



Highly Water-Soluble Matrix Metalloproteinases Inhibitors and Their Effects in a Rat Adjuvant-Induced Arthritis Model

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Abstract—A new series of succinate-based dual inhibitors against matrix metalloproteinases (MMPs) and tumor necrosis factor α converting enzyme (TACE) possessing highly-water solubility was designed, synthesized, and evaluated for enzyme inhibition. Incorporating of acidic or basic functional groups at the P₂' position afforded sufficient water solubility without significant loss of inhibitory potencies. Compound **18e**, which had a guanidino group at the P₂' position as the basic functional group, exhibited broad inhibition against target enzymes for a relatively long period in rat plasma (β $t_{1/2}$; 2.0 h) after sc administration when compared with compounds possessing acidic functional groups (**18a** and **18b**). Consequently, the representative compound **18e** together with compound **18b**, Marimastat and Trocade were evaluated in the rat adjuvant-induced arthritis model, a model of chronic cartilage destruction. It is concluded that the newly synthesized highly water-soluble compound **18e** showed significant activity in suppressing hindpaw swelling and the bone destruction with a minimal administration period (days 3–7). © 2002 Elsevier Science Ltd. All rights reserved.

Introduction

Matrix metalloproteinases (MMPs) are zinc endo-metalloproteinases that are involved in the degradation and remodeling of connective tissues. This family of enzymes shows proteolytic activity towards virtually all of the constituents of the extracellular matrix. The members of this family, currently numbering 28, can be classified into four groups, which are: the collagenases that cleave triple-helical interstitial collagen; the gelatinases that cleave denatured collagen, elastin, and types IV and V collagen; the stromelysins that mainly cleave proteoglycans; and the membrane-type MMPs that have a C-terminal transmembrane domain for anchoring to the cell membrane. The MMPs are involved in crucial physiological and physiopathological events, such as wound healing, nerve growth, angiogenesis, and pregnancy. In these physiological processes, MMP activity is tightly regulated.^{1–5} However, excessive MMPs syntheses and releases can lead to connective tissue degradation and destruction, which occurs in tumor invasion, metastasis,⁶ corneal ulceration,⁷ arthritis disease,⁸ periodontal disease,⁹ and multiple sclero-

sis.¹⁰ For example, increased levels of fibroblast collagenase (MMP-1) and stromelysin-1 (MMP-3) have been observed in the cartilage and synovium of patients with osteoarthritis (OA) or rheumatoid arthritis (RA), and are correlated with the severity of the disease.¹¹

RA, showing chronic and progressive polyarthritis as its main symptoms, is a generalized inflammatory disease with multi organ disorder. Destruction of joint cartilage introduces a serious permanent functional disorder. The cartilage matrix consists mainly of collagen (90% of type II and other types including IX, XI)¹² and aggrecan (chondroitin 6-sulfate, chondroitin 4-sulfate, and keratan sulfate are incorporated as core proteins). The major constituent of type II collagen is degraded by MMP-1, neutrophil collagenase (MMP-8), and collagenase-3 (MMP-13).¹³ MMP-3 can also degrade type II collagen around the N-terminal region which forms intermolecular cross-linkages. Moreover, MMP-3 also destroys the NC2 domain of type IX collagen, which plays an important role in the construction of collagen networks. On the other hand, cleavage of core protein at the G1-G2 domain is conducted by various MMPs [MMP-1, 2, 3, matrilysin (MMP-7), 8, gelatinase B (MMP-9), and 13]. From this evidence, MMP-3 is thought to be one of the key enzymes for RA.¹⁴

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Given the inflammatory phenomenon found in RA, tumor necrosis factor α (TNF- α), a critical pro-inflammatory cytokine produced by monocytes or macrophages, plays an important role by inducing other pro-inflammatory cytokines such as IL-1 β , IL-6, and IL-8. Elevated TNF- α concentrations have been demonstrated in RA disease.¹⁵ TNF- α is initially expressed as a 26 kDa cell-associated form which is then proteolytically processed by TNF- α converting enzyme (TACE) to yield a monomeric soluble form of 17 kDa secreted in a mature form.¹⁶ This secreted protein contains one intrachain disulfide bridge and exists as a dimer or trimer in circulation.¹⁷ The mature TNF- α exerts its multiple biological effects via interaction with two structurally and functionally distinct high-affinity receptors: TNFR1 (p55) and TNFR2 (p75).¹⁸ Binding of TNF- α to these receptors results in the activation of several transduction pathways. From these bindings, suppression of MMPs activity, especially MMP-1 and 3, together with TACE activity could relieve the severity of RA.

Our initial objective was to confirm the efficacy of dual inhibitors of MMPs and TACE in a rat arthritis model using a systemic administration route. Although oral administration is preferable, in order to consider the initial objective of assessing the intrinsic potency of the dual inhibitor a subcutaneous (sc) injection route provides a more direct result by ignoring other biological factors, e.g., intestinal adsorption rate, and hepatic metabolism. Give the sc injection for systemic administration, acquiring a highly water-soluble property is a prerequisite for inhibitors. Generally peptide based drugs are poorly in water soluble. In several MMPs inhibitors progressed into clinical trials (Fig. 1), sulfonamide type compounds, represented as Prinomastat **4**¹⁹ or CGS-27023A **5**²⁰ include a heterocyclic ring which can improve the water solubility. Regarding succinate-based type compound depicted as Batimastat **1**²¹ or Marimastat **2**,^{22,23} efforts to acquire the highly water-soluble derivatives have not been conducted (water solubility were <1 and 1.4 mg/mL,²⁴ respectively). In this study, we have investigated the synthesis of highly water-soluble dual inhibitors based on the chemical structure of succinate-based type compound, without any loss of inhibitory potencies.

Results and Discussion

Design

From a conformational analysis of MMP-3 complexed with a succinyl based MMP inhibitor by X-ray crystallography, the P₁' isobutyl group fits into the S₁' pocket, and the P₁ and P₂' residues have little interaction with MMP-3 and are directed away from the active site into the solvent.²⁵ According to this observation, introduction of hydrophilic functional groups, which interact with the water molecules existing outside the active site, at P₁ and/or P₂' residues could increase the water solubility of succinyl based MMPs inhibitors. As the P₁ residue incorporates a succinate unit with (*S*)-configuration, it seems to be troublesome to resolve diastereoisomers of succinate units after the introduction of various hydrophilic functional groups on the P₁ residue. The P₂' position, which is constructed from an amino acid, is more convenient both for introducing the functional group and obtaining chiral compounds than is the P₁ position. As the P₁ residue and an aromatic ring at the P₂' residue are preferable for *in vitro* activity, we designed dual inhibitors with the new physical characteristics depicted in Figure 2 as a general formula.

Chemistry

The 2,3-disubstituted succinic acid half esters **11** were prepared according to the sequence shown in Scheme 1. Diazotization of the amino group in D-leucine following intracyclization by carboxylate and simultaneous ring opening by bromo anions proceeded predominantly in a sterically conserved reaction that was accompanied by 2–3% racemization. The benzyl esterification was conducted by the removal of water by azeotropic distillation with catalytic *p*-toluenesulfonic acid (TsOH) to give **8**. Then, the C₂–C₃ bond formation at succinate was accomplished by the reaction of α -bromo carboxylate **8** with activated methylene of malonic acid diester to give tricarboxylate **9**.²⁶ After introduction of a cinnamyl group, consequent hydrogenolytic removal of the two benzyl groups and decarboxylation at the malonyl moiety of **10** in the presence of tertiary amine afforded compound **11**.²⁶ The crude **11**, which was obtained as a colorless oil, contained of 3(*R*),2(*S*) and 3(*S*),2(*S*) forms

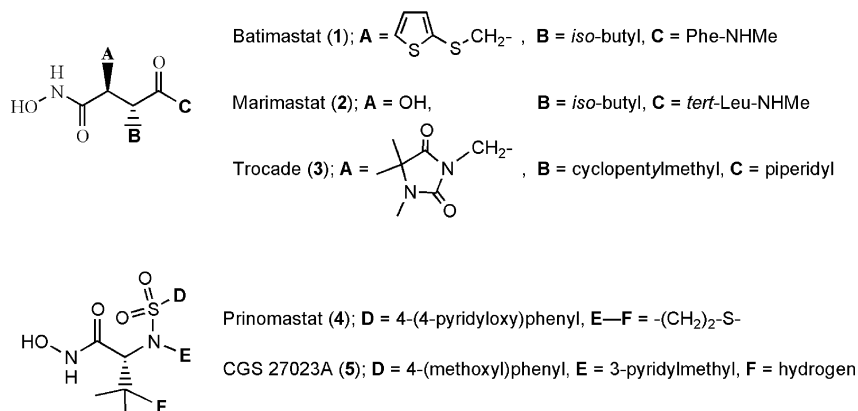


Figure 1. Hydroxamic acid based MMP inhibitors.

as the major products, together with 3(*R*),2(*R*) and 3(*S*),2(*R*) forms as contaminants that were caused by the reaction mechanism of diazotization together with bromination of D-leucine and utilizing a synthetic route for preparation of **11** from **9**. To obtain the pure, desired 3(*R*),2(*S*) form, we examined the crystallization of **11** with 35 kinds of amine including chiral amines. Several amines gave crystals that were subjected to recrystallization from an appropriate solvent. It was found that *tert*-butylamine gave a sufficient diastereomeric excess (de) and enantiomeric excess (ee). The relative stereochemistry was inferred by observation of nuclear Overhauser effect (NOE) between the C-2 methine, the C-3 methine and the one C-4 methine at compound **13**, which was prepared as described by Beckett (Scheme 2).²⁷

The preparation of compounds in which R¹ represents hydrophilic functional groups is summarized in Scheme

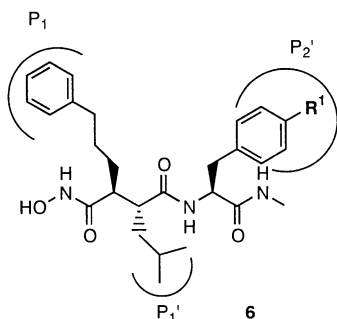
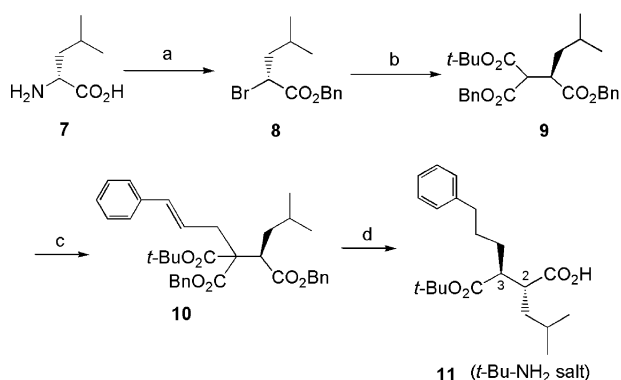
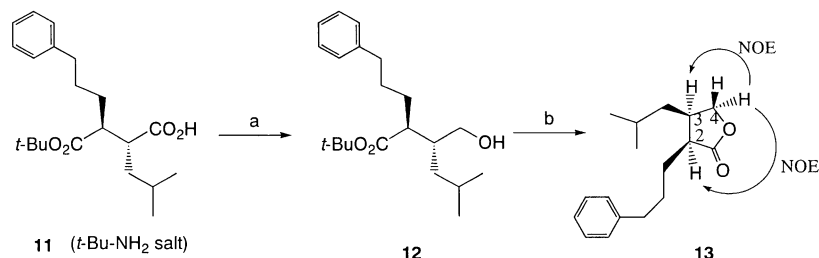


Figure 2. General formula of highly water-soluble inhibitor.



Scheme 1. Preparation of succinate units. Reagents: (a) (1) aq NaNO₂/aq. HBr; (2) BnOH/TsOH in *c*-hexane reflux; (b) benzyl *tert*-butylmalonate/*tert*-BuOK; (c) cinnamyl bromide/NaH; (d) (1) H₂/Pd-C, (2) *N*-ethylmorpholine in toluene reflux, (3) *tert*-butylamine.



Scheme 2. Determination of the relative stereochemistry of **11c**. Reagents: (a) BH₃·THF; (b) TFA/CH₂Cl₂.

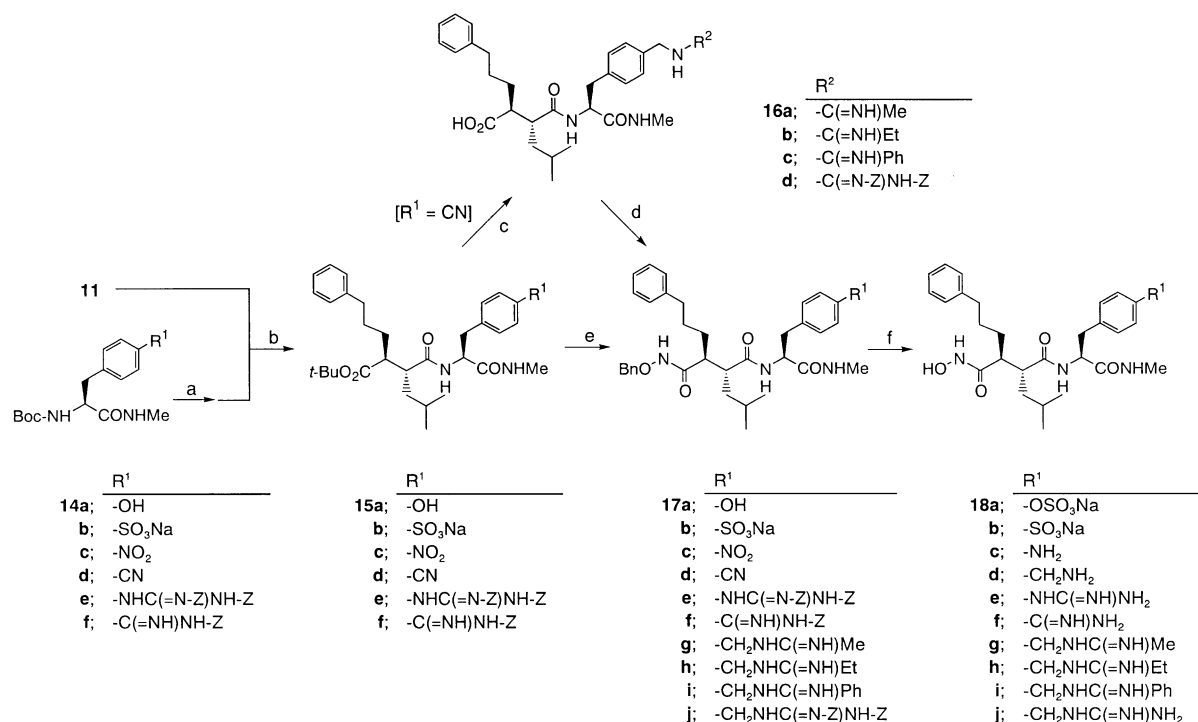
3. The succinate unit **11** was coupled with **14a–f** using EDC/HOBt as the coupling reagent. The *tert*-butyl esters **15a–f** were converted to *O*-benzylhydroxamate **17a–f** by treatment with acid followed by coupling with *O*-benzylhydroxylamine. To construct an acylimidoyliminomethyl group, compounds that had a cyano group on the aromatic ring **15d** were hydrogenated in AcOH and subsequently reacted with ethyl acylimidate to give **16a–c**. *N,N'*-Diprotected guanidinomethyl compound **16d** was also obtained by reaction of the amino methyl intermediate and 1*H*-pyrazole-*N,N'*-bis(benzyloxycarbonyl)carboxamidine,²⁸ instead of ethyl acylimidate. Carboxylic acids **16a–d** were coupled to *O*-benzylhydroxylamine using EDC/HOBt to yield **17g–f**. *O*-sulfonation was carried out on *O*-benzylhydroxamate compound **17a** using sulfur trioxide pyridine complex, which was subsequently hydrogenated to give **18a**. Other target compounds (**18b–j**) were prepared by catalytic hydrogenation of **17b–j**.

Inhibition of MMP-1, 3 and TACE

Initially, the inhibitions of MMP-1, 3 and TACE, which have been assumed to play a crucial role in RA, were evaluated by *in vitro* assays together with the water solubility of those compounds and the results were compared with those for unsubstituted compound (R¹=H) in Table 1. Due to the introduction of hydrophilic functional groups on the aromatic ring of phenylalanine, the inhibitory potency for MMP-1 was decreased, but that for MMP-3 was almost tolerated against Comparative compound. Furthermore, it is noteworthy that after installation of a hydrophilic functional group at the P₂' position, a dramatic increase in water solubility was observed. There was at least a 10-fold increase of water solubility when compared with unsubstituted compounds. In particularly, acidic functional groups represented by sulfonate (**18a** and **18b**) possessing compounds showed at least 100-fold increases in water solubility.

Half-life measurements in rat plasma

Before evaluation of these inhibitors in the rat adjuvant-induced arthritis model, we selected several typical compounds and measured plasma *t*_{1/2} in the β-phase in rats after sc dosing together with the area under the plasma concentration time curve (Table 2). The values in Table 2 indicate the amount of the administered compound plus its active metabolites, since we estimated the value based on *ex vivo* MMP-3 activity



Scheme 3. Syntheses of highly water-soluble MMPs inhibitors **18a–j**. Reagents: (a) 4 N HCl in EtOAc; (b) EDC/HOBt; (c) (1) 95% aq TFA, (2) ethyl acylimidate hydrochloride/Et₃N (for **16a–c**) or 1*H*-pyrazole-*N,N'*-bis(benzyloxycarbonyl)carboxamidide (for **16d**); (d) BnONH₂/EDC/HOBt; (e) (1) 95% aq TFA, (2) BnONH₂/EDC/HOBt; (f) (1) sulfur trioxide pyridine complex (for **18a**), (2) H₂/Pd-C (for **18a–j**).

Table 1. Inhibition of MMP-1, MMP-3 and TACE by hydrophilic MMPs inhibitors and their solubility for water

Compd	R^1	IC ₅₀ (nM) ^a			Solubility (mg/mL)
		MMP-1	MMP-3	TACE	
Comparative compd.	—H	0.15	8	ND ^d	1 >
18a	—OSO ₃ Na	0.4	5	120	> 100
18b	—SO ₃ Na	4	4	270	> 100
18c	—NH ₂	3	1	134	10 ^b
18d	—CH ₂ NH ₂	5	8	210	10 ^b
18e	—NHC(=NH)NH ₂	6	6	130	30 ^b
18f	—C(=NH)NH ₂	5	5	140	80 ^c
18g	—CH ₂ NHC(=NH)Me	7	15	120	70 ^c
18h	—CH ₂ NHC(=NH)Et	6	3	160	70 ^c
18i	—CH ₂ NHC(=NH)Ph	10	10	420	40 ^c
18j	—CH ₂ NHC(=NH)NH ₂	4	10	130	80 ^c

^aConcentration required for 50% inhibition of enzyme activity. Standard deviations are less than 10% of the mean values. Details of the enzyme assays are described in Experimental.

^bAcOH salt.

^cHCl salt.

^dNot done.

measurements. As predicted, acidic sulfonate substituted compounds (**18a** and **18b**) showed a short half-life compared with compounds having substituent of basic functional groups (**18e** and **18g**). Compared with Marimastat and Trocade, compound **18e** was shown to have a superior half-life and almost the same AUC value as Trocade.

Inhibition of MMP-2, 7, 9, metalloelastase (MMP-12), 13 and membrane-type 1 MMP (MMP-14)

In addition to IC_{50} determinations against other MMPs and TACE, **18e** was evaluated together with **18b**, Marimastat, and Trocade (Table 3). As a result, **18b** and **18e** showed similar broad spectrum inhibitory profiles against MMPs and TACE. Marimastat exhibited broad and strong inhibitory potencies against MMPs, and a one order of magnitude decrease in potency against TACE compared with **18e**. On the other hand, Trocade showed relatively MMP-7 and 13 selective inhibitory potencies.

Effect of water-soluble MMP inhibitors on the rat adjuvant-induced arthritis model

Initially, compound **18e** was evaluated in the rat adjuvant-induced arthritis model. **18e** was administered sc in three doses (3, 10, 30 mg/kg/day) from day 0 and continued throughout the 20 days of the experiment with three doses. The time course of hindpaw volumes are depicted in Figure 3. Although, in the saline-administered control group, the hindpaw volume dramatically increased from day 10 after adjuvant injection, **18e** suppressed this swelling efficiently and dose-dependently. The X-ray scoring data, which was utilized by measuring the severity of joint destruction, also improved depending on doses. Representative radiographs of hindpaws from normal rats and rats treated with saline or compound **18e** (30 mg/kg/day) after injection of adjuvant are shown in Figure 4. Compared with the saline-treated control, the hindpaw from a rat treated with compound **18e** shows less bone demineralization, joint space narrowing, and bone erosion at the distal tibia, talus, calcaneus, and metatarsus. To determine the administration point during the experiment, we explored several administration periods (Table 4). Compared to administration every day (days 0–20), early stage administrations (days 0–5 and days 0–7)

showed the similar efficacy except for days 0–3 administration. It is note worthy that the shorter period administration (days 3–7) also caused sufficient suppression. But late stage administrations (days 7–20 and days 14–27) did not improve the swelling. These results indicate that MMPs and/or TACE act at an early stage in this model and play a pivotal role in swelling together with bone destruction. The effects of indomethacin (typical non-steroidal anti-inflammatory drug) and methotrexate (slow-acting anti-rheumatic drug), the common RA therapeutic medicines in this model are also shown in Table 4. Indomethacin showed moderate efficacy (45.7% inhibition of hindpaw swelling), even for late stage administration (days 14–27). On the contrary, at early stage administration, only 10.7% inhibition of hindpaw swelling was observed. Although methotrexate exhibited 51.9% inhibition at early stage administration (days 0–7), hindpaw swelling was exacerbated with late stage administration (–20.9%, days 14–27). From the study of these administration points, it can be concluded that several different pathogenic factors including MMPs and TACE participated in the rat adjuvant-induced arthritis model. Combined administration of compound **18e** and indomethacin might have an additional or synergistic effect in RA patients by suppressing alternative pathogenic factors.

We also measured the efficacy of other developing MMPs inhibitors represented as Marimastat and Trocade together with **18b** and **18e** (Table 5). Under the same administration conditions for route (sc), dose (30 mg/kg), and timing (days 3–7), Trocade which is a MMP-7 and 13 selective inhibitor, showed a lack of efficacy against hindpaw swelling and bone destruction, suggesting that MMP-7 and 13 are not important at an early stage in this model. Probably, due to its short half-life in rat plasma (0.58 h), **18b** showed only 20.6%

Table 2. Effect of hydrophilic functional group on pharmacokinetic properties in the rat after subcutaneous administration

Compd.	R ¹	$t_{1/2\beta}$ (h)	AUC (ng/mL·h)
18a	–OSO ₃ Na	0.33	436
18b	–SO ₃ Na	0.58	1738
18e	–NHC(=NH)NH ₂	2.00	13659
18g	–CH ₂ NHC(=NH)Me	1.83	8148
Marimastat	—	1.15	9790
Trocade	—	1.03	14058

Pharmacokinetic parameter $t_{1/2\beta}$ represents half-lives for second phase. The values were estimated based on the ex vivo MMP-3 activity measurements for plasma concentration. Data shown are mean values ($n=3$). Area under the plasma concentration time curves are extrapolate based on a 0–4 h ($AUC_{(0-4h)}$) value by WinNonlin Standard (Ver. 1.5) software.

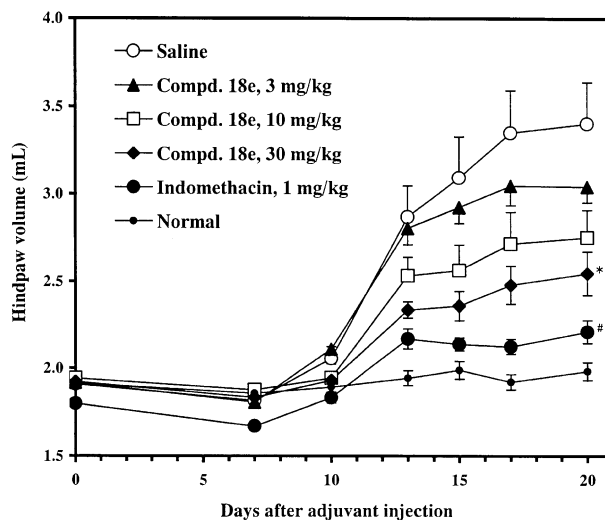


Figure 3. Effect of compound **18e** on hindpaw swelling in adjuvant arthritis rats. Compound **18e** were administered subcutaneously to the back of rats on day 0–20. Indomethacin was given orally daily. Normal rats received no treatment. Data represent mean $\pm$ SE with 6 rats per group. * $P<0.05$ as compared with the control by two-tailed Dunnett's multiple comparison. # $P<0.05$ as compared with the control by two-tailed Student's t -test.

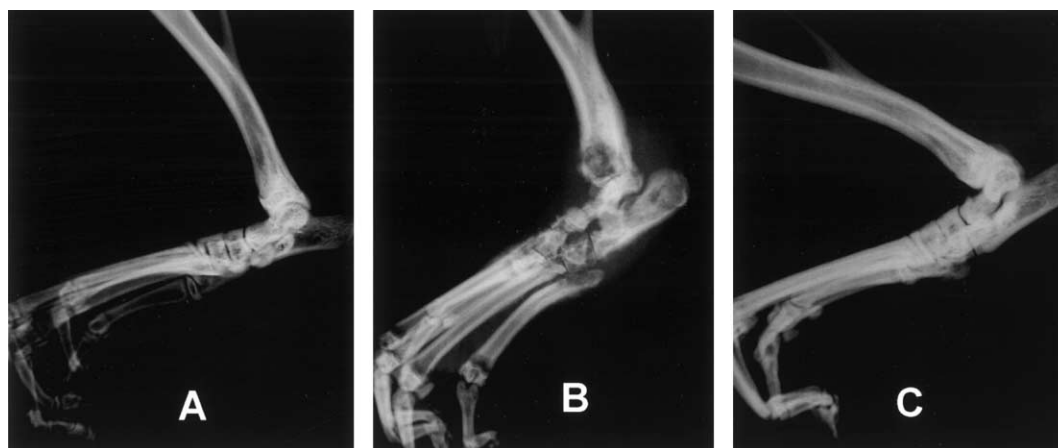


Figure 4. Representative radiographs of hindpaw; (A) normal rat, (B) treated with saline after adjuvant injection, (C) treated with compound **18e** (30 mg/kg/day) after adjuvant injection.

Table 3. Inhibitory of various MMP and TACE by compounds **18b**, **18e**, Marimastat and Trocade

Compd.	IC ₅₀ (nM) ^a								
	MMP-1	MMP-2	MMP-3	MMP-7	MMP-9	MMP-12	MMP-13	MMP-14	TACE
18b	4	1	4	ND ^c	1	ND ^c	0.16	ND ^c	270
18e	6	2	6	0.7	1	0.064	0.07	4	130
Marimastat	2 (5) ^b	13 (6) ^b	50 (200) ^b	2 (20) ^b	9	5	0.74	3	1807
Trocade	23 (7) ^b	314	294 (527) ^b	9	545 (59) ^b	17	1.79	21	> 100,000

^aConcentration required for 50% inhibition of enzyme activity. Standard deviations are less than 10% of the mean values. Details of the enzyme assays are described in Experimental.

^bIC₅₀ values in parentheses are given from references.

^cNot done.

Table 4. Effects of administration timing on hindpaw swelling and bone destruction in adjuvant arthritis rats

Drugs	Dose (mg/kg)	Route	Timing	Hindpaw Swelling inhibition (%)	Bone destruction inhibition (%)
Compd 18e	30	sc	Day 0–3	27.6	28.5
	30	sc	Day 0–7	70.3 ^a	86.2 ^a
	30	sc	Day 3–7	74.4 ^a	89.0 ^a
	30	sc	Day 7–20	15.3	26.6
	30	sc	Day 14–27	–17.4	–24.8
	30	sc	Day 0–20	64.0 ^a	70.4 ^a
Indomethacin	1	po	Day 0–7	10.7	–2.7
	1	po	Day 14–27	45.7 ^b	61.7 ^b
	1	po	Day 0–20	70.3 ^b	86.4 ^b
Methotrexate	0.2	ip	Day 0–7	51.9	58.7
	0.2	ip	Day 14–27	–20.9	–19.2
	0.2	ip	Day 0–20	68.5 ^b	79.8 ^b

sc, subcutaneously; po, per oral; ip, intraperitoneally. The conditions are described in Experimental.

^a $P < 0.05$ as compared with the control by two-tailed Dunnett's multiple comparison.

^b $P < 0.05$ as compared with the control by two-tailed Student's *t*-test.

Table 5. Effects of MMP inhibitors on hindpaw swelling and bone destruction of adjuvant arthritis rats

Compd	Dose (mg/kg)	Route	Timing	Hindpaw swelling % inhibition	Bone destruction % inhibition
18b	30	sc	Day 3–7	20.6	46.9
18e	30	sc	Day 3–7	53.5	62.0
Marimastat	30	sc	Day 3–7	30.3	35.7
Trocade	30	sc	Day 3–7	2.1	–5.4

Drugs were administered subcutaneously to the back of rats once a day from day 3 to 7. sc, Subcutaneously. The conditions are described in Experimental.

inhibition of hindpaw swelling. Marimastat, which has a broad MMPs inhibitory profile and moderate TACE inhibitory potency, exhibited 30% inhibition of hindpaw swelling and improved X-ray scoring. Previously, examples of succinyl-based MMPs inhibitors have been reported to produce anti-arthritis activity in the rat adjuvant-induced arthritis model.^{29–31} Those informations and our result of the major difference between compound **18e** and Trocade suggest that soluble TNF- α might be more operative than MMPs in the rat adjuvant-induced model and suppression of TNF- α release is required for the prevention of bone destruction.

Conclusion

We have developed new dual inhibitors of MMPs and TACE that were highly water-soluble, without significant deletion of their activities. The sc injection of high concentration inhibitors becomes possible through these compounds. The compounds (**18e**), which possesses a basic hydrophilic functional group (guanidino) on the P₂' aromatic ring, showed longer duration in rat plasma level after sc injection than compounds (**18a** and **18b**), which have acidic functional groups at the same position. The representative compound (**18e**) was confirmed as possessing intrinsic efficacy against the rat adjuvant-induced arthritis model, by decreasing the hindpaw swelling and bone destruction. We have found that the best administration point in this model was in the early stage immediately following adjuvant injection; days 3–7 administration gave the shortest period of administration with sufficient efficacy. Treatment with **18e** and then with indomethacin is expected to have an additional or synergistic effect in patients with RA.

Experimental

Chemistry

All commercial chemicals and solvents are reagent grade and used without further purification unless otherwise specified. The following solvents and reagents have been abbreviated: *N,N*-dimethylformamide (DMF), tetrahydrofuran (THF), trifluoroacetic acid (TFA), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), 1-hydroxybenzotriazole (HOBt). Reaction were monitored by thin-layer chromatography on 0.25-mm silica gel plates (Silica gel 60 F₂₅₄, Merck) and visualized with UV light, iodine vapors, 5% phosphomolybdic acid in 95% ethanol, or 0.7% ninhydrin in ethanol. Final compound were typically purified either flash column chromatography on silica gel BW-300 (200–400 mesh, Fuji Silysia Chemical Ltd.) or reverse phase flash column chromatography on C₁₈-bonded silica gel Chromatorex-ODS DM1020T (Fuji Silysia Chemical Ltd.).

Melting points were determined on a Yanagimoto melting point apparatus without correction. Optical rota-

tions were measured in a JASCO DIP-140 apparatus. ¹H NMR was recorded on a JEOL JMN-AL300 spectrometer (300 MHz), and chemical shifts are given in ppm (δ) from tetramethylsilane (TMS), which was used as the internal standard. Mass spectra were obtained on a JEOL JMS 700 spectrometer by fast atom bombardment (FAB) ionization techniques using glycerol as a matrix. All organic extracts were dried with anhydrous MgSO₄ prior to solvent removal on a rotary evaporator under reduced pressure.

Benzyl 2(R)-bromo-4-methylpentanoate (8). To a solution of D-leucine (50.0 g, 381 mmol) in 3 N aq HBr (570 mL), aq sodium nitrite [42.1 g, (610 mmol) was dissolved in 100 mL of water] was added at 0–2 °C and stirred at the same temperature for 3 h. The organic materials were extracted with EtOAc, washed with brine, evaporated to give crude 2(R)-bromo-4-methylpentanoic acid (66.9 g, 90%) as a slightly brown oil. A mixture of crude 2(R)-bromo-4-methylpentanoic acid (66.9 g, 343 mmol), benzyl alcohol (42.6 mL, 412 mmol), *p*-toluenesulfonic acid monohydrate (6.52 g, 34.3 mmol), and toluene (700 mL) was heated under reflux with azeotropic trapping of water for 2 h. The reaction mixture was washed with satd aq NaHCO₃ and brine, then evaporated in vacuo to leave a slightly brown oil, which was purified by flash column chromatography (eluent; *n*-hexane: EtOAc = 50:1) to give **8** (64.6 g, 66%) as a colorless oil: $[\alpha]_{25}^D + 31.8^\circ$ ($c = 1.0$, MeOH). ¹H NMR (CDCl₃) δ ppm; 0.92 (3H, d, $J = 6.5$ Hz), 0.94 (3H, d, $J = 6.4$ Hz), 1.67 (1H, m), 1.90 (2H, m), 4.30 (1H, t, $J = 7.0$ Hz), 5.20 (2H, s), 7.32 (5H, m). HRFABMS calcd for C₁₃H₁₈O₂Br: 285.0490, found: 285.0516.

Dibenzyl 3(RS)-tert-butyloxycarbonyl-2(R)-iso-butylsuccinate (9). To a solution of benzyl *tert*-butylmalonate (24.9 g, 99.6 mmol) in DMF (60 mL), potassium *tert*-butoxide (13.4 g, 120 mmol) was added by portions with stirring at 0 °C. The mixture was stirred for 1 h at room temperature and re-cooled to 0 °C. A solution of **8** (28.4 g, 99.6 mmol) in DMF (60 mL) was added dropwise to the cooled mixture over a period of 1 h. After stirring for 15 h at 5 °C, AcOEt (2 L) was added to the reaction mixture, which was then partitioned and washed successively with brine, 1 N aq HCl, sat. aq NaHCO₃, and brine (twice for each). The organic layer was evaporated in vacuo to leave a slightly yellow oil, which was purified by flash column chromatography (eluent; *n*-hexane: EtOAc = 20:1) to give **9** (40.0 g, 89%) as a colorless oil: $[\alpha]_{25}^D + 16.7^\circ$ ($c = 1.0$, MeOH). ¹H NMR (CDCl₃) δ ppm; 0.82 (3H, d, $J = 6.8$ Hz), 0.84 (3H, d, $J = 6.7$ Hz), 1.15–1.82 (12H, m), 3.21 (1H, m), 3.73 (1H, m), 5.14 (4H, m), 7.32 (10H, m). HRFABMS calcd for C₂₇H₃₅O₆: 455.2434, found: 455.2437.

Dibenzyl 3(RS)-tert-butyloxycarbonyl-3-cinnamyl-2(R)-iso-butylsuccinate (10). To a solution of **9** (9.49 g, 20.9 mmol) in DMF (100 mL) was added 60% sodium hydride (1.0 g, 25.1 mmol) by portions with stirring at room temperature. The mixture was stirred for 2 h at room temperature and cooled to 0 °C. Cinnamyl bromide (5.36 g, 27.2 mmol) was added by portions to the cooled mixture which was then stirred at 5 °C for 15 h.

The solvent was evaporated under reduced pressure, and EtOAc (500 mL) was added to the residue. The mixture was washed successively with brine, 1 N aq HCl, sat. aq NaHCO₃, and brine (twice for each). The organic layer was evaporated in vacuo to leave a slightly brown oil, which was purified by flash column chromatography (eluent; *n*-hexane: EtOAc=20:1) to give **10** (10.8 g, 91%) as a colorless oil: ¹H NMR (CDCl₃) δ ppm; 0.75–0.98 (6H, m), 1.04–1.16 (1H, m), 1.20–1.50 (10H, m), 1.84–1.95 (1H, m), 2.78–2.90 (2H, m), 3.13–3.26 (1H, m), 4.99–5.21 (4H, m), 6.08–6.27 (1H, m), 6.30–6.45 (1H, m), 7.14–7.42 (15H, m). HRFABMS calcd for C₃₆H₄₃O₆: 571.3060, found: 571.3086.

3(S)-tert-Butyloxycarbonyl-6-phenyl-2(R)-isobutylhexanoic acid (11). To a solution of **10** (10.0 g, 17.5 mmol) in methanol (120 mL) was added 5% Pd-C (50% wet catalyst, 2.5 g), and the mixture was vigorously stirred under a hydrogen atmosphere at room temperature for 7 h. The catalyst was filtered off and then methanol was evaporated in vacuo to give dicarboxylic acid intermediate as a colorless oil. The residue was dissolved in toluene (400 mL) and refluxed in a presence of *N*-ethylmorpholine (2.23 mL, 17.5 mmol) for 2 h. The reaction mixture was washed successively with 1 N aq HCl, and brine (twice for each), then evaporated in vacuo to leave a yellow oil. The residue was dissolved in EtOAc (150 mL) and *tert*-butylamine (1.84 mL, 17.5 mmol) was added to form a *tert*-butylamine salt as a colorless solid, which was recrystallized from EtOAc to give **11** (colorless solid, 2.36 g, 32%) as *tert*-butylamine salt. Enantiomeric excess (ee); 97.3%, diastereomeric excess (de); 97.2% [The ee and de were determined by chiral HPLC [column; Chiralpack AD (Daicel Chemical Industries), detection; 220 nm, eluent; *n*-hexane/2-propanol/TFA = 98:2:0.1, column temperature; 30 °C, flow rate; 1 mL/min]. ¹H NMR (CDCl₃) δ ppm; 0.86 (3H, d, *J*=6.6 Hz), 0.89 (3H, d, *J*=6.4 Hz), 1.00–1.13 (1H, m), 1.27 (9H, s), 1.42 (9H, s), 1.46–1.78 (6H, m), 2.40–2.69 (4H, m), 5.37 (3H, brs), 7.08–7.30 (5H, m). HRFABMS calcd for C₂₁H₃₃O₄: 349.2379, found: 349.2371.

tert-Butyl 3(R)-hydroxymethyl-5-methyl-2(S)-phenylpropylhexanoate (12). To a solution of free form of **11** (669 mg, 1.92 mmol) in dry THF (10 mL), borane-THF complex (1.0 M solution in THF, 2.2 mL) was added at –10 °C and stirred at room temperature for 4 h under a nitrogen atmosphere. To the reaction mixture, satd aq NH₄Cl (50 mL) was added and organic materials were extracted with EtOAc followed by washing with 10% aq Na₂CO₃ and brine, and then evaporated in vacuo to leave crude **12** which was purified by flash column chromatography (eluent; *n*-hexane: EtOAc=9:1) to give **12** (440 mg, 69%) as a colorless oil: ¹H NMR (CDCl₃) δ ppm; 0.86 (3H, d, *J*=6.4 Hz), 0.89 (3H, d, *J*=6.4 Hz), 1.01–1.30 (2H, m), 1.34–1.92 (15H, m), 2.38–2.53 (1H, m), 2.63 (2H, t, *J*=6.8 Hz), 3.48–3.67 (2H, m), 7.11–7.34 (5H, m). HRFABMS calcd for C₂₁H₃₅O₃: 335.2586, found: 335.2580.

3(R)-Isobutyl-2(S)-phenylpropyl-γ-butyrolactone (13). To a solution of *tert*-butyl ester **12** (360 mg, 1.08 mmol) in CH₂Cl₂ (7.2 mL), TFA (414 μL, 5.40 mmol) was added

and stirred at 4 °C for 1 h. The reaction mixture was evaporated in vacuo to leave crude **13** which was purified by flash column chromatography (eluent; *n*-hexane: EtOAc=9:1) to give **13** (208 mg, 74%) as a colorless oil: ¹H NMR (CDCl₃) δ ppm; 0.86 (3H, d, *J*=6.4 Hz), 0.90 (3H, d, *J*=6.6 Hz), 1.01–1.25 (2H, m), 1.43–1.91 (5H, m), 2.47–2.59 (2H, m), 2.62–2.76 (2H, m), 4.01 (1H, dd, *J*=3.6, 9.0 Hz), 4.18 (1H, dd, *J*=5.2, 9.0 Hz), 7.13–7.35 (5H, m). HRFABMS calcd for C₁₇H₂₅O₂: 261.1855, found: 261.1825.

Typical procedure for the preparation of compound 15.
***N*-[4-*tert*-Butyloxy-2(R)-isobutyl-3(S)-phenylpropylsuccinyl]-L-tyrosine *N*-methylamide (15a).** To a mixture of **11** (4.21 g, 10 mmol), H-Tyr-NHMe·HCl (2.54 g, 11 mmol) [prepared from Boc-Tyr-NHMe (**14a**) (3.24 g, 11 mmol) and 4 N HCl in EtOAc (60 mL) as usual], HOBT (1.49 g, 11 mmol), and DMF (70 mL), EDC (2.11 g, 11 mmol) was added under stirring at –15 °C. The mixture was stirred at –15 °C for 1 h and further at room temperature overnight. The reaction mixture was diluted with EtOAc (210 mL) and washed successively with brine, 1 N aq HCl, 10% aq Na₂CO₃ and brine (twice for each), then evaporated in vacuo to leave a pale yellow oil. The residue was crystallized from Et₂O to give **15a** (3.57 g, 68%) as a colorless solid: [α]_D²⁵ +9.0° (*c*=0.5, MeOH). ¹H NMR (CDCl₃) δ ppm; 0.78 (3H, d, *J*=6.4 Hz), 0.81 (3H, d, *J*=6.4 Hz), 0.82–1.74 (16H, m), 2.11–2.70 (4H, m), 2.74 (3H, d, *J*=4.7 Hz), 2.81–2.94 (1H, m), 2.94–3.10 (1H, m), 4.65 (1H, m), 6.32 (1H, m), 6.59 (1H, m), 6.80–7.42 (9H, m). HRFABMS calcd for C₃₁H₄₅N₂O₅: 525.3328, found: 525.3350.

***N*-[4-*tert*-Butyloxy-2(R)-isobutyl-3(S)-phenylpropylsuccinyl]-L-4'-sulfophenylalanine *N*-methylamide sodium salt (15b).** Yield; 60%. ¹H NMR (MeOH-*d*₄) δ ppm; 0.74 (3H, d, *J*=6.7 Hz), 0.82 (3H, d, *J*=6.7 Hz), 1.01–1.76 (16H, m), 2.21–2.84 (7H, m), 2.84–3.21 (2H, m), 4.52–4.70 (1H, m), 6.97–7.40 (7H, m), 7.69–7.85 (2H, m). FAB-MS: *m/z* 611 [M+Na]⁺, 589 [M+1]⁺. HRFABMS: calcd for C₃₁H₄₄N₂NaO₇S 611.2767, found 611.2796.

***N*-[4-*tert*-Butyloxy-2(R)-isobutyl-3(S)-phenylpropylsuccinyl]-L-4'-nitrophenylalanine *N*-methylamide (15c).** Yield; 83%. ¹H NMR (CDCl₃) δ ppm; 0.74 (3H, d, *J*=6.4 Hz), 0.80 (3H, d, *J*=6.4 Hz), 0.90–1.74 (16H, m), 2.21–2.60 (4H, m), 2.64 (3H, d, *J*=4.7 Hz), 2.98–3.20 (2H, m), 4.46–4.68 (1H, m), 5.90–6.18 (2H, m), 7.16–7.73 (7H, m), 8.02–8.23 (2H, m). HRFABMS: calcd for C₃₁H₄₄N₃O₆ 554.3230, found 554.3246.

***N*-[4-*tert*-Butyloxy-2(R)-isobutyl-3(S)-phenylpropylsuccinyl]-L-4'-cyanophenylalanine *N*-methylamide (15d).** Yield; 87%. ¹H NMR (CDCl₃) δ ppm; 0.81 (3H, d, *J*=6.4 Hz), 0.84 (3H, d, *J*=6.2 Hz), 0.96–1.82 (16H, m), 2.07–2.70 (4H, m), 2.72 (3H, d, *J*=4.6 Hz), 2.96–3.12 (2H, m), 4.56–4.67 (1H, m), 6.02–6.14 (1H, m), 6.48–6.57 (1H, m), 6.92–7.75 (9H, m). HRFABMS: calcd for C₃₂H₄₄N₃O₄ 534.3332, found 534.3322.

***N*-[4-*tert*-Butyloxy-2(R)-isobutyl-3(S)-phenylpropylsuccinyl]-L-4'-[*N,N'*-bis-(benzyloxycarbonyl)guanidino]phenylalanine *N*-methylamide (15e).** Yield; 70%. ¹H NMR

(CDCl₃) δ ppm; 0.77 (3H, d, J =6.7 Hz), 0.80 (3H, d, J =6.4 Hz), 0.92–1.14 (1H, m), 1.21–1.77 (15H, m), 2.16–2.64 (4H, m), 2.67 (3H, d, J =4.6 Hz), 2.93 (2H, d, J =5.9 Hz), 4.50–4.66 (1H, m), 5.13 (2H, s), 5.18 (2H, s), 5.90–6.11 (1H, m), 6.41–6.53 (1H, m), 6.89–7.65 (21H, m). HRFABMS: calcd for C₄₈H₆₀N₅O₈ 834.4442, found 834.4430.

***N*-[4-*tert*-Butyloxy-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-L-4'-[*N*-(benzyloxycarbonyl)amidino]phenylalanine *N*-methylamide (15f).** Yield; 18%. ¹H NMR (CDCl₃) δ ppm; 0.79 (3H, d, J =6.2 Hz), 0.83 (3H, d, J =6.4 Hz), 0.97–1.12 (1H, m), 1.26–1.69 (15H, m), 2.06–2.48 (4H, m), 2.76 (3H, d, J =4.7 Hz), 3.10 (2H, d, J =6.0 Hz), 4.49–4.62 (1H, m), 5.20 (2H, s), 6.21–6.36 (1H, m), 6.47–6.62 (1H, m), 7.13–7.50 (14H, m), 7.71–7.90 (2H, m). HRFABMS: calcd for C₄₀H₅₃N₄O₆ 685.3965, found 685.3995.

Typical procedure for the preparation of compound 16. ***N*-[4-Hydroxy-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-L-4'-acetimidoyliminomethylphenylalanine *N*-methylamide hydrochloride (16a).** To the compound 15d (1.73 g, 3.24 mmol), ice-cooled 95% aq TFA (16 mL) was added and stirred at 5 °C for 4 h, then evaporated in vacuo. The residue was precipitated from Et₂O followed by filtration to give a carboxylic acid intermediate, *N*-[4-hydroxy-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-L-4'-cyanophenylalanine *N*-methylamide (1.22 g, 79%) as a colorless solid. To a solution of the carboxylic acid intermediate (1.22 g) in EtOH (25 mL)-concn. HCl (1 mL), 5% Pd-C (50% wet, 600 mg) was added and the mixture was vigorously stirred under hydrogen atmosphere at room temperature for 10 h. The catalyst was filtered off and evaporated in vacuo to leave a colorless residue, which was suspended in water (50 mL) and stirred for 30 min. The insoluble material was collected by filtration and dried over P₂O₅ under reduced pressure to give *N*-[4-hydroxy-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-L-4-aminomethylphenylalanine *N*-methylamide hydrochloride (1.15 g, 94%) as colorless solid. To a solution of the hydrochloride salt (1.15 g) in DMF (20 mL), ethyl acetimidate hydrochloride (547 mg, 4.43 mmol) and Et₃N (903 μ L, 6.50 mmol) were added and stirred at 0 °C for 1 h. The reaction mixture was adjusted to pH 2 by adding 1 N aq HCl, which was charged on DIAION HP-20 (Mitsubishi Chemicals; 216 mL) and purified (eluent; 10–80% aq MeOH) to give 16a (1.11 g, 65%) as a colorless amorphous powder upon freeze-drying: $[\alpha]_{25}^D$ –16.5° (c =1.0, MeOH). ¹H NMR (MeOH-*d*₄) δ ppm; 0.76 (3H, d, J =6.3 Hz), 0.80 (3H, d, J =6.2 Hz), 0.94–1.16 (1H, m), 1.29–1.79 (6H, m), 2.17 (3H, s), 2.26–2.77 (7H, m), 2.89–3.10 (2H, m), 4.38 (2H, s), 4.49–4.59 (1H, m), 6.84–7.54 (9H, m). HRFABMS: calcd for C₃₀H₄₃N₄O₄ 523.3284, found 523.3301.

***N*-[4-Hydroxy-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-L-4'-propionimidoyliminomethylphenylalanine *N*-methylamide hydrochloride (16b).** Yield; 59% overall yield from 15d: $[\alpha]_{25}^D$ –14.1° (c =0.2, MeOH). ¹H NMR (MeOH-*d*₄) δ ppm; 0.89 (6H, d, J =6.6 Hz), 1.02–1.84 (10H, m), 2.26–2.75 (9H, m), 2.95 (2H, d, J =5.6 Hz),

4.38 (2H, s), 4.52–4.60 (1H, m), 6.99–7.58 (9H, m). HRFABMS: calcd for C₃₁H₄₅N₄O₄ 537.3441, found 537.3445.

***N*-[4-Hydroxy-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-L-4'-benzimidoyliminomethylphenylalanine *N*-methylamide hydrochloride (16c).** Yield; 53% overall yield from 15d: $[\alpha]_{25}^D$ –9.0° (c =1.0, MeOH). ¹H NMR (MeOH-*d*₄) δ ppm; 0.84 (3H, d, J =6.6 Hz), 0.89 (3H, d, J =6.4 Hz), 0.95–1.80 (7H, m), 2.14–2.81 (7H, m), 2.95 (2H, d, J =5.6 Hz), 4.30 (2H, s), 4.48–4.63 (1H, m), 6.98–7.82 (14H, m). HRFABMS: calcd for C₃₅H₄₅N₄O₄ 585.3441, found 585.3409.

***N*-[4-Hydroxy-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-L-4'-[*N,N'*-bis(benzyloxycarbonyl)guanidinomethyl]phenylalanine *N*-methylamide (16d).** To a solution of *N*-[4-hydroxy-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-L-4-aminomethylphenylalanine *N*-methylamide hydrochloride (500 mg, 0.97 mmol) in DMF (8 mL), 1*H*-pyrazole-*N,N'*-bis(benzyloxycarbonyl)carboxamidine (470 mg, 1.16 mmol) and Et₃N (162 μ L, 1.16 mmol) were added under stirring in an ice bath and stirred at room temperature overnight. The reaction mixture was concentrated under reduced pressure followed by precipitating from Et₂O to give 16d as a colorless solid (650 mg, 86%): mp 141–145 °C, $[\alpha]_{25}^D$ –14.0° (c =0.71, DMF). ¹H NMR (DMSO-*d*₆) δ ppm; 0.74 (3H, d, J =6.7 Hz), 0.78 (3H, d, J =6.7 Hz), 0.91–1.02 (1H, m), 1.31–1.81 (6H, m), 2.14–2.30 (1H, m), 2.30–2.80 (m), 2.85–3.12 (2H, m), 4.32–4.71 (3H, m), 5.03 (2H, s), 5.19 (2H, s), 6.82–7.64 (20H, m), 7.80–7.95 (1H, m), 8.42–8.70 (2H, m). HRFABMS: calcd for C₄₅H₅₄N₅O₈ 792.3972, found 792.3996.

Typical procedure for the preparation of compound 17. ***N*-[4-(*N*-Benzyloxyamino)-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-L-tyrosine *N*-methylamide (17a).** To the compound 15a (8.92 g, 17.0 mmol), ice-cooled 95% aq TFA (30 mL) was added and stirred at 5 °C for 3 h, then evaporated in vacuo. The residue was precipitated from Et₂O followed by filtration to give carboxylic acid intermediate (7.33 g, 92%) as colorless solid. To a mixture of this intermediate (4.68 g, 10 mmol), *O*-benzylhydroxylamine hydrochloride (3.20 g, 20 mmol), HOBT (2.70 g, 20 mmol) and DMF (70 mL), EDC (2.11 g, 11 mmol) was added under stirring at –15 °C followed by adding Et₃N (2.80 mL, 20 mmol). The mixture was stirred at –15 °C for 1 h and further at room temperature overnight. The reaction mixture was diluted with EtOAc (210 mL) and washed successively with brine, 1N aq HCl, satd aq NaHCO₃ and brine (twice for each), then evaporated in vacuo to leave a colorless residue, which was precipitated from Et₂O to give 17a (3.73 g, 65%) as a colorless solid: mp 217–220 °C, $[\alpha]_{25}^D$ +5.6° (c =1.0, DMF). ¹H NMR (MeOH-*d*₄) δ ppm; 0.76 (3H, d, J =6.3 Hz), 0.79 (3H, d, J =6.4 Hz), 0.84–1.03 (1H, m), 1.47–1.74 (6H, m), 2.01–2.12 (1H, m), 2.28–2.70 (3H, m), 2.71 (3H, s), 2.80–2.93 (1H, m), 2.93–3.08 (1H, m), 4.60 (1H, m), 4.84 (2H, s), 6.69–6.97 (4H, m), 7.00–7.52 (10H, m). HRFABMS calcd for C₃₄H₄₄N₃O₅: 574.3281, found: 574.3307.

***N*-[4-(*N*-Benzyloxyamino)-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-*L*-4'-sulfo-phenylalanine *N*-methylamide, monosodium salt (17b).** Yield; 46%. ¹H NMR (MeOH-*d*₄) δ ppm; 0.76–0.98 (6H, m), 1.01–1.19 (1H, m), 1.48–1.86 (6H, m), 2.08–2.20 (1H, m), 2.24–2.67 (3H, m), 2.71 (3H, s), 2.96–3.12 (2H, m), 4.48–4.66 (1H, m), 5.04 (2H, s), 7.06–7.98 (14H, m). FAB-MS: *m/z* 660 [M + Na]⁺, 638 [M + 1]⁺. HRFABMS: calcd for C₃₄H₄₃N₃NaO₇S 660.2719, found 660.2731.

***N*-[4-(*N*-Benzyloxyamino)-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-*L*-4'-nitrophenylalanine *N*-methylamide (17c).** Yield; 63%. ¹H NMR (MeOH-*d*₄) δ ppm; 0.78 (6H, d, *J* = 6.5 Hz), 0.92–1.12 (1H, m), 1.38–1.72 (6H, m), 2.01–2.14 (1H, m), 2.50–2.78 (6H, m), 3.01–3.22 (2H, m), 4.65–4.78 (1H, m), 5.08 (2H, s), 7.26–7.70 (12H, m), 7.92–8.13 (2H, m). HRFABMS: calcd for C₃₄H₄₃N₄O₆ 603.3183, found 603.3191.

***N*-[4-(*N*-Benzyloxyamino)-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-*L*-4'-cyanophenylalanine *N*-methylamide (17d).** Yield; 77%. ¹H NMR (MeOH-*d*₄) δ ppm; 0.79 (3H, d, *J* = 6.4 Hz), 0.81 (3H, d, *J* = 6.4 Hz), 1.01–1.22 (1H, m), 1.36–1.80 (6H, m), 2.08–2.18 (1H, m), 2.20–2.64 (6H, m), 2.71 (3H, s), 3.05–3.22 (2H, m), 4.67–4.85 (m), 7.20–7.81 (14H, m). HRFABMS: calcd for C₃₅H₄₃N₄O₄ 583.3284, found 583.3277.

***N*-[4-(*N*-Benzyloxyamino)-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-*L*-4'-[*N,N'*-bis(benzyloxycarbonyl)guanidinol]-phenylalanine *N*-methylamide (17e).** Yield; 76%. ¹H NMR (DMSO-*d*₆) δ ppm; 0.63–0.89 (6H, m), 1.01–1.20 (1H, m), 1.42–1.78 (6H, m), 2.10–2.24 (1H, m), 2.30–2.58 (m), 2.64 (3H, s), 2.91–3.16 (2H, m), 4.40–4.56 (1H, m), 4.81 (2H, s), 5.12 (2H, s), 5.18 (2H, s), 6.96–7.88 (24H, m). HRFABMS: calcd for C₅₁H₅₉N₆O₈ 883.4394, found 883.4420.

***N*-[4-(*N*-Benzyloxyamino)-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-*L*-4'-[*N*-(benzyloxycarbonyl)amidino]phenylalanine *N*-methylamide (17f).** Yield; 38%. ¹H NMR (CDCl₃) δ ppm; 0.84 (3H, d, *J* = 6.4 Hz), 0.86 (3H, d, *J* = 6.4 Hz), 1.10–1.21 (1H, m), 1.24–1.75 (6H, m), 2.01–2.14 (1H, m), 2.26–2.55 (3H, m), 2.73 (3H, d, *J* = 4.7 Hz), 3.01–3.20 (2H, m), 4.56–4.72 (1H, m), 4.86 (2H, s), 5.02 (2H, s), 5.73–5.96 (2H, m), 6.21–6.37 (1H, m), 6.48–6.65 (1H, m), 6.95–7.80 (20H, m). HRFABMS: calcd for C₄₃H₅₂N₅O₆ 734.3918, found 734.3924.

***N*-[4-(*N*-Benzyloxyamino)-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-*L*-4'-acetimidoyliminomethylphenylalanine *N*-methylamide hydrochloride (17g).** Yield; 76%. ¹H NMR (MeOH-*d*₄) δ ppm; 0.78–0.96 (6H, m), 0.96–1.13 (1H, m), 1.54–1.80 (5H, m), 1.89 (3H, s), 2.03–2.18 (4H, m), 2.54–2.83 (6H, m), 2.98–3.14 (2H, m), 4.36 (2H, s), 4.42–4.61 (1H, m), 6.93–7.69 (14H, m). HRFABMS: calcd for C₃₇H₅₀N₅O₄ 628.3863, found 628.3821.

***N*-[4-(*N*-Benzyloxyamino)-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-*L*-4'-propionimidoyliminomethylphenylalanine *N*-methylamide hydrochloride (17h).** Yield; 76%. ¹H NMR (MeOH-*d*₄) δ ppm; 0.87 (3H, d, *J* = 6.4 Hz),

0.89 (3H, d, *J* = 6.6 Hz), 1.04–1.75 (10H, m), 2.20–2.81 (9H, m), 2.84–2.99 (2H, m), 4.40 (2H, s), 4.56–4.75 (1H, m), 5.02 (2H, s), 6.92–7.65 (14H, m). HRFABMS: calcd for C₃₈H₅₂N₅O₄ 642.4019, found 642.3997.

***N*-[4-(*N*-Benzyloxyamino)-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-*L*-4'-benzimidoyliminomethylphenylalanine *N*-methylamide hydrochloride (17i).** Yield; 92%. ¹H NMR (MeOH-*d*₄) δ ppm; 0.84 (3H, d, *J* = 6.4 Hz), 0.86 (3H, d, *J* = 6.7 Hz), 0.96–1.80 (7H, m), 2.01–2.14 (1H, m), 2.42–2.66 (3H, m), 2.68 (3H, s), 2.83–2.98 (2H, m), 4.40 (2H, s), 4.43–4.54 (1H, m), 5.01 (2H, s), 6.97–8.00 (19H, m). HRFABMS: calcd for C₄₂H₅₂N₅O₄ 690.4019, found 690.4028.

***N*-[4-(*N*-Benzyloxyamino)-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-*L*-4'-[*N,N'*-bis(benzyloxycarbonyl)guanidinomethyl]phenylalanine *N*-methylamide (17j).** Yield; 76%. ¹H NMR (DMSO-*d*₆) δ ppm; 0.59–0.79 (6H, m), 0.90–1.12 (1H, m), 1.54–1.81 (6H, m), 1.85–2.03 (1H, m), 2.40–2.76 (m), 2.81–3.03 (2H, m), 4.25–4.63 (3H, m), 4.76 (2H, s), 5.02 (2H, s), 5.19 (2H, s), 6.81–7.56 (26H, m), 7.81–8.63 (3H, m). HRFABMS: calcd for C₅₂H₆₁N₆O₈ 897.4551, found 897.4564.

***N*-[4-(*N*-Hydroxyamino)-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-*O*-sulfo-*L*-tyrosine *N*-methylamide sodium salt (18a).** To a suspension of 17a (1.96 g, 3.30 mmol) in DMF (20 mL), sulfur trioxide pyridine complex (1.60 g, 9.90 mmol) was added and stirred at room temperature for 1.5 h. The reaction mixture was diluted with 1N aq NaHCO₃ (80 mL) then charged on reverse-phase column chromatography and purified (eluent; 0–50% aq MeOH) to give *N*-[4-(*N*-benzyloxyamino)-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-*O*-sulfo-*L*-tyrosine-*N*-methylamide sodium salt (1.85 g, 83%) as a colorless powder. The obtained compound (1.60 g, 2.37 mmol) was dissolved in MeOH (30 mL) and hydrogenated in the presence of 5% Pd-C (50% wet, 200 mg) at room temperature for 1 h. After the catalyst was filtered off, MeOH was removed by evaporation. The residue was dissolved in water (20 mL) and lyophilized to give 18a (1.28 g, 92%) as a colorless amorphous powder: [α]_D²⁵ –41° (*c* = 1.0, MeOH). ¹H NMR (MeOH-*d*₄) δ ppm; 0.74 (3H, d, *J* = 6.4 Hz), 0.77 (3H, d, *J* = 6.4 Hz), 0.98–1.12 (1H, m), 1.34–1.78 (6H, m), 2.00–2.14 (1H, m), 2.38–2.72 (3H, m), 2.74 (3H, s), 2.84–2.93 (1H, m), 2.93–3.14 (1H, m), 4.44–4.56 (1H, m), 7.14–7.32 (9H, m). FABMS: *m/z* 586 [M + Na]⁺, 564 [M + 1]⁺. Anal. (C₂₇H₃₆N₃NaO₈·0.4H₂O) C, H, N.

Preparation of compound 18b–j. Removal of *O*-benzyl group for compounds 17b–j were performed by the catalytic hydrogenation method utilizing 5% Pd-C as that described for the preparation of 18a.

***N*-[4-(*N*-Hydroxyamino)-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-*L*-4'-sulfo-phenylalanine *N*-methylamide sodium salt (18b).** Yield; 43%. [α]_D²⁵ –3.73° (*c* = 1.0, MeOH). ¹H NMR (MeOH-*d*₄) δ ppm; 0.86 (3H, d, *J* = 6.4 Hz), 0.90 (3H, d, *J* = 6.4 Hz), 0.98–1.08 (1H, m), 1.12–1.48 (6H, m), 1.93–2.07 (1H, m), 2.23–2.51 (3H, m), 2.70 (3H, s), 3.04–3.22 (2H, m), 4.68 (1H, dd, *J* = 5.3,

9.2 Hz), 7.14–7.86 (9H, m). FABMS: m/z 570 $[M + Na]^+$, 548 $[M + 1]^+$. Anal. ($C_{27}H_{36}N_3NaO_7S \cdot 0.3H_2O$) C, H, N.

***N*-[4-(*N*-Hydroxyamino)-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-*L*-4'-aminophenylalanine *N*-methylamide monoacetate (18c).** Yield; 82%. 1H NMR (MeOH- d_4) δ ppm; 0.83 (3H, d, $J=6.3$ Hz), 0.85 (3H, d, $J=6.4$ Hz), 0.96–1.10 (1H, m), 1.14–1.47 (6H, m), 1.90–2.06 (4H, m), 2.22–2.54 (3H, m), 2.71 (3H, s), 2.83 (1H, dd, $J=10.2$, 14.0 Hz), 3.12 (1H, dd, $J=4.4$, 14.0 Hz), 4.51 (1H, dd, $J=4.4$, 10.2 Hz), 7.15–7.32 (7H, m), 7.34–7.42 (2H, m). FABMS: m/z 483 $[M + 1]^+$. Anal. ($C_{29}H_{42}N_4O_6$) C, H, N.

***N*-[4-(*N*-Hydroxyamino)-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-*L*-4'-aminomethylphenylalanine *N*-methylamide monoacetate (18d).** Yield; 80%. 1H NMR (MeOH- d_4) δ ppm; 0.82 (3H, d, $J=6.2$ Hz), 0.86 (3H, d, $J=6.4$ Hz), 0.96–1.09 (1H, m), 1.13–1.50 (6H, m), 1.90–2.08 (4H, m), 2.20–2.56 (3H, m), 2.69 (3H, s), 2.81 (1H, dd, $J=9.6$, 13.9 Hz), 3.10 (1H, dd, $J=4.4$, 13.9 Hz), 3.99 (2H, s), 4.52 (1H, dd, $J=4.4$, 9.6 Hz), 7.12–7.30 (7H, m), 7.33–7.45 (2H, m). FABMS: m/z 497 $[M + 1]^+$. Anal. ($C_{30}H_{44}N_4O_6$) C, H, N.

***N*-[4-(*N*-Hydroxyamino)-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-*L*-4'-guanidinophenylalanine *N*-methylamide monoacetate (18e).** Yield; 93%. $[\alpha]_{25}^D -10.2^\circ$ ($c=1.0$, MeOH). 1H NMR (MeOH- d_4) δ ppm; 0.83 (3H, d, $J=6.7$ Hz), 0.88 (3H, d, $J=6.4$ Hz), 0.92–1.19 (1H, m), 1.22–1.58 (6H, m), 1.89 (3H, s), 1.93–2.08 (1H, m), 2.24–2.56 (3H, m), 2.71 (3H, s), 2.83 (1H, dd, $J=10.1$, 14.0 Hz), 3.12 (1H, dd, $J=4.8$, 14.0 Hz), 4.68 (1H, dd, $J=4.8$, 10.1 Hz), 7.13–7.30 (7H, m), 7.32–7.44 (2H, m). FABMS: m/z 525 $[M + 1]^+$. Anal. ($C_{30}H_{44}N_6O_6$) C, H, N.

***N*-[4-(*N*-Hydroxyamino)-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-*L*-4'-amidinophenylalanine *N*-methylamide monoacetate (18f).** Yield; 96%. $[\alpha]_{25}^D -7.04^\circ$ ($c=1.0$, MeOH). 1H NMR (MeOH- d_4) δ ppm; 0.82 (3H, d, $J=6.5$ Hz), 0.86 (3H, d, $J=6.4$ Hz), 0.92–1.10 (1H, m), 1.33–1.70 (6H, m), 1.91 (3H, s), 1.97–2.11 (1H, m), 2.38–2.59 (3H, m), 2.68 (3H, s), 2.88 (1H, dd, $J=11.0$, 14.2 Hz), 3.13 (1H, dd, $J=4.5$, 14.2 Hz), 4.74 (1H, dd, $J=4.5$, 11.0 Hz), 6.96–7.85 (9H, m). FABMS: m/z 510 $[M + 1]^+$. Anal. ($C_{30}H_{43}N_5O_6$) C, H, N.

***N*-[4-(*N*-Hydroxyamino)-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-*L*-4'-acetimidoyliminomethylphenylalanine *N*-methylamide monoacetate (18g).** Yield; 91%. $[\alpha]_{25}^D -8.9^\circ$ ($c=1.0$, MeOH). 1H NMR (MeOH- d_4) δ ppm; 0.81 (3H, d, $J=6.4$ Hz), 0.85 (3H, d, $J=6.4$ Hz), 0.94–1.13 (1H, m), 1.16–1.54 (6H, m), 1.90 (3H, s), 1.97–2.11 (1H, m), 2.24–2.50 (6H, m), 2.71 (3H, s), 2.90 (1H, dd, $J=11.0$, 13.9 Hz), 3.12 (1H, dd, $J=4.6$, 13.9 Hz), 4.36 (2H, s), 4.77 (1H, dd, $J=4.6$, 11.0 Hz), 6.89–7.62 (9H, m). FABMS: m/z 538 $[M + 1]^+$. Anal. ($C_{32}H_{47}N_5O_6$) C, H, N.

***N*-[4-(*N*-Hydroxyamino)-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-*L*-4'-propionimidoyliminomethylphenylalanine *N*-methylamide monoacetate (18h).** Yield; 90%. $[\alpha]_{25}^D -9.7^\circ$ ($c=1.0$, MeOH). 1H NMR (MeOH- d_4) δ

ppm; 0.82 (3H, d, $J=6.4$ Hz), 0.87 (3H, d, $J=6.6$ Hz), 0.95–1.21 (4H, m), 1.24–1.58 (6H, m), 1.88 (3H, s), 1.96–2.07 (1H, m), 2.24–2.76 (8H, m), 2.89 (1H, dd, $J=10.8$, 14.0 Hz), 3.10 (1H, dd, $J=4.6$, 14.0 Hz), 4.38 (2H, s), 4.71 (1H, dd, $J=4.6$, 10.8 Hz), 6.91–7.55 (9H, m). FABMS: m/z 552 $[M + 1]^+$. Anal. ($C_{33}H_{49}N_5O_6$) C, H, N.

***N*-[4-(*N*-Hydroxyamino)-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-*L*-4'-benzimidoyliminomethylphenylalanine *N*-methylamide monoacetate (18i).** Yield; 79%. $[\alpha]_{25}^D -4.3^\circ$ ($c=1.1$, MeOH). 1H NMR (MeOH- d_4) δ ppm; 0.83 (3H, d, $J=6.4$ Hz), 0.86 (3H, d, $J=6.6$ Hz), 0.94–1.07 (1H, m), 1.22–1.59 (6H, m), 1.93 (3H, s), 2.00–2.14 (1H, m), 2.37–2.62 (3H, m), 2.70 (3H, s), 2.90 (1H, dd, $J=10.7$, 14.0 Hz), 3.12 (1H, dd, $J=5.3$, 14.0 Hz), 4.41 (2H, s), 4.70 (1H, dd, $J=5.3$, 10.7 Hz), 6.92–7.74 (14H, m). FABMS: m/z 600 $[M + 1]^+$. Anal. ($C_{37}H_{49}N_5O_6$) C, H, N.

***N*-[4-(*N*-Hydroxyamino)-2(*R*)-isobutyl-3(*S*)-phenylpropylsuccinyl]-*L*-4'-guanidinomethylphenylalanine *N*-methylamide monoacetate (18j).** Yield; 90%. $[\alpha]_{25}^D -9.3^\circ$ ($c=0.97$, DMF). 1H NMR (MeOH- d_4) δ ppm; 0.81 (3H, d, $J=6.4$ Hz), 0.87 (3H, d, $J=6.6$ Hz), 0.98–1.12 (1H, m), 1.21–1.55 (6H, m), 1.89 (3H, s), 1.96–2.14 (1H, m), 2.24–2.59 (3H, m), 2.69 (3H, s), 2.90 (1H, dd, $J=9.8$, 13.8 Hz), 3.11 (1H, dd, $J=4.7$, 13.8 Hz), 4.36 (2H, s), 4.68 (1H, dd, $J=4.7$, 9.8 Hz), 6.97–7.59 (9H, m). FABMS: m/z 539 $[M + 1]^+$. Anal. ($C_{31}H_{46}N_6O_6$) C, H, N.

MMP inhibition assay

Inhibitory activity of a synthetic compound to MMP-1 and MMP-3 was determined by the method of Nagai et al.³² using a fluorescent isothiocyanate (FITC)-labeled type I collagen (from bovine achilles tendon, Sigma) and that of Twining³³ using a FITC-labeled casein (Sigma, from bovine milk) as substrate, respectively. ProMMP-1 and proMMP-3 were purified from the media of human skin fibroblast NB1RGB cells (RIKEN Cell Bank, RCB0222) stimulated with IL-1 α (Genzyme Corp. MPLS, MN),³⁴ and their precursor enzymes were activated with 1 mM 4-aminophenylmercuric acetate (APMA, Nacalai Tesque, Inc., Kyoto, Japan) at 37°C for 2 h.

MMP-7, MMP-13 and MT1-MMP inhibitory activities were measured by modifying the method of Yamamoto et al.³⁵ using a fluorogenic substrate, (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-(3-[2,4-dinitrophenyl]-*L*-2,3-diaminopropionyl)-Ala-Arg-NH₂ (Peptide Institute, Inc., Osaka, Japan).³⁶ Recombinant human MMP-7 and MMP-13 were purchased from Oriental Yeast Co., Ltd (Shiga, Japan) and Genzyme Corp. (MPLS, MN), respectively. Recombinant human MT1-MMP lacking the transmembrane domain (Ala⁵³⁶-Val⁵⁸²) was expressed and purified from *Escherichia coli*.³⁴ MMP-13, but not MMP-7 and MT1-MMP, was activated by incubation with 1 mM APMA for 2 h at 37°C. One unit of MMP-7, -13 and MT1-MMP was defined as the amount of the enzyme required to degrade 1 pmol of the substrate in

1 min at 37 °C. A mixture of enzyme solution (one unit/40 μ L) and inhibitor solution (10 μ L) was incubated with 50 μ L of substrate solution in 50 mM Tris-HCl (pH 7.5) buffer containing 0.15 M NaCl, 10 mM CaCl₂, 0.05% Brij-35 and 0.02% NaN₃. Each substrate concentration in MMP-7, MMP-13 and MT1-MMP assay were adjusted to 10, 3 and 5 μ M, respectively. Enzyme reaction was performed at 37 °C, for 0.5 h in MMP-7 and MT1-MMP assay and for 2 h in MMP-13 assay, and quenched with 100 μ L of 3% aqueous acetic acid. The emergence of fluorescence intensity (ex 325 nm, em 405 nm) was measured using Biolumin 960 (Molecular Dynamics Pth. Ltd., Key East, VIC).

MMP-2 and MMP-9 inhibitory activities by the synthetic compounds were measured using gelatin zymography.³⁷ Briefly, the media of HT-1080 cells containing MMP-2 and MMP-9 were applied to nonreduced SDS-PAGE using a 7.5% gel containing 0.1% gelatin. After electrophoresis at 4 °C, the gels were soaked in 2.5% Triton X-100 solution with gently shaking for 1 h at room temperature. In the presence or absence of a test compound, the gels were incubated in reaction buffer (50 mM Tris-HCl, 5 mM CaCl₂, 1 μ M ZnCl₂, 0.05% NaN₃, pH 7.5) for 18 h at 37 °C and were stained with 0.1% Coomassie Brilliant Blue. A gelatinolytic activity was visualized as transparent bands on the blue background and was quantified by NIH image software (Ver. 1.6).

TNF α bioassay

An inhibitory effect of a test compound on lipopolysaccharide (LPS, from *E. coli* 0111:B4, DIFCO, Detroit, MI)-induced TNF α release was measured in human acute monocytic leukemia THP-1 cells (ATCC No. TIB202). In the presence of 1 μ g/mL LPS and in the presence or absence of a serially diluted test compound, THP-1 cells were cultured in 24-well flat-bottomed trays (Falcon 3047, Becton Dickinson, Sanjose, CA) at 2×10^6 cells/well (in 1-mL volume) in a humidified atmosphere at 37 °C with 5% CO₂. After 6 h incubation, TNF α levels in the supernatants were measured using mouse fibrosarcoma WEHI-164 cells (ATCC No. CRL1751) lytic assay.³⁸ WEHI-164 cells were cultured in 96 well flat-bottomed trays (Falcon 3075, Becton Dickinson, Sanjose, CA) at 20,000 cells/well (in 0.2-mL volume) in the presence of 1 μ g/mL of actinomycin D-mannitol (Sigma Chemical Co., St Louis, MO) and a serially diluted test sample in a humidified atmosphere at 37 °C with 5% CO₂. Recombinant human TNF α (specific activity 1×10^7 U/mg, Genzyme Corp., MPLS, MN) was used as an internal standard. After 20 h incubation, 20 μ L WST-1 (3.3 mg/mL, DOJINDO, Kumamoto, Japan) was added to the wells and incubated for 4 h, and then optical density of culture assessed at 450 nm.

Pharmacokinetic evaluation

Ex vivo pharmacokinetic studies were carried out in male Lewis rats. A compound was subcutaneously administered at 30 mg/kg, after which blood was taken from the jugular vein under ether anesthesia at various time points (typically 0.5, 1, 2 and 4 h later). The com-

pound in plasma was separated by centrifugation through a 30,000-mol wt cut-off filter (model YMT-30; Amicon Corp., Beverly, MA) for 3 min at 1,500 g.³⁹ The plasma filtrate was serially diluted and tested for inhibition of rabbit MMP-3 using a fluorogenic substrate, (7-methoxycoumarin-4-yl)acetyl-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-N^ε-(2,4-dinitrophenyl)-Lys-NH₂ (Peptide Institute, Inc., Osaka, Japan).⁴⁰ The filtrate dilution necessary for 50% inhibition of rabbit MMP-3 activity was multiplied by the known IC₅₀ concentration to calculate initial plasma drug concentrations (The IC₅₀ values of compound **18b**, **18e**, Marimastat and Trocade against rabbit MMP-3 were 8, 23, 34 and 353 nM, respectively). Control experiments showed no effect of filtrate alone on the MMP-3 assay and <10% binding of the synthetic compounds to plasma filtrate or the filter apparatus. It should be emphasized that this method does not directly quantify the amount of parent compound.

Adjuvant arthritis

Male Lewis rats (Charles River Laboratories, Japan) weighing about 240 g were used. Adjuvant arthritis was induced in the rats by an intradermal injection of 0.6 mg *M. tuberculosis* (H37RA, Difco Laboratories, Detroit, MI) in 0.05 mL liquid paraffin into the base of the tail.⁴⁰ Hindpaw swelling was measured plethymographically by water displacement using a volume meter (TK-101; Muromachi Kikai Co., Ltd., Tokyo, Japan) after adjuvant injection. Area under the hindpaw swelling time curve (AUC) was calculated by the linear trapezoidal method. At the end of the experiment (on day 20 after adjuvant injection), hindpaws were fixed in 10% buffered formalin and those radiographs were taken in Fuji Biomedix Co., Ltd. (Yamanashi, Japan). The scoring of bone degradation in the radiographs was referred to the method by Kashima.⁴¹ The hindpaw joint was divided to four area of the distal tibia, talus, calcaneus and metatarsus bones. Each area were graded by the parameter of bone demineralization, bone erosion and joint space narrowing in a blind fashion from 0 to 4 (0=no change, 1=slight, 2=mild, 3=moderate, 4=marked lesion).

References and Notes

1. Birkedal-Hansen, H.; Moore, W. G. I.; Bodden, M. K.; Windsor, L. J.; Birkedal-Hansen, B.; DeCarlo, A.; Engler, J. A. *Crit. Rev. Oral Biol. Med.* **1993**, *4*, 197.
2. Morphy, J. R.; Millican, T. A.; Porter, J. R. *Curr. Med. Chem.* **1995**, *2*, 743.
3. Hagmann, W. K.; Lark, M. W.; Becker, J. W. *Ann. Rep. Med. Chem.* **1996**, *31*, 231.
4. Babin, R. E.; Bender, S. L. *Chem. Rev.* **1997**, *97*, 1420.
5. Whittaker, M.; Floyd, C. D.; Brown, P.; Gearing, A. J. H. *Chem. Rev.* **1999**, *99*, 2735.
6. Yu, A. E.; Hewitt, R. E.; Connor, E. W.; Stetler-Stevenson, W. G. *Drugs & Aging* **1997**, *11*, 229.
7. Burns, F. R.; Stack, M. S.; Gray, R. D.; Paterson, C. A. *Invest. Ophthalmol. Visual Sci.* **1989**, *32*, 1569.
8. Cawston, T. E. *Pharmacol. Ther.* **1996**, *70*, 163.
9. Overall, C. M.; Wiebkin, O. W.; Thonard, J. C. *J. Periodontal Res.* **1987**, *22*, 81.

10. Liedtke, W.; Cannella, B.; Mazzaccaro, R. J.; Clements, J. M.; Miller, K. M.; Wucherpfennig, K. W.; Andrew, J. H.; Gearing, A. J. H.; Raine, C. S. *Ann. Neurol.* **1998**, *44*, 35.
11. Lohmander, L. S.; Hoerrner, L. A.; Lark, M. W. *Arthritis Rheum.* **1993**, *36*, 181.
12. Henderson, B.; Docherty, A. J. P.; Beeley, N. R. A. *Drugs Future* **1990**, *15*, 495.
13. Wu, J.; Lark, M. W.; Chun, L. E.; Eyre, D. R. *J. Biol. Chem.* **1991**, *266*, 5625.
14. Hasty, K. A.; Reife, R. A.; Kang, A. H.; Stuart, J. M. *Arthritis Rheum.* **1990**, *33*, 388.
15. Feldmann, M.; Brennan, F. M.; Elliott, M.; Katsikis, P.; Maini, R. N. *Circ. Shock* **1994**, *43*, 179.
16. Black, R. A.; Rauch, C. T.; Kozlosky, C. J.; Peschon, J. J.; Slack, J. L.; Wolfson, M. F.; Castner, B. J.; Stocking, K. L.; Reddy, P.; Srinivasan, S.; Nelson, N.; Boiani, N.; Schooley, K. A.; Gerhart, M.; Davis, R.; Fitzner, J. F.; Johnson, R. S.; Paxton, R. J.; March, C. J.; Cerretti, D. P. A. *Nature* **1997**, *385*, 729.
17. Aggarwal, B. B.; Kohr, W. J.; Hass, P. E.; Moffat, B.; Spencer, S. A.; Henzel, W. J.; Bringman, T. S.; Nedwin, G. E.; Goeddel, D. V.; Harkins, R. N. *J. Biol. Chem.* **1985**, *260*, 2345.
18. Nelson, F. C.; Zask, A. *Exp. Opin. Invest. Drugs* **1999**, *8*, 383.
19. Zook, S. E.; Dagnino, R., Jr.; Deason, M. E.; Bender, S. L. Int. Pat. Appl. WO 97/20824, 1997.
20. MacPherson, L. J.; Bayburt, E. K.; Capparelli, M. P.; Carroll, B. J.; Goldstein, R.; Justice, M. R.; Zhu, L.; Hu, S.; Melton, R. A.; Fryer, L.; Goldberg, R. L.; Doughty, J. R.; Spirito, S.; Blancuzzi, V.; Wilson, D.; O'Byrne, E. M.; Ganu, V.; Parker, D. T. *J. Med. Chem.* **1997**, *40*, 2525.
21. Ngo, J.; Castaner, G. J. *Drugs Future* **1996**, *21*, 1215.
22. Pratt, L. M.; Beckett, R. P.; Bellamy, C. L.; Corkill, D. J.; Cossins, C.; Courtney, P. F.; Davies, S. J.; Davidson, A. H.; Drummond, A. H.; Helfrich, K.; Lewis, C. N.; Mangan, M.; Martin, F. M.; Miller, K.; Nayee, P.; Ricketts, M. L.; Thomas, W.; Todd, R. S.; Whittaker, M. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1359.
23. Beckett, R. P.; Davidson, A. H.; Drummond, A. H.; Huxley, P.; Whittaker, M. *Drug Discovery Today* **1996**, *1*, 16.
24. Dickens, J. P.; Crimmin, M. J.; Beckett, R. P. UK Patent 2,287,023, 1995.
25. Xue, C.-B.; He, X.; Roderick, J.; DeGrado, W. F.; Cherney, R. J.; Hardmann, K. D.; Nelson, D. J.; Copeland, R. A.; Jaffee, B. D.; Decicco, C. P. *J. Med. Chem.* **1998**, *41*, 1745.
26. Campion, C.; Davidson, A. H.; Dickens, J. P.; Crimmin, M. J. U.S. Patent 5,240,958, 1993.
27. Beckett, R. P.; Crimmin, M. J.; Davis, M. H.; Spavold, Z. *Synlett* **1993**, 137.
28. Drake, B.; Patek, M.; Lebl, M. *Synthesis* **1994**, 579.
29. DiMartino, M. J.; High, W.; Galloway, W. A.; Crimmin, M. J. *Ann. N.Y. Acad. Sci.* **1994**, *732*, 411.
30. Conway, J. G.; Wakefield, J. A.; Brown, R. H.; Marron, B. E.; Sekut, L.; Stimpson, S. A.; McElroy, A.; Menius, J. A.; Jeffreys, J. J.; Clark, R. L.; McGeehan, G. M.; Connolly, K. M. *J. Exp. Med.* **1995**, *182*, 449.
31. DiMartino, M.; Wolff, C.; High, W.; Stroup, G.; Hoffman, S.; Laydon, J.; Lee, J. C.; Bertolini, D.; Galloway, W. A.; Crimmin, M. J.; Davis, M.; Davies, S. *Inflamm. Res.* **1997**, *46*, 211.
32. Nagai, Y.; Hori, H.; Hattori, S.; Sunada, Y.; Terato, K.; Hashida, R.; Miyamoto, K. *Jpn. J. Inflammation* **1984**, *4* (2), 123.
33. Twining, S. S. *Anal. Biochem.* **1984**, *143*, 30.
34. Aoki, T.; Yonezawa, K.; Ohuchi, E.; Fujimoto, N.; Iwata, K.; Shimada, T.; Shiomi, T.; Okada, Y.; Seiki, M. *J. Immun. Immunochem.* (In press).
35. Yamamoto, M.; Tsujishita, H.; Hori, N.; Ohishi, Y.; Inoue, S.; Ikeda, S.; Okada, Y. *J. Med. Chem.* **1998**, *41*, 1209.
36. Knight, C. G.; Willenbrock, F.; Murphy, G. *FEBS Lett.* **1992**, *296* (3), 263.
37. Ata, N.; Oku, T.; Hattori, M.; Fujii, H.; Nakajima, M.; Saiki, I. *Oncol. Res.* **1996**, *6*, 503.
38. Aggarwal, B. B.; Kohr, W. J.; Hass, P. E.; Moffat, B.; Spencer, S. A.; Henzel, W. J.; Bringman, T. S.; Nedwin, G. E.; Goeddel, D. V.; Harkins, R. N. *J. Biol. Chem.* **1985**, *260* (4), 2345.
39. Conway, J. G.; Wakefield, J. A.; Brown, R. H.; Marron, B. E.; Sekut, L.; Stimpson, S. A.; McElroy, A.; Menius, J. A.; Jeffreys, J. J.; Clark, R. L.; McGeehan, G. M.; Connolly, K. M. *J. Exp. Med.* **1995**, *182*, 449.
40. Nagase, H.; Fields, C. G.; Fields, G. B. *J. Biol. Chem.* **1994**, *269*, 20952.
41. Kashima, E.; Saito, Y.; Sakoe, Y.; Kuriyama, K.; Kudo, S.; Suzue, S. *Ensho (Japanese)* **1996**, *16*, 191.