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Exploration of Cornea Permeable Calpain Inhibitors as Anticataract Agents

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Abstract—To explore cornea permeable calpain inhibitors, four compounds displaying different characteristics were designed and synthesized based on the known potent calpain inhibitor, peptidyl aldehyde SJA6017. Two approaches were adopted; an improvement in the physicochemical properties, and conversion of the active aldehyde. The water-soluble peptidyl aldehyde **1** containing a pyridine ring at the P3 site showed a modest inhibition against calpains and an improvement of corneal permeability in comparison with SJA6017. Replacement of the aldehyde of SJA6017 by an α-ketoamide provided compound **2** that was approximately equipotent with SJA6017, but it was extremely water-insoluble. However, compound **3**, in which the aldehyde was converted into a cyclic hemiacetal, proved to be a less potent calpain inhibitor than SJA6017, but demonstrated excellent transcorneal permeability. Further modification generating the cyclic hemiacetal **4** containing a thiourea linker between the P3 and P2 sites exhibited potent inhibitory activities, high cornea permeability and excellent efficacy in the rat lens culture cataract model.

Introduction

Calpains are nonlysosomal calcium-dependent cysteine proteases and the family of these enzymes has grown rapidly in recent years.[1] These well-known μ-calpain and m-calpain enzymes are ubiquitously found in mammalian cells, and they are implicated in a variety of biological processes and numerous diseases.[2]

Cataract is a major disease of the lens and the leading cause of blindness in the world. Proteolysis of lens proteins, crystallins, by activated calpain has been hypothesized to be one cause of cataract formation, which involves interrupting the normal interaction between lens proteins leading to aggregation, insolubilization and light scattering.[3] Although there is a possibility of topical and oral administrations as the anticataract drugs, topical administration is more advantageous than oral administration from the viewpoint of systemic side effect. The transcorneal permeability of the drug is an important factor to treat intraocular diseases like cataract with ophthalmic solution. In fact, synthetic calpain inhibitors such as SJA6017 and E-64-d have been demonstrated to prevent induced-cataract in lens culture models,[4] they have yet to be launched commercially.

To investigate novel calpain inhibitors as anticataract agents that have the superior cornea permeability, we attempted a synthetic program based on the lead compound SJA6017 using two approaches. The one approach was to obtain a water-soluble calpain inhibitor by conversion of the practically insoluble SJA6017 to incorporate a pyridine ring at the P3 site with the view of studying the physicochemical properties (Fig. 1). It is well known that solubility is the most important factor for studying the absorption of the drug, since only compounds dissolved in solution are able to permeate across the barrier.[5] The other approach was employed to convert the active aldehyde group into a further functional moiety. The aldehyde group is an essential active site and forms a hemithioacetal with the active SH on the cysteine residue of the enzyme. However, the chemical reactivity of the aldehyde is considered to be potentially high, and it may superfluously react with various substances under physiological conditions. Therefore, as an exploration of calpain inhibitors without the aldehyde, α-ketoamide and cyclic hemiacetal moieties were introduced into the SJA6017 backbone. An original α-ketoamide calpain inhibitor, AK-275 was demonstrated to be an effectiveness in an animal model of stroke.[6] Then cyclic hemiacetals have been reported by the Mitsubishi group as

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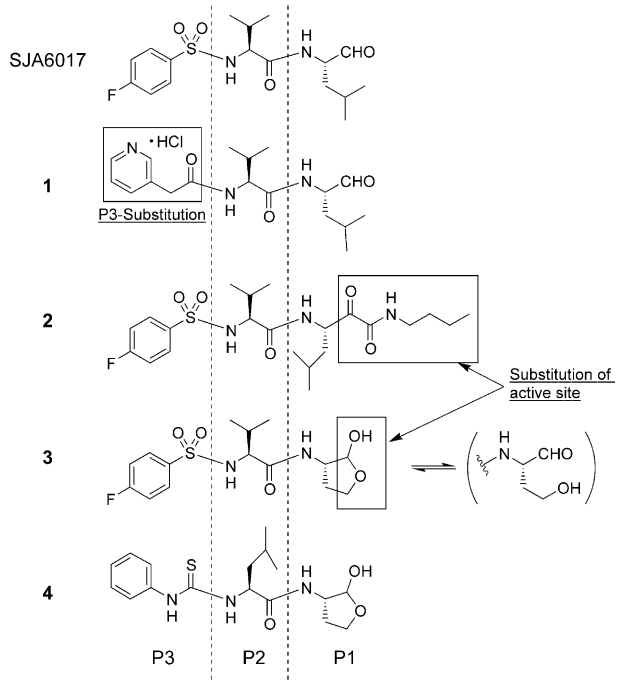


Figure 1. Structures of SJA6017 and calpain inhibitors **1**–**4**.

calpain inhibitors.[7] Since this hemiacetal is capable of forming a cyclized structure between the aldehyde and the hydroxy group of the P1 residue, this formation potentially may offer moderate protection in the reactivity of the aldehyde. We predicted that undesirable chemical reaction of the aldehyde might be prevented and an improvement in the cornea permeability could be achieved by such modifications.

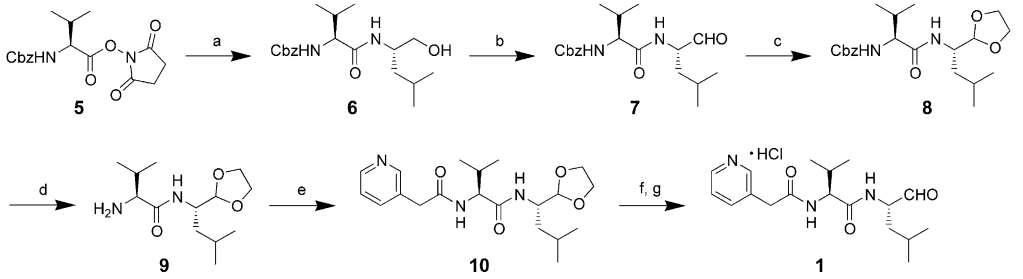
We conducted studies to establish the most effective method for an improvement in cornea permeability, through a change in the physicochemical properties or conversion of the aldehyde as the essential active site of the inhibitor. In this report, we describe the synthesis of SJA6017-based calpain inhibitors, their inhibitory activity and the relationship between the structure and the physicochemical properties in addition to cornea permeability. Furthermore, we report the discovery of a thiourea compound in our efforts to improve the inhibitory activity as well as the cornea permeability and demonstrate its anticataract efficacy in the rat lens culture cataract model.

Results and Discussion

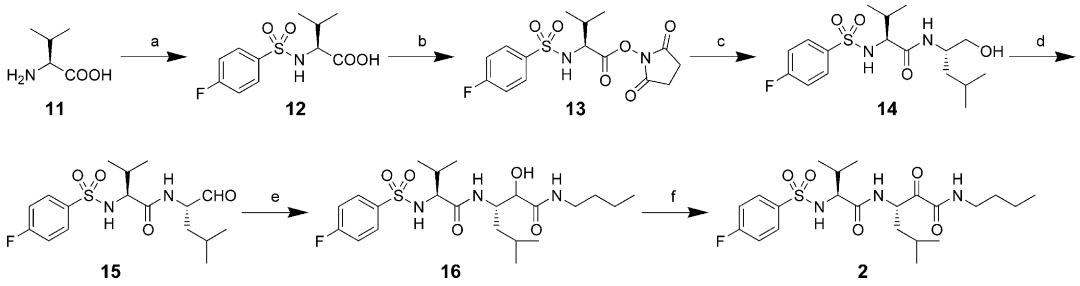
Chemistry

Synthesis of the water-soluble aldehyde **1** is detailed in Scheme 1. Peptidic condensation of commercially available *N*-carbobenzoxy-L-valine *N*-hydroxysuccinimide ester (**5**) and L-leucinol afforded **6**, which was then oxidized with DMSO in the presence of sulfurtrioxide/pyridine complex (SO_3/pyridine) to give peptidyl aldehyde **7**. The aldehyde was protected by the formation of an acetal with ethylene glycol, and subsequent hydrogenolysis of the carbobenzoxy-protecting group afforded intermediate **9**. Acylation of **9** with 3-pyridylacetic acid followed by hydrolysis of the acetal provided aldehyde **1**.

Synthesis of peptidyl α-ketoamide **2** is illustrated in Scheme 2. Sulfonylation of L-valine (**11**) with 4-fluorophenylsulfonylchloride, and condensation of the resulting intermediate **12** and *N*-hydroxysuccinimide (HOSu) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCHCl) provided active ester **13**. Treatment of **13** with L-leucinol followed



Scheme 1. Conditions: (a) L-leucinol, EtOAc; (b) SO_3/pyridine, DIPEA, DMSO; (c) ethylene glycol *p*-TsOH/pyridine, toluene; (d) Pd/C, EtOH; (e) 3-pyridine acetic acid·HCl, HOBt, Et_3N, EDC·HCl, DMF/CH_2Cl_2; (f) 1 M HCl; (g) 4 M HCl/EtOAc.



Scheme 2. Conditions: (a) 4-fluorophenylsulfonylchloride, NaOH, THF/H_2O; (b) HOSu, EDC·HCl, THF/CH_2Cl_2; (c) L-leucinol, EtOAc; (d) SO_3/pyridine, DIPEA, DMSO; (e) *n*-butylisocyanide, TFA, pyridine, CH_2Cl_2; (f) Dess–Martin periodinane, CH_2Cl_2.

by a DMSO oxidation in the presence of SO_3/pyridine provided peptidyl aldehyde **15**. Passerini reaction of aldehyde **15**, butylisocyanide and trifluoroacetic acid and subsequent Dess–Martin oxidation produced the α-ketoamide **2**.[8]

Cyclic hemiacetal **3** was synthesized from active ester **13** as shown in Scheme 3. Thus, treatment of compound **13** with α-amino-γ-butyrolactone gave lactone **17**, and reduction of this compound in the presence of diisobutylaluminum hydride (DIBAL-H) afforded target compound **3**.

The synthesis of cyclic hemiacetal **4** containing a thiourea linker is shown in Scheme 4. Boc-L-leucine (**18**) was coupled with α-amino-γ-butyrolactone to give lactone **20** via the active *N*-hydroxysuccinimide ester **19**. Boc-deprotection of **20** and treatment with phenylisothiocyanate followed by reduction with DIBAL-H provided thiourea compound **4**.

Enzyme inhibition and physicochemical properties

Although SJA6017 is a potent calpain inhibitor, it is poorly soluble in water (Table 1). Replacement of the P3 site of SJA6017 by a pyridine ring provided the water-soluble peptidyl aldehyde **1**, but this compound demonstrated modest inhibition of calpains. Furthermore, compound **1** displayed a slight undesirable inhibitory activity against proteasome.

Compound **2**, which possessed an α-ketoamide in the place of an aldehyde, showed almost identical activity against μ-calpain as SJA6017 and marginally superior activity against m-calpain (2-fold). However, compound **2** was found to be remarkably insoluble in water as compared to SJA6017 (0.0053 vs 0.10 mg/mL). In contrast, conversion to the cyclic hemiacetal **3** resulted in a loss of potency against μ- and m-calpain. Interestingly, compound **3** has a 15-fold higher water-solubility than SJA6017, even though it is an uncharged compound with a very similar structure to SJA6017. This phenomenon appears to be a consequence of the presence of a tetrahydrofuran moiety in the structure of **3**. Therefore, we took an interest in cyclic hemiacetal and applied it in the synthesis with the objective of producing more potent calpain inhibitors.

In the next stage, a cyclic hemiacetal containing a thiourea linker between the P3 and P2 sites, compound **4** was synthesized and tested. This resulting compound was about 10-fold more potent against both μ- and m-calpain than compound **3**. An investigation into the physicochemical properties of hemiacetals **3** and **4** revealed almost identical solubilities and log*P* (partition coefficient) values for both compounds. Therefore, we have successfully produced various types of calpain inhibitors by altering the physicochemical properties or by replacement of the aldehyde for use in cornea permeability experiments.

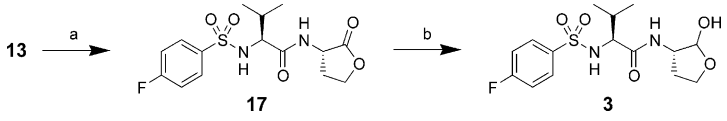
Transcorneal permeation

Plots of the aqueous humor concentration of drugs after topical administration to albino rabbits are shown in Figure 2. The area under the curves ($AUC_{0\rightarrow 3\ h}$) of aldehyde SJA6017, water-soluble aldehyde **1**, hemiacetal **3** and thiourea-contained hemiacetal **4** were 0.051, 0.31, 0.39 and 1.7 μg/mLh, respectively. The α-ketoamide **2** was not tested since its extremely low solubility would be expected to result in low permeability.

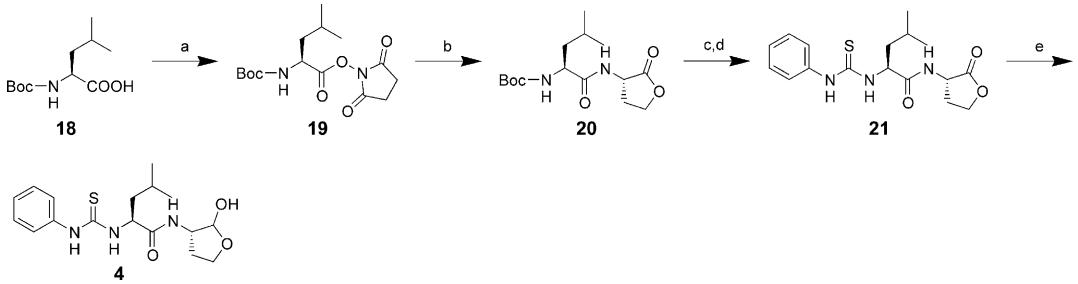
Table 1. Inhibitory activity and physicochemical properties

Compd	IC_{50} (μM)			Water-solubility[d] (mg/mL)	Log*P*
	μ-Calpain[a]	m-Calpain[b]	20S Proteasome[c]		
1	0.083	0.33	15	>100	0.025
2	0.021	0.021	>100	0.0053	>3
3	0.88	2.6	>100	1.5	0.60
4	0.086	0.19	>100	1.5	0.70
SJA6017	0.022	0.049	>100	0.10	1.7

[a]Human erythrocyte μ-calpain.
[b]Porcine kidney m-calpain.
[c]Human recombinant 20S proteasome.
[d]Water-solubility in pH 7 buffer, at 25 °C.



Scheme 3. Conditions: (a) *S*-α-amino-γ-butyrolactone·HCl, Et_3N, DMF; (b) DIBAL-H, CH_2Cl_2.



Scheme 4. Conditions: (a) HOSu, EDC·HCl, THF/CH_2Cl_2; (b) *S*-α-amino-γ-butyrolactone·HBr, Et_3N, DMF; (c) 4 M HCl/EtOAc; (d) phenylisothiocyanate, Et_3N, EtOAc; (e) DIBAL-H, CH_2Cl_2.

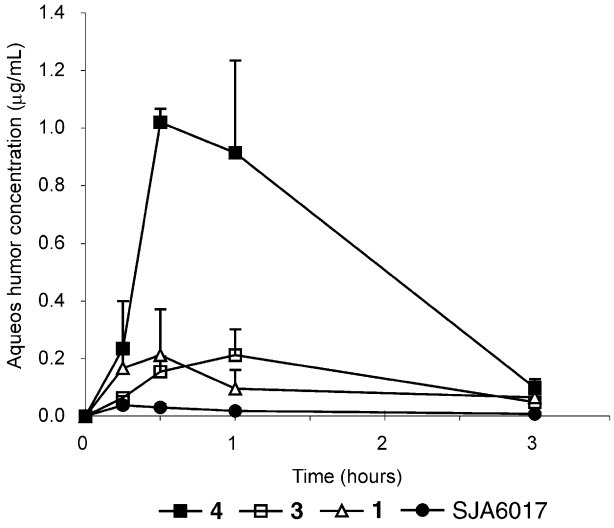


Figure 2. Mean aqueous humor levels after topically administration of 0.5% compound **1** in aqueous solution, 0.5% compound **3** in aqueous suspension and 0.5% compound **4** in aqueous suspension. Each dosing volume was 50 μL (single instillation, $n = 3$ eyes).

Water-soluble aldehyde **1** and hemiacetal **3** showed an improved permeability as compared to SJA6017, and the AUC values increased to approximately 6-fold and 8-fold, respectively. Both the increased solubility and the conversion of the aldehyde may have contributed to this increase in transcorneal permeability. It appears that while SJA6017 has limiting physicochemical properties, the aldehyde lowers the permeability by adsorbing to certain other substances present in cornea. However, the improvement of permeability of hemiacetal **3**, was considered to be a consequence of not only removal of the aldehyde, but also the effect of increased solubility. Interestingly, the thiourea-contained hemiacetal **4** revealed a marked improvement in the permeability, with the increase in AUC values of about 33-fold of SJA6017 and 4-fold of hemiacetal **3**. Although hemiacetals **3** and **4** have a similar structure and comparable physicochemical properties such as water-solubility and log*P* (Table 1), the two compounds have significantly different melting points (**3**: mp 162–164 °C, **4**: mp 62–64 °C). A transdermal absorption study has reported a correlation between the melting point and transdermal absorption.[9] Similarly to transdermal absorption, transcorneal permeation through ophthalmic aqueous suspension might be also affected by the melting point of drugs, and the higher permeation of hemiacetal **4** might be attributed to the lower melting point.

Cultured lens study

The effectiveness of hemiacetal **4** as anticataract agent was evaluated on the calcium ionophore (A23187) induced rat lens culture cataract model, and the results are summarized in Figure 3. Lenses that were cultured for 5 days in the medium remained clear and were similar in appearance to fresh lenses (Fig. 3, Normal). On the other hand, the addition of 10 μM A23187 caused a dense opacity to appear in the central region of the lens by day 5 of culture (Fig. 3, A23187). Most importantly, hemiacetal **4** (100 μM) delayed the appearance of the dense nuclear opacity against A23187-induced cataract (Fig. 3, A23187 + **4**). Thus, we have demonstrated the efficiency of hemiacetal **4** as an anticataract agent in the rat lens culture model.

Conclusions

We have provided a novel strategy for exploration of cornea permeable calpain inhibitors. Among the four synthesized inhibitors with different characteristics, α-ketoamide **2** was the most potent calpain inhibitor, however this compound was extremely water-insoluble. Although water-soluble aldehyde **1** having a pyridine ring at the P3 site, and compound **3** in which the aldehyde of SJA6017 was replaced with a cyclic hemiacetal, both displayed reduced inhibitory activities against calpains, they showed improved transcorneal permeability. From these results, it was concluded that existence of the aldehyde and the solubility affected the cornea permeability in the case of SJA6017. Hemiacetal **4** containing a thiourea linker between P3 and P2 sites was superior to hemiacetal **3** in view of both the inhibitory activity (μ-calpain: 10-fold, m-calpain: 14-fold) and corneal permeability (AUC: 3-fold). Furthermore, hemiacetal **4** exhibited the efficacy against A23187-induced nuclear cataract in the rat lens culture model. Future work is aimed at generating inhibitors that demonstrate superior activity combined with excellent cornea permeability based on our knowledge presented in this paper. The expansion of this series of calpain inhibitors will be reported in due course.

Experimental

General. Melting points were determined on a Yanaco micro melting point apparatus without correction. ^{1}H NMR spectra were recorded on a Varian Gemini-2000 spectrometer. Chemical shifts are reported in parts per million, and coupling constants (J) are reported in Hertz. The NMR spectra of compounds **3** and **4** were extremely complicated due to the presence of anomeric carbon. The major and minor peaks of the same proton were separately listed, in case each peak was identified. Elemental analyses were performed on an Elementar Vario EL analyzer. FAB high-resolution mass spectra were obtained by Jeol JMS-700T mass spectrometer. Matrix-assisted laser desorption ionization time-of-flight mass spectra (MALDI-TOF MS) were obtained on a Perseptive Voyager DE PRO mass spectrometer using α-cyano-4-hydroxycinamic acid as the matrix. Optical rotations were measured in a Horiba SEPA-2000 model.

(2*S*)-2-(Benzyloxycarbonylamino)-*N*-((1*S*)-1-(hydroxymethyl)-3-methylbutyl)-3-methylbutanamide (6). To a solution of *N*-carbobenzoxy-L-valine *N*-hydroxysuccinimide ester (30 g, 86 mmol) in EtOAc (400 mL)

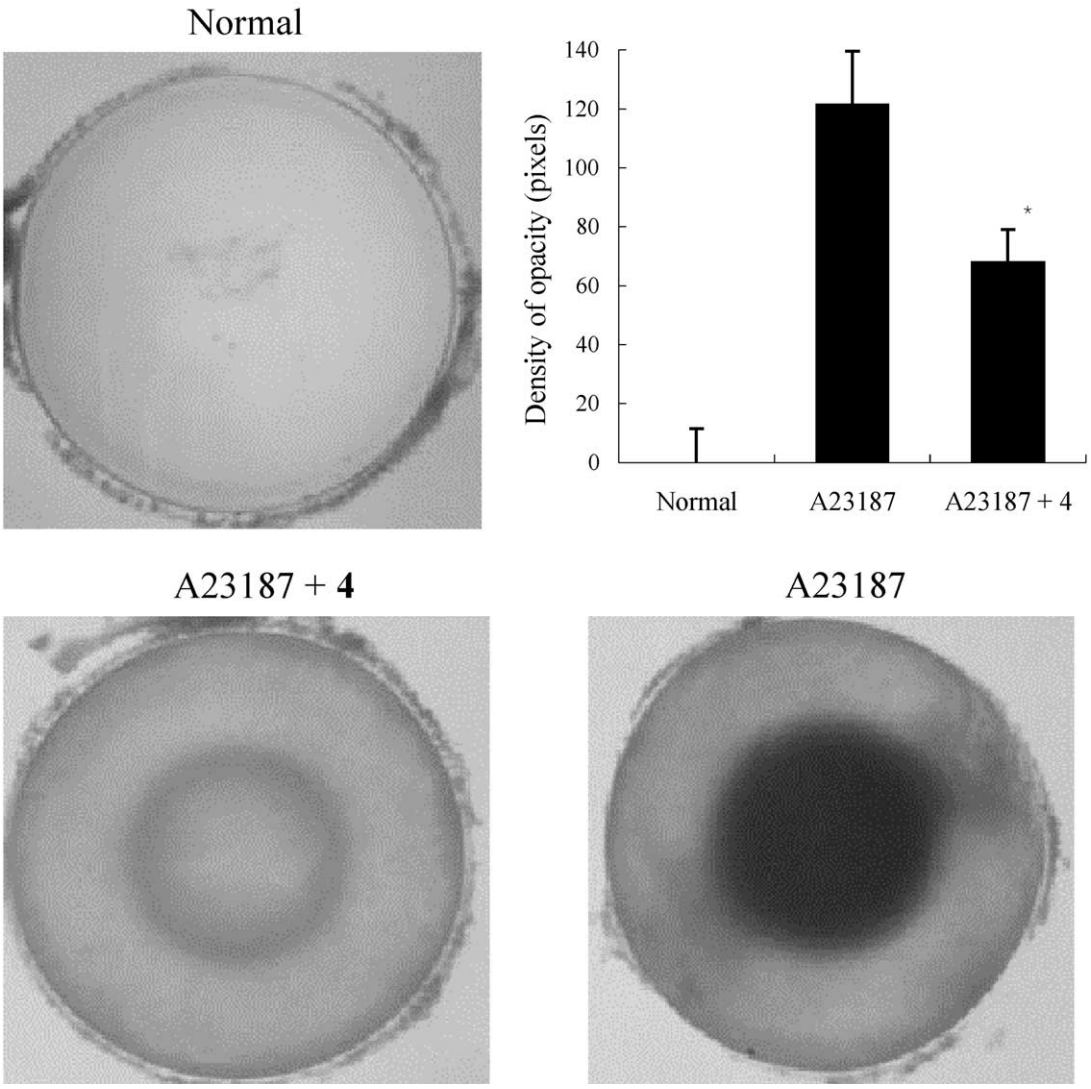


Figure 3. Representative photomicroscopy of A23187-induced cataract and inhibition by hemiacetal **4** (100 μM) on day 5 of culture. Darker areas are cataracts in these backlit lenses. Insert bar graph shows increased density of nuclear opacity in cultured lenses treated with A23187 and reduction of opacity by hemiacetal **4**. Data are mean ±SD ($n=5$). *$P<0.01$ relative to A23187.

was added L-leucinol (12 g, 100 mmol). The mixture was stirred at room temperature for 18 h. Resulting precipitate was dissolved by addition of EtOAc, and the solution was washed with 1 M HCl, saturated $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo. The residue was crystallized from hexane to give **6** (19 g, 63%) as colorless crystals. Mp 65–66 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.80–0.87 (m, 12H), 1.26–1.37 (m, 2H), 1.59 (m, 1H), 1.92 (m, 1H), 3.18 (m, 1H), 3.30 (m, 1H), 3.77–3.83 (m, 2H), 4.61 (m, 1H), 4.99–5.08 (m, 2H), 7.21 (d, 1H, $J=8.7$), 7.31–7.36 (m, 5H), 7.51 (d, 1H, $J=8.7$). Anal. calcd for $C_{19}H_{30}N_2O_4$: C, 65.12; H, 8.63; N, 7.99; found: C, 65.06; H, 8.44; N, 7.79.

(2*S*)-2-(Benzyloxycarbonylamino)-*N*-((1*S*)-1-formyl-3-methylbutyl)-3-methylbutanamide (7). Compound **6** (19 g, 54 mmol) was dissolved in DMSO (150 mL) and CH_2Cl_2 (70 mL). *N*,*N*-Diisopropylethylamine (28 g, 220 mmol) and a suspension of SO_3/pyridine (35 g, 220 mmol) in DMSO (70 mL) were added under the ice-cool condition. The mixture was stirred for 30 min under the same condition. The reaction mixture was diluted with EtOAc, and the solution was washed with 1 M HCl, saturated $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo. The resulting white solid was crystallized from EtOAc/hexane to give **7** (12 g, 63%) as colorless crystals. Mp 123–125 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.84–0.91 (m, 12H), 1.37–1.65 (m, 3H), 2.00 (m, 1H), 3.90 (m, 1H), 4.12 (m, 1H), 5.04 (s, 2H), 7.31–7.37 (m, 6H), 8.34 (d, 1H, $J=6.9$), 9.39 (s, 1H). FAB-HRMS calcd for $C_{19}H_{29}N_2O_4$ $[M+H]^+$: 349.2127, found: 349.2119.

(2*S*)-*N*-((1*S*)-1-(2,5-Dioxolanyl)-3-methylbutyl)-3-methyl-2-(benzyloxycarbonylamino)butanamide (8). To a solution of **7** (11 g, 31 mmol) in toluene (300 mL) was added ethylene glycol (9.8 g, 160 mmol) and pyridinium toluenesulfonate (1.6 g, 6.3 mmol). The mixture was stirred at 80 °C for 4 h. The mixture was washed with

1 M HCl, saturated $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo. Resulting white solid was crystallized from EtOAc/hexane to give **8** (12 g, 97%) as colorless crystals. Mp 106–107 °C. ^{1}H NMR (300 MHz, DMSO-d_6) 0.79–0.87 (m, 12H), 1.21 (m, 1H), 1.38 (m, 1H), 1.57 (m, 1H), 1.93 (m, 1H), 3.73–3.9 (m, 5H), 4.01 (m, 1H), 4.71 (d, 1H, J = 2.4), 4.98-5.09 (m, 2H), 7.18 (d, 1H, J = 9.0), 7.31–7.35 (m, 5H), 7.54 (d, 1H, J = 9.3). Anal. calcd for $C_{21}H_{32}N_2O_5$: C, 64.26; H, 8.22; N, 7.14; found: C, 64.17; H, 7.95; N, 7.00.

(2*S*)-2-Amino-*N*-((1*S*)-1-(2,5-dioxolanyl)-3-methylbutyl)-3-methylbutanamide (9). Compound **8** (12 g, 31 mmol) was dissolved in EtOH (250 mL) and hydrogenated at room temperature under atmosphere pressure over palladium carbon powder (Pd: 10%) (2.0 g). After stirring for 72 h, palladium carbon was filtered off, and the mixture was concentrated in vacuo to give **9** (7.5 g, quant) as colorless oil. ^{1}H NMR (300 MHz, DMSO-d_6) d 0.76 (d, 3H, J = 6.9), 0.82 (d, 3H, J = 6.6), 0.87 (d, 6H, J = 6.6), 1.23 (m, 1H), 1.37 (m, 1H), 1.55 (m, 1H), 1.68 (s, 2H), 1.90 (m, 1H), 2.96 (d, 1H, J = 5.1), 3.74–3.91 (m, 4H), 4.01 (m, 1H), 4.72 (d, 1H, J = 3.3), 7.62 (d, 1H, J = 9.6).

(2*S*)-*N*-((1*S*)-1-(2,5-Dioxolanyl)-3-methylbutyl)-3-methyl - 2 - ((3 - pyridylmethyl)carbonylamino)butanamide (10). Compound **9** (0.80 g, 3.1 mmol), 3-pyridylacetic acid hydrochloride (0.59 g, 3.4 mmol), 1-hydroxybenzotriazole (HOBt) (0.46 g, 3.4 mmol) and Et_3N (0.34 g, 3.4 mmol) were dissolved in DMF (20 mL). A solution of EDCHCl (0.65 g, 3.4 mmol) in CH_2Cl_2 (20 mL) was added under the ice-cool condition. The mixture was stirred at room temperature for 18 h and concentrated in vacuo. The residue was dissolved in EtOAc, and the solution was washed with saturated $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo. The residue was crystallized from hexane to give **10** (0.92 g, 79%) as colorless crystals. Mp 90–91 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.77–0.84 (m, 12H), 1.21 (m, 1H), 1.35 (m, 1H), 1.51 (m, 1H), 1.92 (m, 1H), 3.51 (d, 1H, J = 14.1), 3.58 (d, 1H, J = 14.1), 3.73–3.87 (m, 4H), 3.99 (m, 1H), 4.18 (dd, 1H, J = 8.9, 7.2), 4.69 (d, 1H, J = 3.6), 7.31 (m, 1H), 7.65–7.71 (m, 2H), 8.19 (d, 1H, J = 8.9), 8.41–8.46 (m, 2H). FAB-HRMS calcd for $C_{20}H_{32}N_3O_4$ $[M+H]^+$: 378.2393, found: 378.2403.

(2*S*)-*N*-((1*S*)-1-Formyl-3-methylbutyl)-3-methyl-2-((3-pyridylmethyl)carbonylamino)butanamide hydrochloride (1). Compound **10** (0.89 g, 2.4 mmol) was dissolved in 1 M HCl (20 mL). The mixture was stirred at 50 °C for 4 h and purified by HPLC system (column: YMC-Pack ODS-A 250×20 mm, mobile phase CH_3CN/H_2O/TFA = 20:80:0.1). The main fractions were collected, neutralized by addition of $NaHCO_3$ and extracted with EtOAc. The organic layer was washed with saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo. The residue was crystallized from hexane to give pyridine free of **1** (0.50 g, 64%) as colorless crystals. Mp 109–111 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.82–0.88 (m, 12H), 1.40 (m, 1H), 1.49–1.61 (m, 2H), 1.99 (m, 1H), 3.51 (d, 1H, J = 14.1), 3.60 (d, 1H, J = 14.1), 4.12 (m, 1H), 4.21 (m, 1H), 7.32 (m, 1H), 7.67 (d, 1H, J = 7.5), 8.28 (d, 1H, J = 9.0), 8.41–8.47 (m, 3H), 9.39 (s, 1H). $[\alpha]_D^{25}$ −24.1° (c = 0.203). This compound was dissolved in EtOAc, and 4 M HCl/EtOAc (0.40 mL) was added under the ice-cool condition. Resulting precipitate was collected and washed with EtOAc to give **1** (0.40 g, 86%) as colorless crystals. Mp 79–80 °C. Anal. calcd for $C_{18}H_{27}N_3O_3HClH_2O$: C, 55.73; H, 7.80; N, 10.83; found: C, 55.58; H, 7.66; N, 10.63. MALDI-TOF MS calcd for $C_{18}H_{27}N_3O_3Na$ $[M+Na]^+$: 356.195, found: 356.196.

***N*-((4-Fluorophenyl)sulfonyl)-L-valine (12).** To a solution of L-valine (**11**) (82 g, 700 mmol) in 1 M NaOH (700 mL), THF (500 mL) and water (500 mL), a solution of 4-fluorobenzenesulfonylchloride (135 g, 700 mmol) in THF (700 mL) and 1 M NaOH (700 mL) were added slowly at the same time under the ice-cool condition. The mixture was stirred at room temperature for 18 h, and diluted with EtOAc. The solution was washed with 1 M HCl, saturated $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo. The residue was crystallized from hexane to give **12** (193 g, 76%) as colorless crystals. Mp 116–118 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.79 (d, 3H, J = 6.6), 0.83 (d, 3H, J = 6.6), 1.94 (m, 1H), 3.52 (dd, 1H, J = 9.3, 6.0), 7.35–7.43 (m, 2H), 7.80–7.87 (m, 2H), 8.08 (d, 1H, J = 9.3), 12.61 (brs, 1H). Anal. calcd for $C_{11}H_{14}FNO_4S$: C, 47.99; H, 5.12; N, 5.09; found: C, 47.86; H, 5.06; N, 5.06.

***N*-((4-Fluorophenyl)sulfonyl)-L-valine *N*-Hydroxysuccinimide ester (13).** Compound **12** (146 g, 530 mmol) and HOSu (73 g, 636 mmol) were dissolved in THF (1200 mL), and a suspension of EDCHCl (122 g, 636 mmol) in CH_2Cl_2 (1200 mL) was added thereto under the ice-cool condition. The mixture was stirred at room temperature for 18 h. After concentration in vacuo, the residue was dissolved in EtOAc, and the solution was washed with 1 M HCl, saturated $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo. The residue was crystallized from hexane to give **13** (197 g, 94%) as colorless crystals. Mp 147–149 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.88 (d, 3H, J = 6.9), 0.91 (d, 3H, J = 6.9), 2.10 (m, 1H), 2.79 (s, 4H), 4.14 (m, 1H), 7.36–7.41 (m, 2H), 7.85–7.89 (m, 2H), 8.67 (d, 1H, J = 8.7). Anal. calcd for $C_{15}H_{17}FN_2O_6S$: C, 48.38; H, 4.60; N, 7.52; found: C, 48.22; H, 4.63; N, 7.41.

(2*S*)-2-((4-Fluorophenyl)sulfonylamino)-*N*-((1*S*)-1-(hydroxymethyl)-3-methylbutyl)-3-methylbutanamide (14). To a solution of **13** (185 g, 496 mmol) in EtOAc (4000 mL) was added L-leucinol (70 g, 590 mmol). The mixture was stirred at room temperature for 18 h and washed with 1 M HCl, saturated $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo. Resulting white solid was crystallized from hexane/EtOAc (9:1) to give **14** (183 g, 98%) as colorless crystals. Mp 120–122 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.66 (d, 3H, J = 6.0), 0.75–0.85 (m, 9H), 0.98 (m, 1H), 1.10–1.19 (m, 2H), 1.81 (m, 1H), 3.06 (m, 1H), 3.18 (m, 1H), 3.48–3.57 (m, 2H), 4.55 (t, 1H, J = 5.4), 7.32–7.41 (m, 2H), 7.50 (d, 1H, J = 8.4), 7.74 (d, 1H, J = 9.0),

7.80–7.89 (m, 2H). Anal. calcd for $C_{17}H_{27}FN_2O_4S$: C, 54.53; H, 7.26; N, 7.48; found: C, 54.21; H, 7.22; N, 7.45.

(2*S*)-2-((4-Fluorophenyl)sulfonylamino)-*N*-((1*S*)-1-formyl-3-methylbutyl)-3-methylbutanamide (15). Compound **14** (182 g, 486 mmol) was dissolved in DMSO (800 mL) and CH_2Cl_2 (400 mL). *N,N*-Diisopropylethylamine (251 g, 1940 mmol) and a suspension of SO_3/pyridine (309 g, 1940 mmol) in DMSO (500 mL) were added under the ice-cool condition. The mixture was stirred for 30 min under the same condition. The reaction mixture was diluted with EtOAc, and the solution was washed with 1 M HCl, saturated $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo. Resulting white solid was washed with hexane, and crystallized from EtOAc to give **15** (67 g, 37%) as colorless crystals. Mp 154 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.74 (d, 3H, J = 6.3), 0.81–0.88 (m, 9H), 1.15–1.45 (m, 3H), 1.87 (m, 1H), 3.59 (dd, 1H, J = 9.2, 6.6), 3.84 (m, 1H), 7.34–7.42 (m, 2H), 7.79–7.85 (m, 2H), 7.96 (d, 1H, J = 9.2), 8.27 (d, 1H, J = 7.2), 9.14 (s, 1H). Anal. calcd for $C_{17}H_{25}FN_2O_4S$: C, 54.82; H, 6.77; N, 7.52; found: C, 54.67; H, 6.63; N, 7.41.

(3*S*)-*N*-Butyl-3-((2*S*)-2-((4-fluorophenyl)sulfonylamino)-3-methylbutanoylamino)-2-hydroxy-5-methylhexanamide (16). TFA (2.4 g, 21 mmol) was added dropwise to a cooled solution of **15** (4.0 g, 11 mmol), *n*-butylisocyanide (1.0 g, 12 mmol) and pyridine (3.4 g, 43 mmol) in CH_2Cl_2 (100 mL). The mixture was stirred at room temperature for 18 h. After concentration in vacuo, the residue was dissolved in EtOAc, and the solution was washed with 1 M HCl, saturated $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by HPLC system (column: YMC Pack ODS-A 250×20 mm, mobile phase: CH_3CN/H_2O/TFA = 40:60:0.1), and crystallized from hexane to give **16** (1.0 g, 20%) as colorless crystals. Mp 192–194 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.69 (d, 3H, J = 6.9), 0.70 (d, 3H, J = 5.4), 0.75 (d, 3H, J = 6.0), 0.79 (d, 3H, J = 6.6), 0.85 (t, 3H, J = 7.4), 0.94 (m, 1H), 1.05–1.09 (m, 2H), 1.18–1.27 (m, 2H), 1.30–1.39 (m, 2H), 1.82 (m, 1H), 2.92–3.07 (m, 2H), 3.63 (dd, 1H, J = 9.2, 5.7), 3.72 (dd, 1H, J = 6.0, 2.6), 3.93 (m, 1H), 5.65 (d, 1H, J = 6.0), 7.31–7.36 (m, 3H), 7.53 (t, 1H, J = 5.9), 7.59 (d, 1H, J = 9.2), 7.80–7.85 (m, 2H). Anal. calcd for $C_{22}H_{36}FN_3O_5S$: C, 55.79; H, 7.66; N, 8.87; found: C, 55.60; H, 7.40; N, 8.73.

(3*S*)-*N*-Butyl-3-((2*S*)-2-((4-fluorophenyl)sulfonylamino)-3-methylbutanoylamino)-5-methyl-2-oxohexanamide (2). To a solution of **16** (1.0 g, 2.1 mmol) in CH_2Cl_2 (100 mL) was added Dess–Martin periodinane (1.3 g, 3.2 mmol). The mixture was stirred at room temperature for 18 h. Aqueous 10% $Na_2S_2O_3$ (50 mL) and aqueous 10% $NaHCO_3$ (50 mL) were added, and the mixture was stirred for 10 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 crystallized from EtOAc/hexane to give **2** (0.80 g, 80%) as colorless crystals. Mp 108–110 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.73 (d, 3H, J = 5.7), 0.79–0.88 (m, 12H), 1.15–1.45 (m, 7H), 1.82 (m, 1H), 3.04-3.11 (m, 2H), 3.59 (m, 1H), 4.75 (m, 1H), 7.33–7.39 (m, 2H), 7.78–7.83 (m, 2H), 7.88 (d, 1H, J = 9.3), 8.15 (d, 1H, J = 6.3), 8.65 (t, 1H, J = 5.9). Anal. calcd for $C_{22}H_{34}FN_3O_5S$: C, 56.03; H, 7.27; N, 8.91; found: C, 55.99; H, 7.00; N, 8.91. MALDI-TOF MS calcd for $C_{22}H_{34}FN_3O_5SNa$ $[M+Na]^+$: 494.210, found: 494.243. $[\alpha]_D^{25}$ +42.4° (c = 0.203, DMSO).

(2*S*)-2-((4-Fluorophenyl)sulfonylamino)-3-methyl-*N*-((3*S*)-tetrahydro-2-oxo-3-furanyl)butanamide (17). To a solution of compound **13** (4.8 g, 13 mmol) in DMF (80 mL) was added *S*-α-amino-γ-butyrolactone hydrochloride (1.4 g, 14 mmol) and Et_3N (2.6 g, 16 mmol). The mixture was stirred at room temperature for 18 h. After dilution with EtOAc, the solution was washed with 1 M HCl, saturated $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo. The residue was crystallized from hexane to give **17** (2.0 g, 43%) as colorless crystals. Mp 116–118 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.84 (d, 6H, J = 6.6), 1.76–1.95 (m, 2H), 2.16 (m, 1H), 3.45 (t, 1H, J = 8.1), 4.17 (m, 1H), 4.30 (m, 1H), 4.48 (m, 1H), 7.34–7.42 (m, 2H), 7.77–7.84 (m, 2H), 7.99 (d, 1H, J = 8.7), 8.38 (d, 1H, J = 8.1). Anal. calcd for $C_{15}H_{19}FN_2O_5S$: C, 50.27; H, 5.34; N, 7.82; found: C, 50.14; H, 5.34; N, 7.65.

(2*S*)-2-((4-Fluorophenyl)sulfonylamino)-3-methyl-*N*-((3*S*)-tetrahydro-2-hydroxy-3-furanyl)butanamide (3). To a solution of compound **17** (1.9 g, 5.3 mmol) in CH_2Cl_2 (250 mL) was added DIBAL-H (1.0 M solution in toluene) (17 mL, 17 mmol) at −78 °C. The mixture was kept below −70 °C and stirred for 3 h, and then aqueous saturated NH_4Cl (4.0 mL) was added and stirred for 30 min. $MgSO_4$ and EtOAc (100 mL) were added at room temperature, and inorganic was filtered off. After concentration in vacuo, the residue was purified by HPLC system (column: YMC-Pack ODS-A 250×20 mm, mobile phase: CH_3CN/H_2O/TFA = 20:80:0.1). The main fractions were collected, and extracted with EtOAc. The solution was washed with saturated $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo, and crystallized from hexane to give **3** (0.19 g, 9.9%) as colorless crystals. Mp 162–164 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ (major/minor) 0.77/0.76–0.84 (d/m, 3H, J = 6.3), 0.80/0.76–0.84 (d/m, 3H, J = 6.6), 1.53 (m, 1H), 1.75–1.87 (m, 2H,), 3.53–3.87 (m, 4H), 4.99/4.83 (t/d, 1H, J = 4.4/4.8), 6.34–6.35/6.08 (m/d, 1H, J = 4.5), 7.35–7.41 (m, 2H), 7.68/7.90 (d/d, 1H, J = 7.8/6.3), 7.77–7.84 (m, 3H). major/minor = 3.3:1.0. Anal. calcd for $C_{15}H_{21}FN_2O_5S$: C, 49.99; H, 5.87; N, 7.77; found: C, 49.89; H, 5.97; N, 7.55. MALDI-TOF MS calcd for $C_{15}H_{21}FN_2O_5SNa$ $[M+Na]^+$: 383.105, found: 383.103. $[\alpha]_D^{25}$ −35.2° (c = 0.203).

***N*-(*tert*-Butoxy)carbonyl-L-leucine *N*-hydroxysuccinimide ester (19).** Boc-L-leucine (27 g, 110 mmol) and HOSu (16 g, 140 mmol) were dissolved in THF (180 mL), and a suspension of EDCHCl (27 g, 140 mmol) in CH_2Cl_2 (180 mL) was added thereto under the ice-cool condition. The mixture was stirred at room temperature for 18 h, and concentrated in vacuo. The residue was dis-

solved in EtOAc, and the solution was washed with 1 M HCl, saturated $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo. Resulting white solid was crystallized from hexane to give **19** (33 g, 93%) as colorless crystals. Mp 74–76 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.88 (d, 3H, J = 6.3), 0.91 (d, 3H, J = 6.3), 1.39 (s, 9H), 1.54–1.75 (m, 3H), 2.80 (s, 4H), 4.32 (m, 1H), 7.62 (d, 1H, J = 8.4). FAB-HRMS calcd for $C_{15}H_{25}N_2O_6$ $[M+H]^+$: 329.1713, found: 329.1695.

(2*S*)-2-((*tert*-Butoxy)carbonylamino)-4-methyl-*N*-((3*S*)-tetrahydro-2-oxo-3-furanyl)pentanamide (20). To a solution of **19** (20 g, 61 mmol) in DMF (120 mL) was added *S*-α-amino-γ-butyrolactone hydrobromide (17 g, 91 mmol) and Et_3N (18 g, 180 mmol) under the ice-cool condition. The mixture was stirred at room temperature for 18 h. The mixture was diluted with EtOAc, and the solution was washed with 1 M HCl, saturated $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo. The residue was crystallized from hexane to give **20** (19 g, 99%) as colorless crystals. Mp 92–93 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.85 (d, 3H, J = 6.3), 0.88 (d, 3H, J = 6.6), 1.28–1.50 (m, 2H), 1.38 (s, 9H), 1.61 (m, 1H), 2.14 (m, 1H), 2.39 (m, 1H), 3.96 (m, 1H), 4.20 (m, 1H), 4.34 (m, 1H), 4.60 (m, 1H), 6.88 (d, 1H, J = 8.4), 8.30 (d, 1H, J = 8.4). FAB-HRMS calcd for $C_{15}H_{27}N_2O_5$ $[M+H]^+$: 315.1920, found: 315.1925.

(2*S*)-4-Methyl-2-(3-phenylthioureido)-*N*-((3*S*)-tetrahydro-2-oxo-3-furanyl)pentanamide (21). To a solution of **20** (3.4 g, 11 mmol) in EtOAc (100 mL) was added 4 M HCl/EtOAc (8.0 mL) under the ice-cool condition. The mixture was stirred at room temperature for 18 h, and concentrated in vacuo. EtOAc was added, and then removed in vacuo (twice). The residue was suspended with EtOAc, and phenylisothiocyanate (1.5 g, 11 mmol) and Et_3N (3.3 g, 32 mmol) were added thereto. The mixture was stirred at room temperature for 3 h, and washed with 1 M HCl, saturated $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo. The residue was crystallized from hexane, and washed with hexane to give **21** (1.8 g, 48%) as colorless crystals. Mp 78–79 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.92 (d, 3H, J = 6.3), 0.93 (d, 3H, J = 6.6), 1.55–1.70 (m, 3H), 2.20 (m, 1H), 2.40 (m, 1H), 4.22 (m, 1H), 4.36 (m, 1H), 4.62 (m, 1H), 4.96 (m, 1H), 7.10 (m, 1H), 7.29–7.34 (m, 2H), 7.51–7.54 (m, 2H), 7.78 (d, 1H, J = 8.1), 8.64 (d, 1H, J = 8.1), 9.70 (s, 1H). FAB-HRMS calcd for $C_{17}H_{24}N_3O_3S$ $[M+H]^+$: 350.1538, found: 350.1542.

(2*S*)-4-Methyl-2-(3-phenylthioureido)-*N*-((3*S*)-tetrahydro-2-hydroxy-3-furanyl)pentanamide (4). To a solution of **21** (1.7 g, 4.9 mmol) in CH_2Cl_2 (200 mL) was added DIBAL-H (1.0 M solution in toluene) (17 mL, 17 mmol) at −78 °C. The mixture was kept below −70 °C and stirred for 3 h, and then saturated NH_4Cl solution (4.0 mL) was added and stirred for 30 min. $MgSO_4$ and EtOAc (150 mL) were added at room temperature, and inorganic was filtered off using Celite. After concentration in vacuo, the residue was purified by HPLC system (column: YMC-Pack ODS-A 250×20 mm, mobile phase: CH_3CN/H_2O/TFA = 30:70:0.1). The main fractions were collected, and extracted with EtOAc. The solution was washed with saturated $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo. The residue was crystallized from hexane to give **4** (0.46 g, 27%) as colorless crystals. Mp 62–64 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.91 (d, 6H, J = 6.0), 1.49–1.91 (m, 4H), 2.14 (m, 1H), 3.64–4.00 (m, 3H), 4.91 (m, 1H), 5.00/5.11 (d/t, 1H, J = 4.5/4.7), 6.16/6.34 (d/d, 1H, J = 4.8/4.8), 7.10 (m, 1H), 7.29–7.34 (m, 2H), 7.49–7.53 (m, 2H), 7.72/7.78 (d/d, 1H, J = 8.4/8.7), 8.26/7.87 (d/d, 1H, J = 6.3/7.5), 9.69 (s, 1H), major:minor = 1.0:0.85. Anal. calcd for $C_{17}H_{25}N_3O_3S$: C, 58.09; H, 7.17; N, 11.96; found: C, 58.03; H, 7.26; N, 11.60. MALDI-TOF MS calcd for $C_{17}H_{25}N_3O_3SNa$ $[M+Na]^+$: 358.174, found: 358.169. $[\alpha]_D^{25}$ +23.6° (c = 0.211).

Inhibition assay for calpains. This inhibition assay was performed as described in the previous literature[10] using commercially μ-calpain (human erythrocyte, Cosmo Bio Co., Ltd.) and m-calpain (porcine kidney, Cosmo Bio Co., Ltd.). Assay solution including 0.5 mg/mL casein, 20 mM DTT, 50 mM Tris–HCl (pH 7.4) and 1.0 nmol of enzyme was used. In each well was placed 200 μL of assay solution and 2.5 μL of DMSO including inhibitor of different concentration. Reaction was started by addition of 50 μL of 20 mM $CaCl_2$ in test well and 50 μL of 1 mM EDTA in blank well. After incubation for 60 min at 30 °C, the mixture (100 μL) was transferred to another plate in which 100 μL of H_2O and 50 μL of Bio-Rad protein assay dye reagent were placed in each well. After incubation at room temperature for 15 min, the mixture was read OD at 595 nm with plate reader (Multiskan Multisoft, Labsystems). The percentage 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.

Inhibition assay for proteasome. 20S proteasome assay kit (QunatiZyme® Assay System AK-740, BIOMOL Research Lab., Inc.) was purchased, and the procedure was performed according to the guided method. In each well was placed 5.0 μL of 10% DMSO including inhibitor of different concentration, 90 μL of assay buffer (25 mM HEPES, pH 7.5, 0.5 mM EDTA, 0.05% NP-40, 0.03% SDS) and 5.0 μL of enzyme solution (0.02 mg/mL 20S proteasome from human erythrocyte in assay buffer). The reaction was started by addition of 10 μL substrate solution (0.29 mg/mL Suc-LLVY-AMC in assay buffer) at 30 °C, and the fluorescence measurements were performed with plate reader (excitation: 360 nm, emission: 450 nm, CYTOFLUOR series 4000, Perseptive Biosystems) for 60 min. The percent inhibition was calculated from the difference between the inhibitor slope and control slope (no inhibitor). The IC_{50} was obtained from the graphical analysis of the concentration and the inhibition.

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 HPLC.

Determination of partition coefficient (water/octanol). To a solution of inhibitor (generally 0.1 mg/mL) in pH 7 buffer solution (2.0 mL) was added octanol (2.0 mL). The mixture was shook at 25 °C for 1 h, and centrifuged (3000 rpm). The concentration of inhibitor in each phase was determined by HPLC.

Transcorneal permeation. Eye drops of compound **1** was prepared as 0.5% aqueous solution. Eye drops of compounds **3** and **4** were prepared as 0.5% aqueous suspensions. Each eye drops (50 μL, 1 drop) was exactly applied to eyes of albino rabbits with micropipet (single instillation, $n=3$ eyes). At 0.5, 1, 2 and 4 h after instillation, the animals were sacrificed with pentobarbital overdosage and the aqueous humor (250 μL) was sampled from each eye. The aqueous humor specimens were subjected to measurement of drug concentration by HPLC analysis.

Lens culture. Lenses from 5-week-old Sprague–Dawley rats were cultured at 37 °C under 5% CO_2 in 4 mL Eagle's minimum essential medium (MEM Gibco) with 10% fetal bovine serum (Gibco) (Normal group). Calcium ionophore (A23187) (10 μM) was present on day 1 only (A23187 group), and 100 μM hemiacetal **4** was present continuously (A23187 + **4**). After 5 days of culture, lenses were photographed under a dissecting microscope, and density of lens opacity was quantitated using computerized image analysis (Image 1.31 software, Twilight clone BBC, Silver Springs, MD).

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