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Cyclopropenone-containing Cysteine Proteinase Inhibitors. Synthesis and Enzyme Inhibitory Activities

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Abstract—By focusing on the amphiphilic properties of cyclopropenone (e.g. a good electrophile and a precursor for a stable 2π-aromatic hydroxycyclopropenium cation), a new class of cysteine proteinase inhibitors containing a cyclopropenone moiety was designed. For the purpose of the present research, we needed to devise a new method to introduce a peptide-related moiety as a substituent on the cyclopropenone residue. We investigated the reaction of metalated cyclopropenone acetal derivatives (**2**, R^2 = metal) with *N*-protected α-aminoaldehydes **4** to obtain the adduct **5**, and succeeded in the preparation of highly potentiated cysteine proteinase inhibitors **8** after several steps transformations. They showed strong inhibitory activities only to cysteine proteinases such as calpain, papain, cathepsin B, and cathepsin L and not to serine (e.g. thrombin and cathepsin G) and asparatic protainases (e.g. cathepsin D). Kinetic studies indicated that they are competitive inhibitors, and by the examinations of their inhibitory mechanism it became clear that they are reversible inhibitors. © 1999 Elsevier Science Ltd. All rights reserved.

Introduction

Cyclopropenone has amphiphilic properties; it can act as a good electrophile for 1,2- or 1,4-addition receiving various nucleophiles and as a precursor for a stable 2π-aromatic hydroxycyclopropenium cation that can be generated readily by protonation of the carbonyl group.[1] The former property may be amenable to the design of irreversible inhibitors by taking advantage of the reaction of the cyclopropenone group with a cysteine or serine residue at the active site of enzyme, or that of antitumor agents by the reaction with a nucleic base in DNA. The latter property may be applied to the design of reversible enzyme inhibitors, receptor agonists or antagonists by the use of strong electrostatic interactions between hydroxycyclopropenium cation and the surface of the target enzyme or receptor. However, the repertoire of the biologically active cyclopropenone derivatives reported so far involves only an antibiotic penitoricin[2] and its congeners,[3] which showed antimicrobial activity and cytotoxicity (Fig. 1).

We focused our attention on the possibility of using cyclopropenone derivatives as a candidate of cysteine proteinase inhibitors. A thiol residue of cysteine at the catalytic site of the enzyme may be reactive enough to add to the cyclopropenone, or since thiol is quite acidic, it would protonate the cyclopropenone if these two residues exist at a suitable distance on the surface of the enzyme. Cysteine proteinase inhibitors are good targets in medicinal research. For example, a calpain inhibitor may be used as a therapeutic agent for stroke or cardiac ischaemia.[4] A cathepsin B inhibitor may be used as a drug for inflammation[5] or prevent metastasis of cancer.[6] A cathepsin L inhibitor may become a drug for osteoporosis.[7] A inhibitor of caspase-1 (interleukin-1β-converting enzyme) may prevent cell apoptosis.[8]

For an active-site-directed inhibitor of an enzyme, a peptide-like moiety might be needed for the interaction with the enzyme. This concept has appeared in some known cysteine proteinase inhibitors such as peptidyl halomethylketones or peptidyl aldehydes.[9] We have previously developed some reactions of 2-lithiated cyclopropenone acetals (**2**, R^2 = Li) with simple electrophiles such as aldehydes, ketones or halogenated compounds.[10] For the purpose of the present research, we needed to devise a new method to introduce a peptide-related moiety as a substituent on the cyclopropenone residue. We investigated the reaction of metalated

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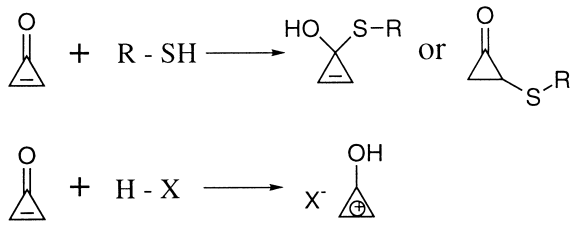


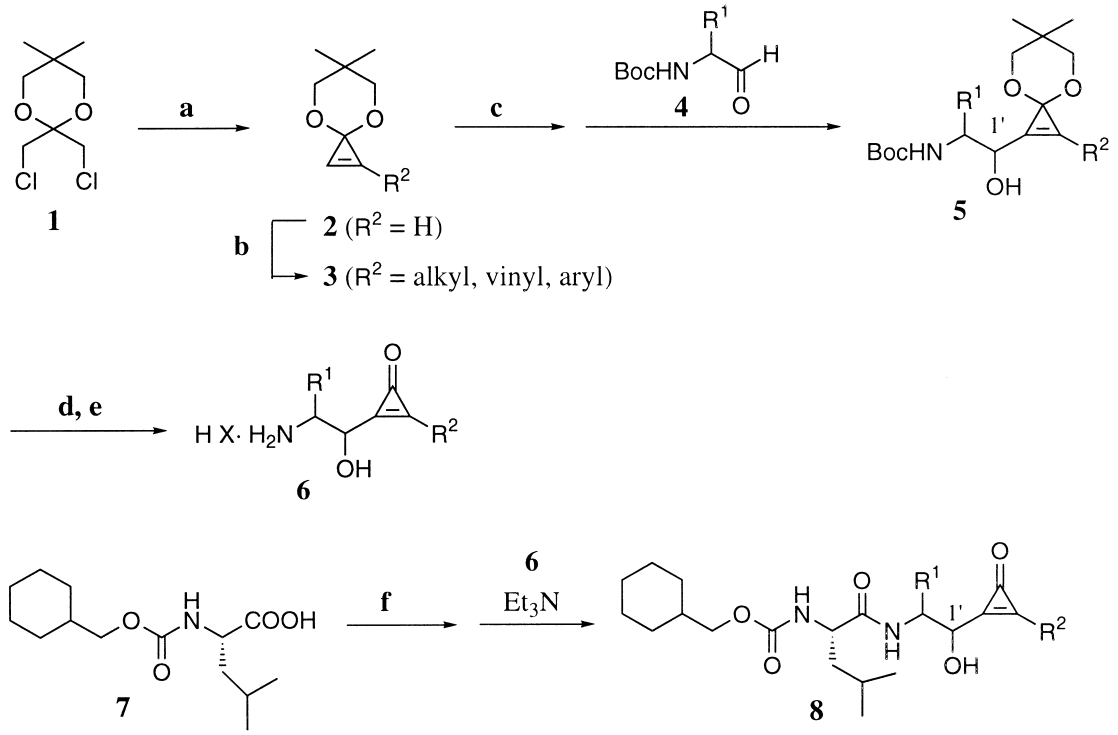
Figure 1. Amphiphilic properties of cyclopropenone.

cyclopropenone acetal derivatives (**2**, R^2 = metal) with *N*-protected α-aminoaldehydes,[11] and succeeded in the preparation of a highly potentiated cysteine proteinase inhibitor by overcoming several synthetic problems.[12] We report herein the synthesis of novel cysteine proteinase inhibitors and their inhibitory activities against calpain with some discussion about structure activity relationship.

Results and Discussion

Chemistry

A typical synthetic route is summarized in Scheme 1. Cyclopropenone acetals **2** and **3** were prepared in one step by the treatment of 1,3-dichloroacetone acetal **1** with metallic sodium in liquid ammonia containing diethyl ether followed by addition of an R^2 souse (i.e. H^+, R^{2+}).[13] As an alternative route of **3**, the acetal **2** was lithiated by the reaction with butyllithium in the presence of HMPA,[14] and the resulting vinyllithium reagent was alkylated, arylated, or vinylated to afford the 2-substituted derivatives **3**.[10] To obtain the target compound **5**, the cyclopropenone acetal **2** or **3** was lithiated a second time by the above method in the presence of TMEDA instead of HMPA, and, after transmetallation to a dichrolocerium salt by the addition of anhydrous $CeCl_3$ suspension in THF,[15] this salt was allowed to react with *N*-Boc-aminoaldehyde **4** to obtain the alcohol **5**.[11] The ratio of stereoisomers at the 1′-carbon atom was 85:15 to 70:30 as determined by ^1H NMR. In general, a compound of R^2 = H showed good diastereoselectivity. In the next step, deprotections were achieved in a stepwise manner. First, the acetal moiety was removed under a mild acidic condition by the treatment with Amberlist 15® in the presence of 2,6-di-*tert*-butylpyridine in hexane, diluted sulfuric acid or diluted hydrochloric acid. Then the *N*-Boc group was removed by the reaction with one equivalent of $TsOH \cdot H_2O$ or excess HCl to obtain the amine **6** as its TsOH or HCl salt. In the case of R^2 = aryl, deprotection of both acetal and Boc groups was achieved simultaneously by treatment of 3N HCl:dioxane in the presence of one equivalent of water. The final coupling reaction between **6** and **7** was performed as follows. The carboxylic acid moiety of (*S*)-*N*-cyclohexylmethoxycarbonylleucine **7** was activated by isobutyl chloroformate in the presence of triethylamine, and the resulting mixed anhydride was allowed to react with the amine **6** to afford the designed compound **8**. In some cases, stereoisomers could be separated readily by a silica gel chromatography at this stage.



Scheme 1. Reagents: (a) (i) Na, liqued NH_3-Et_2O; (ii) NH_4Cl or electrophile; (b) (i) n-BuLi, HMPA, THF; (ii) electrophile; (c) (i) n-BuLi, TMEDA, THF; (ii) $CeCl_3$, THF; (d) Amberlist 15®, 2,6-di-*tert*-butylpyridine, hexane or 0.1 N H_2SO_4, AcOEt-H_2O or 0.1 N HCl, AcOEt-H_2O; (e) $TsOH \cdot H_2O$, CH_2Cl_2 or 3 N HCl, dioxane; (f) *i*-BuOCOCl, Et_3N, CH_2Cl_2.

Proteinase inhibitory activity

Inhibitory activity against m-calpain was assayed by using synthetic substrate (Suc-Leu-Leu-Val-Tyr-MCA) under a manner similar to the one described in the literature.[16] First we examined the effects of several kinds of the substituents on the cyclopropenone ring for the inhibitory activity, and results are summarized in Table 1. The inhibitory activity of alkyl substituted derivatives **8c** and **8d** were much less as compared to the non-substituted compound **8a** or **8b**. However, the activity of the conjugated 1-hexenyl substituted derivative **8e** was stronger than the less bulky compound **8d**, and the aromatic substituted derivatives **8f**–**8l**, except for the compound **8k**, showed quite good activities in spite of their bulkiness.

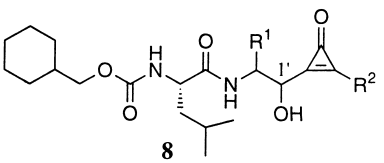
One of our hypotheses has been that the hydroxycyclopropenium cation generated by protonation of the cyclopropenone might play an important role for the enzyme inhibitory activity. It was reported that the electron donating σ-inductive effect of an attached alkyl group is more significant for the stabilization of the resultant hydroxycyclopropenium cation than the electron donating π-conjugative effect of an attached aromatic group.[17] Furthermore, di-*n*-propylcyclopropenone is slightly more basic than the diphenylcyclopropenone,[18] and hence alkyl-substituted derivatives might show better inhibitory activities. However, alkyl-substituted derivatives in Table 1 showed lower inhibitory activities than phenyl-substituted derivatives, suggesting that the electronic effect of substituents does not seem to be important.

Conformational effects were considered next. The conformation of the cyclopropenone and phenyl ring moieties in compounds **8f**, **8g** and **8h** must be nearly planner as indicated by the X-ray analysis of **8g** as well as 1,2-diphenylcyclopropenone[19] and bis(*p*-chlorophenyl)cyclopropenone.[20] The lack of potency in compound **8k** is possibly due to the twist between the cyclopropenone ring and the phenyl group, which would be caused by the *o*-methyl group. Thus it seems that the planarity of the aryl cyclopropenone unit is essential for the inhibitory activity. The deleterious effects of bulky alkyl substituents seemed to confirm this view.

Although it was generally difficult to separate two diastereomers of **8** at the 1′-carbon atom, we could separate the diasteroisomers in the compound R^1 = isopropyl by column chromatography on silica gel. The absolute configuration of the minor isomer **8g** was determined by X-ray crystallography which proved that it is in (1′*R*)-configuration. In all examined cases, the activity of (1′*S*)-isomer is stronger then the (1′*R*)-isomer two to fourfold (**8f** versus **8g**, **8i** versus **8j**).

The binding site of the compound **8** to calpain is not clear at the present time, but we guessed that the R^1 binds to the P_1 site and leucine moiety binds to the P_2 site. In order to certify this hypothesis, we examined the substituent effect of R^3 group in the compound **9** which are summarized in Table 2. The isobutyl group (leucine) showed most potent activity in the examined four substituents. It is said that calpain recognizes its substrate by leucine at the S_2 position, as supported by the fact that many potent inhibitors of calpain have leucine at their P_2 position.[21] On the contrary the inhibitory activities against cathepsin B had different tendency from those against calpain, so the results against calpain were characteristic of the order of calpain. According to these results, hypothetical binding sites of **8** to calpain may be formulated as follows; cyclohexylmethyl as P_3, isobutyl as P_2, R^1 as P_1, R^2 may be as P'_1, yet unclear from these data. Simple cyclopropenone derivatives such as 2-phenylcyclopropenone or 2-(1-hydroxyhexyl)-3-phenylcyclopropenone were inactive even at higher concentration (200 μM), and hence the important role of the peptide mimetic moiety is apparent.

Table 1. m-Calpain inhibitory activity of the compound **8**

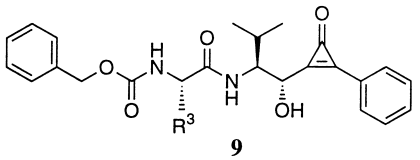


8

Compound	R^1	R^2	IC_{50} (μM)	(1′*S*):(1′*R*)[a]
8a	$CH(CH_3)_2$	H	0.50	7:3
8b	$CH_2CH_2CH_2CH_3$	H	0.81	8:2
8c	$CH_2CH_2CH_2CH_3$	CH_3	40.0	6:4
8d	$CH_2CH_2CH_2CH_3$	$CH_2CH(CH_3)_2$	>200	6:4
8e	$CH_2CH_2CH_2CH_3$	(*Z*)-1-Hexenyl	56.0	(1′*S*)[c]
8f	$CH(CH_3)_2$	Phenyl	1.62	(1′*S*)[c]
8g	$CH(CH_3)_2$	Phenyl	3.25	(1′*R*)[b]
8h	$CH_2CH_2CH_2CH_3$	Phenyl	2.70	7:3
8i	$CH(CH_3)_2$	4-F-Phenyl	1.51	(1′*S*)[c]
8j	$CH(CH_3)_2$	4-F-Phenyl	7.00	(1′*R*)[c]
8k	$CH_2CH_2CH_2CH_3$	2-Me-Phenyl	29.0	7:3
8l	$CH_2CH_2CH_2CH_3$	5-Trimethylsilyl-2-thienyl	11.0	7:3

[a]Determined by ^{1}H NMR.
[b]Determined by X-ray crystallography.
[c]Assumed by analogy to the configuration of **8g**.

Table 2. Inhibitory activity of the compound **9**



9

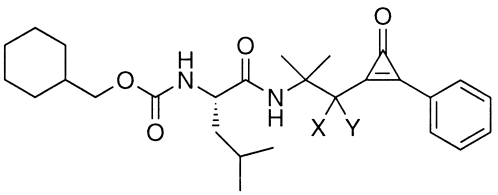
Compound	R^3	IC_{50} (μM) Calpain	Cathepsin B
9a	CH_3	48.0	22.9
9b	$CH(CH_3)_2$	8.20	9.45
9c	$CH_2CH(CH_3)_2$	2.10	29.0
9d	CH_2Ph	16.2	>100

Finally we examined the modification of 1′-hydroxyl group. 1′-Methoxy and 1′-acetoxy derivatives of **8** showed no activity. Although we tried to synthesize a 1′-keto derivative of **8** by various methods, all attempts were unsuccessful because of the presence of the 2′-hydrogen atom. Thus we prepared the 2′-dimethyl derivative **10a** and oxidized the hydroxyl group to obtain the 1′-keto derivative **10b** (Fig. 2). The activity of the compound **10a** was not strong perhaps because of the substituent effect at the 2′-position, and that of **10b** was much weaker than **10a**. Based on these results, we concluded that the 1′-hydroxyl group plays an important role for m-calpain inhibitory activity.

The inhibitory activities against other cysteine proteinases were measured in the compound **8f** and **8g** which are summarized in Table 3. The (1′*S*) isomer **8f** showed strong activities against papain,[22] cathepsin B,[22] and cathepsin L.[22] On the contrary, the (1′*R*) isomer **8g** inhibited the latter two strongly, but showed much weak inhibitory activity against papain. This result indicates that the configuration of 1′-hydroxyl group is very important for the inhibition of papain. Examination of inhibitory activity of **8f** against other types of proteinases revealed interesting trend. Namely, **8f** inhibited neither serine proteinase (thrombin[23] and cathepsin G[24]) nor asparatic proteinase (cathepsin D[25]) even at high concentration (100 μM). Thus, the compounds of this series are selective inhibitors of cysteine proteinases.

Study on inhibitory mechanism

Lineweaver–Burk plot of m-calpain inhibition by the compound **8f** was performed in a usual method and its



10a: X = H, Y = OH; IC_{50} = 42.0 μM
10b: X, Y = (=O); IC_{50} >100 μM

Figure 2. Calpain inhibitory activity of the compound **10**.

Table 3. Cysteine proteinase inhibitory activity of **8f** and **8g**

Enzyme	IC_{50} (μM) **8f**	**8g**	**8g**/**8f**
m-Calpain[a]	1.62	3.25	2.01
Papain[b]	0.054	22	407
Cathepsin B[c]	0.71	0.044	0.06
Cathepsin L[d]	0.00086	0.0013	1.52

[a]Purified from rat brain.
[b]Purified from papaya.
[c]Purified from bovine spleen.
[d]Purified from rat kidney.

result is shown in Figure 3. This plot shows that the compound **8f** is a competitive inhibitor of m-calpain with a K_i of 0.50 ± 0.126 μM. Lineweaver–Burk plot of papain inhibition also showed a competitive inhibition with a K_i of 0.055 ± 0.229 μM.[12]

In the light of its diverse reactivities, cyclopropenone may inhibit enzymes both reversibly and irreversibly. In order to make this point clear, we performed the following experiment.[26] m-Calpain, synthetic substrate and an inhibitor were incubated in high concentration for a while after the addition of $CaCl_2$. Then EDTA (a calcium chelator) was added to stop the activity of enzyme. Next, the solution was diluted with buffer, and $CaCl_2$ and excess amount of substrate were added again to regenerate the activity of the enzyme and the remaining activity was measured. The result is shown in Figure 4.

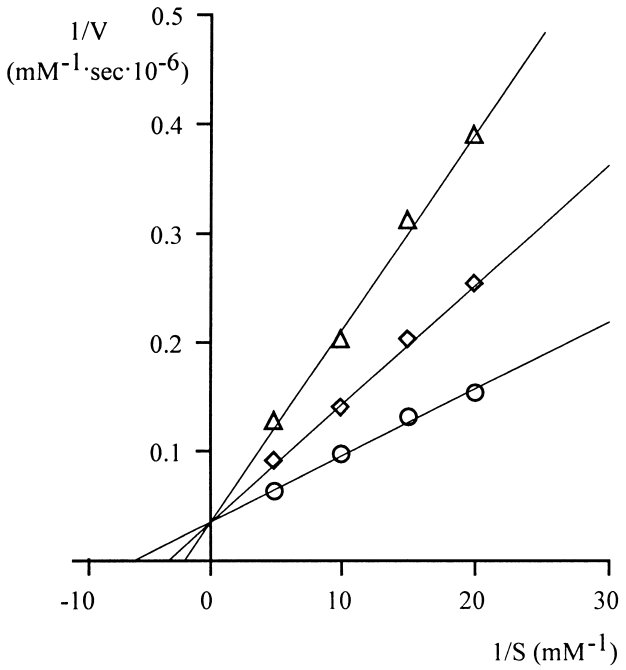


Figure 3. Lineweaver–Burk Plot of m-calpain inhibition by the compound **8f** with Suc-Leu-Leu-Val-Tyr-Mec. A K_m of the substate is 0.13 mM. Concentrations of the inhibitor were (○) 0, (◇) 0.5 μM and (△) 1.0 μM. The inhibitor **8f** was dissolved in dimethyl sulfoxide and was added to the imidazole–HCl buffer (pH 7.3) containing m-calpain, L-cysteine, 2-mercaptoethanol and the substrate. Then $CaCl_2$ was added to activate the enzyme.

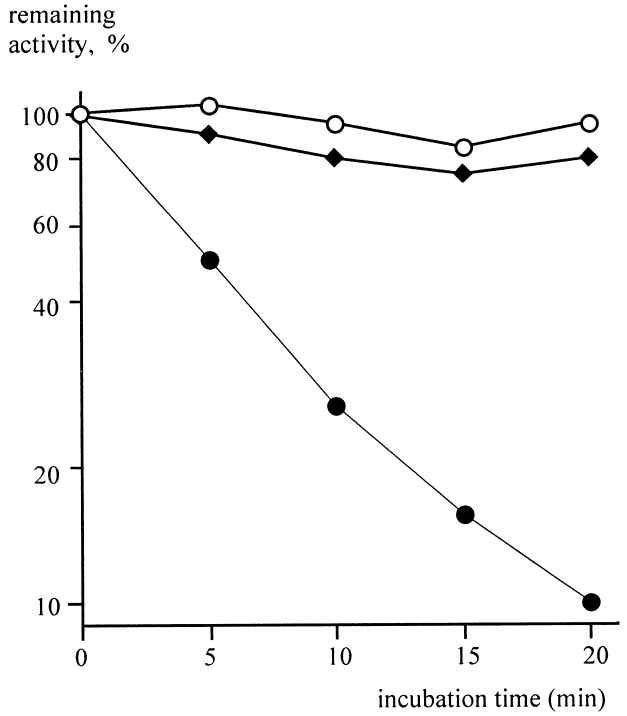


Figure 4. Examination for the reversible or irreversible inhibition. After m-calpain, the substrate, an inhibitor and $CaCl_2$ were incubated for a while, EDTA was added to stop the activity of m-calpain. Then the substrate and $CaCl_2$ were added and the remaining enzyme activity was measured. Examined compounds were (◆) **8h** and (●) E-64, which were compared with (○) control.

In the case of E-64,[27] a well-known irreversible inhibitor, the remaining enzyme activity decreased in response to the incubation time. In contrast, the remaining enzyme activity in the case of the compound **8h** was virtually unchanged as compared with the activity of the control run.[28] Thus the compound **8h** has been shown to be a reversible inhibitor of calpain. In a similar experiment, the compound **8f** also showed the reversible property.

In spite of the reversibility of the compound **8**, the cysteine residue at the active site of calpain might react with the inhibitor. A similar example has been seen in peptidyl aldehydes, reversible inhibitors of cysteine or serine proteinases. The hydroxyl residue at the active site of a serine proteinase reacted with the carbonyl moiety of an inhibitor to form a reversible hemiacetal, which was confirmed by X-ray crystallography[29] or ^{1}H NMR.[30] Although the cyclopropenone containing compounds in this report were stable under acidic or neutral conditions, they are quite unstable under basic conditions. For example, treatment of the compound **8f** with 1 equivalent of sodium methoxide in methanol gave a complex mixture presumably due to a nucleophilic addition of methoxide followed by various reactions or intramolecular reactions under a basic condition. From this result it is apparent that the cyclopropenone moiety of the compound **8f** is a good electrophile, so at the active site of calpain a reversible adduct of the thiol residue and the cyclopropenone might be formed. Thus it is unclear at this point whether the reaction of the inhibitor and the cysteine residue is proceeding or not. Investigations about the inhibitory mechanism are ongoing.

Conclusion

We designed a new class of cysteine proteinase inhibitors containing a cyclopropenone moiety by focusing on its amphiphilic properties (e.g. a good electrophile and a precursor for a stable 2π-aromatic hydroxycyclopropenium cation). We investigated the reaction of metalated cyclopropenone acetal derivatives (**2**, R^2 = metal) with *N*-protected α-aminoaldehydes **4** to obtain the adduct **5**, and succeeded in the preparation of highly potentiated cysteine proteinase inhibitors **8** after several steps transformations. They showed strong inhibitory activities only to cysteine proteinases such as calpain, papain, cathepsin B, and cathepsin L and not to serine and asparatic proteinases. By the examinations of their inhibitory mechanism, it became clear that they are competitive and reversible inhibitors.

Experimental

General

All ^{1}H NMR spectra taken at 250 MHz were measured on a Bruker AC-250 instrument, and are reported in parts per million (ppm) from internal tetramethylsilane. IR spectra were recorded on a JASCO FT/IR-5300 instrument; absorptions are reported in cm^{-1}. Fluorescence for enzyme inhibition assays was recorded on a Hitachi Fluorescence Spectrophotometer F-3000. Most of reagents were purchased from Tokyo Chemical Industry, and used without further purification. Suc-Leu-Leu-Val-Tyr-MCA was purchased from Peptide Institute. m-Calpain was purified from rat brain, and cathepsin L was purified from rat kidney according to the reported method. Z-Phe-Arg-MCA, Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide, H-D-Phe-Pip-Arg-*p*-nitroanilide, papain, cathepsin B, cathepsin D, cathepsin G and thrombin were purchased from Sigma.

2-((2*S*)-2-*tert*-Butoxycarbonylamino-1-hydroxy-3-methylbutyl)-3-phenylcyclopropenone 2,2-dimethyl-1,3-propanediyl acetal 5 (R^1 = isopropyl; R^2 = Ph). 2-Phenylcyclopropenone 2,2-dimethyl-1,3-propanediyl acetal **3** (R^2 = Ph)[13] (3.51 g) was dissolved in THF (30 mL), and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (3.8 g) was added. Then the resulting solution was cooled to −78 °C, and *n*-BuLi (1.55 mol/L solution in *n*-hexane, 10.5 mL) was added. After stirring for 20 min at that temperature, a cooled suspension of anhydrous $CeCl_3$, which was prepared from $CeCl_3 \cdot 7H_2O$ (8.0 g) by drying in vacuo at 140 °C for 2 h, in THF (30 mL) was added, and the resulting suspension was stirred for 20 min. Next (*S*)-*N*-*tert*-butoxycarbonylvalinal **4** (R^1 = isopropyl) (1.82 g) in THF (20 mL) was added, and stirred at −78 °C for 2 h. The reaction mixture was quenched by the addition of water (1 mL) in THF (5 mL) at −78 °C, and the temperature of it was raised to 20 °C. Then the resulting

suspension was filtered by Celite and washed well with ethyl acetate. After the filtrate was dried over Na_2SO_4, the solvent was evaporated to afford the crude mixture, which was purified by column chromatography (hexane: ethyl acetate = 4:1) to give the titled compound (2.71 g, 72%). IR (KBr) 3430, 2960, 1855, 1800, 1710, 1690 cm^{-1}; NMR (CD_3OD) δ 0.95–1.60 (m, 12H), 1.38 (s, 3H), 1.42 (s, 6H), 1.95–2,15 (m, 1H), 3.60–3.75 (m, 1H), 3.75–3.95 (m, 4H), 5.00–5.10 (m, 1H), 6.03 (d, J = 10 Hz, 0.33H), 6.23 (d, J = 10 Hz, 0.67H), 7.40–7.65 (m, 3H), 7.70–7.90 (m, 2H). Anal. calcd for $C_{24}H_{35}NO_5$: C, 68.59; H, 8.89; N, 3.28. Found: C, 68.20; H, 8.77; N, 3.24.

2-((2*S*)-2-Amino-1-hydroxy-3-methylbutyl)-3-phenylcyclopropenone hydrochloride 6 (R^1 = isopropyl; R^2 = Ph). To a solution of the above acetal **5** (3.12 g) in 1,4-dioxane (8 mL) and water (0.2 mL), 4 N HCl in 1.4-dioxane (24 mL) was added at room temperature. After stirring for 20 min, the formed precipitates were filtered and washed with 1,4-dioxane to afford the titled compound (1.87 g, 94%) as a highly hygroscopic solid. IR (KBr) 3200, 1855, 1618 cm^{-1}; NMR (CD_3OD) δ 1.20 (d, J = 7.0 Hz, 2.25H), 1.22 (d, J = 7.0 Hz, 2.25H), 1.23 (d, J = 6.0 Hz, 0.75H), 1.25 (d, J = 6.0 Hz, 0.75H), 2.10 (m, 0.25H), 2.30 (m, 0.75H), 3.38 (dd, J = 6.0 Hz, 6.0 Hz, 0.25H), 3.45 (dd, J = 6.0 Hz, 6.0 Hz, 0.75H), 5.21 (d, J = 6.0 Hz, 0.75H), 5.45 (d, J = 6.0 Hz, 0.25H), 7.60–7.80 (m, 3H), 8.10–8.20 (m, 2H). Anal. calcd for $C_{14}H_{17}NO_2 \cdot 1.1HCl \cdot 0.6H_2O$: C, 59.97; H, 6.92; N, 5.00; Cl, 13.28. Found: C, 60.00; H, 6.66; N, 5.20; Cl, 13.35.

2-((1*S*,2*S*)-2-((*S*)-2-Cyclohexylmethoxycarbonylamino-4-methylvalerylamino)-1-hydroxy-3-methylbutyl)-3-phenylcyclopropenone (8f). To a solution of (*S*)-*N*-cyclohexylmethoxycarbonylleucine **7** (2.46 g) in CH_2Cl_2 (40 mL), were added triethylamine (0.92 g) and isobutyl chloroformate (1.15 g) at −5 °C and stirred at that temperature for 15 min. Then the amine **6** (1.87 g) and triethylamine (0.97 g) were added and the resulting solution was stirred for 1 h. After adding 0.5 N HCl solution (10 mL), the aqueous layer was extracted with CH_2Cl_2. The combined extracts were washed successively with water, satd $NaHCO_3$ solution, and satd NaCl solution, and dried over $MgSO_4$. Removal of the solvent gave a crude solid, which was purified by column chromatography (hexane:ethyl acetate = 1:1) to give the titled compound (1.86 g, 55%). Mp 85–89 °C; IR (KBr) 3330, 1858, 1695, 1658, 1625 cm^{-1}; NMR ($CDCl_3$) δ 0.77 (d, J = 5.0 Hz, 6H), 0.80–1.05 (m, 2H), 1.01 (d, J = 6.6 Hz, 3H), 1.10 (d, J = 6.6 Hz, 3H), 1.10–1.80 (m, 12H), 2.40 (m, 1H), 3.50–3.88 (m, 3H), 4.09 (m, 1H), 5.11 (d, J = 4.4 Hz, 1H), 5.31 (m,1H), 7.36–7.62 (m, 4H), 7.98 (dd, J = 7.5 Hz, 1.7 Hz, 2H). Anal. calcd for $C_{28}H_{40}N_2O_5 \cdot H_2O$: C, 66.91; H, 8.42; N, 5.57. Found: C, 66.96; H, 8.14; N, 5.46.

2-((1*R*,2*S*)-2-((*S*)-2-Cyclohexylmethoxycarbonylamino-4-methylvalerylamino)-1-hydroxy-3-methylbutyl)-3-phenylcyclopropenone (8g). This compound was obtained by the above column chromatography (0.61 mg, 18%). Mp 148–149 °C; IR (KBr) 3350, 3260, 1860, 1825, 1715, 1653, 1625 cm^{-1}; NMR ($CDCl_3$) δ 0.80–1.00 (m, 8H), 1.08 (d, J = 6.6 Hz, 3H), 1.15 (d, J = 6.7 Hz, 3H), 1.10–1.80 (m, 12H), 2.16 (m, 1H), 3.68 (m, 1H), 3.75 (m, 1H), 4.02–4.22 (m, 2H), 5.14 (d, J = 2.8 Hz, 1H), 5.19 (d, J = 6.5 Hz, 1H), 6.92 (d, J = 6.0 Hz, 1H), 7.42–7.65 (m, 3H), 7.99 (dd, J = 6.7 Hz, 1.5 Hz, 2H). Anal. calcd for $C_{28}H_{40}N_2O_5 \cdot 0.2H_2O$: C, 68.88; H, 8.34; N, 5.74. Found: C, 68.85; H, 8.55; N, 5.67.

2-((2*S*)-2-((*S*)-2-Cyclohexylmethoxycarbonylamino-4-methylvalerylamino)-1-hydroxy-3-methylbutyl)cyclopropenone (8a). IR (KBr) 3310, 1825, 1690, 1650 cm^{-1}; NMR ($CDCl_3$) δ 0.80–1.80 (m, 26H), 1.90–2.35 (m, 2H), 3.60–4.25 (m, 4H), 4.87–5.05 (m, 1H), 5.05–5.45 (m, 1H), 6.85–7.03 (m, 0.3H), 7.35–7.52 (m, 0.7H), 8.54 (s, 0.7H), 8.62 (s, 0.3H). Anal. calcd for $C_{22}H_{36}N_2O_5 \cdot 0.7H_2O$: C, 62.74; H, 8.95; N, 6.65. Found: C, 62.72; H, 8.81; N, 6.50.

2-((2*S*)-2-((*S*)-2-Cyclohexylmethoxycarbonylamino-4-methylvalerylamino)-1-hydroxyhexyl)cyclopropenone (8b). IR (KBr) 3340, 1830, 1690, 1650 cm^{-1}; NMR ($CDCl_3$) δ 0.80–1.05 (m, 11H), 1.10–1.88 (m, 18H), 2.24 (s, 1H), 3.73–3.95 (m, 2H), 4.05–4.30 (m, 2H), 4.80–4.93 (m, 1H), 5.20–5.35 (m, 0.2H), 5.35–5.55 (m, 0.8H), 5.55–5.75 (m, 0.8H), 6.95–7.05 (m, 0.2H), 8.57 (s, 0.8H), 8.68 (s, 0.2H). Anal. calcd for $C_{23}H_{38}N_2O_5$: C, 65.38; H, 9.06; N, 6.63. Found: C, 65.37; H, 9.28; N, 6.51.

2-((2*S*)-2-((*S*)-2-Cyclohexylmethoxycarbonylamino-4-methylvalerylamino)-1-hydroxyhexyl)-3-methylcyclopropenone (8c). IR (KBr) 3320, 1845, 1690, 1653, 1630 cm^{-1}; NMR ($CDCl_3$) δ 0.85–1.10 (m, 11H), 1.10–1.95 (m, 18H), 2.33 (s, 3H), 2.49 (s, 1H), 3.75–3.97 (m, 2H), 3.97–4.28 (m, 2H), 4.73–4.90 (m, 1H), 5.20–5.85 (m, 1.6H), 7.10–7.50 (m, 0.4H). Anal. calcd for $C_{24}H_{40}N_2O_5 \cdot 0.3H_2O$: C, 65.22; H, 9.26; N, 6.34. Found: C, 65.14; H, 9.26; N, 6.07.

2-((2*S*)-2-((*S*)-2-Cyclohexylmethoxycarbonylamino-4-methylvalerylamino)-1-hydroxyhexyl)-3-isopentylcyclopropenone (8d). IR (KBr) 3310, 1838, 1720, 1698, 1652, 1620 cm^{-1}; NMR ($CDCl_3$) δ 0.85–1.10 (m, 21H), 1.10–1.95 (m, 21H), 2.45–2.55 (m, 1H), 2.55–2.70 (m, 2H), 3.73–3.93 (m, 2H), 3.93–4.25 (m, 2H), 4.73–4.85 (m, 1H), 5.25–5.85 (m, 1.6H), 7.30–7.45 (m, 0.4H). Anal. calcd for $C_{28}H_{48}N_2O_5 \cdot 0.5H_2O$: C, 67.03; H, 9.84; N, 5.58. Found: C, 67.26; H, 9.81; N, 5.28.

2-((1*S*,2*S*)-2-((*S*)-2-Cyclohexylmethoxycarbonylamino-4-methylvalerylamino)-1-hydroxyhexyl)-3-((*Z*)-1-hexenyl)-cyclopropenone (8e). Mp 138–140 °C; IR (KBr) 3290, 1838, 1711, 1655, 1630 cm^{-1}; NMR ($CDCl_3$) δ 0.83–1.08 (m, 14H), 1.10–2.05 (m, 22H), 2.56 (m, 2H), 3.85 (s, 2H), 4.02 (m, 1H), 4.15 (m, 1H), 4.82 (m, 1H), 5.22–5.48 (m, 2H), 6.26 (d, J = 10 Hz, 1H), 6.48 (m, 1H), 7.08 (m, 1H). Anal. calcd for $C_{29}H_{42}N_2O_5 \cdot 0.2H_2O$: C, 69.35; H, 8.51; N, 5.58. Found: C, 69.33; H, 8.56; N, 5.49.

2-((2*S*)-2-((*S*)-2-Cyclohexylmethoxycarbonylamino-4-methylvalerylamino)-1-hydroxyhexyl)-3-phenylcyclopropenone (8h). IR (KBr) 3310, 1855, 1690, 1655, 1625 cm^{-1}; NMR ($CDCl_3$) δ 0.65–0.75 (m, 1.2H), 0.75–1.05 (m, 7.8H), 1.10–2.00 (m, 20H), 2.07 (s, 1H), 3.65–3.90 (m, 2H), 4.00–4.20 (m, 1.7H), 4.20–4.35 (m, 0.3H), 4.95–5.05 (m, 1H), 5.10–5.30 (m, 0.3H), 5.45–5.60 (m, 0.7H), 5.70–

5.85 (m, 1H), 7.00–7.25 (m, 0.7H), 7.45–7.56 (m, 3.3H), 7.95–8.05 (m, 2H). Anal. calcd for $C_{29}H_{42}N_2O_5 \cdot 0.2H_2O$: C, 69.35; H, 8.51; N, 5.58. Found: C, 69.45; H, 8.45; N, 5.38.

2-((1*S*,2*S*)-2-((*S*)-2-Cyclohexylmethoxycarbonylamino-4-methylvalerylamino)-1-hydroxy-3-methylbutyl)-3-(4-fluorophenyl)cyclopropenone (8i). Mp 86–90 °C; IR (KBr) 3320, 1858, 1693, 1656, 1625, 1602 cm^{-1}; NMR ($CDCl_3$) δ 0.79 (d, J = 5.9 Hz, 6H), 1.01 (d, J = 6.6 Hz, 3H), 1.09 (d, J = 6.6 Hz, 3H), 0.70–1.45 (m, 7H), 1.45–1.83 (m, 7H), 2.39 (m, 1H), 3.50–3.85 (m, 3H), 4.08 (m, 1H), 5.10 (dd, J = 8.6 Hz, 4.7 Hz, 1H), 5.25 (m, 1H), 6.23 (m, 1H), 7.19 (dd, J = 8.5 Hz, 8.5 Hz, 2H), 7.47 (m, 1H), 8.01 (dd, J = 8.5 Hz, 5.5 Hz, 2H). Anal. calcd for $C_{28}H_{39}FN_2O_5 \cdot 0.4H_2O$: C, 65.96; H, 7.87; N, 5.49. Found: C, 65.88; H, 7.99; N, 5.67.

2-((1*R*,2*S*)-2-((*S*)-2-Cyclohexylmethoxycarbonylamino-4-methylvalerylamino)-1-hydroxy-3-methylbutyl)-3-(4-fluorophenyl)cyclopropenone (8j). Mp 166–168 °C; IR (KBr) 3340, 3260, 1861, 1714, 1655, 1625 cm^{-1}; NMR ($CDCl_3$) δ 0.63–1.03 (m, 8H), 1.07 (d, J = 6.6 Hz, 3H), 1.14 (d, J = 6.6 Hz, 3H), 1.03–1.80 (m, 12H), 2.14 (m, 1H), 3.55–3.85 (m, 2H), 3.95–4.15 (m, 2H), 5.00–5.23 (m, 2H), 5.29 (d, J = 5.7 Hz, 1H), 6.90 (d, J = 7.0 Hz, 1H), 7.18 (dd, J = 8.5 Hz, 8.5 Hz, 2H), 8.04 (dd, J = 8.5 Hz, 5.8 Hz, 2H). Anal. calcd for $C_{28}H_{39}FN_2O_5 \cdot H_2O$: C, 64.60; H, 7.94; N, 5.38. Found: C, 64.38; H, 7.63; N, 5.35.

2-((2*S*)-2-((*S*)-2-Cyclohexylmethoxycarbonylamino-4-methylvalerylamino)-1-hydroxyhexyl)-3-(1-methylphenyl)cyclopropenone (8k). IR (KBr) 3320, 1845, 1692, 1655, 1615 cm^{-1}; NMR ($CDCl_3$) δ 0.60–1.05 (m, 11H), 1.05–2.00 (m, 18H), 2.66 (s, 2.1H), 2.68 (s, 0.9H), 3.65–3.95 (m, 2H), 3.95–4.35 (m, 2H), 4.95–5.12 (m, 1H), 5.24 (m, 0.3H), 5.55 (m, 0.7H), 7.05–7.35 (m, 6H), 7.35–7.50 (m, 3H), 8.05–8.20 (m, 2H). Anal. calcd for $C_{30}H_{44}N_2O_5 \cdot 0.2H_2O$: C, 69.79; H, 8.67; N, 5.43. Found: C, 69.96; H, 9.09; N, 5.20.

2-((2*S*)-2-((*S*)-2-Cyclohexylmethoxycarbonylamino-4-methylvalerylamino)-1-hydroxyhexyl)-3-(5-trimethylsilyl-2-thienyl)cyclopropenone (8l). IR (KBr) 3320, 1853, 1655, 1615 cm^{-1}; NMR ($CDCl_3$) δ 0.35 (s, 9H), 0.70–1.05 (m, 11H), 1.10–2.00 (m, 18H), 3.73–3.90 (m, 2H), 3.99–4.15 (m, 2H), 4.92 (d, J = 4.8 Hz, 1H), 4.95–5.10 (m, 0.3H), 5.10–5.30 (m, 0.7H), 6.50–6.65 (m, 0.3H), 6.80–6.95 (m, 0.7H), 7.29 (d, J = 3.6 Hz, 1H), 7.82 (d, J = 3.6 Hz, 0.7H), 7.84 (d, J = 3.6 Hz, 0.3H). Anal. calcd for $C_{30}H_{48}N_2O_5SSi \cdot 0.3H_2O$: C, 61.88; H, 8.41; N, 4.81. Found: C, 61.62; H, 8.43; N, 4.72.

2-((1*S*,2*S*)-2-((*S*)-2-Benzyloxycarbonylaminopropionylamino)-1-hydroxy-3-methylbutyl)-3-phenylcyclopropenone (9a). Mp 156–158 °C; IR (KBr) 3290, 1850, 1690, 1643, 1618 cm^{-1}; NMR ($CDCl_3$) δ 0.98 (d, J = 5.9 Hz, 3H), 1.07 (d, J = 6.6 Hz, 3H), 1.19 (d, J = 7.0 Hz, 3H), 2.36 (m, 1H), 3.61 (m, 1H), 4.18 (m, 1H), 4.90–5.15 (m, 3H), 5.47 (m, 1H), 7.31 (s, 5H), 7.25–7.65 (m, 5H), 7.95 (d, J = 7.4 Hz, 2H). Anal. calcd for $C_{25}H_{28}N_2O_5 \cdot 0.5H_2O$: C, 67.40; H, 6.56; N, 6.29. Found: C, 67.49; H, 6.48; N, 6.24.

2-((1*S*,2*S*)-2-((*S*)-2-Benzyloxycarbonylamino-3-methylbutyrylamino)-1-hydroxy-3-methylbutyl)-3-phenylcyclopropenone (9b). Mp 157–158 °C; IR (KBr) 3310, 1850, 1697, 1643, 1618 cm^{-1}; NMR ($CDCl_3$) δ 0.74 (d, J = 6.8 Hz, 3H), 0.85 (d, J = 6.7 Hz, 3H), 0.98 (d, J = 6.5 Hz, 3H), 1.08 (d, J = 6.6 Hz, 3H), 2.04 (m, 1H), 2.36 (m, 1H), 3.69 (m, 1H), 4.01 (m, 1H), 4.90–5.10 (m, 3H), 5.47 (d, J = 8.2 Hz, 1H), 6.15 (d, J = 8.2 Hz, 1H), 7.30 (s, 5H), 7.40–7.60 (m, 4H), 7.93–8.00 (m, 2H). Anal. calcd for $C_{27}H_{32}N_2O_5 \cdot 0.2H_2O$: C, 69.27; H, 6.98; N, 5.98. Found: C, 69.39; H, 7.09; N, 5.96.

2-((1*S*,2*S*)-2-((*S*)-2-Benzyloxycarbonylamino-4-methylvalerylamino)-1-hydroxy-3-methylbutyl)-3-phenylcyclopropenone (9c). Mp 84–94 °C; IR (KBr) 3320, 1855, 1700, 1655, 1625 cm^{-1}; NMR ($CDCl_3$) δ 0.60–0.85 (m, 6H), 0.97 (d, J = 6.0 Hz, 3H), 1.07 (d, J = 6.0 Hz, 3H), 1.20–1.60 (m, 3H), 1.85–2.25 (m, 1H), 2.25–2.45 (m, 1H), 3.55–3.70 (m, 1H), 4.00–4.10 (m, 1H), 4.85–5.15 (m, 3H), 5.53 (d, J = 8.0 Hz, 1H), 7.29 (s, 5H), 7.40–7.60 (m, 4H), 7.93–8.05 (m, 2H). Anal. calcd for $C_{28}H_{34}N_2O_5 \cdot 0.3H_2O$: C, 69.49; H, 7.21; N, 5.79. Found: C, 69.41; H, 7.30; N, 5.66.

2-((1*S*,2*S*)-2-((*S*)-2-Benzyloxycarbonylamino-3-phenylpropionylamino)-1-hydroxy-3-methylbutyl)-3-phenylcyclopropenone (9d). Mp 175–177 °C; IR (KBr) 3300, 1850, 1697, 1648, 1620 cm^{-1}; NMR ($CDCl_3$) δ 0.85 (d, J = 6.7 Hz, 3H), 0.95 (d, J = 6.6 Hz, 3H), 2.28 (m, 1H), 2.80 (dd, J = 14 Hz, 7.9 Hz, 1H), 2.99 (dd, J = 14 Hz, 7.9 Hz, 1H), 3.60 (m, 1H), 4.42 (m, 1H), 4.85–5.05 (m, 3H), 5.42 (d, J = 6.2 Hz, 1H), 5.99 (d, J = 8.0 Hz, 1H), 7.05–7.35 (m, 10H), 7.35–7.60 (m, 4H), 7.92–8.03 (m, 2H). Anal. calcd for $C_{31}H_{32}N_2O_5$: C, 72.64; H, 6.29; N, 5.46. Found: C, 72.78; H, 6.37; N, 5.46.

2-(2-((*S*)-2-Cyclohexylmethoxycarbonylamino-4-methylvalerylamino)-1-hydroxy-2-methylpropyl)-3-phenylcyclopropenone (10a). IR (KBr) 3300, 1857, 1695, 1648, 1623 cm^{-1}; NMR ($CDCl_3$) δ 0.65–1.05 (m, 8H), 1.05–1.90 (m, 18H), 3.68–3.95 (m, 2H), 4.00 (m, 1H), 4.70–4.88 (m, 1H), 4.99 (m, 1H), 6.50–6.93 (m, 2H), 7.40–7.60 (m, 2H), 7.40–7.60 (m, 3H), 8.01 (dd, J = 5.8 Hz, 1.8 Hz, 2H). Anal. calcd for $C_{27}H_{38}N_2O_5 \cdot 0.3H_2O$: C, 68.13; H, 8.17; N, 5.89. Found: C, 68.08; H, 8.17; N, 5.80.

2-(2-((*S*)-2-Cyclohexylmethoxycarbonylamino-4-methylvalerylamino)-2-methylpropionyl)-3-phenylcyclopropenone (10b). To a solution of oxalyl chloride (25 μL) in CH_2Cl_2 (2 mL), dimethyl sulfoxide (41 μL) was added at −78 °C. After stirring for 15 min, the alcohol **10a** (110 mg) was added, and 15 min later triethylamine (0.26 mL) was added. Then the resulting solution was raised to room temperature during 1.5 h, and diluted hydrochloric acid (2 mL) was added. After extraction with CH_2Cl_2, the combined extracts were washed with satd NaCl solution, and dried over $MgSO_4$. Removal of the solvent gave a crude product, which was purified by column chromatography (hexane:ethyl acetate = 4:1) to afford the titled compound (56 mg, 52%). Mp 42–49 °C; IR (KBr) 3350, 1768, 1720, 1680 cm^{-1}; NMR ($CDCl_3$) δ 0.85–1.35 (m, 14H), 1.50 (s, 3H), 1.50–1.87 (m, 9H), 3.91 (m, 2H), 4.52 (m, 1H), 4.58–4.63 (m, 1H), 5.09 (m,

1H), 7.30–7.55 (m, 3H), 7.93–8.06 (m, 2H). Anal. calcd for $C_{27}H_{36}N_2O_5 \cdot 0.3H_2O$: C, 68.42; H, 7.78; N, 5.91. Found: C, 68.53; H, 7.99; N, 5.88.

m-Calpain inhibitory activity

To a imidazole–HCl buffer, pH 7.3, (910 μL) containing 5 mM L-cysteine and 2.5 mM 2-mercaptoethanol, were added Suc-Leu-Leu-Val-Tyr-MCA (5 mM in DMSO, 40 μL), DMSO solution of an inhibitor (10 μL), and m-calpain (30 μL). Then immediately after the addition of 500 mM $CaCl_2$ (10 μL), the resulting solution was incubated for 10 min at 30 °C, during that time increase of fluorescence (excitation at 380 nm; emission at 460 nm) was recorded. The inhibitory activity was calculated by the comparison of average hydrosis rate with control run which was performed in the absence of the inhibitor. Lineweaver–Burk plots were calculated from the following assays; the inhibitor concentrations were 0, 0.5, 1.0 μM, and substrate concentrations were 0, 0.05, 0.067, 0.1, 0.2 mM.

Papain inhibitory activity

To a 400 mM sodium potassium phosphate buffer, pH 6.8 (120 μL) containing 8 mM dithiothreitol and 4 mM EDTA, were added DMSO solution of an inhibitor (5 μL) and papain in 0.1% Brij 35 solution (250 μL). The resulting solution was incubated for 5 min at 40 °C, and 20 mM Z-Phe-Arg-MCA in 0.23% DMSO solution (125 μL) was added. After incubating for 10 min at 40 °C, 100 mM sodium chloroacetate in 100 mM sodium acetate buffer, pH 4.3 (500 μL) was added to it, and the fluorescence (excitation at 380 nm; emission at 460 nm) was recorded. The inhibitory activity was calculated by the comparison of the control run which was performed in the absence of the inhibitor.

Cathepsin B inhibitory activity

It was assayed under a similar manner to the papain inhibitory activity, except that the pH of the buffer was 6.0.

Cathepsin L inhibitory activity

It was assayed under a similar manner to the papain inhibitory activity, except that the pH of the buffer was 5.5 and the incubation temperature was 30 °C.

Cathepsin G inhibitory activity

To a 0.1 M HEPES buffer, pH 7.5 (425 μL) containing 0.5 M NaCl and 0.1% Brij 35, were added DMSO solution of an inhibitor (5 μL) and cathepsin G solution (30 μL). The resulting solution was incubated for 5 min at 37 °C, and 125 mM Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide in DMSO (40 μL) was added. After incubating for 15 min at 37 °C, 100 mM sodium acetate buffer, pH 4.3 (500 μL) was added to it, and the absorbance at 410 nm was recorded. The inhibitory activity was calculated by the comparison of the control run which was performed in the absence of the inhibitor.

Thrombin inhibitory activity

It was assayed under a similar manner to the cathepsin G inhibitory activity, except that the buffer was 0.1 M Tris-HCl buffer, pH 8.0, and the substrate was H-D-Phe-Pip-Arg-*p*-nitroanilide.

Cathepsin D inhibitory activity

Hemoglobin substrate powder (16 g) was dissolved in water (100 mL), and adjusted to pH 7.5 with a 4 N NaOH solution. The pH was maintained at 7.5 with NaOH while succinic anhydride (5 g) was added to the solution. After the reaction had ceased, the hemoglobin was acidified to pH 2.5 and dialyzed at room temperature against 0.1 N acetic acid (100 volumes). Final adjustment to a 4% solution was made with 0.1 M sodium acetate buffer, pH 4.0. Four percent substrate buffer (100 μL) and an inhibitor were placed into a test tube and incubated for 5 min at 37 °C. Cathepsin D in sodium acetate buffer, pH 4.0 (100 μL) was added, and the resulting solution was incubated for 10 min at 37 °C. Then 10 μL was withdrawn and placed into 0.1 M phosphate buffer, pH 6.8 (2 mL). Then the tube received 0.1 mg/mL fluorescamine in acetone (1 mL) followed by rapid mixing. The emission at 475 nm was measured and the inhibitory activity was calculated by the comparison of the control run which was performed in the absence of the inhibitor.

Investigation of reversible or irreversible inhibition

To a imidazole-HCl buffer, pH 7.3 (54 μL) containing 5 mM L-cysteine and 2.5 mM 2-mercaptoethanol, were added Suc-Leu-Leu-Val-Tyr-MCA (5 mM in DMSO, 4 μL), DMSO solution of an inhibitor (1 μL), and m-calpain (40 μL). Then 500 mM $CaCl_2$ (1 μL) was added, and the resulting solution was incubated for a while (0, 5, 10, 15, 20 min) at 30 °C. After stopping the reaction by the addition of 100 mM EDTA (5 μL), 50 μL was separated and were added Suc-Leu-Leu-Val-Tyr-MCA (5 mM in DMSO, 40 μL) and imidazole–HCl buffer, pH 7.3 (900 μL). Immediately after the addition of 500 mM $CaCl_2$ (10 μL), the resulting solution was incubated for 10 min at 30 °C, during that time increase of fluorescence (excitation at 380 nm; emission at 460 nm) was recorded and the average hydrosis rate was plotted.

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