a flow rate of 0.20 mL/min. Data reduction was performed using Sciex Analyst ver. 1.4 software. The non-compartmental model was used to calculate pharmacokinetic parameters from the resulting plasma or retinal concentration versus time profiles using WinNonLin.

4.9. Structure elucidation of metabolites

The structure prediction of in vitro metabolites of ketoamides **8**–**10** was performed on LC/MS (API 4000 triple–quadrupole mass spectrometer, turbo ion spray) with a turbo ion spray source. The human S9 incubation for determination of metabolic stability described above was used. The LC/MS conditions were designed to determine the MW and the unique fragmentation pattern of each metabolite. The LC system and column are the same as those in pharmacokinetic studies described above. The mobile phase consisted of 0.1% aqueous formic acid (solvent A) and 0.1% formic acid in MeOH (solvent B). After sample injection (10 μL), the solvent B was increased linearly from 10% to 70% in 20 min and then to 90% in 30 min followed by a 5-min hold at a flow rate of 0.20 mL/min. Positive- and negative-ion LC/MS spectra were recorded using Q1 MS (scan range: m/z 350–560).

4.10. Solubility determination

A suspension of test compound in 2.0 mL of buffer solution (pH 7.0) or aqueous HCl (pH 1.2) 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 made exactly 10 mL by addition of mobile phase as a sample solution. The measurement was performed with 20 μL each of the sample solution and standard solution under the analytical HPLC according to the following condition: wavelength; UV 250 nm, column; YMC-Pack ODS A-303 (250 × 4.6 mm, 5 μm), column temperature; 45 °C, mobile phase; $CH_3CN/H_2O/TFA$ = 40:60:0.1, and flow rate; 1.0 mL/min. The concentration of test compound in sample solution was calculated from the ratio of the peak areas in the sample solution and the standard solution.

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