

## **SUPPORTING INFORMATION**

### **Coupling protein engineering with probe design to inhibit and image matrix metallo proteases with controlled specificity**

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## Supplementary methods

### Chemical Synthesis

All reagents were purchased and used without further purification. The LC-MS data were acquired using an Agilent HPLC in tandem with an API 150 mass spectrometer (Applied Biosystems/SCIEX) equipped with an electrospray interface. The HRMS data were acquired using the Micromass Q-ToF mass spectrometer. The NMR data were acquired using a 300 MHz or 500 MHz Varian Inova NMR spectrometer.

**Synthesis of (S)-4-benzyl-3-(4-methylpentanoyl)oxazolidin-2-one (1).** To the solution of s-4-benzyl-2-oxazolidnone (1 eq) in dry THF under argon at -78°C (using acetone dry ice bath) was added n-BuLi (1.1 eq) drop wise for 10 min and allowed to stirrer for 1 h. After stirring the reaction mixture at -78°C for 1 h, the methylvaleroyl chloride (1 eq) was added over 5 min and let stirred for 1 h. The reaction mixture was then moved out of ice bath and let stirred for another hour at RT. After confirming completion of reaction by TLC, saturated NH<sub>4</sub>Cl was added to the reaction mixture and it was concentrated to remove THF. The aqueous layer was then washed with DCM three times. The combined organic layer was dried over sodium sulphate and the crude product was then purified by flash column chromatography (eluent: 1:1 Hex:EtOAc) to afford pure product 1 as a colorless oil, yield 88.7%. <sup>1</sup>H NMR (300 MHz, Chloroform-d) δ 7.35 – 7.27 (m, 3H), 7.25 – 7.19 (m, 2H), 4.75 – 4.60 (m, 1H), 4.21 – 4.15 (m, 2H), 3.29 (dd, J = 13.4, 3.3 Hz, 1H), 2.95 (ddd, J = 10.2, 8.6, 6.5 Hz, 2H), 2.85 – 2.71 (m, 1H), 1.67 – 1.52 (m, 3H), 0.95 (d, J = 6.2 Hz, 6H); <sup>13</sup>C NMR (300 MHz, Chloroform-d) δ 173.41, 153.27, 135.20, 129.27, 128.75, 127.13, 65.96, 54.97, 37.71, 33.46, 32.94, 27.50, 22.23, 22.22.

**Synthesis of (R)-tert-butyl 3-((S)-4-benzyl-2-oxooxazolidine-3-carbonyl)-5-methylhexanoate (2).** LHDMS (1 eq) was cooled under argon at -78°C (using acetone/dry ice bath). To this compound, a solution of **1** (1 eq) in dry THF was added

drop wise for 10 min and allowed to stir for 1 h. Afterwards, the t-butyl bromoacetate (2.5 eq) was added slowly over 10 min. After the reaction mixture was stirred for 1 h and then allowed to warm at -40°C for another hour. The reaction mixture was then allowed to warm to room temperature and quenched with saturated solution of NH<sub>4</sub>Cl (50% reaction volume). The reaction mixture was concentrated to remove THF and the aqueous layer was then washed with DCM (3x). The combined organic layer was dried over sodium sulphate and the crude product was then purified by flash column chromatography (85:15 Hex:EtOAc) to afford pure product **2** as a white solid, yield 70%. <sup>1</sup>H NMR (300 MHz, Chloroform-d) δ 7.22 – 7.15 (m, 2H), 4.54 – 4.45 (m, 1H), 4.10 (ddt, J = 14.4, 9.5, 4.6 Hz, 1H), 4.05 – 3.96 (m, 2H), 3.19 (dd, J = 13.5, 3.3 Hz, 1H), 2.64 – 2.54 (m, 2H), 2.34 (dd, J = 16.6, 4.6 Hz, 1H), 1.50 – 1.34 (m, 2H), 1.28 (s, 9H), 1.24 – 1.14 (m, 1H), 0.81 – 0.76 (m, 6H), 7.15 – 7.10 (m, 3H); <sup>13</sup>C NMR (300 MHz, Chloroform-d) δ 176.66, 171.61, 153.16, 136.03, 129.76, 129.15, 127.42, 80.92, 66.09, 55.88, 41.16, 37.79, 37.41, 28.32, 25.99, 23.63, 21.95.

**Synthesis of (R)-2-(2-(tert-butoxy)-2-oxoethyl)-4-methylpentanoic acid (3).** To a solution of **2** (1 eq) in THF:Water (4:1) at 0°C was added 30% H<sub>2</sub>O<sub>2</sub> (3.6 eq) and reaction stirred for 5 min. After LiOH (3.3 eq) (pre-dissolved in a small amount of water) was added to the reaction mixture and then stirred for 2 h. The reaction was then cooled at 0°C and NaNO<sub>2</sub> (3 eq) was added. The reaction mixture was then allowed to warm back up at room temperature for 20 min and concentrated to remove THF. After the addition of 1 M NaOH, the reaction mixture was washed with DCM (2x). The combined aqueous layers were acidified using 1 M HCl until pH 6 and extracted with EtOAc (3x), dried over sodium sulphate and concentrated under vacuum to afford pure product **3** as a slight yellow oil, yield 87%. <sup>1</sup>H NMR (500 MHz, Methanol-d<sub>4</sub>) δ 2.81 – 2.75 (m, 1H), 2.51 (dd, J = 16.2, 9.4 Hz, 1H), 2.36 (dd, J = 16.2, 5.3 Hz, 1H), 1.68 –

1.50 (m, 2H), 1.44 (s, 9H), 1.32 – 1.25 (m, 1H), 0.93 (dd,  $J = 17.4, 6.6$  Hz, 6H);  $^{13}\text{C}$  NMR (500 MHz, Methanol- $d_4$ )  $\delta$  178.97, 172.96, 81.87, 42.37, 40.99, 38.89, 28.27, 26.95, 23.03, 22.65.

**Synthesis of 3-azidopropan-1-amine (4).** To a solution of bromopropylamine (1 eq) in water LiN<sub>3</sub> (3 eq) was added and the reaction mixture was stirred at RT for 10 min. Afterwards, it was heated at 80°C under reflux for 12 h. Then, the reaction mixture was brought at room temperature and 2/3 of water was evaporated under vacuum. The residue was diluted with ether and the resulting biphasic mixture was cooled down to 0°C. KOH pellets were added and the aqueous layer was extracted with Ether (2x). All organic layers were combined and dried over Na<sub>2</sub>SO<sub>4</sub> evaporation of solvent to give product **4** as color less oil, yield 81%.  $^1\text{H}$  NMR (500 MHz, Chloroform- $d$ )  $\delta$  4.86 (s, 1H), 3.38 (t,  $J = 6.7$  Hz, 2H), 2.81 (t,  $J = 6.9$  Hz, 2H), 1.78  $\delta$  1.70 (m, 2H);  $^{13}\text{C}$  NMR (500 MHz, CDCl<sub>3</sub>)  $\delta$  48.99, 39.15, 32.23.

**Synthesis of N-(3-azidopropyl)-2-chloroacetamide (5).** A solution of azidopropylamine (**4**) (1 eq) in dry DCM was cooled down at 0°C under argon atmosphere. To the above, triethylamine (2 eq) was added followed by dropwise addition of chloroacetic acid anhydride (2.5 eq) for 10 min. Then the reaction mixture was allowed to warm to room temperature and was stirred overnight. After completion of reaction, a sodium bicarbonate solution was added to the reaction mixture and the resulting solution was extracted with DCM (2x). The combined organic layers were dried over Na<sub>2</sub>SO<sub>4</sub> and evaporation of the solvent gave a crude product, which was then purified by flash column chromatography (1-5% MeOH in DCM) to afford **5** as colorless oil, yield 75%.  $^1\text{H}$  NMR (500 MHz, Chloroform- $d$ )  $\delta$  4.08 (s, 2H), 3.47  $\delta$  3.38 (m, 4H), 1.85 (m,  $J = 6.6$  Hz, 2H);  $^{13}\text{C}$  NMR (500 MHz, Chloroform- $d$ )  $\delta$  166.24, 49.25, 42.57, 37.57, 28.43.

**Synthesis of (9H-fluoren-9-yl) methyl (5,6-diamino-6-oxohexyl)carbamate (6).** This

compound was synthesized using a Rink resin (Advanced Chemtech) following standard solid phase peptide synthesis methods. The resin was deprotected with 20% piperidine solution and loaded with Boc-Lys(Fmoc)-OH (2 eq), Hydroxybenzotriazole (HOBT; 2 eq) and diisopropylcarbodiimide (DIC; 2 eq.) dissolved in anhydrous DMF for 4 h. Then, the resin was washed with DCM and DMF and the product was cleaved from resin by addition of 95% TFA and 5% DCM. The solution was concentrated under vacuum to afford the product **6** as a colorless solid. The crude product was purified by recrystallization using Ether/Hexane, yield 63%. <sup>1</sup>H NMR (500 MHz, Methanol-d<sub>4</sub>) δ 7.82 (d, J = 7.6 Hz, 2H), 7.66 (d, J = 7.7 Hz, 2H), 7.41 (t, J = 7.5 Hz, 2H), 7.33 (t, J = 7.5 Hz, 2H), 4.38 (d, J = 6.7 Hz, 2H), 4.22 (t, J = 6.7 Hz, 1H), 3.86 (t, J = 6.6 Hz, 1H), 3.15 (t, J = 7.0 Hz, 2H), 1.97 – 1.80 (m, 2H), 1.57 (p, J = 7.4 Hz, 2H), 1.45 (p, J = 14.8, 7.5 Hz, 2H); [M+H]<sup>+</sup> calcd. 368.4; found 368.2.

**Synthesis of 7.** To a solution of **6** (1 eq) in dry DMF under argon was added N-BOC-Propargylglycine (1 eq), Hydroxybenzotriazole (HOBT; 1 eq) and N-Ethyl-N'-(3-dimethylaminopropyl)carbodiimide HCl, (EDC; 1.2 eq.). The reaction mixture was stirred over night at room temperature and the solvent was evaporated under vacuum. The crude product mixture was purified by flash column chromatography (2-10 % of MeOH in DCM) to afford **7** as a colorless solid, yield 62 %. <sup>1</sup>H NMR (300 MHz, DMSO-d<sub>6</sub>) δ 7.89 (d, 2H), 7.69 (dd, J = 7.5 Hz, 2H), 7.42 (td, 2H), 7.34 (td, J = 7.4, 1.3 Hz, 2H), 4.29 (d, J = 7.5 Hz, 2H), 4.26 – 4.11 (m, 2H), 4.13 – 4.05 (m, 1H), 2.94 (q, J = 6.5 Hz, 2H), 2.85 – 2.82 (m, 1H), 2.63 – 2.53 (m, 1H), 2.43 (ddd, J = 16.7, 8.8, 2.6 Hz, 1H), 1.72 – 1.57 (m, 1H), 1.57 – 1.46 (m, 1H), 1.39 (s, 9H), 1.31 – 1.18 (m, 2H); [M+H]<sup>+</sup> calcd. 563.6; found 563.0.

**Synthesis of (9H-fluoren-9-yl)methyl (6-amino-5-(2-aminopent-4-ynamido)-6-oxohexyl)carbamate (8).** The Boc group was removed by incubation with 30%

TFA/DCM(v/v) for 3hrs. Then the solvent was evaporated under vacuum and the product was co-evaporated with toluene to afford **8** as a colorless solid, yield, 98%. <sup>1</sup>H NMR (500 MHz, Methanol-d<sub>4</sub>) δ 7.78 (d, J = 7.5 Hz, 2H), 7.63 (d, J = 7.5 Hz, 2H), 7.41 – 7.35 (m, 2H), 7.33 – 7.27 (m, 2H), 4.34 (d, J = 6.8 Hz, 2H), 4.18 (t, J = 6.7 Hz, 1H), 3.87 – 3.72 (m, 2H), 3.12 (t, J = 6.9 Hz, 1H), 1.96 – 1.76 (m, 2H), 1.54 (p, J = 7.1 Hz, 2H), 1.43 (qd, J = 9.2, 8.7, 6.2 Hz, 2H); <sup>13</sup>C NMR (500 MHz, Methanol-d<sub>4</sub>) δ 216.63, 171.24, 163.70, 157.85, 156.51, 144.14, 141.44, 126.98, 124.96, 119.80, 66.48, 47.33 (d, J = 7.3 Hz), 40.04, 35.81, 31.60, 31.03, 30.50, 29.36, 21.84. [M+H]<sup>+</sup> calcd. 463.5; found 462.4.

**Synthesis of tert-butyl 9-carbamoyl-1-(9H-fluoren-9-yl)-15-isobutyl-3,11,14-trioxo-12-(prop-2-yn-1-yl)-2-oxa-4,10,13-triazaheptadecan-17-oate (9).** To a solution of **8** (1 eq) in dry DMF under argon was added **3** (1 eq), Hydroxybenzotriazole (HOBT; 1 eq) and N-Ethyl-N'-(3-dimethylaminopropyl)carbodiimide HCl, (EDC; 1.2 eq.). The reaction mixture was stirred over night at room temperature and the solvent was evaporated under vacuum. The crude product mixture was then purified by flash column chromatography (2-10 % of MeOH in DCM) to afford **9** as a colorless solid, yield 53 %. <sup>1</sup>H NMR (500 MHz, Methanol-d<sub>4</sub>) δ 7.82 – 7.77 (d, 2H), 7.65 (d, J = 7.5 Hz, 2H), 7.38 (t, J = 7.4 Hz, 2H), 7.30 (t, J = 7.5 Hz, 2H), 4.46 – 4.42 (m, 1H), 4.37 – 4.29 (m, 3H), 4.19 (t, J = 7.1 Hz, 1H), 3.11 (m, J = 11.4, 6.4 Hz, 2H), 2.83 – 2.62 (m, 4H), 2.59 – 2.29 (m, 4H), 1.91 – 1.82 (m, 1H), 1.73 – 1.65 (m, 1H), 1.60 – 1.47 (m, 4H), 1.42 (s, 9H), 1.24 – 1.18 (m, 1H), 0.90 (dd, J = 27.3, 6.2 Hz, 6H). [M+H]<sup>+</sup> calcd. 675.8; found 675.0.

**Synthesis of 9-carbamoyl-1-(9H-fluoren-9-yl)-15-isobutyl-3,11,14-trioxo-12-(prop-2-yn-1-yl)-2-oxa-4,10,13-triazaheptadecan-17-oic acid (10).** Boc group was removed by incubation with 30% TFA/DCM (v/v) for 3 h. Then the solvent was evaporated under vacuum and the product was co-evaporated with toluene to afford **10** as a colorless

solid, yield, 95%.  $^1\text{H}$  NMR (500 MHz, Methanol- $d_4$ )  $\delta$  4.39 – 4.27 (m, 2H), 3.21 – 3.15 (m, 1H), 2.94 – 2.90 (m, 3H), 2.79 (ddt,  $J$  = 10.4, 6.7, 4.2 Hz, 2H), 2.44 – 2.39 (m, 1H), 2.22 (dd,  $J$  = 14.7, 5.4 Hz, 1H), 1.91 – 1.84 (m, 1H), 1.81 – 1.73 (m, 1H), 1.72 – 1.59 (m, 5H), 1.26 – 1.20 (m, 2H), 0.92 (dd,  $J$  = 21.9, 6.4 Hz, 6H).  $[\text{M}+\text{H}]^+$  calcd. 619.7; found 619.6.

**Synthesis of *N1-((1,6-diamino-1-oxohexan-2-yl)amino)-1-oxopent-4-yn-2-yl)-N4-hydroxy-2-isobutylsuccinamide (11)*.** It was synthesized using a Hydroxylamine Wang resin (Novabiochem) and following standard solid phase peptide synthesis methods. The resin was loaded with product **10** (1.1 eq), Hydroxybenzotriazole (HOBt; 1.1 eq) and diisopropylcarbodiimide (DIC; 1.1 eq.) dissolved in anhydrous DMF for 4 h. Then, the Fmoc protecting group was removed with 20% piperidine in DMF (v/v) for 20 min, followed by cleavage from Hydroxylamine Wang resin by addition of 95% TFA, 2.5% DCM and 2.5% water. The evaporation of the solvent under vacuum afforded the product **11** as colorless oil, yield 85.  $[\text{M}+\text{H}]^+$  calcd 412.5; found 411.7.

**Synthesis of *6-amino-2-(2-(2-(2-(aminooxy)-2-oxoethyl)-4-methylpentanamido)-3-(1-(3-(2-chloroacetamido)propyl)-1H-1,2,3-triazol-4-yl)propanamido)hexanamide (12)*.**

The alkyne precursor **11** (1 eq), product **5** (1.2 eq), copper (II) sulfate pentahydrate (0.05 eq) and sodium ascorbate (0.1 eq) were dissolved in 2:1 tBuOH:H<sub>2</sub>O. The resulting mixture was stirred at room temperature for 12 h and the solvent was removed under reduced pressure. The residue was purified using C18 reverse phase HPLC with a water/acetonitrile gradient, yield 19%.  $[\text{M}+\text{H}]^+$  calcd. 589.0; found 588.3.

**Synthesis of *TND124 (13)*.** The product **12** (1 eq), Cy5 succinimidyl ester (1 eq) and DIPEA (2 eq) were dissolved in DMSO. The reaction mixture was stirred and protected from light for 30 min. TND124 was purified using C18 reverse phase HPLC with a water/acetonitrile gradient, yield 48%.  $[\text{M}+\text{H}]^+$  calculated for  $\text{C}_{57}\text{H}_{81}\text{ClN}_{11}\text{O}_{13}\text{S}_2^+$

1227.9; found 1227.5.

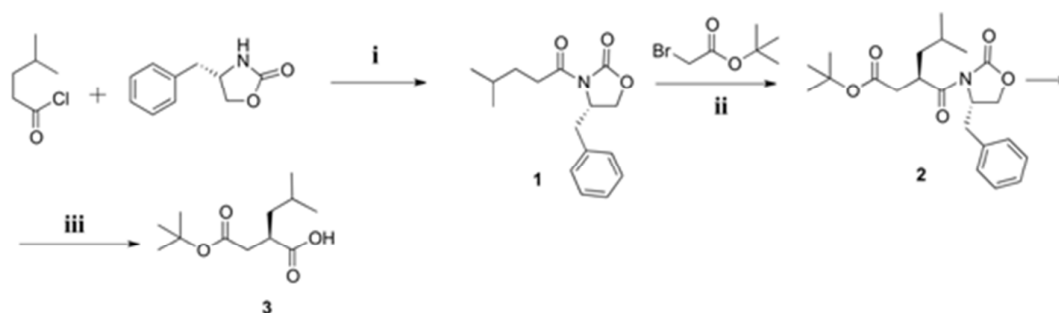
**Synthesis 2-(2-(2-acetamido-4-methylpentanamido)-3-(1-(3-(2-chloroacetamido)propyl)-1H-1,2,3-triazol-4-yl)propanamido)-6-aminohexanamide (14).** This compound was synthesized using a Rink resin (Advanced Chemtech) and following standard solid phase peptide synthesis methods. The resin was deprotected with 20% piperidine solution and loaded with Fmoc-Lys(Boc)-OH (3 eq), hydroxybenzotriazole (HOB T; 3 eq) and diisopropylcarbodiimide (DIC; 3 eq.) dissolved in anhydrous DMF for four hours. Then, the resin was washed with DCM and DMF and the amine group was deprotected by Fmoc cleavage using 20% piperidine in DMF. The same procedure was performed in the loading of Fmoc-Pra-OH and Fmoc-Leu-OH. After the Fmoc deprotection of the leucine, the acetylation of the amine is performed by the addition of 10eq of acetic anhydride and 12 eq of N,N-diisopropylethylamine in dry DCM for 1 h. After checking the completion of the reaction using Kaiser test, a click chemistry on resin is performed. The product **5** (2 eq) is dissolved in dry THF and loaded onto the resin. Afterwards, 2 eq of CuI and 25 eq of N,N-diisopropylethylamine (DIEA) are added. The reaction mixture is shaken overnight. Then, the resin is washed with DMF, DCM and a solution of 0.5% sodium diethyldithiocarbamate ( $\text{Et}_2\text{NCSSNa}$ ) and 0.5% DIEA in DMF in order to remove the copper. The next step is the cleavage of the product from the resin using 95% TFA, 2.5% DCM and 2.5% triisopropylsilane (TIS). TFA is evaporated under vacuum and the crude mixture is purified using C18 reverse phase HPLC with a water/acetonitrile gradient, yield 50%.  $[\text{M}+\text{H}]^+$  calcd 573.1; found 572.6.

**Synthesis TND126 (15).** The product **14** (1 eq), Cy5 succinimidyl ester (1 eq) and DIPEA (2 eq) were dissolved in DMSO. The reaction mixture was stirred and protected from light for 30 min. TND124 was purified using C18 reverse phase HPLC with a

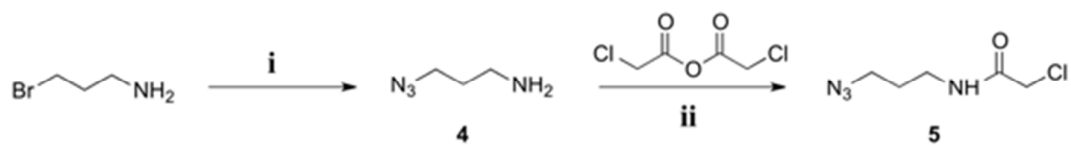


water/acetonitrile gradient, yield 70%.  $[M+H]^+$  calculated for  $C_{57}H_{81}ClN_{11}O_{12}S_2^+$   
1211.9; found 1211.7.

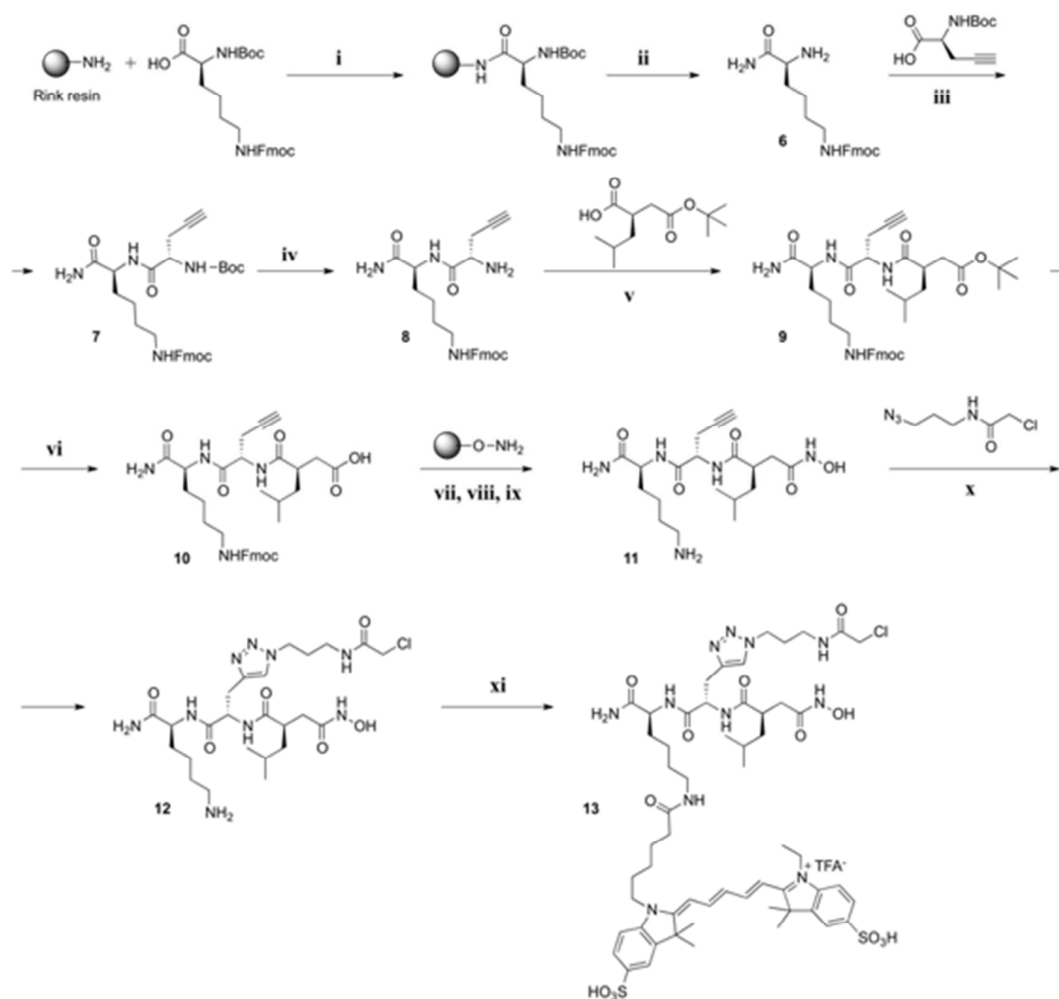
## Supplementary Synthetic Scheme



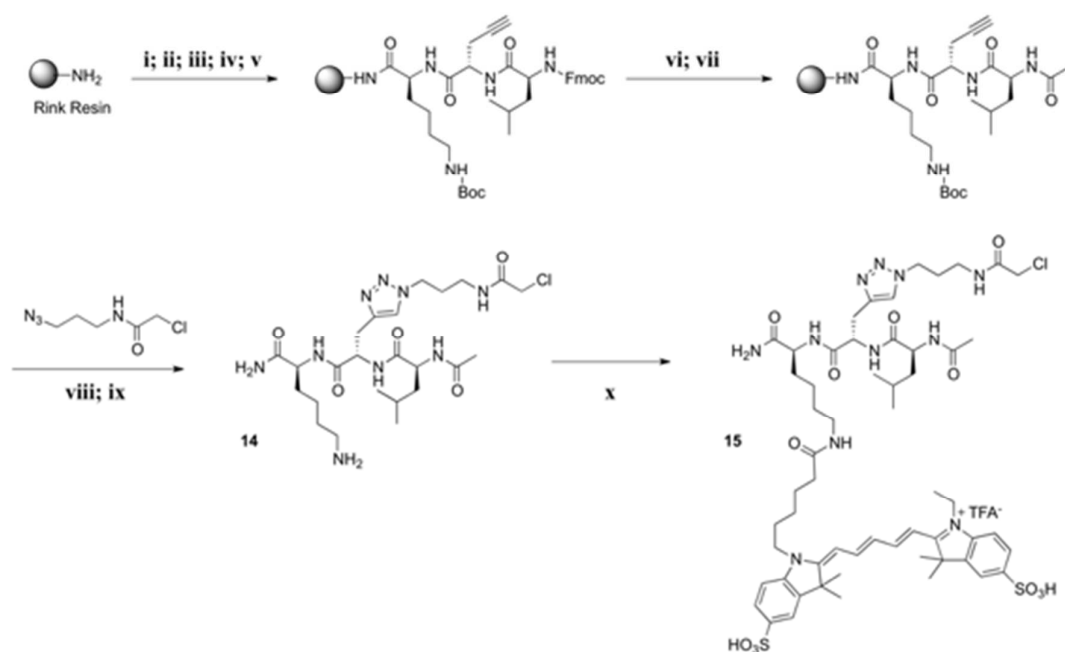
**Supplementary scheme 1.** Synthesis of (R)-2-(2-(tert-butoxy)-2-oxoethyl)-4-methylpentanoic acid. i) n-BuLi, THF, -78<sup>0</sup>C; ii) t-butyl bromoacetate, LHMDS, THF, -78<sup>0</sup>C; iii) LiOH, H<sub>2</sub>O<sub>2</sub>, 4:1 THF:H<sub>2</sub>O



**Supplementary scheme 2.** Synthesis of N-(3-azidopropyl)-2-chloroacetamide. i)  $\text{LiN}_3$ ,  $\text{H}_2\text{O}$ , reflux; ii) chloroacetic acid anhydride,  $\text{Et}_3\text{N}$ , DCM,  $0^\circ\text{C}$



**Supplementary scheme 3.** Synthesis of TND124. i) Rink resin, HOBT, DIC, DMF; ii) 95% TFA, DCM; iii) N-Boc-Propargylglycine, HOBT, EDC, DMF; iv) 30% TFA, DCM; v) (R)-2-(2-(tert-butoxy)-2-oxoethyl)-4-methylpentanoic acid, HOBT, EDC, DMF; vi) 30% TFA, DCM; vii) Hydroxylamine Wang resin, HOBT, DIC, DMF; viii) 20% piperidine in DMF; ix) 95% TFA in DCM; x) N-(3-azidopropyl)-2-chloroacetamide, CuSO<sub>4</sub>, ascorbic acid, 2:1 tBOH/H<sub>2</sub>O; xi) Cy5-NHS, DIPEA, DMF



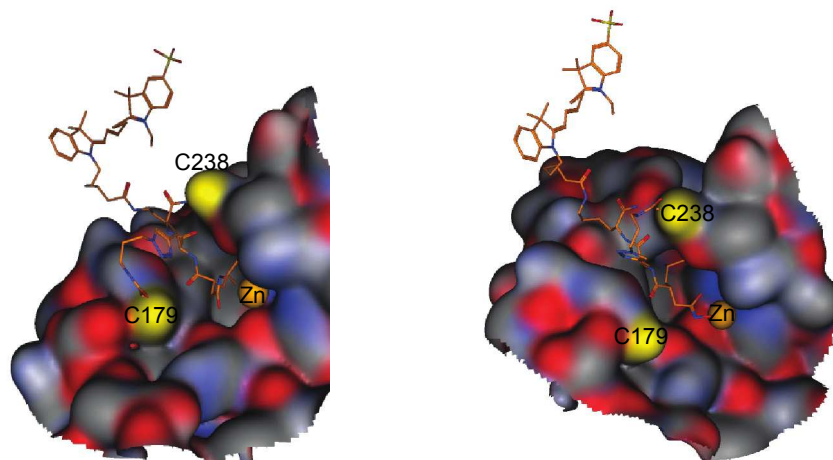
**Supplementary scheme 4.** Synthesis of TND126 . i) Fmoc-Lys(Boc)-OH, HOBT, DIC, DMF; ii) 20% piperidine, DMF; iii) Fmoc-Pra-OH, HOBT, DIC, DMF; iv) 20% piperidine, DMF; v) Fmoc-Leu-OH, HOBT, DIC, DMF; vi) 20% piperidine, DMF; vii) acetic anhydride, DIPEA, DMF; viii) N-(3-azidopropyl)-2-chloroacetamide, CuI, DIPEA, THF; ix) 95% TFA in DCM; x) Cy5-NHS, DIPEA, DMF

## Supplementary table

**Table S1.** List of primers used for plasmid construction

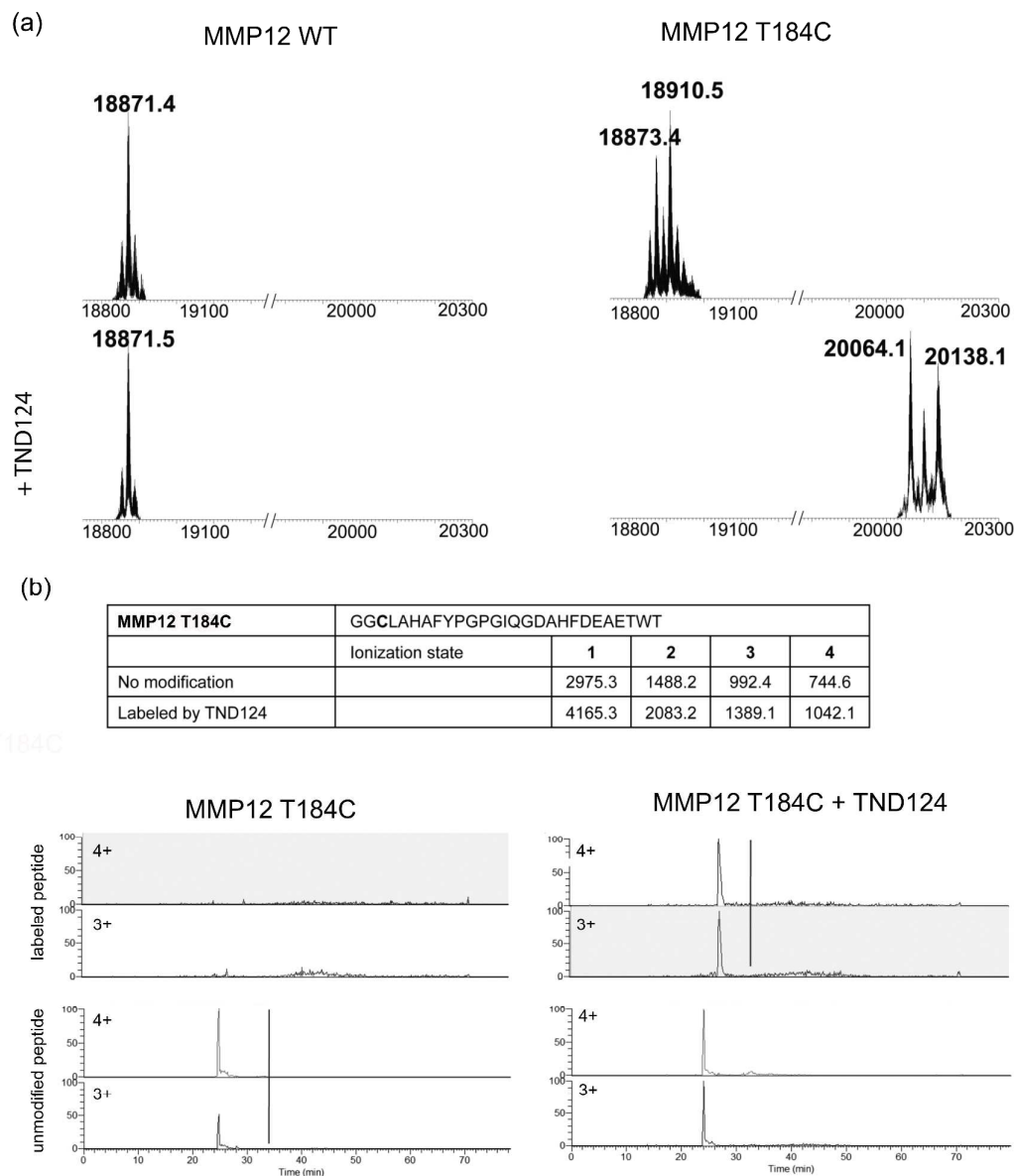
	Sequence (5' to 3')
MMP12-5'	CTTCCATATGGCTCCCATG
MMP12-3'	CTTCCATATGGCCTCCCTC
MMP12-T184C-5'	AAAAGCATGGGCTAGACAACCACCTTTGCCATC
MMP12-T184C-3'	GATGGCAAAGGTGGTTGTCTAGCCCATGCTTTT
MMP12-T243C-5'	GGGTAAAGGTATCTGTAGCAGGGGTACATTATTGAC
MMP12-T243C-3'	GTCAATAATGTACCCCTGCTACAGATACCTTAACCC
MT1-MMPcat-5'	CTTCCATATGGCCCAAGGCAGCAACTTC
MT1-MMPcat-3'	ACGGAGCTCGATGTTGGGCCCATAG
MT1-MMPcat-F198C5'	GGTGAAGGAGGGTGCCTGGCTCATGCC
MT1-MMPcat-F198C3'	GGCATGAGCCAGGCACCCTCCTTCACC
MT1-MMPcat-F260C5'	TGGCCCCCTGCTACCAGTGGATGG
MT1-MMPcat-F260C3'	CCATCCACTGGTAGCAGGGGGCCA
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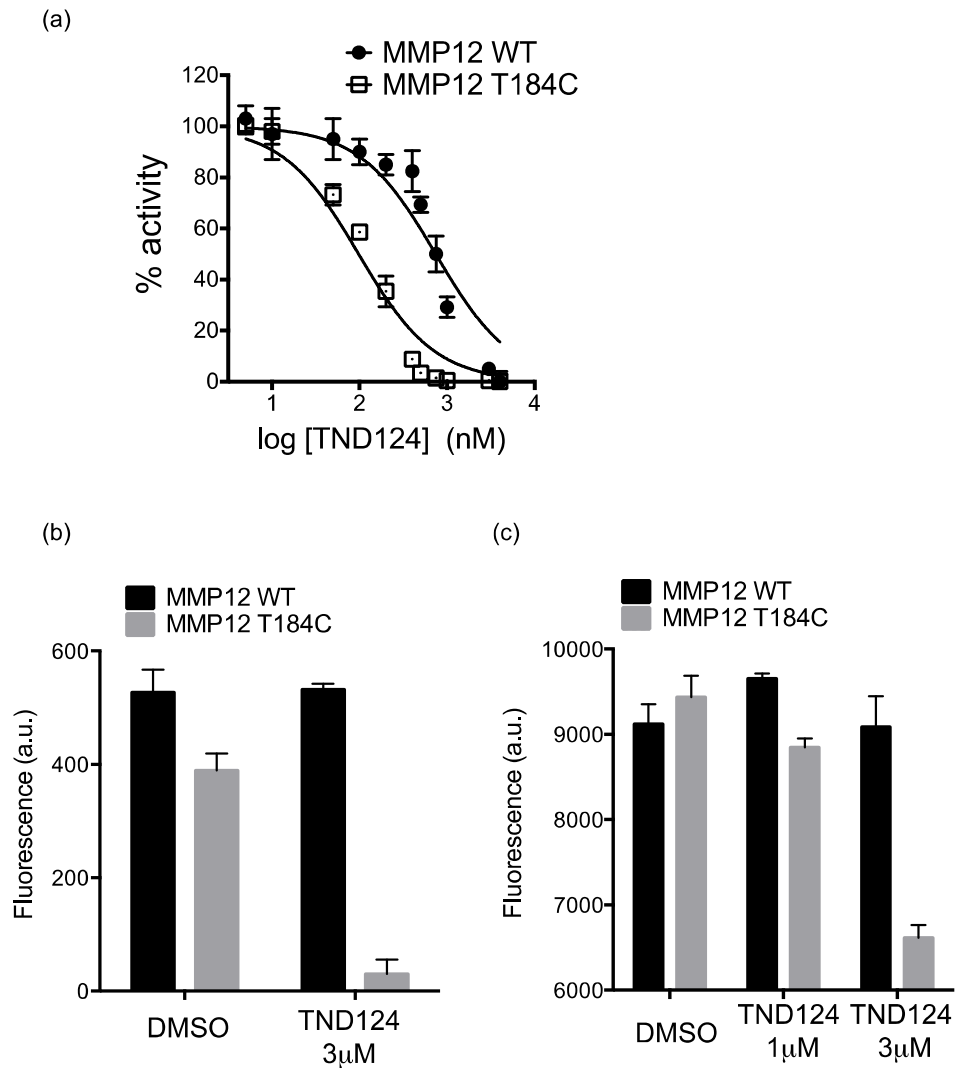


**Figure S2.** Docking of TND124 into the active site of MMP8. We used MMP8 because of the availability of high-resolution crystal structures of the enzyme bound to a hydroxamate inhibitor (PDB 1A85). For this docking we covalently attached the electrophile to either C179 (left) or C238 (right) residues and then used energy minimization to determine if the probe could still access the active site zinc atom.

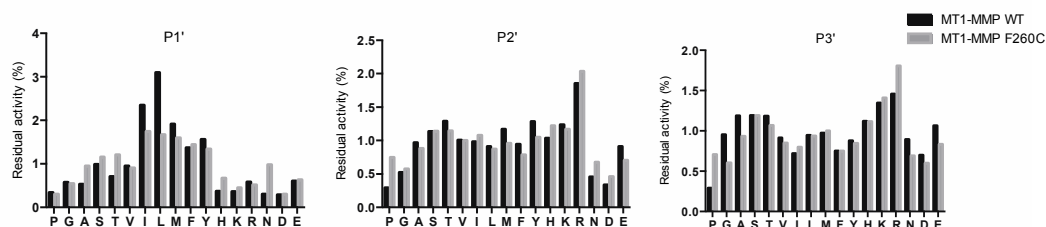




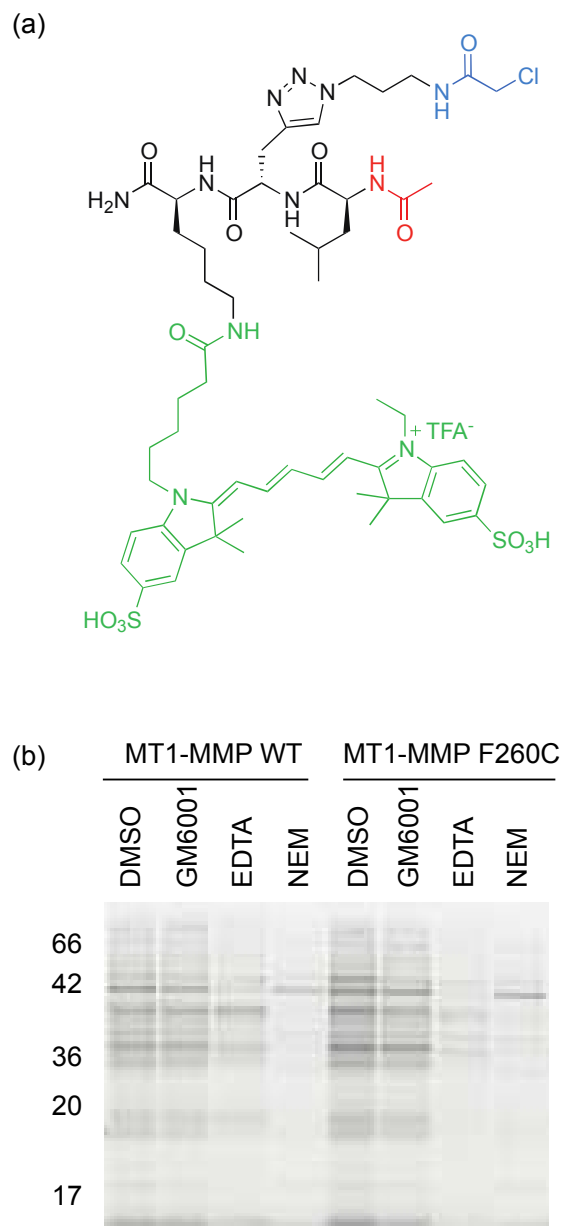
**Figure S3.** Confirmation of specific covalent probe labeling of MMP12. (a) Intact mass spectrometry analysis of the WT or T184C MMP12 alone or incubated with the probe. Labeling by the probe results in a shift in the mass by 1193amu. (b) T184C MMP12 was incubated with DMSO or TND124 and digested with trypsin and peptides were analyzed by LCMS. The +3 and +4 charged peptide containing the engineered cysteine was detected in the unmodified form in both the DMSO and TND124 treated samples (bottom spectra) and was detected as the probe labeled peptide (mass increase of 1193 amu) in the sample treated with TND124 (top spectra).



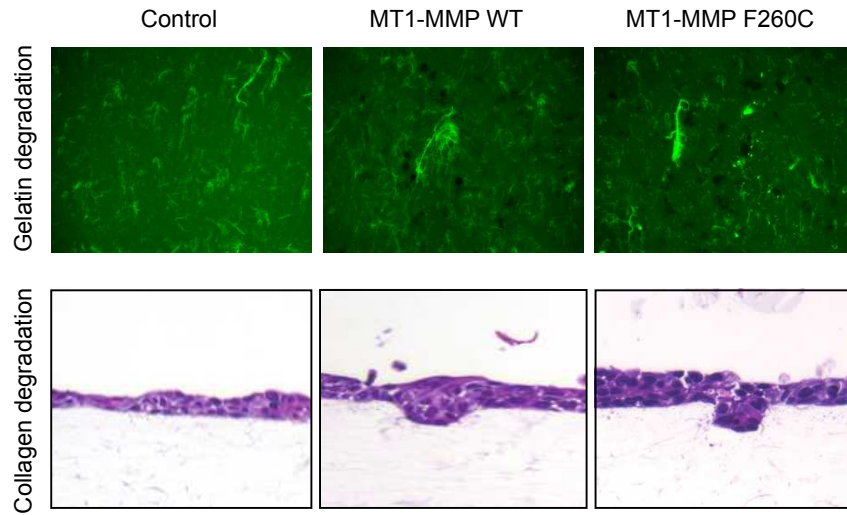
**Figure S4.** Specific inhibition of the mutant MMPs by TND124. (a) WT or T184C MMP12 was incubated with TND124 at the indicated concentrations and hydrolysis activity of the enzyme was measured over time using the substrate Mca - PLGL-Dpa-AR-NH<sub>2</sub> (where MCA is the fluorophore (7-methoxycoumarin-4-yl)acetate and Dnp is the quencher dinitrophenyl). Data is plotted as percent activity relative to enzyme incubated with DMSO. (b) Recombinant WT or T184C MMP12 catalytic domains were pre-incubated with DMSO or TND124 for 30 minutes at the indicated concentration and the samples were diluted 10 fold into a solution containing FAM conjugated collagen IV. Substrate processing was determined by centrifugation to pellet the unprocessed substrate followed by measuring FAM fragments in the supernatant after 48 hr incubation. (c) Recombinant WT or T184C MMP-12 enzymes were pre-incubated with DMSO or TND124 for 30 minutes at the indicated concentrations and activity against FITC conjugated gelatin was measured as described in (b).



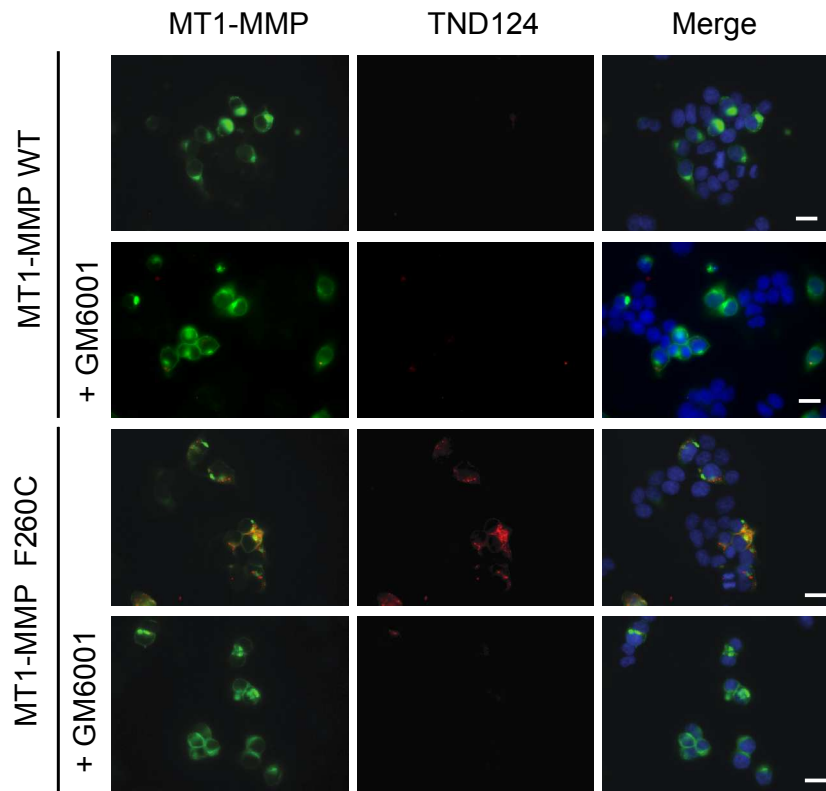
**Figure S5.** Profiling substrate specificity of WT and F260C MT1-MMP using libraries of peptide substrates. A library of synthetic peptides containing the sequence Ac-X-X-X-X-X-X-X-X-[D-Lys]-[D-Lys] (where X is an equimolar mixture of the 19 amino acids excluding Cys) was digested by addition of either WT or F260C MT1-MMP. After completion of the cleavage reaction, the pool of resulting peptides was subjected to N-terminal sequencing. The abundance of each amino acid found at the P1', P2' and P3' positions directly C-terminal to the newly formed N-termini is shown for both proteases. Values are plotted as selectivity values in which the data is normalized as described in the methods section so that the value for average representation is 1.0



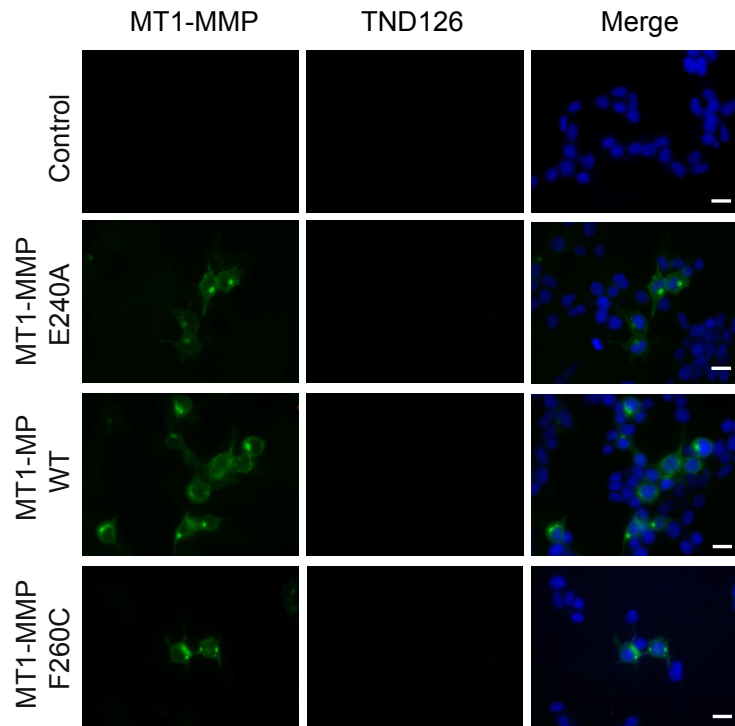
**Figure S6.** The control probe TND126 does not label the WT or engineered MT1-MMP. (a) Structure of the control probe TND126. The electrophile (chloromethyl amide) is shown in blue; the control for the electrophile in red and the fluorescent tag (Cy5) in green. (b) WT or F260C MT1-MMP was added to rat liver lysates. The general MMP inhibitor GM6001 (10 $\mu$ M), EDTA (10mM), NEM (1mM) were added prior to labeling with TND126 (1 $\mu$ M) for 1 hour. Samples were analyzed by SDS-PAGE followed by scanning of the gel with a flatbed laser scanner. The predicted location of the labeled catalytic domain is shown with an arrowhead.



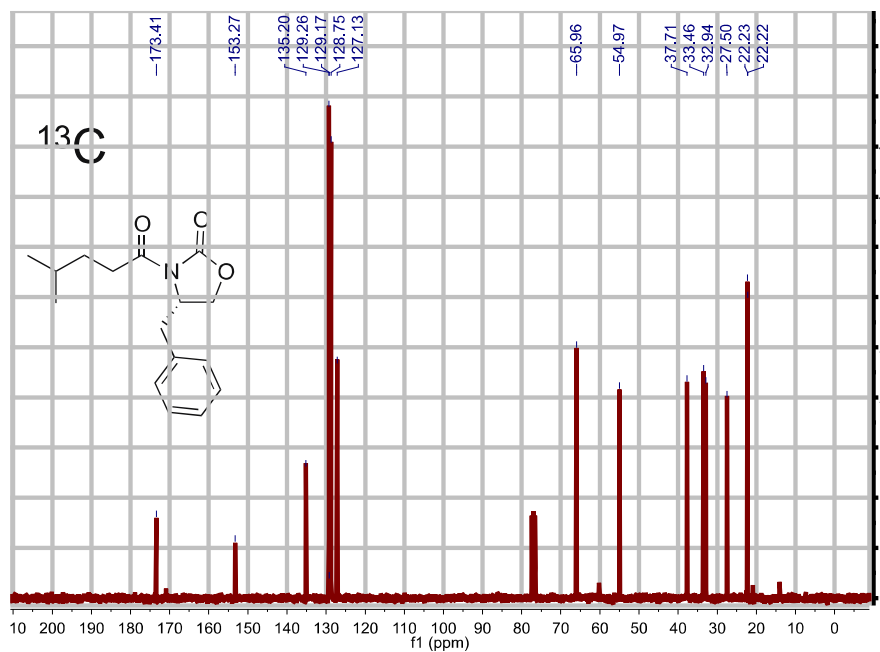
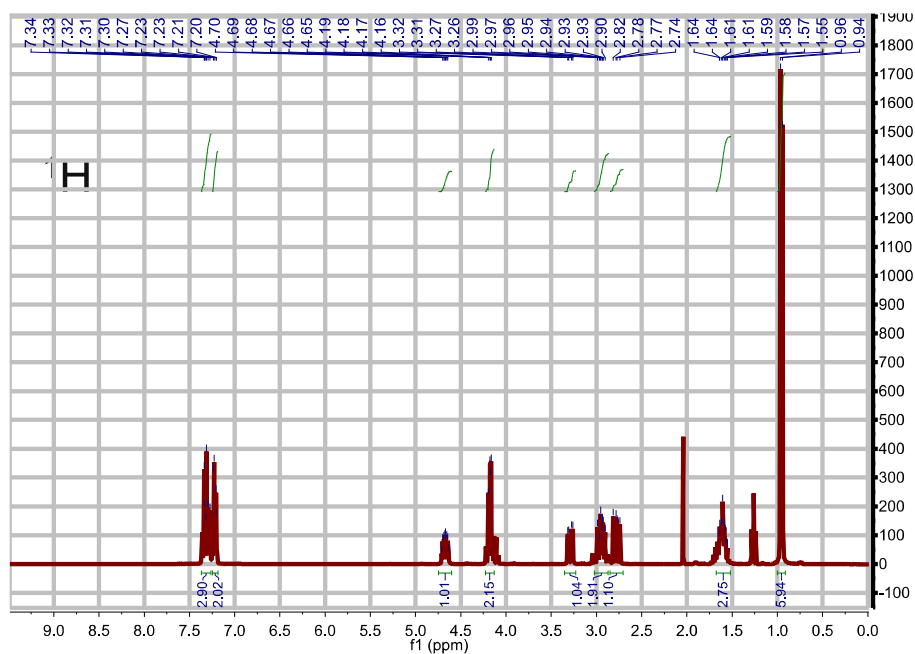
**Figure S7.** The activity of the full length F260C MT1-MMP was confirmed by gelatin or collagen invasion assays. MCF7 cells transiently transfected with empty plasmid (control), WT or F260C MT1-MMP were plated on fluorescent-labeled gelatin or collagen film. Only the cells expressing the WT or the F260C mutant could degrade both substrates.



**Figure S8.** Specificity of TND124 labeling in live cells. Microscopy images of HEK293T cells transiently transfected with HA tagged WT or F260C mutant MT1-MMP that were pre-incubated with GM6001 (10 $\mu$ M) or DMSO and then labeled with TND124 (1.5 $\mu$ M; red). Cells were washed, fixed and stained with DAPI (blue) and an antibody against HA tag (green). Scale bar represents 20 $\mu$ m.



**Figure S9.** Labeling of cells with the control probe TND126. HEK293T were transiently transfected with an empty plasmid (control), full length WT, E240A or F260C MT1-MMP (all of them tagged with an HA tag) and incubated with TND126 (1.5 $\mu$ M, red). Cells were washed, fixed and stained with DAPI and an antibody against the HA tag (green). Scale bar represents 20 $\mu$ m.

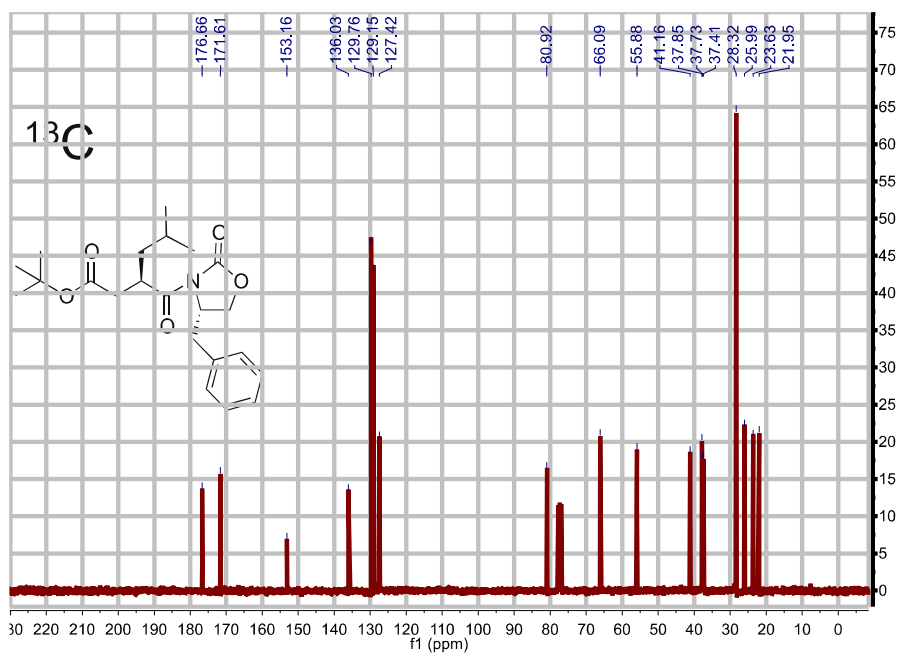
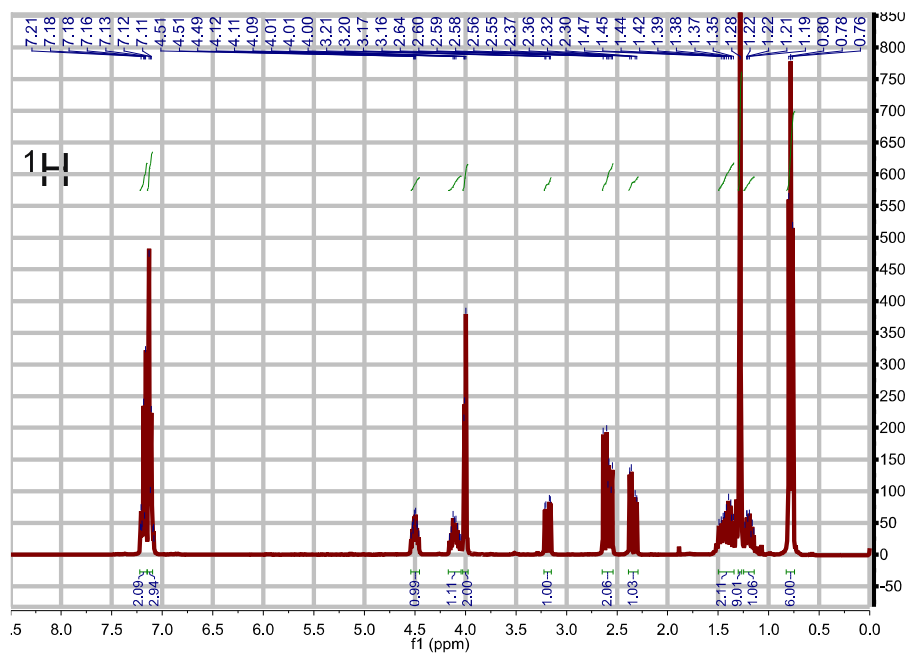


**<sup>1</sup>H and <sup>13</sup>C NMR spectra of (S)-4-benzyl-3-(4-methylpentanoyl)oxazolidin-2-one**

<sup>1</sup>H NMR (300 MHz, Chloroform-d) δ 7.35 – 7.27 (m, 3H), 7.25 – 7.19 (m, 2H), 4.75 – 4.60 (m, 1H), 4.21 – 4.15 (m, 2H), 3.29 (dd, J = 13.4, 3.3 Hz, 1H), 2.95 (ddd, J = 10.2, 8.6, 6.5 Hz, 2H), 2.85 – 2.71 (m, 1H), 1.67 – 1.52 (m, 3H), 0.95 (d, J = 6.2 Hz, 6H).

<sup>13</sup>C NMR (75 MHz, Chloroform-d) δ 173.41, 153.27, 135.20, 129.27, 128.75, 127.13, 65.96, 54.97, 37.71, 33.46, 32.94, 27.50, 22.23, 22.22

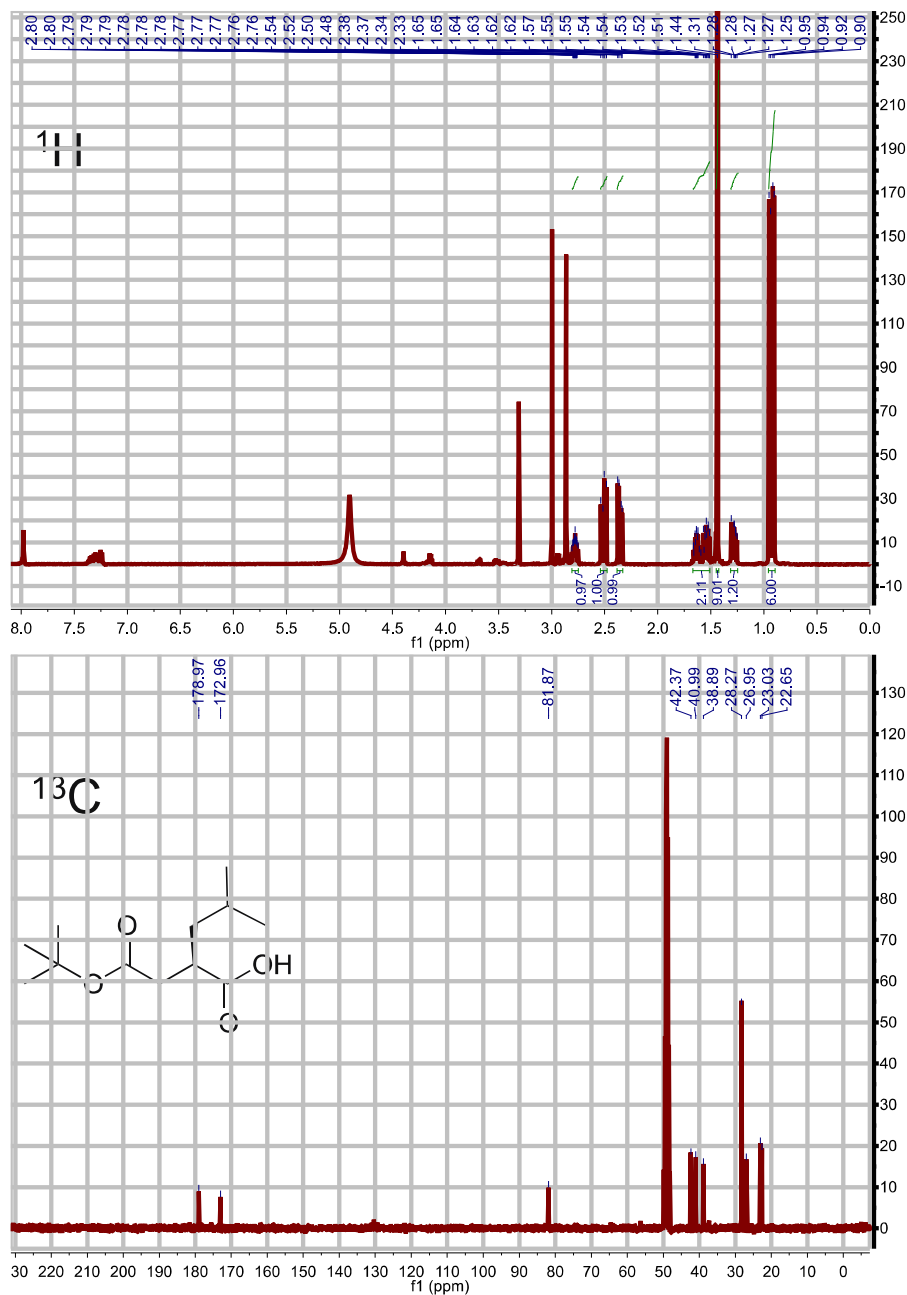




**<sup>1</sup>H and <sup>13</sup>C NMR spectra of (R)-tert-butyl 3-((S)-4-benzyl-2-oxooxazolidine-3-carbonyl)-5-methylhexanoate**

<sup>1</sup>H NMR (300 MHz, Chloroform-d) δ 7.22 – 7.15 (m, 2H), 4.54 – 4.45 (m, 1H), 4.10 (ddt, J = 14.4, 9.5, 4.6 Hz, 1H), 4.05 – 3.96 (m, 2H), 3.19 (dd, J = 13.5, 3.3 Hz, 1H), 2.64 – 2.54 (m, 2H), 2.34 (dd, J = 16.6, 4.6 Hz, 1H), 1.50 – 1.34 (m, 2H), 1.28 (s, 9H), 1.24 – 1.14 (m, 1H), 0.81 – 0.76 (m, 6H), 7.15 – 7.10 (m, 3H).

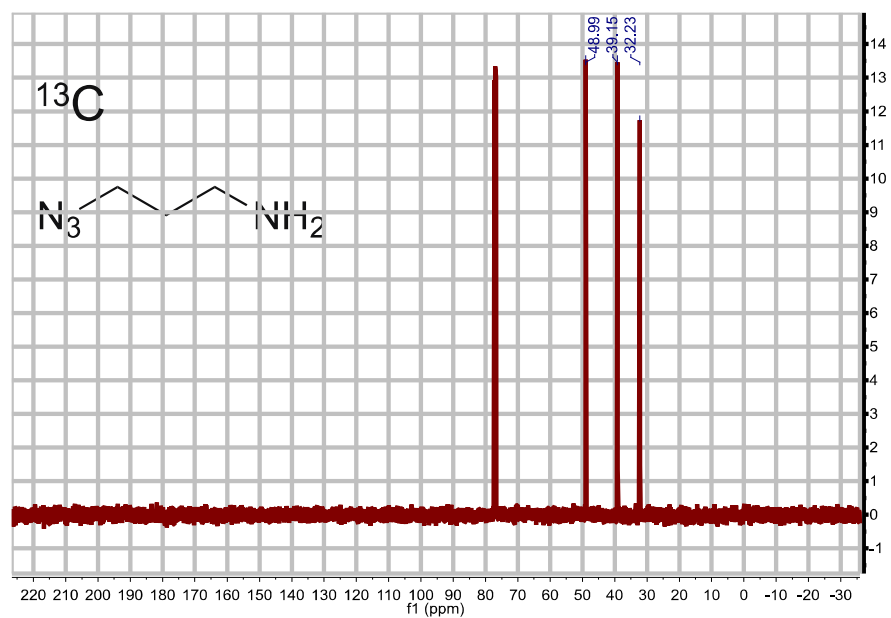
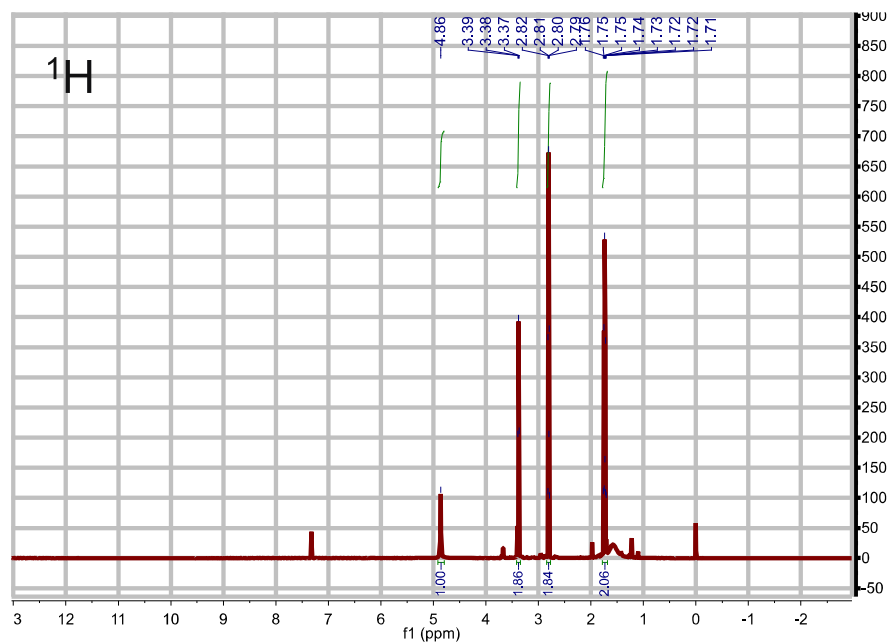
<sup>13</sup>C NMR (75 MHz, Chloroform-d) δ 176.66, 171.61, 153.16, 136.03, 129.76, 129.15, 127.42, 80.92, 66.09, 55.88, 41.16, 37.79, 37.41, 28.32, 25.99, 23.63, 21.95.



**<sup>1</sup>H and <sup>13</sup>C NMR spectra of (R)-2-(2-(tert-butoxy)-2-oxoethyl)-4-methylpentanoic acid**

<sup>1</sup>H NMR (500 MHz, Methanol-d<sub>4</sub>) δ 2.81 – 2.75 (m, 1H), 2.51 (dd, J = 16.2, 9.4 Hz, 1H), 2.36 (dd, J = 16.2, 5.3 Hz, 1H), 1.68 – 1.50 (m, 2H), 1.44 (s, 9H), 1.32 – 1.25 (m, 1H), 0.93 (dd, J = 17.4, 6.6 Hz, 6H).

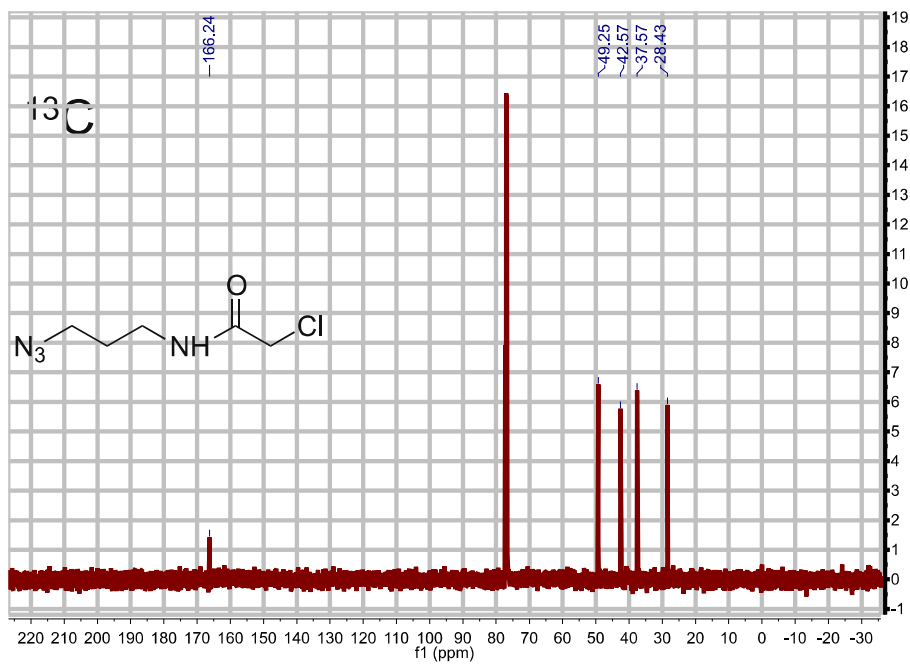
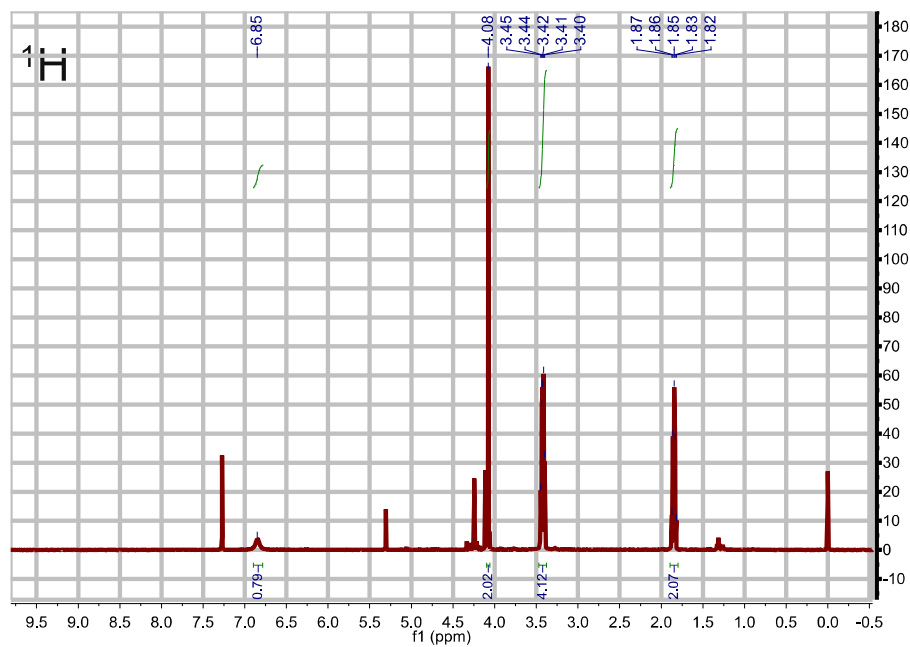
<sup>13</sup>C NMR (75 MHz, Methanol-d<sub>4</sub>) δ 178.97, 172.96, 81.87, 42.37, 40.99, 38.89, 28.27, 26.95, 23.03, 22.65.



**$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 3-azidopropan-1-amine**

$^1\text{H}$  NMR (500 MHz, Chloroform- $d$ )  $\delta$  4.86 (s, 1H), 3.38 (t,  $J$  = 6.7 Hz, 2H), 2.81 (t,  $J$  = 6.9 Hz, 2H), 1.78  $\delta$  1.70 (m, 2H).

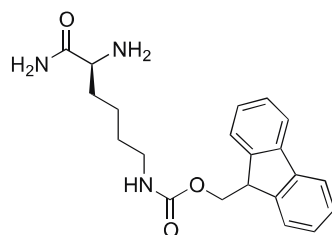
$^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ )  $\delta$  48.99, 39.15, 32.23.



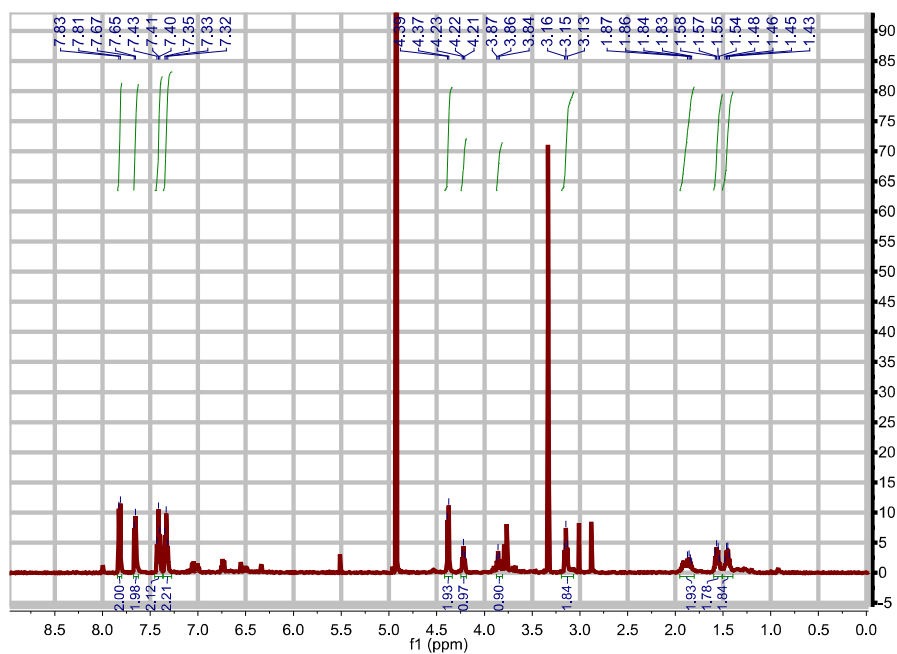
**<sup>1</sup>H and <sup>13</sup>C NMR spectra of N-(3-azidopropyl)-2-chloroacetamide**

<sup>1</sup>H NMR (500 MHz, Chloroform-d)  $\delta$  4.08 (s, 2H), 3.47  $\delta$  3.38 (m, 4H), 1.85 (m,  $J$  = 6.6 Hz, 2H).

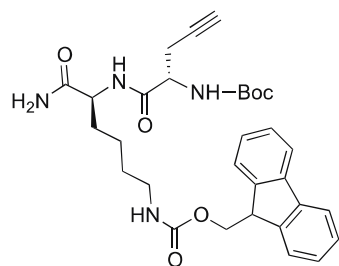
<sup>13</sup>C NMR (126 MHz, Chloroform-d)  $\delta$  166.24, 49.25, 42.57, 37.57, 28.43.



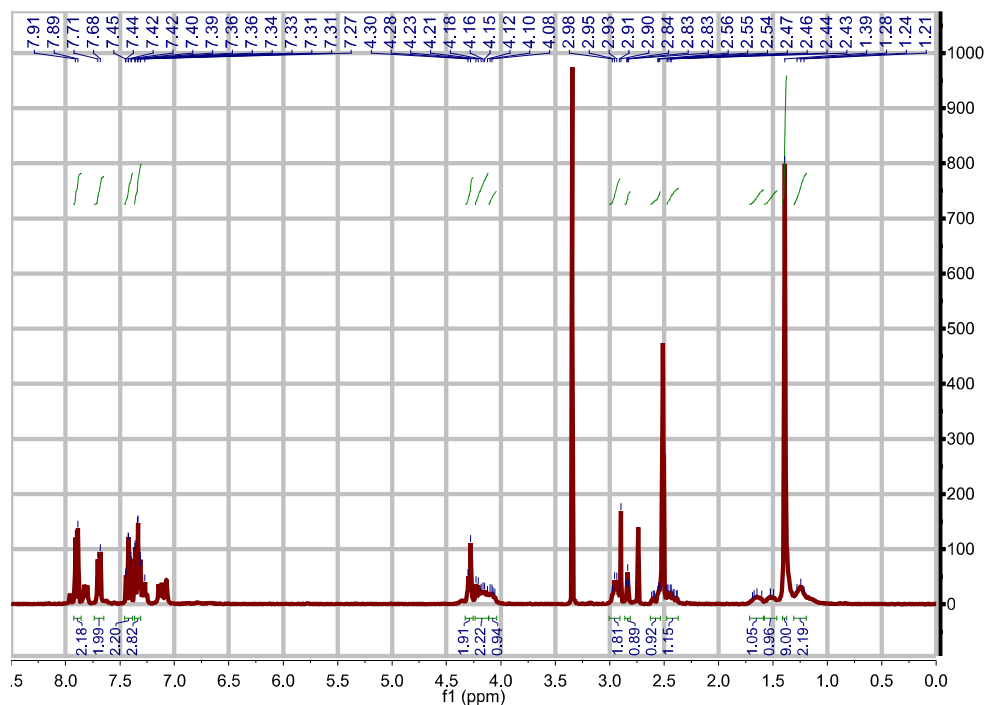
$^1\text{H}$



**$^1\text{H}$  NMR spectra of (9H-fluoren-9-yl) methyl (5,6-diamino-6-oxohexyl)carbamate**  
 $^1\text{H}$  NMR (500 MHz, Methanol- $\text{d}_4$ )  $\delta$  7.82 (d,  $J$  = 7.6 Hz, 2H), 7.66 (d,  $J$  = 7.7 Hz, 2H), 7.41 (t,  $J$  = 7.5 Hz, 2H), 7.33 (t,  $J$  = 7.5 Hz, 2H), 4.38 (d,  $J$  = 6.7 Hz, 2H), 4.22 (t,  $J$  = 6.7 Hz, 1H), 3.86 (t,  $J$  = 6.6 Hz, 1H), 3.15 (t,  $J$  = 7.0 Hz, 2H), 1.97 – 1.80 (m, 2H), 1.57 (p,  $J$  = 7.4 Hz, 2H), 1.45 (p,  $J$  = 14.8, 7.5 Hz, 2H).

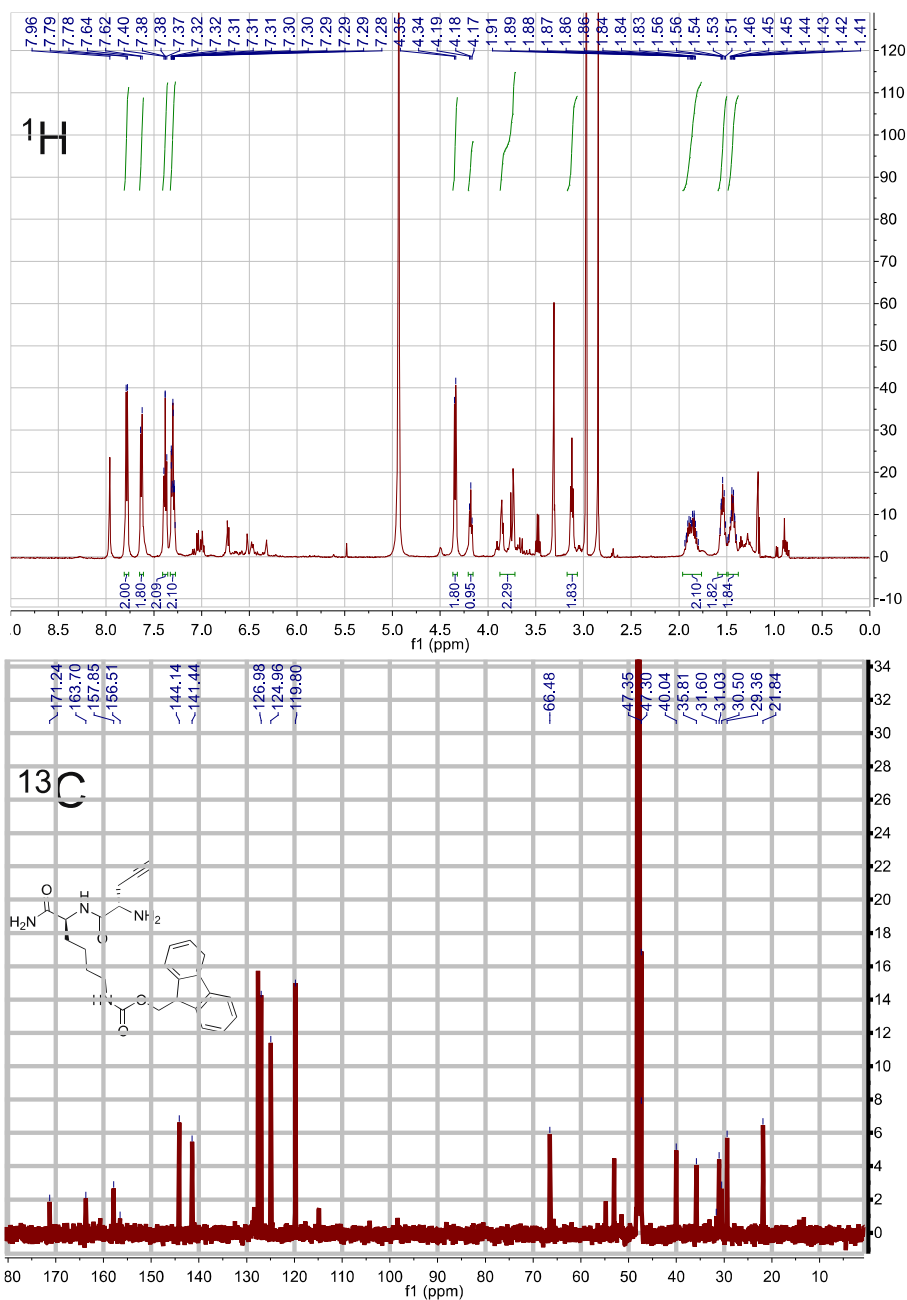


$^1\text{H}$



### $^1\text{H}$ NMR spectra of compound 7

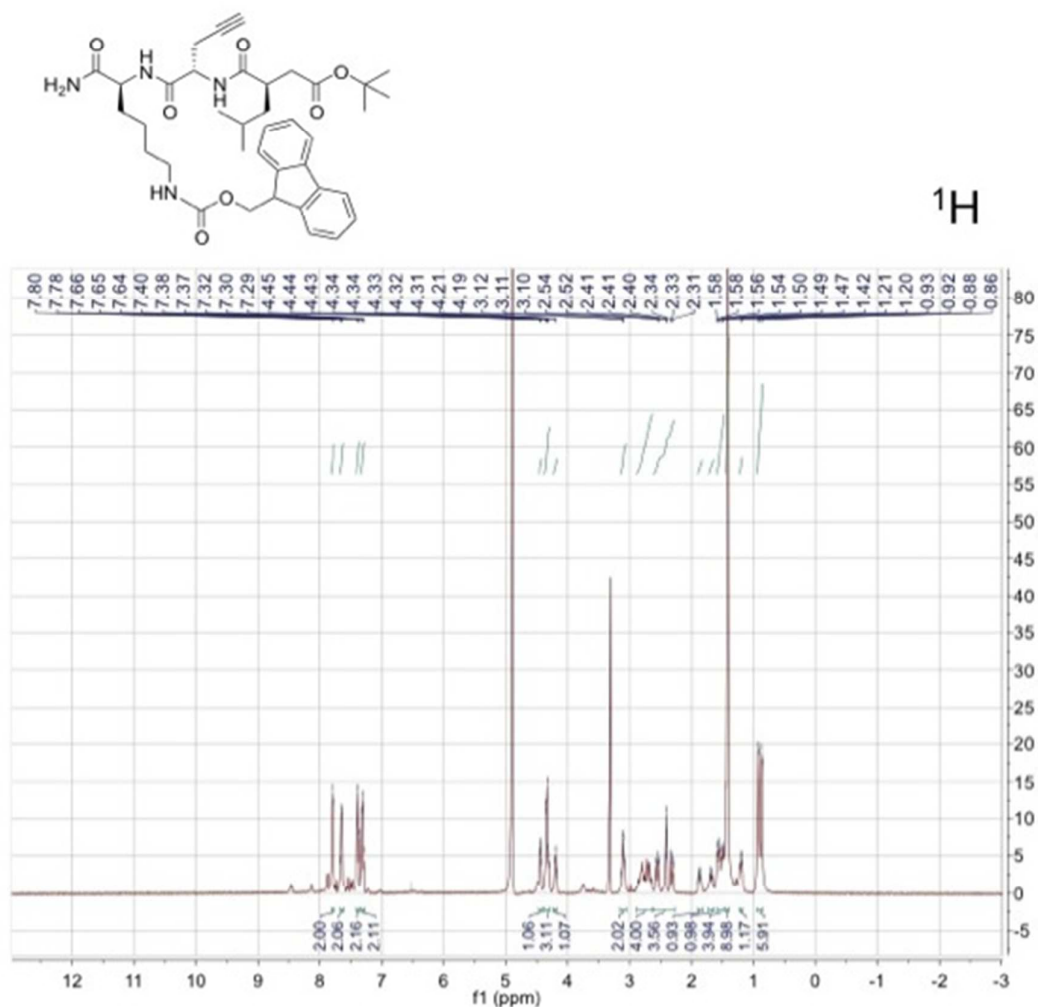
$^1\text{H}$  NMR (300 MHz, DMSO- $d_6$ )  $\delta$  7.89 (d, 2H), 7.69 (dd,  $J$  = 7.5 Hz, 2H), 7.42 (td, 2H), 7.34 (td,  $J$  = 7.4, 1.3 Hz, 2H), 4.29 (d,  $J$  = 7.5 Hz, 2H), 4.26 – 4.11 (m, 2H), 4.13 – 4.05 (m, 1H), 2.94 (q,  $J$  = 6.5 Hz, 2H), 2.85 – 2.82 (m, 1H), 2.63 – 2.53 (m, 1H), 2.43 (ddd,  $J$  = 16.7, 8.8, 2.6 Hz, 1H), 1.72 – 1.57 (m, 1H), 1.57 – 1.46 (m, 1H), 1.39 (s, 9H), 1.31 – 1.18 (m, 2H).



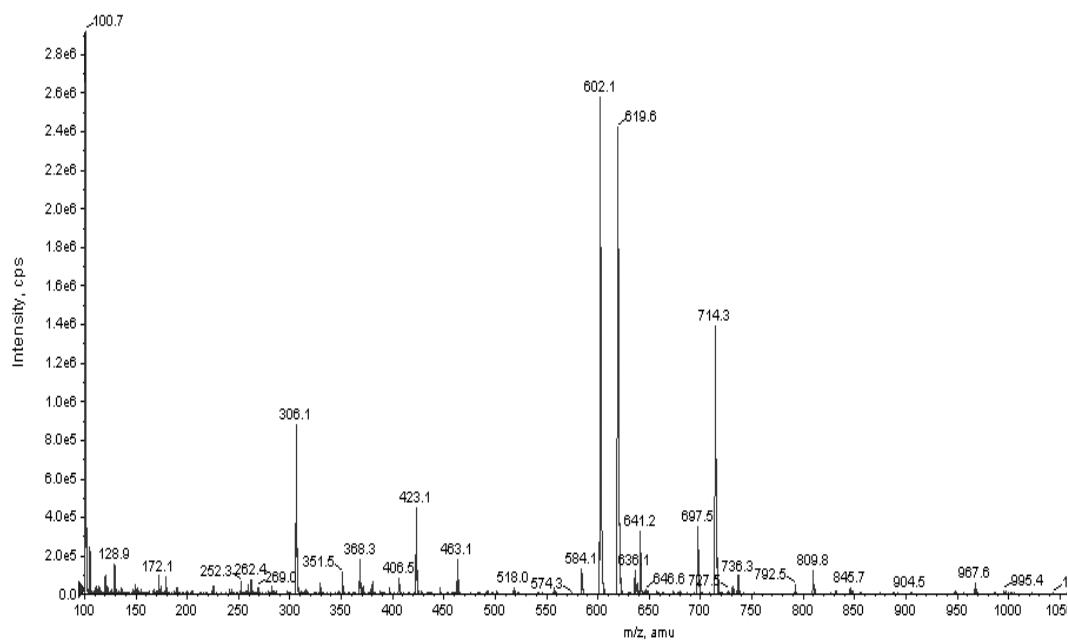
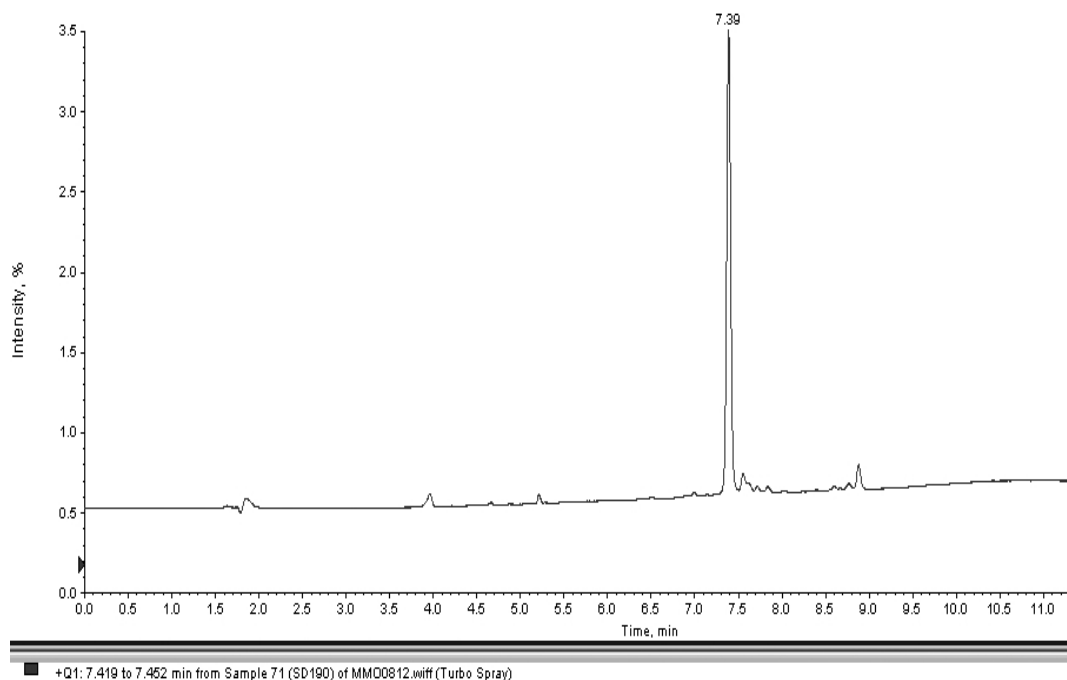
**$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of (9H-fluoren-9-yl)methyl (6-amino-5-(2-aminopent-4-ynamido)-6-oxohexyl)carbamate**

$^1\text{H}$  NMR (500 MHz, Methanol- $d_4$ )  $\delta$  7.78 (d,  $J$  = 7.5 Hz, 2H), 7.63 (d,  $J$  = 7.5 Hz, 2H), 7.41 – 7.35 (m, 2H), 7.33 – 7.27 (m, 2H), 4.34 (d,  $J$  = 6.8 Hz, 2H), 4.18 (t,  $J$  = 6.7 Hz, 1H), 3.87 – 3.72 (m, 2H), 3.12 (t,  $J$  = 6.9 Hz, 1H), 1.96 – 1.76 (m, 2H), 1.54 (p,  $J$  = 7.1 Hz, 2H), 1.43 (qd,  $J$  = 9.2, 8.7, 6.2 Hz, 2H).

$^{13}\text{C}$  NMR (126 MHz, Methanol- $d_4$ )  $\delta$  216.63, 171.24, 163.70, 157.85, 156.51, 144.14, 141.44, 126.98, 124.96, 119.80, 66.48, 47.33 (d,  $J$  = 7.3 Hz), 40.04, 35.81, 31.60, 31.03, 30.50, 29.36, 21.84.





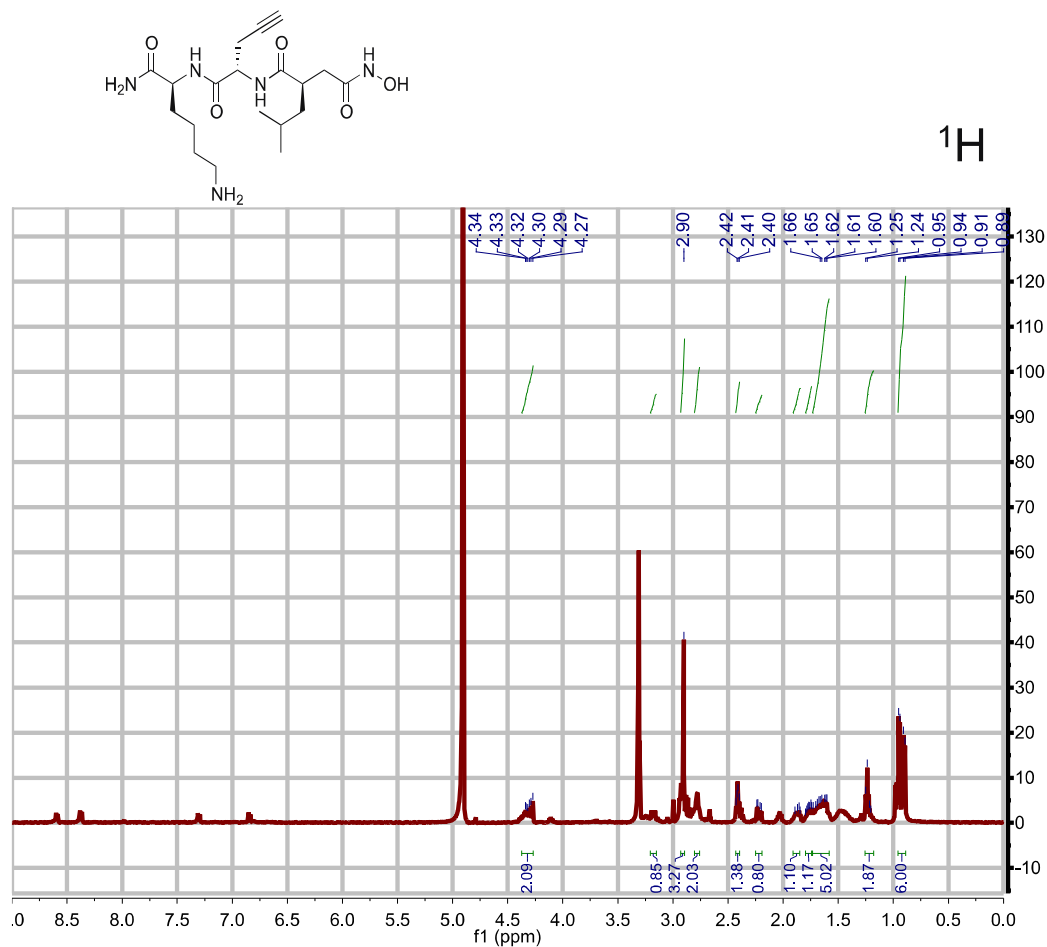


**LC-MS spectra of 9-carbamoyl-1-(9H-fluoren-9-yl)-15-isobutyl-3,11,14-trioxo-12-(prop-2-yn-1-yl)-2-oxa-4,10,13-triazaheptadecan-17-oic acid**

UV trace of the compound (above)

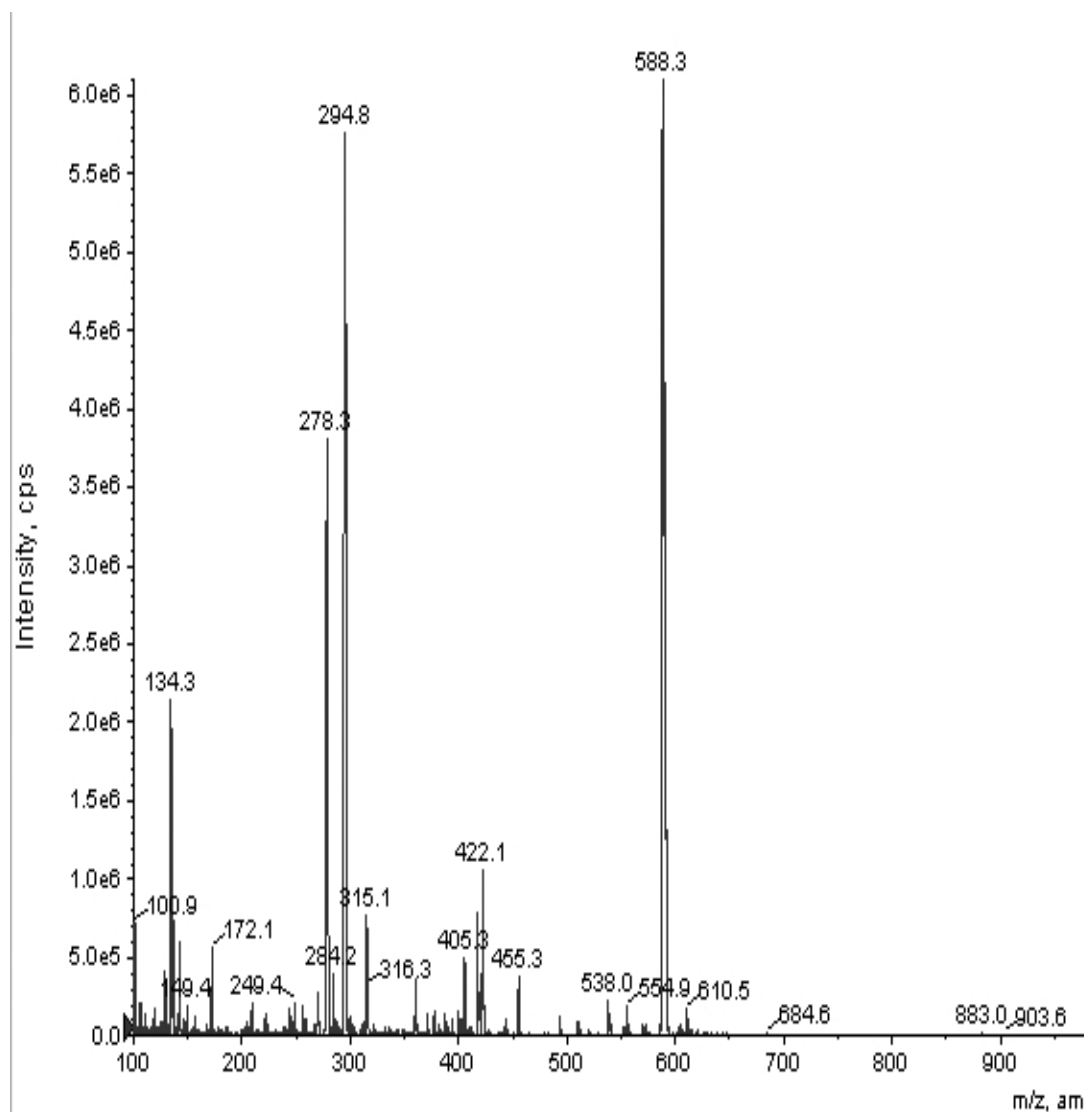
Mass spectra of the compound.  $[M+H]^+$  calculated for  $C_{34}H_{42}N_4O_7$  618.7; found 619.6 (below)





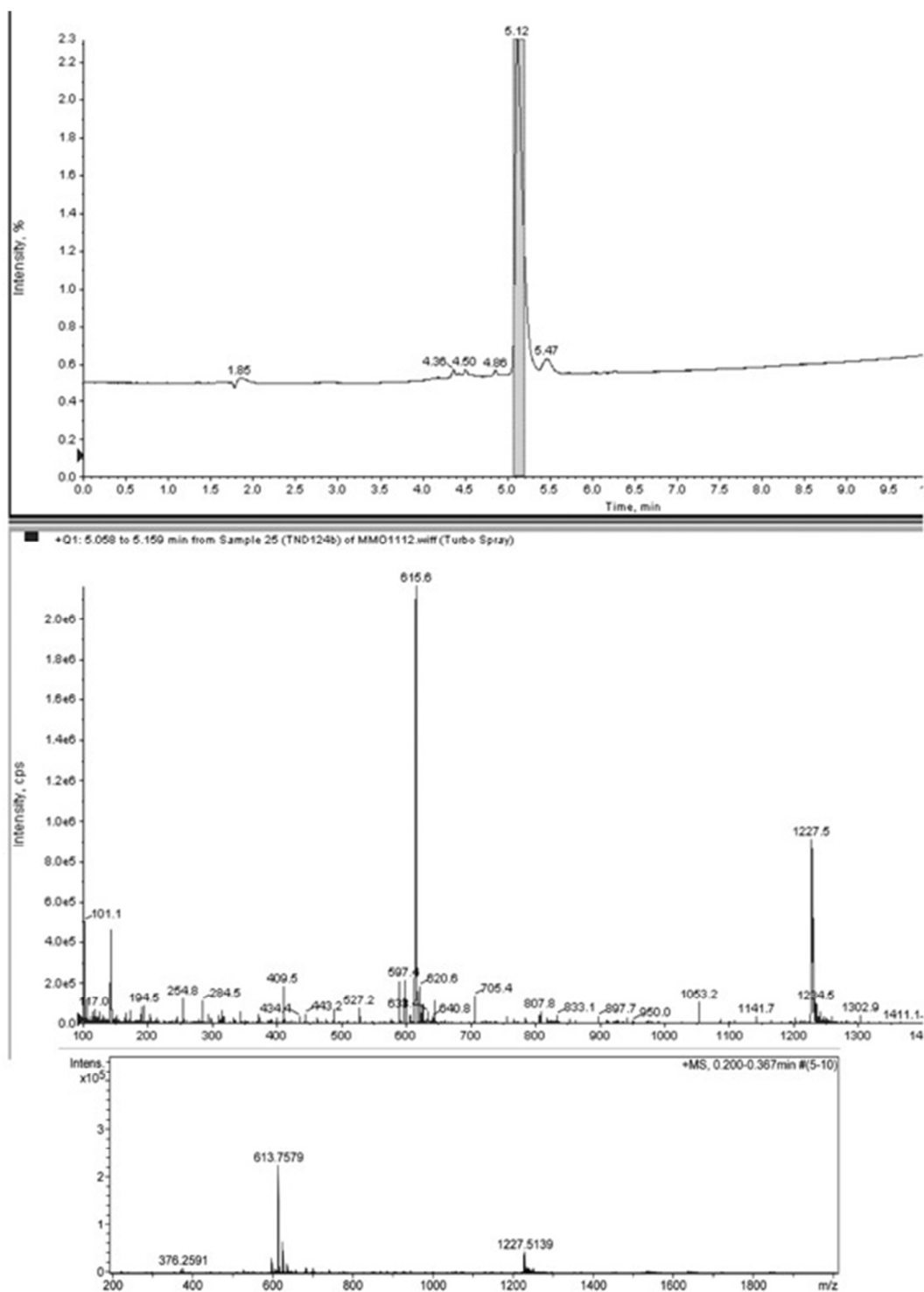
**<sup>1</sup>H NMR spectra of N1-(1-((1,6-diamino-1-oxohexan-2-yl)amino)-1-oxopent-4-yn-2-yl)-N4-hydroxy-2-isobutylsuccinamide**

<sup>1</sup>H NMR (500 MHz, Methanol-d<sub>4</sub>) δ 4.39 – 4.27 (m, 2H), 3.21 – 3.15 (m, 1H), 2.94 – 2.90 (m, 3H), 2.79 (ddt, J = 10.4, 6.7, 4.2 Hz, 2H), 2.44 – 2.39 (m, 1H), 2.22 (dd, J = 14.7, 5.4 Hz, 1H), 1.91 – 1.84 (m, 1H), 1.81 – 1.73 (m, 1H), 1.72 – 1.59 (m, 5H), 1.26 – 1.20 (m, 2H), 0.92 (dd, J = 21.9, 6.4 Hz, 6H).



**MS spectra of 6-amino-2-(2-(2-(2-(aminoxy)-2-oxoethyl)-4-methylpentanamido)-3-(1-(3-(2-chloroacetamido)propyl)-1H-1,2,3-triazol-4-yl)propanamido)hexanamide**

Mass spectrum of the compound.  $[M+H]^+$  calculated for  $C_{24}H_{42}ClN_9O_6$  588.1; found 588.3

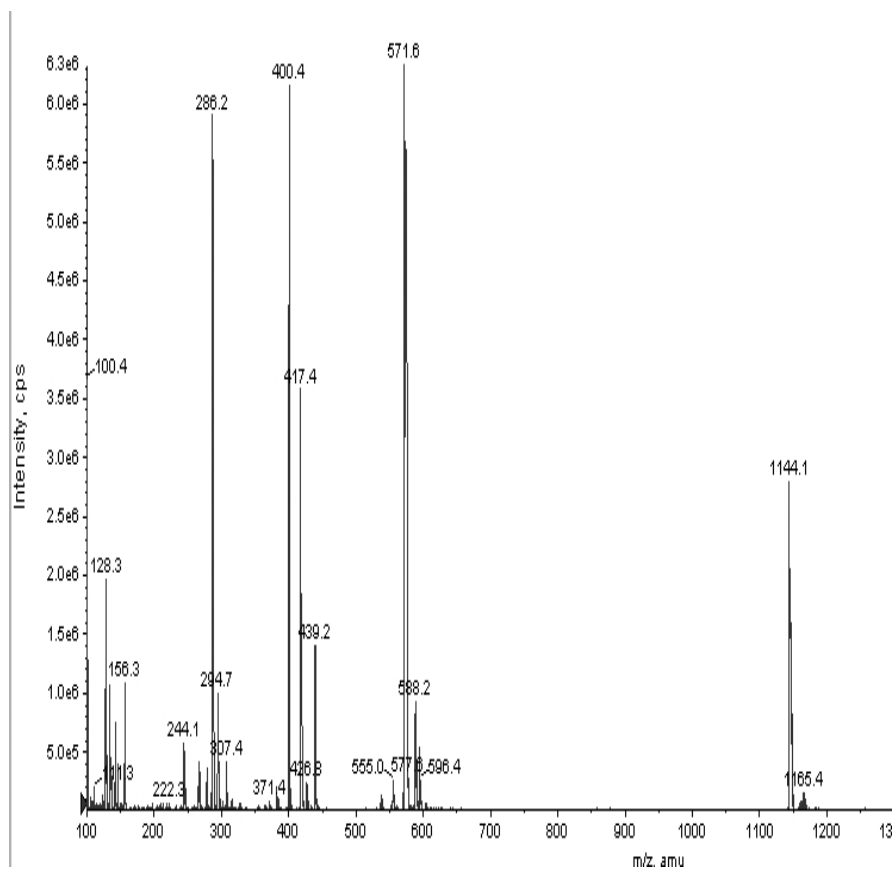


### LC-MS and HRMS spectra of TND124

UV trace of the compound (above)

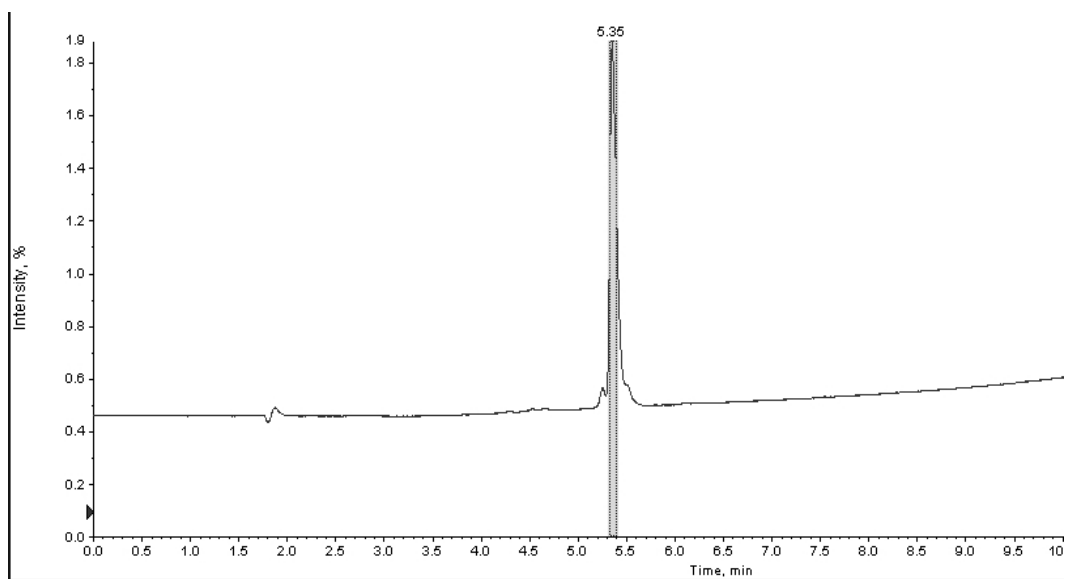
Mass spectra of the compound.  $[M+H]^+$  calculated for  $C_{57}H_{81}ClN_{11}O_{13}S_2^+$  1227.9; found 1227.5. (middle)

High resolution mass spectrometry.  $[M+H]^+$  calculated for  $C_{57}H_{81}ClN_{11}O_{13}S_2^+$  1226.5140; found 1226.5126.  $[M+H]^+$  calculated for  $C_{57}H_{82}ClN_{11}O_{13}S_2^+$  613.7606; found 613.7579 (below)

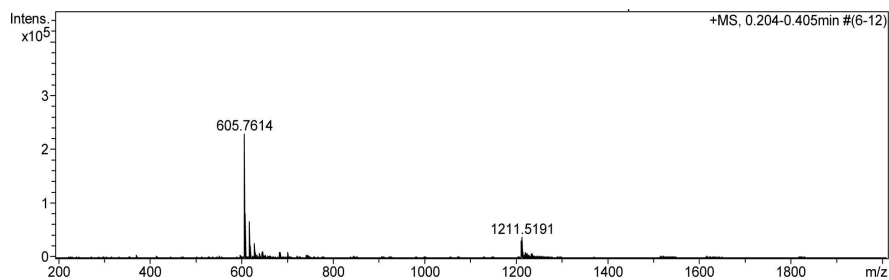
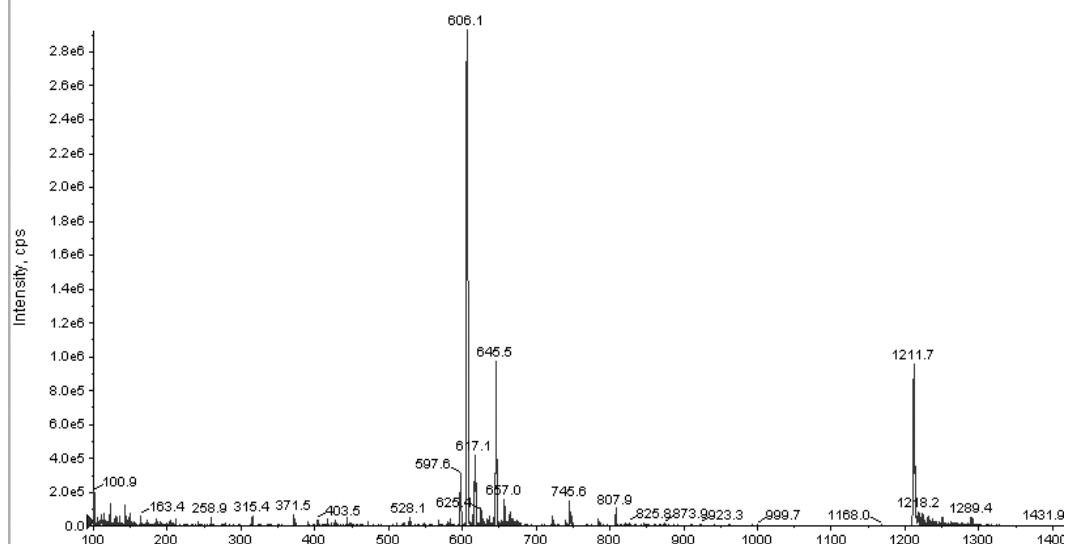


**MS spectra of 2-(2-(2-acetamido-4-methylpentanamido)-3-(1-(3-(2-chloroacetamido)propyl)-1H-1,2,3-triazol-4-yl)propanamido)-6-aminohexanamide**

Mass spectrum of the compound.  $[M+H]^+$  calculated for  $C_{24}H_{42}ClN_9O_5$  572.1; found 571.6



+Q1: 5.309 to 5.359 min from Sample 61 (TND126f11b) of MM00812.wiff (Turbo Spray)



### LC-MS and HRMS spectra of TND126

UV trace of the compound (above)

Mass spectra of the compound.  $[M+H]^+$  calculated for  $C_{57}H_{81}ClN_{11}O_{12}S_2^+$  1211.9; found 1211.7 (middle)

High resolution mass spectrometry.  $[M+H]^+$  calculated for  $C_{57}H_{81}ClN_{11}O_{12}S_2^+$  1210.5191; found 1210.5180.  $[M+H]^+$  calculated for  $C_{57}H_{82}ClN_{11}O_{12}S_2^+$  605.7632; found 605.7614.