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ARTICLE

A Caged Substrate Peptide for Matrix Metalloproteinases

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Based on the widely applied fluorogenic peptide FS-6 (Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2; Mca = methoxycoumarin-4-acetyl; Dpa = *N*-3-(2,4-dinitrophenyl)l-α,β-diaminopropionyl) a caged substrate peptide Ac-Lys-Pro-Leu-Gly-Lys*-Lys-Ala-Arg-NH_2 (*, position of the cage group) for matrix metalloproteinases was synthesized and characterized. The synthesis implies the modification of a carbamidated lysine side-chain amine with a photocleavable 2-nitrobenzyl group. Mass spectrometry upon UV irradiation demonstrated the complete photolytic cleavage of the protecting group. Time-resolved laser-flash photolysis at 355 nm in combination with transient absorption spectroscopy determined the biphasic decomposition with τ_a = 171 ± 3 ms (79%) and τ_b = 2.9 ± 0.2ms (21%) at pH 6.0 of the photo induced release of 2-nitrobenzyl group. The recombinantly expressed catalytic domain of human membrane type I matrix metalloproteinase (MT1-MMP or MMP-14) was used to determine the hydrolysis efficiency for the caged peptide before and after photolysis. It turned out that the cage group sufficiently shields the peptide from peptidase activity, which can be thus controlled by UV light.

Introduction

The use of caged compounds for the light triggered release of biologically active compounds has been demonstrated for many cases.[1-3] The development of fully optical control for effector activation has proven to be useful in a number of applications where *in situ* modulation of substrate concentration with spatial and temporal resolution is important.[3-5] In specific cases, fast decomposition of photoactivated compounds has allowed to overcome the temporal limitations imposed by rapid mixing techniques.[6] However, the application of light triggered activation of the large group of hydrolytic enzymes called peptidases is a yet underrepresented issue. An important class of peptidases is comprised by the zinc-dependent matrix metalloproteinases (MMPs), of which more than 20 different isoforms are expressed in humans. They play a major role in the degradation and remodeling of extracellular matrix substrates and are, therefore, involved in processes like wound healing, bone resorption, embryonic development, tissue development etc.[7-10] Consequently, diseases like arthritis, rheumatism, and cancer are often linked to MMP dysfunction.[6,8] Even if MMPs share the zinc binding motif conserved sequence HEXGHXXGXXH and show essentially identical three dimensional structure, their substrate specificity is very different.[11] Therefore, the design of pharmaceutically applicable MMP inhibitors requires the detailed knowledge about the substrate-enzyme interaction and their catalytic process.

Recently, the study of the role of water dynamics on the substrate-enzyme interaction of the membrane type I matrix metalloproteinase (MMP14 or MT1-MMP) revealed a strong coupling between water and protein motion being crucial for the substrate recognition.[10,12,13] MT1-MMP has been reported to cleave several types of collagen, e.g. type I, II, III collagens, and to hydrolyze a variety of different substrates such as κ-elastin, gelatin, β-casein, and other extracellular matrix components.[14] The active site contains a catalytic zinc atom bound to three hystidines and exposed to the solvent. The structure of the substrate binding site is characterized by a pocket located next to the zinc atom called "S1 pocket", which is probably a determining factor of substrate specificity. This hydrophobic pocket has different depth among MMP isoforms and dictates the substrate docking.[15] The suggested catalytic mechanism of action for MMPs involves the nucleophilic attack of water molecule polarized by the catalytic Zn^{2+} and the conserved glutamate on the scissile bond at close-to-neutral pH. For this to happen, a substrate must be bound and form a Michaelis complex.[6,16] Even if several specificity studies using collagen-model peptides have been published[11], the specific mechanism of MMPs-substrate interaction and recognition remains an important unresolved issue. Here we report on the design and synthesis of a MT1-MMP caged substrate peptide.

The products of light decomposition are characterized by mass spectrometry. Laser-flash photolysis experiments allow determining an upper limit for substrate release by light. The applicability of the photolabile caged MT1-MMP is demonstrated by fluorescence and HPLC detection.

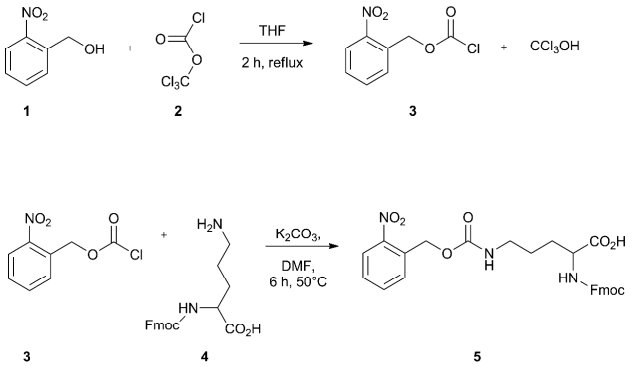
Materials and Methods

Materials

The expression plasmid for the catalytic domain of human MT1-MMP (residues 112–292) was transformed into *Escherichia coli* BL21(DE3) (Novagen). Expression and purification of the enzyme was carried out as described previously.[17]

Synthesis of nitrobenzyl-L-lysine

Synthesis of 2-nitrobenzylcarbonyl chloride (**3**) (3.13 g light brown crystals). 1.68 g (11 mmol) of 2-nitrobenzylalcohol (**1**) and 4.36 g (22 mmol) of diphosgen (**2**) were dissolved in THF and refluxed for 2 h. The solvent was removed under reduced pressure at 50°C. mp 59°C (from 3:2 diethyl ether/pentane); *m/z* 169 (M^+, 17%), 152 (2), 136 (44), 125 (37), 121 (11), 104 (32), 99 (12), 91 (72), 78 (100), 63 (74), 51 (49), 44 (26), 39 (17), 30 (5); NMR: δ_H(500 MHz; CD_2Cl_2; $(CH_3)_4Si$) 5.73 (2 H, s, CH_2), 7.59 (1 H, m, Ph-C^4H), 7.66 (1 H, m, Ph-C^5H), 7.75 (1 H, d, Ph-C^6H), 8.18 (1 H, d, Ph-C^3H); $\delta_{^{13}C}$(400 MHz; $CDCl_3$; $CDCl_3$) 67.8 (CH_2), 125.4 (Ph-C^3), 128.8 (Ph-C^4), 129.6 (Ph-C^6), 130.1 (Ph-C^1), 134.2 (Ph-C^6), 147.2 (C–NO_2), 147.5 (COCl).



Scheme 1 Synthetic route for the preparation of N^2-(9-fluoromethoxycarbonyl)-N^6-2-nitrobenzyloxycarbonyl-L-lysine as a precursor for peptide synthesis.

Synthesis of N^2-(9-fluoromethoxycarbonyl)-N^6-2-nitrobenzyloxycarbonyl-L-lysine (**5**) (2.8 g, 15.5% light yellow crystals). All steps were carried out in the dark. 12.33 g (33.5 mmol) of N^2-(9-fluoromethoxycarbonyl)-L-lysine (**4**) (Fmoc–Lys–OH) (Bachem) was suspended in 200 mL of DMF and incubated with 1.1 g (33.5 mmol) of **3** and 4.63 g (33.5 mmol) of K_2CO_3. The mixture was stirred for 6 h at 50°C. Afterwards 600 mL of CH_2Cl_2 were added and extracted with saturated $NaHCO_3$ solution and twice with saturated NaCl solution. Upon filtration the solution was dried over $MgSO_4$ and the solvent removed under reduced pressure. The brown liquid was dissolved in 95:5 CH_2Cl_2/CH_3OH and purified by silica gel chromatography. Elution was achieved with 85:15 CH_2Cl_2/CH_3OH. NMR: δ_H(400 MHz; d_6-DMSO; $(CH_3)Si_4$) 8.08 (1 H, d, C^3H–CNO_2), 7.86 (2 H, d, Fmoc-$C^{4/5}$H), 7.77 (1 H, t, Fmoc-C^9H), 7.30 (2 H, m, Fmoc-$C^{2/7}$H), 7.40 (4 H, m, Fmoc-$C^{1/3/6/8}$H), 7.6 (3 H, m, Ph-$C^{4/5/6}$H), 5.33 (3 H, s, NH), 4.2 (3 H, m, Fmoc-CH_2/Lys-C^α), 3.32 (broad, OH), 3.16 (2 H, s, CH_2–C–CNO_2), 3.95 (2 H, m, Lys-$C^\varepsilon H_2$), 1,6 (2 H, m, Lys-$C^\beta H_2$), 1.38 (2 H, m, Lys-$C^\delta H_2$), 1.26 (2 H, m, Lys-$C^\gamma H_2$).

Peptide Synthesis

The peptides Fluo-LK, Fluo-KK, KK, and Ø-KK presented in Scheme 2 were synthesized by standard solid phase synthesis (SPS; Rink amide ChemMatrix, Aldrich) using Fmoc chemistry and *N*-methylpyrolidon as the solvent. In case of the caged peptide Ø-KK the coupling efficiency of the modified Lys was 84.7%, as estimated from UV absorption of the fulvene adduct formed upon removal of the N_δ-Fmoc protecting group with 25% piperidine/DMF for 20 min at room temperature. For the synthesis of Ø-KK and the fluorogenic peptides FS-1 and FS-6 care was taken to minimize exposure to light. All peptides were purified by C_{18}-RPC (Phenomenex Gemini C18, 110 Å, 5 µm, 250 mm × 21.2 mm) using HPLC and a linear gradient of CH_3CN from 0 to 90% in 0.1% trifluoroacetic acid (TFA). Peptides were characterized by ESI MS: Fluo-LK: *m/z* Found: 1263.7 $[M + H]^+$. Calc.: 1263.6; Fluo-KK: *m/z* Found: 1278.6, Calc.: 1278.7; KK: *m/z* Found: 938.6, Calc.: 939.6; Ø-KK: *m/z* Found: 1117.7, Calc.: 1117.6; For Ø-KK: λ_{max}(water)/nm 266 (ε/dm^3 mol^{-1} cm^{-1} 3914);

```
                                   Dnp
                                   |
Fluo-LK: Mca-Lys-Pro-Leu-Gly~Leu-Lys-Ala-Arg-NH2

                                   Dnp
                                   |
Fluo-KK: Mca-Lys-Pro-Leu-Gly~Lys-Lys-Ala-Arg-NH2

KK:      Ac-Lys-Pro-Leu-Gly~Lys-Lys-Ala-Arg-NH2

                                   Ø
                                   |
Ø-KK:    Ac-Lys-Pro-Leu-Gly~Lys-Lys-Ala-Arg-NH2

FS-1:       Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH2

FS-6:    Mca-Lys-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH2
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Scheme 2 Substrate peptides for MT1-MMP referred to in this study. Mca = methoxycoumarin-4-acetyl; Dnp = 2,4-dinitrophenyl; Ø = 2-nitrobenzyloxycarbonyl; Dpa = *N*-3-(2,4-dinitrophenyl)-L-α,β-diaminopropionyl. ~ denotes the hydrolyzed peptide bond.

Enzyme kinetics of fluorescent peptide substrates

Enzyme kinetic parameters were determined by monitoring the increase in fluorescence intensity of Mca due to the decrease of FRET quenching[18] by Dnp upon hydrolysis of the fluorogenic peptides Fluo-KK and Fluo-LK with a 96-well plate reader connected to a fluorescence spectrophotometer (Varian Cary Eclipse) at 25°C. Fluorescence at λ_{em} = 400 nm was monitored upon excitation at λ_{ex} = 340 nm. To measure the rate of substrate cleavage, enzyme was added to the reaction mixture in 50 mM Tris/HCl (pH 7.4), 100 mM NaCl, 5 mM $CaCl_2$ and varying concentrations of fluorescent substrate (2 - 15 µM). The kinetic data were fitted by a pseudo-first-order reaction rate (**Eq. [1]**) using ORIGINLAB 8.6 (Microcal)

Eq. 1 $$F(t) = F_{max}[1\text{-}e^{-kt}] + F_0$$

where $F(t)$ is the fluorescence at time t, F_0 and F_{max} are the initial and the final fluorescence intensities, respectively, and k is the pseudo-first-rate constant $k = [E]_{tot}$ (k_{cat}/K_M).[19] Concentration of MT1-MMP and fluorescent substrates was determined by UV-VIS absorption ($\epsilon_{275\ nm}$ = 35410 M^{-1} cm^{-1} and $\epsilon_{410\ nm}$= 7500 M^{-1} cm^{-1} for the enzyme and fluorogenic substrates respectively).[20]

Analysis of the hydrolysis of non-fluorescent peptides

2.5 µM MT1-MMP in 50 mM Tris/HCl (pH 7.4), 100 mM NaCl, 5 mM $CaCl_2$ was incubated with 1 mM of either KK or Ø-KK for 2.5 h at 25°C. Aliquots of 150 µL were taken every 30 min and immediately frozen in liquid nitrogen. Quantification of peptides was performed by C_{18}-RPC (Phenomenex Gemin, C18, 110 Å, 5 µm, 250 mm × 4.6 mm) using HPLC and absorbance detection at 202 and 265 nm. A linear gradient of CH_3CN from 0 to 90% in 0.1% TFA, 1.0 ml/min was applied.

Laser Flash Photolysis

The kinetics of Ø-KK photodecomposition were studied by means of transient absorption.[21] A solution of Ø-KK in 50 mM Tris/HCl (pH 7.4), 100 mM NaCl, 5 mM $CaCl_2$ at 25°C in a quartz cuvette was photo excited by the third harmonic (λ = 355 nm) of a nanosecond, Q-switched Nd:YAG laser (Surelite II – 10, Continuum, FWHM 5 ns, 10 mJ)[22] which entered the cuvette at 90° to the detection beam. The monitoring beam was provided by the cw output of a 75 W Xe arc lamp (Oriel) that was filtered with a 405 nm interference filter. The beam was then passed through a monochromator (Oriel MS257), its intensity monitored by a 5-stages photomultiplier (Applied Photophysics) and digitized by a digital oscilloscope (LeCroy LT374). The experiment was repeated with Ø-KK in N_2 saturated water whose pH was adjusted by adding small amounts of concentrated NaOH or HCl solutions, and in 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)/NaOH (pH 6.0)

Design of a photocaged MMP substrate

According to the nomenclature of Schechter and Berger[23], the main subsite for substrate recognition in MMPs is the specificity pocket S1´.[6,24] As the structure of S1´ varies between MMP family members, modification of the P1´ side-chain allows the modification of substrate specificity.[10,25-27] Thus, it was our aim to inhibit substrate cleavage by a sterically demanding modification of P1' using a photocleavable group so that photolytic release provides the substrate peptide.

A widely used substrate peptide for the study of MMP reaction kinetics is the fluorogenic peptide FS-6 (see Scheme 2)[20] with the non-natural amino acid *N*-3-(2,4-dinitrophenyl)-L-α,β-diaminopropionyl (Dpa) as the quencher group in position P2'. It was previously demonstrated that substitution of Dpa by N^6-2,4-dinitrophenyl-L-lysine (Lys(Dnp)) results in a substrate for MT1-MMP[10] termed Fluo-LK (Scheme 2). We determined the enzyme kinetic constants for Fluo-KK applying Michaelis-Menten kinetics (Fig. 1). As a result, both the secondary rate constant k_{cat}/K_M as well as the Michaelis constant K_M are comparable to the values obtained for FS-6 (Table 1), indicating that substitution of Dpa by Lys(Dnp) is well tolerable.

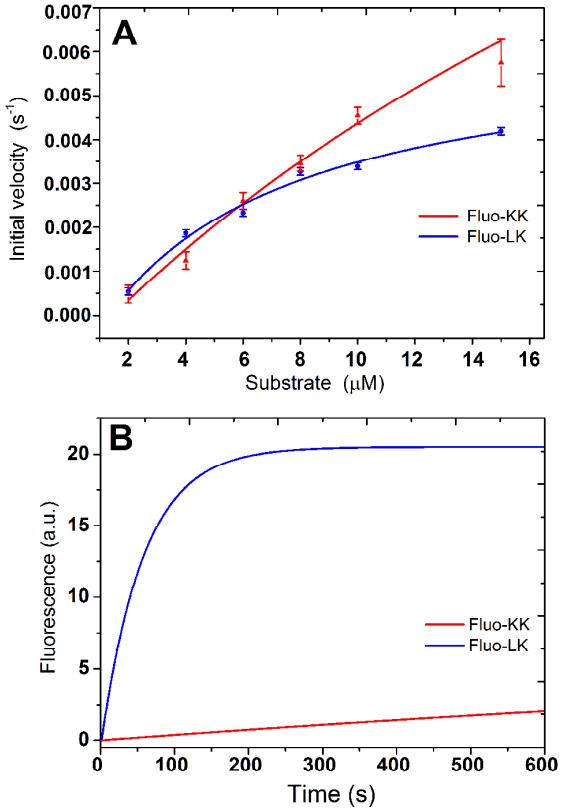

Fig. 1 (A) Kinetic parameters k_{cat} and K_M were obtained for the hydrolysis of different concentration of Fluo-LK (blue line) and Fluo-KK (red line) by MT1-MMP in 50 mM Tris/HCl (pH 7.4), 100 mM NaCl, 5 mM $CaCl_2$ at 25°C. Data were fitted by the Michaelis-Menten equation. **(B)** Hydrolysis of Fluo-LK (blue line) and Fluo-KK (red line) by MT1-MMP. ($[E_0]$ = 0.35 µM in 50 mM Tris/HCl (pH 7.4), 100 mM NaCl, 5 mM $CaCl_2$ at 25°C, [S] = 10 µM). First-order rate constants, k_{obs}, were obtained by fitting eq. [1], second order rate constants k_{cat}/K_M were calculated from $k_{obs}/[E_0]$ (see Table 1).

For the modification of the P1' side-chain, the insertion of a chemical functionality is mandatory. Because a structure of MT1-MMP with a substrate peptide or product peptides is currently not available[17], a superposition of its inhibitor bound X-ray structure (PDB code 1BQQ)[28] with the X-ray structure of MMP12 containing the two product peptides Pro–Gln–Gly and Ile–Ala–Gly in the catalytic site (PDB code 2OXZ)[29] was performed (Fig. 2). It should be noted that MMP12 and MT1-MMP share a high degree of similarity in the active site architecture. Fig 2 suggests that the side-chain of P1' of the product peptide Ile–Ala–Gly is directed toward the interior of S1'. However, S1' is comprised of a very deep pocket, much larger than it would be required to host the isobutyl side-chain of P1', suggesting that a long side-chain with a functional group like Lys could substitute this position and still the peptide being cleaved. To the best of our knowledge, despite large efforts in the design of optimal synthetic substrate for MMPs, Lys in P1´ was usually not considered.[10,30]

Table 1 Second-order rate constant k_{cat}/K_M, Michaelis constant K_M for the hydrolysis of Fluo-LK and Fluo-KK in comparison to commercially available fluorogenic peptides (FS-1 and FS-6, see Scheme 1) for MT1-MMP at 25 °C.

Substrate	pH	k_{cat}/K_M (M^{-1} s^{-1})	K_M (μM)
FS-1[20]	7.5	3.9×10^5	n.d
FS-6[20]	7.5	1.3×10^6	7.9
Fluo-KK[a]	7.4	$(0.7 \pm 0.5) \times 10^5$	36 ± 15
Fluo-KK[a]	6	$(0.11 \pm 0.02) \times 10^4$	40 ± 18
Fluo-LK[a]	7.4	$(2.2 \pm 0.2) \times 10^6$	5.7 ± 1.3

n.d. not determined.

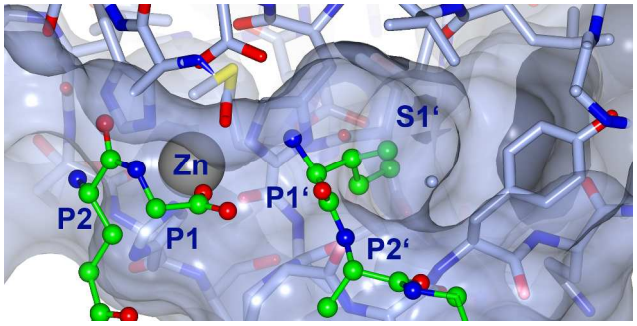

Fig. 2 Superposition of the X-ray structure of MT1-MMP (PDB code 1BQQ)[28] with that of MMP12 (PDB code 2OXZ)[29] with soaked in product peptides Pro–Gln–Gly (P1 and P2) and Ile–Ala–Gly (P1' and P2').

To test if Lys can be inserted into site P1', the peptide Fluo-KK was synthesized as a derivative of Fluo-LK (Scheme 2). As can be seen in Fig. 1, Fluo-KK is a reasonable substrate for MT1-MMP. Determination of the enzyme kinetic constants demonstrates that its affinity and specificity are approx. one order of magnitude smaller compared to Fluo-LK (Table 1). However, the parameters are very similar to another widely applied MMP substrate peptide FS-1 (see Scheme 2). As a result, Lys at P1' can indeed be used and the amine group is available for modification with a photoremovable protecting group.

Results and discussions

Synthesis of a caged substrate peptide

In comparison to the direct coupling of the 2-nitrobenzyl group to a side-chain amine, linkage via a carbamido group requires less synthetic effort and higher yields are obtained. Such a peptide was previously reported to release an inhibitory peptide against the calmodulin dependent protein kinase II upon photoactivation.[31] Scheme 2 shows that the synthesis of the caged Fmoc-L-lysine **5** requires only two steps of decent yield and can be easily inserted as a resin in routine SPS as was demonstrated here in the case of Ø-KK.

ESI MS demonstrated the integrity of Ø-KK (Fig. 3). Moreover, upon UV irradiation the mass peak corresponded to the peptide without the cage group (KK, Scheme 2), demonstrating that photoconversion was indeed obtained.

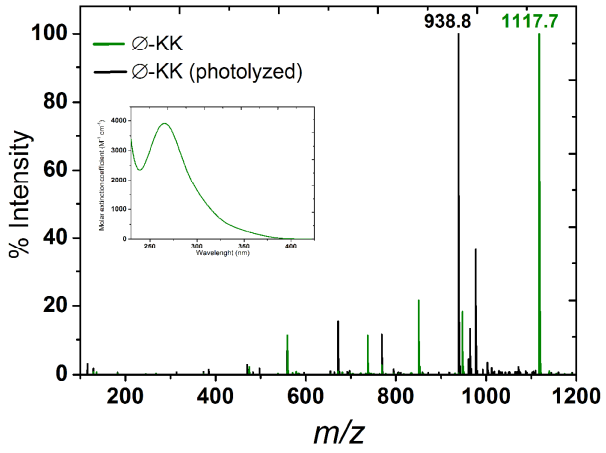

Fig. 3 ESI MS of Ø-KK before (green) and after photolysis (black). 1mg of Ø-KK was dissolved in 3 mL of 5 mM *N*-ethylmorpholine/acetic acid (pH 7.4). Photocleavage was performed through irradiation at 366 nm (8 W, ≈1.2 mW/m^2) for 1 h. Ø-KK: *m/z*: obs. 1117.7, calc. 1117.6; KK: *m/z*: obs. 938.8, calc. 939.6. The inset shows the absorption spectrum of Ø-KK.

Mechanism of photolysis

A usable caged compound requires a significantly reduced inhibitory activity and fast recovery of the activity after cleavage of the photolabile group by light.[1,4,32,33] On the basis of previous experimental evidence on related systems, we propose that the release mechanism of the photocleavable group is described by Scheme 3. Experimental evidence for this mechanism will be presented and discussed in the following section.

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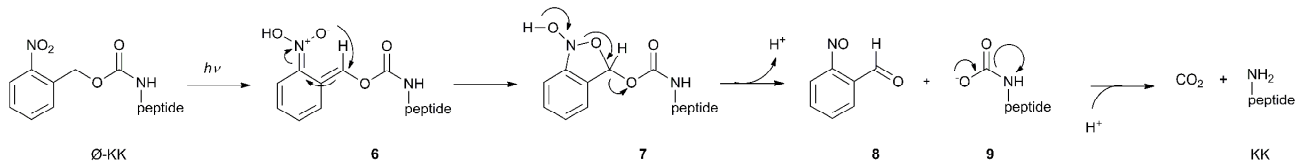

Scheme 3 Reaction mechanism of the photolysis of Ø-KK.

Flash photolysis experiments indicate that the photogenerated nitronic acid intermediates **6** of 2-nitrobenzyl compounds undergo deprotonation in water at neutral pH with rates in the 10^7-10^8 s^{-1} range.[34-36] The deprotonation rate is essentially determined by the pK_a of the particular nitronic acid intermediate, with the more acidic compounds having higher dissociation rates.[37-39] For instance, the nitronic acid generated by photolysis of 2-nitrobenzaldehyde has a pK_a value of 2.1±0.1 with a deprotonation rate exceeding 10^8 s^{-1}, whereas for 1-(2-nitrophenyl)ethyl sulfate (caged sulfate) the pK_a of the corresponding nitronic acid is 3.69 ± 0.05, with a deprotonation rate of (1.58 ± 0.09) × 10^7 s^{-1}.[21] The decomposition reaction proceeds from **6** through the bicyclic intermediate **7**. The lifetime of this intermediate can be very different depending on the leaving group and is dramatically increased in the presence of a buffer at neutral pH.[21,40,41] Upon decomposition of **7**, the carbamidated peptide **9** is autoconverted to the final product KK under protonation of the Lys nitrogen and the release of CO_2. The carbamate decarboxylation occurs with a rate of 10^9 to 10^6 M^{-1} s^{-1} which was determined for numerous different carbamidates at pH 6.8-13.0.[42] Detailed studies of the caged glutamate *N*-1-(2-nitrophenyl)ethoxycarbonyl-L-glutamate revealed that the decarboxylation is not rate limiting between pH 5 and 8.[43] Fig 4 shows the absorbance changes at 405 nm of a buffered (pH 7.4) aqueous solution of Ø-KK, following a single, 15 mJ laser pulse at 355 nm. For comparison, Fig 4 also shows the signal observed after photolysis of caged sulfate under identical conditions. After a fast, unresolved rising phase due to formation of the nitronic acid, we observe a slower rising kinetics characterized by a lifetime of (96±9) ns. This transient is most likely associated with deprotonation of the nitronic acid to give the *aci*-nitro anion, as suggested by studies on related compounds.[44,45] Similarly, the rise in the signal measured on caged sulfate occurs with a lifetime of (64 ± 7) ns, as previously reported.[21] At about 1 µs, equilibrium is reached for the *aci*-nitro intermediate for both compounds. The *aci*-nitro intermediates for Ø-KK and for caged sulfate decay with lifetimes of (840 ± 20) ms and (61 ± 1) ms, respectively. This sets a lower limit for the release of **9** in well buffered neutral solutions. When the *aci*-nitro intermediate was monitored in N_2 saturated water (pH 5.8), decay was found to occur through a biexponential relaxation, with lifetimes (3.9 ± 0.1) ms (31%) and (414 ± 15) ms 69%). In a buffered solution at lower pH (10 mM MES/NaOH, pH 6.0) a similar biexponential behavior was obtained (τ_a = (171 ± 3) ms (79%); τ_b = (2.9 ± 0.2) ms (21%)). The reason for the biexponential relaxation is at present not understood and will require future investigations.

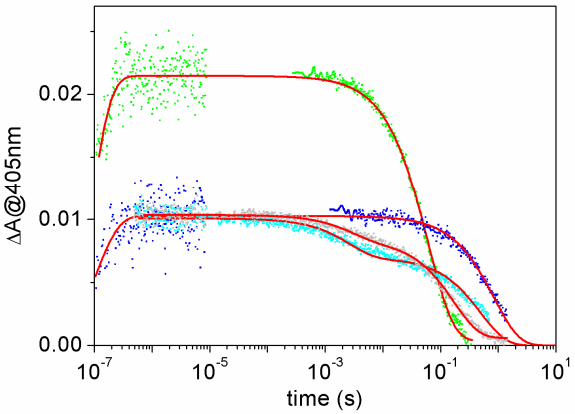

Fig. 4 Time resolved absorbance changes at 405 nm following exposure to 15 mJ laser shots for caged sulfate (green) and Ø-KK in 50mM Tris/HCl (pH 7.41), 100 mM NaCl, 5 mM $CaCl_2$ (blue) and in 10 mM MES/NaOH (pH 6.0) (grey). The cyan curve shows the *aci*-nitro transient absorbance for Ø-KK in nitrogen saturated water (pH 7.0). Red solid lines are the results of a fit to an exponential rise in the nanoseconds and a mono- or bi-exponential decay in the long milliseconds. For the cases of Ø-KK in nitrogen saturated water and in MES buffer it was not possible to observe the rise of the signal in the nanoseconds, due to intense scattered light. See text for fitting parameters.

Using caged sulfate as an actinometer, and considering that the chromophoric properties of Ø-KK and caged sulfate are essentially identical, the absorbance changes at 1 µs can be used to estimate yield for formation of the *aci*-nitro intermediate. Using the yield determined for caged sulfate (0.47) we can estimate an upper limit for the yield of the *aci*-nitro intermediate of Ø-KK as 0.24±0.05.

Cleavage of non-fluorescent peptides

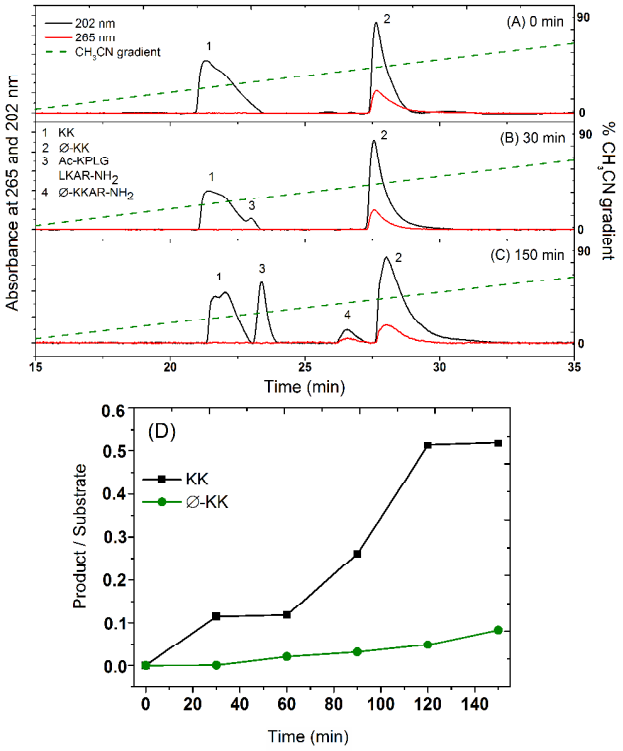

Fig. 5 HPLC profiles upon incubation of MT1-MMP with KK and Ø-KK (A) before addition of the enzyme, (B) after 30 min and (C) after 2.5 h. The peaks were assigned as follows: (1) KK, (2) Ø-KK, (3) KK fragment Ac–Lys–Pro–Leu–Gly or Leu–Lys–Ala–Arg–NH_2, (4) Ø-KK fragment Lys(Ø)–Lys–Ala–Arg–NH_2. (D): product-substrate ratio *versus* incubation time.

The qualitative comparison of the hydrolysis rate for the case of the caged and non-caged substrate is important to demonstrate the utility of Ø-KK as caged-compound in experiments in presence of an enzyme. This study was done by time course C_{18}-RPC measurements of the MT1-MMP + Ø-KK + KK mixture. Simultaneous detection of absorbencies at 202 nm and 265 nm allowed distinguishing between peptide containing a 2-nitrobenzyl group and those without. Assignment of the peaks was performed by chromatographic separation of KK and Ø-KK. The peaks corresponding to the product peptides were obtained from the extended incubation of KK and Ø-KK with MT1-MMP in the dark.

Fig. 5A shows the chromatogram of the intact substrates before addition of the enzyme in which KK and Ø-KK are well separated. Fig. 5B and C exhibit the chromatogram of a sample upon incubation with MT1-MMP for 30 min and 2.5 h, respectively, at 25°C. An additional peak is observed after 30 min and corresponds to one the of KK fragments. After 1 h, a fourth peak appeared with absorption at 265 nm, that has been attributed to the Lys(Ø)–Lys–Ala–Arg–NH_2 fragment of Ø-KK (Scheme 2). The time course of the peptide cleavage is shown in Fig.5 (bottom) demonstrating that Ø-KK cleavage is occurring with a much smaller rate compared to KK (>10-fold) and can be neglected in presence of uncaged compound.

Despite the negligible hydrolysis of the caged-substrate compared to the KK-substrate, the fact that the protein preserves a slight ability to cleave the caged-peptide indicates that the caged-peptide is still able to get into the active pocket and get cleaved. This is demonstrated by the presence of peak 4 in Fig. 5A, corresponding to the peptide fragment bound to the cage group. At this stage it seems difficult to assess whether the decreased activity is due to a weaker binding of the substrate or to a lower enzymatic activity at the binding site. However, it seems plausible that entrance and exit from the active site is possible but hampered by steric hindrance of the cage group, resulting in a high inhibition of hydrolysis. The hypothesis that the caged-peptide may act as an inhibitor due to non-specific interaction in other sites of the protein is very unlikely.

Conclusions

In the present study, a new caged-peptide for the study of enzyme-substrate interactions of MMPs was synthesized and characterized. Enzyme kinetic studies demonstrated that the substrate resin P1', which is typically Leu can be substituted by Lys and being still effectively cleaved. The introduction of the Lys5 amine function allowed the attachment of a photolabile *o*-nitrobenzyl group in the substrate peptide Ac–Lys–Pro–Leu–Gly–Lys–Lys–Ala–Arg–NH_2. The new compound Ø-KK (Scheme 1) is easy to synthesize and highly soluble in water. ESI MS demonstrated that light induced removal of the nitrobenzyl group was successful and that no significant side reaction had occurred. The kinetics of photodecomposition after nanosecond laser photolysis has been characterized by time-resolved transient absorbance spectroscopy. The protolytic dissociation of the *aci*-nitro compound **6** through the bicyclic intermediate **7** is pH dependent and occurs with τ_a = 171 ms in buffered solutions at pH 6.0. At this pH, a second faster decay (21%) occurs with τ_b = 2.9 ms (Fig 4).

Analysis of the enzymatic hydrolysis by MT1-MMP using C_{18}-RPC demonstrated that the cleavage of the caged-substrate can be neglected in presence of the non-caged peptide. This demonstrates that Ø-KK is sufficiently sterically hindered to allow incubation and activation by UV light.

To the best of our knowledge, this is the first light-controlled caged peptide suitable for matrix metalloproteinases.

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Notes and references

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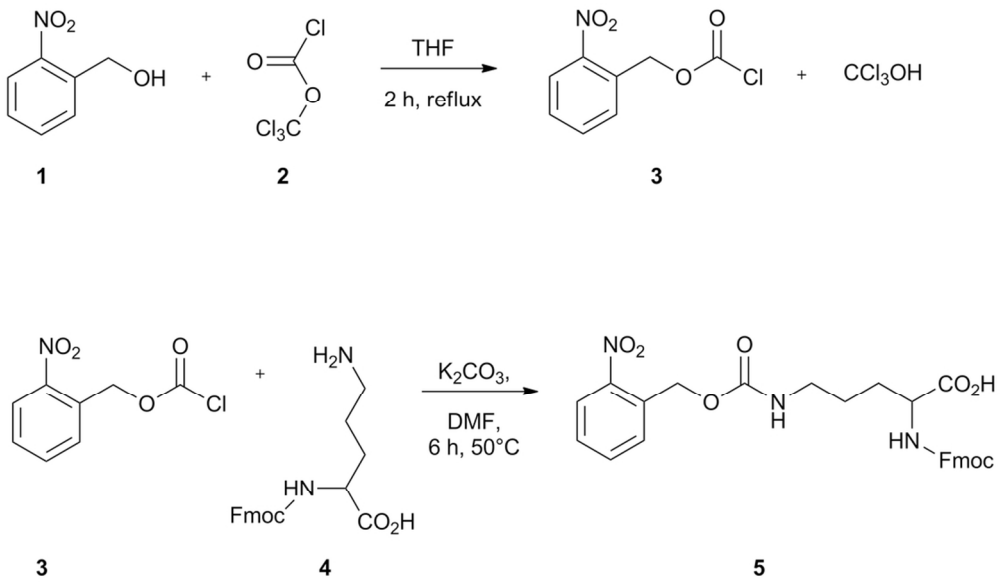

Scheme 1 Synthetic route for the preparation of N2-(9-fluoromethoxycarbonyl)-N6-2-nitrobenzyloxycarbonyl-L-lysine as a precursor for peptide synthesis.
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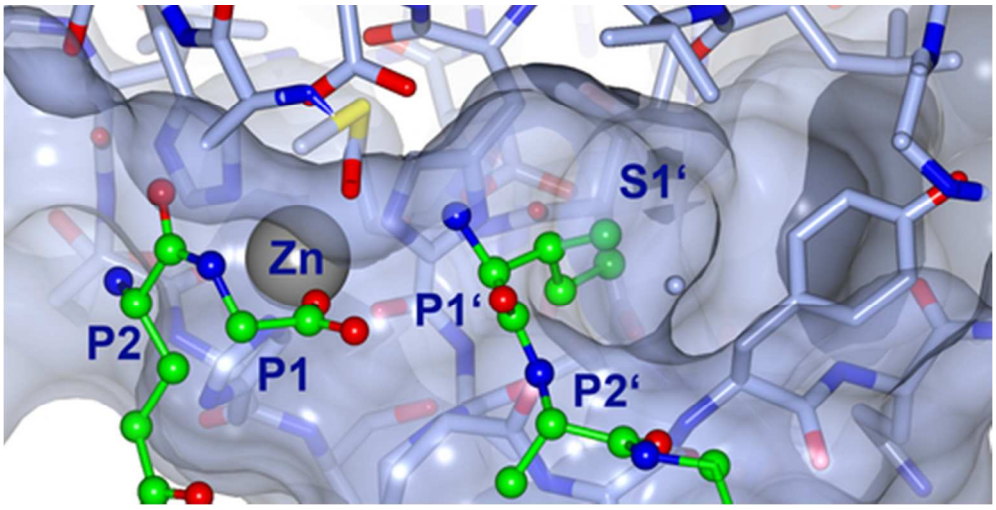


Fig. 2 Superposition of the X-ray structure of MT1-MMP (PDB code 1BQQ)28 with that of MMP12 (PDB code 2OXZ)29 with soaked in product peptides Pro–Gln–Gly (P1 and P2) and Ile–Ala–Gly (P1’ and P2’).
41x21mm (300 x 300 DPI)

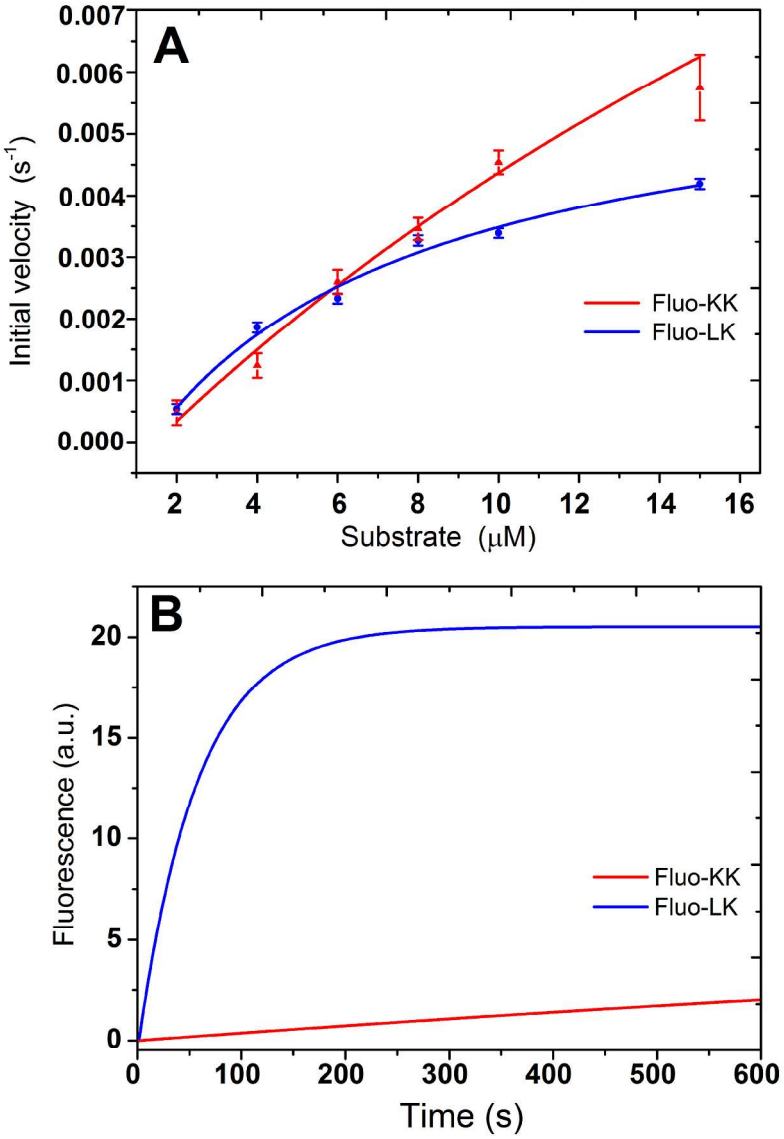


Fig. 1 (A) Kinetic parameters kcat and KM were obtained for the hydrolysis of different concentration of Fluo-LK (blue line) and Fluo-KK (red line) by MT1-MMP in 50 mM Tris/HCl (pH 7.4), 100 mM NaCl, 5 mM CaCl2 at 25°C. Data were fitted by the Michaelis-Menten equation. (B) Hydrolysis of Fluo-LK (blue line) and Fluo-KK (red line) by MT1-MMP. ([E0] = 0.35 μM in 50 mM Tris/HCl (pH 7.4), 100 mM NaCl, 5 mM CaCl2 at 25°C, [S] = 10 μM). First-order rate constants, kobs, were obtained by fitting eq. [1], second order rate constants kcat/KM were calculated from kobs/[E0] (see Table 1).
112x153mm (600 x 600 DPI)

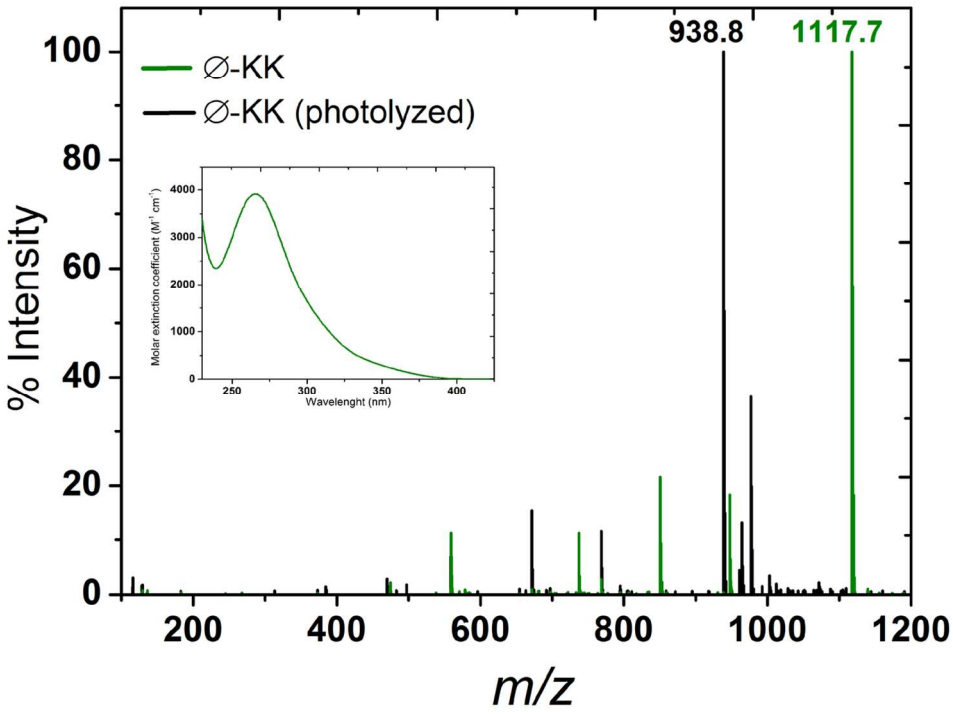
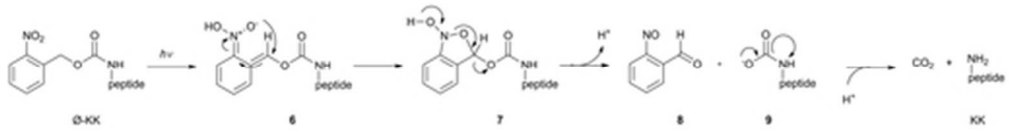


Fig. 3 ESI MS of Ø-KK before (green) and after photolysis (black). 1mg of Ø-KK was dissolved in 3 mL of 5 mM N-ethylmorpholine/acetic acid (pH 7.4). Photocleavage was performed through irradiation at 366 nm (8 W, ≈1.2 mW/m2) for 1 h. Ø-KK: m/z: obs. 1117.7, calc. 1117.6; KK: m/z: obs. 938.8, calc. 939.6. The inset shows the absorption spectrum of Ø-KK.
61x45mm (600 x 600 DPI)



Scheme 3 Reaction mechanism of the photolysis of Ø-KK.
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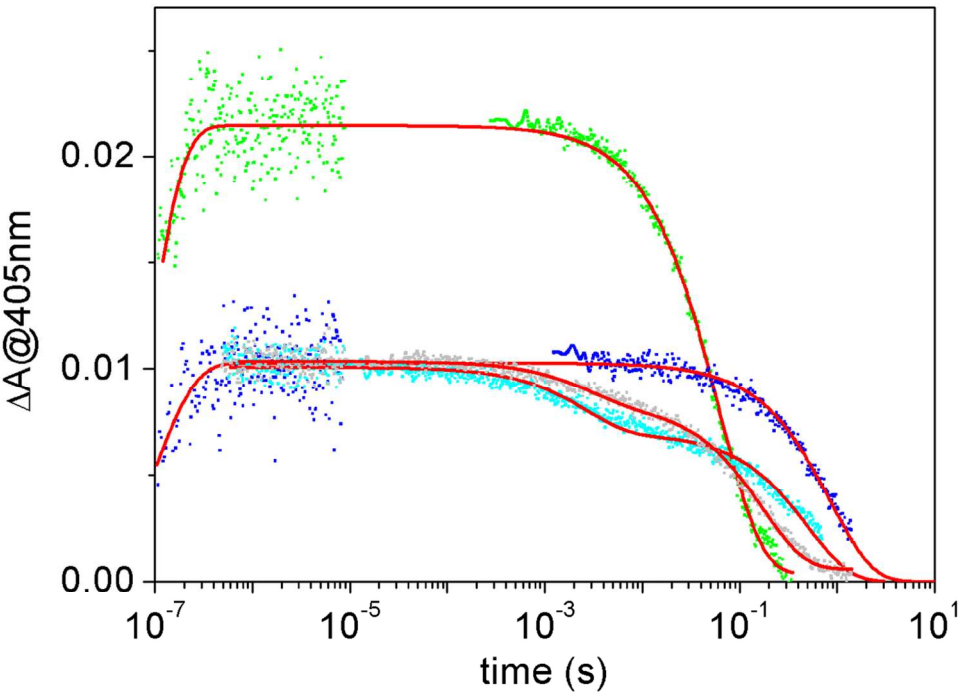


Fig. 4 Time resolved absorbance changes at 405 nm following exposure to 15 mJ laser shots for caged sulfate (green) and Ø-KK in 50 mM Tris/HCl (pH 7.41), 100 mM NaCl, 5 mM CaCl2 (blue) and in 10 mM MES/NaOH (pH 6.0) (grey). The cyan curve shows the aci-nitro transient absorbance for Ø-KK in nitrogen saturated water (pH 7.0). Red solid lines are the results of a fit to an exponential rise in the nanoseconds and a mono- or bi-exponential decay in the long milliseconds. For the cases of Ø-KK in nitrogen saturated water and in MES buffer it was not possible to observe the rise of the signal in the nanoseconds, due to intense scattered light. See text for fitting parameters.
59x42mm (600 x 600 DPI)

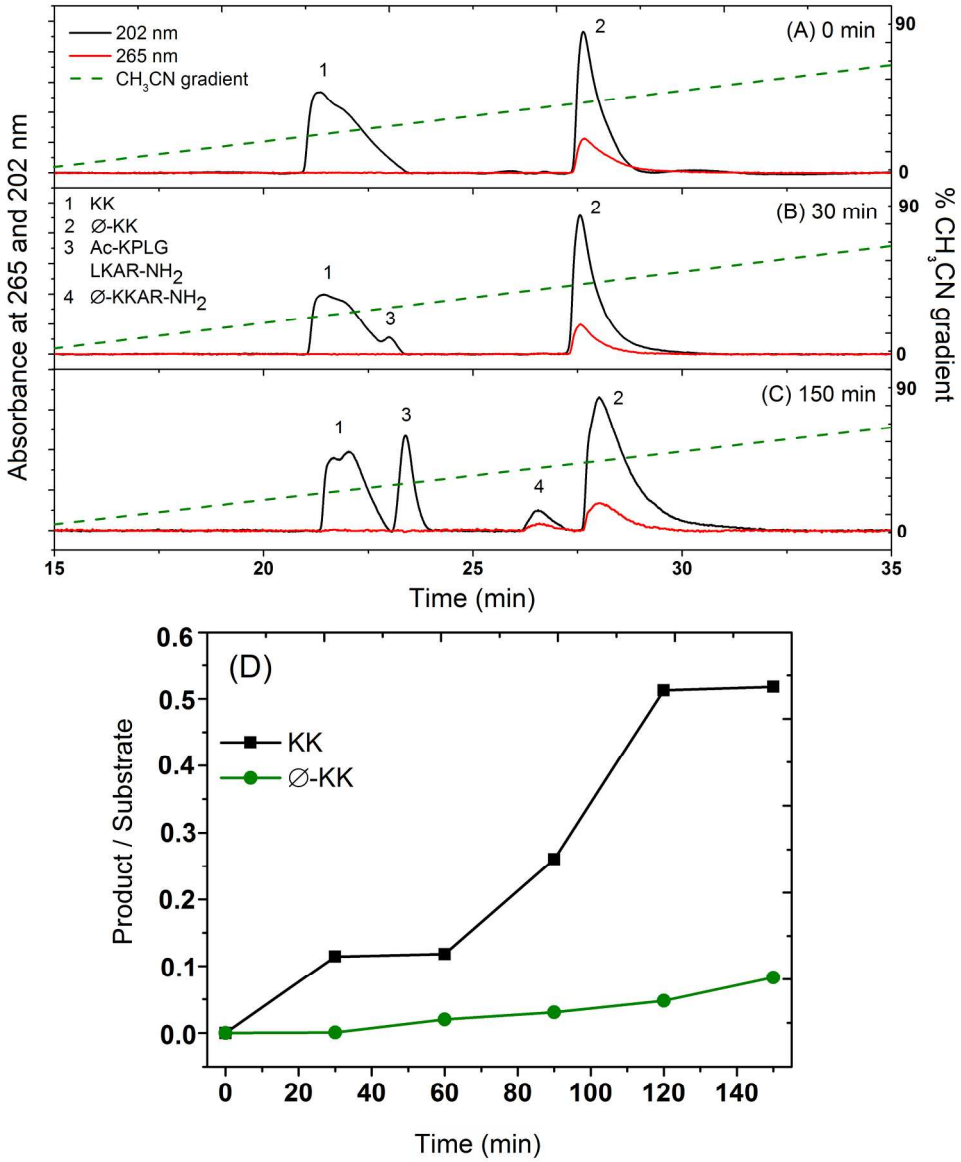
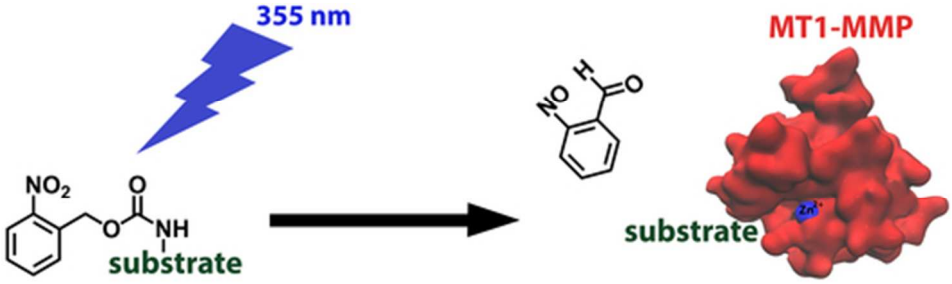


Fig. 5 HPLC profiles upon incubation of MT1-MMP with KK and Ø-KK (A) before addition of the enzyme, (B) after 30 min and (C) after 2.5 h. The peaks were assigned as follows: (1) KK, (2) Ø-KK, (3) KK fragment Ac–Lys–Pro–Leu–Gly or Leu–Lys–Ala–Arg–NH2, (4) Ø-KK fragment Lys(Ø)–Lys–Ala–Arg–NH2. (D): product-substrate ratio versus incubation time.
101x125mm (600 x 600 DPI)



Graphical Abstract
23x6mm (600 x 600 DPI)

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Elena Decaneto*, Stefania Abbruzzetti, Inge Heise, Wolfgang Lubitz, Cristiano Viappiani* and Markus Knipp

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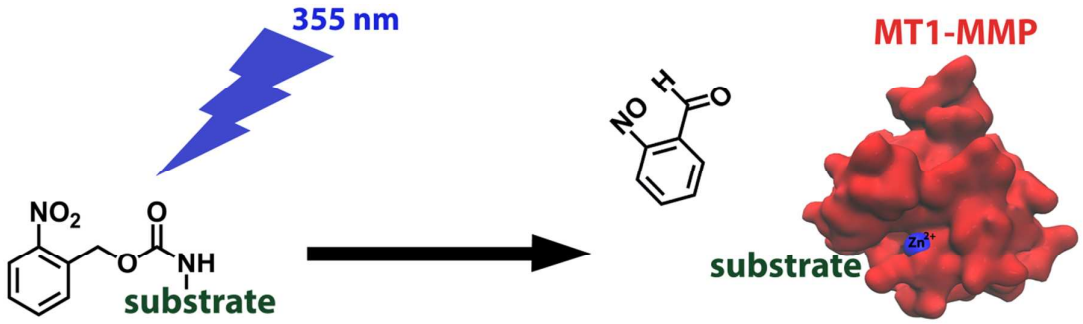

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