Supporting Information

"Click" Synthesis of Small Molecule Probes for Activity-Based Fingerprinting of Matrix Metalloproteases

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1. General Information:

All chemicals were purchased from commercial vendors and used without further purification, unless otherwise noted. ^{1}H NMR spectra were recorded on a Bruker 300 MHz, 800 MHz or DPX-300 NMR spectrometer. Chemical shifts are reported in parts per million referenced with respect to residual solvent ($CHCl_3$ = 7.26 ppm and DMSO-d6 = 2.50 ppm) or from internal standard tetramethylsilane (TMS = 0.00 ppm). The following abbreviations were used in reporting spectra: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets. All reactions were carried out under N_2 atmosphere, unless otherwise stated. HPLC grade solvents were used for all the reactions. ESI mass spectra were acquired in the positive mode using a Finnigan/Mat TSQ7000 spectrometer. Analytical and semi-prep RP-HPLC separations were performed on Phenomex C18 columns (150 x 3.0 mm and 250 x 21.2 mm, respectively), using either a Shimadzu Prominence HPLC system equipped with a Shimadzu SPD-20A detector or a Waters Delta 600 HPLC system. Eluents A (0.1 % TFA/acetonitrile) and B (0.1 % TFA/water) were used as the mobile phases.

Enzymes were obtained from commercial sources at the highest grade available and used directly without further purification. We used in this study a repertoire of enzymes including a range of bacterial metalloproteases, namely thermolysin from *Bacillus thermoproteolyticus* (Calbiochem, USA. Cat# 58656), collagenase from *Clostridium histolyticum* (Sigma-Aldrich, USA Cat#C-7657), anthrax lethal factor (Calbiochem, USA. 176900), bovine carbonic anhydrase (Sigma-Aldrich, USA Cat# C-3934) as well as a range of human matrix metalloproteases, namely MMP-7 (Calbiochem, USA. Cat# 444270), MMP-3 (Calbiochem, USA. Cat# 444217) and MT1-MMP (i.e. MMP-14) (Calbiochem, USA. Cat# 475935). β-Chymotrypsin was also used as a negative control for the microarray experiment (Sigma-Aldrich, USA Cat# C-4629).

2. Chemical Synthesis

2.1 Synthesis of warheads 1 and 2

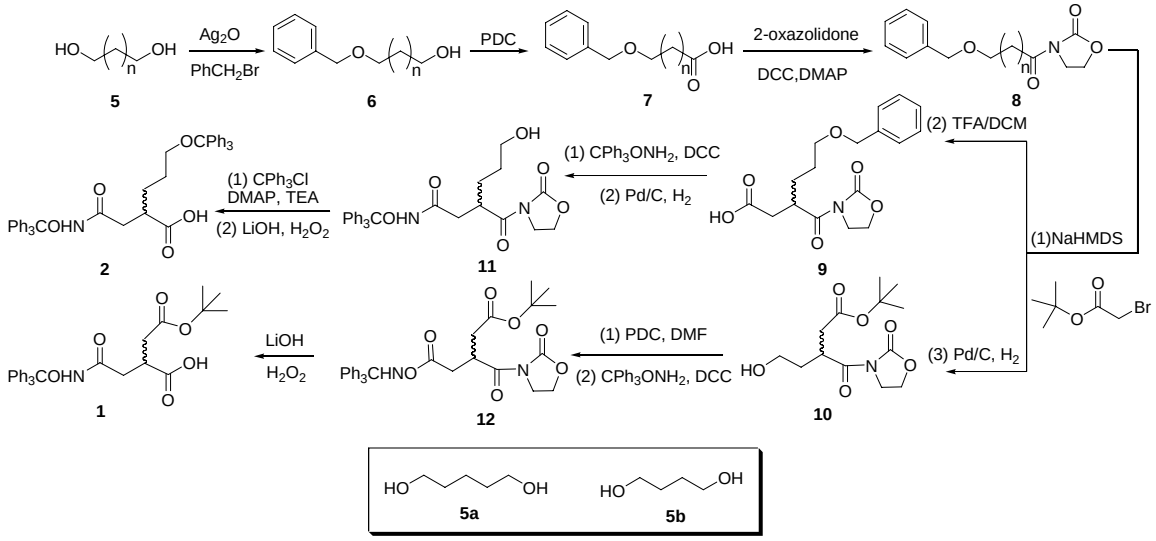


Figure S1. Synthesis of the acidic and hydrophilic MMP warheads (**1** and **2**, respectively).

Synthesis of hydrophilic and acidic P1' substituted warheads involves 7 steps. Both of them started with mono-protection of the diol **5**. This was followed by PDC oxidation of alcohol **6** to give carboxylic acid **7**. Upon installing oxazolidinone, enolate chemistry was carried out to introduce the succinyl template, giving rise to **9** and **10**, respectively. To synthesize warhead **2**, trityl-protected hydroxylamine was first coupled to **9** using standard DCC procedures, followed by selective removal of the benzyl group on the alcohol using Pd/C with H_2 to give **11**. Subsequently, the free alcohol was protected with the acid-labile trityl group, followed by base hydrolysis of oxazolidinone to give the final warhead **2**. To synthesize warhead **1**, the alcohol moiety in **10** was oxidized to the corresponding acid, followed by coupling with trityl-protected hydroxylamine, giving **12**. Finally, base hydrolysis of oxazolidinone furnished the final warhead **1**.

*General procedure for the conversion of **5a-b** to **6a-b**:*[1]

To a solution of the diol **5** (**a** or **b**; 120 mmol) in DCM (300 ml) was added Ag_2O (41.7 g, 180 mmol) and benzyl bromide (15.7 ml, 132 mmol) at room temperature under a nitrogen atmosphere. The reaction was stirred further for 4 hrs before the solid was removed by filtration. The resulting filtrate was concentrated *in vacuo* and purified by flash chromatography (10 – 30% EA/hexane) to give **6** as a colorless oil.

5-(benzyloxy)pentan-1-ol **(6a)**:

Yield = 84%. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.40-7.25 (m, 5H), 4.50 (s, 2H), 3.58 (t, J = 12.2 Hz, 2H), 3.48 (t, J = 12.9 Hz, 1H), 2.28 (s, 1H), 1.67-1.54 (m, 4H), 1.51-1.40 (m, 2H); ESI-MS: m/z $[M+Na]^+$ = 217.3.

4-(benzyloxy)butan-1-ol **(6b)**:

Yield = 80%. ^{1}H-NMR (300 MHz, $CDCl_3$) δ7.48 – 7.21, (m, 5H), 4.49, (s, 2H), 3.57 (t, *J* = 6.48 Hz, 2H), 3.48 (t, *J* = 5.93 Hz, 2H), 2.91 (br s, 1H), 1.70 – 1.57 (m, 4H); ESI-MS: m/z $[M+Na]^+$ = 203.2

*General procedure for oxidation of **6a-b** to **7a-b**:*[2]

To a solution of **6** (**a** or **b**; 96 mmol) in DMF (250 ml) at 0 ºC under a nitrogen atmosphere was added pyridinium dichromate (170.6 g, 453 mmol). The mixture was gradually raised to room temperature and stirred overnight. Water was subsequently added and the resulting mixture was extracted with ether. The combined ether layers were washed with brine, dried over Na_2SO_4, filtered and concentrated. The oil obtained was purified by flash chromatography (10 – 20% EA/hexane) to give **7**.

5-(benzyloxy)pentanoic acid **(7a)**:

Yield = 50%. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 9.96 (br s, 1H), 7.42-7.25 (m, 5H), 4.51 (s, 2H), 3.50 (t, *J* = 10.8 Hz, 2H), 2.38 (t, *J* = 12.6 Hz, 1H), 2.71 (m, 4H); ESI-MS: m/z $[M-H]^-$ = 207.2.

4-(benzyloxy)butanoic acid **(7b)**:

Yield = 64%. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 10.10 (br s, 1H), 7.42-7.30 (m, 5H), 4.54 (s, 2H), 3.56 (t, *J* = 5.94 Hz, 2H), 2.51 (t, *J* = 6.96 Hz, 2H), 1.98 (t, *J* = 6.63 Hz, 2H). ESI-MS: m/z $[M-H]^-$ = 193.1.

*Procedures for conversion of **7a-b** to **8a-b**, then to **9 & 10**:*

The synthesis of **9** and **10** from **7a-b** was accomplished using procedures described previously,[1] and below.

3-(5-(benzyloxy)pentanoyl)oxazolidin-2-one **(8a)**:

Synthesized based on previously published procedures.[3] Yield = 80%. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.33-7.22 (m, 5H), 4.60 (s, 2H), 4.35 (t, *J* = 16.2 Hz, 2H), 3.90 (t, *J* = 15.9 Hz, 2H), 3.50 (t, *J* = 12.9Hz, 2H), 2.96 (t, *J* = 12.9 Hz, 2H), 1.82-1.67 (m, 4H); ESI-MS: m/z $[M+Na]^+$ = 300.2.

3-(4-(benzyloxy)butanoyl)oxazolidin-2-one **(8b)**:

Synthesized based on previously published procedures.[3] Yield = 82%. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.38-7.26 (m, 5H), 4.47 (s, 2H), 3.56 (t, *J* = 5.94Hz, 2H), 2.51 (t, *J* = 6.96 Hz, 2H), 1.98 (t, *J* = 6.63 Hz, 2H). ESI-MS: m/z $[M+Na]^+$ = 286.2.

6-(benzyloxy)-3-(2-oxooxazolidine-3-carbonyl)hexanoic acid **(9)**:

The oxazolidine precursor of **9** was synthesized from **8a** accordingly (Yield = 71%),[3] and characterized by NMR and MS. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.38-7.26 (m, 5H), 4.47 (s, 2H), 4.38-4.30 (m, 2H), 4.25-4.16 (m, 1H), 4.08-3.92 (m, 2H), 3.46 (m, 2H), 2.83-2.74 (dd, *J* = 17.97 Hz, *J* = 10.68 Hz, 1H), 2.45-2.38 (dd, *J* = 16.70 Hz, *J* = 4.11 Hz, 1H), 1.78-1.56 (m, 4H); ESI-MS: m/z $[M+Na]^+$ = 414.2.

The above intermediate (13.3 g, 34 mmol) was treated with TFA (26.2 ml, 340 mmol) in DCM (170 ml), based on a previously published procedure,[3] to give **9** (11.3 g, 33.7 mmol) in 99% yield. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.38-7.26 (m, 5H), 4.38 (s, 2H), 4.42-4.28 (m, 2H), 4.24-4.12 (m, 1H), 4.08-3.92 (m, 2H), 3.50 (m, 2H), 2.83-2.74 (dd, *J* = 17.26 Hz, *J* = 10.18 Hz, 1H), 2.56-2.42 (dd, *J* = 16.70 Hz, *J* = 4.11 Hz, 1H), 1.78-1.56 (m, 4H); ESI-MS: m/z $[M-H]^+$ = 334.3.

tert-butyl 5-hydroxy-3-(2-oxooxazolidine-3-carbonyl)pentanoate **(10)**:

The oxazolidine precursor of **10** was synthesized from **8b** accordingly (Yield = 78%),[3] and characterized by NMR and MS. ^{1}H-NMR (300 MHz, $CDCl_3$) δ7.35 – 7.20 (m, 5H), 4.50 – 4.30 (m, 2H), 4.30 – 4.20 (m, 2H), 4.17 – 3.81 (m, 2H), 3.69 – 3.52 (m, 3H), 2.87 – 2.76 (dd, *J* = 16.5 Hz, *J* = 10.44 Hz, 1H), 2.85 – 2.76 (dd, *J* = 16.71 Hz, *J* = 4.53Hz, 1H), 2.09 – 1.98 (m, 1H), 1.35 (s, 9H); ESI-MS: m/z $[M+Na]^+$ = 414.3.

Hydrogenolysis of above intermediate (13.2 g, 35 mmol) was accomplished in THF (175 ml) with Pd/C (1.32 g) by stirring the mixture at room temperature under a hydrogen atmosphere for 1.5 hrs. The mixture was filtered, and the filtrate concentrated and purified by flash chromatography (80-100% EA/hexane) to yield **10** (7.96 g, 27.7 mmol, 79%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 4.43 – 4.35 (m, 2H), 4.24 – 4.14 (m, 2H), 4.05 – 3.96 (m, 2H), 3.65 – 3.60 (t, *J* = 5.94 Hz, 2H), 2.83 – 2.74 (dd, *J* = 16.89 Hz, *J* = 10.47 Hz, 1H), 2.46 – 2.39 (dd, *J* = 16.89 Hz, *J* = 4.20 Hz, 1H), 1.92 – 1.65 (m, 2H), 1.35 (s, 9H); ESI-MS: m/z $[M+Na]^+$ = 310.2.

6-hydroxy-3-(2-oxooxazolidine-3-carbonyl)-N-(trityloxy)hexanamide **(11)**:

Coupling of acid **9** (11.4 g, 34.0 mmol) with trityl-protected hydroxylamine (11.2 g, 40.8 mmol) and DCC (8.42 g, 40.8 mmol) in DCM (120 ml), following previously published procedures,[3] yielded the corresponding intermediate (80%) after purification by flash chromatography (40-60% EA/hexane). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.68 (br s, 1H), 7.38-7.26 (m, 20H), 4.45 (s, 2H), 4.38-4.22 (m, 2H), 4.04-3.82 (m, 3H), 3.37 (m, 2H), 2.40-2.37 (m, 1H), 2.12-2.01 (m, 1H), 1.58-1.32 (m, 4H); ESI-MS: m/z $[M+Na]^+$ = 615.1.

The above intermediate (16.1 g, 27.2 mmol) in THF (130 ml) and Pd/C (1.61 g) was stirred at room temperature under a hydrogen atmosphere for 1.5 hrs. The mixture was filtered, and the filtrate concentrated and purified by column chromatography (80-100% EA/hexane) to afford **11** (10.8g, 21.5 mmol, 75%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.68 (br s, 1H), 7.38-7.26 (m, 15H), 4.47-4.34 (m, 2H), 4.06-3.85 (m, 3H), 3.56-3.42 (m, 2H), 2.43-2.34 (m, 1H), 2.02-1.88 (m, 1H), 1.48-1.20 (m, 4H); ESI-MS: m/z $[M+Na]^+$ = 525.1.

2-(2-oxo-2-(trityloxyamino)ethyl)-5-(trityloxy)pentanoic acid **(2)**:

To a solution of **11** (10.8 g, 21.5 mmol), DMAP (0.26 g, 2.15 mmol) and TEA (5.36 ml, 38.7 mmol) in DCM (240 ml) was added CPh_3Cl (6.00 g, 21.5 mmol) and the reaction was stirred at room temperature overnight. DCM was removed *in vacuo* to yield a yellow semi-solid. Subsequently, 20% EA/hexane was added to the residue. The white solid obtained was filtered off and washed again with 20% EA/hexane to afford the pure intermediate (15.6 g, 21.0 mmol, 98%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.18-7.56 (m, 31H), 4.22 (t, *J* = 7.83 Hz, 2H), 3.61 (t, *J* = 8.73 Hz, 2H), 3.14-3.06 (q, 2H), 2.99-2.97 (m,

1H), 2.38-2.22 (m, 1H), 1.96-1.78 (m, 1H), 1.48-1.20 (m, 4H); ESI-MS: m/z $[M+Na]^+$ = 767.8.

The above intermediate (15.6 g, 21.0 mmol) was treated with LiOH (1.51 g, 63.0 mmol) and 30 % H_2O_2 (11.8 ml, 113 mmol) using previously published procedures,[3] followed by purification with flash chromatography (5-10% MeOH) to give **2** (9.66 g, 14.3 mmol, 68%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.25-7.16 (m, 31H), 3.18-2.82 (m, 2H), 2.71-2.55 (m, 1H), 2.48-2.25 (m, 1H), 2.18-2.02 (m, 1H), 1.72-1.25 (m, 4H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 176.0, 174.5, 146.8, 129.2-126.4 (m), 88.9, 82.0, 68.6, 36.2, 35.4, 24.7, 22.1; ESI-MS: m/z $[M-H]^-$ = 674.3.

tert-butyl 5-oxo-3-(2-oxooxazolidine-3-carbonyl)-5-(trityloxyamino)pentanoate **(12):**

Oxidation of alcohol **10** (7.96 g, 27.7 mmol) was accomplished by following the procedure described for the conversion of **6a-b** to **7a-b**, using PDC (31.3 g, 83.1 mmol) in DMF (70 ml), to furnish the corresponding intermediate (6.51 g, 21.6 mmol, 78%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 4.48 – 4.34 (m, 3H), 4.08 – 3.93 (m, 2H), 2.84 – 2.69 (m, 2H), 2.54 – 2.38 (m, 2H), 1.40 (br s, 9H); ESI-MS: m/z $[M-H]^-$ = 300.3.

Above intermediate (65.1 g, 21.6 mmol) was coupled with trityl-protected hydoxylamine (7.13 g, 25.9 mmol) and DCC (5.34 g, 25.9 mmol) in DCM (65 ml), following previously published procedures.[3] Upon purification by flash chromatography (50-70% EA/hexane), the desired product **12** was isolated (7.37 g, 13.2 mmol, 61%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.47 – 7.32 (m, 16H), 4.37 (t, J = 8.01 Hz, 2H), 4.12 (q, J = 7.32 Hz, 1H), 3.96 (t, J = 7.32 Hz, 2H), 2.39 – 1.85 (m, 4H), 1.40 (s, 9H); ESI-MS: m/z $[M+Na]^+$ = 581.5.

2-(2-tert-butoxy-2-oxoethyl)-4-oxo-4-(trityloxyamino)butanoic acid **(1)**:

Hydrolytic cleavage of the oxazolidinone moiety in **12** (7.37 g, 13.2 mmol) was accomplished by LiOH (0.95 g, 39.6 mmol) and 30 % H_2O_2 (6.36 ml, 60.7 mmol) using previously published procedures,[3] which upon purification by flash chromatography (70-80% EA/hexane) yielded **1** (4.72 g, 9.64 mmol, 73%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.43 – 7.29 (m, 16H), 3.02-2.87 (m, 1H), 2.79-2.63 (m, 1H), 2.48-2.25 (m, 2H), 2.18-2.02 (m, 1H), 1.43 (s, 9H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 177.0, 172.2, 171.9, 141.8, 140.8, 81.0, 65.0, 40.6, 36.7, 29.8, 27.9; ESI-MS: m/z $[M+Na]^+$ = 512.4.

2.2 Synthesis of warhead 3

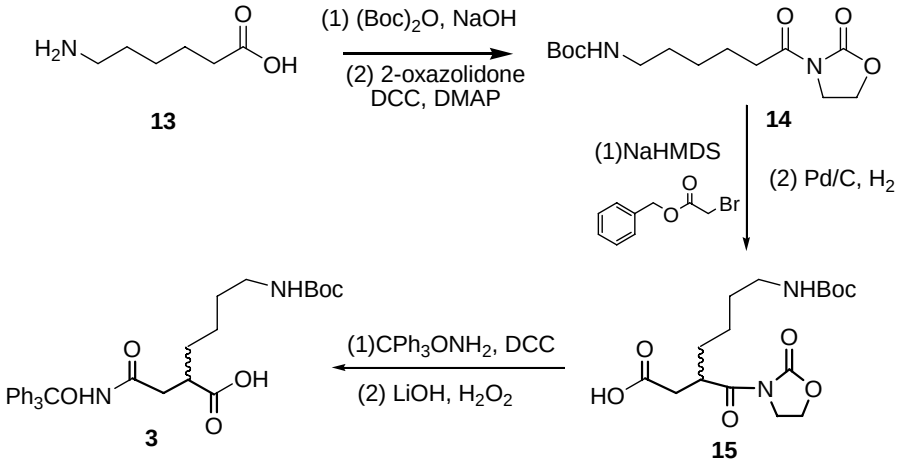


Figure S2. Synthesis route towards the basic warhead, **3**.

tert-butyl 6-oxo-6-(2-oxooxazolidin-3-yl)hexylcarbamate **(14)**

To a solution of 6-aminohexanoic acid **13** (7.87 g, 60 mmol) and NaOH (2 M solution, 50ml) in dioxane/water (2:1; 180 ml) at 0 °C was added $(Boc)_2O$ (15.7 g, 72 mmol). The reaction was allowed to proceed at room temperature overnight. Subsequently, dioxane was removed under reduced pressure. The resulting mixture was acidified to pH 2 with 1 M HCl, then extracted with EA (3 x 80 ml). The combined organic extracts were dried over Na_2SO_4, filtered and concentrated to furnish the desired product as colorless oil (13.8 g, 99%), which was further reacted with 2-oxazolidinone (4.35 g, 50 mmol), DMAP (0.92 g, 7.5 mmol) and DCC (15.5g, 75 mmol) based on the general procedures described for the synthesis of **8a-b**.[3] Upon column purification (20 - 40% EA/hexane), the desired product **14** was isolated as white solid (15.6 g, 52.0 mmol, 65%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 4.55 (br s, 1H), δ 4.39 (t, J = 8.01 Hz, 2H), 3.99 (t, J = 8.19 Hz, 2H), 3.19 – 3.02 (m, 2H), 2.89 (t, J = 7.49 Hz, 2H), 1.72 – 1.58 (m, 2H), 1.57 – 1.35 (m, 4H), 1.41 (s, 9H); ESI-MS: m/z $[M+Na]^+$ = 323.2.

7-(tert-butoxycarbonyl)-3-(2-oxooxazolidine-3-carbonyl)heptanoic acid **(15)**

Compound **14** (15.6 g, 52.0 mmol) was alkylated with benzyl 2-bromoacetate (15.5 g, 67.6 mmol) using NaHMDS (67.6 ml, 67.6 mmol) in 180 ml THF following previously described procedures.[1] Upon column purification (20 – 35% EA/hexane), the product/intermediate was isolated as colorless oil (16.6 g, 36.9 mmol, 71%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.30 (m, 5H), δ 5.11 – 4.97 (m, 2H), 4.56 (br s, 1H), 4.42-4.28 (m, 2H), 4.26-4.16 (m, 1H), 4.02-3.78 (m, 2H), 3.07-3.05 (m, 2H), 2.98-2.82 (dd, J = 17.07Hz, J = 6.27Hz , 1H), 2.55-2.48 (dd, J = 16.91Hz, J = 3.84Hz , 1H), 1.65 (m, 2H), 1.41 (m, 11H), 1.26 (m, 2H). ESI-MS; m/z $[M+Na]^+$ = 437.3.

Hydrogenolysis of the above intermediate (16.6 g, 36.9 mmol) in THF (130 ml) with Pd/C (1.66 g) was accomplished using procedures described earlier for the synthesis of **11**. The desired product **15** was obtained without column purification (13.2 g, 36.9 mmol, 99%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 4.63 (br s, 1H), 4.39 (t, J = 8.01 Hz, 2H), 4.18-4.10 (m, 1H), 3.98 (t, J = 8.15 Hz, 2H), 3.07-3.05 (m, 2H), 2.91-2.80 (dd, J =

17.07Hz, J = 6.27 Hz , 1H), 2.53-2.45 (dd, J = 16.91 Hz, J = 3.84Hz , 1H), 1.68-1.63 (m, 2H), 1.43-1.38 (m, 13H); ESI-MS: m/z $[M-H]^-$ = 357.3.

6-(tert-butoxycarbonyl)-2-(2-oxo-2-(trityloxyamino)ethyl)hexanoic acid **(3)**

Acid **15** (13.2 g, 36.9 mmol) was coupled with trityl-protected hydroxylamine (13.2 g, 48.0 mmol) and DCC (9.90 g, 48.0 mmol) in DCM (180 ml) using procedures previously described.[1] Upon column purification (30 – 50% EA/hexane), the intermediate was obtained as a white solid (18.2 g, 29.5 mmol, 80%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.72 (br s, 1H), 7.34 (m, 15H), 4.52 (br s, 1H), 4.33 (t, J = 16.38Hz, 2H), 4.04-3.88 (m, 3H), 3.04-3.052 (m, 2H), 2.40-2.30 (m, 1H), 2.08-2.02 (m, 1H), 1.48-1.26 (m, 15H); ESI-MS: m/z $[M-H]^-$ = 615.2.

Hydrolysis of above intermediate was achieved by the treatment with LiOH (2.12 g, 88.5 mmol), H_2O_2 (12.4 ml, 118 mmol) in THF/H_2O (3/1; 360 ml) using procedures previously described,[1] and purified to give the desired product **3** as a while solid (10.9 g, 20.0 mmol, 68%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.30 (br s, 1H), 7.33 (m, 15H), 4.59 (br s, 1H), 3.02-2.95 (m, 2H), 2.52-2.40 (m, 1H), 2.27-2.16 (m, 1H), 1.50-1.26 (m, 15H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 174.9, 171.8, 146.8, 144.5, 130.0 – 126.5 (m), 82.2, 79.6, 40.4, 36.9, 31.5, 30.5, 28.4, 23.2; ESI-MS: m/z $[M+Na]^+$ = 569.1.

2.3 Synthesis of warhead 4

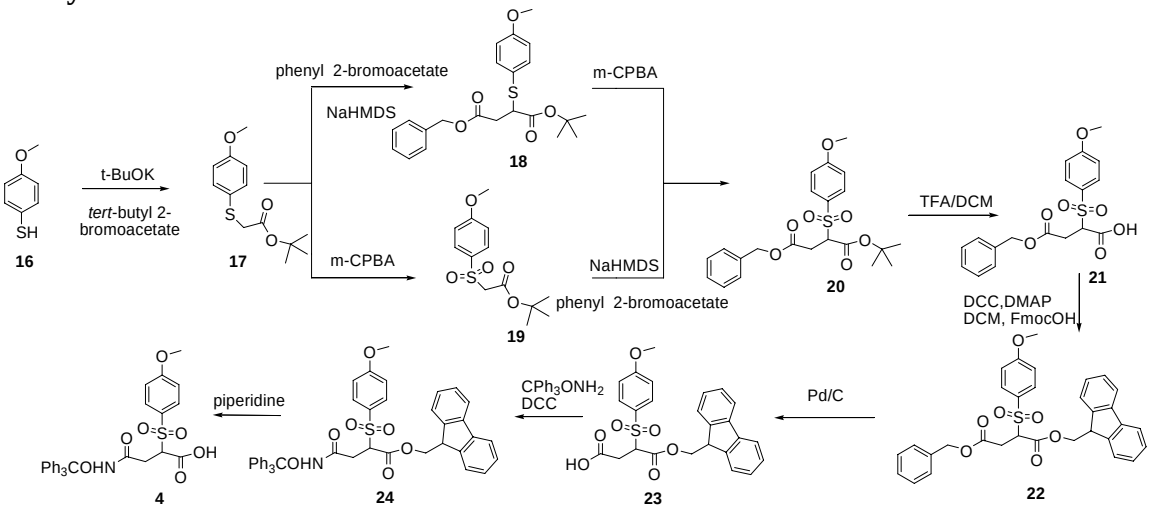


Figure S3. Synthesis route of the sulfone warhead **4**.

tert-butyl 2-(4-methoxyphenylthio)acetate **(17)**:

To a stirred solution of **16** (8.61 ml, 70 mmol) in THF (110 ml) under a nitrogen atmosphere at 0 ºC was added t-BuOK over 5 min. Tert-butyl bromoacetate was then added dropwise. The reaction mixture was stirred for another 3 hrs, after which the white solid formed was filtered away. The filtrate was concentrated and purified by column chromatography (100% hexane – 5% DCM/hexane) to furnish **17** as colorless oil (15.0 g, 58.8 mmol, 84%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.40 (d, J = 9.06 Hz, 2H), 6.82 (d, J = 9.06 Hz, 2H), 3.76 (s, 3H), 3.41 (s, 2H), 1.38 (s, 9H); ESI-MS: m/z $[M+Na]^+$ = 277.2.

4-benzyl 1-tert-butyl 2-(4-methoxyphenylthio)succinate **(18)**:

Compound **17** (7.12 g, 28.0 mmol) was alkylated with benzyl 2-bromoacetate (12.8 g, 56.0 mmol) using NaHMDS (56.0 ml, 56.0 mmol) in 180 ml THF following general procedures described previously.[1] Upon column purification (2 – 7% EA/hexane), the desired product **18** was obtained as colorless oil (8.69 g, 21.6 mmol, 77%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.43 (d, *J* = 8.70 Hz, 2H), 7.34 (s, 5H), 6.84 (d, *J* = 8.70 Hz, 2H), 5.12 (s, 2H), 3.79 (m, 4H), 2.94-2.85 (dd, *J* = 16.89 Hz, *J* = 9.75 Hz , 1H), 2.74-2.67 (dd, *J* =17.07 Hz, *J* = 5.55 Hz , 1H), 1.39 (s, 9H); ESI-MS: m/z $[M+Na]^+$ = 425.0.

tert-butyl 2-(4-methoxyphenylsulfonyl)acetate **(19)**:

To a solution of **17** (7.12 g, 28.0 mmol) in DCM (140 ml) under a nitrogen atmosphere at 0 ºC was added m-CPBA (12.1 g, 70.0 mmol) in small portions. The reaction mixture was stirred overnight. The white solid formed was filtered away, and the filtrate was concentrated and purified by column chromatography (15 – 40% DCM/hexane), to furnish **19** (5.61 g, 19.6 mmol, 70%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.79 (d, *J* = 8.71 Hz, 2H), 7.00 (d, *J* = 8.72 Hz, 2H), 3.99 (s, 2H), 3.87 (s, 3H), 1.37 (s, 9H); ESI-MS: m/z $[M+Na]^+$ = 308.9.

4-benzyl 1-tert-butyl 2-(4-methoxyphenylsulfonyl)succinate **(20)**:

Compound **18** (8.69 g, 21.6 mmol) was oxidized with m-CPBA (9.32 g, 54.0 mol) using procedures described for the conversion of **17** to **19**. Upon column purification (60 – 80% EA/hexane), the desired product **20** was isolated as colorless oil (7.13 g, 16.4 mmol, 76%)

Sulfone **19** (5.61 g, 19.6 mmol) was alkylated with benzyl 2-bromoacetate (8.98 g, 39.2 mmol) using NaHMDS (39.2 ml, 39.2 mmol) in 100 ml THF following procedures described earlier. Upon column purification (60-80% EA/hexane), the desired product **20** was isolated (6.39 g, 14.7 mmol, 75%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.79 (d, *J* = 9.06 Hz, 2H), 7.34 (s, 5H), 7.01 (d, *J* = 9.06 Hz, 2H), 5.11 (s, 2H), 4.33 (t, *J* = 14.97Hz, 1H), 3.88 (s, 3H), 3.10 (d, *J* = 7.32 Hz, 2H), 1.34 (s, 9H); ESI-MS: m/z $[M+Na]^+$ = 457.0.

4-(benzyloxy)-2-(4-methoxyphenylsulfonyl)-4-oxobutanoic acid **(21)**:

Cleavage of the *tert*-butyl group in **20** (13.0 g, 30 mmol) with TFA (23.1 ml, 300 mmol) in DCM (150 ml) was accomplished using procedures described previously[1] to give **21** (11.1 g, 29.4 mmol, 98%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.78 (d, *J* = 8.73 Hz, 2H), 7.33 (s, 5H), 7.01 (d, *J* = 8.73 Hz, 2H), 5.64 (br s, 1H), 5.10 (q, 2H), 4.46 (t, *J* = 14.61 Hz, 1H), 3.87 (s, 3H), 3.11 (d, *J* = 7.68 Hz, 2H); ESI-MS: m/z $[M+Na]^+$ = 401.1.

1-(9H-fluoren-9-yl)methyl 4-benzyl 2-(4-methoxyphenylsulfonyl)succinate **(22)**:

Acid **21** (11.1 g, 29.4 mmol) was coupled with Fmoc-OH (4.35 g, 29.4 mmol), using DCC (7.28 g, 35.3 mmol) and DMAP (0.43 g, 3.53 mmol) following procedures described previously.[1] Upon column purification (10 - 25% EA/hexane), the desired product **22** was isolated (15.5 g, 27.9 mmol, 95%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.77 (q, 4H), 7.60 (d, *J* = 7.28 Hz, 2H), 7.52 (d, *J* = 7.28 Hz, 2H), 7.42 (t, *J* = 12.28 Hz, 2H), 7.30 (m, 7H), 6.96 (d, *J* = 8.73 Hz, 2H), 5.11 (q, 2H), 4.56 (t, *J* = 14.97 Hz, 1H), 4.38 (q, 1H), 4.23 (q, 1H), 4.10 (q, 1H), 3.81 (s, 3H), 3.19 (d, *J* = 7.32 Hz, 2H); ESI-MS: m/z $[M+Na]^+$ = 579.2.

4-((9H-fluoren-9-yl)methoxy)-3-(4-methoxyphenylsulfonyl)-4-oxobutanoic acid **(23)**:

Hydrogenolysis of **22** (15.5 g, 27.9 mmol) in THF (140 ml) with Pd/C (1.55 g) was accomplished using procedures described for the synthesis of **11**. Upon column purification (5-10% DCM/hexane), the desired product **23** was isolated (12.2 g, 26.2 mmol, 94%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.78 (q, 4H), 7.60 (d, J = 7.28 Hz, 1H), 7.52 (d, J = 7.28 Hz, 1H), 7.42 (t, J = 12.28 Hz, 2H), 7.30 (m, 2H), 6.96 (d, J = 8.73 Hz, 2H), 4.50 (m, 2H), 4.25 (t, J = 17.76 Hz, 1H), 3.81 (s, 3H), 3.15 (d, 1H), 3.81 (s, J = 9.09 Hz, 2H); ESI-MS: m/z $[M+Na]^+$ = 489.1.

(9H-fluoren-9-yl)methyl 2-(4-methoxyphenylsulfonyl)-4-(trityloxyamino)butanoate **(24)**:

Acid **23** (12.2 g, 26.2 mmol) was coupled with trityl-protected hydroxylamine (8.65 g, 31.4 mmol) and DCC (6.48 g, 31.4 mmol) in DCM (130 ml) using procedure described previously[1]. After column purification (20 – 30% EA/hexane), **24** (13.8 g, 19.1 mmol, 73%) was isolated as a white solid. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.78 (q, 4H), 7.60 (d, J = 7.28 Hz, 1H), 7.52 (d, J = 7.28 Hz, 1H), 7.35 (m, 20H), 6.93 (d, J = 9.06 Hz, 2H), 4.48-4.35 (m, 1H), 4.30-4.08 (m, 3H), 3.83 (s, 3H), 2.50 (m, 1H), 2.08 (m, 1H); ESI-MS: m/z $[M+Na]^+$ = 746.2.

2-(4-methoxyphenylsulfonyl)-4-oxo-4-(trityloxyamino)butanoic acid **(4)**:

24 (13.8 g, 19.1 mmol) was stirred in 20% piperidine (in DMF; 100ml) at room temperature for 15 min. The reaction mixture was concentrated *in vacuo* and purified by column chromatography (5-10% MeOH/DCM) to furnish **4** as an off-white solid (10.1 g, 18.5 mmol, 97%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.72 (d, J = 8.34 Hz, 2H), 7.35-7.24 (m, 16H), 4.10-4.02 (m, 1H), 3.87 (s, 3H), 2.58-2.49 (m, 1H), 2.47-2.32 (m, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 164.2, 146.8, 140.6, 131.4, 129.0 – 127.5 (m), 114.3, 65.7, 55.7, 45.8, 29.7. ESI-MS: m/z $[M+Na]^+$ = 568.2.

2.4 Synthesis of azide (29)

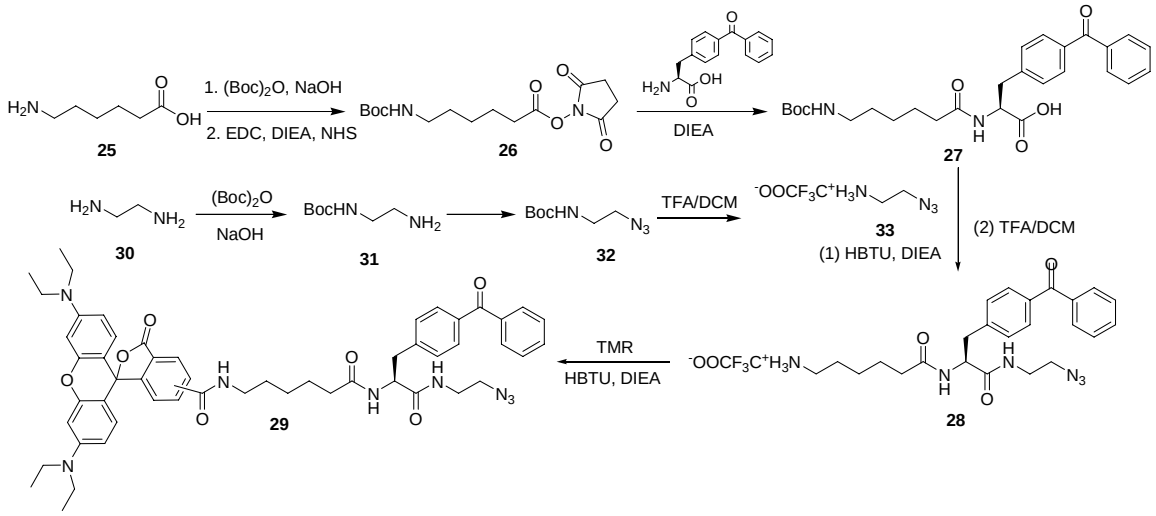


Figure S4. Synthetic scheme of the azide **29**.

NHS ester **(26)**:

To a solution of 6-aminohexanoic acid **25** (7.87 g, 60 mmol) and NaOH (2 M, 50 ml) in dioxane/water (2/1; 180 ml) at 0 °C was added $(Boc)_2O$ (15.7 g, 72 mmol). The reaction was allowed to proceed at room temperature overnight. Subsequently, dioxane was removed under reduced pressure and the resulting mixture was acidified to pH 2 with 1 M HCl, followed by extraction with EA (3 x 80 ml). The combined organic layers were dried over Na_2SO_4, filtered and concentrated to furnish a colorless oil, which was subsequently reacted with NHS (8.06 g, 70 mmol) and EDC (13.42 g, 70 mmol) in DMF at room temperature overnight. Upon column purification (20 - 40% EA/hexane), the desired product **26** was obtained as colorless oil (13.8 g, 42.0 mmol, 70%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 4.60 (br s, 1H), 3.16 – 3.05 (m, 2H), 2.82 (s, 4H), 2.60 (t, J = 7.49 Hz, 2H), 1.81 – 1.70 (m, 2H), 1.51 – 1.40 (m, 13H); ESI-MS: m/z $[M+Na]^+$ = 351.3.

3-(4-benzoylphenyl)-2-(6-(tert-butoxycarbonyl)hexanamido)propanoic acid **(27)**:

26 (1.64 g, 5 mmol) dissolved in 15ml DMF was added H-p-Bz-Phe-OH (1.35 g, 5 mmol) and DIEA (1.05 ml, 6 mmol). The reaction mixture was stirred under N_2 overnight. After that, DMF was removed *in vacuo*. The residue was taken into EA and washed with 1 M HCl. The EA layer was dried over Na_2SO_4, filtered and concentrated. Upon column chromatography (5-10% MeOH/DCM), the final product **27** was obtained as colorless oil (2.05 g, 4.25 mmol, 85%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.08 (s, 1H), 7.74 – 7.66 (m, 4H), 7.63 – 7.56 (m, 1H), 7.49 – 7.44 (m, 2H), 7.31 – 7.26 (m, 2H), 4.83 (br s, 1H), 3.41 – 2.96 (m, 2H), 2.21 – 2.05 (m, 2H), 1.61 – 1.47 (m, 2H), 1.47 – 1.32 (m, 11H), 1.30 – 1.14 (m, 2H); ESI-MS: m/z $[M-1]^-$ = 481.5.

tert-butyl 2-aminoethylcarbamate **(31)**:

A solution of di-tert-butyl dicarbonate (6.1 g, 28 mmol) in DCM (400 ml) was added dropwise over 6 h to a solution of ethylenediamine (11.2 ml, 166.7 mmol) predissolved in DCM (50 ml) while maintaining vigorous stirring. The reaction was continued with stirring for another 24 h at room temperature. Upon concentration, the resulting oil was taken into aqueous sodium carbonate (600 ml) and extracted with dichloromethane (2 x 300 ml). The organic layer was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to yield **5** as colourless oil (4.47 g, 100%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 5.07 (br s, 1H), 3.17 – 3.06 (m, 2H), 2.74 (t, J = 5.91 Hz, 2H), 1.39 (s, 9H); ESI-MS: m/z $[M+1]^+$ = 161.2.

tert-butyl 2-azidoethylcarbamate **(32)**:[4]

Sodium azide (2.25 g, 34.7 mmol) was dissolved in a mixture of H_2O (5.7 ml) and CH_2Cl_2 (9.5 ml) at 0 ^oC. Triflyl anhydride (1.18 ml, 7.04 mmol) was subsequently added dropwise to the solution. The reaction was continued for 2 h. The CH_2Cl_2 layer was removed and the aqueous portion was extracted with CH_2Cl_2 (2 x 4.75 ml). The combined organic fractions, which contain triflyl azide, were washed once with saturated Na_2CO_3 and directly added to a solution containing **31** (564 mg, 3.52 mmol), K_2CO_3 (731 mg, 5.3 mmol), $CuSO_4$ pentahydrate (8.8 mg, 35.2 μmol), distilled H_2O (11.4 ml) and CH_3OH (22.7 ml). The resulting mixture was stirred at ambient temperature overnight. Subsequently, the organic solvents were removed under reduced pressure and the aqueous slurry was diluted with H_2O (75 ml) and acidified to pH 2 with 6 N HCl.

Following extraction with DCM (3 × 50 ml), the combined organic layers were washed with H_2O, dried over $MgSO_4$, and concentrated *in vacuo*. Upon column purification, the desired product **32** was isolated as colorless oil (590 mg, 3.17 mmol, 90%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 5.14 (br s, 1H), 3.22 – 3.11 (m, 2H), 2.79 (t, *J* = 5.91 Hz, 2H), 1.45 (s, 9H); ESI-MS: m/z $[M+1]^+$ = 187.1.

2-azidoethanamine **(33)**:

32 was deprotected by TFA/DCM as described above to give **33** (97% yield). ^{1}H-NMR (300 MHz, MeOD) δ 3.67 (t, *J* = 5.75 Hz, 2H), 3.07 (t, *J* = 5.58 Hz, 2H); ESI-MS: m/z $[M+1]^+$ = 87.1.

6-amino-N-(1-(2-azidoethylamino)-3-(4-benzoylphenyl)-1-oxopropan-2-yl) hexanamide **(28)**:

To compound **27** (1.93 g, 4 mmol) dissolved in DMF was added HBTU (1.90 g, 5 mmol) and DIEA (0.87 ml, 5 mmol) at 0 ^{o}C. The mixture was stirred for 10 min before addition of **33** (0.43 g, 5 mmol). The mixture was further agitated for another 12 h at room temperature, after which DMF was removed *in vacuo* and the residue was taken into ethyl acetate (50 ml). The organic layer was washed with saturated $NaHCO_3$ (2 x 30 ml), 1 M HCl (2 x 30ml), brine (2 x 30 ml), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to afford a yellow oily product. Purification of this compound by flash chromatography (silica gel, ethyl acetate/hexane = 3:1) furnished the intermediate as a white solid (1.65g, 3mmol, 75%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.81 – 7.65 (m, 4H), 7.53 (t, *J* = 7.32 Hz, 1H), 7.41 (t, *J* = 7.50 Hz, 2H), 7.28 (m, 2H), 4.88 – 4.72 (m, 1H), 3.55 – 3.36 (m, 4H), 3.36 – 3.04 (m, 4H), 2.14 (t, *J* = 7.49 Hz, 2H), 1.62 – 1.48 (m, 2H), 1.47 – 1.31 (m, 11H), 1.27 – 1.22 (m, 2H); ESI-MS: m/z $[M+23]^+$ = 573.4.

Deprotection of above intermediate (1.65 g, 3 mmol) with TFA (10 eq) in DCM gave **28** as yellow oil (1.35 g, 3 mmol, ~100%). ^{1}H-NMR (300 MHz, MeOD) δ 7.91 – 7.75 (m, 4H), 7.66 (t, *J* = 7.32 Hz, 1H) 7.54 (t, *J* = 7.49 Hz, 2H), 7.44 (d, *J* = 8.37 Hz, 2H), 4.73 – 4.68 (m, 1H), 3.41 – 3.31 (m, 4H), 3.28 – 3.21 (m, 1H), 3.04 – 2.97 (m, 1H), 2.91 – 2.85 (m, 2H), 2.25 – 2.20 (m, 2H), 1.66 – 1.50 (m, 4H), 1.36 – 1.23 (m, 2H); ESI-MS: m/z $[M+1]^+$ = 451.4.

Azide **(29)**:

Coupling of **28** with Rhodamine followed the same procedures as conversion **27** to **28**. Column purification (5%-10%-15% MeOH/DCM) was carried out to isolate the pure product (50% yield). ^{1}H-NMR (300 MHz, MeOD) δ 8.60 – 8.42 (m, 1H), 8.26 – 8.08 (m, 1H), 7.82 (s, 1H), 7.71 – 7.37 (m, 10H), 7.15 – 6.97 (m, 6H), 4.70 – 4.65 (m, 1H), 3.78 – 3.61 (m, 8H), 3.35 – 3.32 (m, 2H), 3.31 – 3.19, (m, 1H), 2.99 – 2.89 (m, 1H), 2.19 (t, *J* = 7.49 Hz, 2H), 1.63 – 1.46 (m, 4H), 1.39 – 1.20 (m, 14H); ESI-MS: m/z $[M+1]^+$ = 920.2.

3. Construction of MMP probes using "Click Chemistry"

3.1. Synthesis of probes (A-H):

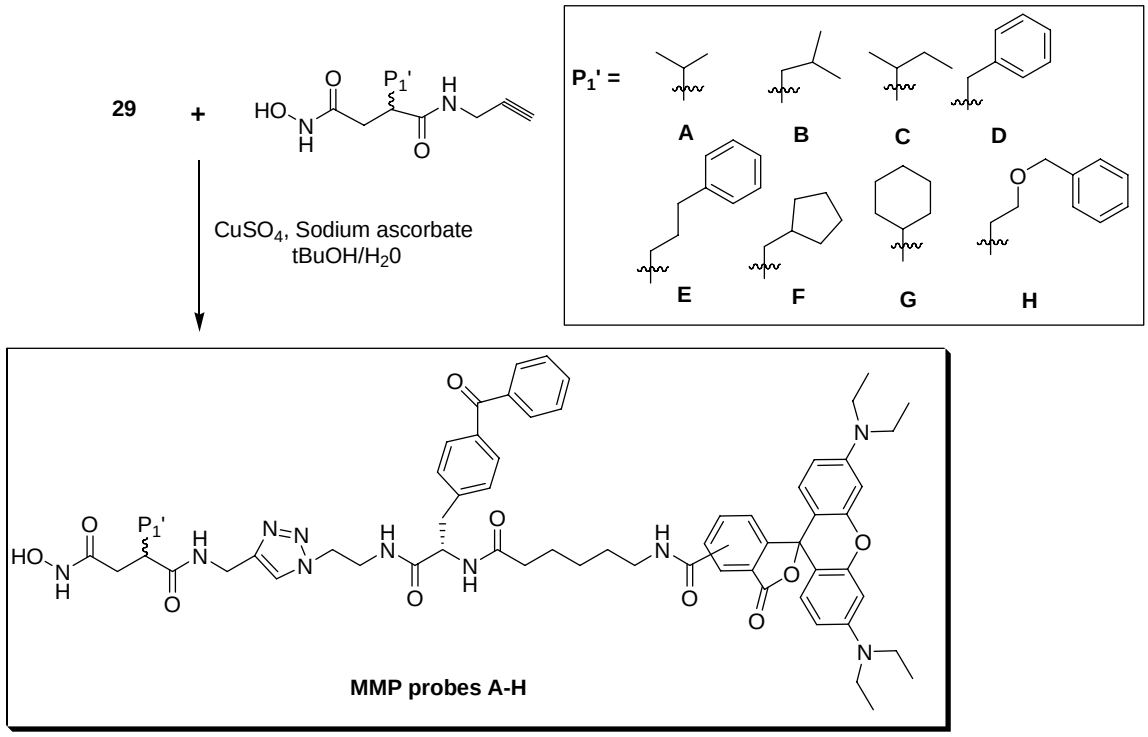


Figure S5. Synthesis of probes (**A**-**H**).

Synthesis of the above 8 hydrophobic alkyne-containing warheads followed procedures similar to ones previously,[3] and details will be reported elsewhere. As shown in **Figure S5**, the alkyne (24 µmol, 1.2 eq) and the azide **29** (20 µmol, 1 eq) were dissolved in a minimal amount of DMSO. A mixture of *t*BuOH/H_2O solution (1:1; 1 ml) was subsequently added and the reaction was shaken for a few minutes to obtain a clear solution. The "click chemistry" was initiated by sequential addition of catalytic amounts of sodium ascorbate (0.1 eq) and $CuSO_4$ (0.01 eq). The reaction was continued with shaking at room temperature for another 12 hrs. Upon further dilution with DMSO (1 ml), the reaction product was directly injected on LC-MS; results indicated the complete consumption of the azide and quantitative formation of the triazole final product in all cases. The final probes (**A-H**) were subsequently purified by semi-prep reverse phase HPLC and characterized by MS. Semi-prep HPLC conditions: 30% to 80% A in 60 min gradients.

3.2. Synthesis of probes (I-L):

The synthesis of probes (**I-L**) following scheme shown in **Figure S5** was problematic, due to the polar nature of the unprotected side chains which rendered the warheads difficult to purify before the "Click Chemistry" step. Consequently, the

scheme below (**Figure S6**) was used to synthesize the probes instead. Briefly, warheads **1**-**4** were converted to the corresponding alkynes, **i**-**l**. Subsequently, "Click Chemistry" was carried out with the protected warheads, followed by direct TFA treatment to remove the protecting groups. Upon HPLC purification, the final probes (**I**-**L**) were obtained in pure form and characterized by LC-MS.

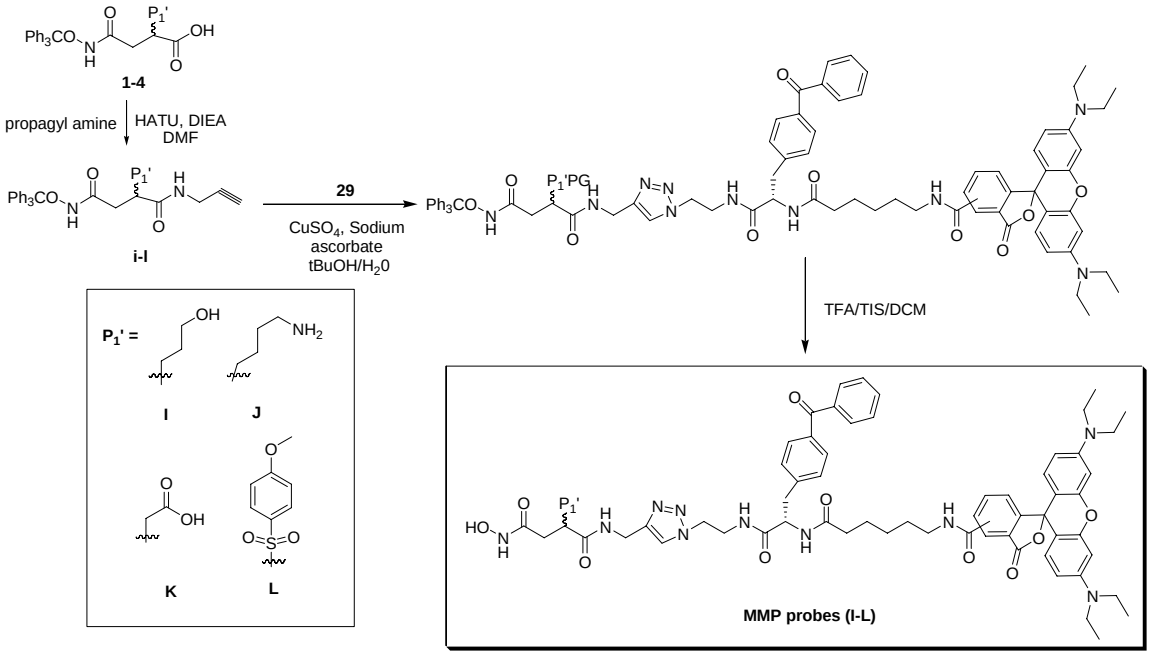


Figure S6. Synthesis route of probes (**I**-**L**).

General procedure for the coupling of **1**-**4** with propargyl amine

To a solution of acid (**1** to **4**) (5 mmol), HATU (6 mmol) and DIEA (6 mmol) in DMF (10 ml) was added propargyl amine (6 mmol) at room temperature under a nitrogen atmosphere. The mixture was stirred for 2-4 hrs. The solvent was removed *in vacuo* and the resulting oil residue was diluted with DCM and extracted with water. The combined DCM layers were dried over Na_2SO_4, filtered and concentrated. The crude product was purified by column chromatography (80% DCM/hexane – 10% MeOH/DCM) to afford the alkyne (**i** to **l**) in 75 – 90% yield, typically as a white or off-white solid.

N^1-(prop-2-ynyl)-N^4-(trityloxy)-2-(3-(trityloxy)propyl)succinamide **(i)**

Yield = 85%. ^{1}H-NMR (300 MHz, MeOD) δ 7.42-7.27 (m, 30H), 3.99-3.78 (m, 2H), 3.12-2.95 (m, 2H), 2.89 (s, 1H), 2.62-2.52 (m, 1H), 2.50-2.38 (m, 1H), 2.25-2.10 (m, 1H), 1.50-1.21 (m, 4H); ESI-MS: m/z $[M+23]^+$ = 736.1.

tert-butyl 7-oxo-5-(prop-2-ynylcarbamoyl)-7-(trityloxyamino) heptylcarbamate **(j)**

Yield = 80%. ^{1}H-NMR (300 MHz, MeOD) δ 7.46-7.33 (m, 15H), 4.00-3.75 (m, 2H), 3.09-2.92 (m, 2H), 2.62-2.58 (m, 1H), 2.52-2.45 (m, 1H), 2.25-2.11 (m, 1H), 2.09-2.02 (m, 1H), 1.50 (s, 9H), 1.48-1.25 (m, 4H), 1.25-1.15 (m, 4H); ESI-MS: m/z $[M+23]^+$ = 606.3.

tert-butyl 5-oxo-3-(prop-2-ynylcarbamoyl)-5-(trityloxyamino) pentanoate **(k)**

Yield = 90%. ^{1}H-NMR (300 MHz, MeOD) δ 7.43-7.26 (m, 15H), 3.99-3.57 (m, 4H), 2.80 (s, 1H), 2.48-2.32 (m, 1H), 2.30-2.15 (m, 2H), 1.42 (s, 9H); ESI-MS: m/z $[M+23]^+ = 549.3$.

2-(4-methoxyphenylsulfonyl)-N^1-(prop-2-ynyl)-N^4-(trityloxy)succinamide **(l)**

Yield = 75%. ^{1}H-NMR (300 MHz, MeOD) δ 7.42-7.22 (m, 19H), 4.04-3.62 (m, 4H), 3.05 (s, 3H), 2.35-2.22 (m, 1H), 2.18 (s, 1H), 2.12-1.90 (m, 2H); ESI-MS: m/z $[M+23]^+ = 605.2$.

"Click Chemistry" followed by TFA deprotection

The alkyne **i-l** (24 µmol, 1.2 eq) and the azide **29** (20 µmol, 1 eq) were dissolved in a minimal amount of DMSO. A mixture of ^{t}BuOH/H_2O solution (1:1; 1 ml) was subsequently added and the reaction was shaken for a few minutes to obtain a clear solution. The "click chemistry" was initiated by sequential addition of catalytic amounts of sodium ascorbate (0.1 eq) and $CuSO_4$ (0.01 eq). The reaction was continued with shaking at room temperature for another 12 hrs. Upon further dilution with DMSO (1 ml), the reaction product was directly injected on LC-MS; results indicated the complete consumption of the azide and quantitative formation of the triazole final product in all cases. Subsequently, TFA (4 ml) and TIS (200 µl) were added to the reaction mixtures. The mixture was stirred for 1 hr, following which the solvent was removed *in vacuo*. The crude product was directly purified by semi-prep RP-HPLC (conditions: 30% to 100% A in 60 min).

4. LC-MS characterization of the final probes (A-L):

Due to the presence of two isomeric forms of the Rhodamine dye used (e.g. 5-and 6-Tetraethylrhodamine) in the synthesis, both isomers of all 12 final probes were produced as a result (**Figure S7**). They were unambiguously confirmed by LC-MS profiles which indicate, in every final probe, two peaks with equal intensity and identical molecular weight, but differing slightly in the retention time, were produced. We were able to isolate both isomeric forms in all but one cases (**A-L**).

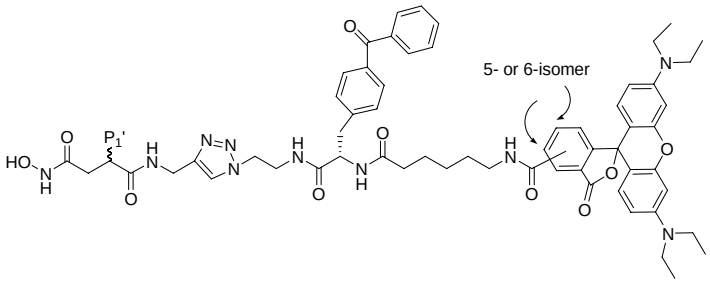
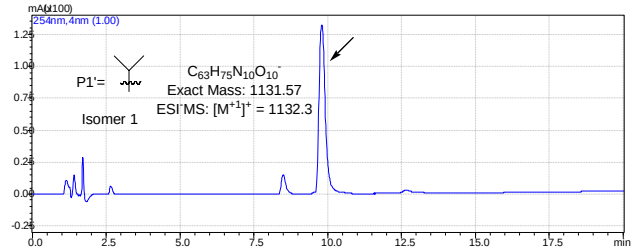
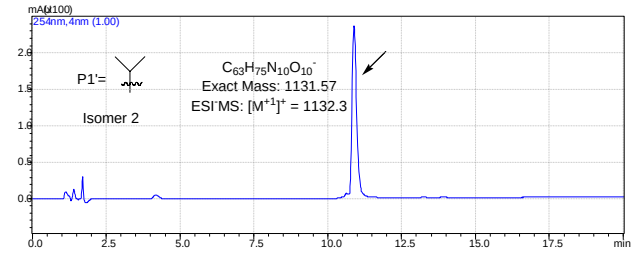


Figure S7. Two isomeric forms of the final probes as a result of the Tetraethylrhodamine dye

Probe A:

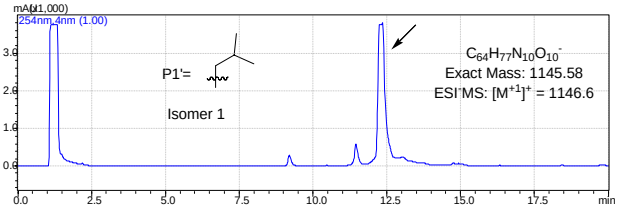
LC conditions: 30% → 80% A in 20 min

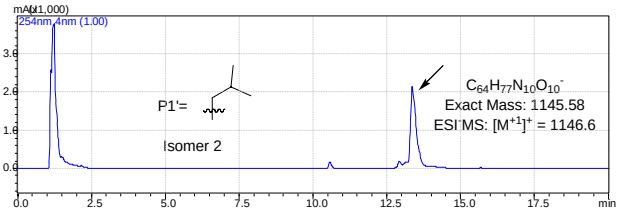




Probe B:

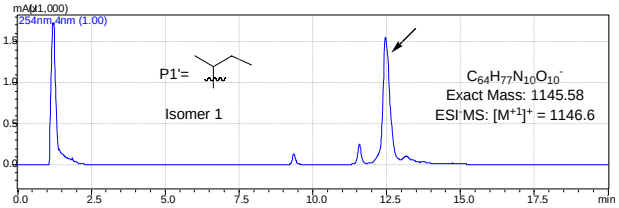
LC conditions: 30% → 80% A in 20 min

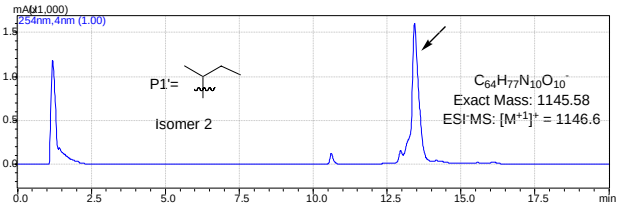




Probe C:

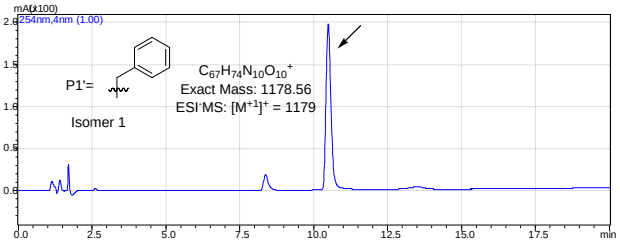
LC conditions: 30% → 80% A in 20 min

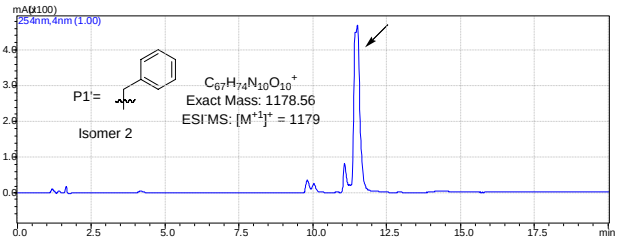




Probe D:

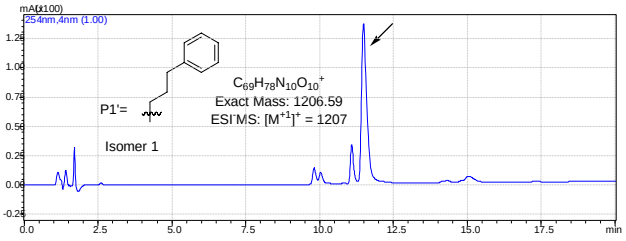
LC conditions: 30% → 80% A in 20 min

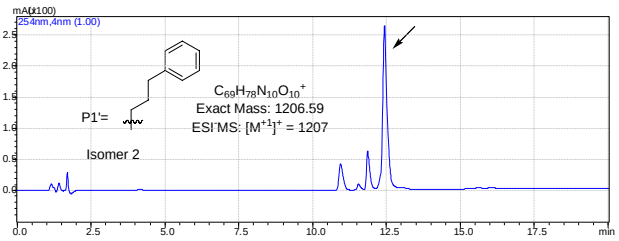




Probe E:

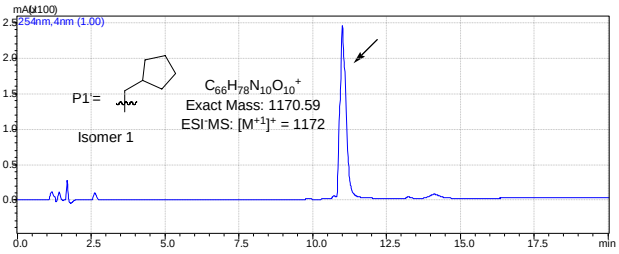
LC conditions: 30% → 80% A in 20 min

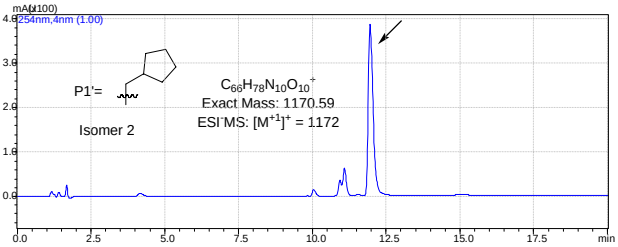




Probe F:

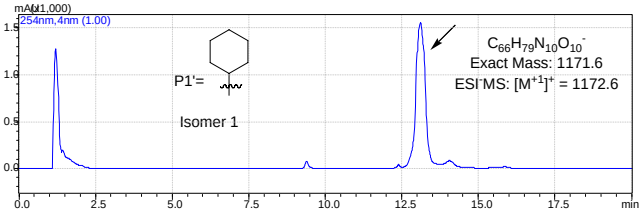
LC conditions: 30% → 80% A in 20 min

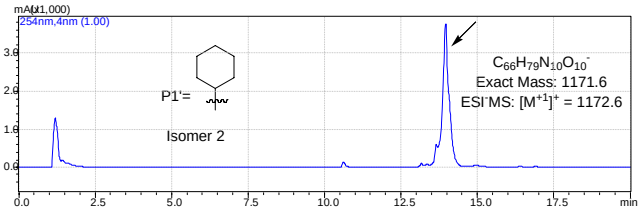




Probe G:

LC conditions: 30% → 80% A in 20 min





Probe H:

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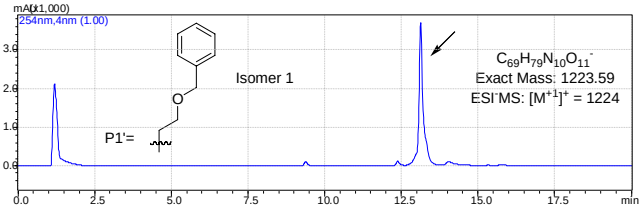
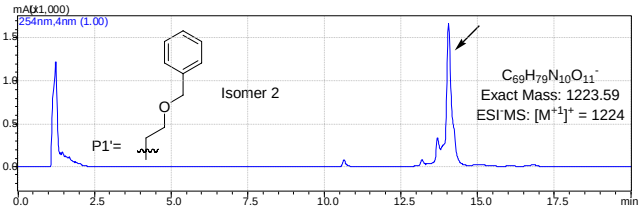
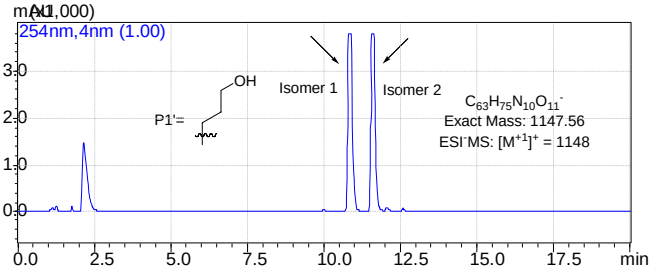




Probe I:

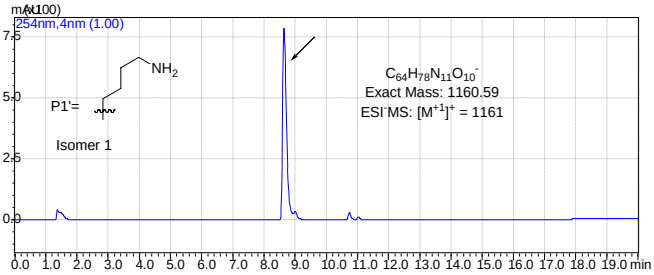
LC conditions: 30% → 100% A in 20 min

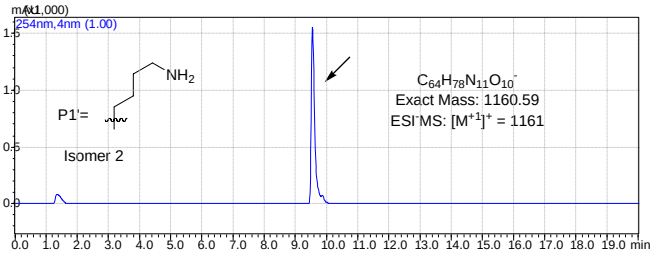


*We did not manage to isolate the two isomeric forms of probe **I** by semi-prep HPLC, as shown above.

Probe J:

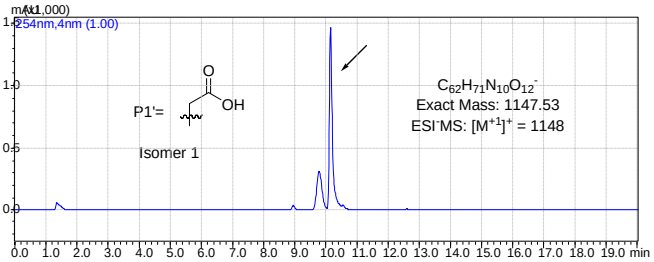
LC conditions: 30% → 100% A in 20 min

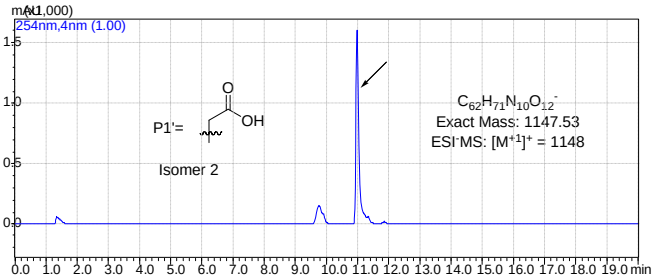




Probe K:

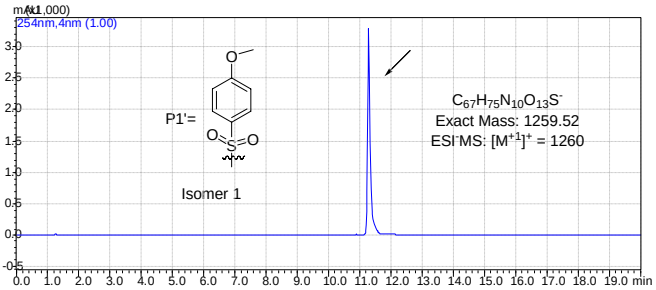
LC conditions: 30% → 100% A in 20 min

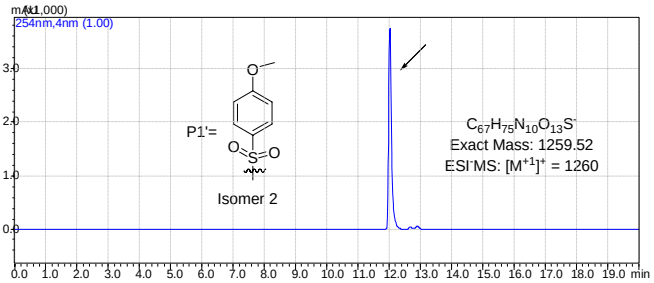




Probe L:

LC conditions: 30% → 100% A in 20 min

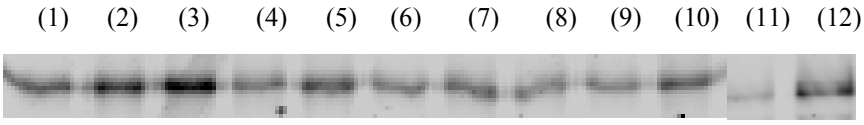




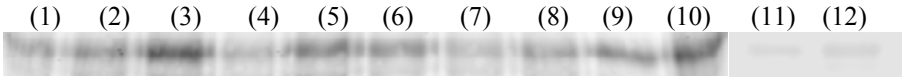
5. Enzyme Fingerprinting Procedures:

Fingerprints against various metalloproteases were obtained using gel-based experiments, as previously described.[5] Briefly, each probe was added at a final concentration of 500 nM to various enzymes in 20 µl reaction volumes buffered with 50 mM Tris.HCl (pH 8). Following a short incubation (30 min) at room temperature in the dark, the samples were irradiated under low-wavelength UV irradiation for a further 20 min. The reaction was quenched with the addition of SDS loading dye and subsequent boiling at 95 ^{o}C for 10 min. Samples were resolved on a 10% denaturing SDS-PAGE gel and fluorescence was detected on a Typhoon fluorescence gel scanner (GE Healthcare, USA). Observed fluorescence bands corresponding to labelled proteins were quantitated using the ImageQuant software (GE Healthcare, USA). The representative gel profiles against our panel of enzymes are shown below (**Figure S8**). The fingerprints are presented in **Figure 1** (in the maintext) as intensity heat-maps by normalizing against the highest signals within each dataset. Cluster analysis was performed using Gene Cluster (http://rana.stanford.edu/software) using average linkage clustering and visualized using the TreeView software (http://rana.stanford.edu/software). We further confirmed that heat-denatured enzymes did not show labelling with our probes, indicating that the observed labelling was dependant on enzyme activity (data not shown).

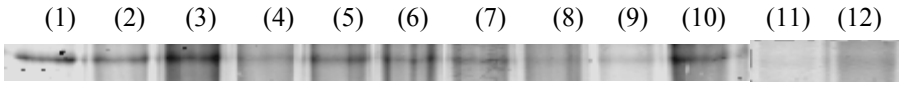
Thermolysin 1 μg/lane



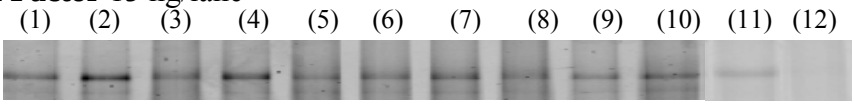
Carbonic Anhydrase 1 μg/lane



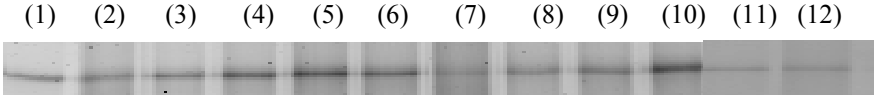
Bacterial Collagenase 1 μg/lane



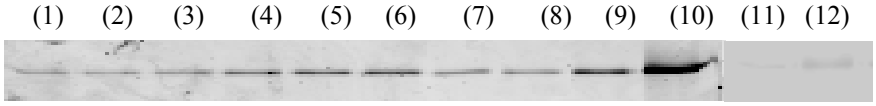
Anthrax Lethal Factor 13 ng/lane



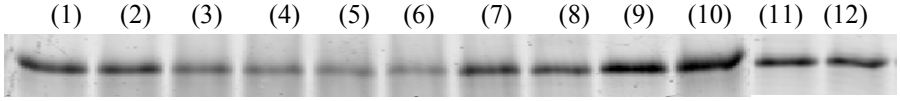
MMP-7 96 ng/lane



MT1-MMP 30 ng/lane



MMP3 10 ng/lane



Probes: (1) **Leu** (**B**); (2) **Ile** (**C**); (3) **Phe** (**D**); (4) **Long-Phe** (**E**); (5) **Val** (**A**); (6) **Cyclohexyl** (**F**); (7) C**yclopentyl** (**G**); (8) **O-Ph** (**H**); (9) **Long-OH** (**I**); (10) **Lys** (**J**); (11) **Asp** (**K**); (12) **Sulfone** (**L**).

Figure S8

6. UV-Dependent Labeling:

A control experiment was performed with a representative enzyme to ensure that cross-linking was UV-dependant. Using the standard conditions indicated above, a 500 nM final concentration of the **Sulfone** probe was used to label 1 μg of thermolysin in the presence and absence of UV irradiation. As would be expected, labeling was only observed upon activation with no detectable non-specific labeling (**Figure S9**).

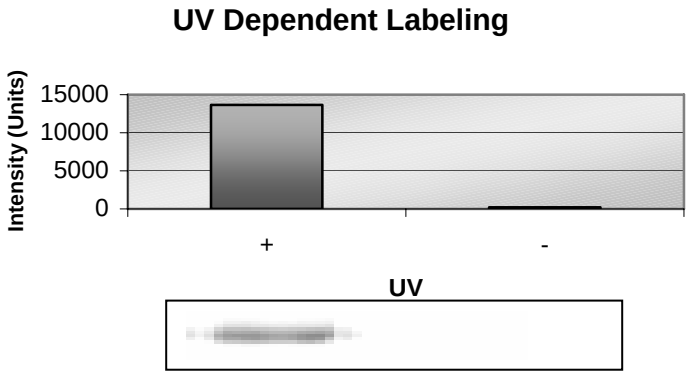


Figure S9. UV-dependent labelling experiment with Thermolysin and the Sulfone probe (**L**).

7. Concentration dependent labeling:

Protein concentration-dependent labeling

A two-fold dilution series of thermolysin was prepared from 50 µg/ml to 3.125 µg/ml. Labeling was performed using the protocol described with the **Leu** probe at a 500 nM concentration. The results from these experiments shown below (**Figure S10**) indicate that the probes bind in a concentration-dependant manner.

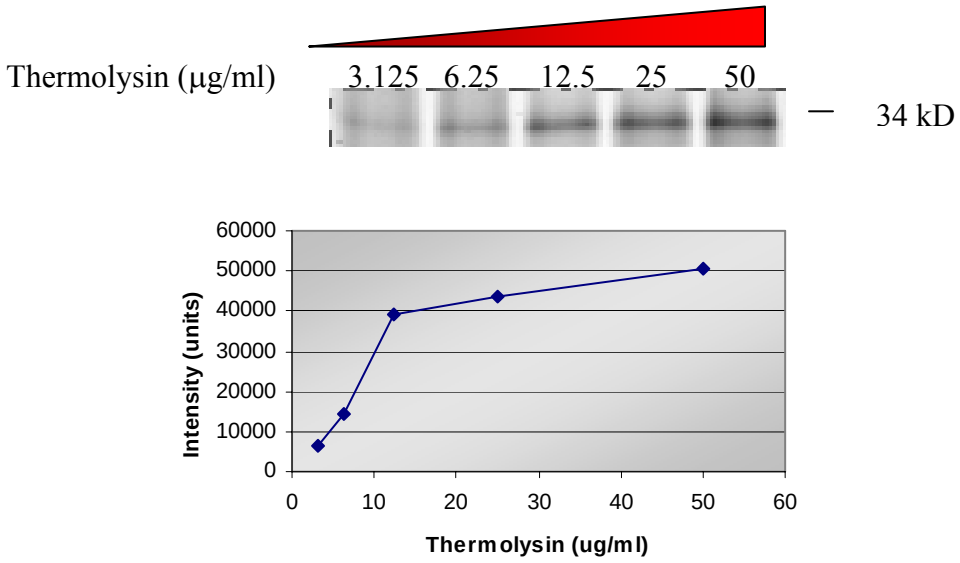
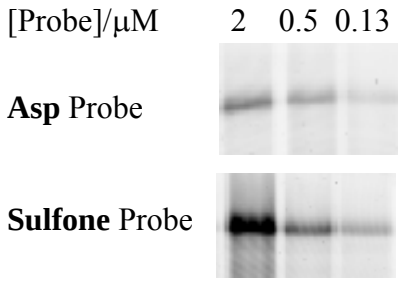


Figure S10. Protein concentration-dependent labeling experiment.

Probe concentration-dependent labeling

A series of probe concentrations ranging from 2 μM to 0.13 μM was used to label thermolysin (5 μg/lane). The representative results obtained with the **Asp** and the **Sulfone** probe are shown below to highlight that labeling was exhibited in a concentration-dependant manner (**Figure S11**).



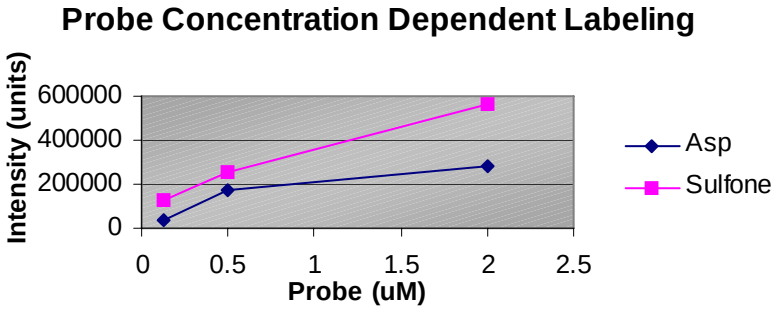


Figure S11. Protein concentration-dependent labeling experiment.

8. Labeling in the presence of complex cellular lysates:

We tested the ability of our probes to selectively label our proteins of interest within a complex cellular mileu, following similar procedures described previously.[5] *E. coli* cell lysates were derived by growing XL1 Blue Gold cells to OD_{600} 0.5 in Luria Bertani (LB) Broth. The cells were pelleted and resuspended in PBS (containing 0.1% Tween) before being lysed by sonication. The protein extract was separated from cellular debris through subsequent centrifugation for 40 min at 20,000g and was found to produce only faint bands when screened with some of the probe library members. We then spiked thermolysin to 100 μg/ml in a concentration gradient of protein extract. The **Leu** probe was then added to a final concentration of 1 μM and the mixture was incubated for 30 min and irradiated with UV as previously described.[5] The results indicate that thermolysin was readily labeled by the **Leu** probe even when diluted over 10 fold in the bacterial lysate, demonstrating that future experiments could be performed directly with cellular extracts (**Figure S12**). Similar results have been independently verified by Cravatt *et al*.[6]

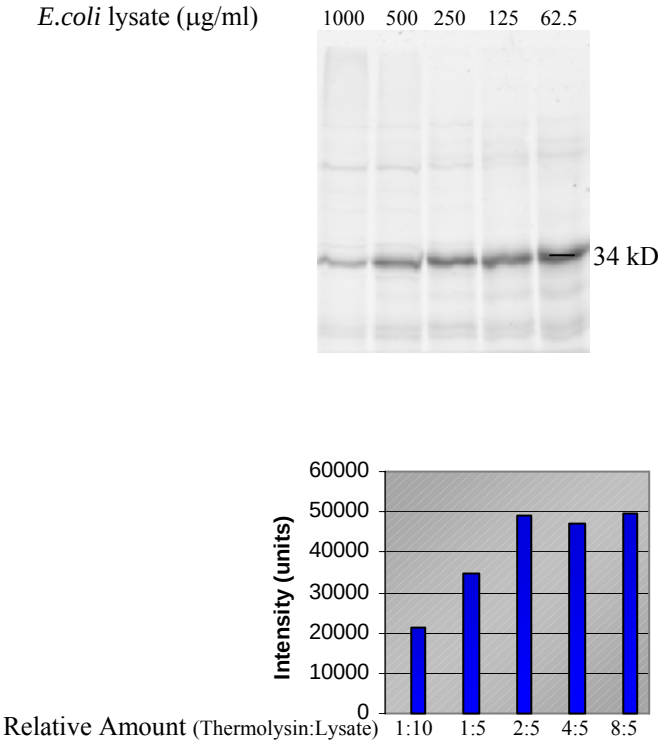


Figure S12. Cell lysate experiments in which thermolysin was spiked in bacterial cell lysates and labeled by the **Leu** probe.

9. Labeling with probes on a protein microarray platform:

We tested the feasibility of screening proteins rapidly in a high-throughput format with our probe library, following protocols modified based on previously published procedures.[7] The protein microarray offers multiple applications in protein characterization and annotation and we tested the potential of using our probes with this platform in detecting enzymatic activity of metalloproteases in a miniaturized format. In a proof of concept experiment, selected proteins were spotted using an ESI SMA™ arrayer (Ontario, Canada) on hydrogel slides (Perkin Elmer, USA). Samples were spotted in 0.1 M $NaHCO_3$ (pH 9) in triplicate using SMP15B Pins (TeleChem International Inc, USA) with a spacing of 750 μm between spots (the pins produced 9.4 nl spots with a diameter of ~550 μm). The pins were rinsed between samples using two cycles of wash (for 10 s) and sonication (for 10 s) in reservoirs containing 70% ethanol followed by drying under reduced pressure (for 10 s). The slides were incubated overnight at 4 ^{o}C before blocking with 1% BSA in phosphate buffered saline (PBS) (pH 7.4) for 1 hr (according to manufacturer's protocols). They were treated with 10 μM of the **Leu** probe in 100 μl PBS containing 1% BSA (pH 7.4) under coverslip for 30 min in a dark humid chamber at room temperature. Following irradiation under short wave UV for 20 min and rinsing with PBS for 10min, the slides were scanned on an ArrayWoRx™

microarray scanner (Applied Precision, USA) equipped with the relevant filters for rhodamine ($\lambda_{ex/em}$ = 548/595 nM). The resulting images from duplicated grids are shown in the maintext (**Fig. 3**). From the results, we observed strong labeling with protein spotted on 3D hydrogel slides whilst screens with proteins immobilized covalently on NHS surfaces gave consistently inferior results. Positive signals were observed for several metalloproteases/zinc-binding proteins, namely Collagenase, Thermolysin and Carbonic anhydrase. Anthrax Lethal Factor, as well as several MMPs, failed to label under these conditions presumably because of the relatively low enzyme concentration (from the original stock from commercial vendor) used in the experiment. As expected, a serine protease, β-chymotrypsin spotted alongside as a negative control did not label with our MMP probe. We also observed that when 20 mM EDTA was incorporated in the probe incubation buffer, no labeling was observed (data not shown), indicating that labeling of the probe to the enzymes is zinc-dependent. Collectively these results demonstrate that the protein microarray platform may be utilized in future experiments for high-throughput protein fingerprinting using our library of probes. Detailed studies of this work will be reported in due course.

10. Docking Simulations:

In order to visualize the binding configurations, we docked several of the probes that successfully labelled the MMP-7 using the Sybyl™ software, on the FlexX suite.[8] Coordinates for the MMP-7 protein were obtained from the Protein Data Bank accession 1MMQ.[9] Probe structures were drawn and using the "Sketch Molecule" option and hydrogens were added. The structures were minimized using 100 iterations at 0.05 kcal/mol Å to relieve any torsional strain and formal charges were assigned. Water molecules were removed from the protein structure, and the docking sphere was set at 15 Å and centered on the zinc residue in the enzyme active site. Applying these criteria, the docking was performed for 30 iterations, with the most optimized configuration for selected probes displayed in **Figure S13**. Protein surface representation was rendered by MOLCAD. As would be expected, the optimized docking configuration of the inhibitor/enzyme complex shows the probes adopt an extended conformation, fitting comfortably in the enzyme active site (**Figure S13**). The hydroxamate group from the inhibitor was shown to chelate to the bound zinc atom. Probes having small side chains (**Leu**, **Phe** and **Val**) fit nicely into the S_1' pocket of MMP-7 (**Figure S13**, A-C). As expected in the original design, the extended probe structure makes several contacts with the available subsites of the enzymes, ameliorating the interaction. In addition, a number of favorable hydrogen bonds were also evident in the complex.

A

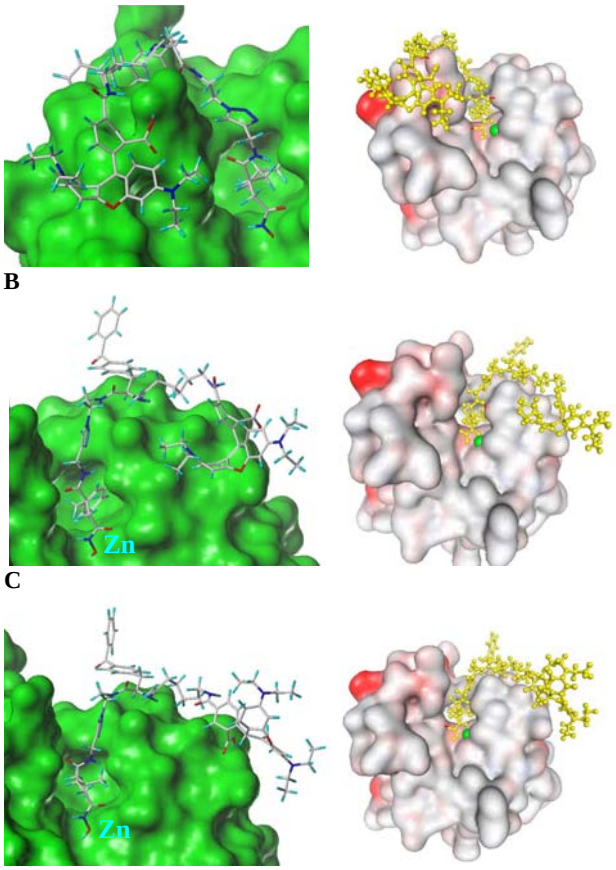


Figure S13. *In silico* docking displays the possible binding mode of the MMP-7 probe complex. The hydroxamic group in the inhibitor chelates with the zinc atom (shown as a green sphere) in the enzyme active site, with the P_1' probe position projecting into the S_1' pockets of the enzyme **A**. **Leu** probe. **B**. **Phe** Probe **C Val** probe. Docking was performed using Sybyl v7.2 (Tripos, Missouri, USA) with electrostatic surface images generated using WebLab ViewerLite (Accelrys, San Diego, USA). Hydrogen bonds are shown as red lines.

11. References:

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