Synthesis of Peptide Aldehyde Derivatives as Selective Inhibitors of Human Cathepsin L and Their Inhibitory Effect on Bone Resorption[1]

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Cathepsin L, a lysosomal cysteine protease, is secreted by osteoclasts and participates in bone collagen degradation. In a search for cathepsin L inhibitors as antiosteoporotic agents, a series of peptide aldehyde derivatives were prepared by two synthetic approaches, DMSO oxidation of the corresponding alcohol derivatives and DIBAL-H reduction of the corresponding *N*,*O*-dimethylhydroxylamide derivatives, and evaluated for inhibitory activity against human cathepsin L and for inhibitory effects on bone resorption. Some of the peptide aldehyde derivatives including α-acylamino aldehyde derivatives showed potent activities. Among these compounds, *N*-(1-naphthalenylsulfonyl-L-isoleucyl-L-tryptophanal (**12**) was selected as a candidate for further investigation. Compound **12**, a potent, selective, and reversible inhibitor of human cathepsin L with an IC_{50} of 1.9 nM, inhibited the release of Ca^{2+} and hydroxyproline from bone in in vitro bone culture system and also prevented bone loss in ovariectomized mice at an oral dose of 50 mg/kg.

Introduction

Osteoporosis is characterized by low bone mass and increased risk of fracture. Bone loss is most often the result of an increase in bone resorption over bone formation. Bone resorption is accomplished by osteoclasts, which are large, hemopoietically derived, and multinucleated cells formed by the fusion of precursors related to monocytes/macrophages. Osteoclasts attach to bone surface and form a sealed extracellular compartment which is acidified by H^+-ATPase, thus forming an acidic environment into which lysosomal proteases are secreted. These lysosomal proteases, including cathepsins B, K, and L, digest the matrix, 90% of which is type I collagen.[2] Cathepsin K is a recently identified cysteine protease of the papain/cathepsin superfamily including cathepsins B and L and is expressed specifically in osteoclasts with properties similar to cathepsin L.[3] Although cathepsin K has attracted recent interest, which of the lysosomal proteases plays the principal direct role in the bone resorption process is unclear.

Katunuma et al.[4] reported that cathepsin L, a lysosomal cysteine protease, is the protease principally responsible for bone collagen degradation. It has also been reported that, of members of the cathepsin family, cathepsin L possesses the most potent type I collagen-degrading activity.[5] These findings led us to attempt to develop a potent and selective cathepsin L inhibitor as a new type of antiosteoporotic agent. Recently, Woo et al.[6] reported that intraperitoneal administration of *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal as a synthetic cathepsin L inhibitor at a dose of 5−10 mg/kg/day for 4 weeks suppressed bone weight loss in ovariectomized mice, an animal model of osteoporosis. Although their study follows the lines of our research, the development of an orally active cathepsin L inhibitor as an antiosteoporotic drug is our final aim.

Two microbial products, E-64 and leupeptin (Chart 1), are known to be inhibitors of cysteine proteases including cathepsin L. E-64[7] and leupeptin[8] exhibit bone resorption inhibitory effects in vitro.[9] However, E-64, which has an epoxysuccinyl moiety, is an irreversible cysteine protease inhibitor presuming the unexpected and undesirable side effects. On the other hand, the tripeptide aldehyde derivative leupeptin is a reversible but nonselective inhibitor of serine and cysteine proteases but is not clinically usable as a therapeutic drug. In our search for potent and selective human cathepsin L inhibitors, we synthesized a series of peptide aldehyde derivatives including α-acylamino aldehyde derivatives and evaluated their inhibitory activity against recombinant human cathepsin L. The effects of some aldehyde derivatives on bone resorption were also examined through the inhibition of the release of Ca^{2+} and hydroxyproline from bone. Finally, the in vivo effect of the peptide aldehyde derivatives on prevention of bone loss in ovariectomized mice was confirmed suggesting the possibility of the use of cathepsin L inhibitors as therapeutic drugs for the treatment of osteoporosis. This report details some structure−activity relationship (SAR) studies on the peptide aldehydes.

Chemistry

Peptide aldehyde derivatives including α-acylamino aldehyde derivatives were prepared by two synthetic methods: DMSO-SO_3·pyridine oxidation[10] of the corresponding alcohols (Scheme 1) and DIBAL-H reduction[11] of the corresponding *N*-methoxy-*N*-methylcarboxamides (Scheme 2). Most of the aldehyde derivatives were obtained using the DMSO-SO_3·pyridine oxidation method.

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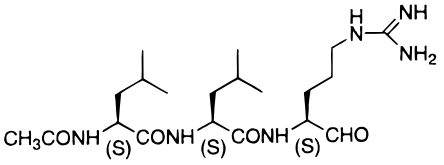
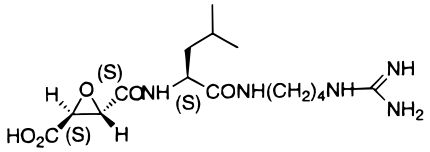
Chart 1. Microbial Cysteine Protease Inhibitors



Leupeptin



E-64

Since racemization at the α-position of the formyl moiety occurred during SiO_2 chromatography performed to eliminate dimethyl sulfide, an alternative DIBAL-H reduction method was also used. In the reduction of a series of dipeptide *N*-methoxy-*N*-methylcarboxamide derivatives with tryptophan on the P_1 position, more than 4 equiv of DIBAL-H was needed to complete the reaction, due to trapping of 3 equiv of DIBAL-H by three N-H protons (P_1 and P_2 amino acids and indole ring).

Results and Discussion

The aldehyde compounds prepared were tested for inhibitory activity against recombinant human cathepsin L.[12] The activity against human cathepsin B was also measured to evaluate selectivity. Cathepsin B is a lysosomal cysteine protease of the papain/cathepsin superfamily including cathepsins K and L but does not play a role in the mechanism of bone resorption.[13]

First, the effect of variation of the N-terminal substituent (P_3 position) was investigated for a series of isoleucyl-tryptophanal derivatives (Table 1). Most of these compounds exhibited potent inhibitory activity against cathepsin L, with IC_{50} values in the nanomolar range. Of these compounds, compound **12** with a 1-naphthalenylsulfonyl moiety had an IC_{50} of 1.9 nM and exhibited excellent selectivity for cathepsin L, with a ratio (IC_{50} for cathepsin B/IC_{50} for cathepsin L) of 789. Replacement of the 1-naphthalenylsulfonyl moiety with a 2-naphthalenylsulfonyl moiety (compound **13**) did not affect activity but lowered selectivity (B/L = 6). The small alkyl moiety (compound **5**) lowered the activity for cathepsin L.

Next, the P_2 amino acid was optimized in a series of *N*-(1-naphthalenylsulfonyl) derivatives (Table 2). Generally, potent activity was observed for compounds with L-amino acids at the P_2 position. Of these compounds, inhibitors with hydrophobic and moderate-sized side chain (Ile, Leu, Val) showed more potent activity against cathepsin L than inhibitors with a large-sized side chain (Phe) or a small-sized side chain (Ala). L-Leu (**15**) and L-Phe (**18**) derivatives also exhibited favorable selectivity, comparable to that of **12**.

The effects of variation of the P_1 substituent were studied as shown in Table 3. Hydrophobic α-amino aldehyde derivatives containing an aromatic ring in the side chain such as Trp, Phe, and Tyr had enhanced cathepsin L selectivity, whereas glycinal and unnatural *R*-configuration tryptophanal derivatives had reduced potency.

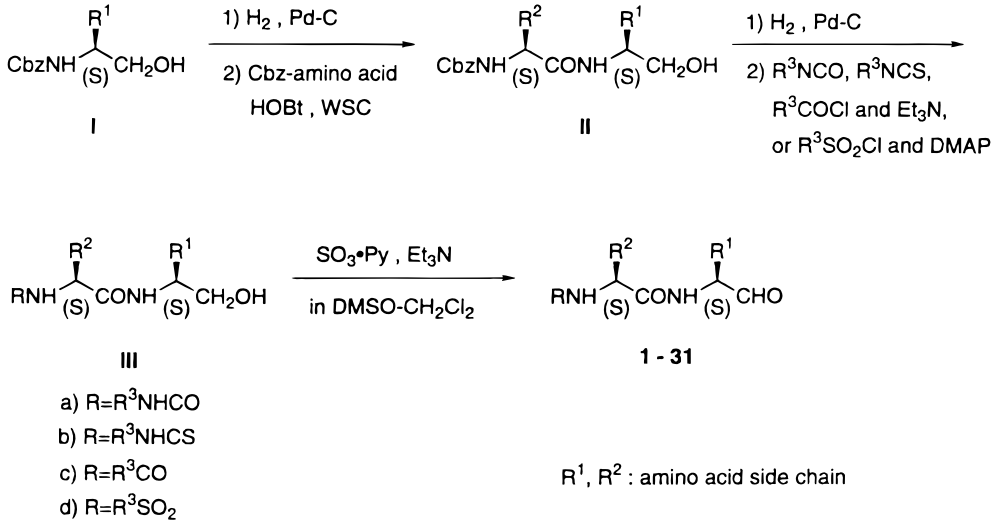
α-Acylamino aldehyde derivatives were synthesized in a process to develop nonpeptide cathepsin L inhibitors (Table 4). Although these derivatives were less potent than dipeptide aldehydes, compounds **28**−**31** with hydrophobic and steric hindered acyl moieties exhibited moderate inhibition of cathepsin L activity. Compound **31**, in particular, with a dibenzylacetyl moiety, exhibited excellent activity with an IC_{50} of 32 nM. This finding suggests that the dibenzylacetyl substituent mimics the *N*-benzoyl-phenylalanyl moiety; this finding may be useful for the development of a nonpeptide cathepsin L inhibitor.

On the basis of the findings of this structure−activity study, we postulated the active site of human cathepsin L to exhibit the following characteristics. The steric factor of the side chain at the P_1 site rarely affected activity. In addition, the conversion of *S*-configuration to *R*-configuration at the P_1 site markedly decreased potency, suggesting that the amino acid side chain at the P_1 site plays a role in regulating the orientation of the formyl group but does not interact with the S_1 position of cathepsin L.

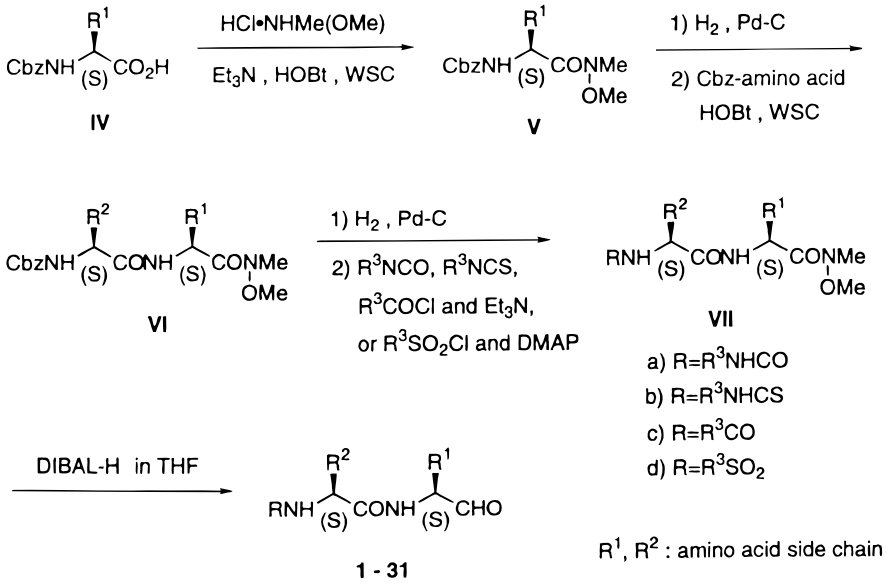
The amino acids at the P_2 site with an α-branched alkyl chain, Ile, Leu, and Val, exhibited favorable activity, whereas Phe and Ala had reduced activity. The S_2 pocket is considered to be a hydrophobic and moderate-sized cavity. Concerning the N-terminal substituent (P_3 position), hydrophobic and bulky moieties such as 1- and 2-naphthalenylsulfonyl substituents are suitable in cathepsin L. On the other hand, cathepsin B accommodates a 2-naphthalenylsulfonyl substituent at the P_3 position, but not 1-naphthalenylsulfonyl moiety. These findings suggest that the S_3 pocket in the active site of human cathepsin L possesses a hydrophobic and deep cavity, while cathepsin B has a wide and shallow cavity in the S_3 pocket. Our postulation in the S_2 and S_3 subsites of human cathepsin L completely coincided with the result of the crystal structure of human cathepsin L complexed with E-64, reported by Fujishima et al.,[14] who described that the S_2 pocket of human cathepsin L is shallow and narrow compared to that of cathepsin B, whereas the S_3 subsite of cathepsin L accommodates a more bulky group than that of cathepsin B.

Inhibition of Bone Resorption in Vitro and in Vivo. Of the dipeptide aldehyde and α-acylamino aldehyde derivatives prepared, four compounds (**6**, **12**, **21**, **31**) which exhibited potent and selective cathepsin L inhibitory activity were evaluated for inhibitory effects on bone resorption in vitro and in vivo (Table 5). All of these compounds suppressed serum-stimulated $^{45}Ca^{2+}$ release at a concentration of 30 μM in a fetal long bone culture system (Raisz's assay).[15] These compounds also prevented bone loss in ovariectomized mice when administered orally at 50 mg/kg for 3 weeks (Table 5). The effects of these inhibitors on bone resorption in vitro and in vivo are considered to be due to inhibition of cathepsin L, leading to prevention of bone collagen degradation. However, mature human cathepsins L and K share high sequence identity (60%). Pairwise sequence alignment and mapping of the active site residues from the three-dimensional structures[14,16] indicate that cathepsin K is much more like cathepsin L than cathepsin B (data not presented). Especially, most of the residues

Scheme 1



Scheme 2



spatially proximal to Cys[139] in the active site are conserved between cathepsins L and K. Therefore, although we did not examine the cathepsin K inhibitory activity of these inhibitors, they are expected to be potent inhibitors against cathepsin K as well as cathepsin L, and there is a possibility that dual inhibition of cathepsins L and K is important to show the in vitro and in vivo bone resorption inhibitory effects.

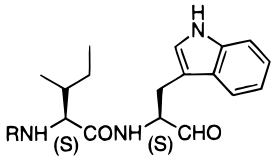
On the basis of the results of further pharmacological and toxicological studies of these compounds, the tryptophanal derivative **12**, *N*-(1-naphthalenylsulfonyl)-L-isoleucyl-L-tryptophanal, was selected as a candidate for further investigation. Compound **12** showed potent inhibitory activity against human cathepsin L with an IC_{50} of 1.9 nM and selectivity (cathepsin L vs B) superior to those of E-64 and leupeptin (Table 6). Compound **12** also inhibited the degradation of bovine type I collagen by cathepsin L with an IC_{50} of 2.0 nM, comparable to the potency of leupeptin with an IC_{50} of 2.5 nM (Table 7). In a mouse calvaria culture system, compound **12** suppressed both enhancements of cathepsin L-like protease activity and hydroxyproline release by 1α,25-$(OH)_2D_3$ (Table 8). These findings suggest that compound **12**, a selective cathepsin L inhibitor, inhibited the degradation of bone matrix (type I collagen) and bone mineral (Ca^{2+}) release, which resulted in prevention of bone loss.

Compound **12** is the first example of an orally active cathepsin L inhibitor and should be useful for the treatment of osteoporosis.

Experimental Section

Chemistry. Melting points (uncorrected) were determined in an open capillary with a Yanaco micro melting point apparatus MP model. Infrared (IR) spectra were taken on a Jasco IR-810 spectrophotometer in KBr disks for solids and liquid films for oils. Nuclear magnetic resonance (^1H NMR) spectra were recorded on a Varian Gemini-200 (200 MHz) spectrometer containing tetramethylsilane as an internal standard. Optical rotations were measured at 20 °C on a Jasco DIP-181 digital polarimeter. Elemental analyses (C, H, and N) were performed by Takeda Analytical Research Laboratories, Ltd. Chromatography was done using the flash column technique on silica gel (Merck Kieselgel 60: 230−400 mesh). The following abbreviations were used: DMF, *N*,*N*-dimethylformamide; THF, tetrahydrofuran; DMSO, dimethyl sulfoxide; MeOH, methyl alcohol; Cbz, carbobenzyloxy; HOBt, 1-hy-

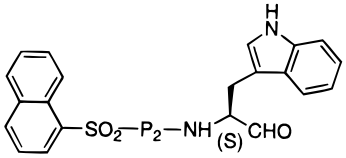
Table 1. Variation of the P_3 Substituent: Inhibitory Activities of Dipeptide Aldehyde Derivatives Against Human Cathepsins L and B



compd	R	mp (°C)	$[\alpha]_D$ (*c*, solvent)[a]	formula	anal.[b]	cathepsin L[c]	cathepsin B[c]	B/L
1	Boc	114−115	+22.3° (0.78, $CHCl_3$)	$C_{22}H_{31}N_3O_4$	C,H,N	3.3	460	139
2	Cbz	137−140	+28.5° (0.40, MeOH)	$C_{25}H_{29}N_3O_4$	C,H,N	7.1	530	74
3	3-Me-PhNHCO	202−204[d]	−20.8° (0.32, DMSO)	$C_{25}H_{30}N_4O_3 \cdot 1/4H_2O$	C,H,N	3.3	270	81
4	$PhCH_2NHCO$	183−185	−16.9° (0.40, DMSO)	$C_{25}H_{30}N_4O_3 \cdot 1/2H_2O$	C,H,N	6.1	270	44
5	*i*-PrNHCO	201−204	−15.9° (0.57, DMSO)	$C_{21}H_{30}N_4O_3 \cdot 1/4H_2O$	C,H,N	30	580	81
6	2-CF_3-PhNHCO	185−186[c]	−18.7° (0.50, DMSO)	$C_{25}H_{27}N_4O_3F_3 \cdot 1/2H_2O$	C,H,N	2.0	220	110
7	1-NapNHCO[e]	192−193	+4.4° (0.40, DMSO)	$C_{28}H_{30}N_4O_3 \cdot 1/2H_2O$	C,H,N	2.0	370	185
8	$PhCH_2NHCS$	130−131	−12.9° (0.34, DMSO)	$C_{25}H_{30}N_4O_2S$	C,H,N	2.2	410	186
9	1-NapNHCS[e]	amorphous	+34.2° (0.33, $CHCl_3$)	$C_{28}H_{30}N_4O_2S \cdot H_2O$	C,H,N[f]	4.5	310	69
10	PhNHCS	amorphous	+18.8° (0.49, $CHCl_3$)	$C_{24}H_{28}N_4O_2S$	C,H,N[g]	2.2	440	20
11	4-Me-$PhSO_2$	199−201	−32.7° (0.51, MeOH)	$C_{24}H_{29}N_3O_4S$	C,H,N[h]	2.2	110	50
12	1-$NapSO_2$[e]	145−146	−48.9° (0.50, $CHCl_3$)	$C_{27}H_{29}N_3O_4S$	C,H,N	1.9	1500	789
13	2-$NapSO_2$[e]	172−173	+5.9° (0.43, $CHCl_3$)	$C_{27}H_{29}N_3O_4S$	C,H,N	3.9	23	6
14	PhCO	188−190	+45.4° (0.26, $CHCl_3$)	$C_{24}H_{27}N_3O_3 \cdot 1/2H_2O$	C,H,N	2.1	52	24

[a] Measured at 20 °C. [b] Elemental analyses were within ±0.4% of theoretical values, unless stated otherwise. [c] IC_{50} value, nM. [d] Decomposition. [e] Nap, naphthyl. [f] N: calcd, 11.10; found, 10.64. [g] N: calcd, 12.32; found, 11.82. [h] N: calcd, 9.22; found, 8.74.

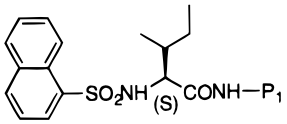
Table 2. Variation of the P_2 Residue: Inhibitory Activities of Dipeptide Aldehyde Derivatives Against Human Cathepsins L and B



compd	P_2	mp (°C)	$[\alpha]_D$ (*c*, solvent)[a]	formula	anal.[b]	cathepsin L[c]	cathepsin B[c]	B/L
12	L-Ile	145−146	−48.9° (0.50, $CHCl_3$)	$C_{27}H_{29}N_3O_4S$	C,H,N	1.9	1500	789
15	L-Leu	81−82	−40.5° (0.50, $CHCl_3$)	$C_{27}H_{29}N_3O_4S$	C,H,N	2.2	>1000	<454
16	L-Ala	119−120	+26.7° (0.50, MeOH)	$C_{24}H_{23}N_3O_4S \cdot 1/2Et_2O$	C,H,N	5.3	530	100
17	L-Val	117−118	−17.2° (0.50, MeOH)	$C_{26}H_{27}N_3O_4S$	C,H,N	0.97	30	30
18	L-Phe	amorphous	−71.5° (0.75, $CHCl_3$)	$C_{30}H_{27}N_3O_4S$[d]	C,H,N	4.5	3900	867
19	L-Asp(OMe)	amorphous	−87.4° (0.33, MeOH)	$C_{26}H_{25}N_3O_6S \cdot 1/2H_2O$	C,H,N[e]	4.3	65	15
20	Gly	amorphous	−54.8° (0.50, $CHCl_3$)	$C_{23}H_{21}N_3O_4S \cdot 1/2Et_2O$	C,H,N	150	>1000	<6

[a] Measured at 20 °C. [b] Elemental analyses were within ±0.4% of theoretical values, unless stated otherwise. [c] IC_{50} value, nM. [d] Additive: $1/2H_2O \cdot 1/2i\text{-}Pr_2O$. [e] N: calcd, 8.13; found, 7.70.

Table 3. Variation of the P_1 Residue: Inhibitory Activities of Dipeptide Aldehyde Derivatives Against Human Cathepsins L and B



compd	P_1	mp (°C)	$[\alpha]_D$ (*c*, solvent)[a]	formula	anal.[b]	cathepsin L[c]	cathepsin B[c]	B/L
12	L-Trp-H	145−146	−48.9° (0.50, $CHCl_3$)	$C_{27}H_{29}N_3O_4S$	C,H,N	1.9	1500	789
21	L-Phe-H	138−139	−24.3° (0.60, DMSO)	$C_{25}H_{28}N_2O_4S$	C,H,N	0.95	1400	1473
22	L-Tyr-H	amorphous	−20.4° (0.31, $CHCl_3$)	$C_{25}H_{28}N_2O_5S \cdot 1/2H_2O$	C,H,N	1.4	1300	929
23	L-Leu-H	155−156	+12.9° (0.82, DMSO)	$C_{22}H_{30}N_2O_4S$	C,H,N	0.95	100	105
24	L-Val-H	145−146	+24.3° (0.52, DMSO)	$C_{21}H_{29}N_2O_4S$	C,H,N	0.74	120	162
25	L-Ala-H	150−151	+36.7° (0.45, DMSO)	$C_{10}H_{24}N_2O_4S$	C,H,N	3.1	480	155
26	Gly-H	amorphous	−27.1° (0.42, MeOH)	$C_{18}H_{22}N_2O_4S$	C,H,N	95	3200	34

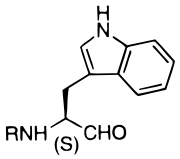
[a] Measured at 20 °C. [b] Elemental analyses were within ±0.4% of theoretical values, unless stated otherwise. [c] IC_{50} value, nM.

droxybenzotriazole; WSC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride.

General Procedure for the Deprotection of Cbz-Protected Amine. A mixture of Cbz-protected amine (10 mmol) and 5% Pd−C ($^1/_3$ weight of Cbz-amine, 50% wet) in THF (15 mL)−MeOH (15 mL) was hydrogenated at 1 atm and room temperature for 3 h. The catalyst was removed by filtration and washed with THF. The filtrate was concentrated under reduced pressure, and the residual MeOH was removed completely by chasing several times with ethyl acetate to give the corresponding amine, which was used in the following reaction without further purification.

General Procedure for the Coupling Reaction of Amino Alcohols with Cbz-Amino Acids. *N*-Cbz-L-isoleucyl-L-tryptophanol. Cbz group of *N*-Cbz-L-tryptophanol (35 g, 99 mmol), prepared by the method of Hamada et al.,[10] was deprotected by the general procedure. The obtained L-tryptophanol and Cbz-L-isoleucine (28 g, 106 mmol) were dissolved in DMF (300 mL), and the solution was cooled on an ice bath. HOBt (17 g, 111 mmol) and WSC (21 g, 110 mmol)

Table 4. Inhibitory Activities of α-Acylamino Aldehyde Derivatives Against Human Cathepsins L and B



compd	R	mp (°C)	$[\alpha]_D$ (*c*, solvent)[a]	formula	anal.[b]	cathepsin L[c]	cathepsin B[c]	B/L
27	Cbz	105−106	+49.0° (0.50, $CHCl_3$)	$C_{19}H_{18}N_2O_3$	C,H,N	>1000	>1000	
28	$PhCH_2CH_2CO$	124−125	+60.0° (0.58, $CHCl_3$)	$C_{20}H_{20}N_2O_2 \cdot 1/4H_2O$	C,H,N	110	>1000	>9
29	cyclohexylCO	138−139	−41.5° (0.50, MeOH)	$C_{18}H_{22}N_2O_2$	C,H,N	200	780	4
30	$(n\text{-}Pr)_2CHCO$	135−136	−86.8° (0.50, $CHCl_3$)	$C_{19}H_{26}N_2O_2 \cdot 1/4H_2O$	C,H,N	290	>1000	>3
31	$(PhCH_2)_2CHCO$	142−144	+14.1° (0.57, $CHCl_3$)	$C_{20}H_{20}N_2O_2 \cdot 1/4H_2O$	C,H,N	32	>1000	>31

[a] Measured at 20 °C. [b] Elemental analyses were within ±0.4% of theoretical values, unless stated otherwise. [c] IC_{50} value, nM.

Table 5. Inhibitory Effects of Peptide Aldehyde Derivatives on Bone Resorption

	Raisz's assay[a]	mouse OVX model[b]
compd	^{45}Ca release suppression (% of control)	bone loss suppression (% of inhibition)
6	59.2**[c]	88.8**[d]
12	57.0***	50.6*
21	59.8***	58.2*
31	55.0***	52.9**

[a] Concentration: 30 μM. [b] Dose: 50 mg/kg po. [c] Statistically significant at $^{**}p < 0.01$ and $^{***}p < 0.001$, by Student's *t*-test. [d] Statistically significant at $^{*}p < 0.05$ and $^{**}p < 0.01$, by Student's *t*-test compared with the OVX control.

Table 6. Inhibitory Activities of Enzyme Inhibitors Against Human Cathepsins L and B

	enzyme inhibition (IC_{50}, nM)		selectivity
inhibitor	cathepsin L	cathepsin B	(B/L)
E-64	47	16	0.33
leupeptin	6.6	9.4	1.4
12	1.9	1500	789

Table 7. Inhibition of Collagenolytic Activity of Human Cathepsin L

inhibitor	IC_{50} (nM)
leupeptin	2.5
12	2.0

Table 8. Inhibitory Activities of Bone Resorption Stimulated by $1\alpha,25\text{-}(OH)_2D_3$ on Mouse Calvarial Organ Culture[a]

	cathepsin L activity (mU/mL)	hydroxyproline release (μg/mL)
control	0.957 ± 0.067	1.541 ± 0.080
$1\alpha,25\text{-}(OH)_2D_3$	1.959 ± 0.037	2.106 ± 0.045
12 (10^{-7} M)	1.548 ± 0.063*	1.945 ± 0.023*
(10^{-6} M)	1.069 ± 0.019**	1.868 ± 0.027**
(10^{-5} M)	1.004 ± 0.032**	1.683 ± 0.190**

[a] Statistically significant at $^{*}p < 0.05$ and $^{**}p < 0.01$ by ANOVA compared with $1\alpha,25\text{-}(OH)_2D_3$.

were added to the solution, and the whole was stirred at room temperature for 14 h. The reaction mixture was concentrated in vacuo, and the residue was suspended in ethyl acetate. The suspension was washed successively with aqueous citric acid, H_2O, aqueous $NaHCO_3$, and brine and dried over $MgSO_4$. Removal of the solvent gave a white solid, which was washed with ethyl acetate−hexane to afford the title compound as white crystals (36.7 g, 84%): mp 184−185 °C; $[\alpha]_D$ −36.0° (*c* = 0.50, DMSO); ^1H NMR (DMSO-d_6) δ 0.7−0.9 (6H,m), 0.9−1.2 (1H,m), 1.3−1.5 (1H,m), 1.6−1.8 (1H,m), 2.75 (1H,dd,*J* = 6.8 & 14.0Hz), 2.93 (1H,dd,*J* = 6.8 & 14.0Hz), 3.2−3.5 (2H,m), 3.87 (1H,t,*J* = 8.4Hz), 3.9−4.1 (1H,m), 4.72 (1H,t,*J* = 5.4Hz), 5.04 (2H,s), 6.9−7.4 (9H,m), 7.60 (1H,d,*J* = 7.6Hz), 7.72 (1H,d,*J* = 8.0Hz), 10.77 (1H,s). Anal. ($C_{25}H_{31}N_3O_4 \cdot 1/4H_2O$) C, H, N.

General Procedure for the Introduction of *N*-(Thio)-carbamoyl Group. *N*-(3-Methylphenylcarbamoyl)-L-isoleucyl-L-tryptophanol. Cbz group of *N*-Cbz-L-isoleucyl-L-tryptophanol (0.70 g, 1.60 mmol) was deprotected by the general procedure. The obtained amino compound was dissolved in CH_2Cl_2 (15 mL)−THF (5 mL), and 3-methylphenyl isocyanate (0.21 mL,1.69 mmol) was added to the solution with cooling. After stirring at room temperature for 90 min, the reaction mixture was diluted with $CHCl_3$−MeOH and filtered through Celite. The filtrate was concentrated under reduced pressure, and the resulting solid was recrystallized from CH_2Cl_2−MeOH to give the title compound as a white solid (0.43 g, 61%): mp 185−186 °C; $[\alpha]_D$ −35.0° (*c* = 0.38, DMSO); ^1H NMR (DMSO-d_6) δ 0.8−1.8 (9H,m), 2.25 (3H,s), 2.77 (1H,dd,*J* = 6.8 & 14.6Hz), 2.95 (1H,dd,*J* = 6.8 & 14.6Hz), 3.3−3.5 (2H,m), 4.0−4.1 (1H,m), 4.14 (1H,dd,*J* = 6.8 & 8.8Hz), 4.66 (1H,t,*J* = 5.0Hz), 6.31 (1H,d,*J* = 9.0Hz), 6.71 (1H,d,*J* = 6.8Hz), 6.9−7.3 (6H,m), 7.31 (1H,d,*J* = 7.4Hz), 7.89 (1H,d,*J* = 7.8Hz), 8.57 (1H,s), 10.74 (1H,s). Anal. ($C_{25}H_{32}N_4O_3$) C, H, N.

***N*-(2-Trifluoromethylphenylcarbamoyl)-L-isoleucyl-L-tryptophanol.** The title compound was synthesized in the same manner as described for preparation of *N*-(3-methylphenylcarbamoyl)-L-isoleucyl-L-tryptophanol: faint pink solid; yield 85%; mp 232−234 °C; $[\alpha]_D$ −35.0° (*c* = 0.43, DMSO); ^1H NMR (DMSO-d_6) δ 0.7−1.2 (7H,m), 1.3−1.6 (1H,m), 1.6−1.8 (1H,m), 2.77 (1H,dd,*J* = 6.6 & 14.4Hz), 2.96 (1H,dd,*J* = 6.6 & 14.4Hz), 3.2−3.5 (2H,m), 4.03 (1H,q,*J* = 7.1Hz), 4.16 (1H,dd,*J* = 6.6 & 14.4Hz), 4.69 (1H,brs), 6.9−8.0 (11H,m), 8.08 (1H,s), 10.77 (1H,brs). Anal. ($C_{25}H_{29}N_4O_3F_3$) C, H, N.

General Procedure for the Introduction of *N*-Acyl Group. *N*-Benzoyl-L-isoleucyl-L-tryptophanol. Cbz group of *N*-Cbz-L-isoleucyl-L-tryptophanol (8.0 g, 18.3 mmol) was deprotected by the general procedure. The obtained amino compound was dissolved in DMF (40 mL), and benzoyl chloride (2.8 g, 19.9 mmol) and 4-*N*,*N*-dimethylaminopyridine (2.5 g, 20.5 mmol) were added to the solution with cooling. The whole was stirred at the same temperature for 90 min and then concentrated in vacuo. The residue was suspended in ethyl acetate, and the suspension was washed successively with aqueous citric acid, H_2O, aqueous $NaHCO_3$, and brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residual yellow oil was crystallized from ethyl acetate−hexane to afford the title compound as a pale pink solid (3.9 g, 52%): mp 142−143 °C; $[\alpha]_D$ −27.2° (*c* = 0.26, $CHCl_3$); ^1H NMR (DMSO-d_6) δ 0.7−1.0 (6H,m), 1.0−1.3 (1H,m), 1.4−1.7 (1H,m), 1.8−2.0 (1H,m), 2.77 (1H,dd,*J* = 7.0 & 14.8Hz), 2.95 (1H,dd,*J* = 6.4 & 14.8Hz), 3.2−3.5 (2H,m), 3.9−4.1 (1H,m), 4.35 (1H,t,*J* = 8.6Hz), 4.68 (1H,brt,*J* = 5.2Hz), 6.9−7.1 (2H,m), 7.13 (1H,s), 7.30 (1H,d,*J* = 7.4Hz), 7.4−7.7 (4H,m), 7.8−8.0 (3H,m), 8.21 (1H,d,*J* = 8.4Hz), 10.74 (1H,s). Anal. ($C_{24}H_{29}N_3O_3$) C, H, N.

***N*-(2-Benzyl-3-phenylpropionyl)-L-tryptophanol.** The title compound was synthesized in the same manner as described for preparation of *N*-benzoyl-L-isoleucyl-L-tryptophanol: colorless crystals; yield 72%; mp 120−122 °C; $[\alpha]_D$ −13.9° (*c* = 0.62, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 2.02 (1H,t,*J* = 6.0Hz), 2.3−2.5 (1H,m), 2.7−3.1 (4H,m), 3.2−3.4 (2H,m), 3.9−4.1

(1H,m), 5.13 (1H,d,J = 7.0Hz), 6.42 (1H,d,J = 2.6Hz), 7.0−7.9 (14H,m), 7.93 (1H,brs). Anal. ($C_{27}H_{28}N_2O_2$·1/4H_2O) C, H, N.

General Procedure for the Introduction of *N*-Sulfonyl Group. *N*-(1-Naphthalenylsulfonyl)-L-isoleucyl-L-tryptophanol. Cbz group of *N*-Cbz-L-isoleucyl-L-tryptophanol (55 g, 0.13 mol) was deprotected by the general procedure. The obtained amino compound was dissolved in DMF (300 mL), and 1-naphthalenylsulfonyl chloride (30 g, 0.13 mol) and 4-*N*,*N*-dimethylaminopyridine (17 g, 0.14 mol) were added to the solution with cooling. The whole was stirred at the same temperature for 2 h and then concentrated in vacuo. The residue was suspended in ethyl acetate, and the suspension was washed successively with aqueous citric acid, H_2O, aqueous $NaHCO_3$, and brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residual light brown oil was crystallized from CH_2Cl_2−ether to afford the title compound as a white solid (51 g, 82%): mp 150−151 °C; $[\alpha]_D$ −77.7° (c = 0.57, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.42 (3H,d,J = 6.6Hz), 0.52 (3H,t,J = 6.6Hz), 0.5−0.8 (1H,m), 0.9−1.1 (1H,m), 1.5−1.8 (1H,m), 2.5−2.7 (2H,m), 2.82 (1H,dd,J = 7.2 & 14.4Hz), 3.3−3.6 (3H,m), 4.0−4.2 (1H,m), 5.36 (1H,d,J = 6.6Hz), 6.37 (1H,d,J = 8.2Hz), 6.99 (1H,d,J = 2.2Hz), 7.1−7.7 (7H,m), 7.94 (1H,d,J = 7.8Hz), 8.06 (1H,d,J = 8.0Hz), 8.17 (1H,brs), 8.22 (1H,d,J = 7.4Hz), 8.66 (1H,d,J = 8.8Hz). Anal. ($C_{27}H_{31}N_3O_4S$) C, H, N.

***N*-(1-Naphthalenylsulfonyl)-L-isoleucyl-L-phenylalaninol.** The title compound was synthesized in the same manner as described for preparation of *N*-(1-naphthalenylsulfonyl)-L-isoleucyl-L-tryptophanol: colorless crystals; yield 87%; mp 169−170 °C; $[\alpha]_D$ −108.3° (c = 0.65, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.39 (3H,d,J = 5.0Hz), 0.54 (3H,t,J = 6.8Hz), 0.6−1.1 (2H,m), 1.6−1.8 (1H,m), 2.47 (1H,dd,J = 8.2 & 14.0Hz), 2.57 (1H,t,J = 6.0Hz), 2.73 (1H,dd,J = 7.0 & 14.0Hz), 3.2−3.6 (3H,m), 4.0−4.2 (1H,m), 5.34 (1H,d,J = 6.0Hz), 6.43 (1H,dJ = 8.2Hz), 7.1−7.4 (5H,m), 7.5−7.8 (3H,m), 7.97 (1H,d,J = 7.8Hz), 8.10 (1H,d,J = 8.4Hz), 8.25 (1H,dd,J = 1.2 & 7.2Hz), 8.68 (1H,d,J = 8.6Hz). Anal. ($C_{25}H_{30}N_2O_4S$) C, H, N.

General Procedure for the Py·SO_3-DMSO Oxidation. *N*-(1-Naphthalenylsulfonyl)-L-isoleucyl-L-tryptophanal (12). To an ice-cooled and stirred solution of *N*-(1-naphthalenylsulfonyl)-L-isoleucyl-L-tryptophanol (19.4 g, 39.3 mmol) and triethylamine (11.0 mL, 78.9 mmol) in DMSO (140 mL) was added portionwise a solution of SO_3·Py (12.5 g, 78.5 mmol) in DMSO (60 mL), and the whole was stirred at room temperature for 50 min. The reaction mixture was poured into ice−water and extracted with ethyl acetate. The extract was washed successively with aqueous citric acid, H_2O, aqueous $NaHCO_3$, and brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residual pale yellow oil was crystallized from ethyl acetate−hexane to afford **12** as a pale yellow solid (9.6 g, 50%): mp 145−146 °C; $[\alpha]_D$ −48.9° (c = 0.50, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.61 (3H,d,J = 6.6Hz), 0.63 (3H,t,J = 6.2Hz), 0.8−0.9 (1H,m), 1.1−1.3 (1H,m), 1.6−1.7 (1H,m), 2.87 (2H,d,J = 6.8Hz), 3.56 (1H,dd,J = 5.4 & 8.2Hz), 4.48 (1H,dd,J = 6.8 & 13.6Hz), 5.38 (1H,d,J = 8.0Hz), 6.29 (1H,d,J = 6.6Hz), 6.95 (1H,d,J = 2.4Hz), 7.1−7.8 (7H,m), 7.9−8.3 (4H,m), 8.67 (1H,d,J = 8.8Hz), 9.30 (1H,s). Anal. ($C_{27}H_{29}N_3O_4S$) C, H, N.

***N*-(2-Trifluoromethylphenylcarbamoyl)-L-isoleucyl-L-tryptophanal (6).** **6** was synthesized in the same manner as described for preparation of **12**: pale yellow solid; yield 36%; mp 185−186 °C dec; $[\alpha]_D$ −18.7° (c = 0.50, DMSO); ^{1}H NMR (DMSO-d_6) δ 0.7−1.2 (7H,m), 1.3−1.5 (1H,m), 1.6−1.8 (1H,m), 3.02 (1H,dd,J = 8.2 & 15.0Hz), 3.25 (1H,dd,J = 5.4 & 15.0Hz), 4.27 (1H,dd,J = 6.2 & 8.2Hz), 4.43 (1H,q,J = 7.0Hz), 6.9−7.7 (9H,m), 7.93 (1H,d,J = 8.4Hz), 8.06 (1H,s), 8.60 (1H,d,J = 6.6Hz), 9.50 (1H,s), 10.88 (1H,brs). Anal. ($C_{25}H_{27}N_4O_3F_3$·1/2H_2O) C, H, N.

***N*-(1-Naphthalenylsulfonyl)-L-isoleucyl-L-phenylalaninal (21).** **21** was synthesized in the same manner as described for preparation of **12**: colorless needles; yield 67%; mp 138−139 °C; $[\alpha]_D$ −24.3° (c = 0.60, DMSO); ^{1}H NMR (DMSO-d_6) δ 0.5−0.7 (6H,m), 0.8−1.0 (1H,m), 1.1−1.4 (1H,m), 1.4−1.6 (1H,m), 2.55 (1H,dd,J = 7.4 & 14.8Hz), 2.90 (1H,dd,J = 14.8Hz), 3.4−3.6 (1H,m), 3.7−3.9 (1H,m), 7.0−7.3 (5H,m), 7.5−7.7 (3H,m), 8.0−8.4 (4H,m), 8.68 (1H,d,J = 8.2Hz), 8.74 (1H,s). Anal. ($C_{25}H_{28}N_2O_4S$) C, H, N.

***N*-(2-Benzyl-3-phenylpropionyl)-L-tryptophanal (31).** **31** was synthesized in the same manner as described for preparation of **12**: colorless prisms; yield 69%; mp 142−144 °C; $[\alpha]_D$ +14.1° (c = 0.57, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 2.5−2.7 (1H,m), 2.7−3.2 (6H,m), 4.50 (1H,q,J = 6.0Hz), 5.55 (1H,d,J = 6.6Hz), 6.36 (1H,d,J = 2.4Hz), 7.0−7.4 (14H,m), 7.89 (1H,brs), 9.19 (1H,s). Anal. ($C_{20}H_{20}N_2O_2$·1/4H_2O) C, H, N.

***N*-Cbz-L-tryptophan *N*,*O*-Dimethylhydroxylamide.** To an ice-cooled and stirred solution of *N*-Cbz-L-tryptophan (40.0 g, 118 mmol), *N*,*O*-dimethylhydroxylamine hydrochloride (12.0 g, 123 mmol), and triethylamine (17.6 mL,126 mmol) in DMF (300 mL) were added HOBt (20.0 g, 131 mmol) and WSC (24.8 g, 129 mmol), and the whole was stirred at room temperature for 15 h. The reaction mixture was concentrated in vacuo. The residue was suspended in ethyl acetate, and the suspension was washed successively with aqueous citric acid, H_2O, aqueous $NaHCO_3$, and brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residual pale brown solid was washed with ethyl acetate−hexane to afford the title compound as a white solid (42 g, 93%): mp 131−132 °C; $[\alpha]_D$ −21.0° (c = 0.56, MeOH); ^{1}H NMR ($CDCl_3$) δ 3.0−3.2 (1H,m), 3.15 (3H,s), 3.27 (1H,dd,J = 5.6 & 15.0Hz), 3.66 (3H,s), 5.00 (1H,d,J = 11.4Hz), 5.13 (1H,d,J = 11.4Hz), 5.51 (1H,d,J = 8.2Hz), 6.98 (1H,d,J = 2.2Hz), 7.0−7.4 (8H,m), 7.58 (1H,d,J = 7.4Hz), 8.11 (1H,brs). Anal. ($C_{21}H_{23}N_3O_4$) C, H, N.

***N*-Cbz-L-isoleucyl-L-tryptophan *N*,*O*-Dimethylhydroxylamide.** The title compound was synthesized in the same manner as described for preparation of *N*-Cbz-L-isoleucyl-L-tryptophanol: colorless amorphous solid; yield 92%; $[\alpha]_D$ −36.6° (c = 0.55, MeOH); ^{1}H NMR ($CDCl_3$) δ 0.7−1.2 (7H,m), 1.2−1.5 (1H,m), 1.7−1.9 (1H,m), 3.1−3.2 (1H,m), 3.15 (3H,s), 3.27 (1H,dd,J = 6.0 & 14.8Hz), 3.69 (3H,s), 4.1−4.2 (1H,m), 5.11 (2H,s), 5.2−5.4 (2H,m), 6.55 (1H,d,J = 8.2Hz), 6.99 (1H,d,J = 1.8Hz), 7.0−7.4 (8H,m), 7.55 (1H,d,J = 8.2Hz), 7.92 (1H,brs). Anal. ($C_{27}H_{34}N_4O_5$·1/2H_2O) C, H, N.

***N*-(1-Naphthalenylsulfonyl)-L-isoleucyl-L-tryptophan *N*,*O*-Dimethylhydroxylamide.** The title compound was synthesized in the same manner as described for preparation of *N*-(1-naphthalenylsulfonyl)-L-isoleucyl-L-tryptophanol. The obtained product was dissolved in toluene, and the solution was concentrated in vacuo to give a pale yellow amorphous solid; yield 83%; $[\alpha]_D$ +30.2° (c = 0.75, MeOH); ^{1}H NMR ($CDCl_3$) δ 0.5−1.0 (7H,m), 1.1−1.4 (1H,m), 1.5−1.7 (1H,m), 2.77 (1H,dd,J = 5.6 & 15.0Hz), 2.92 (1H,dd,J = 6.0 & 15.0Hz), 3.04 (3H,s), 3.44 (3H,s), 3.5−3.6 (1H,m), 4.8−5.0 (1H,m), 5.74 (1H,d,J = 8.8Hz), 6.48 (1H,d,J = 7.2Hz), 6.90 (1H,s), 7.0−7.9 (9H,m), 8.18 (1H,d,J = 7.4Hz), 8.20 (1H,brs), 8.68 (1H,d,J = 8.6Hz). Anal. ($C_{29}H_{34}N_4O_5S$·1/2toluene) C, H, N.

***N*-(1-Naphthalenylsulfonyl)-L-isoleucyl-L-tryptophanal (12).** To a stirred solution of *N*-(1-naphthalenylsulfonyl)-L-isoleucyl-L-tryptophan *N*,*O*-dimethylhydroxylamide (21 g, 38.1 mmol) in absolute THF (200 mL) was added dropwise 1.5 M diisobutylaluminum hydride toluene solution (107 mL,161 mmol) at −60 °C. After stirring at −50 °C for 4 h, the reaction mixture was poured into aqueous citric acid, extracted with AcOEt, washed successively with aqueous citric acid, H_2O, aqueous $NaHCO_3$, and brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residual yellow oil was crystallized from ethyl acetate−hexane to give **12** as a pale yellow solid (15.3 g, 81%).

Biological Procedures. 1. Determination of Human Cathepsin L Inhibitory Activity. Recombinant human cathepsin L was diluted with a diluent [0.1% Brij 35 (Sigma Chemical Co.)] to a concentration of 1 μg/mL. To 1 μL of this enzyme dilution were added 46 μL of the diluent, 2 μL of 0.1 M DTT, and 25 μL of an activator/buffer (340 mM sodium acetate, 60 mM acetic acid, 4 mM disodium EDTA, pH 5.5). To this mixture were added a 1 μL sample, diluted to 10^{-2} M with dimethyl sulfoxide (DMSO), and 25 μL of 20 μM Z-Phe-Arg-NMec (enzyme substrate solution), followed by incubation

at 30 °C for 10 min, after which 100 μL of a reaction stopper (100 mM sodium monochloroacetate, 30 mM sodium acetate, 70 mM acetic acid, pH 4.3) was added. This reaction was carried out on a 96-well fluoroplate (Labo Systems).

After the reaction was stopped, the fluorescence intensity of free aminomethylcoumarin was determined at a wavelength of 450 nm (excitation wavelength = 365 nm), using a fluorometer FCA (Baxter Healthcare Co.). For a control, 1 μL of sample-free DMSO was added instead; the fluorometric value obtained from this control reaction was taken as 100% activity. When the residual activity was not higher than 10%, the sample solution was further diluted and then assayed for residual activity in the same procedure as above to obtain the IC_{50} value.

2. Determination of Human Cathepsin B Inhibitory Activity. Human cathepsin B (Athens Research and Rechnology, Inc.) was diluted with 100 nM sodium acetate buffer (pH 5.5) to a concentration of 30 μg/mL. To 1 μL of this enzyme dilution were added 46 μL of the diluent, 2 μL of 0.1 M DTT, and 25 μL of a 250 mM sodium phosphate buffer (pH 6.5). To this mixture were added a 1 μL sample, diluted to 10^{-2} M with DMSO, and 25 μL of 20 μM Z-Arg-Arg-NMec (enzyme substrate solution), followed by incubation at 37 °C for 20 min, after which 100 μL of a reaction stopper (100 mM sodium monochloroacetate, 30 mM sodium acetate, 70 mM acetic acid, pH 4.3) was added. This reaction was carried out on a 96-well fluoroplate (Labo Systems).

After the reaction was stopped, the human cathepsin B inhibitory activity was determined by the same procedure as described above.

3. Assay of Bone Resorption (Raisz's Method). Bone resorption inhibitory effect was determined by Raisz's method.[15] That is, ^{45}Ca (radioisotope of calcium in $^{45}CaCl_2$ solution) (50 μCi) was subcutaneously injected into a Sprague−Dawley rat on the 18th day of pregnancy. On the next day, the abdomen was opened, and a fetus was aseptically removed. The left and right humeri (radii and ulnae) were removed under a dissecting microscope, and as much connective tissue and cartilage as possible were removed. Thus, bone culture samples were prepared. The bone was incubated in a medium (0.6 mL) of BCJb Medium (Fitton-Jackson modification; GIBCO Laboratories) containing 2 mg/mL bovine serum albumin at 37 °C for 24 h in atmosphere of 5% CO_2 in air. The bone was cultured for an additional 2 days in the above medium containing a final concentration of 30 μM test compound. This bone was cultivated for 2 days in the resulting medium. The ^{45}Ca radioactivity in the medium and the ^{45}Ca radioactivity in the bone were determined. The ratio (%) of ^{45}Ca released from the bone into the medium was calculated according to the following equation:

$$\text{ratio of } {}^{45}\text{Ca released from bone into medium (\%)} = [({}^{45}\text{Ca released into medium})/({}^{45}\text{Ca released into medium} + {}^{45}\text{Ca incorporated in bone})] \times 100$$

Bones from the same litter were cultured for 2 days in the same manner without addition of the compound and used as a control. The mean of the values for five bones for each group was calculated. The ratio (%) of this value to the control value was calculated.

4. Assay of Bone Resorption in Ovariectomized Mice. Thirteen-week-old female C3H mice underwent bilateral ovariectomy. Test compound was administered orally once a day for 3 weeks after the operation. On the day following the last dosing, right femur was dissected and cleaned of soft tissue. To determine the dry weight, each bone was cut in one-third from the distal end, dried for 12 h at 110 °C, and weighed.

5. Determination of Inhibitory Activity for Collagenolytic Activity of Cathepsin L. Collagenolytic activity was determined essentially as described by Etherington.[17] Bovine tendon collagen was suspended at 10 mg/mL in a solution 25% glycerol/0.35% acetic acid. The collagen was dispersed with the Polytron homogenizer. Collagenolytic activity was assayed at pH 3.5 in 1.5 mL Eppendorf tubes. Each tube contained 100 μL of collagen suspension, 150 μL of 100 mM sodium formate buffer containing 4 mM DTT, 15 μL of each concentration of test compound, and 25 μL of enzyme solution (100 μg/mL). The tubes were incubated at 37 °C for 16 h. At the end of the incubation period the tubes were centrifuged at 12 000 rpm for 10 min to remove the residual collagen. Blank readings were obtained from assay mixtures that had been prepared on ice and then centrifuged without prior incubation.

The amount of collagen degraded was calculated from concentration of hydroxyproline in solution, which was assumed to be 14% (w/w) of collagen. A sample (100 mL) of supernatant was transferred to a 10 mL centrifuge glass tube (Iwaki Glass), and 100 mL of concentrated HCl was added. The tubes were then sealed with screw caps, and the hydrolysis was performed at 130 °C for 5 h. After cooling, 2 mL of 40 mM citric acid−0.32 M NaOH was added to each tube. Hydroxyproline was determined by the method of Woessner.[18]

6. Assay of Bone Resorption in Calvarial Organ Culture. Measurement of Hydroxyproline Release. According to the method described by Kream et al.,[19] in brief, calvaria were dissected from 21-day-old fetal rats, cultured in a 35 mm plastic tissue culture well in 2 mL of BGJ_b medium containing 1 mg/mL BSA, 100 μg/mL ascorbic acid, and 1 mM proline. The culture dishes were placed on a rocking platform in a incubator equilibrated with 5% CO_2 at 37 °C. After 3 h, the calvaria were placed into new culture media with or without 1α,25-$(OH)_2D_3$ (10 nM) for 96 h. After cultivation, an aliquot of the culture media was hydrolyzed by adding HCl to a final concentration of 6 N for 3 h at 130 °C. The hydrolyzed samples were neutralized (pH 7) by adding 2.5 N NaOH followed by the addition of distilled water to the final volume of 600 μL. Chloramine T solution (300 μL, 0.05 M) was added to the sample and incubated for 20 min at room temperature. After addition of *p*-dimethylaminobenzaldehyde solution (300 μL, 20%), the sample mixture was placed in a water bath at 60 °C for 20 min. The developed color was spectrophotometrically measured at 557 nm.

Measurement of Cathepsin L-like Protease Activity. Enzymatic activity was assayed using 5 μM Z-Phe-Arg-MCA (Sigma Chemical Co.) as the substrate, as described by Barrett and Kirschke.[20] The fluorescence intensity of free aminomethylcumarin was determined at a wavelength of 450 nm (excitation wavelength = 365 nm), using a fluorometer FCA (Baxter Healthcare Co.).

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