Design and Synthesis of Phosphinamide-Based Hydroxamic Acids as Inhibitors of Matrix Metalloproteinases

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A new series of hydroxamic acid-based matrix metalloproteinase (MMP) inhibitors containing a unique phosphinamide motif derived from D-amino acid was designed, synthesized, and tested for enzyme inhibition. Compounds with an *R* configuration at phosphorus were found to be potent MMP inhibitors while molecules with the *S* configuration were almost inactive. Structure−activity relationship studies of the series led to the discovery of the potent inhibitor **16** with IC_{50} = 20.5 nM and 24.4 nM against fibroblast collagenase (MMP-1) and stromelysin (MMP-3), respectively. The binding mode of this novel phosphinamide-based series of MMP inhibitors was established based on X-ray crystallography of the complex of stromelysin and **16**.

Introduction

The matrix metalloproteinases (MMPs) are a family of zinc-containing enzymes that are capable of degrading many proteinaceous components of the extracellular matrix.[1] Important members of this family are collagenase (MMP-1), which cleaves all three α-chains of native interstitial collagens; 72-kDa gelatinase (MMP-2) and 92-kDa gelatinase (MMP-9), which cleave denatured collagen (gelatin) and basement membrane; and stromelysin (MMP-3), which degrades a wider variety of protein substrates including gelatin, fibronectin, and laminin as well as the core protein of cartilage proteoglycans.

Matrix metalloproteinases have been implicated in several pathological processes including arthritis,[2,3] tumor growth, metastasis,[4] periodontal disease,[5] and multiple sclerosis;[6] thus, the discovery of potent and selective inhibitors of the enzymes is a highly attractive endeavor both scientifically and commercially. The growing interest in this field is reflected by an increasing number of inhibitors that are being discovered in various industrial and academic laboratories.[7] Data from phase II pancreatic cancer trial of marimastat, the most clinically advanced MMP inhibitor, have also been discussed.[8,9]

Design

CGS27023A (Ciba-Geigy, Figure 1) was the first potent, nonpeptidic MMP inhibitor to be described.[10] Structure−activity relationship (SAR) data[11,12] obtained from this novel series of hydroxamic acids suggested that the sulfonamide group is essential for potent inhibition of stromelysin. Introduction of the amide group in place of the sulfonamide linkage, as in molecule **A**, eliminated all in vitro activity. On the basis of this observation, the authors suggested[11] that the oxygen atoms of the sulfonamide group might develop two distinct hydrogen bonds leading to strong binding with the enzyme. The authors also contemplated that the increased conformational freedom of the sulfonamide bond could account for the observed increase in potency. We thought that the tetrahedral geometry at the sulfur atom, as opposed to planarity of the amide linkage, could also be a factor playing an important role in determining potency. Searching for experimental evidence, we have designed analogues of CGS27023A and molecule **A** that mimic the geometry and conformational freedom of the sulfonamide bond yet provide only one oxygen atom for possible interactions with the enzyme. We proposed that a phosphinamide linkage, as represented by molecule **B**, might be a good candidate to provide the required structural properties. Indeed, this idea has led to the discovery of a novel series of MMP inhibitors with unique SAR.

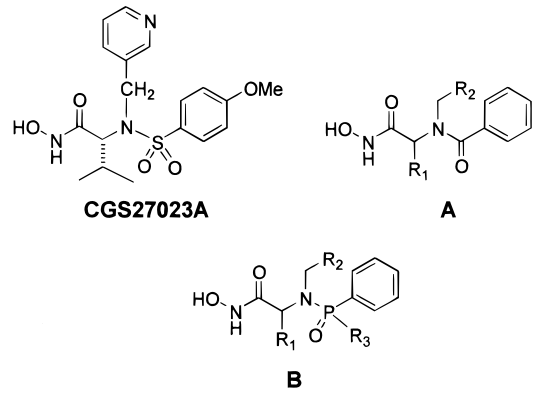

Figure 1. Design of phosphinamide-based MMP inhibitors.

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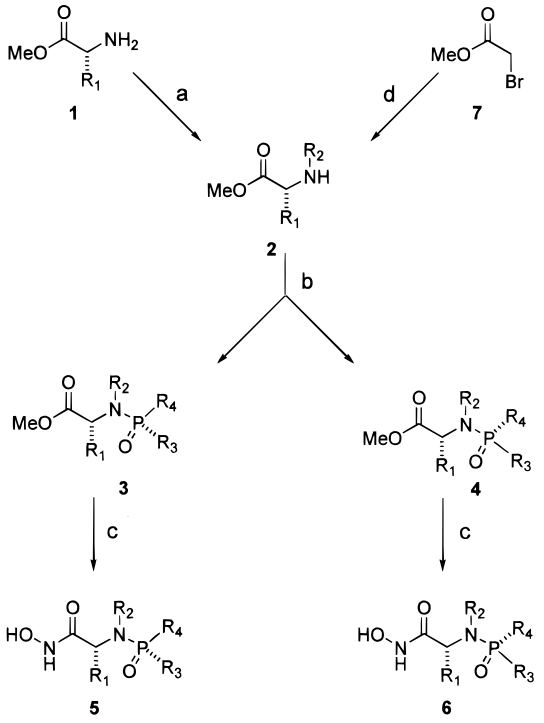
Scheme 1[a]



[a] Reagents: (a) R_2CHO, $NaBH_3(CN)$; (b) $R_3R_4P(O)Cl$, Et_3N, silica gel chromatography; (c) NH_2OH, KOH, MeOH; (d) R_2NH_2, Et_3N, DMF.

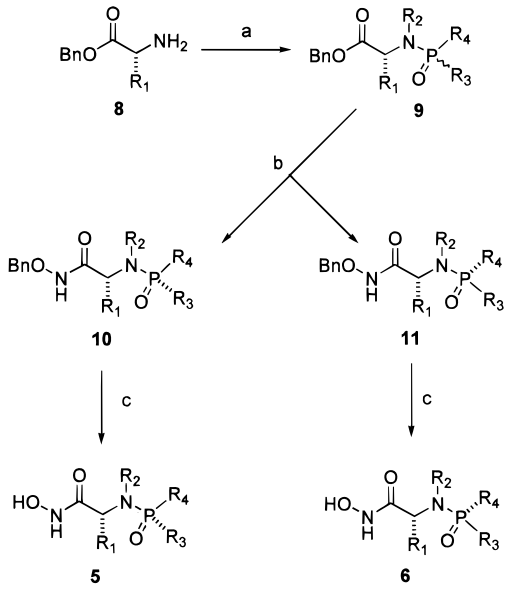
Chemistry

The preparation of hydroxamic acids **5** and **6** was carried out as shown in Scheme 1. The D-amino acid methyl ester **1** was subjected to the reductive alkylation procedure using an appropriate aldehyde and sodium cyanoborohydride. The resulting N-alkylated amino acid **2** was then allowed to react with alkylaryl (diaryl) phosphinyl chloride in the presence of triethylamine to produce the diastereomeric phosphinamides **3** and **4** which were separated by silica gel column chromatography. The diastereomerically pure esters were converted to the corresponding hydroxamic acids **5** and **6** using methanolic hydroxylamine generated by the treatment of its hydrochloride salt with methanolic potassium hydroxide.[13]

In the case of the glycine-derived inhibitors ($R_1 = H$), intermediate **2** was prepared using methyl bromoacetate (**7**) as the starting material. The reaction of **7** with the corresponding primary amine in the presence of triethylamine in *N*,*N*-dimethylformamide proceeded cleanly to give the corresponding N-alkylated product **2**. Further steps toward the target hydroxamic acid were identical to those described above.

An alternative synthetic sequence involving the stepwise introduction of the hydroxamic acid was also developed and is shown in Scheme 2. The amino acid benzyl ester **8** was converted into a mixture of diastereomeric phosphinamides **9** using the methodology described above (see Scheme 1). The free acids, obtained after removal of the benzyl protecting group by hydrogenolysis, were then coupled with *O*-benzylhydroxylamine. The diastereomerically pure benzyl hydroxamates **10** and **11** were obtained after silica gel chromatography. The target hydroxamic acids **5** and **6** were

Scheme 2[a]



[a] Reagents: (a) see Scheme 1, steps (a) and (b); (b) 1. H_2, Pd/C, 2. $BnONH_2$, HOBT, EDAC, NMM; (c) H_2, Pd/C.

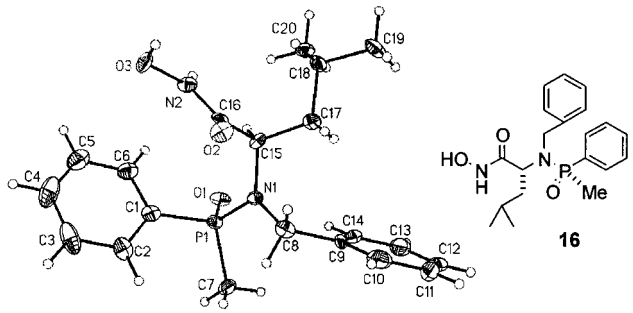


Figure 2. An ORTEP view of phosphinamide **16**.

prepared by palladium-catalyzed hydrogenolysis of the benzyl hydroxamate.

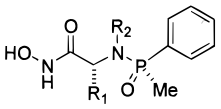
The absolute configuration at the phosphorus center was established using high-resolution X-ray data obtained for compound **16** shown in Figure 2.[14] Because **16** and the corresponding benzyl hydroxamate showed higher mobility on silica gel chromatography plates compared to their diastereomers, the *R* configuration on phosphorus was assigned by analogy to the more mobile compounds for all diastereomeric pairs in the series. This assignment was also supported by a characteristic signal pattern in ^1H NMR which was similar for all diastereomeric pairs.

All hydroxamic acids were found to be stable in the solid state and in aqueous solutions at pH > 4.0. However, at low pH we observed a slow decomposition due to hydrolysis of the phosphinamide bond. The results of detailed studies of this process will be published separately.

Results and Discussion

SAR of R_1 and R_2 Position. All compounds were tested for the inhibition of truncated collagenase-1 (MMP-1[15]) and stromelysin (MMP-3[16]), and the data are summarized in Tables 1, 2, and 3. The first molecule prepared to test the concept of the sulfonamide-to-phosphinamide switch was the simple glycine derivative **12**. This compound, as a racemic mixture, showed

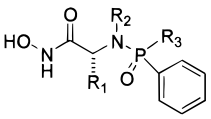
Table 1. SAR at R_1 and R_2 Position in the Series with *R* Configuration on the Phosphorus



compd	R_1	R_2	IC_{50} (nM)[a] MMP-1	MMP-3
12[b]	H	$(CH_2)_2C_6H_5$	525	700
13	CH_3	$CH_2C_6H_5$	120	67.9
14	CH_3	*n*-C_6H_{13}	1290	1600
15	CH_2*i*-Pr	H	2510	2550
16	CH_2*i*-Pr	$CH_2C_6H_5$	20.5	24.4
CGS27023A			49.5	16.9 (43)[c]

[a] See Experimental Section for details of the enzyme assays. [b] Racemic mixture. [c] K_i values reported[12] for CGS27023A are given in parentheses.

Table 2. Effect of Configuration at the Phosphorus Atom on Inhibition of Collagenase and Stromelysin



compd[a]	R_1	R_2	R_3	IC_{50} (nM)[b] MMP-1	MMP-3
15 (*R*)	CH_2*i*-Pr	H	CH_3	2510	2550
17 (*S*)	CH_2*i*-Pr	H	CH_3	>100000	130500
16 (*R*)	CH_2*i*-Pr	$CH_2C_6H_5$	CH_3	20.5	24.4
18 (*S*)	CH_2*i*-Pr	$CH_2C_6H_5$	CH_3	7120	9170
19 (*R*)	CH_3	$CH_2C_6H_5$	C_2H_5	608	700
20 (*S*)	CH_3	$CH_2C_6H_5$	C_2H_5	33300	49300

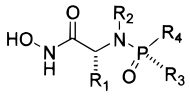
[a] Configuration at the phosphorus center. [b] See Experimental Section for details of the enzyme assays.

promising potency of less than 1 μM against both collagenase and stromelysin. A brief SAR study of a series of inhibitors with the *R* configuration at the phosphorus center (Table 1) pointed to the positive effect of a bulky substituent R_1 on the inhibitory potency (e.g. compare compounds **12**, **13**, and **16**). Appropriate substitution at R_2 was also very important for in vitro activity, with alkylaryl substituents giving the best results (e.g. **13** versus **14** and **16** versus **15**).

As shown in Table 2, the stereochemistry at the phosphorus atom has a major influence on the potency of the phosphinamide-based MMP inhibitors. A decrease in potency of about 2 orders of magnitude was observed when switching the configuration at phosphorus from *R* to *S*. Some insight into the conformational requirements for such an effect was gained from X-ray studies of the stromelysin−**16** complex and is discussed below.

SAR of the R_3 and R_4 Positions. After establishing that the correct absolute configuration of the phosphorus atom is essential for inhibitory activity, we briefly studied the SAR of substituents R_3 and R_4 (see Table 3). Clearly an increase in size of R_3 has a negative effect on activity. This effect is especially pronounced for compounds with substitution at the α-position to the hydroxamic acid ($R_1 \neq H$). Exchanging the methyl group at R_3 for a bulkier phenyl led to a 2-fold decrease in potency in the glycine series (compare compound **12** versus **21**), but a 2 orders of magnitude difference was observed in the alanine series (compound **13** versus **22**).

Table 3. SAR at the R_3 and R_4 Position



compd	R_1	R_2	R_3	R_4	IC_{50} (nM)[a] MMP-1	MMP-3
12[b]	H	$(CH_2)_2C_6H_5$	Me	Ph	525	700
21[b]	H	$(CH_2)_2C_6H_5$	Ph	Ph	854	1750
13	Me	$CH_2C_6H_5$	Me	Ph	120	67.9
19	Me	$CH_2C_6H_5$	Et	Ph	608	700
22	Me	$CH_2C_6H_5$	Ph	Ph	6790	10300
16	CH_2*i*-Pr	$CH_2C_6H_5$	Me	Ph	20.5	24.4
23	CH_2*i*-Pr	$CH_2C_6H_5$	Me	Me	518	1040

[a] See Experimental Section for details of the enzyme assays. [b] Racemic mixture.

Both enzymes seem to have little tolerance for changes in the R_3 position. In the case of MMP-3, substituting the methyl group (compound **13**) by a slightly larger ethyl group (compound **19**) decreased the potency by 1 order of magnitude.

The presence of the α-phenyl group at position R_4 is essential for the high potency observed with this series of MMP inhibitors. This is illustrated by a 20−30-fold drop in potency observed for compound **23** (versus **16**) where the phenyl group is replaced by a small methyl substituent. However, it is remarkable that **23** is still a high nanomolar inhibitor with such a small P1′ substituent.

Inhibition of Gelatinase, Matrilysin, Neutrophil Collagenase, Gelatinase B and Collagenase-3.[15] Selected molecules from the phosphinamide series have also been tested for activity against several other matrix metalloproteinases: gelatinase A (MMP-2), matrilysin (MMP-7), neutrophil collagenase (MMP-8), gelatinase B (MMP-9), and collagenase-3 (MMP-13) (Table 4). They inhibited other matrix metalloproteinases with potencies matching those observed for collagenase and stromelysin. The only exception was matrilysin for which considerably weaker inhibition at a low micromolar level was observed.

Structure of Stromelysin−Inhibitor Complex.[17] To better understand the binding interactions between the phosphinamide-based hydroxamic acids and the MMP enzymes we obtained a crystal of the truncated stromelysin−**16** complex and solved its structure using the molecular replacement method. The catalytic site of stromelysin with the bound inhibitor is shown schematically in Figure 3. In general, interactions of the hydroxamic acid with stromelysin were consistent with those already reported in the literature.[18] The hydroxamic acid acted as a bidentate ligand with the two oxygen atoms in binding distance of 1.83 and 2.07 Å from the active site Zn^{2+} ion. The terminal OH oxygen and the nitrogen atom of the hydroxamic acid formed additional hydrogen bonds with the oxygen Oϵ2 (2.80 Å) of the carboxyl group of Glu-202 and the carbonyl group (3.09 Å) of Ala-165, respectively (see Figures 3 and 4).

The most interesting feature of the enzyme−inhibitor interactions was revealed by analysis of the environment around the phosphorus atom. The phosphinic acid oxygen was clearly within the hydrogen-bonding distance to the N−H of Leu-164 (2.70 Å) and Ala-165 (3.19

Table 4. Inhibition of MMP-2, MMP-7, MMP-8, MMP-9, and MMP-13

	IC_{50} (nM)[a]						
compd	MMP-1	MMP-2	MMP-3	MMP-7	MMP-8	MMP-9	MMP-13
13	120	nd	67.9	1860	nd	32.5	nd
16	20.5	13.3	24.4	886	5.3	20.6	7.4
CGS27023A	49.5 (33)[b]	9.1 (20)[b]	16.9 (43)[b]	106	4.4	4.3 (8)[b]	4.3

[a] See Experimental Section for details of the enzyme assays. nd = not determined. [b] K_i values reported[12] for CGS27023A are given in parentheses.

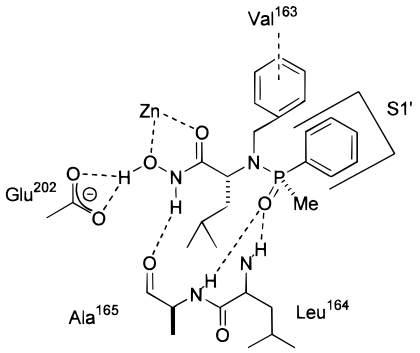


Figure 3. Binding interactions between **16** and stromelysin.

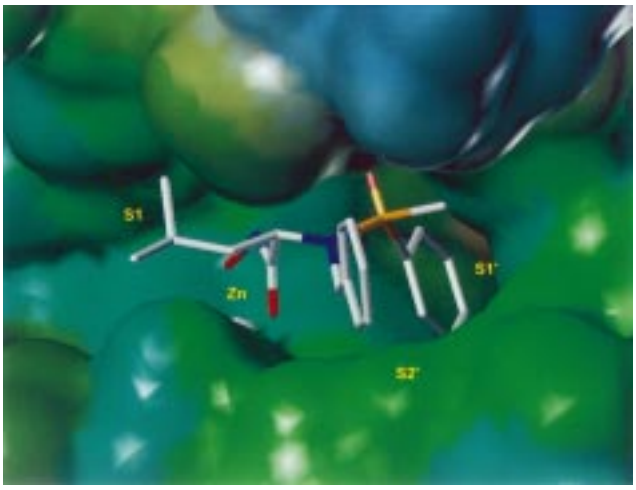


Figure 4. Active site of stromelysin with bound **16**.

Å). Additionally, the phosphorus bound methyl group was within van der Waals distance to Leu-164. Such an arrangement of substituents at the phosphorus center seemed to be quite optimal since the methyl-to-ethyl switch at the phosphorus center resulted in a 6−10-fold decrease of potency against both target enzymes (compare compound **13** versus **19**). The enzyme's sensitivity to changes in the phosphorus environment was further confirmed by a loss in potency of about 2 orders of magnitude upon the inversion of configuration (see Table 2). In fact this is the area where CGS27023A may experience some unfavorable interactions with MMP enzymes by placing one of the sulfonyl oxygen atoms close to the hydrophobic isobutyl chain of Leu-164.

The phosphorus bound phenyl group was comfortably placed in the S1′ pocket of stromelysin, similar to that of CGS27023A,[18] and no additional substituent on the aryl ring was necessary to achieve low nanomolar potency. Interestingly, replacement of the entire aromatic system bound to phosphorus by a methyl group resulted in only a 20−30-fold drop in potency. The *N*-benzyl and the isobutyl groups of **16** were within van der Waals distance to the hydrophobic side chains of Val-163, Leu-164, and His-166, providing additional positive interactions of the inhibitor with stromelysin.

Conclusions

In summary, we have designed a phosphinamide-based compounds group as structural probes of enzyme−inhibitor binding interactions in the region of Leu-164 and Ala-165 of stromelysin. The phosphinamide linkage was introduced as a mimic of tetrahedral geometry and conformational freedom of the sulfonamide group present in a series of potent MMP inhibitors. It was also a mimic of inactive amides by providing only one oxygen atom (bound to phosphorus) capable of developing hydrogen bond interactions with Leu-164 and/or Ala-165. Following this design a series of phosphinamide-based hydroxamic acids was synthesized and tested for inhibitory activity of several matrix metalloproteinases. Many of the analogues were found to be potent inhibitors of fibroblast collagenase (MMP-1), gelatinase A (MMP-2), stromelysin (MMP-3), neutrophil collagenase (MMP-8), gelatinase B (MMP-9), and collagenase-3 (MMP-13) and moderate inhibitors of matrilysin (MMP-7). Optimum enzyme inhibitory activity in the series was associated with the *R* configuration at the phosphorus chiral center, allowing for optimal hydrogen bond interactions between the phosphinic acid oxygen atom and Leu-164 and Ala-165 of stromelysin. The presence of this interaction was confirmed by X-ray crystallography of an inhibitor−enzyme complex. All the SAR and the X-ray crystallography data show that (1) one oxygen atom can provide sufficient binding interaction with Leu-164 and Ala-165 of stromelysin and (2) the tetrahedral geometry of the sulfonamide or the phosphinamide linkage is critical for the potent inhibition of matrix metalloproteinases.

Experimental Section

General Methods and Materials. ^1H and ^{13}C NMR spectra were measured at 300 and 75.4 MHz, respectively, on a Bruker or GE instrument using chloroform as solvent unless otherwise indicated. Mass spectra were measured at 70 eV (CI mode). Analytical thin-layer chromatography (TLC) analysis was performed using Merck DC-F_{254} silica gel plates. Flash chromatography was performed as described by Still[19] using kieselgel 60 (230−400 mesh) silica gel. All organic extracts were dried with anhydrous $MgSO_4$ or Na_2SO_4 prior to solvent removal on a rotary evaporator under reduced pressure. Human synovial proMMP-3 was obtained from Dr. Hideaki Nagase, University of Kansas Medical Center, Kansas City, KS. Human fibroblast proMMP-1, human MMP-9, and human recombinant MMP-7 catalytic domain were obtained from Dr. Howard Welgus, Jewish Hospital, St. Louis, MO. Human recombinant MMP-8 catalytic domain was obtained from Dr. Harald Tschesche, University Bielefeld, Bielefeld, Germany. Human recombinant proMMP-2 was purified from CHO cells as described below. Human recombinant truncated MMP-3 and truncated MMP-1 were purified from *Escherichia coli* cells

as described below. Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 was purchased from Bachem Bioscience, King of Prussia, PA.

***N*-(2-Phenethyl)glycine Methyl Ester Hydrochloride.** A solution of phenylethylalanine (6.63 mL, 52.8 mmol) and triethylamine (7.39 mL, 53 mmol) in anhydrous *N*,*N*-dimethylformamide (80 mL) was cooled to 0 °C, and to this mixture was dropwise added a solution of methylbromoacetate (5 mL, 52.8 mmol) in anhydrous *N*,*N*-dimethylformamide (40 mL). The reaction was stirred for 20 min at 0 °C. The mixture was poured into 250 mL of ethyl acetate, washed with water (3×), dried over sodium sulfate, and evaporated to give a colorless oil. To prepare the hydrochloride the crude oil was dissolved in ether (75 mL). In a separate flask, acetyl chloride (3.8 mL) was added dropwise to 2.5 mL of methanol at 0 °C. This solution was added dropwise to the ether solution. The precipitated solids were collected by filtration to give 9.2 g (76% yield) of *N*-(2-phenethyl)glycine methyl ester hydrochloride as a colorless solid: ^{1}H NMR ($CDCl_3$) δ 3.31 (m, 4H), 3.89 (t, 2H, J = 5.5 Hz), 3.76 (s, 3H), 7.27 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 36.23, 50.55, 51.54, 54.83, 126.03, 128.27, 128.47, 139.45, 172.55.

***N*-(Diphenylphosphinyl)-*N*-(2-phenylethyl)glycine Methyl Ester.** Diphenylphosphinic chloride (0.42 mL, 2.2 mmol) was dissolved in dichloromethane (5 mL) and cooled to 0 °C. To this mixture was added a solution of ester *N*-(2-phenethyl)glycine methyl ester hydrochloride (500 mg, 2.2 mmol) and *N*-methylmorpholine (0.73 mL, 6.6 mmol) in dichloromethane (5 mL). The reaction was stirred for 16 h at room temperature, washed with water and brine, dried over sodium sulfate, and concentrated to give *N*-(diphenylphosphinyl)-*N*-(2-phenylethyl)glycine methyl ester as a colorless solid: ^{1}H NMR ($CDCl_3$) δ 2.72 (m, 2H), 3.19 (m, 2H), 3.61 (s, 3H), 3.78 (d, 2H, J = 6.0 Hz), 6.87 (m, 2H), 7.13 (m, 3H), 7.42 (m, 5H), 7.70−7.95 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 34.97, 47.27, 49.67, 51.86, 126.26, 128.42, 128.58, 131.97, 132.46, 132.59, 138.62, 176.56.

***N*-Hydroxy-2-[[diphenylphosphinyl](2-phenylethyl)-amino]-acetamide (21)**. *N*-(Diphenylphosphinyl)-*N*-(2-phenylethyl)glycine methyl ester (160 mg, 0.41 mmol) was dissolved in methanol (2.5 mL). To this mixture was added hydroxylamine hydrochloride (57 mg, 0.81 mmol), followed by 2 mmol of a 25% methanolic solution of sodium methoxide. The reaction was stirred for 16 h, neutralized with 1 N hydrochloric acid, and concentrated. The crude product was purified by silica gel flash chromatography to give 41.6 mg (26% yield) of hydroxamic acid **21** as a colorless solid: ^{1}H NMR (CD_3OD) δ 2.83 (m, 2H), 3.18 (m, 2H), 3.68 (d, 2H, J = 9.9 Hz), 6.96 (m, 2H), 7.16 (m, 3H), 7.52−7.65 (m, 7H), 7.94 (m, 3H); ^{13}C NMR (CD_3OD) δ 35.92, 47.67, 51.00, 127.47, 129.56, 129.95, 130.12, 130.59, 132.38, 133.67, 133.80, 139.91, 168.83; ^{31}P NMR (CD_3OD) δ 35.19; MS-IS *m*/*z* 395 $[M + H]^+$, 417 $[M + Na]^+$, 433 $[M + K]^+$; HRMS calcd for $C_{22}H_{24}N_2O_3P$ $(M + H)^+$, 395.1525; Found, 395.1527. Anal. ($C_{22}H_{23}N_2O_3P \cdot 0.25H_2O$) C, H, N.

***N*-Hydroxy-2-[[methylphenylphosphinyl](2-phenylethyl)-amino]-acetamide (12).** Following the route described above and using methylphenylphosphinic chloride compound **12** was obtained with 47% yield as a colorless solid: ^{1}H NMR (CD_3OD) δ 1.80 (d, 3H, J = 13.9 Hz), 2.75 (t, 2H, J = 7.0 Hz), 3.13 (m, 2H), 3.71 (ABX, 2H, J_{AB} = 10 Hz, J_{AX} = 13 Hz, J_{BX} = 13 Hz), 7.03 (m, 2H), 7.17 (m, 3H), 7.54 (m, 3H), 7.82 (m, 3H); ^{13}C NMR (CD_3OD) δ 14.05, 15.28, 35.79, 47.41, 50.25, 127.48, 129.56, 129.80, 130.00, 132.49, 132.63, 133.59, 140.05; ^{31}P NMR (CD_3OD) δ 43.85; MS-IS *m*/*z* 333 $[M + H]^+$, 350 $[M + NH_4]^+$, 355 $[M + Na]^+$, 371 $[M + K]^+$. Anal. ($C_{17}H_{21}N_2O_3P$) C, H, N.

Ethyl Ethylphenylphosphinate. A mixture of diethyl phenylphosphonite (4.5 g, 22.70 mmol), ethyl iodide (0.24 mL, 3 mmol), and benzene (100 mL) was stirred and heated at 85 °C for 24 h. Another portion of ethyl iodide (0.30 mL, 3.75 mmol) was added, and the reaction was stirred for an additional 36 h at 85 °C. The volatiles were removed on a rotary evaporator to give ethyl ethylphenylphosphinate as an oil: ^{1}H NMR (CD_3OD) δ 1.08 (m, 3H), 1.27 (t, 3H, J = 6.0 Hz), 1.88 (m, 2H), 3.84 (m, 1H), 4.09 (m, 1H), 7.43 (m, 3H), 7.78 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 5.65, 16.19, 16.28, 21.85, 23.20, 60.27, 128.23, 128.38, 129.60, 131.39, 131.90; ^{31}P NMR (CD_3OD) δ 46.72.

Ethylphenylphosphinic Chloride. To a solution of ethyl ethylphenylphosphinate (2 g, 10 mmol) in benzene (200 mL) was added oxalyl chloride (1.3 mL, 15 mmol). The mixture was stirred for 3 h at room temperature and concentrated on a rotary evaporator, and the product was dried under vacuum for 12 h to give ethylphenylphosphinic chloride as an oil: ^{1}H NMR ($CDCl_3$) δ 1.23 (m, 3H), 2.38 (m, 2H), 2.59 (m, 3H), 7.88 (m, 2H).

***N*-Benzyl D-Alanine Methyl Ester.** D-Alanine methyl ester (4 g, 28.66 mmol) was dissolved in methanol (100 mL), and sodium acetate (5.88 g, 71.65 mmol) and benzaldehyde (2.9 mL, 28.66 mmol) were added. The mixture was stirred for 15 min, and then a solution of sodium cyanoborohydride (1.08 g, 17.2 mmol) in methanol (5 mL) was added dropwise. After the mixture was stirred for 2 h, the methanol was evaporated under reduced pressure, and the product was extracted into ether. The organic phase was washed with water (2×). The crude product was purified by flash silica gel chromatography (8:2 hexane:ethyl acetate) to give 3.3 g (44% yield) of *N*-benzyl D-alanine methyl ester as an oil: ^{1}H NMR (CD_3OD) δ 1.33 (d, 3H, J = 7.1 Hz), 3.40 (q, 1H, J = 7.0 Hz), 3.73 (s, 3H), 3.74 (AB, 2H, J = 13.0, 39.7 Hz), 7.23−7.56 (m, 5H); ^{13}C NMR (CD_3OD) δ 18.95, 51.71, 51.88, 55.85, 127.08, 128.22, 128.38, 139.98, 176.52.

***N*-((*R* and *S*)-Ethylphenylphosphinyl)-*N*-benzyl-D-alanine Methyl Ester.** To a solution of ethylphenylphosphinic chloride (1.04 g, 5.5 mmol) in dichloromethane (15 mL) was added a solution of *N*-benzyl D-alanine methyl ester (1.37 g, 7.1 mmol) and *N*-methylmorpholine (1.36 mL, 12.4 mmol) in dichloromethane (15 mL). A catalytic amount of 4-(dimethylamino)pyridine was added, and the reaction was stirred 90 h at room temperature. Two spots were observed on TLC. These compounds were separated by flash silica gel chromatography (95:5 ethyl acetate:methanol) to give two diastereomeric products.

***N*-((*R*)-Ethylphenylphosphinyl)-*N*-benzyl-D-alanine Methyl Ester:** 41% yield; R_f 0.35, 100% ethyl acetate; ^{1}H NMR (CD_3OD) δ 1.04 (m, 3H), 1.31 (d, 3H, J = 7.3 Hz), 1.93 (m, 2H), 3.43 (s, 3H), 4.19−4.33 (m, 3H), 7.21−7.35 (m, 5H), 7.48 (m, 3H), 7.80 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 5.78, 16.68, 20.14, 21.33, 47.45, 51.50, 53.22, 126.99, 127.54, 128.16, 128.28, 131.31, 131.43, 139.10 173.98; ^{31}P NMR ($CDCl_3$) δ 43.40.

***N*-((*S*)-Ethylphenylphosphinyl)-*N*-benzyl-D-alanine Methyl Ester:** 25% yield; R_f 0.25, 100% ethyl acetate; ^{1}H NMR (CD_3OD) δ 1.12 (m, 3H), 1.32 (d, 3H, J = 7.3 Hz), 2.07 (m, 2H), 3.55 (s, 3H), 4.24 (AB, 2H, J_{AB} = 9.9 Hz, J_{AX} = J_{BX} = 3.7 Hz), 4.37 (m, 1H), 7.26 (m, 5H), 7.50 (m, 3H), 7.82 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 6.06, 16.80, 20.04, 21.25, 47.53, 51.66, 53.04, 126.94, 127.60, 128.03, 128.19, 131.24, 131.36, 131.50, 138.80, 173.29; ^{31}P NMR ($CDCl_3$) δ 43.35.

***N*-Hydroxy-2(*R*)-[[(*R*)-ethylphenylphosphinyl]amino]-propionamide (19).** *N*-((*R*)-Ethylphenylphosphinyl)-*N*-benzyl-D-alanine methyl ester (333 mg, 0.96 mmol) was treated with a solution of NH_2OH[13] (3.3 mL, 1.76 M in methanol), and the reaction was stirred at room temperature for 16 h. The reaction mixture was neutralized with 1 M aqueous HCl and concentrated on a rotary evaporator. The residue was purified by flash silica gel chromatography (95:5 ethyl acetate:methanol) to give 110 mg (33% yield) of **19** as a colorless solid: ^{1}H NMR (CD_3OD) δ 1.06 (m, 3H), 1.27 (d, 3H, J = 7.0 Hz), 2.03 (m, 2H), 3.95 (m, 1H), 4.52 (m, 2H), 7.25 (m, 5H), 7.59 (m, 3H), 7.82 (m, 2H); ^{13}C NMR (CD_3OD) δ 6.26, 18.12, 21.11, 22.31, 53.63, 128.11, 128.25, 128.49, 129.41, 129.79, 129.95, 132.74, 132.87, 133.50, 141.32, 171.98; ^{31}P NMR (CD_3OD) δ 48.14; MS-IS *m*/*z* 347 $[M + H]^+$, 369 $[M + Na]^+$. Anal. ($C_{18}H_{23}N_2O_3P \cdot 0.15H_2O$) C, H, N.

***N*-Hydroxy-2(*R*)-[[(*S*)-ethylphenylphosphinyl]amino]-propionamide (20).** Following the method described for the preparation of **19**, compound **13** was obtained from the corresponding methyl ester in 54% yield as a colorless solid:

^{1}H NMR (CD_3OD) δ 0.94 (m, 3H), 1.17 (d, 3H, J = 7.0 Hz), 2.02 (m, 2H), 4.04 (m, 1H), 4.43 (ABX, 2H, J_{AB} = 13.4 Hz, J_{AX} = J_{BX} = 17.0 Hz); ^{13}C NMR (CD_3OD) δ 6.25, 16.92, 20.92, 22.35, 53.87, 128.27, 129.54, 129.94, 130.10, 132.93, 133.06, 133.68, 140.86, 173.10;^{31}P NMR (CD_3OD) δ 52.58; MS-IS *m/z* 347 $[M + H]^+$, 369 $[M + Na]^+$. Anal. ($C_{18}H_{23}N_2O_3P\cdot0.2H_2O$) C, H, N.

The following compounds were prepared according to the procedure described above for **19** and **20**.

***N*-Hydroxy-2(*R*)-[[(*R*)-methylphenylphosphinyl]-benzylamino]-propionamide (13):** 42% yield; colorless solid; ^{1}H NMR (CD_3OD) δ 1.28 (d, 3H, J = 10.3 Hz), 1.77 (d, 3H, J = 13.9 Hz), 3.95 (m, 1H), 4.50 (m, 2H), 7.20−7.35 (m, 5H), 7.47−7.60 (m, 3H), 7.8 (m, 2H); ^{13}C NMR (CD_3OD) δ 14.81, 16.02, 18.19, 53.63, 128.13, 128.46, 129.44, 129.79, 129.69, 132.26, 132.40, 133.50, 141.43, 171.74; ^{31}P NMR (CD_3OD) δ 44.13; MS-IS *m/z* 333 $[M + H]^+$, 355 $[M + Na]^+$. Anal. ($C_{17}H_{21}N_2O_3P\cdot0.25H_2O$) C, H, N.

***N*-Hydroxy-2(*R*)-[[(*R*)-methylphenylphosphinyl]-hexylamino]-propionamide (14):** 56% yield; colorless solid; ^{1}H NMR (CD_3OD) δ 0.86 (t, 3H, J = 7.0 Hz), 1.16 (m, 6H), 1.39 (d, 3H, J = 7.1 Hz), 1.47 (m, 2H), 1.87 (d, 3H, J = 13.9 Hz), 3.06 (m, 2H), 3.99 (m, 1H), 7.60 (m, 3H), 7.84 (m, 2H); ^{13}C NMR (CD_3OD) δ 14.31, 14.52, 16.66, 17.31, 23.59, 27.82, 32.46, 32.59, 45.50, 53.60, 129.85, 130.02, 132.27, 132.41, 133.55, 172.45; ^{31}P NMR (CD_3OD) δ 43.23; MS-IS *m/z* 327 $[M + H]^+$, 349 $[M + Na]^+$. Anal. ($C_{16}H_{27}N_2O_3P$) C, H, N.

***N*-Hydroxy-2(*R*)-[[(*R*)-methylphenylphosphinyl]amino]-4-methylpentanamide (15).** Compound **15** was prepared from the corresponding methyl ester (R_f = 0.25, 100% ethyl acetate): 49% yield; colorless solid; ^{1}H NMR (CD_3OD) δ 0.95 (d, 3H, J = 6 Hz), 0.98 (d, 3H, J = 6.0 Hz), 1.20−1.41 (m, 3H), 1.61 (d, 3H, J = 14.0 Hz), 3.39 (m, 1H), 7.60 (m, 3H), 7.82 (m, 2H); ^{13}C NMR (CD_3OD) δ 15.86, 17.10, 22.63, 23.13, 25.54, 45.53, 64.43, 129.71, 129.88, 132.52, 132.66, 133.25, 177.91; ^{31}P NMR (CD_3OD) δ 35.38; MS-IS *m/z* 283 $[M + H]^+$. Anal. ($C_{13}H_{19}N_2O_3P$) C, H, N.

***N*-Hydroxy-2(*R*)-[[(*S*)-methylphenylphosphinyl]amino]-4-methylpentanamide (17).** Compound **17** was prepared from the corresponding methyl ester (R_f = 0.14, 100% ethyl acetate): 53% yield; colorless solid; ^{1}H NMR (CD_3OD) δ 1.64 (d, 3H, 6.6 Hz), 1.81 (d, 3H, 6.6 Hz), 1.20−1.42 (m, 3H), 1.64 (d, 3H, J = 14.7 Hz), 3.35 (m, 1H), 7.54 (m, 3H), 7.86 (m, 2H); ^{13}C NMR (CD_3OD) δ 15.32, 16.98, 22.35, 22.93, 25.46, 51.49, 129.67, 129.84, 132.53, 132.67, 133.34, 176.50; ^{31}P NMR (CD_3OD) δ 34.98; MS-IS *m/z* 283 $[M + H]^+$. Anal. ($C_{13}H_{19}N_2O_3P$) C, H, N.

***N*-Hydroxy-2(*R*)-[[diphenylphosphinyl]benzylamino]-propionamide (22):** 39% yield; oil; ^{1}H NMR (CD_3OD) δ 1.25 (d, 3H, J = 7.0 Hz), 4.04 (m, 1H), 4.39 (m, 2H), 7.16 (m, 4H), 7.40−7.61 (m, 6H), 7.80 (m, 3H), 7.92 (m, 2H); ^{13}C NMR (CD_3OD) δ 18.01, 54.59, 128.01, 128.72, 129.21, 129.91, 130.04, 132.60, 132.75, 133.49, 133.62, 133.71, 140.59, 171.43; ^{31}P NMR (CD_3OD) δ 36.52; MS-IS *m/z* 395 $[M + H]^+$, 417 $[M + Na]^+$; HRMS calcd for $C_{22}H_{24}N_2O_3P$ $(M + H)^+$, 395.1525; found, 395.1525. Anal. ($C_{22}H_{23}N_2O_3P$) C, H, N.

***N*-Hydroxy-2(*R*)-[[dimethylphosphinyl]benzylamino]-4-methylpentanamide (23):** 62% yield; colorless solid; ^{1}H NMR (DMSO) δ 0.58 (d, 3H, 6.4 Hz), 0.70 (d, 3H, J = 6.4 Hz), 1.20−140 (m, 2H), 1.33 (d, 3H, J = 12.5 Hz), 1.38 (d, 3H, J = 12.5 Hz), 1.70 (m, 1H), 3.78 (m, 1H), 4.20−4.50 (m, 2H), 7.10−7.40 (m, 5H); ^{13}C NMR (DMSO) δ 15.61, 16.05, 16.52, 17.21, 22.11, 22.56, 24.09, 45.57, 53.20, 126.42, 127.23, 128.03, 141.85, 168.41; ^{31}P NMR (DMSO) δ 47.78; MS-IS *m/z* 313 $[M + H]^+$, 335 $[M + Na]^+$, 357 $[M + K]^+$; HRMS calcd for $C_{15}H_{26}N_2O_3P$ $(M + H)^+$, 313.1681; found, 313.1688. Anal. ($C_{15}H_{25}N_2O_3P$) C, H, N.

***N*-Benzyl D-Leucine Benzyl Ester.** D-Leucine benzyl ester (3 g, 11.66 mmol) was dissolved in methanol, and to this mixture was added sodium acetate (1.9 g, 23.3 mmol) followed by benzaldehyde (1.2 mL, 11.66 mmol). The mixture was stirred for 10 min, and a solution of sodium cyanoborohydride (427 mg, 6.8 mmol) in methanol (4 mL) was added dropwise. The reaction was stirred for 3 h, 10% aqueous $NaHCO_3$ was added with stirring, the reaction mixture was concentrated using a rotary evaporator, and the product was extracted into ether (3×). The combined organic phases were washed with water (2×), dried over sodium sulfate, and evaporated to give 2.15 g (51% yield) of *N*-benzyl D-leucine benzyl ester as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 0.84 (d, 3H, J = 6.6 Hz), 0.91 (d, 3H, J = 6.6 Hz), 1.50 (t, 2H, J = 7.3 Hz), 1.79 (m, 1H), 3.35 (t, 1H, J = 7.2 Hz), 3.70 (AB, 2H, J = 12.8, 56.6 Hz), 5.17 (s, 2H), 7.22−7.47(m, 10H); ^{13}C NMR ($CDCl_3$) δ 22.02, 22.59, 24.69, 42.56, 51.94, 59.18, 66.15, 126.84, 127.41, 128.13, 128.39, 128.81, 129.08, 139.66, 175.72.

***N*-Benzyloxy-2(*R*)-[[(*R*)-methylphenylphosphinyl]-benzylamino]-4-methylpentanamide and *N*-Benzyloxy-2-(*R*)-[[(*S*)-methylphenylphosphinyl] benzylamino]-4-methylpentanamide.** Methyl phenyl phosphinic chloride (0.89 mL, 6.42 mmol) was dissolved in dichloromethane and cooled to 0 °C. To this mixture was added a solution of *N*-benzyl D-leucine benzyl ester (2 g, 6.42 mmol), and *N*-methyl morpholine (1.5 mL, 13.48 mmol) in dichloromethane. A catalytic amount of 4-(dimethylamino)pyridine was added, and the reaction was stirred at room temperature for 22 h. The mixture was concentrated using a rotary evaporator, and the residue was dissolved with ethyl acetate. This mixture was washed with water and brine, dried over sodium sulfate, and concentrated. The product was isolated by flash silica gel chromatography (100% ethyl acetate) in 76% yield as a mixture of diastereomers.

A mixture of diastereomeric benzyl esters (2.04 g, 4.53 mmol) was dissolved in methanol, 10% Pd/C (500 mg) was added, and the reaction mixture was stirred under a hydrogen atmosphere for 45 min. The mixture was filtered through Celite, and the filtrate was collected and concentrated to quantitatively give *N*-(*R*/*S*-methylphenylphosphinyl)-*N*-benzyl-D-leucine as a white glassy substance.

A portion of this material (1.5 g, 4.17 mmol) was dissolved in *N*,*N*-dimethylformamide and cooled to 0 °C. To this mixture was added sequentially hydroxybenzotriazole hydrate (1.69 g, 12.5 mmol), *N*-methylmorpholine (1.37 mL, 12.5 mmol), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC, 959 mg, 5 mmol). After the mixture was stirred for 10 min, *O*-benzylhydroxylamine hydrochloride (666 mg, 4.17 mmol) was added, and the reaction was stirred for 3 h, warming to room temperature. Two diastereomers were observed by TLC. To the mixture was added water, and the mixture was extracted with ethyl acetate. The organic phases were combined, washed with water and brine, dried over sodium sulfate, and concentrated to give an oil. The diastereomers were then isolated by flash silica gel chromatography (1:1 hexane:ethyl acetate) to give *N*-benzyloxy-2(*R*)-[[(*R*)-methylphenylphosphinyl]benzylamino]-4-methylpentanamide [37% yield; R_f = 0.25 (1:1 hexane:ethyl acetate); ^{31}P NMR (CD_3OD) δ 43.89] and *N*-benzyloxy-2(*R*)-[[(*S*)-methylphenylphosphinyl]benzylamino]-4-methylpentanamide [22% yield; R_f = 0.15 (1:1 hexane:ethyl acetate); ^{31}P NMR (CD_3OD) δ 43.93].

***N*-Hydroxy-2(*R*)-[[(*R*)-methylphenylphosphinyl]-benzylamino]-4-methylpentanamide (16).** *N*-Benzyloxy-2(*R*)-[[(*R*)-methylphenylphosphinyl]benzylamino]-4-methylpentanamide (460 mg, 0.99 mmol) was dissolved in methanol (10 mL), 10% Pd/C (100 mg) was added, and the reaction mixture was stirred under a hydrogen atmosphere for 2 h. The mixture was filtered through Celite, and the filtrate was collected and concentrated to give a white glassy solid. The product was crystallized from ethyl acetate−hexane to give 278 mg (75% yield) of **16** as a white crystalline solid: ^{1}H NMR (CD_3OD) δ 0.61 (d, 3H, J = 6.4 Hz), 0.68 (d, 3H, J = 6.4 Hz), 1.36−1.60 (m, 2H), 1.78 (m, 1H), 1.79 (d, 3H, J = 13.7 Hz), 3.75 (m, 1H), 4.50 (ABX, 2H, J_{AB} = 12.4 Hz, J_{AX} = J_{BX} = 16.7 Hz), 7.19−7.33 (m, 5H), 7.54 (m, 3H), 7.78 (m, 2H); ^{13}C NMR (CD_3OD) δ 14.92, 26.62, 22.35, 22.94, 25.87, 41.48, 56.19, 128.12, 128.87, 129.36, 129.83, 130.00, 132.17, 132.31, 133.50, 141.00, 171.29; ^{31}P NMR (CD_3OD) δ 44.28; MS-IS *m/z* 375 $[M + H]^+$, 397 $[M + Na]^+$, 413 $[M + K]^+$. Anal. ($C_{20}H_{27}N_2O_3P\cdot0.5H_2O$) C, H, N.

***N*-Hydroxy-2(*R*)-[[(*S*)-methylphenylphosphinyl]benzylamino]-4-methylpentanamide (18).** Following the method described for the preparation of **16**, compound **18** was obtained in 72% yield from the corresponding benzyl ester as a colorless solid: ^{1}H NMR (CD_3OD) δ 0.47 (d, 3H, J = 6.6 Hz), 0.64 (d, 3H, J = 6.6 Hz), 1.10 (m, 1H), 1.29 (m, 1H), 1.70 (m, 1H), 1.71 (d, 3H, J = 13.7 Hz), 3.84 (m, 1H), 4.35−4.87 (m, 2H), 7.24 (m, 5H), 7.56 (m, 3H), 7.83 (m, 2H); ^{13}C NMR (CD_3OD) δ 12.73, 13.94, 20.47, 21.50, 24.22, 38.68, 54.09, 126.60, 126.82, 127.94, 128.30, 128.47, 130.92, 131.05, 132.04, 139.75, 169.23; ^{31}P NMR (CD_3OD) δ 43.02; MS-IS *m/z* 375 $[M + H]^+$, 397 $[M + Na]^+$, 413 $[M + K]^+$. Anal. ($C_{20}H_{27}N_2O_3P \cdot 0.25H_2O$) C, H, N.

Expression and Purification of Human Recombinant Truncated MMP-1. Briefly, DNA sequence coding for Val82-Pro249 of proMMP-1 was amplified by polymerase chain reaction from a commercially available plasmid, p35-1 (ATCC, Rockville, MD; N. S. Templeton et al. *Cancer Res.* **1990** *50*, 5431−5437) encoding human interstitial MMP-1. The PCR fragment was ligated into the expression vector, pET-11a (Novagen, Madison, WI), and expressed in *E. coli* BL21(DE3) cells. The protein was solubilized from inclusion bodies in 6 M urea and 0.15 M NaCl (pH 7.5); refolded in 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, and 0.1 mM zinc acetate; and then purified to homogeneity over a hydroxamic acid inhibitor affinity column as previously described (Moore, W. A.; Spilburg, C. A. *Biochemistry* **1986**, *25*, 5189−5195). N-Terminal sequence analysis confirmed the presence of three N-terminal sequences of Val-Leu-Thr-Glu-Gly-Asn, Met-Val-Leu-Thr-Glu-Gly-Asn, and Leu-Thr-Glu-Gly-Asn (minor).

Expression and Purification of Human Recombinant proMMP-2. A partial cDNA clone for MMP-2 known as K-121 (Tryggvason, K. *J. Biol. Chem.* **1990**, *265*, 11077−11082.), was obtained from ATCC and subcloned into the pBlueScript SK^- (pBS) plasmid. Sanger dideoxy sequencing revealed that the first 134 bp of the coding sequence were missing from the 5′ end of K-121. To restore the missing sequence, two overlapping 90+-mer oligonucleotides were designed and synthesized. These, along with a 3′ antisense oligonucleotide, were used as primers to the K-121 template in a series of polymerase chain reaction (PCR) experiments to synthesize a full-length MMP-2 cDNA. The PCR product was then subcloned into pBS. To express MMP-2 in the mammalian CHO D^- cell system, it was first subcloned into the mammalian expression vector pJT1 (J. Ting, CRD), which contains the DHFR gene. Recombinant MMP-2/pJT1 was Polybrene transfected into CHO D^- cells. Clonal cell populations were isolated and screened for production of MMP-2 mRNA using specific oligonucleotide primers and reverse transcription PCR. Ten clones producing the highest levels of MMP-2 mRNA were selected, and the DHFR/MMP-2 construct was amplified by gradually increasing media methotrexate (MTX, a DHFR inhibitor) concentration. Clonal selection was further narrowed after assessing MMP bioactivity in an MMP fluorescence assay (M. Anastasio, CRD) and on zymogram gels. Those clones showing activity were tested for MMP-2 protein production by sequential Edman degradation protein sequencing (F. Wang, P&GP Cell & Molecular Biology Core) and Western blot. On the basis of all results, one clone was expanded for growth in roller bottles. Approximately 9 L of serum-free conditioned media with an MMP-2 concentration of ∼35 mG/L were generated. ProMMP-2 was purified from conditioned media as previously described (T. Crabbe et al. *Eur. J. Biochem.* **1993** *218*, 431−438) with the following modifications. Conditioned serum-free media was reduced in volume with an Amicon S1Y30 spiral-wound cartridge prior to chromatography on a gelatin-Sepharose 4B column equilibrated in 25 mM Tris/HCl, 30 mM NaCl, 10 mM $CaCl_2$, pH 7.5 (TNC buffer). The column was washed with equilibration buffer before elution of the bound protein by TNC buffer containing 1 M NaCl followed by 1 M NaCl and 10% (by vol) dimethyl sulfoxide (DMSO) in TNC buffer. ProMMP-2 fractions were concentrated in an Amicon ultrafiltration cell with a YM30 membrane and diafiltered against 25 mM MES/NaOH, 30 mM NaCl, 10 mM $CaCl_2$, pH 6.0 (MNC buffer). The concentrate was then chromatographed over a second gelatin-Sepharose 4B column equilibrated in MNC buffer, washed with equilibration buffer to remove nonbinding proteins, and eluted with MNC buffer containing 0.3 M NaCl and 10% (by vol) DMSO. Elution fractions containing progelatinase A were pooled, diluted, and loaded onto an S-Sepharose Fast Flow column equilibrated in MNC buffer and eluted with MNC buffer containing 0.3 M NaCl. The concentrated fractions of purified proMMP-2 were combined and stored in MNC buffer containing 0.3 M NaCl at −70 °C. N-Terminal sequence, amino acid, and mass spectrophotometric analysis confirmed the identity of the purified protein with the expected sequence for progelatinase A.

Expression and Purification of Human Recombinant Truncated MMP-3. Briefly, DNA sequence coding for Ala^{-1}−Thr^{255} of proMMP-3 was amplified by polymerase chain reaction from a recombinant plasmid encoding human synovial preproMMP-3 kindly provided by Dr. Hideaki Nagase (University of Kansas Medical Center, Kansas City, KS) and Dr. Markku Kurkinen (Department of Medicine, UMDNJ-Robert Wood Johnson Medical School). After DNA sequence verification, this DNA fragment was ligated into the expression vector, pET-3d (Novagen, Madison, WI), and expressed in *E. coli* BL21 (DE3) cells as previously described (Marcy, A. I. et al. *Biochemistry* **1991**, *30*, 6476−6483). ProMMP-3 was solubilized form inclusion bodies in 8 M guanidine−HCl, refolded in 100 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 0.5 mM zinc acetate, and activated overnight at 37 °C with 1.5 mM *p*-aminophenylmercuric acetate. Active, truncated MMP-3 was purified to homogeneity over a hydroxamic acid inhibitor affinity column as described (Moore, W. A.; Spilburg, C. A. *Biochemistry* **1986**, *25*, 5189−5195). N-Terminal sequence analysis confirmed the sequence to be consistent with the catalytic domain, Phe^{83}-Thr^{255}, of proMMP-3.

Collagenase (MMP-1) Inhibition Assay. ProMMP-1 was activated prior to assay by treatment with trypsin. MMP-1 activity was monitored using a fluorescence assay previously described.[15] In a Dynatech MicroFLUOR plate, 10 nM of activated collagenase was incubated with 8 μM Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 in 50 mM Tris-HCl, 10 mM $CaCl_2$, 0.15 M NaCl, 0.05% Brij for 20−30 min at 37 °C in the presence of varying concentrations of inhibitor. The reaction was then quenched with 50 mM EDTA and the fluorescence increase monitored on a Perkin-Elmer LS50B spectrofluorometer (λex 328 nm, λem 393 nM). Activity was measured as a percentage of control activity in the absence of inhibitor. Inhibitor concentrations were run in triplicate, and IC_{50} determinations were calculated from a four-parameter logistic fit of the data within a single experiment.

Stromelysin (MMP-3) Inhibition Assay. ProMMP-3 was activated prior to assay by treatment with *p*-aminophenylmercuric acetate or trypsin. MMP-3 activity was measured by following the degradation of ^{3}[H]-reduced, carboxymethylated transferrin.[16] In a Multiscreen DP filtration plate (Millipore), 50 ng of activated MMP-3, 30 μg of ^{3}[H]-transferrin, and varying concentrations of inhibitor were incubated in a buffer of 50 mM Tris-HCl, 10 mM $CaCl_2$, 0.15 M NaCl, 0.05% Brij (pH 7.5), at 37 °C for 3 h. The reaction was quenched by addition of 4.4% TCA, and TCA-soluble fragments were counted for radioactivity. Activity was measured as a percentage of control activity in the absence of inhibitor. Inhibitor concentrations were run in triplicate, and IC_{50} determinations were calculated from a four-parameter logistic fit of the data within a single experiment.

Gelatinase A (MMP-2), Matrilysin (MMP-7), Neutrophil Collagenase (MMP-8), Gelatinase B (MMP-9), and Rat Collagenase-3 (MMP-13) Inhibition Assay. Inhibition of all enzymes was measured according to the representative procedure described below for MMP-2. ProMMP-2 was activated prior to assay by treatment with 1 mM *p*-aminophenylmercuric acetate for 45 min at 37 °C. ProMMP-9 was activated with MMP-3 (1:20 MMP-3:MMP-9) and stored at −80 °C until use. MMP-7, MMP-8, and rat collagenase were all supplied as active enzymes and stored frozen until use. MMP activity was monitored using a fluorescence assay previously

described,[15] modified for a microtiter plate format. In a Dynatech MicroFLUOR plate, active enzyme was incubated with 8 μM Mca-Pro-Leu-Dpa-Ala-Arg-NH_2 in 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 0.15 M NaCl, 0.05% Brij, for 20−30 min at 37 °C in the presence of varying concentrations of inhibitor. The reaction was then quenched with 50 mM EDTA, and the relative fluorescence was monitored on a Perkin-Elmer LS50B spectrofluorometer (λex 328 nm, λem 393 nM) fitted with a microplate reader attachment. Activity was measured as a percentage of control activity in the absence of inhibitor. Inhibitor concentrations were run in triplicate, and IC_{50} determinations were calculated from a four-parameter logistic fit of the data within a single experiment.

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Supporting Information Available: X-ray crystallographic data for **16** (8 pages). Ordering information is given on any current masthead page.

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