Synthesis and Structure–Activity Relationships of Gelatinase Inhibitors Derived from Matlystatins

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To investigate a series of new inhibitors of gelatinases based on matlystatin B (1b), extensive structure–activity relationship studies were performed. The new derivatives were evaluated *in vitro* for the ability to inhibit gelatinases. The inhibitory activities against thermolysin were also assayed to test the compounds' selectivity. Among the compounds modified at the P'_3 moiety, the *N*-methylamide derivative 5g was virtually twice as effective on gelatinase B as the parent compound 1b (5g, $IC_{50}=0.27\ \mu M$ *vs.* 1b, $IC_{50}=0.57\ \mu M$). Other derivatives, including 1) esters 7a and 7b having the ester portions P'_2 and P'_3, 2) the cyclic amino acids, L-proline or L-pipecolinic acid (13a and 13b) bearing P'_2, and 3) compounds 29a and 29b representing an attachment of the pentyl side chain at C3′ (P'_1 side chain) instead of C2′, all showed decreased potencies. The key discovery was the observation that the introduction of a nonyl group at the P'_1 position yielded a compound (31f, $IC_{50}=0.0012\ \mu M$) with high inhibitory activity against gelatinases and high selectivity over thermolysin. This result suggested that the S'_1 subsites of the gelatinases have a locally deep hydrophobic structure, since on the basis of the optimum inhibitory activity in the alkyl series, the nonyl group seems to fit best into this hydrophobic pocket. Thus 31f exhibited a 475-fold more potent inhibitory activity than 1b towards gelatinase B.

Key words matlystatin; matrix metalloproteinase; gelatinase inhibitor; structure–activity relationship

Matrix metalloproteinases (MMPs) are a family of Zn^{2+}-dependent enzymes responsible for degradation of the protein components of connective tissue. Based on their substrate specificity, MMPs can be classified into three groups; *i.e.*, interstitial collagenase (MMP-1),[1] gelatinases (gelatinase A (MMP-2) and gelatinase B (MMP-9)),[2] and stromelysins (stromelysin-1 (MMP-3),[3] stromelysin-2 and stromelysin-3). MMP-1 degrades interstitial fibrillar collagens such as type I, II, and III collagens. MMP-2 and MMP-9 degrade type IV and V collagens. MMP-3 degrades type IV and X collagens as well as gelatin, proteoglycans, fibronectin, and laminin. MMPs play an important role in the pathogenesis of rheumatoid arthritis, tumor invasion and metastasis, and other diseases. We are interested in low-molecular inhibitors of gelatinases among the family of MMPs, because many reports have revealed a positive correlation between tumor metastatic potential and net increase in enzyme activity.[4] The rational design of a low-molecular inhibitor of collagenase, based on the sequence of cleavage sites in the enzyme, has been carried out and reviewed,[5] but the sequence disparity in the case of gelatinases do not afford any rational basis for inhibitor design. Thus our strategy to design a low-molecular inhibitor of gelatinases had to rely on the structure–activity relationships of certain natural products. In the course of our studies, matlystatins (**1a**, **b**) were isolated from *Actinomadura atramentaria*.[6] Recently a similar inhibitor, BE16627B (**2**) [L-*N*-(*N*-hydroxy-2-isobutylsuccinamoyl)-seryl-L-valine (IC_{50} values reported[7b] were 0.85 and 0.58 μM against gelatinase B and gelatinase A respectively), was isolated from *Streptomyces* sp. by a Banyu group.[7] Compound **2** inhibited metalloproteinase-dependent human tumor growth in nude mice. Thus, gelatinase inhibitors are thought to hold promise as antitumor agents. In the previous report, we described the total synthesis of matlystatins A (**1a**),[8] B (**1b**)[9] and stereo isomers of **1b**.[9b] From studies on the structure–activity relationships of **1b**

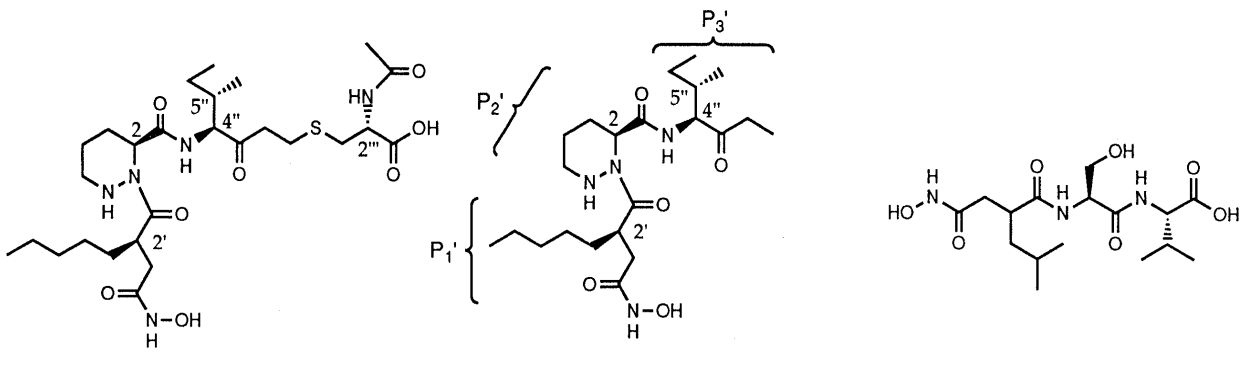

Chart 1

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and its stereo isomers, it is clear that the absolute configuration of C2′*R* is important for potent inhibitory activity.[9b] In this report, we describe the synthesis and structure–activity relationships of gelatinase inhibitors based on matlystatins.

Chemistry

The P'_3-modified derivatives **5a**—**g** were prepared as outlined in Chart 2. The synthesis of the key intermediate **4** in excellent optical purity has already been reported in a paper on the total synthesis of **1b**.[9] Known *N*-protected amino ketones, **3a**[10a] $[[\alpha]_D^{26}$ +32.9° (*c*=0.993, $CHCl_3$), 96% ee by HPLC analysis using a Daicel Chiralcel OJ] and **3b**[10a] $[[\alpha]_D^{26}$ +74.9° (*c*=0.991), >99% ee by HPLC analysis using a Daicel Chiralcel OD],were prepared from the corresponding L-leucine and L-valine derivatives, respectively, *via* Weinreb's amides in 2 steps.[10] After hydrogenation of **3a** to generate the amino ketone, the coupling of the latter to **4** in the presence of diethylphosphoryl cyanide (DEPC)[11] in tetrahydrofuran (THF)–*N*,*N*-dimethylformamide (DMF) (3:1) followed by hydrogenation over 10% Pd–C, afforded **5a** (77% overall yield) as a single diastereomer by HPLC analysis. In the same manner, **5b** was prepared from **4** and **3b** in 44% overall yield after silica gel chromatography and its diastereomeric purity was confirmed by HPLC analysis. Other amide derivatives (**5c**—**g**) were prepared from the corresponding amines **3c**—**g** and the carboxylic acid **4** by using essentially the same procedure.

Syntheses of the ester derivatives, **7a** and **7b**, are summarized in Chart 3. Hydrogenation of the known *tert*-butyl ester **6a**, an intermediate of the total synthesis of **1b**,[9] afforded **7a** in 66% yield. Conversion of the carboxylic acid **4** to the methyl ester **6b** (88% yield) with diazomethane followed by hydrogenation over 10% Pd–C in methanol (MeOH) afforded the desired methyl ester **7b** (64% yield).

Conversion of the P'_2 amino acid moiety of **5f** to other cyclic amino acids was performed. Preparations of the L-proline derivative **13a** and L-pipecolinic acid derivative **13b** and are depicted in Chart 4. The synthesis of (2*R*)-2-[(2,2,2,-trichloroethoxycarbonyl)methyl]heptanoic acid (**8**) has already been reported.[9,12] Conversion of **8** to the acid chloride by treatment with oxalyl chloride in benzene at 60 °C was followed by coupling with L-pipecolinic acid *tert*-butyl ester (**9b**) in the presence of *N*-ethylmorpholine in THF to provide **10b** as a single diastereomer (by ^{1}H-NMR analysis) in 63% yield, after silica gel chromatographic separation.[13] Removal of the 2,2,2-trichloroethyl (Tce) group from **10b** with Zn–1 M NH_4OAc in THF[14] followed by coupling of the resulting carboxylic acid **11b** with *O*-benzylhydroxylamine in THF–DMF (5:1) using DEPC afforded **12b** in 78% overall yield. Conversion of **12b** to the L-pipecolinic acid derivative **13b** was carried out by three subsequent steps as follows. Acid hydrolysis of the *tert*-butyl ester in **12b** with trifluoroacetic acid (TFA) in CH_2Cl_2 was followed by conversion to the *N*,*N*-dimethylamide using DEPC as a coupling reagent. Subsequent hydrogenation over 10% Pd–C in MeOH provided **13b** in 22% yield for the three-step process. The desired L-proline derivative **13a** was prepared from **8** and **9a** by essentially the same

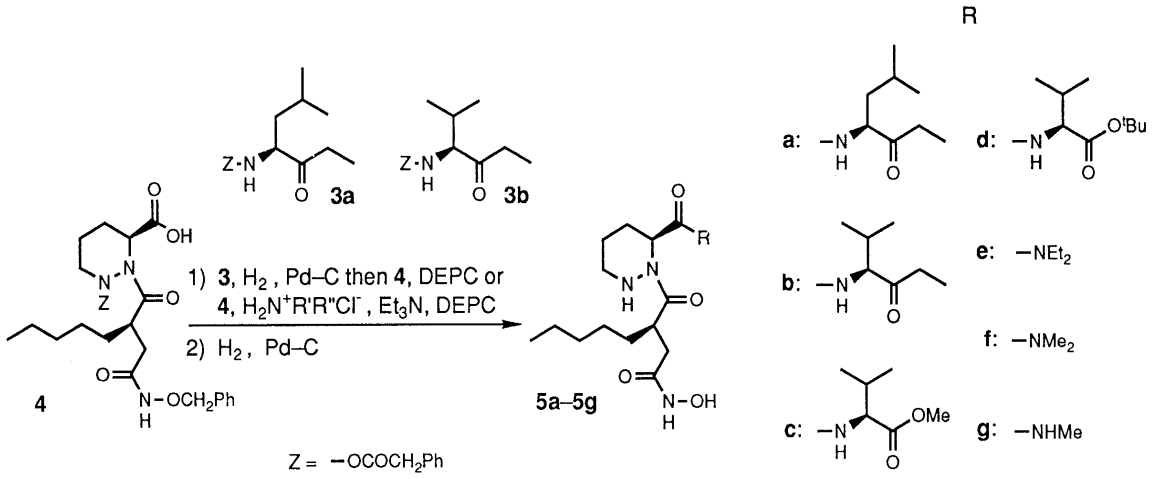


Chart 2

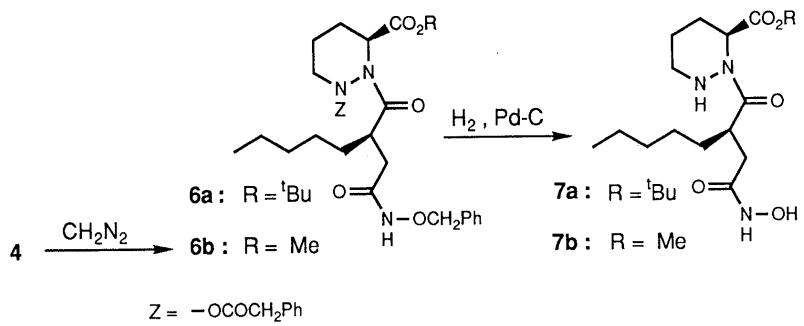


Chart 3

procedure as for the preparation of **13b**.

Compounds **31a** and **31b** containing a *n*-pentyl group at the C3′ position instead of the C2′ position in the case of **5g** and compounds (**31c—g**), containing various normal alkyl groups at the C2′ position with an *R* configuration, were prepared according to the route depicted in Charts 5—7. In Charts 5 and 6, syntheses of intermediates **17** and **24** are depicted. (4*S*)-4-Isopropyl-3-[(2*R*)-2-(*tert*-butoxycarbonylmethyl)-1-oxoheptyl]-2-oxazolidinone (**14a**), prepared according to a previously reported method,[9] was hydrolyzed with lithium hydroperoxide in THF at 0 °C, giving **15a** in 62% yield.[15] Conversion to the Tce ester **16a** was carried out by treating **15a** with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), 1-hydroxybenzotriazole (HOBt), and 2,2,2-trichloroethanol in the presence of pyridine, with a 64% yield.[16] Acid hydrolysis of the *tert*-butyl ester with 4 N HCl in 1,4-dioxane afforded **17a** in quantitative yield. According to the same procedure, **17b** was synthesized from known **14b**[9] (Chart 5). At this stage the enantiomeric purity of **17a** and **17b** is unresolved, but both **27a** and **27b**, resulting from the coupling reaction in the subsequent step, appeared to retain enantiomeric integrity on the basis of HPLC analysis (*vide infra*).

(2*R*)-2-[(2,2,2-Trichloroethoxycarbonyl)methyl]undecanoic acid (**24d**) was synthesized using Evans' asymmetric alkylation method as depicted in Chart 6.[17] The starting material **19d** was prepared in 96% yield by lithiation of (4*S*)-4-isopropyl-2-oxazolidinone (**18**) and

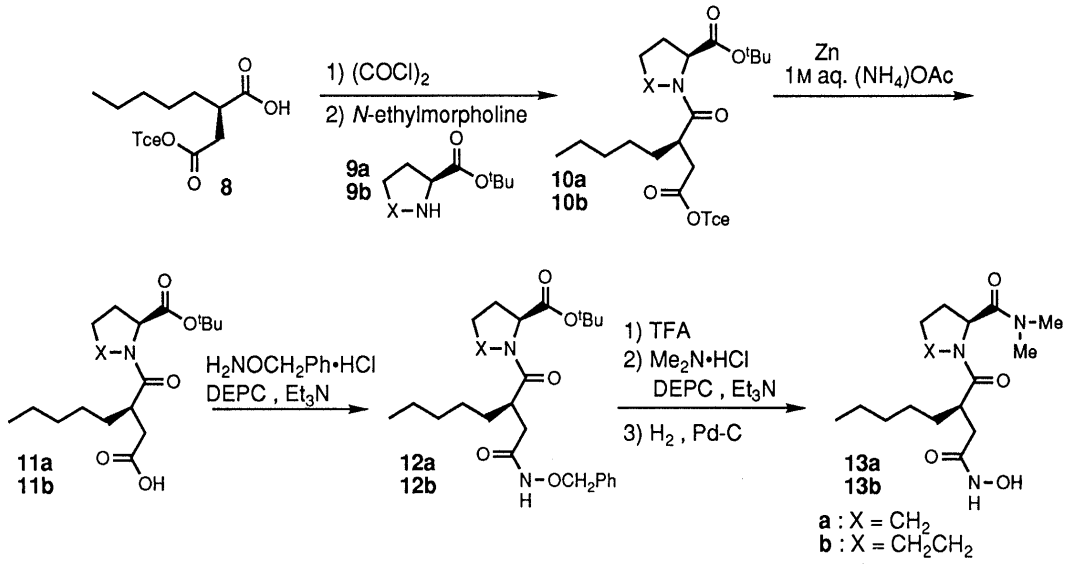


Chart 4

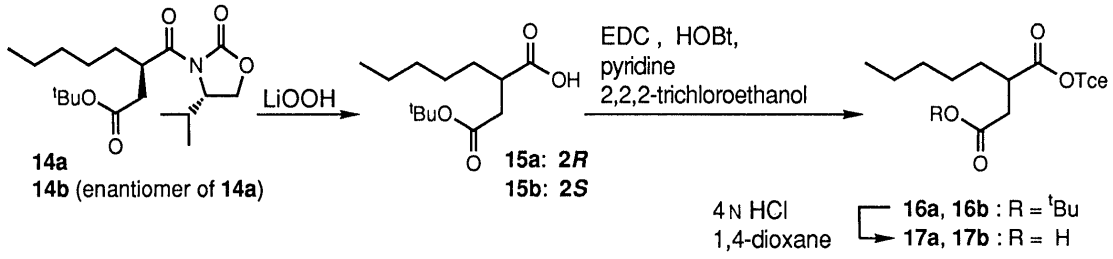


Chart 5

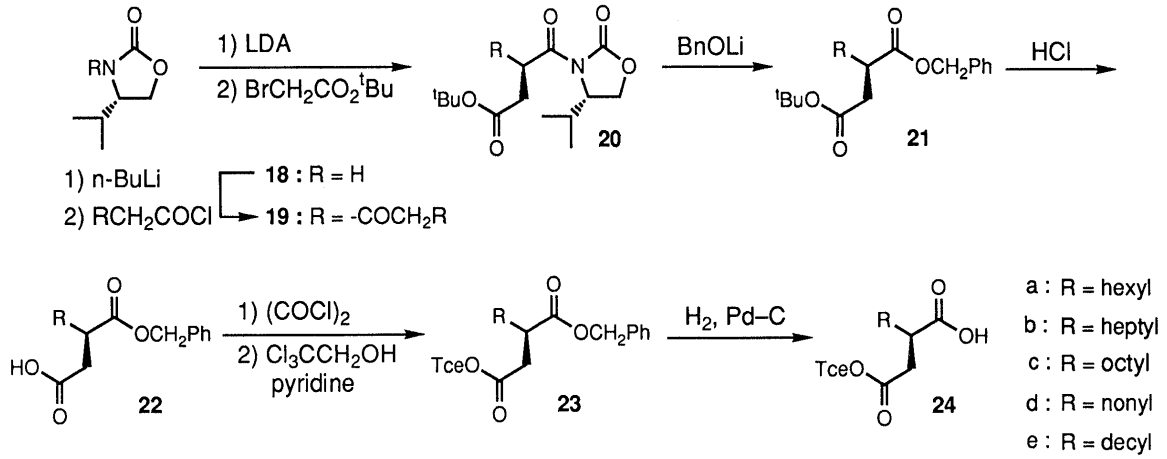
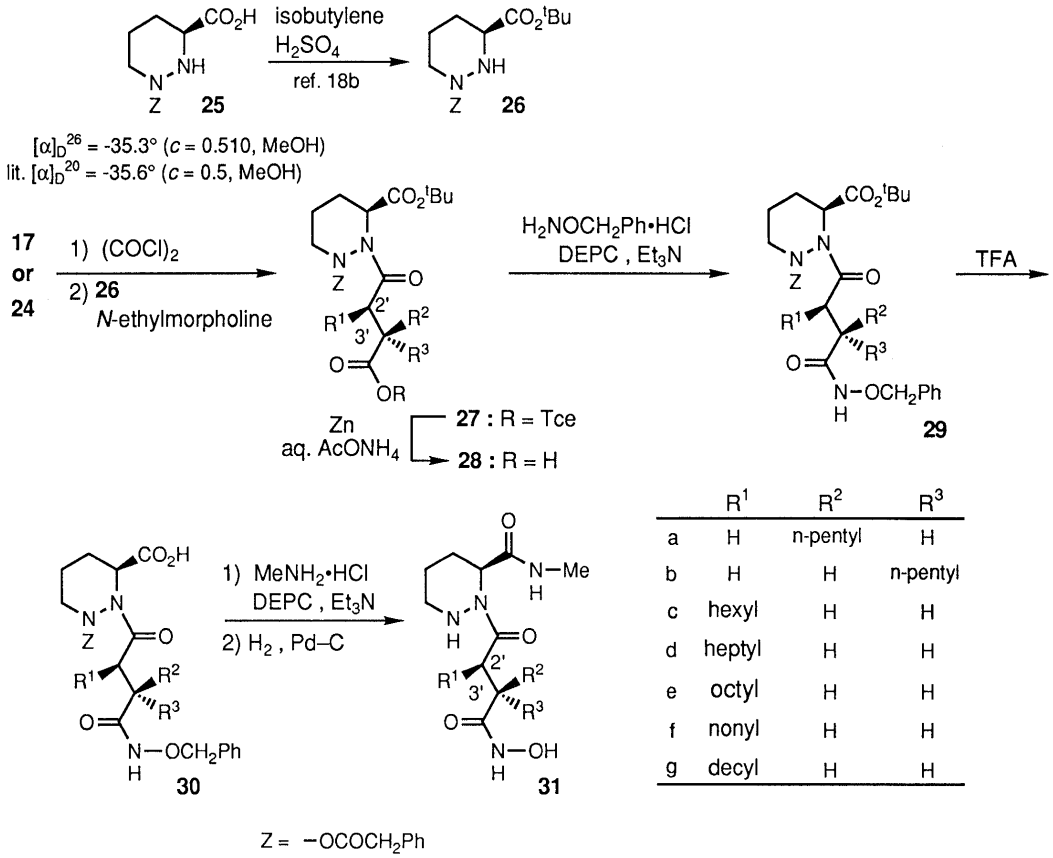


Chart 6

subsequent reaction with undecanoyl chloride. The lithium enolate prepared from **19d** and lithium diisopropylamide (LDA) in THF at −78 °C was treated with *tert*-butyl bromoacetate at the same temperature to provide crude **20d**, the de of which was determined to be 92% by HPLC analysis. Diastereomerically pure **20d** was easily obtained after silica gel chromatographic separation of the crude **20d** (hexane : EtOAc, 80 : 1—10 : 1) in 79% conversion yield, and it was recrystallized from H_2O–MeOH. Removal of the chiral auxiliary was accomplished with lithium benzyloxide, reported as a racemization—free method by Evans *et al.*,[17] to provide the diester **21d** in 91% yield. Acid hydrolysis of the *tert*-butyl ester in **21d** was followed by conversion to the Tce ester to provide the diester **23d** in 75% overall yield. The desired **24d** was prepared by hydrogenation of **23d** with 10% Pd–C in MeOH in 81% yield. According to the same procedure, **24a—c** and **24e** having C2′*R* configuration were synthesized from **18** and the corresponding acid chlorides. At this juncture the enantiomeric purity of **24a—e** was not determined, however, as in the cases of **17a** and **17b**, this was not of any concern in terms of decrease in ee and de of **27c—g** resulting from the coupling reaction in the subsequent step (*vide infra*). Syntheses of **31** from **17** or **24** are shown in Chart 7. The known *tert*-butyl (3*S*)-1-benzyloxycarbonylhexahydropyridazine-3-carboxylate (**26**)[18] {prepared by esterification of **25**[18b] $[[\alpha]_D^{26} = -35.3°$ ($c = 0.510$, MeOH), lit. $[\alpha]_D^{20} = -35.6°$ ($c = 0.5$, MeOH)] by treatment with isobutylene and sulfuric acid} was coupled with **17** or **24** using the acid chloride method to provide pure **27** as follows. The coupling of **17a** or **17b** with **26** afforded **27a** (2*S*,3′*R* form) or **27b** (2*S*,3′*S* form) as the only detectable product by TLC analysis. After silica gel chromatography (hexane–EtOAc, 7 : 1), pure **27a** or **27b** was easily obtained as a single diasteromer in 73% or 95% yield respectively. The purity was established by HPLC analysis: column, Tosoh TSK-Gel® Silica-60, 7.8 × 300 mm; eluent, hexane–isopropanol (10 : 1). At a flow rate of 1.5 ml/min (detection UV at 254 nm) **27a** and **27b** are eluted at different retention times, **27a**, 8.52 min; **27b**, 9.31 min. In the case of the coupling of **24d** with **26**, the de of crude **27f** was shown to be 92% by HPLC analysis, and diastereomerically pure **27f** (2*S*,2′*R* form) was easily obtained after silica gel chromatographic separation (hexane–EtOAc, 80 : 1—10 : 1) in 88% yield. Its purity was established by HPLC analysis: column, TSK-Gel® Silica-60, 7.8 × 300 mm; eluent, hexane–isopropanol (60 : 1). At a flow rate of 0.6 ml/min (detection UV at 210 nm) **27f** and its diastereomer are eluted at different retention times, **27f**, 24.33 min; diastereomer of **27f**, 26.69 min. Similary, in the remaining alkyl series, **27c—e** and **27g** (2*S*,2′*R* form) were detected as almost sole products by TLC analysis. After silica gel chromatography, the desired **27c—e** and **27g** (>99% de by HPLC analysis) were easily obtained by silica gel chromatographic separation. To ascertain that the coupling products **27c—e** and **27g** are diastereomerically pure and have the expected stereochemistry, coupling of (3*RS*)-1-benzyloxycarbonylhexahydropyridazine-3-carboxylate (racemic **26**) to the carboxylic acids **24a—c** and **24e** was carried out. In all cases, the resulting diastereomerically mixed products were detected as two distinct peaks by HPLC analysis, and these peaks were



	R^1	R^2	R^3
a	H	n-pentyl	H
b	H	H	n-pentyl
c	hexyl	H	H
d	heptyl	H	H
e	octyl	H	H
f	nonyl	H	H
g	decyl	H	H

Chart 7

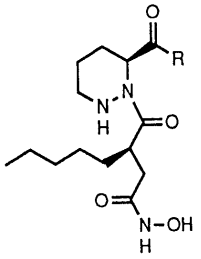
easily separated to afford a single product (*vide supra*). Purified **27a—g** were thus obtained, and the remaining steps to **31a—g** were carried out using essentially the same procedure as described to synthesize **13**, as follows. Treatment of **27** with Zn–1 M NH_4OAc afforded the carboxylic acids **28**.[14] Coupling of **28** with *O*-benzylhydroxylamine using DEPC provided **29**. The target compounds **31** were prepared from **29** by three-step conversion (acid hydrolysis, *N*-methylamidation, and hydrogenation).

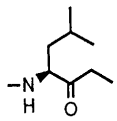
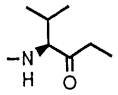
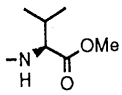
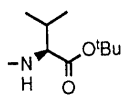
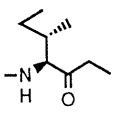
Results and Discussion

Considering the results of previous studies on interstitial collagenase inhibitors and the homology in the active site between interstitial collagenase and gelatinase B, it seems likely that matlystatins interact with the Zn^{2+} atom in the active site of gelatinases with their hydroxamic acid moiety in a bidentate manner, and that matlystatin B (**1b**) acts as a mimic of the tripeptide P'_1 to P'_3.[5] Assays were carried out according to the procedure described previously.[6b]

Firstly, modifications of the hexahydropyridazine-3-carboxamide portion (P'_2–P'_3) (**5a—g** and **13**) were carried out to investigate the effects of the P'_3 residues (Table 1).

Table 1. *In Vitro* Inhibitory Activities of the Amide (**5a—f**) and Ester (**7a, b**) Derivatives against Gelatinases A, B and Thermolysin



Compound	R	IC_{50} (μM) Gelatinase A	Gelatinase B	Thermolysin
5a		3.8	1.7	6.8
5b		0.82	0.33	2.8
5c		0.49	0.35	2.0
5d		1.1	0.36	2.2
5e	$-NEt_2$	12	3.4	22
5f	$-NMe_2$	3.1	0.60	4.3
5g	–NHMe	0.27	0.27	6.8
7a	$-O^tBu$	15	6.8	35
7b	–OMe	3.6	2.4	14
Matlystatin B (**1b**)		1.7	0.57	3.3

Replacement of the isoleucine residue at P'_3 in **1b** with a leucine residue caused about a 3-fold decrease in potency (**5a**, $IC_{50}=1.7\,\mu M$ *vs.* **1b**, $IC_{50}=0.57\,\mu M$). However, replacement of this residue with a valine residue enhanced the potency to a certain extent (**5b**, $IC_{50}=0.33\,\mu M$). Other analogues containing valine residues at the same position, **5c** and **5d**, had almost the same IC_{50} values (**5c**, $IC_{50}=0.35\,\mu M$ and **5d**, $IC_{50}=0.36\,\mu M$). To develop inhibitors with simpler structures, the amide compounds **5e—g** were synthesized. The potency of the *N,N*-diethylamide derivative **5e** ($IC_{50}=3.4\,\mu M$) was one-tenth of that of **5b**, although the *N,N*-dimethylamide derivative **5f** ($IC_{50}=0.60\,\mu M$) exhibited nearly the same inhibitory activities as the parent compound **1b**. With an IC_{50} value of $0.27\,\mu M$, the *N*-methylamide derivative **5g** was approximately twice as potent as **1b**. In summary, the P'_3 position isoleucine residue does not seem to play a crucial role in inhibiting gelatinase B, and *N*-methylamide is preferred at this position.

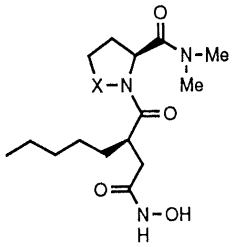
Compounds containing an ester bond at P'_2–P'_3 (**7a** and **7b**) were prepared to investigate the effect of the P'_2–P'_3 amide bond. As shown in Table 1, this conversion apparently resulted in reduced gelatinase B inhibitory potency (**5g** *vs.* **7a**, $IC_{50}=6.8\,\mu M$ and **7b**, $IC_{50}=2.4\,\mu M$). This result reveals the importance of the P'_2–P'_3 amide bond, and suggests the existence of hydrogen bonding between gelatinase B and the amide at P'_2–P'_3.

To investigate the effect of the hexahydropyridazine ring, **13a** and **13b**, having a pyrrolidine or piperidine ring, were prepared. As shown in Table 2, these replacements led to substantial losses of gelatinase B inhibitory activity (**5f** *vs.* **13a**, $IC_{50}=73\,\mu M$ and **13b**, $IC_{50}=7.9\,\mu M$). Thus, P'_2 hexahydropyridazine-3-carboxamide is an essential structural feature for matlystatin derivatives to inhibit gelatinase B.

As for the inhibitory activities against gelatinase A, the results were nearly parallel to those obtained (Table 1) in the case of gelatinase B. It is noteworthy that **5g** ($IC_{50}=0.27\,\mu M$) was approximately 6 times more potent than **1b**.

Our attention was then turned to the P'_1 residue. Most of the reported synthetic MMP inhibitors with hydroxamic acid structures possess an isobutyl group corresponding to the leucine residue at the P'_1 position, because interstitial

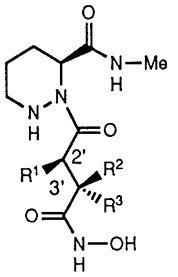
Table 2. *In Vitro* Inhibitory Activities of Compounds **5f**, **13a**, and **13b** against Gelatinase B



5f: X = $NHCH_2$
13a: X = CH_2
13b: X = CH_2CH_2

Compound	IC_{50} (μM)
5f	0.60
13a	73
13b	7.9

Table 3. *In Vitro* Inhibitory Activities of Compounds Containing Various P_1' Residues against Gelatinases and Thermolysin



31a—g, 5g

Compound	R^1	R^2	R^3	IC_{50} (μM) Gelatinase A	Gelatinase B	Thermolysin
31a	H	*n*-Pentyl	H	N.A.	49	N.A.
31b	H	H	*n*-Pentyl	N.A.	87	N.A.
5g	*n*-Pentyl	H	H	0.27	0.27	6.8
31c	Hexyl	H	H	0.11	0.082	130
31d	Heptyl	H	H	0.34	0.042	34% inhibition at 140
31e	Octyl	H	H	0.14	0.017	No inhibition at 270
31f	Nonyl	H	H	0.038	0.0012	No inhibition at 260
31g	Decyl	H	H	0.27	0.027	No inhibition at 250
Matlystatin B (**1b**)	*n*-Pentyl	H	H	1.7	0.57	3.3

N.A. = not assayed.

collagenase is well known to cleave Gly–Leu or Gly–Ile amide bonds.[5] As the sequences of the cleavage sites are not fully known in the case of gelatinase B, suitable structures for the P_1' residue were studied empirically. First, to examine the positional effect of the C2′ side chain, **31a** and **31b** were tested (Table 3). With IC_{50} values of 49 μM (**31a**) and 87 μM (**31b**), it was clear that switching of the pentyl group at C2′ in the case of matlystatins to C3′ greatly reduced the activity against gelatinase B (**31a** and **31b** *vs.* **5g**, $IC_{50} = 0.27$ μM). This result reveals that positioning of the pentyl side chain at C2′ is necessary for potent activity.

On the basis of the structure—activity relationships of **1b** and its stereo isomers, it is already known that the absolute configuration of *R* at C2′ is important for exhibiting the desired inhibitory activity.[9b] Thus, in the subsequent step, compounds **31c—g** with *R* configuration side chains of different lengths at C2′ (P_1' residue) were synthesized. As for the inhibitory activities against gelatinase B, compound **31c** with a hexyl side chain was approximately 3 times more potent than the pentyl derivative (**31c**: $IC_{50} = 82$ nM *vs.* **5g**). Furthermore, a heptyl side chain enhanced the inhibitory potency (**31c** *vs.* **31d**, $IC_{50} = 42$ nM). As shown in Table 3, there is a tendency for derivatives with longer hydrocarbon side chains from pentyl to nonyl to have more potent activities. The best results were obtained by replacing the pentyl side chain with a nonyl side chain. With an IC_{50} value of 1.2 nM, the nonyl derivative **31f** was 225 times more potent than **5g** and 475 times more potent than the parent compound **1b**. As shown with the derivative **31g**, however, placing a decyl group on P_1' led to a considerable loss of potency (**31g**, $IC_{50} = 27$ nM).

As for the inhibitory activities against gelatinase A, similar results were observed to those with gelatinase B. With an IC_{50} value of 38 nM, **31f** exhibited the most potent activity among the derivatives synthesized in this study.

Next, inhibitory activities against thermolysin, a Zn^{2+}-dependent bacterial endopeptidase, were investigated. This enzyme was employed as a control because its complexation structures with inhibitors have been the most extensively investigated among the metalloproteinases.[19] The selectivity ratio (IC_{50} for thermolysin/IC_{50} for gelatinase B) was 25 in the case of **1b**. Insertion of one methylene into the C2′ side chain in **5g** resulted in an increase of the IC_{50} value of **31c** to 130 nM. Furthermore, **31f** did not inhibit this enzyme at all even at the concentration of 260 μM. This means that the selectivity ratio of **31f** is over 2×10^5. From these results, the nonyl group with *R* absolute configuration is the best P_1' residue among the *n*-alkyl groups examined. These results suggested the S_1' subsites of the gelatinases are sharply defined and have a locally deep hydrophobic structure. The nonyl group seems to be fit well to these subsites, interacting favorably with the deep structure. Thus, **31f** exhibited a 475-fold more potent inhibitory activity than **1b** against gelatinase B. In contrast, the poor activity of **31f** on thermolysin is probably caused by steric repulsion between the S_1' subsite of thermolysin and the nonyl group in **31f**. Further investigations into the mechanisms of binding of the compounds to the enzymes are under way.

Experimental

All melting points were determined on a Yanagimoto micro melting point apparatus without correction. IR spectra were recorded on a JASCO FT-IR 8900, JASCO FT-IR 8300, or JASCO A-102 spectrometer, and ^{1}H-NMR spectra were recorded on a JEOL GX-270 or JEOL JNM-EX 270 spectrometer using tetramethylsilane (TMS) as an internal standard. Mass spectra (MS) and high-resolution mass spectra (HRMS) were obtained on a JEOL JMS-AX 505H spectrometer for electron-impact ionization (EI) or a JEOL JMS-SX/SX 102A spectrometer for

fast atom bombardment ionization (FAB). Optical rotations were measured with a Perkin–Elmer 241 polarimeter. Silica gel 60 (230—400 mesh ASTM Merck) was used as the adsorbent for column chromatography. Preparative TLC was performed on Merck precoated Silica gel 60 F_{254} (0.5 or 2.0 mm) plates or Merck precoated Silica gel 60 silanized F_{254} (0.25 mm) plates.

***N*-[(1*S*)-1-Isopropyl-2-oxobutyl]-(3*S*)-2-[(2*R*)-2-hydroxyaminocarbonylmethyl-1-oxoheptyl]hexahydropyridazine-3-carboxamide (5b)** A solution of **3b** (61 mg, 0.23 mmol) in THF (2.0 ml) was subjected to hydrogenation over 10% Pd–C (6 mg) at room temperature for 30 min. The catalyst was filtered off and the filtrate was concentrated *in vacuo*, to afford the α-amino ketone. This α-amino ketone in THF (4.0 ml) was added along with DEPC (60 μl, 0.37 mmol) to a stirred solution of **4** (47 mg, 87 mmol) in THF–DMF (10 ml, 3 : 1) at 0 °C. Stirring was continued at the same temperature for 5 h, then the mixture was poured into 5% aqueous $KHSO_4$ and extracted with EtOAc. The extract was washed successively with H_2O and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was subjected to preparative TLC ($CHCl_3$: MeOH, 15 : 1) to give a crude coupling product (75 mg). This was dissolved in MeOH (3.0 ml) and subjected to hydrogenation over 10% Pd–C (8 mg) at room temperature for 1.5 h. The catalyst was filtered off and the filtrate was concentrated *in vacuo*. The residue was purified by preparative TLC ($CHCl_3$–MeOH, 10 : 1) to give **5b** (28 mg, 77% in 2 steps) as a single diasteromer. HPLC analysis: column, Waters Radial-Pak 8NVC18, 8 × 100 mm; eluent, 70% MeOH–0.2% (v/v) Et_3N–H_3PO_4 buffer pH 3.3; flow rate 1.7 ml/min; detection UV at 210 nm; elution time, 6.91 min. Crystals: mp 52—54 °C, $[\alpha]_D^{26}$ −30.6° (*c* = 1.00, EtOH). IR (film): 3300, 1715, 1660, 1625 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.77 (3H, d, *J* = 6.6 Hz), 0.84 (3H, t, *J* = 7.3 Hz), 0.93 (3H, d, *J* = 6.6 Hz), 1.09 (3H, t, *J* = 7.3 Hz), 1.13—2.68 (15H, m), 2.57 (2H, q, *J* = 7.3 Hz), 2.70—3.18 (2H, m), 3.98 (1H, br d, *J* = 5.9 Hz), 4.64 (1H, dd, *J* = 8.6, 4.6 Hz), 4.85 (1H, d, *J* = 12.5 Hz), 5.37 (1H, br s), 7.64 (1H, d, *J* = 8.6 Hz), 9.91 (1H, m). HRMS (EI) *m/z* $(M)^+$: Calcd for $C_{21}H_{38}N_4O_5$: 426.2842. Found: 426.2851.

***N*-[(1*R*)-1-(2-Methylpropyl)-2-oxobutyl]-(3*S*)-2-[(2*R*)-2-hydroxyaminocarbonylmethyl-1-oxoheptyl]hexahydropyridazine-3-carboxamide (5a)** Compound **5a** was prepared from **3a** and **4** by the same procedure as described for the synthesis of **5b** (44% yield in 2 steps as an oil), $[\alpha]_D^{26}$ −39.5° (*c* = 1.00, EtOH). IR (film): 3300, 1720, 1660, 1625 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.78—0.98 (9H, m), 1.09 (3H, t, *J* = 6.9 Hz), 1.12—2.11 (15H, m), 2.18—2.50 (2H, m), 2.59 (2H, m), 2.70—3.12 (2H, m), 4.00 (1H, m), 4.65 (1H, br d, *J* = 5.3 Hz), 4.87 (1H, d, *J* = 11.9 Hz), 5.28 (1H, br s), 7.68 (1H, d, *J* = 5.3 Hz), 9.90 (1H, m). HRMS (EI) *m/z* $(M+H)^+$: Calcd for $C_{22}H_{41}N_4O_5$: 441.3069. Found: 441.3077.

General Procedure for the Preparation of 5c—g An amine hydrochloride (0.23 mmol), Et_3N (25 μl, 0.18 mmol) and DEPC (40 μl, 0.21 mmol) were added to a stirred solution of **4** (76 μmol) in THF–DMF (3.6 ml, 3 : 1) at 0 °C. The mixture was stirred at 0 °C for 15 min then at room temperature overnight, poured into 5% aqueous $KHSO_4$ and extracted with EtOAc. The extract was successively washed with H_2O and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was subjected to preparative TLC ($CHCl_3$–MeOH, 15 : 1) to give the corresponding crude coupling product. The crude coupling product was dissolved in MeOH (2.5 ml) and subjected to hydrogenation over 10% Pd–C (9 mg) at room temperature for 2 h. The catalyst was filtered off and the filtrate was concentrated *in vacuo*. The residue was purified by preparative TLC ($CHCl_3$–MeOH, 20 : 1) to give **5**.

5c: 61% yield in 2 steps, an oil, $[\alpha]_D^{26}$ −27.0° (*c* = 1.02, EtOH). IR (film): 3300, 1730, 1660, 1625 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.67—1.02 (9H, m), 1.04—2.60 (15H, m), 2.82 (1H, m), 3.01 (1H, br d, *J* = 12.5 Hz), 3.77 (3H, s), 3.97 (1H, m), 4.57 (1H, dd, *J* = 7.9, 5.3 Hz), 4.92 (1H, d, *J* = 11.9 Hz), 5.38 (1H, s), 7.74 (1H, br d, *J* = 7.3 Hz), 9.82 (1H, m). HRMS (EI) *m/z* $(M)^+$: Calcd for $C_{20}H_{36}N_4O_6$: 428.2635. Found: 428.2631.

5d: 49% yield in 2 steps, crystals, mp 136—139 °C, $[\alpha]_D^{26}$ −36.6° (*c* = 1.00, EtOH). IR (film): 3320, 1720, 1665, 1630 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.72—0.97 (9H, m), 1.11—2.38 (14H, m), 1.48 (9H, s), 2.49 (1H, dd, *J* = 12.5, 11.2 Hz), 2.83 (1H, m), 3.01 (1H, br d, *J* = 11.9 Hz), 3.96 (1H, m), 4.47 (1H, m), 4.89 (1H, d, *J* = 11.2 Hz), 5.38 (1H, br s), 7.54 (1H, br d, *J* = 7.9 Hz), 9.86 (1H, m). HRMS (EI) *m/z* $(M)^+$: Calcd for $C_{23}H_{42}N_4O_6$: 470.3104. Found: 470.3110.

5e: 66% yield in 2 steps, an oil, $[\alpha]_D^{26}$ −19.7° (*c* = 1.00, EtOH). IR (film): 3255, 1620 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.85 (3H, t, *J* = 6.6 Hz), 1.11 (3H, t, *J* = 6.9 Hz), 1.13—2.08 (15H, m), 2.29 (1H, dd, *J* = 14.5, 4.0 Hz), 2.52 (1H, dd, *J* = 14.5, 11.0 Hz), 2.81 (1H, dd, *J* = 13.9, 11.9 Hz), 3.04 (1H, br d, *J* = 13.9 Hz), 3.08—3.47 (3H, m), 3.53 (1H, m), 3.90 (1H, m), 5.32 (1H, d, *J* = 11.2 Hz), 5.43 (1H, d, *J* = 5.3 Hz), 7.97—8.62 (1H, br), 9.25—9.69 (1H, br). HRMS (EI) *m/z* $(M)^+$: Calcd for $C_{18}H_{34}N_4O_4$: 370.2580. Found: 370.2584.

5f: 79% yield in 2 steps, an oil, $[\alpha]_D^{26}$ +4.7° (*c* = 1.00, EtOH). IR (film): 3265, 1635, 1625 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.85 (3H, t, *J* = 6.6 Hz), 1.12—2.09 (12H, m), 2.38 (1H, dd, *J* = 14.2, 4.0 Hz), 2.53 (1H, dd, *J* = 14.2, 10.8 Hz), 2.70—3.13 (2H, m), 2.93 (3H, s), 3.05 (3H, s), 3.92 (1H, m), 5.26 (1H, d, *J* = 11.9 Hz), 5.51 (1H, br s), 9.28—9.92 (1H, br). HRMS (EI) *m/z* $(M)^+$: Calcd for $C_{16}H_{30}N_4O_4$: 342.2267. Found: 342.2284.

5g: 21% yield in 2 steps, an oil, $[\alpha]_D^{26}$ −8.8° (*c* = 0.45, EtOH). IR (film): 3270, 1655, 1625 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.87 (3H, t, *J* = 6.6 Hz), 1.00—2.13 (12H, m), 2.30 (1H, m), 2.52 (1H, br t, *J* = 12.5 Hz), 2.79 (3H, d, *J* = 4.5 Hz), 2.82 (1H, m), 3.02 (1H, br d, *J* = 13.2 Hz), 3.85 (1H, m), 4.61 (1H, d, *J* = 11.9 Hz), 5.05 (1H, br s), 6.59 (1H, br s). HRMS (EI) *m/z* $(M)^+$: Calcd for $C_{15}H_{28}N_4O_4$: 328.2111. Found: 328.2094.

***tert*-Butyl (3*S*)-2-[(2*R*)-2-Hydroxyaminocarbonylmethyl-1-oxoheptyl]-hexahydropyridazine-3-carboxylate (7a)** A suspension of **6a** (127 mg, 259 μmol) and 10% Pd–C (10 mg) in MeOH (4.0 ml) was subjected to hydrogenation at room temperature for 5 h. The catalyst was filtered off and the filtrate was concentrated *in vacuo*. The residue was purified by preparative TLC ($CHCl_3$–MeOH, 15 : 1) to give **7a** (63 mg, 66%) as an oil, $[\alpha]_D^{26}$ −10.7° (*c* = 1.00, EtOH). IR (film): 3225, 1725, 1640 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.86 (3H, t, *J* = 6.6 Hz), 1.11—1.70 (10H, m), 1.48 (9H, s), 1.89 (1H, m), 2.03—2.38 (2H, m), 2.52 (1H, m), 2.72—3.13 (2H, m), 3.98 (1H, br s), 4.28 (1H, br d, *J* = 10.6 Hz), 5.20 (1H, s). HRMS (EI) *m/z* $(M+H)^+$: Calcd for $C_{18}H_{34}N_3O_5$: 372.2499. Found: 372.2517.

Methyl (3*S*)-1-Benzyloxycarbonyl-2-[(2*R*)-2-benzyloxyaminocarbonylmethyl-1-oxoheptyl]hexahydropyridazine-3-carboxylate (6b) A solution of **4** (263 mg, 488 μmol) in EtOAc (4.0 ml) was treated with a slight excess of diazomethane solution in diethyl ether at room temperature. The volatiles were removed *in vacuo*, and the residue was chromatographed on silica gel (hexane–EtOAc, 1 : 1) to give **6b** (237 mg, 88%) as an oil, $[\alpha]_D^{26}$ −44.2° (*c* = 1.43, $CHCl_3$). IR (film): 3256, 1738, 1675 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.80 (3H, t, *J* = 6.6 Hz), 0.70—2.60 (14H, m), 3.21 (1H, m), 3.35—3.70 (1H, m, overlapped with δ 3.54), 3.54 (3H, br s), 4.25 (1H, m), 4.83 (1H, d, *J* = 11.9 Hz), 4.89 (1H, d, *J* = 11.9 Hz), 5.12 (1H, d, *J* = 11.9 Hz), 5.18 (1H, d, *J* = 11.9 Hz), 5.36 (1H, m), 7.20—7.50 (10H, m), 8.16 (1H, br s). HRMS (FAB) *m/z* $(M+H)^+$: Calcd for $C_{30}H_{40}N_3O_7$: 554.2866. Found: 554.2873.

Methyl (3*S*)-2-[(2*R*)-2-Hydroxyaminocarbonylmethyl-1-oxoheptyl]-hexahydropyridazine-3-carboxylate (7b) Compound **7b** was prepared from **6b** by the same procedure as described for the synthesis of **7a**.

7b: 64% yield, an oil, $[\alpha]_D^{26}$ −15° (*c* = 0.35, EtOH). IR (film): 3245, 1735, 1630 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.83 (3H, t, *J* = 6.6 Hz), 1.10—2.01 (11H, m), 2.22 (1H, m), 2.30 (1H, dd, *J* = 14.2, 3.6 Hz), 2.54 (1H, dd, *J* = 14.2, 11.2 Hz), 2.73—3.14 (2H, m), 3.77 (3H, s), 3.95 (1H, m), 4.20 (1H, d, *J* = 11.9 Hz), 5.34 (1H, d, *J* = 4.0 Hz), 7.35—8.20 (1H, br), 9.18 (1H, m). HRMS (EI) *m/z* $(M)^+$: Calcd for $C_{15}H_{27}N_3O_5$: 329.1950. Found: 329.1965.

***tert*-Butyl (2*S*)-1-[(2*R*)-1-Oxo-2-(2,2,2-trichloroethoxycarbonyl)methylheptyl]piperidine-2-carboxylate (10b)** A solution of **8** (525 mg, 1.64 mmol) in benzene (8.0 ml) was treated with oxalyl chloride (560 μl, 6.42 mmol) under N_2 at 60 °C for 2 h. The mixture was concentrated *in vacuo* to give the acid chloride as a pale yellow oil. Then a solution of the acid chloride in THF (9.0 ml) was added using a cannula to a stirred solution of **9b** (291 mg, 1.58 mmol) and *N*-ethylmorpholine (320 μl, 2.51 mmol) in THF (9.0 ml) under N_2 at −15 °C. The mixture was gradually warmed to room temperature and stirred overnight. The mixture was poured into 0.2 N aqueous HCl and extracted with EtOAc. The extract was successively washed with H_2O and then brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane–EtOAc, 6 : 1) to afford **10b** (482 mg, 63%) as a colorless oil, $[\alpha]_D^{25}$ −29.7° (*c* = 1.00, $CHCl_3$). IR (film): 1756, 1734, 1645 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, *J* = 6.6 Hz), 1.18—1.79 (13H, m), 1.46 (9H, s), 2.22 (1H, br d, *J* = 13.2 Hz), 2.58 (1H, dd, *J* = 17.2, 4.0 Hz), 3.04 (1H, dd, *J* = 17.2, 9.9 Hz), 3.15—3.37 (2H, m), 3.92 (1H, br d, *J* = 12.5 Hz), 4.61 (1H, d, *J* = 11.9 Hz), 4.84 (1H, d, *J* = 11.9 Hz), 5.32 (1H, br d, *J* = 4.0 Hz). HRMS (EI) *m/z* $(M)^+$: Calcd for $C_{21}H_{34}NO_5(^{35}Cl)_3$: 485.1502. Found: 485.1515.

***tert*-Butyl (2*S*)-1-[(2*R*)-2-Benzyloxyaminocarbonylmethyl-1-oxoheptyl]piperidine-2-carboxylate (12b)** Zinc powder (1.25 g, 19.1 mmol) and

1 M aqueous NH_4OAc (1.5 ml) were added to a vigorously stirred solution of **10b** (463 mg, 0.951 mmol) in THF (15 ml) at room temperature. After 3 h the zinc powder was removed by filtration. The filtrate was poured into 1 N aqueous HCl and extracted with EtOAc. The extract was dried over Na_2SO_4, and concentrated *in vacuo*. The residue was chromatographed on silica gel ($CHCl_3$–MeOH, 25 : 1) to afford the crude carboxylic acid (265 mg). A stirred solution of this carboxylic acid in THF–DMF (9.0 ml, 3 : 1) was treated with *O*-benzylhydroxylamine hydrochloride (183 mg, 1.15 mmol), Et_3N (240 μl, 1.72 mmol), and DEPC (240 μl, 1.47 mmol) under N_2 at −15 °C. After 3.5 h, the mixture was poured into 5% aqueous $KHSO_4$ and the whole was extracted with EtOAc. The extract was washed successively with H_2O and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was chromatographed on silica gel ($CHCl_3$–MeOH, 100 : 1) to afford **12b** (340 mg, 78% in 2 steps) as a colorless oil; $[\alpha]_D^{25}$ −51.4° (c = 1.00, $CHCl_3$). IR (film): 1732, 1638, 1619 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.87 (3H, t, J = 6.6 Hz), 1.12—1.81 (13H, m), 1.45 (9H, s), 2.10—2.31 (2H, m), 2.46 (1H, br t, J = 11.2 Hz), 3.12—3.36 (2H, m), 3.91 (1H, br d, J = 12.5 Hz), 4.86 (2H, s), 5.25 (1H, br s), 7.27—7.48 (5H, m), 8.90 (1H, br s). HRMS (EI) m/z $(M)^+$: Calcd for $C_{26}H_{40}N_2O_5$: 460.2937. Found: 460.2945.

***N,N*-Dimethyl-(2*S*)-1-[(2*R*)-2-hydroxyaminocarbonylmethyl-1-oxoheptyl]piperidine-2-carboxamide (13b)** A mixture of **12b** (330 mg, 0.717 mmol), trifluoroacetic acid (1.3 ml, 17.0 mmol), and CH_2Cl_2 (17 ml) was stirred at room temperature overnight, then concentrated *in vacuo*. The residue was chromatographed on silica gel ($CHCl_3$–MeOH, 40 : 1) to afford the crude carboxylic acid (232 mg). Dimethylamine hydrochloride (28 mg, 0.34 mmol), Et_3N (45 μl, 0.32 mmol), and DEPC (50 μl, 0.31 mmol) were added to a solution of this carboxylic acid (46 mg) in THF–DMF (3.0 ml, 5 : 1) under N_2 at −15 °C. After 4.5 h, the mixture was poured into 5% aqueous $KHSO_4$ and the whole was extracted with EtOAc. The extract was successively washed with H_2O and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was subjected to preparative TLC ($CHCl_3$–MeOH, 20 : 1) to give a crude coupling product. This was dissolved in MeOH (2.5 ml) and subjected to hydrogenation over 10% Pd–C (9 mg) at room temperature for 2.5 h. The catalyst was filtered off and the filtrate was concentrated *in vacuo*. The residue was purified successively by preparative TLC ($CHCl_3$–MeOH, 15 : 1) and Silica gel 60 silanized preparative TLC (MeOH–H_2O, 1 : 1) to give **13b** (11 mg, 22% in 3 steps based on consumed **12b**) as an amorphous powder, $[\alpha]_D^{26}$ −43.8° (c = 0.925, EtOH). IR (film): 3457, 3253, 1640, 1630 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.87 (3H, t, J = 6.6 Hz), 1.01—2.09 (14H, m), 2.32 (1H, dd, J = 13.1, 5.2 Hz), 2.59 (1H, br t, J = 13.1 Hz), 2.92 (3H, s), 3.04 (3H, s), 3.28 (1H, m), 3.60—3.93 (2H, m), 5.42 (1H, s), 9.23—9.73 (1H, br). HRMS (FAB) m/z $(M+H)^+$: Calcd for $C_{17}H_{32}N_3O_4$ 342.2392. Found: 342.2365.

N,N-Dimethyl-(2*S*)-1-[(2*R*)-2-hydroxyaminocarbonylmethyl-1-oxoheptyl]pyrrolidine-2-carboxamide (**13a**) was prepared from **8** and L-proline *tert*-butyl ester (**9a**) by the same procedure as described for the synthesis **13b**.

13a: An oil, $[\alpha]_D^{26}$ −45.5° (c = 1.00, EtOH). IR (film): 3224, 1650, 1637 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J = 6.6 Hz), 1.11—2.22 (12H, m), 2.30 (1H, dd, J = 14.5, 4.0 Hz), 2.55 (1H, dd, J = 14.5, 10.6 Hz), 2.94 (3H, s), 3.08 (3H, s), 3.10 (1H, m), 3.58—3.99 (2H, m), 4.85 (1H, m), 10.25 (1H, br s). HRMS (FAB) m/z $(M+H)^+$: Calcd for $C_{16}H_{30}N_3O_4$: 328.2237. Found: 328.2211.

(2*R*)-2-*tert*-Butoxycarbonylmethylheptanoic Acid (15a) A mixture of **14a** (2.51 g, 7.07 mmol), lithium hydroxide monohydrate (593 mg, 14.1 mmol), 31% aqueous H_2O_2 (3.5 ml, 35 mmol) and THF–H_2O (170 ml, 3 : 1) was stirred at 0 °C for 1.5 h. Then 1.5 M aqueous Na_2SO_3 (26 ml) was added, and the mixture was carefully poured into 0.5 N aqueous HCl and extracted with EtOAc. The extract was chromatographed on silica gel ($CHCl_3$–MeOH, 45 : 1) to afford **15a** (1.07 g, 62%) as a colorless oil, $[\alpha]_D^{26}$ +14.5° (c = 1.97, EtOH). IR (film): 1734, 1709 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J = 7.4 Hz), 1.21—1.41 (6H, m), 1.43 (9H, s), 1.52 (1H,m), 1.65 (1H, m), 2.38 (1H, dd, J = 16.5, 5.3 Hz), 2.62 (1H, dd, J = 16.5, 9.2 Hz), 2.80 (1H, m). HRMS (EI) m/z $(M+H)^+$: Calcd for $C_{13}H_{25}O_4$: 245.1752. Found: 245.1752.

***tert*-Butyl (3*R*)-3-(2,2,2-Trichloroethoxycarbonyl)octanoate (16a)** A mixture of **15a** (1.07 g, 4.39 mmol), 2,2,2-trichloroethanol (0.46 ml, 4.79 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (868 mg, 4.53 mmol), 1-hydroxybenzotriazole (583 mg, 4.31 mmol), pyridine (0.36 ml, 4.45 mmol), Et_3N (0.62 ml, 4.45 mmol), and CH_2Cl_2 was stirred at 0 °C. After 1 h, the mixture was warmed to room temperature, and stirred overnight. The mixture was poured into 5% aqueous $KHSO_4$ and extracted with EtOAc. The extract was successively washed with H_2O and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane–EtOAc, 20 : 1) to afford **16a** (1.05 g, 64%) as a colorless oil, $[\alpha]_D^{25}$ +2.0° (c = 3.0, $CHCl_3$). IR (film): 1756, 1732 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J = 6.6 Hz), 1.19—1.46 (6H, m), 1.43 (9H, s), 1.48—1.79 (2H, m), 2.43 (1H, dd, J = 16.5, 5.3 Hz), 2.70 (1H, dd, J = 16.5, 8.6 Hz), 2.93 (1H, m), 4.66 (1H, d, J = 12.2 Hz), 4.84 (1H, d, J = 12.2 Hz). HRMS (EI) m/z $(M+H)^+$: Calcd for $C_{15}H_{26}(^{35}Cl)_3O_4$: 375.0907. Found: 375.0890.

(3*R*)-3-(2,2,2-Trichloroethoxycarbonyl)octanoic Acid (17a) A mixture of **16a** (1.03 g, 2.75 mmol) and 4 N aqueous HCl in 1,4-dioxane (20 ml) was stirred at room temperature overnight, then concentrated *in vacuo*. The residue was chromatographed on silica gel ($CHCl_3$–MeOH, 25 : 1) to afford **17a** (893 mg, quant.) as a colorless oil, $[\alpha]_D^{25}$ +4.3° (c = 5.1, EtOH). IR (film): 1754, 1713 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J = 6.3 Hz), 1.13—1.46 (6H, m), 1.49—1.85 (2H, m), 2.55 (1H, dd, J = 17.2, 4.6 Hz), 2.83 (1H, dd, J = 17.2, 9.2 Hz), 2.98 (1H, m), 4.69 (1H, d, J = 11.9 Hz), 4.82 (1H, d, J = 11.9 Hz). MS (EI) m/z $(M+H)^+$: 319. HRMS (EI) m/z $(M+H-H_2O)^+$: Calcd for $C_{11}H_{16}(^{35}Cl)_3O_3$: 301.0176. Found: 301.0161.

(3*S*)-3-(2,2,2-Trichloroethoxycarbonyl)octanoic Acid (17b) Enantiomer of **17a**: A colorless oil, $[\alpha]_D^{25}$ −4.1° (c = 5.4, EtOH). HRMS (EI) m/z $(M+H)^+$: Calcd for $C_{11}H_{18}(^{35}Cl)_3O_4$: 319.0271. Found: 319.0264.

(4*S*)-4-Isopropyl-3-[(2*R*)-2-1-oxoundecyl]-2-oxazolidinone (19d) *n*-Butyl lithium (15.0 ml of a 1.68 M solution in hexane, 25.2 mmol) was added to a stirred solution of **18** (3.06 g, 23.7 mmol) in THF (85 ml) at −78 °C under N_2. This mixture was stirred for 25 min, then undecanoyl chloride (5.12 g, 25.0 mmol) was added at the same temperature over 3 min. The mixture was stirred for 2.5 h at the same temperature, then poured into 5% aqueous NH_4Cl solution, and the whole was extracted with EtOAc. The extract was successively washed with H_2O and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane–EtOAc, 4 : 1) to afford **19d** (6.77 g, 96%) as a colorless oil, $[\alpha]_D^{26}$ +61.2° (c = 1.00, $CHCl_3$). IR (film): 1784, 1704 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.87 (3H, t, J = 6.6 Hz), 0.88 (3H, t, J = 6.8 Hz), 0.92 (3H, d, J = 6.6 Hz), 1.18—1.42 (14H, m), 1.54—1.74 (2H, m), 2.38 (1H, m), 2.85 (1H, ddd, J = 16.4, 8.4, 6.8 Hz), 2.98 (1H, ddd, J = 16.4, 8.5, 6.7 Hz), 4.20 (1H, dd, J = 9.1, 3.2 Hz), 4.26 (1H, t, J = 9.1 Hz), 4.43 (1H, dt, J = 9.1, 3.2 Hz). HRMS (EI) m/z $(M+H)^+$: Calcd for $C_{17}H_{31}NO_3$: 297.2296. Found: 297.2304.

(4*S*)-4-Isopropyl-3-[(2*R*)-2-(*tert*-butoxycarbonylmethyl)-1-oxoundecyl]-2-oxazolidinone (20d) A stirred solution of **19d** (291 mg, 980 μmol) in THF (5.0 ml) was treated with LDA (3.28 ml of a 0.323 M solution in THF, 1.06 mmol) at −78 °C under N_2. This mixture was stirred for 15 min, then *tert*-butyl bromoacetate (500 μl, 310 mmol) was added at the same temperature over 3 min. The mixture was gradually warmed to −68 °C over 3.5 h with stirring, then poured into saturated aqueous NH_4Cl solution, and extracted with EtOAc. The extract was successively washed with H_2O and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The diastereoselectivity of the coupling was determined to be 96 : 4 by HPLC analysis. The residue was chromatographed on silica gel (hexane–EtOAc, 80 : 1—10 : 1) to afford **20d** (>99% de, 248 mg, 62%, converting yield 79%) as a single diasteromer (**20d**: Rf = 0.51, diastereomer of **20d**: Rf = 0.37, hexane–EtOAc = 5 : 1). This was recrystallized from MeOH–H_2O to give colorless crystals, mp 57—58 °C, $[\alpha]_D^{26}$ +49.4° (c = 0.991, $CHCl_3$). IR (KBr): 1764, 1731, 1699 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.87 (3H, t, J = 6.9 Hz), 0.88 (3H, d, J = 7.0 Hz), 0.91 (3H, d, J = 7.0 Hz), 1.17—1.48 (15H, m), 1.41 (9H, s), 1.60 (1H, m), 2.37 (1H, m), 2.43 (1H, dd, J = 16.7, 4.6 Hz), 2.74 (1H, dd, J = 16.7, 10.3 Hz), 4.10—4.29 (3H, m), 4.43 (1H, dt, J = 7.0, 3.7 Hz). MS (EI) m/z $(M+H)^+$ 412; *Anal.* Calcd for $C_{23}H_{41}NO_5$: C, 67.12; H, 10.04; N, 3.40. Found: C, 66.82; H, 10.13; N, 3.43.

The starting material **19a** (30 mg, 26%) was also recovered from the silica gel chromatography eluates.

***tert*-Butyl (3*R*)-3-Benzyloxycarbonyldodecanoate (21d)** A solution of **20d** (5.51 g, 13.4 mmol) in THF (40 ml) was treated with a THF solution of lithium benzyloxide (54 ml) [prepared from benzyl alcohol (2.8 ml, 27.1 mmol) and *n*-butyl lithium (12.0 ml, 1.68 M solution in hexane, 20.2 mmol) in THF (40 ml) at 0 °C under N_2] at 0 °C for 1 h under N_2. The mixture was poured into 5% aqueous $KHSO_4$ and the whole was extracted with EtOAc. The extract was successively washed with H_2O and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane–EtOAc, 20 : 1) to afford **21d** (4.74 g, 91%) as a colorless oil, $[\alpha]_D^{26}$ +1.0° (c = 6.0, $CHCl_3$). IR (film):

1732 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=6.6 Hz), 1.16—1.33 (14H, m), 1.40 (9H, s), 1.42—1.71 (2H, m), 2.36 (1H, dd, J=16.5, 5.6 Hz), 2.64 (1H, dd, J=16.5, 9.2 Hz), 2.83 (1H, m), 5.09 (1H, d, J=12.2 Hz), 5.17 (1H, d, J=12.2 Hz), 7.25—7.39 (5H, m). HRMS (EI) m/z $(M+H)^+$: Calcd for $C_{24}H_{39}O_4$: 391.2848. Found: 391.2837.

(3*R*)-3-Benzyloxycarbonyldodecanoic Acid (22d) A solution of **21d** (2.85 g, 7.30 mmol) in 3 N HCl–1,4-dioxane (45 ml) was stirred overnight at room temperature. The mixture was carefully poured into cold 0.5 N aqueous NaOH (180 ml) and the whole was extracted with EtOAc. The extract was dried over Na_2SO_4, and concentrated *in vacuo*. The residue was chromatographed on silica gel ($CHCl_3$–MeOH, 40 : 1) to afford **22d** (2.43 g, 100%) as a colorless oil, $[\alpha]_D^{26}$ +3.6° (c=1.0, EtOH). IR (film): 1736, 1713 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=6.6 Hz), 1.13—1.39 (14H, m), 1.42—1.76 (2H, m), 2.48 (1H, dd, J=16.2, 4.0 Hz), 2.78 (1H, dd, J=16.2, 9.2 Hz), 2.88 (1H, m), 5.12 (1H, d, J=12.5 Hz), 5.17 (1H, d, J=12.5 Hz), 7.25—7.42 (5H, m). HRMS (EI) m/z $(M)^+$: Calcd for $C_{20}H_{30}O_4$: 334.2144. Found: 334.2152.

2,2,2-Trichloroethyl (3*R*)-3-Benzyloxycarbonyldodecanoate (23d) A solution of **22d** (2.43 g, 7.28 mmol) and oxalyl chloride (2.5 ml, 28.7 mmol) in toluene (50 ml) was stirred at 60 °C for 2 h under N_2. The volatiles were removed *in vacuo* to give the acid chloride. Pyridine (700 μl, 7.06 mmol) and 2,2,2-trichloroethanol (3.2 ml, 33.3 mmol) were added to a solution of this acid chloride in THF (40 ml) under N_2 at 0 °C. The mixture was stirred at the same temperature for 2.5 h, then poured into 5% aqueous $KHSO_4$, and the whole was extracted with EtOAc. The extract was dried over Na_2SO_4, and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane–EtOAc, 30 : 1) to afford **23d** (2.53 g, 75%) as a colorless oil, $[\alpha]_D^{26}$ −0.35° (c=6.0, $CHCl_3$). IR (film): 1759, 1736 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.91 (3H, t, J=6.9 Hz), 1.11—1.36 (14H, m), 1.43—1.81 (2H, m), 2.63 (1H, dd, J=15.2, 3.3 Hz), 2.92 (1H, dd, J=15.2, 9.2 Hz), 2.95 (1H, m), 4.68 (1H, d, J=11.9 Hz), 4.75 (1H, d, J=11.9 Hz), 5.14 (1H, d, J=12.5 Hz), 5.21 (1H, d, J=12.5 Hz), 7.26—7.42 (5H, m). HRMS (EI) m/z $(M+H)^+$: Calcd for $C_{22}H_{32}(^{35}Cl)_3O_4$: 465.1366. Found: 465.1351.

(2*R*)-2-[(2,2,2-Trichloroethoxycarbonyl)methyl]undecanoic Acid (24d) A suspension of **23d** (2.53 g, 5.45 mmol) and 10% Pd–C (130 mg) in MeOH (30 ml) was hydrogenated at room temperature for 1.5 h. The catalyst was filtered off and the filtrate was concentrated *in vacuo*. The residue was chromatographed on silica gel ($CHCl_3$–MeOH, 40 : 1—30 : 1) to afford **24d** (1.65 g, 81%) as a colorless oil, $[\alpha]_D^{26}$ +11.0° (c=6.41, EtOH). IR (film): 1759, 1710 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=6.9 Hz), 1.15—1.46 (14H, m), 1.60 (1H, m), 1.72 (1H, m), 2.61 (1H, dd, J=16.0, 4.2 Hz), 2.87 (1H, dd, J=16.0, 9.2 Hz), 2.93 (1H, m), 4.72 (1H, d, J=12.0 Hz), 4.78 (1H, d, J=12.0 Hz). HRMS (EI) m/z $(M+H)^+$: Calcd for $C_{15}H_{26}(^{35}Cl)_3O_4$: 375.0897. Found: 375.0909.

Compounds **31a—g** were prepared from **17a** and **17b** (for **31a** and **31b**) or **24a—e** (for **31c—g**) and **26** by the same procedure as described for the synthesis of **13b**. Spectral properties of intermediates and P$'_1$-modified compounds (**31a—g**) are as follows.

***tert*-Butyl (3*S*)-1-Benzyloxycarbonyl-2-[(3*R*)-3-(2,2,2-trichloroethoxycarbonyl)-1-oxooctyl]hexahydropyridazine-3-carboxylate (27a)** A colorless oil, $[\alpha]_D^{25}$ −13.9° (c=1.00, $CHCl_3$). IR (film): 1733, 1683 cm^{-1}, ^{1}H-NMR ($CDCl_3$) δ: 0.86 (3H, t, J=6.8 Hz), 1.10—2.50 (13H, m), 1.42 (9H, s), 2.75 (1H, dd, J=17.2, 10.6 Hz), 2.87—3.20 (2H, m), 4.32 (1H, m), 4.55 (1H, d, J=12.2 Hz), 4.89 (1H, d, J=12.2 Hz), 4.80—5.50 (3H, m), 7.23—7.43 (5H, m). HRMS (EI) m/z $(M)^+$: Calcd for $C_{28}H_{39}(^{35}Cl)_3N_2O_7$: 620.1823. Found: 620.1801.

***tert*-Butyl (3*S*)-1-Benzyloxycarbonyl-2-[(3*R*)-3-carboxy-1-oxooctyl]-hexahydropyridazine-3-carboxylate (28a)** An amorphous powder, $[\alpha]_D^{25}$ −22.9° (c=1.00, EtOH). IR (film): 3193, 1732, 1683 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.86 (3H, t, J=6.3 Hz), 1.02—2.40 (13H, m), 1.42 (9H, s), 2.68 (1H, dd, J=16.5, 10.6 Hz), 2.77—3.20 (2H, m), 4.30 (1H, m), 4.85—5.50 (3H, m), 7.21—7.41 (5H, m). HRMS (EI) m/z $(M+H)^+$: Calcd for $C_{26}H_{39}N_2O_7$: 491.2757. Found: 491.2784.

***tert*-Butyl (3*S*)-1-Benzyloxycarbonyl-2-[(3*R*)-3-benzyloxyaminocarbonyl-1-oxooctyl]hexahydropyridazine-3-carboxylate (29a)** A colorless oil, $[\alpha]_D^{25}$ −44.6° (c=1.00, EtOH). IR (film): 3232, 1730, 1678 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.86 (3H, t, J=6.7 Hz), 0.95—2.10 (12H, m), 1.41 (9H, s), 2.12—2.58 (2H, m), 2.73 (1H, dd, J=16.2, 10.9 Hz), 3.03 (1H, m), 4.28 (1H, m), 4.77—5.50 (5H, m), 7.25—7.50 (10H, m), 8.25—8.70 (1H, br). HRMS (EI) m/z $(M+H)^+$: Calcd for $C_{33}H_{46}N_3O_7$: 596.3336. Found: 596.3324.

(3*S*)-1-Benzyloxycarbonyl-2-[(3*R*)-3-benzyloxyaminocarbonyl-1-oxooctyl]hexahydropyridazine-3-carboxylic Acid (30a) An amorphous powder, $[\alpha]_D^{25}$ −20.2° (c=1.00, EtOH). IR (film): 3230, 1721, 1674 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.84 (3H, t, J=6.9 Hz), 0.85—3.40 (16H, m), 4.20 (1H, m), 4.70—5.60 (5H, m), 7.10—7.60 (10H, m), 8.75 (1H, m), 9.40 (1H, m). HRMS (EI) m/z $(M+H)^+$: Calcd for $C_{29}H_{38}N_3O_7$: 540.2709. Found: 540.2690. *Anal.* Calcd for $C_{29}H_{37}N_3O_7 \cdot 0.3H_2O$: C, 63.91; H, 6.95; N, 7.71. Found: C, 63.62; H, 6.83; N, 7.71.

***tert*-Butyl (3*S*)-1-Benzyloxycarbonyl-2-{(2*R*)-1-oxo-2-[(2,2,2-trichloroethoxycarbonyl)methyl]undecyl}hexahydropyridazine-3-carboxylate (27f)** A colorless oil, $[\alpha]_D^{26}$ −7.0° (c=1.0, $CHCl_3$). IR (film): 1740, 1678 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=6.6 Hz), 0.93—1.70 (18H, m), 1.43 (9H, s), 1.78—2.12 (2H, m), 2.60 (1H, dd, J=17.2, 3.3 Hz), 2.94 (1H, dd, J=17.2, 10.6 Hz), 3.12 (1H, m), 3.42 (1H, m), 4.27 (1H, m), 4.61 (1H, d, J=11.9 Hz), 4.77 (1H, d, J=11.9 Hz), 5.14 (1H, d, J=12.5 Hz), 5.21 (1H, d, J=12.5 Hz), 5.27 (1H, t, J=4.0 Hz), 7.23—7.42 (5H, m). HRMS (EI) m/z $(M)^+$: Calcd for $C_{32}H_{47}(^{35}Cl)_3N_2O_7$: 676.2451. Found: 676.2449.

***tert*-Butyl (3*S*)-1-Benzyloxycarbonyl-2-[(2*R*)-2-carboxymethyl-1-oxoundecyl]hexahydropyridazine-3-carboxylate (28f)** A colorless oil, $[\alpha]_D^{26}$ −22.0° (c=1.00, EtOH). IR (film): 3193, 1733, 1679, 1651 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=6.6 Hz), 0.93—1.58 (18H, m), 1.43 (9H, s), 1.69—2.12 (2H, m), 2.48 (1H, dd, J=17.2, 3.3 Hz), 2.81 (1H, dd, J=17.2, 10.6 Hz), 3.07 (1H, m), 3.31 (1H, m), 4.25 (1H, m), 5.13 (1H, d, J=11.9 Hz), 5.20 (1H, d, J=11.9 Hz), 5.26 (1H, br t, J=4.7 Hz), 7.23—7.42 (5H, m). HRMS (EI) m/z $(M+H)^+$: Calcd for $C_{30}H_{47}N_2O_7$: 547.3383. Found: 547.3361.

***tert*-Butyl (3*S*)-1-Benzyloxycarbonyl-2-[(2*R*)-2-benzyloxyaminocarbonylmethyl-1-oxoundecyl]hexahydropyridazine-3-carboxylate (29f)** A colorless oil, $[\alpha]_D^{26}$ −38.2° (c=1.00, $CHCl_3$). IR (film): 3249, 1733, 1675 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=6.9 Hz), 0.90—2.50 (22H, m), 1.42 (9H, s), 3.21 (1H, m), 3.46 (1H, m), 4.25 (1H, m), 4.82 (1H, d, J=11.9 Hz), 4.88 (1H, d, J=11.9 Hz), 5.13 (1H, d, J=12.5 Hz), 5.20 (1H, d, J=12.5 Hz), 5.25 (1H, m), 7.21—7.48 (10H, m), 8.12 (1H, m). HRMS (EI) m/z $(M+H)^+$: Calcd for $C_{37}H_{54}N_3O_7$: 652.3970. Found: 652.3945.

(3*S*)-1-Benzyloxycarbonyl-2-[(2*R*)-2-benzyloxyaminocarbonylmethyl-1-oxoundecyl]hexahydropyridazine-3-carboxylic Acid (30f) A colorless oil, $[\alpha]_D^{26}$ −24.3° (c=1.00, EtOH). IR (film): 3223, 1714, 1671, 1602 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=6.6 Hz), 0.87—2.59 (22H, m), 2.88—3.27 (2H, m), 4.10 (1H, br d, J=11.2 Hz), 4.69—5.39 (5H, m), 7.13 (1H, br s), 7.24—7.51 (10H, m), 12.30 (1H, s). *Anal.* Calcd for $C_{33}H_{45}N_3O_7 \cdot H_2O$: C, 64.58; H, 7.71; N, 6.85. Found: C, 64.77; H, 7.31; N, 6.96.

***N*-Methyl-(3*S*)-2-[(3*R*)-3-hydroxyaminocarbonyl-1-oxooctyl]hexahydropyridazine-3-carboxyamide (31a)** A colorless oil, $[\alpha]_D^{25}$ −17.8° (c=1.00, EtOH). IR (film): 3264, 1644 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.87 (3H, t, J=6.6 Hz), 1.03—1.90 (11H, m), 2.09 (1H, d, J=8.6 Hz), 2.25—3.00 (4H, m), 2.79 (3H, s), 3.05 (1H, d, J=13.0 Hz), 4.80 (1H, br d, J=12.5 Hz), 4.90 (1H, d, J=3.3 Hz), 7.27 (1H, br s), 8.14—8.78 (1H, br), 9.51 (1H, br s). HRMS (EI) m/z $(M+H)^+$: Calcd for $C_{15}H_{29}N_4O_4$: 329.2189. Found: 329.2182.

***N*-Methyl-(3*S*)-2-[(3*S*)-3-hydroxyaminocarbonyl-1-oxooctyl]hexahydropyridazine-3-carboxyamide (31b)** A colorless oil, $[\alpha]_D^{25}$ −40.7° (c=1.00, EtOH). IR (film): 3270, 1640 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.87 (3H, t, J=6.6 Hz), 1.09—1.83 (11H, m), 2.22 (1H, br d, J=8.6 Hz), 2.40—2.88 (3H, m), 2.74 (3H, s), 3.03 (1H, br d, J=12.5 Hz), 3.15 (1H, m), 4.56 (1H, br d, J=12.5 Hz), 5.04 (1H, br s), 7.30 (1H, m). HRMS (EI) m/z $(M)^+$: Calcd for $C_{15}H_{28}N_4O_4$: 328.2111. Found: 328.2097.

***N*-Methyl-(3*S*)-2-[(2*R*)-2-hydroxyaminocarbonylmethyl-1-oxoocyl]-hexahydropyridazine-3-carboxamide (31c)** A colorless oil, $[\alpha]_D^{26}$ −9.3° (c=0.90, EtOH). IR (film): 3271, 1648, 1626 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.87 (3H, t, J=6.5 Hz), 0.99—1.95 (13H, m), 2.05 (1H, br d, J=10.6 Hz), 2.29 (1H, dd, J=13.9, 3.3 Hz), 2.52 (1H, br t, J=13.9 Hz), 2.79 (3H, d, J=4.6 Hz), 2.81 (1H, m), 3.02 (1H, d, J=13.2 Hz), 3.88 (1H, m), 4.67 (1H, d, J=11.9 Hz), 5.06 (1H, s), 6.75 (1H, d, J=4.6 Hz), 9.52—9.91 (1H, br). HRMS (EI) m/z $(M)^+$: Calcd for $C_{16}H_{30}N_4O_4$: 342.2267. Found: 342.2256.

***N*-Methyl-(3*S*)-2-[(2R)-2-hydroxyaminocarbonylmethyl-1-oxononyl]-hexahydropyridazine-3-carboxamide (31d)** An amorphous powder, $[\alpha]_D^{26}$ −9.5° (c=1.0, EtOH). IR (film): 3268, 1652, 1626 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.87 (3H, t, J=6.6 Hz), 1.00—1.95 (15H, m), 2.05 (1H, br d, J=3.3 Hz), 2.28 (1H, dd, J=13.9, 4.0 Hz), 2.51 (1H, dd, J=13.9, 11.2 Hz), 2.78 (3H, d, J=4.6 Hz), 2.82 (1H, m), 3.02 (1H, br d, J=12.5 Hz), 3.87 (1H, m), 4.73 (1H, d, J=11.9 Hz), 5.06 (1H, br s), 6.93 (1H, m), 9.91—10.12 (1H, br). HRMS (EI) m/z $(M+H)^+$: Calcd for

$C_{17}H_{33}N_4O_4$: 357.2502. Found: 357.2493.

***N*-Methyl-(3*S*)-2-[(2*R*)-2-hydroxyaminocarbonylmethyl-1-oxodecyl]-hexahydropyridazine-3-carboxamide (31e)** An amorphous powder, $[\alpha]_D^{25}$ −7.9° (c=0.50, EtOH). IR (film): 3260, 1650, 1625 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=6.6 Hz), 1.05—1.95 (17H, m), 2.05 (1H, br d, J=10.6 Hz), 2.29 (1H, dd, J=13.9, 3.3 Hz), 2.52 (1H, br t, J=13.9 Hz), 2.80 (3H, d, J=4.6 Hz), 2.83 (1H, m), 3.03 (1H, d, J=11.9 Hz), 3.84 (1H, m), 4.64 (1H, d, J=9.9 Hz), 5.05 (1H, br s), 6.55 (1H, br s), 9.20—9.80 (1H, br). HRMS (FAB) m/z $(M+H)^+$: Calcd for $C_{18}H_{35}N_4O_4$: 371.2658. Found: 371.2667.

***N*-Methyl-(3*S*)-2-[(2*R*)-2-hydroxyaminocarbonylmethyl-1-oxoundecyl]hexahydropyridazine-3-carboxamide (31f)** An amorphous powder, $[\alpha]_D^{26}$ −7.6° (c=1.0, EtOH). IR (film): 3270, 1653, 1626 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.87 (3H, t, J=6.6 Hz), 1.05—1.93 (19H, m), 2.04 (1H, br d, J=11.9 Hz), 2.28 (1H, dd, J=13.9, 3.3 Hz), 2.51 (1H, dd, J=13.9, 11.2 Hz), 2.78 (3H, d, J=4.6 Hz), 2.82 (1H, m), 3.01 (1H, br d, J=12.5 Hz), 3.87 (1H, m), 4.74 (1H, d, J=11.9 Hz), 5.06 (1H, s), 6.96 (1H, m), 9.81—10.19 (1H, br). HRMS (FAB) m/z $(M+H)^+$: Calcd for $C_{19}H_{37}N_4O_4$: 385.2815. Found: 385.2802.

***N*-Methyl-(3*S*)-2-[(2*R*)-2-hydroxyaminocarbonylmethyl-1-oxododecyl]hexahydropyridazine-3-carboxamide (31g)** An amorphous powder, $[\alpha]_D^{26}$ −8.9° (c=0.61, EtOH). IR (film): 3267, 1652, 1625 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=6.6 Hz), 1.04—2.12 (22H, m), 2.19—2.63 (2H, m), 2.79 (3H, d, J=3.3 Hz), 2.80 (1H, m), 3.03 (1H, br d, J=13.2 Hz), 3.85 (1H, m), 4.64 (1H, d, J=11.9 Hz), 5.05 (1H, s), 6.68 (1H, m), 9.48—9.76 (1H, br). MS (EI) m/z $(M)^+$: 398. HRMS (EI) m/z $(M-H_2O)^+$: Calcd for $C_{20}H_{36}N_4O_3$: 380.2787. Found: 380.2809.

Purification of Gelatinases Gelatinase A and gelatinase B were purified from cultured cells of human fibrosarcoma HT 1080 as described previously.[6b] Briefly, the cells were cultured in Ham's F-12-Dulbecco's modified Eagle's medium (DMEM) 1 : 1 mixture, and the subconfluent cell cultures were incubated for 5 d in serum-free medium containing 100 units/ml recombinant human TNF α (Genzyme, U.S.A.).[20] The conditioned medium was adjusted to pH 8.0 and passed through a DEAE-cellulose column preequilibrated in 50 mM Tris–HCl buffer, pH 8.0. The flow—through fraction was applied to Green A Matrex Gel preequilibrated in buffer A (50 mM Tris–HCl buffer, pH 7.6, 10 mM $CaCl_2$, 0.05% Brij35, and 0.02% NaN_3), and the enzyme was eluted with buffer A containing 1.0 M NaCl. The fractions containing gelatinases A and B were pooled and applied to a gelatin–Sepharose (Pharmacia) column preequilibrated in buffer A containing 0.5 M NaCl. The column was extensively washed with the same buffer, and the enzyme was eluted using a 0—10% DMSO gradient. This step separated gelatinase A and gelatinase B. The activities of gelatinases during purification were monitored by gelatin zymography.

Gelatinase Assay Type I collagen-derived gelatin was used as the substrate. Type I collagen was purified from rat tail. After ^{3}H-acetylation, it was denatured by heat treatment at 60 °C for 30 min. Gelatinase B was activated with 1 mM aminophenylmercuric acetate (APMA) at 37 °C for 3 h, and APMA was eliminated by dialysis prior to assay. The assay was carried out in a total volume of 200 μl containing 50 mM Tris–HCl buffer, pH 7.5, 10 mM $CaCl_2$, 0.15 M NaCl, 0.05% Brij35, 0.02% NaN_3, 2 μg of ^{3}H-acetyl gelatin, and appropriate amounts of the enzyme. For gelatinase A, the reaction mixture contained 1 mM APMA. Assays were carried out at 37 °C for 0.5—3 h, and were terminated as described.[21] In all experiments, conditions were chosen such that the reaction was a linear function of time.

Thermolysin Assay Thermolysin was assayed by the method of Komiyama *et al.*, using *N*-benzyloxycarbonyl-glycyl-L-leucine-amide as a substrate.[22]

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References and Notes

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