Membrane Type-1 Matrix Metalloprotease and Stromelysin-3 Cleave More Efficiently Synthetic Substrates Containing Unusual Amino Acids in Their P$_1^\prime$ Positions*

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The influence of the substrate P$_1^\prime$ position on the specificity of two zinc metalloproteases, membrane type-1 matrix metalloprotease (MT1-MMP) and stromelysin-3 (ST3), was evaluated by synthesizing a series of fluorogenic substrates of general formula dansyl-Pro-Leu-Ala-Xaa-Trp-Ala-Arg-NH$_2$, where Xaa in the P$_1^\prime$ position represents unusual amino acids containing either long arylalkyl or alkyl side chains. Our data demonstrate that both MT1-MMP and ST3 cleave substrates containing in their P$_1^\prime$ position unusual amino acids with extremely long side chains more efficiently than the corresponding substrates with natural phenylalanine or leucine amino acids. In this series of substrates, the replacement of leucine by S-para-methoxybenzyl cysteine increased the $k_{cat}/K_M$ ratio by a factor of 37 for MT1-MMP and 9 for ST3. The substrate with a S-para-methoxybenzyl cysteine residue in the P$_1^\prime$ position displayed a $k_{cat}/K_M$ value of 1.59 $\times 10^6$ M$^{-1}$ s$^{-1}$ and 1.67 $\times 10^4$ M$^{-1}$ s$^{-1}$, when assayed with MT1-MPP and ST3, respectively. This substrate is thus one of the most rapidly hydrolyzed substrates so far reported for matrixins, and is the first synthetic peptide efficiently cleaved by ST3. These unexpected results for these two matrixins suggest that extracellular proteins may be cleaved by matrixins at sites containing amino acids with unusual long side chains, like those generated in vivo by some post-translational modifications.

Matrix metalloproteases (MMPs), also known as matrixins, form a group of structurally related zinc endopeptidases collectively able to degrade all components of the extracellular matrix (1). MMPs are believed to be mediators of both normal and pathological tissue remodeling processes, and their increased expression has been observed in a variety of human disorders (2–4). In particular, membrane type-1 (MT1)-MMP, a gelatinase A activator (5), and stromelysin-3 (ST3), a matrixin with unusual functional properties (6), are expressed in most human carcinomas (7–9). MT1-MMP and ST3 both belong to a subgroup of MMPs which are believed to be intracellularly activated by furin or furin-like convertases, thereby suggesting that MT1-MMP and ST3 are present in tissues in an active form, in contrast to other MMPs which are secreted as inactivezymogens (10–12). As part of a program aimed at developing therapeutic inhibitors for these two MMPs, the specificity of these enzymes in cleaving synthetic substrates has been investigated. Current data on the specificity of MT1-MMP toward the degradation of synthetic substrates are extremely limited (13), while no synthetic substrate has yet been reported to be cleaved by ST3.

In contrast to MT1-MMP and ST3, several studies have been devoted to the delineation of the specificity of other matrixin family members by developing both synthetic substrates (14–18) and inhibitors for these enzymes (19, 20). Such approaches have been greatly facilitated by the resolution of the crystal structures of several MMP catalytic domains (21–27), allowing structure-based design strategies to be used (28). According to these structural studies, the S$_1^\prime$ subsite of these enzymes appears as a cavity of variable size, depending on the nature of the amino acid residue located near the bottom of this cavity. In stromelysin-1 (ST1), which contains leucine in this particular position, the S$_1^\prime$ pocket is a deep cavity, forming a channel that extends through the whole body of the enzyme catalytic domain (29, 30). X-ray structure analysis of a complex between ST1 and a carboxyalkyl inhibitor harboring a homophenylalanine in the P$_1^\prime$ position has consistently shown that the homophenylalanine side chain only fills half of the S$_1^\prime$ pocket of ST1 (29). A comparable situation is likely to occur in most other MMPs, including MT1-MMP, collagenase-2 (COL2), stromelysin-1 (ST1), stromelysin-2 (ST2), gelatinase A, and gelatinase B, due to the presence in their S$_1^\prime$ pocket of a leucine at the same position. The high potency of several inhibitors, substituted in their P$_1^\prime$ position by side chains longer than homophenylalanine, toward this subgroup of matrixins is consistent with this proposal (28, 31–35). In contrast to this subgroup of matrixins, collagenase-1 (COL1), matrixin, and ST3 possess in their S$_1^\prime$ phenylalanine; HPLC, high performance liquid chromatography; Fmoc, N-(9-fluorenylmethoxycarbonyl).
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subsite a residue other than leucine. COL1, matrilisin, and ST3 possess in this particular position an arginine, a tyrosine and a glutamine, respectively. In the case of this matrixin subgroup, x-ray structures of COL1 and matrilisin have demonstrated that the presence of either arginine or tyrosine reduces the size of their $S_1'$ subsite (24, 26, 27). While no crystal structure is presently available for ST3, a similar situation has been suggested to occur in this matrixin (36).

While the particular shape of the $S_1'$ pocket in matrixins has been extensively exploited for the design of matrixin inhibitors, only one study so far examined the influence of this $S_1'$ cavity on the cleavage of synthetic substrates with unusual amino acids in their $P_1'$ position (37). It is worth remembering that most of the matrixin inhibitors developed to date and harboring long side chains in their $P_1'$ position are not transition-state analogues. Therefore, the ability of matrixins to cleave substrates containing in their $P_1'$ position amino acids with unusual side chains cannot be predicted from these inhibitor studies. To address this issue more systematically in the case of MT1-MMP and ST3, synthetic heptapeptides containing in their $P_1'$ position amino acids with arylalkyl or alkyl side chains of varying size were synthesized. The amino acid sequence being retained as a fluorophore. An arginine residue was added at the C-terminal extremity of this sequence to improve peptide solubility. In the present report, these fluorogenic peptides were evaluated as substrates for MT1-MMP and ST3. Based on this study, a fluorogenic compound, containing dinitrophenyl-coumarin as a quencher-fluophore pair, was also synthesized and examined for comparison.

MATERIALS AND METHODS

Rink amide resin, 2-(4H-benzotiazol-1-yl)-1H,1.3,3-tetramethyluroni

hexafluorophosphate (HBTU) and all Fmoc natural amino acid derivatives were purchased from Novabiochem. The unusual Fmoc amino acids were from Novabiochem and Advanced Chemtech. 7-Me

thoxycoumarin-2-acetic acid (McaOH) and 5-dimethylamino-1-naph

thalenesulfonyl chloride (dansyl chloride, dnsCl) were from Aldrich.

Chemistry—Unusual Fmoc amino acids not commercially available (namely, 2-aminoheptanoic acid and 2-amino-5-phenyl-pentanoic acid) were synthesized by catalytic phase transfer alkylation of ethyl diphe

nylmethyleneglycinate with the appropriate alkyl bromides followed by hydrolysis, as described by O'Donnell et al. (40) and O'Donnell and Eckrich (41). 2-Aminoheptanoic, 2-aminooctanoic (Aldrich) and 2-amino-5-phenyl-pentanoic acids were converted into their Fmoc derivatives by the action of Fmoc-Cl in water/Na$_2$CO$_3$/dioxane solution according to the standard literature procedure (42). The Fmoc amino acids were obtained as white, crystalline compounds after their purification by flash chromatography on silica gel using ethyl acetate/hexane/acetonic acid eluents. Their structure and the purity were confirmed by NMR and mass spectroscopy analysis. These unusual Fmoc amino acids were used for peptide synthesis as mixtures of enantiomers. For these pep

tides, the diastereoisomers were separated by HPLC. N$^\text{Fmoc}$-N$^\text{Fmoc}$-N$^\text{Fmoc}$-2,4-dinitrophenyl-1,2,3-diaminopropionic acid (Fmoc-DpaOH) was synthe

sized starting from Fmoc-1-AsnOH, as described by Knight et al. (14).

Substrate Synthesis and Characterization—Solid phase synthesis of the substrates was performed in a model 357 Advanced Chemtech multiple peptide synthesizer on a Rink amide resin. Typically, three equivalents of an amino acid, the alternate Fmoc-Leu-Ala-Leu-Arg-NH$_2$, Fmoc-Leu-Ala-Trp-Ala-Arg-NH$_2$, Fmoc-Leu-Ala-Trp-Ala-Arg-NH$_2$, or Fmoc-Leu-Ala-Trp-Ala-Arg-NH$_2$ was added. The resulting Fmoc N-protection group was removed with a 30% solution of piperidine in N-methylpyrrolidone. N-terminal acylation of the pep

tides was achieved either with excess dansyl chloride (20 equivalents) in the presence of diisopropylphosphorylamine or with triple coupling of McaOH, under the conditions described above. Cleavage of the peptides from the resin, together with the cleavage of the side chain protection groups, were performed by the action of trifluoroacetic acid containing 5% trisopropylsilane.

Peptides were purified by preparative HPLC column (Yvdac, 218TP1022) performed on a Gilson system equipped with a variable wavelength detector. Gradient elutions were performed using solutions A (10% acetonitrile in 0.1% trifluoroacetic acid in water) and B (90% acetonitrile in 0.1% trifluoroacetic acid in water). All peptides were recovered by lyophilization. Peptide purities were checked by amino acid analyses, analytical HPLC (Yvdac, 218TP104 column) and mass spectrometry.

Enzymes—cDNAs corresponding to the catalytic domains of mouse ST3 (Phe-102 to Ser-276) and human MT1-MMP (Tyr-111 to Arg-298) were introduced into the expression vector pET-3b, expressed in Escherichia coli BL21 (DF3) cells after isopropyl-1-thio-β-D-galactopyrano

side induction and purified essentially as described in Noël et al. (43). Briefly, both MMP catalytic domains were solubilized from bacterial inclusion bodies with 8 M urea in the presence of 100 mM dithiothreitol and purified on a Q-Sepharose anion-exchange column (Pharmacia Biotech Inc.). Purified catalytic domains were then slowly refolded at a protein concentration of 50 μg/ml by dialysis to dilute out the urea. The refolding step was followed by size exclusion chromatography, using a gel filtration column (Superdex-200, Pharmacia) to eliminate the aggregates, and to retain the active monomeric protein alone.

Enzyme Kinetics—Substrate specificity assays were performed in 50 mM Tris/HCl buffer, pH 7.5, 10 mM CaCl$_2$, in the absence (MT1-MMP) or presence of 0.2 mM NaCl (ST3), at 25 °C. Substrate concentrations were determined spectrophotometrically using ε$_{430}$nm = 4300 M$^{-1}$ cm$^{-1}$ for dansyl peptides (44) and ε$_{328}$nm = 12900 M$^{-1}$ cm$^{-1}$ for coumarin peptides (14). Substrates were prepared as 1 mM stock solutions in di

methyl sulfoxide. Enzyme concentrations were determined from optical density, using the method of Gill and von Hippel (45) to calculate the extinction coefficient of two matrixins. In the case of MT1-MMP, values of h$_{cat}$/K$_{m}$ were determined from first-order full-time course reaction curves obtained at [S] $\ll$ K$_m$ (S = 0.2 μM), at 10 mM final enzyme concentration. These progress curves were monitored by follow

ing the increase in fluorescence at 340 nm (λ$_{ex}$, 280 nm), induced by the cleavage of the dns substrates, in a Biologic PMS 200 spectropho

tometer. Due to the lower efficiency of ST3 in cleaving this series of substrates, the observation of full-time course reactions for ST3 has required the use of higher enzyme concentrations, leading to a high fluorescence background. Thus, the kinetic parameters for ST3 were based on HPLC (Thermo Separation Products system) allowing the separation of the unreacted substrate from the cleavage products and its quantification. Immediately after the initiation of the reaction, aliquots were withdrawn from this reaction solution by the autosampler (Spectra System AS300), at predetermined time intervals, and injected onto the column. Substrate and products (S = 0.2 μM, ST3 concentration from 50 to 200 nM) were separated on a C18 column (Yvdac, 218TP1014), eluted with a linear acetonitrile gradient in 0.1% trifluoro

acetic acid. Products were detected using an FL-300 Spectra System fluorescence detector (λ$_{ex}$, 280 nm; λ$_{em}$, 340 nm) and were identified by mass spectroscopy analysis. Data analysis was performed with a Thermo Separation Products dataget integrator.

The two approaches used for MT1-MMP and ST3 yielded product progress curves, from which values for the specificity constant h$_{cat}$/K$_{m}$ were determined by fitting these curves with the integrated Michaelis-Menten Equation 1, by nonlinear regression (46)

$$[P] = [S_0] (1 - \exp(-kt))$$

(1) where $k_{cat}$/K$_m$ = [total enzyme]. For each $k_{cat}$/K$_m$ determina

tion, three independent experiments were taken into consideration.

Individual kinetic parameters ($h_{cat}$ and K$_m$) for MT1-MMP and ST3 were obtained from analysis of fluorescence curves, under steady-state rate conditions, over 0.2 to 5 K$_m$ substrate concentration ranges. The experiments were carried out in a 5 × 5 × 45-mm fluorescence cell, using excitation at 280 or 300 nm (slit width, 5 nm) and emission at 360 nm (slit width, 5 nm) for the dns derivatives, and excitation at 328 nm (slit width, 5 nm) and emission at 400 nm (slit width, 5 nm) for the Mca derivatives. Cleavage of the following substrates: dns-Pro-Leu-Ala-Cys(OMeBzl)-Trp-Ala-Arg-NH$_2$, dns-Pro-Leu-Ala-Cys(OMeBzl)-Trp-Ala-Arg-NH$_2$, and Mca-Pro-Leu-Ala-Cys(OMeBzl)-Trp-Ala-Arg-Dpa-NH$_2$, was accom

panied by a 12-, 13-, and 16-fold increase in fluorescence, respectively. Initial rates measurements for twelve different substrate concentra

tions were performed. K$_m$ and h$_{cat}$ values were determined by fitting these data to the equation of Michaelis-Menten by nonlinear regression.
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**RESULTS**

The different side chains in the $P_1'$ position of our series of fluorogenic substrates are described in Fig. 1. Arylalkyl side chains longer than that of phenylalanine were selected to assess the effect of the side chain's length on MT1-MMP and ST3 cleaving activities. In addition, substrates with arylalkyl side chains containing an oxygen or sulfur heteroatom were also examined. The effects of alkyl side chains longer than leucine were also studied.

Products resulting from the cleavage of this series of substrates by MT1-MMP or ST3 were characterized both by HPLC and mass spectroscopy. For each substrate, we observed a single cleavage site between the expected Ala-Xaa peptide bond, with no inhibition of the reaction by the released products (Fig. 2, and data not shown). Values of the specificity constant $k_{cat}/K_m$ that were determined from the first-order progress curves for the hydrolysis of substrates by MT1-MMP are reported in Table I. The most rapidly cleaved substrate by this enzyme was that harboring the longest side chain in the $P_1'$ position (Cys(OMeBzl)). In the arylalkyl series, the lengthening of the side chain by one (hPhe) and two (pPhe) methylene(s) resulted in a marked increase in catalytic efficiency, as well as the replacement of the Pro-Leu sequence by a slower rate than their counterparts in the arylalkyl series (Table I). The substitution Leu $\rightarrow$ Cys(OMeBzl) in these substrates led to a 37-fold increase in the $k_{cat}/K_m$ ratio (Table I). In the case of ST3, the preferred side chain in the $P_1'$ position of the substrate was also the longest arylalkyl one (Cys(OMeBzl)) (Table I). However, the substrates containing leucine, methionine or n-hexyl in the $P_1'$ position were cleaved more rapidly than that with an n-hexyl side chain. As compared with MT1-MMP, the former three substrates were rather well cleaved by ST3, while the n-hexyl compound was a poor substrate.

The $K_m$, $k_{cat}$ and $k_{cat}/K_m$ values determined for two substrates (Xaa = Leu and Cys(OMeBzl)) are reported in Table II. For these matrixins, the substitution Leu $\rightarrow$ Cys(OMeBzl) was found to increase both the substrate affinity and the $k_{cat}$ value. However, while these two substrates displayed similar $K_m$ for MT1-MMP and ST3, the $k_{cat}$ values determined for ST3 on these substrates were about two orders of magnitude lower than those measured for MT1-MMP. The free energy difference ($\Delta G^\circ$) associated with the substitution Leu $\rightarrow$ Cys(OMeBzl) was evaluated for each enzyme from the ratio of the $k_{cat}/K_m$ values determined for these two substrates. For MT1-MMP, this substitution corresponds to a free energy change of 2 kcal/mol, while the same modification causes a free energy change of 1.55 kcal/mol for ST3.

The substitution of alanine by phenylalanine in the $P_1$ position, as well as the replacement of the Pro-Leu sequence by alanine residues in the $P_3$ and $P_2$ positions, led to substrates which were poorly hydrolyzed by MT1-MMP (Table I). In contrast, these two substrates were rather well cleaved by ST3. Thus, based on these preliminary data, the specificity requirement of the non-primed subsites of ST3 appears quite different from that observed for MT1-MMP.

The finding that the best substrate in this series was that containing a Cys(OMeBzl) residue in the $P_1'$ position led us to develop a fluorogenic substrate, Mca-Pro-Leu-Ala-Cys(OMeBzl)/Trp-Ala-Arg-Dpa-NH$_2$, characterized by the presence of the Mca-Dpa fluorophore-quencher pair. Such a fluorophore-quencher pair has been demonstrated to provide more sensitive fluorescent assays than those based on the dns-Trp pair. Using the Mca-Pro-Leu-Ala-Cys(OMeBzl)/Trp-Ala-Arg-Dpa-NH$_2$ substrate, the enzymatic activity of a 5 nM ST3 solution can be determined with high accuracy, in less than 20 min. On this time scale, only a very small fraction of the commercially available Mca-Dpa substrate for matrixins (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH$_2$) was cleaved with the same amount of ST3,
even at a 5 μM final concentration of this substrate. The Mca-Pro-Leu-Ala-Cys(OMeBzl)-Trp-Ala-Arg-Dpa-NH₂ displayed a \( k_{\text{cat}}/K_m \) value of 1.59 \( \times 10^6 \) M\(^{-1}\) s\(^{-1}\) when tested with MT1-MMP, at 25 °C. This substrate is therefore one of the most rapidly cleaved synthetic substrates so far reported for matrixins. For example, a \( k_{\text{cat}}/K_m \) value of 1.57 \( \times 10^5 \) M\(^{-1}\) s\(^{-1}\) has been determined for the cleavage of the Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ substrate by MT1-MMP, at the same pH, but at 37 °C (13). Furthermore, the first synthetic substrates cleaved by ST3 were identified in this series. The cleavage of the Mca-Pro-Leu-Ala-Cys(OMeBzl)-Trp-Ala-Arg-Dpa-NH₂ fluorogenic peptide provides a simple test for routine assay of ST3, useful for ST3 inhibitor screens. The influence of unusual amino acid substitutions in the P₁ position of synthetic substrates was previously evaluated for two matrixins, COL1 and GEL B (37). A small increase (4-fold) in the \( k_{\text{cat}}/K_m \) value was observed for substrates containing γ-S substituted cysteine in the P₁ position, as compared with the parent compound with leucine in the P₁ position. Based on the present work, this modest effect may be due to the fact that only γ-S substituents of reduced size, with a methyl and an ethyl group, were investigated in this study.

The substitution Leu → Cys(OMeBzl) in the P₁ position of the substrate improved the \( K_m \) value of the substrate for MT1-MMP, a finding in agreement with x-ray data (25, 29, 30) and inhibitors studies (31–35). This suggests that in all matrixins containing a leucine residue in their S₁p pocket, this pocket should be a deep cavity able to accommodate extremely long amino acid side chains. In contrast, the increase in substrate affinity resulting from the Leu → Cys(OMeBzl) substitution for ST3 is unexpected. ST3 is characterized by the presence of
glutamine in its S1 \textsuperscript{*} subsite, a property which was predicted to confer a shallow S1 \textsuperscript{*} pocket to ST3 (36), paralleling the situation observed in the x-ray structures of COL1 and matrixin (24, 26, 27). A possible interpretation of our results is that the glutamine side chain in the ST3 S1 \textsuperscript{*} pocket can move, allowing this matrixin to bind and cleave substrates harboring long side chains in the P1 \textsuperscript{*} position. Such a scenario has been proposed during inhibitor binding for the arginine side chain of COL1, since this matrixin was unexpectedly found to be potently inhibited by synthetic compounds with long side chains at the P1 \textsuperscript{*} position (34). A shift of the glutamine side chain in ST3, allowing substrate binding, may explain why the free energy change associated with the Leu \rightarrow Cys(OmeBzl) substitution is lower for ST3 than MT1-MMP. In the case of ST3, the interactions engaged between the P1 \textsuperscript{*} position of the substrate and the S1 \textsuperscript{*} pocket would have to compensate for the energy associated with the displacement of the glutamine side chain.

Interestingly, the modification Leu \rightarrow Cys(OmeBzl) not only improves the $K_m$, but also the $k_{cat}$ value for the substrate for both MT1-MMP and ST3 enzymes. Therefore, for these two matrixins, the binding energy associated with the interaction of substrates containing an unusual amino acid side chain in the P1 \textsuperscript{*} position with the enzyme catalytic site is used to stabilize the energy of the substrate-enzyme complex both in the ground and transition-states. This last effect, as mentioned in the introduction, could not be predicted from previous studies aimed at evaluating matrixin-inhibitor interactions, since most inhibitors so far developed were not analogues of the substrate in the transition-state (20). For a long time, the functional significance of a deep S1 \textsuperscript{*} subsite in matrixins able to accommodate long P1 \textsuperscript{*} groups of inhibitors was a subject of debate. In this respect, our data clearly indicate that the filling of this S1 \textsuperscript{*} subsite by an appropriate group in the P1 \textsuperscript{*} position yields substrates with high $k_{cat}/K_m$ values.

The $k_{cat}$ values reported in this study for two substrates are two orders of magnitude lower for ST3 than those observed for MT1-MMP. These low turnover numbers are in agreement with the very weak proteolytic activities reported for ST3 against most usual matrixin substrates, with the exception of α1 proteinase inhibitor (43, 47, 48). Arguments pointing to ST3 as a particular member of the matrixin family have been presented before and include, in addition to unusual proteolytic properties, both unusual proform processing (10, 49) and regulation of gene expression (50, 51). However, our observations do not rule out the possibility that ST3 may rapidly hydrolyze in vivo some, as yet unexamined, substrates. Our preliminary data on the specificity of ST3 for the nonprimed residues of synthetic substrates, in comparison to MT1-MMP, support the possibility that ST3 could cleave substrates which are not presently considered as matrixin substrates. Many reports have documented the preference of matrixins for small residues in vivo (104), probably increase the $K_m$ value, as this involves a gain of only a few kilocalories/mol in the enzyme-substrate binding energy (Table II). An important issue is to determine whether it is possible to identify specific peptide sequences that are hydrolyzed by ST3 with an efficiency similar to that reported for the other matrixins. Our data showing that ST3 efficiently cleaves substrates harboring unusual long side chains in their sequence suggest that this matrixin may hydrolyze, in vivo, proteins also containing unusual amino acids. In this respect, extracellular proteins undergoing particular post-translational modifications may represent potential targets for ST3. Although a large number of protein substrates have already been described for MT1-MMP, as well as for the other matrixins containing a leucine residue in their S1 \textsuperscript{*} subsite, our observations raise the possibility that these matrixins may also cleave,

### Table I

| Substrates | $K_m$ (μM) | $k_{cat}$ (μM s$^{-1}$) | $k_{cat}/K_m$ | $K_m$ (μM) | $k_{cat}$ (μM s$^{-1}$) | $k_{cat}/K_m$ |
|------------|-----------|----------------------|--------------|-----------|----------------------|--------------|
| Dns-Pro-Leu-Ala-Cys(OmeBzl)-Trp-Ala-Arg-NH$_2$ | 159 ± 0.44 | 1.67 ± 0.04 | | | | |
| Dns-Pro-Leu-Ala-Cys(Bzl)-Trp-Ala-Arg-NH$_2$ | 65 ± 0.32 | 1.12 ± 0.09 | | | | |
| Dns-Pro-Leu-Ala-Ser(Bzl)-Trp-Ala-Arg-NH$_2$ | 25 ± 0.05 | 0.70 ± 0.01 | | | | |
| Dns-Pro-Leu-Ala-pPhe-Trp-Ala-Arg-NH$_2$ | 24 ± 0.07 | 0.18 ± 0.009 | | | | |
| Dns-Pro-Leu-Ala-hPhe-Trp-Ala-Arg-NH$_2$ | 9.5 ± 0.06 | 0.27 ± 0.03 | | | | |
| Dns-Pro-Leu-Ala-nHex-Trp-Ala-Arg-NH$_2$ | 6.3 ± 0.04 | 0.001 ± 0.004 | | | | |
| Dns-Pro-Leu-Ala-Leu-Trp-Ala-Arg-NH$_2$ | 4.2 ± 0.03 | 0.18 ± 0.0015 | | | | |
| Dns-Pro-Leu-Ala-Met-Trp-Ala-Arg-NH$_2$ | 3.6 ± 0.03 | 0.52 ± 0.006 | | | | |
| Dns-Pro-Leu-Ala-nPent-Trp-Ala-Arg-NH$_2$ | 3.1 ± 0.02 | 0.30 ± 0.005 | | | | |
| Dns-Pro-Leu-Ala-Nle-Trp-Ala-Arg-NH$_2$ | 1.8 ± 0.01 | 0.13 ± 0.005 | | | | |
| Dns-Pro-Leu-Ala-Phe-Trp-Ala-Arg-NH$_2$ | 1.1 ± 0.01 | 0.02 ± 0.006 | | | | |
| Dns-Pro-Leu-Phe-Cys(Bzl)-Trp-Ala-Arg-NH$_2$ | <1 | 0.52 ± 0.03 | | | | |
| Dns-Ala-Ala-Ala-Cys(Bzl)-Trp-Ala-Arg-NH$_2$ | <1 | 0.90 ± 0.02 | | | | |

### Table II

| Substrate | $K_m$ (μM) | $k_{cat}$ (μM s$^{-1}$) | $k_{cat}/K_m$ | $K_m$ (μM) | $k_{cat}$ (μM s$^{-1}$) | $k_{cat}/K_m$ |
|-----------|-----------|----------------------|--------------|-----------|----------------------|--------------|
| Xaa = Cys(OmeBzl) | 2.79 ± 0.8 | 4.3 ± 0.7 | 1541218 | 1.5 ± 0.17 | 0.033 ± 0.003 | 22900 |
| Xaa = Leu | 13.7 ± 0.32 | 0.73 ± 0.01 | 53284 | 6.8 ± 1.1 | 0.011 ± 0.001 | 1617 |
| $\Delta G^{b}$ | 2.00 kcal/mol | | | 1.55 kcal/mol | | |

$^{a}$ Calculated from the ratio of the $k_{cat}$ and $K_m$ values.

$^{b} \Delta G^b = RT \ln(k_{cat}/K_m)$, $T = 298K$. 

Assays were carried out in 50 mM Tris/HCl, pH 7.5, 10 mM CaCl$_2$, in the presence (ST3) or absence (MT1-MMP) of 200 mM NaCl.
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in vivo, proteins at sites characterized by the presence of amino acids with unusual long side chains.

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Membrane Type-1 Matrix Metalloprotease and Stromelysin-3 Cleave More Efficiently Synthetic Substrates Containing Unusual Amino Acids in Their P1\textsuperscript{′} Positions

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