# Hydroxamate derivatives of substrate-analogous peptides containing aminomalonic acid are potent inhibitors of matrix metalloproteinases

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**Abstract Novel peptides containing the sequence -Pro-Leu-Ama(NHOH)- were synthesized and characterized by spectroscopic techniques. Their inhibitory properties towards the activated form of native human gelatinase B (MMP-9) and the catalytic domain of neutrophil collagenase (cdMMP-8) were determined. The most effective inhibitor synthesized exhibits $K_i$ values of $2\times10^{-6}$ M (cdMMP-8) and $5\times10^{-9}$ M (MMP-9) thus attaining interesting discrimination between the tested metalloproteinases. A most important feature of this type of inhibitor is its peptide nature making the compounds similar to natural substrates. In spite of the peptide character of the inhibitors synthesized, the $P_1$-$P_1'$-peptide bond shows a high resistance to cleavage by the proteinases.**



*Key words:* Matrix metalloproteinase; Inhibition; Peptide inhibitor; Hydroxamate; Aminomalonic acid

## 1. Introduction

Matrix metalloproteinases (MMPs) are a family of at least 15 zinc-containing proteinases playing a fundamental role in the degradation and remodelling of connective tissue by hydrolysis of matrix proteins such as collagens, gelatins and proteoglycans [1]. In this way MMPs are involved in many pathophysiological processes connected to extracellular matrix disintegration, e.g. rheumatoid arthritis or tumor invasion and metastasis. Matrix metalloproteinases have similar domain structures including as major domains an N-terminal prodomain, the catalytic domain and, in most cases, a hemopexin domain in some species involved in substrate specificity. The metalloproteinases are produced as zymogens and activated by removal of the prodomain, e.g. by organomercuric agents and autolytic cleavage or by other proteases. MMPs are divided into several subgroups based on their domain structures, sequence homologies and their substrate preferences. Tissue inhibitors of metalloproteinases (TIMPs), naturally occurring proteins specifically inhibiting these proteinases, control MMPs in vivo. An imbalance between MMPs and their natural antagonists, the TIMPs, can result in pathophysiological destructive processes such as rheumatoid arthritis, parodontosis or fibrosis. This may be compensated with potent synthetic inhibitors. Numerous synthetic low-molecular weight MMP inhibitors have been synthesized [2,3] and research is currently being carried out on their in vivo efficiency in different kinds of cancer or destructive joint diseases. Most of the synthetic MMP inhibitors developed so far are small molecular compounds binding to the catalytic site of the enzymes. Several X-ray crystal structures of the catalytic domains of collagenases complexed with an inhibitor have been published [4–7]. Effective inhibitors are equipped with a zinc chelator group and a peptide or non-peptide backbone mimicking a natural substrate. The hydroxamic acid function is a very good zinc-chelating group thus providing the most potent inhibitors of MMPs. In general the hydroxamic acid-based inhibitors are of the C-terminal type, binding at S′-subsites of the enzyme (nomenclature $S_n...S_n'$, $P_n...P_n'$ according to Schechter and Berger [8]). N-terminal inhibitors are much less effective. For the most part, C-terminal hydroxamic acid inhibitors are succinyl derivatives with various aliphatic or aromatic substituents on either the α- or β-carbon or both and residues at the γ-carboxyl function, attached by a peptide linkage.

In this paper a novel type of hydroxamic acid-based peptide inhibitor is presented. The residue carrying the hydroxamate function is an aminomalonic acid (Ama) in position $P_1$. This allows the addition of various residues suitable to gain maximum attachment to the entire binding site of the target enzyme on both S- and S′-subsites. The most important feature of this type of inhibitor is the peptide backbone built up by α-amino acids to comprise the positions $P_3$ to $P_1'$ at least, giving these compounds the same basic frame as a peptide substrate. In accordance with the specificity of the metalloproteinases tested the $P_3$ position of these inhibitors is occupied by a proline; $P_2$ is an aliphatic amino acid, whereby leucine or alanine is favored. The best inhibitory properties were obtained with the bulky tyrosine benzylether moiety in the $P_1'$ position (Fig. 1).

## 2. Materials and methods

### 2.1. Chemicals and equipment

Reagents and solvents were of p.a. quality from Fluka (Buchs), Merck (Darmstadt) and Baker (Gross-Gerau). A Waters chromatography system with Baker wide pore octadecyl column (C18, 5 μm) was used for analytical HPLC (5 min water with 0.1% TFA, gradient: 5–45 min from 0 to 100% acetonitrile, water (80:20, v/v) with 0.09% TFA). Analytical TLC was carried out on KG 60 $F_{254}$ alu foil and preparative layer chromatography on KG 60 $F_{254}$ plates, 2 mm layer, both from Merck.

Characterization of the synthesized inhibitors and precursors was carried out using NMR (Bruker AC 250 P), ion spray mass spectroscopy (API III Quadrupol mass spectrometer with ion spray interface from Sciex) or FAB mass spectroscopy (liquid-SIMS, positive ionization, Autospec VG by Fisons). Enzyme inhibition assays were performed using a spectrofluorometer Jasco FP-550 or a luminescence spectrometer Perkin-Elmer LS 50B.

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*Abbreviations:* Ama, aminomalonic acid; cdMMP, catalytic domain of an MMP; MMP, matrix metalloproteinase; DCC, dicyclohexyl carbodiimide; THF, tetrahydrofuran; TIMP, tissue inhibitor of metalloproteinases; TLC, thin layer chromatography

Dedicated to Prof. Dr. Klaus Burger on the occasion of his 60th birthday.


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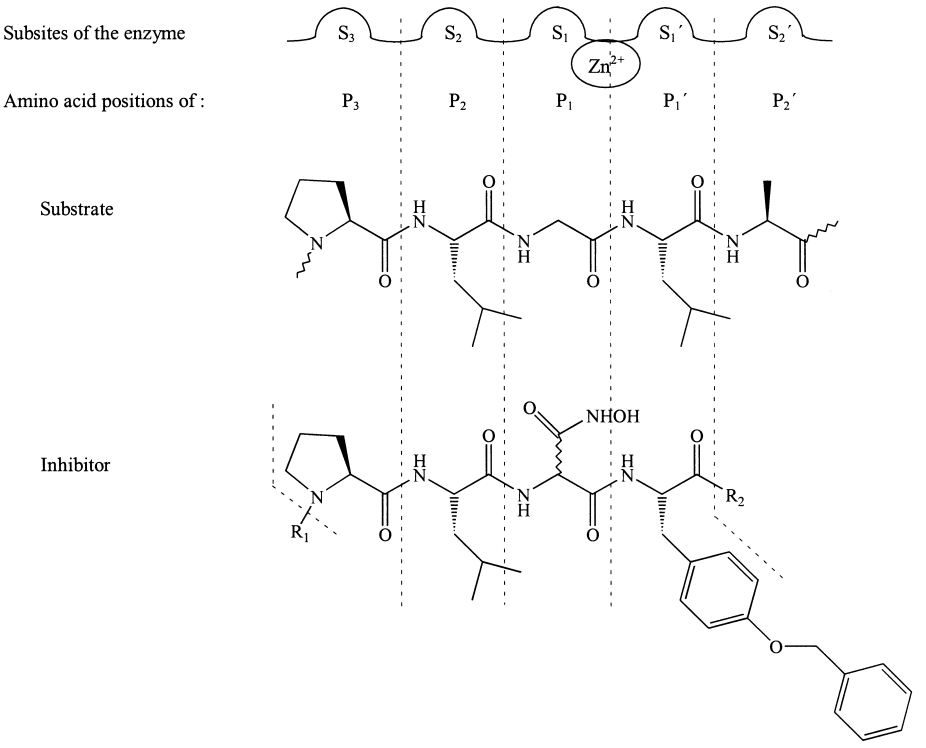



Fig. 1. Partial sequence of a typical substrate of gelatinase B (MMP-9) with the amino acid residues numbered according to Schechter and Berger [8] compared with the structure of an inhibitor of the type $R_1$-Pro-Leu-Ama(NHOH)-Tyr(Bzl)-$R_2$ presumably binding at the subsites of the enzyme. $R_1$ is a protecting group, H, acetyl or an amino acid, $R_2$ is an amine, an alcohol, NHOH or a C-terminal protected amino acid or peptide.

*2.2. Synthesis of the inhibitors*

The peptides containing an Ama(OMe) residue were synthesized by segment condensation of N-protected tripeptides of the type $R_1$-Pro-Aaa-Ama(OMe)OH and H-Tyr(Bzl)-$R_2$, where $R_1$ is a Boc protecting group or acetyl, Aaa is glycine, alanine or leucine and $R_2$ is an amide-bonded *S*(−)-α-methylbenzylamine, *R*(+)-α-methylbenzylamine or *R*(−)-α-methylcyclohexanemethylamine. Peptide coupling reactions were carried out in organic solvents following standard procedures. Alternatively an N-protected dipeptide Boc-Aaa-Ama(OMe)OH was coupled with the C-terminal fragment and, after removal of the Boc group, the $R_1$-Pro was added using an active ester. The conversion of the aminomalonic acid methyl ester function into the corresponding hydroxamic acid was effected via hydrolysis of the ester to the free malonic acid and treatment with hydroxylamine hydrochloride, a tertiary amine and DCC or directly by aminolysis of the methyl ester with hydroxylamine [9].

The synthesis of Boc-Leu-Ama(OMe)OH described below is a typical procedure for the preparation of N-protected di or tripeptides containing Ama(OMe)OH.

Preparation of Boc-Leu-Ama$(OMe)_2$: Boc-Leu-OH (5 mmol), aminomalonic acid dimethyl ester hydrochloride (5 mmol) and triethyl amine (5 mmol) were suspended in 50 ml of a mixture of THF, ethyl acetate and dichloromethane (2:1:1, v/v). A solution of DCC (5 mmol) in 10 ml dichloromethane was added dropwise at 0°C. The mixture was stirred for about one hour at this temperature and then at 5°C until the end of the reaction (TLC control). After filtration the solvent was removed in vacuo and the residue dissolved in ethyl acetate. The solution was extracted with 5% aqueous $Na_2CO_3$, 10% $NaHSO_4$ and 5% $NaHCO_3$ solutions, dried with $Na_2SO_4$ and evaporated. The crude product was dissolved in 15 ml dichloromethane and stored at 0°C for one day. The precipitated dicyclohexyl urea was removed by filtration and the solution was evaporated to dryness. About 4.75 mmol (95%) Boc-Leu-Ama$(OMe)_2$ was obtained. TLC: $R_f$ 0.7 (chloroform/methanol, 10:1, v/v).

NMR: $^1$H (250 MHz, $CDCl_3$): 0.95 (*m*, 6 H, $CH_3$, Leu); 1.45 (*s*, 9 H, $CH_3$, Boc); 1.52 (*m*, 1 H, Leu); 1.69 (*m*, 2 H, Leu); 3.81 (*s*, 6 H, $(OMe)_2$); 4.22 (*m*, 1 H, αCH, Leu); 4.94 (*d*, 1 H); 5.19 (*d*, 1 H); 7.18 (*w*, 1 H, NH, Ama).

$^{13}$C (63 MHz, $CDCl_3$): 21.86; 22.97; 24.73; 28.30; 41.05 ($CH_2$); 53.38; 56.08; 58.10; 81.05 ($C_q$); 166.50; 166.53; 172.59.

For the analogous synthesis of a tripeptide of the type Boc-Pro-Aaa-Ama$(OMe)_2$ the corresponding dipeptide Boc-Pro-Aaa-OH was used.

Preparation of Boc-Leu-Ama(OMe)OH: To a solution of Boc-Leu-Ama$(OMe)_2$ (3 mmol) in 15 ml anhydrous methanol a solution of KOH (2.9 mmol) in 2 ml methanol was added dropwise at 0°C. The reaction was carried out under nitrogen atmosphere. The mixture was stirred at 0°C for 3 h, then stirring was continued overnight at room temperature. After evaporation, the residue was dissolved in 25 ml 5% aqueous $NaHCO_3$. The solution was extracted with ether, cooled to 0°C, acidified with $NaHSO_4$ and finally extracted with ethyl acetate several times. The combined extracts containing the malonic acid derivative were dried with $Na_2SO_4$ and the solvent was removed in vacuo at 25°C maximum. Yield 2.4 mmol (80%). TLC: $R_f$ 0.45 (chloroform/methanol/32% acetic acid/ethyl acetate, 5:3:1:4, v/v). HPLC: $R_t$ 34.9 min.

NMR: $^1$H (250 MHz, DMSO-$D_6$): 0.86 (*m*, 6 H, $CH_3$, Leu); 1.46 (*s*, 9 H, $CH_3$, Boc); 1.53 (*m*, 2 H, Leu); 1.62 (*m*, 1 H, Leu); 3.69 (*s*, 3 H, OMe); 4.10 (*m*, 1 H, αCH, Leu); 4.98 (*d*, 1 H, αCH, Ama); 6.95 (*t*, 1 H); 8.39 (*m*, 1 H).

$^{13}$C (63 MHz, DMSO-$D_6$): 21.29; 22.93; 24.14; 28.09; 40.43 ($CH_2$); 52.32; 52.53; 55.99; 56.04; 78.05 ($C_q$); 155.24; 167.24; 167.30; 172.81.

The coupling of Boc-Aaa-Ama(OMe)OH or Boc-Pro-Aaa-Ama(OMe)OH derivatives with the C-terminal fragment was carried out in dichloromethane with DCC and the product isolated as described above. If necessary the crude product subsequently should be separated by chromatography over Sephadex LH20 in methanol.

Deprotection of the peptides of the type Boc-Aaa-Ama(OMe)-Tyr(Bzl)-$R_2$ and subsequent addition of the N-protected proline occurred via acid catalyzed hydrolysis of the Boc-protecting group: the N-protected peptide (about 0.5 mmol) was dissolved or suspended in

7 ml 2,2,2-trifluoroethanol and 0.7 ml half concentrated HCl was added. The mixture was stirred until the end of the reaction (TLC-control), than poured into cold 5% aqueous $NaHCO_3$. The aqueous solution was extracted with ethyl acetate several times, the organic solutions were dried with $Na_2SO_4$ and Boc-Pro-OSu was added. After extraction of the organic solution and chromatographical separation the pure peptide was obtained in high yields.

Preparation of the free aminomalonic acid derivatives occurred via hydrolysis of the methyl ester dissolved in methanol with 2 M NaOH at 0°C. When TLC indicated total disappearance of the methyl ester, the mixture was poured into 5% $NaHCO_3$ and the product was extracted with ethyl acetate.

General procedures for the synthesis of the hydroxamic acid derivatives:

(i) The peptide of the type Boc-Pro-Aaa-Ama(OH)-Tyr(Bzl)-$R_2$ (0.1 mmol) was dissolved in 5 ml of a mixture of dichloromethane and ethyl acetate (4:1, v/v), a tertiary amine (preferably triethyl amine, *N*-ethyl or *N*-methyl morpholine, 0.12 mmol) and hydroxylamine hydrochloride (0.1 mmol) in DMF and DCC (0.11 mmol) were added at 0°C. The mixture was stirred at 5°C overnight, filtered and evaporated. The solution of the residue in ethyl acetate was extracted with 10% $KHSO_4$, 5% $NaHCO_3$ (which in some cases resulted in precipitation of the hydroxamic acid salt in the organic layer) and 10% $KHSO_4$, dried with $Na_2SO_4$ and the solvent was removed in vacuo. The crude product was purified via preparative layer chromatography (chloroform/methanol, 10:1, v/v).

(ii) The peptide containing aminomalonic acid methyl ester was dissolved in anhydrous methanol. To the solution hydroxylamine hydrochloride (about 5 molar equivalents) was added and the mixture was made strongly alkaline with sodium methanolate, added in small portions. Stirring at 5°C overnight caused total aminolysis of the methyl ester by hydroxylamine. At 0°C 10% $NaHSO_4$ was added and the product was extracted with ethyl acetate.

Properties of the compounds listed in Table 1:

(**1**) TLC: $R_f$ 0.65 (chloroform/methanol/32% acetic acid/ethyl acetate, 5:3:1:4, v/v), $R_f$ 0.2 (chloroform/methanol, 10:1, v/v). HPLC: $R_t$ 42.5 min. MS: ion spray *m/z* 767.4 $(M+Na)^+$, 745.4 $(M+H)^+$, 645.4 $(M\text{-}Boc+H)^+$.

(**2**) TLC: $R_f$ 0.63 (chloroform/methanol/32% acetic acid/ethyl acetate, 5:3:1:4, v/v), $R_f$ 0.17 (chloroform/methanol, 10:1, v/v). HPLC: $R_t$ 42.2 min. MS: ion spray *m/z* 767.4 $(M+Na)^+$, 745.4 $(M+H)^+$, 645.4 $(M\text{-}Boc+H)^+$.

(**3**) TLC: $R_f$ 0.7 (chloroform/methanol/32% acetic acid/ethyl acetate, 5:3:1:4, v/v), $R_f$ 0.42 (chloroform/methanol, 10:1, v/v). HPLC: $R_t$ 44.8 min. MS: $FAB^+$ (matrix glycerol, CsI admixture) *m/z* 883 $(M+Cs)^+$, 751 $(M+H)^+$, 651 $(M\text{-}Boc+H)^+$; ion spray *m/z* 773.4 $(M+Na)^+$, 751.4 $(M+H)^+$, 651.4 $(M\text{-}Boc+H)^+$.

(**4**) TLC: $R_f$ 0.75 (chloroform/methanol/32% acetic acid/ethyl acetate, 5:3:1:4, v/v), $R_f$ 0.3 (chloroform, methanol, 10:1, v/v). HPLC: $R_t$ 42.6 min. MS: ion spray *m/z* 781.5 $(M+Na)^+$, 659.5 $(M\text{-}Boc+H)^+$.

(**5**) TLC: $R_f$ 0.75 (chloroform/methanol/32% acetic acid/ethyl acetate, 5:3:1:4, v/v), $R_f$ 0.5 (chloroform/methanol, 10:1, v/v). HPLC: $R_t$ 46.9 min. MS: ion spray *m/z* 823.6 $(M+Na)^+$, 801.4 $(M+H)^+$, 701.4 $(M\text{-}Boc+H)^+$. NMR: $^1H$ (250 MHz, DMSO-$D_6$): 0.85 (*m*, 6 H, $CH_3$, Leu); 1.21–1.37 (*m*, 12 H, $CH_3$: Boc, $CH(Ph)CH_3$); 1.4–2.15 (*m*, 7 H: $CH_2$, $C_tH$, Leu; $\beta CH_2$, $\gamma CH_2$, Pro); 2.75–3.0 (*m*, 2 H, $CH_2$, Tyr); 3.15–3.38 (*m*, 2 H, $\delta CH_2$, Pro); 4.13 (*m*, 1 H); 4.34 (*m*, 1 H); 4.46 (*m*, 1 H); 4.78–4.90 (*m*, 2 H); 5.05 (*s*, 2 H, $CH_2$, Bzl); 6.90 (*d*, 2 H, arom.); 7.12 (*t*, 2 H, arom.); 7.20–7.45 (*m*, 10 H, arom.); 7.90 (*m*, 1 H); 8.05–8.16 (*m*, 2 H); 8.29 (*d*, 1 H); 9.10, 9.14 (*s*, 1 H); 10.56, 10.63 (*s*, 1 H).

(**6**) TLC: $R_f$ 0.75 (chloroform/methanol/32% acetic acid/ethyl acetate, 5:3:1:4, v/v), $R_f$ 0.5 (chloroform/methanol, 10:1, v/v). HPLC: $R_t$ 46.7 min. MS: ion spray *m/z* 823.5 $(M+Na)^+$, 801.5 $(M+H)^+$, 701.5 $(M\text{-}Boc+H)^+$.

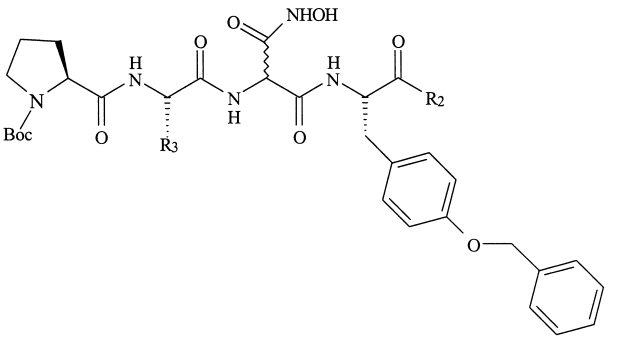
(**7**) TLC: $R_f$ 0.75 (chloroform/methanol/32% acetic acid/ethyl acetate, 5:3:1:4, v/v), $R_f$ 0.5 (chloroform/methanol, 10:1, v/v). HPLC: $R_t$ 49.7 min. MS: ion spray *m/z* 829.5 $(M+Na)^+$, 807.5 $(M+H)^+$, 707.5 $(M\text{-}Boc+H)^+$.

### 2.3. Enzyme inhibition assays

The inhibition constants $K_i$ of the inhibitors were determined for the proteolytically activated form of native human gelatinase B (MMP-9) [10] and the catalytic domain of neutrophil collagenase (cdMMP-8) [11]. The substrate used, (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-(3-(2,4-dinitrophenyl)-L-2,3-diamino-propionyl)-Ala-Arg-$NH_2$ [12], is an internally quenched fluorescent peptide, which is cleaved at the Gly-Leu bond by the tested metalloproteinases. The resulting increase of fluorescence was measured at an excitation wavelength of 328 nm and an emission wavelength of 393 nm.

The assay mixtures contained constant concentrations of the enzyme and 3% DMSO (as solvent for inhibitors and substrate) in 2 ml of buffer (0.1 M Tris-HCl, pH 7.5, 0.1 M NaCl, 10 mM $CaCl_2$, 0.05% Brij 35). For at least three series with constant concentrations of substrate (approx. 0.2–1 μM) and different concentrations of the inhibitor every initial fluorescence increase, which is proportional to the substrate concentration and corresponds to the remaining proteolytic activity of the enzyme, was measured at 25°C. The substrate

Table 1
Tested inhibitors and their $K_i$ values determined with gelatinase B (MMP-9) and the catalytic domain of the neutrophil collagenase (MMP-8) in mol/l




| Compound no. | $R_2$ | $R_3$ | MMP-9 | cdMMP-8 |
|---|---|---|---|---|
| **1** | NH-*R*-CH(Ph)$CH_3$ | H | $4.0\times10^{-7}$ | $6.5\times10^{-6}$ |
| **2** | NH-*S*-CH(Ph)$CH_3$ | H | $>10^{-7}$ | $1.8\times10^{-5}$ |
| **3** | NH-*R*-CH(c-$C_6H_{11}$)$CH_3$ | H | $>10^{-7}$ | $1.4\times10^{-5}$ |
| **4** | NH-*S*-CH(Ph)$CH_3$ | $CH_3$ | $5.0\times10^{-9}$ | $1.9\times10^{-6}$ |
| **5** | NH-*R*-CH(Ph)$CH_3$ | $CH_2$-CH-$(CH_3)_2$ | $5.0\times10^{-9}$ | $8.0\times10^{-7}$ |
| **6** | NH-*S*-CH(Ph)$CH_3$ | $CH_2$-CH-$(CH_3)_2$ | $5.0\times10^{-9}$ | $8.0\times10^{-7}$ |
| **7** | NH-*R*-CH(c-$C_6H_{11}$)$CH_3$ | $CH_2$-CH-$(CH_3)_2$ | $1.8\times10^{-8}$ | $2.5\times10^{-6}$ |

NH-*R*-CH(Ph)$CH_3$, NH-*S*-CH(Ph)$CH_3$ and NH-*R*-CH($C_6H_{11}$)$CH_3$ are N-bounded residues of *R*(+)-α-methylbenzylamine, *S*(−)-α-methylbenzylamine, and *R*(−)-α-methylcyclohexanemethylamine.

(predissolved 0.1 mg in 1 ml DMSO) was added after incubation of the enzyme with the inhibitor in the buffer for at least 10 min. $K_i$ values were determined by the established method of Dixon [13].

Activation of the latent progelatinase B (MMP-9), isolated from human neutrophils, was carried out in assay buffer by adding bovine trypsin (50 μl, 0.6 mg/ml) to the proenzyme (0.45 ml, 120 μg/ml) and incubating at 37°C for 10 min. The trypsin was then inactivated with aprotinin (50 μl, 1.2 mg/ml).

## 3. Results and discussion

The $K_i$ values of the synthesized MMP inhibitors determined in quantitative fluorometric assays are shown in Table 1. Some of these compounds exhibit very good inhibitory properties against the enzymes tested. Analogous derivatives with free carboxyl function, methyl or ethyl esters at the aminomalonic acid instead of a hydroxamate in this position show virtually no inhibitory effect [9]. Thus it is obvious that a zinc-complexing function is essential for a high affinity to the metalloproteinases. This observation confirms the assumption that these inhibitors bind competitively to the catalytic center, with the hydroxamic acid in a proper position to chelate the zinc ion in the active site. The complexing group, representing the complete side chain of the amino acid in positon $P_1$, is fixed as close as possible to the peptide backbone and to the reactive-site bond.

Apart from this favorable conformational arrangement of the chelating group towards the metal ion another relevant criterion for effective inhibition is an optimal adjustment of the backbone to a natural substrate. The $P_1$-residue in a natural substrate is glycine. Its substitution by the aminomalonic acid derivative only adds a small chelating function to the peptide. Except for the Ama(NHOH)-residue in $P_1$ position this type of peptide inhibitor is highly variable on both N- and C-terminal sides, thus allowing design of the rest of the structure for an optimal fit into to the enzyme subsites.

Corresponding to the substrate specificity of the tested metalloproteinases, the $P_3$-position of all inhibitors is occupied by a proline. A substitution of the proline in compound **1** by a Boc protecting group results in a decrease of the inhibition constant by about two orders of magnitude for the MMP-9 and one order for the cdMMP-8.

Compounds **5**, **6** and **7** with leucine at $P_2$-position have the strongest inhibitory effect toward the catalytic domain of MMP-8, in agreement with the sequences of various synthetic peptide substrates of collagenases [14–16]. The inhibition constants of these compounds for the gelatinase B are about two orders of magnitude lower than those for the collagenase with its high substrate discrimination.

A more distinct selectivity between MMP-9 and cdMMP-8 is shown by compound **4** with alanine at the $P_2$-site ($K_i = 5 \times 10^{-9}$ M for MMP-9, $1.9 \times 10^{-6}$ for cdMMP-8). Whereas the $K_i$ value of **4** for the MMP-9 equals those of the analogous leucine derivatives, a distinct decrease can be observed for the cdMMP-8. There is a nearly 400-fold inhibitory discrimination of **4** between the two enzymes.

The glycine-containing derivates **1**–**3** inhibit the tested enzymes one to two orders of magnitude less efficiently. Thus an amino acid with a chiral center in position $P_2$ seems to be favorable. This effect is more pronounced for MMP-9, therefore these inhibitors are less selective.

The compounds with the bulky α-methylcyclohexanemethylamine at position $P_2'$ have weaker inhibitory effects than the aromatic analogues.

Despite their peptide character the inhibitors were resistant to hydrolysis by the proteases as confirmed by HPLC investigations after incubation of the inhibitors with the metalloproteinases for several hours. None of the possible fragments from cleavage of the Ama-Tyr-bond could be detected. Thus, these inhibitors and especially the $P_1$-$P_1'$-peptide bond exhibit a high resistance to cleavage by the proteinases.

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