Supplemental Information

Synthesis and in Vitro and in Vivo Evaluation of MMP-12 Selective Optical Probes.

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EXPERIMENTAL SECTION

General information. All chemicals were commercial products of analytical grade and were used without further purification unless otherwise specified. Universal polyethylene glycol (PEG) NovaTag™ resin (code: 855058, substitution: 0.24 mmol/g), Fmoc-(L)-Glu(OtBu)-OH (code: 852009) and Boc-NH-PEG_{27}-COOH (code: 851083) were purchased from Merck Millipore (Darmstadt, Germany). Cy®5 Mono NHS ester and Cy®5.5 Mono NHS ester were purchased from Sigma Aldrich (Saint Louis, MO, USA, code: GEPA15100 and code: GEPA15601 respectively). 6S-IDCC-NHS ester was purchased from IC Discovery (Berlin, Germany, code: 222010). IRDye® 800CW NHS ester was purchased from LI-COR Biotechnology (Lincoln, NE, USA, code: 929-70020). All other reagents were purchased from Sigma Aldrich (Saint Louis, MO, USA). Pseudo peptide synthesis was performed manually in polypropylene syringe equipped with a polyethylene frit and a stopper. Microwave experiments were performed on a Discover apparatus (CEM μWave, Matthews, NC, USA) using open vessel mode with SPS kit. For compounds **6** to **12**, ^{1}H (200 MHz), ^{13}C (50 MHz) and ^{31}P (81 MHz) NMR spectra were recorded on a Varian 200 MHz Mercury spectrometer. For probes **1** to **4**, ^{1}H NMR spectra were recorded on a Avance Brucker 600 MHz with a TCi Cryo-probe. ^{1}H and ^{13}C spectra are referenced according to the residual peak of the solvent based on literature data[1]. ^{31}P NMR chemical shifts are reported in ppm downfield from 85% H_3PO_4 (external standard). ^{13}C and ^{31}P NMR spectra are fully proton decoupled. Column silica gel plates (E. Merck silica gel $60F_{254}$) and components were visualized by the following methods: UV light absorbance, and/or charring after spraying with a solution of $NH_4^+HSO_4^-$. A $CHCl_3$/MeOH/AcOH 7:2:1 solvent system was used for all R_f values reported in the experimental section, unless otherwise noted. Melting points (measured on an Electrothermal apparatus) are uncorrected.

Compound analysis by Reverse Phase High Pressure Liquid Chromatography (RP-HPLC) was performed on a Supelco Ascentis® Express C18 column (100 × 4.6 mm, 2.7 μm, flow rate = 1.2 mL/min), a Grace Vision HT HL C18 column (150 × 4.6 mm, 3 μm, flow rate = 1.2 mL/min) or an Agilent XDB C18 column (150 × 4.6 mm, 5 μm, flow rate = 0.6 mL/min). Compound purification was performed on a Supelco Ascentis® C18 column (150 × 10 mm, 5 μm, flow rate = 3 mL/min). UV detection was performed at 230 nm, 280 nm or at the wavelength corresponding to the maximum of fluorochrome absorption. A solvent system consisting of (A) 0.1% TFA in water and (B) 0.09% TFA in acetonitrile was used. Retention times (Rt) are reported in minutes. UV absorption and fluorescence measurements were performed on a UV-1800 spectrophotometer (Shimadzu, Kyoto Japan), and on a Cary Eclipse apparatus (Varian, Palo Alto, CA), respectively. Mass spectrometry data were collected using a 4800 MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA) or an ESI ion trap Esquire HCT spectrometer (Bruker

Daltonics, Billerica, MA). Amino acid composition was characterized using an aminoTac JLC-500/V amino acid analyser (JEOL, Tokyo, Japan). The identity and purity of each synthesized compound was assessed by NMR, analytical HPLC (two conditions of elution) and mass spectrometry. Fluorescensce measurements were performed at room temperature. Extinction coefficients were calculated from absorbance measurements and solution concentrations determined by amino acid compositions.

Synthetic Methods.

3'-chloro-[1,1'-biphenyl]-4-carbaldehyde (**6**). In a solution of 4-bromobenzaldehyde (69.4 g, 375 mmol) in toluene (900 mL), 3-chlorophenylboronic acid (64.5 g, 414 mmol) and K_2CO_3 (85.8 g, 621 mmol) were added. The resulting mixture was first degassed with Argon, $Pd(PPh_3)_4$ (10.8 g, 9.36 mmol) was added and the reaction mixture was refluxed under Argon atmosphere. After 24 h, complete consumption of the aldehyde was observed and the reaction mixture was washed with 5% $NaCO_3$ (2 × 200 mL). The organic phase was stirred in an open flask overnight, dried over Na_2SO_4 and then evaporated under vacuum. The resulting dark residue was dissolved in a petroleum ether (PE)/methanol 3:1 mixture upon heating and the hot solution was filtered. The filtrates were slowly cooled to room temperature and a crystalline solid was obtained. After filtration, a second crop of crystals were collected from the filtrates upon cooling at 0°C. The combined solids were recrystallized from PE/Et_2O 9.5:0.5 to afford 53.3 g of an off-white solid. The filtrates from the above procedure were concentrated and purified by column chromatography (PE/Et_2O 9.9:0.1 → 9.5:0.5) to afford 23.4 g of compound **6** as a white solid (94%). M. p. 57-60 °C; R_f = 0.40 (PE/Et_2O 9:1); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.31-7.50 (m, 2H), 7.53-7.62 (m, 1H), 7.64-7.71 (m, 1H), 7.78 (d, J = 8.1 Hz, 2H), 7.91 (d, J = 8.1 Hz, 2H); ^{13}C NMR (50 MHz, d_6-DMSO) δ 125.7, 126.8, 127.4, 128.3, 130.0, 130.7, 134.0, 135.4, 140.8, 144.1, 192.4; HRMS (MALDI-TOF$^+$) m/z for $[C_{13}H_{10}ClO]^+$ calcd 217.0420; found: 217.0430. Corresponding spectroscopic data can be found in the literature[2].

3'-Chloro-[1,1'-biphenyl]-4-carbaldehyde oxime (**7**). To a solution of aldehyde **6** (26.9 g, 124 mmol) and $H_2NOH{\cdot}HCl$ (12.9 g, 186 mmol) in EtOH (500 mL), pyridine (37 ml, 460 mmol) was slowly added. The resulting solution was refluxed for 3 h and then concentrated under vacuum. The residue was partitioned in Et_2O (200 mL) and H_2O (200 ml), the organic phase was separated and washed with H_2O (2 × 100 mL), 1 M HCl (2 × 100 mL) and H_2O (100 mL). The organic layer was then dried over Na_2SO_4 and the solvent was evaporated. Compound **7** (28.1 g, 98%) was obtained as a white solid. A crystalline fraction of **7** can be obtained after re crystallization in AcOEt. M.p. 114-116 °C; R_f = 0.55 (PE/AcOEt 9:1); ^{1}H NMR (200 MHz, d_6-DMSO) δ 6.86-7.42 (m, 8H), 7.76 (s, 1H); ^{13}C NMR (50 MHz, d_6-DMSO/$CDCl_3$ 1:1) δ 124.5, 126.2, 126.5, 126.5, 126.8, 129.6, 132.1, 133.8, 139.5, 141.5, 147.4; HRMS (MALDI-TOF$^+$) m/z for $[C_{13}H_{11}ClNO]^+$ calcd 232.0529; found: 232.0533. Corresponding spectroscopic data can be found in the literature[3].

Diethyl 2-{[3-(3'-chloro-[1,1'-biphenyl]-4-yl)isoxazol-5-yl]methyl}malonate (**8**). To a solution of oxime **7** (26.0 g, 112 mmol) in $CHCl_3$ (400 mL), pyridine (6.2 mL, 77 mmol) was added. NCS (16.5 g, 124 mmol) was added at room temperature and the resulting mixture was stirred at 45 °C for 4 h. To this solution, diethyl propargyl malonate (20.6 g, 104 mmol) was added, followed by the addition of Et_3N (14.5 mL, 104 mmol) at the same temperature during 2 h. The reaction mixture was stirred for 48 h at 45 °C. the resulting mixture was concentrated under vacuum. The residue was diluted with AcOEt (500 mL) and washed with 1 M HCl (2 × 200 ml), H_2O (200 ml) and brine (50 ml). The organic phase was dried over Na_2SO_4 and evaporated under vacuum. The crude compound was purified by column chromatography (PE/AcOEt 9.5:0.5→3:1) to afford compound **8** (38.2 g, 86%) as a colourless thick slurry. A crystalline fraction of **8** was obtained after crystallization by a minimum quantity of PE/Et_2O 1:1 (21.1 g). M.p. 51-53 °C; R_f = 0.66 (PE/AcOEt 4:1); ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.27 (t, J = 7.1 Hz, 6H), 3.44 (d, J = 7.6 Hz, 2H), 3.44 (t, J = 7.6 Hz, 1H), 4.23 (t, J = 7.1 Hz, 6H), 6.43 (s, 1H), 7.29-7.43 (m, 2H), 7.44-7.53 (m, 1H), 7.57-7.61 (m, 1H), 7.84 (d, J = 8.1 Hz, 2H), 7.63 (d, J = 8.1 Hz, 2H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 14.1, 26.1, 50.2, 62.1, 100.6, 125.3, 127.3, 127.4, 127.6, 127.8, 128.5, 130.2, 134.9, 141.3, 142.1, 162.1, 168.0, 169.9; HRMS (MALDI-TOF$^+$) m/z for $[C_{23}H_{22}ClNO_5]^+$ calcd. 428.1265; found: 428.1275.

Ethyl 2-{[3-(3'-chloro-[1,1'-biphenyl]-4-yl)isoxazol-5-yl]methyl}acrylate (**9**). Compound **8** (14.0 g, 32.7 mmol) was dissolved in EtOH (130 mL) and the solution was cooled to 0 °C. A solution of KOH (2.38 g, 42.4 mmol) in EtOH (130 mL) was added drop-wise during 1 h. The resulting mixture was stirred at room temperature for 4 h and was then concentrated under vacuum. The residue was diluted with H_2O (700 mL) and the aqueous phase was washed with Et_2O (2 × 150 ml), acidified with 2 M HCl to pH 1 and extracted with AcOEt (3 × 200 mL). The organic layers were dried over Na_2SO_4 and evaporated under vacuum. The resulting white solid (12.6 g) was not isolated and directly suspended in cold AcOEt (126 mL) and Et_2NH (3.75 mL, 36.3 mmol) was slowly added at 0 °C. Paraformaldehyde (1.33 g, 44.3 mmol) was then added and the final mixture was refluxed for 4 h. Evaporation of the volatiles was realized under high vacuum and the residue was partitioned in Et_2O (200 mL) and H_2O (200 ml). The organic layer was washed with 1M HCl (2 × 100 mL), H_2O (100 mL), 5% $NaHCO_3$ (2 × 100 mL), H_2O (50 ml) and brine (50 ml), dried over Na_2SO_4 and evaporated under vacuum. The crude was purified by column chromatography (PE/AcOEt 9.5:0.5→8:2) to afford compound **9** (8.4 g, 86% for two steps) as a colourless thick oil. R_f = 0.68 (PE/AcOEt 4:1); ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.29 (t, J = 7.1 Hz, 3H), 3.84 (s, 2H), 4.23 (t, J = 7.1 Hz, 2H), 5.73-5.77 (m, 1H), 6.36-6.41 (m, 2H), 7.27-7.42 (m, 2H), 7.43-7.51 (m, 1H), 7.56-7.66 (m, 3H), 7.85 (d, J = 8.7 Hz, 2H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 14.1, 29.6, 61.2, 100.2, 125.2, 127.1, 127.2, 127.4, 127.7, 128.0, 128.6, 130.1, 134.7, 135.4, 141.0, 142.0, 161.9, 165.9, 170.8.

(4-Bromophenyl)(2-{[3-(3'-chloro-[1,1'-biphenyl]-4-yl)isoxazol-5-yl]methyl}-3-ethoxy-3-oxopropyl)phosphinic acid (**10**). A solution dichloromethane (147 ml) containing (4-Bromophenyl) phosphinic acid[4] (4.34 g, 19.6 mmol), compound **9** (8.3 g, 22.6 mmol) and Hunig's base (23.9 mL, 137 mmol) in a Schlenk flask was degassed by applying three freeze-pump-thaw cycles. The mixture was then cooled to -78 °C and purged with argon for 15 min. Then, pre cooled at -78 °C freshly distilled TMSCl (17.4 mL, 137 mmol) was added to the reaction vessel at once. The temperature was slowly raised to 25 °C and the clear solution was stirred for 48 h at room temperature. After the end of the reaction, the mixture was cooled to 0°C, abs. EtOH (20 mL) was added drop-wise and stirring at room temperature for 30 min followed. Removal of volatiles under vacuum affords a viscous oily residue that was sonicated in presence of AcOEt (10 mL) and HCl 1M (100 mL) for 5 min. The resulting white precipitate was filtered, washed with 1M HCl, H_2O and cold AcOEt/Et_2O 1:1 and dried over P_2O_5 to afford compound **10** (10.5 g, 91%) as a white solid. M.p. 165-167 °C; R_f = 0.47 ($CHCl_3$/MeOH/AcOH 7:0.5:0.5); ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.15 (t, J = 7.1 Hz, 3H), 2.05 (td, J = 6.1, 15.2 Hz, 1H), 2.38 (td, J = 6.6, 14.4 Hz, 1H), 2.98-3.26 (m, 3H), 3.88-4.14 (m, 2H), 5.73-5.77 (m, 1H), 6.33 (s, 1H), 7.28-7.67 (m, 10H), 7.71-7.86 (m. 2H), 10.3 (br s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 14.1, 29.9 (d, J = 7.1 Hz), 31.6 (d, J = 101.4 Hz), 38.2, 61.5, 100.8, 125.3, 127.3, 127.4, 127.6, 127.9, 128.0, 128.5, 128.9, 130.2, 131.5, 131.8, 132.1, 132.7, 133.0, 134.9, 141.3, 142.1, 161.9, 170.1, 172.7 (d, J = 9.4 Hz); ^{31}P NMR (81 MHz, $CDCl^3$) δ 41.9; HRMS (MALDI-TOF$^+$) m/z for $[C_{27}H_{25}BrClNO_5P]^+$ calcd 588.0342 ; found: 588.0331.

Ethyl 3-{[(adamantan-1-yl)oxy](4-bromophenyl)phosphoryl}-2-{[3-(3'-chloro-[1,1'-biphenyl]-4-*yl)isoxazol-5-yl]methyl}propanoate* (**11**). To a refluxing solution of phosphinic derivative **10** (5.53 g, 9.40 mmol) and 1-adamantylbromide (2.12 g, 9.87 mmol) in $CHCl_3$ (130 mL), silver (I) oxide (2.51 g, 10.8 mmol) was added portion-wise over 1 h. After 6 h of reflux, the solvent was removed under vacuum and the residue was treated with Et_2O (10 mL). The resulting mixture was filtered through celite and the filtrates were evaporated. The residue was purified by silica gel column chromatography, using PE (40-60 °C)/AcOEt 9:→1:1 as solvent system to afford compound **11** (6.3 g, 93%) as a colourless viscous gum. R_f = 0.52 (PE/AcOEt 1:1); ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.08-1.23 (2 × t, J = 7.1 Hz, 3H), 1.51 (br s, 6H), 1.75-2.56 (m, 11H), 2.97-3.32 (m, 3H), 3.84-4.24 (m, 2H), 6.34 and 6.37 (two s, 1H), 7.22-7.85 (m, 12H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 14.1, 14.1, 29.6 (d, J = 8.4 Hz), 29.9 (d, J = 9.6 Hz), 31.1, 32.7 (d, J = 104.4 Hz), 32.9 (d, J = 104.4 Hz), 35.6, 38.2 (d, J = 1.3 Hz), 38.9 (d, J = 3.1 Hz), 44.4 (t, J = 3.6 Hz), 61.3, 61.3, 83.8 (t, J = 9.5 Hz), 100.6, 125.2, 127.1, 127.2, 127.5, 127.7, 128.5, 128.5, 130.1, 131.5, 131.7, 131.8, 132.0, 132.9, 133.1, 133.2, 134.0, 134.4, 134.7, 141.1, 142.0, 161.8, 170.3, 170.4, 172.9 (d, J = 10.3 Hz), 173.0 (d, J = 9.0 Hz); ^{31}P NMR (81 MHz, $CDCl_3$) δ 36.0, 36.7; HRMS (MALDI-TOF$^+$) m/z for $[C_{37}H_{39}BrClNO_5P]^+$ calcd 722.1438; found: 722.1312.

3-{[(Adamantan-1-yl)oxy](4-bromophenyl)phosphoryl}-2-{[3-(3'-chloro-[1,1'-biphenyl]-4- yl)isoxazol-5-yl]methyl}propanoic acid (**12**). To an ice-cold, stirred solution of adamantyl ester **11** (3.90 g, 5.39 mmol) in EtOH (70 mL), a 4 M aqueous solution of NaOH (13.5 mL, 54 mmol) was added drop-wise. The reaction mixture was stirred for 3 h. Then the solvent was removed and the residue was diluted with water and acidified with 0.5M HCl in an ice water bath. This aqueous solution was extracted with AcOEt (2 × 100 mL) and the combined organic layers were dried over Na_2SO_4 and concentrated. The residue was treated with cold PE/Et_2O 1:2 to afford after filtration compound **12** (3.6 g, 97%) as a white solid. R_f = 0.63 ($CHCl_3$/MeOH 95:5); ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.50 (br s, 6H), 1.76-2.70 (m, 11H), 2.94-3.49 (m, 3H), 6.31and 6.48 (two s, 1H), 7.22-7.86 (m, 12H), 11.08 (br s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 29.7, 30.0, 31.1, 32.4 (d, J = 103.4 Hz), 32.5 (d, J = 103.8 Hz), 35.5, 38.1, 38.2, 44.3 (t, J = 3.7 Hz), 84.9 (d, J = 9.4 Hz), 85.0 (d, J = 9.6 Hz), 100.5, 100.7, 125.2, 127.1, 127.3, 127.5, 127.7, 128.4, 128.5, 130.1, 130.7, 131.9, 132.1, 132.7, 132.9, 133.0, 133.1, 133.2, 134.8, 141.1, 142.0, 161.8, 161.9, 170.5, 170.7, 174.4, 174.6, 174.7; ^{31}P NMR (81 MHz, $CDCl_3$) δ 38.5, 38.9; HRMS (MALDI-TOF$^+$) m/z for $[C_{35}H_{34}BrClNO_5P]^+$ calcd 694.1125; found: 694.1181.

General protocol for solid phase synthesis and access to compounds (**13**) *and* (**14**). Standard Fmoc methodology was used to build the amino acid sequence on a Universal PEG NovaTag™ resin (300 mg, 0.074 mmol). Fmoc-Glu(OtBu)-OH (0.315 g, 0.74 mmol, 10 equivalents) and phosphinic building block **12** (0.062 g, 0.089 mmol, 1.2 equivalents) were incorporated on solid support following a standard protocol in the presence of N,N'-Diisopropylcarbodiimide (DIC) and of 6-Chloro-1-Hydroxybenzotriazole dihydrate (ClHOBt) in Dimethylformamide (DMF). The coupling reactions for Fmoc-Glu(OtBu)-OH amino acid and phosphinic building blocks were performed under microwave irradiation (45 W) at 60 °C for 10 min and 60 min, respectively. To access compound **14** with a longer PEG linker, an additional elongation step on the amino function present at the *C*-terminal position was implemented. In this respect, the resin was first split in two portions and one of which was treated with a solution of 0.6 M 1-Hydroxybenzotriazole (Trifluoroethanol/Dichloromethane 1:1) at room temperature for 30 min. The resulting resin was then washed with DMF (2 × 5 min) and DCM (2 × 5 min). Boc-NH-PEG_{27}-COOH (0.263 g, 0.185 mmol, 2.5 equivalents) was first activated for 5 minutes in the presence of DIC (0.289 mL, 10 equivalents) and HOBt (0.316 g, 10 equivalents) and then incorporated onto the solid support. The coupling reaction was run overnight at room temperature. Compounds **13** and **14** were then cleaved from the support with Trifluoroacetic acid/Triisopropylsilane/water 95:2.5:2.5 cocktail (5 mL, 3 × 45 min) and with Trifluoroacetic acid/DCM solution 1:1 (5 mL, 2 × 45 min). The two crude pseudo peptides as a diastereomer mixture were purified by RP-HPLC on a Supelco Ascentis® C18 column (0 to 100% B in 30 min) to lead to compounds **13** (0.0088 g, 17%) and **14** (0.014 g, 34%) as white powders after freeze drying.

Compound **13**: $\varepsilon_{272\ nm}$ = 33 550 cm^{-1}. M^{-1} in methanol; R_t = 6.28 min (Supelco Ascentis® Express C18 column, 0 to 100% B in 10 min) and R_t = 7.07 min (Grace Vision HT C18 HL column, 0 to 100%B in 10 min); ^{1}H NMR (600 MHz, d_6-DMSO) δ; 1.22 (s, 0.5H), 1.58 (m, 2H), 1.73 (m, 4H), 1.83-2.00 (m, 3H), 2.10-2.35 (m, 4H), 2.83 (m, 2H), 3.05 (m, 4H), 3.28-3.48 (m, 14H), 4.04 (m, 1H), 4.14 (m, 1H), 6.80 (s, 1H), 7.4-7.55 (m, 3H), 7.60-7.72 (m, 6H), 7.75-7.85 (m, 3H), 7.90 (m, 2H), 8.20 (bs, 1H), 8.40 (m, 1H); MS (MALDI TOF) m/z for $[C_{45}H_{55}BrClN_5O_{13}P]^-$ calcd 1018.2411, found 1018.3916.

Compound **14**: $\varepsilon_{272\ nm}$ = 33 550 $cm^{-1}.M^{-1}$ in methanol; R_t = 6.47 min (Supelco Ascentis® Express C18 column, 0 to 100% B in 10 min) and R_t = 7.47 min (Grace Vision HT C18 HL column, 0 to 100% B in 10 min); MS (MALDI TOF) m/z for $[C_{104}H_{172}BrClN_6O_{42}P]^-$ calcd 2322.0123, found 2322.2065.

*General procedure for fluorescent probe synthesis **1** to **5***. To a solution of compounds **13** or **14** (1 equivalent) in DMF (C = 2 mM) were added successively N,N-diisopropylethylamine (10 equivalents) and a solution of the appropriate NHS-activated fluorescent dye in DMF (1.5 equivalents, C = 3 mM). The resulting solution was stirred at room temperature and the progress of the reaction was monitored by RP-HPLC (Supelco Ascentis® Express C18 column, 0 to 100% B in 15 min). The reaction mixture was quenched with water and the crude material was purified by RP-HPLC (Supelco Ascentis® C18 column, 0 to 100% B in 30 min). All synthesized fluorescent probes (**1**-**5**) were obtained as solids after freeze drying and stored at -20°C away from light.

RXP470-PEG$_2$-Cy55, **1.** A blue solid was obtained (60%). In PBS: $\varepsilon_{272\ nm}$ = 42,600 M^{-1}. cm^{-1}, $\varepsilon_{680\ nm}$ = 262,600 M^{-1}. cm^{-1}; Rt = 6.38 min (Supelco Ascentis® Express C18 column, 0 to 100% B in 10 min) and Rt = 6.55 min (Grace Vision HT C18 HL column, 0 to 100% B in 10 min); ^{1}H NMR (600 MHz, d_6-DMSO) δ; 1.24 (m, 5H), 1.36 (m, 5H), 1.54-1.59 (m, 5H), 1.76 (m, 4H), 1.96 (s, 12H), 2.05 (m, 3H), 2.12-2.21 (m, 6H), 3.04 (m, 8H), 4.07-4.16 (m, 3H), 4.23-4.29 (m, 3H), 6.36 (m, 3H), 6.64 (m, 1H), 6.79 (s, 1H), 7.46 (m, 2H), 7.52 (m, 3H), 7.63-7.84 (m, 8H), 7.91 (m, 2H), 8.12 (m, 2H), 8.20 (s, 1H), 8.39 (m, 1H), 8.45 (m, 3H), 9.02 (m, 2H); MS (MALDI TOF) m/z for $[C_{86}H_{97}BrClN_7O_{26}PS_4]^-$ calcd 1916.3981, found 1916.4143.

RXP470-PEG$_2$-Cy5, **2**. A blue solid was obtained (55%). In PBS: $\varepsilon_{278\ nm}$ = 35,300 M^{-1}. cm^{-1}, $\varepsilon_{653\ nm}$= 256,900 M^{-1}. cm^{-1}; Rt = 6.37 min (Supelco Ascentis® Express C18 column, 0 to 100% B in 10 min) and Rt = 7.44 min (Grace Vision HT C18 HL column, 0 to 100% B in 10 min). ^{1}H NMR (600 MHz, d_6-DMSO) δ; 1.24-1.27 (m, 4H), 1.31-1.33 (m, 2H), 1.52-1.60 (m, 8H), 1.69 (s, 12H), 1.75-1.82 (m, 3H), 1.85-2.00 (m, 3H), 2.04 (m, 2H), 2.15-2.39 (m, 6H), 2.98 (m, 1H), 3.02-3.10 (m, 7H), 3.33-3.36 (m, 4H), 3.40-3.47 (m, 4H), 4.08 (m, 3H), 4.15 (m, 3H), 6.29 (m, 2H), 6.58 (m, 1H), 6.79 (s, 1H), 7.32 (m, 2H), 7.48 (m, 1H), 7.53 (m, 2H), 7.63-7.66 (m, 4H), 7.69-7.72 (m, 4H), 7.76 (m, 1H), 7.80-7.85 (m, 4H), 7.91

(m, 2H), 8.12 (m, 1H), 8.34-8.40 (m, 3H); MS (MALDI TOF) m/z for $[C_{78}H_{93}BrClN_7O_{20}PS_2]^-$ calcd 1656.4532, found 1656.2805.

RXP470-PEG$_4$-6SIDCC, **3**. A bleu solid was obtained (34%). In PBS: $\varepsilon_{280\ nm}$ = 27,000 M^{-1}. cm^{-1}, $\varepsilon_{680\ nm}$= 233,200 M^{-1}. cm^{-1}; Rt = 8.75 min (Supelco Ascentis® Express C18 column, 0 to 100% B in 20 min) and Rt = 10.55 min (Grace Vision HT C18 HL column, 0 to 100% B in 20 min); ^{1}H NMR (600 MHz, d_6-DMSO) δ; 1.58-1.78 (m, 18H), 1.92-2.03 (m, 18H), 2.15-2.30 (m, 10H), 2.93 (m, 3H), 3.04-3.10 (m, 7H), 3.22 (m, 2H), 3.39 (m, 4H), 3.41-3.51 (m, 12H), 3.87 (m, 2H), 4.07 (m, 1H), 4.15 (m, 1H), 5.80 (m, 2H), 6.79 (s, 1H), 7.01 (s, 1H), 7.10 (s, 1H), 7.18 (s, 1H) 7.28 (m, 2H), 7.47 (m, 2H), 7.52 (m, 1H), 7.63-7.92 (m, 13H), 8.12 (m, 1H), 8.20 (s, 2H), 8.39 (m, 2H), 8.48 (s, 2H), 8.55 (m, 2H), 8.99 (m, 2H); MS (MALDI TOF) m/z for $[C_{104}H_{124}BrClN_8O_{36}PS_6]^-$ calcd 2397.5057, found 2397.4805.

RXP470-PEG$_2$-CW800, **4**. A green solid was obtained (62%). In PBS: $\varepsilon_{278\ nm}$ = 24,700 M^{-1}. cm^{-1}, $\varepsilon_{780\ nm}$= 217,600 M^{-1}. cm^{-1}; Rt = 9.23 min (Supelco Ascentis® Express C18 column, 0 to 100% B in 20 min) and Rt = 10.75 min (Grace Vision HT C18 HL column, 0 to 100% B in 20 min); ^{1}H NMR (600 MHz, d_6-DMSO) δ; 1.25 (s, 2H), 1.31 (s, 12H), 1.54 (m, 2H), 1.60 (m, 5H), 1.66 (m, 2H), 1.76 (m, 5H), 1.95 (m, 3H), 2.05 (m, 2H), 2.10-2.39 (m, 6H), 2.72 (m, 5H), 3.05 (m, 7H), 3.35 (m, 3H), 4.07-4.15 (m, 6H), 6.14 (m, 2H), 6.27 (m, 2H), 6.80 (s, 1H), 7.00 (s, 1H), 7.10 (m, 3H), 7.17 (s, 1H) 7.26 (m, 1H), 7.37 (m, 1H), 7.48 (m, 1H), 7.53 (m, 1H), 7.60-7.85 (m, 15H), 7.93 (m, 2H), 8.14 (m, 1H), 8.40 (m, 1H); MS (MALDI TOF) m/z for $[C_{91}H_{107}BrClN_7O_{27}PS_4]^-$ calcd 2002.4713, found 2002.2283.

RXP470-PEG$_{29}$-Cy5.5, ***6***. A bleu solid was obtained (67%). In PBS: $\varepsilon_{278\ nm}$ = 42,300 M^{-1}. cm^{-1}, $\varepsilon_{680\ nm}$ = 264,300 M^{-1}. cm^{-1}; Rt = 7.20 min (Supelco Ascentis® Express C18 column, 0 to 100% B in 20 min) and Rt = 6.55 min (Grace Vision HT C18 HL column, 0 to 100% B in 20 min); MS (MALDI TOF) m/z for $[C_{145}H_{214}BrClN_8O_{55}PS_4]^-$ calcd 3320.1692, found 3320.1614.

Protein preparation. The expression and purification for human MMP-12 was carried out as previously described[5]. In brief, the synthetic gene used for the crystallization of the catalytic domain codes for residues 106–263, with Phe67 mutated to Asp, as this mutation improves solubility and reduces non-specific aggregation. Plasmids were propagated in the *Escherichia coli* strain XL1-Blue and the recombinant catalytic domains expressed in *E. coli* cells BL21 (DE3 star). After induction, the cells were harvested by centrifugation and the pellets re-suspended. The cell suspension was disrupted and then centrifuged. The pellets were washed and then re dissolved. Refolding, purification and dialysis steps were carried out as previously described[5]. Acetohydroxamic acid (AHA), 10 mM, was added to prevent self-degradation during refolding, and during concentration using an Amicon stirred ultra-filtration cell with a 3500 Da molecular weight cut-off ultra-filtration regenerated cellulose membrane (Millipore, Billerica, MA, USA). The purity was found to be higher than 95% by SDS–PAGE with a catalytic activity higher than 90%.

Crystallization and structure determination. For crystallization experiments, the catalytic domain of MMP-12-F171D was concentrated to 0.689 mM in a buffer consisting of 3 mM $CaCl_2$, 10 mM hydroxamic acid (AHA), 200 mM NaCl, 20 mM Tris-HCl, pH 7.5 while the MMP-12-F171D-K241A mutant was used at 0.661 mM in the same buffer. Probe **1** (RXP470.1-PEG_2-Cy5.5) was dissolved in DMSO at 10 mM and added to the protein in a volumetric ratio 1:10, just before setting up the sitting drops in CrysChem plates for equilibration by vapour diffusion. The screening consisted of six drop with conditions that had given crystals in previous trials using the MMP-12-F171D mutant. The drops were streak seeded[6] immediately after setting up the drops with crystals of the $P2_12_12$ space group, following the strategy previously described[7]. The crystals used for structure determination were obtained using 1 µL protein-ligand drops and 1 µL reservoir solution consisting of 40% polyethylene glycol (PEG) 4,000, 0.18 M imidazole piperidine, 10% dioxane, pH 8.5. Since crystals obtained showed no electron density for the fluorophore, the crystallization conditions were modified: seeding was eliminated, pH and buffers were varied and the MMP-12-F171D-K241A was also used. After three iterations of crystal growth, cryoprotectant screening and data collection on beamlines Proxima 1 and 2A at the Soleil storage ring in St. Aubin, France, suitable conditions to obtain some weak electron density for the fluorophore were determined (Table S1). The best data from crystals of the $P2_1P2_12$ crystal form were obtained from MMP-12-F171D with a reservoir solution consisting of 20% PEG 4,000, 2% dioxane 0.2 M Tris-HCl, pH 9.5. Most crystals grow in the $P2_1$ crystal form, both for the MMP-12-F171D and the MMP-12-F171D-K241A mutants. The best crystals for this second mutant were obtained with a reservoir solution consisting of 20% PEG 4,000, 2% γ-valerolactone, 0.2 M Tris-HCl, pH 9.5. In both cases the crystals grew spontaneously and were suitable for data collection. The crystals were transferred to various pre-mixed cryo-protecting solutions CryoProtX[8] and CryoSol[9] (Molecular Dimensions) for a few seconds, then picked up into a loop and finally cryo-cooled in liquid nitrogen, either with a buffer, or without, until optimal conditions were determined (Table S1).

Table S1: Crystallization conditions and data collection statistics

PDB code	5L79				5L7F			
Protein	MMP-12 (F171D) 689 µM in 200 mM NaCl, 3 mM CaCl2, 1mM ZnCl2, 20 mM Tris-HCl, pH 7.5, 10 mM hydroxamic acid, 10% DMSO				MMP-12 (F171D-K241A) 661 µM in 200 mM NaCl, 3 mM CaCl2, 1mM ZnCl2, 20 mM Tris-HCl, pH 7.5, 10 mM hydroxamic acid, 10% DMSO			
Ligand	**RXP470.1-PEG2-Cy5.5** *R47-6PJ* **(1mM)**				**RXP470.1-PEG2-Cy5.5** *R47-6PJ* **(0.5 mM)**			
Crystallization	20% PEG 4,000, 2% dioxane, 20 mM TRIS pH 9.5				20% PEG 4000, 2% γ-valerolactone, 20 mM Tris-HCl, pH 9.5			
Cryoprotectant	40% CM26, 25% Monomethyl-PEG 6,000, 100 mM Bis-Tris-propane/Na citrate pH 6.8				40% CM26, 25% Monomethyl-PEG 6,000.			
Data Collection								
Source	Soleil Proxima-1				Soleil Proxima-1			
Wavelength (Å)	0.97857				0.97857			
Space group	$P2_12_12$				$P2_1$			
Unit-cell (Å/°)	70.90 62.79 37.74 / 90.0 90.0 90.0				48.92 70.37 52.22 / 90.0 104.6 90.0			
Molec./asym.	1				2			
Resolution (Å)	47-2.07 / 2.13-2.07				48-1.8 / 1.84-1.79			
$CC_{1/2}$	99.7 / 77.6				98.8 / 61.4			
$<I/\sigma(I)>^{\&}$	10.15 / 1.67				6.87 / 1.43			
R_{merge}(%)	15.3 / 127.8				14.5 / 94.7			
$R_{p.i.m.}$(%)	13.6 / 113.8				11.3 / 73.8			
Completeness (%)	99.94 / 100.0				96.9 / 98.4			
Multiplicity	4.69 / 4.74 (anomalous)				2.4 / 2.4 (anomalous)			
Refinement								
Resolution (Å)	47-2.07 / 2.18-2.07				43-1.8 / 1.36-1.8			
No. of reflections (anomalous)	19713 / 2667				55377			
No. of reflections (merged)	10703 / -							
R_{work} (%)	20.6 / 32.3				14.3 / 33.7			
R_{free} (%)	24.1 / 36.4				19.3 / 35.4			
R.m.s. deviations								
Bond lengths (Å)	0.003				0.028			
Bond angles (°)	1.239				2.452			
Ramachandran[ɨ]								
favored (%)	98.7				98.1			
outliers (#)	0				0			
Ligands	RSCC	RSR	LLDF	B-factors(Å)	RSCC	RSR	LLDF	B-factors(Å)
PDB ligand code **R47** (RXP470.1)	A 0.89	0.15	0.28	23,40,62,64	A 0.95 B 0.95	0.09 0.09	0.68 0.57	10, 17, 36, 52 11, 16, 37, 42
PDB ligand code **6PJ** (PEG2-Cy5.5)	A 0.83	0.32	3.17	42,85,119,143	A 0.80 B 0.86	0.25 0.24	6.96 4.85	30, 81, 111, 130 31, 60, 94, 97

[a]$R_{sym} = \sum_{hkl}\sum_i|I_i - \langle I\rangle|/\sum_{hkl}\sum_i I_i$ / [b]Rpim = · [c]$R_{work} = \sum_{hkl}||F_{obs}|-k|F_{calc}||/\sum_{hkl}|F_{obs}|$, / [d]$R_{free}$ was calculated using 5% of data excluded from refinement.

[e]Cryomix CM-26 consists of 12.5 % diethylene glycol + 12.5 % ethylene glycol + 12.5 % glycerol + 25 % 2,3-butanediol + 12.5 % DMSO[9].

The final data were collected on beamline Proxima-1 where the crystals were robotically mounted. Data processing was carried out at the synchrotron facility using XDS[10] with the 'xdsme' script (*https://**github**.com/legrandp/xdsme*) to optimize data quality. The resolution cut-off was selected on the basis of the $CC^{1/2}$ correlation coefficient[11]. The $P2_12_12$ structure was solved by rigid body refinement using REFMAC5[12] starting with a MMP-12 coordinate set with similar cell parameters without inhibitor and refined using the Phenix package[13]. The electron density maps were viewed and fitted in COOT[14] and the inhibitor portion RXP470.1 (PDB ligand code R47) was obtained from PDB entry 4GQL[15] while the PEG2-Cy5.5 (PDB ligand code 6PJ) portion was built using the monomer library sketcher from the CCP4[16] program suite and placed in the electron density as it developed during refinement. The two moieties were linked together in the PDB file with a LINK statement, a strategy to obtain independent fit

statistics for R47 and 6PJ. The $P2_12_12$ structure was subjected to twenty four cycles of rebuilding and refinement with REFMAC5[12]. The $P2_1$ structure of the F171D-K241A solved by molecular replacement from the $P2_12_12$ refined structure required eight rebuilding cycles, both operations were carried out with the PHENIX GUI[13].

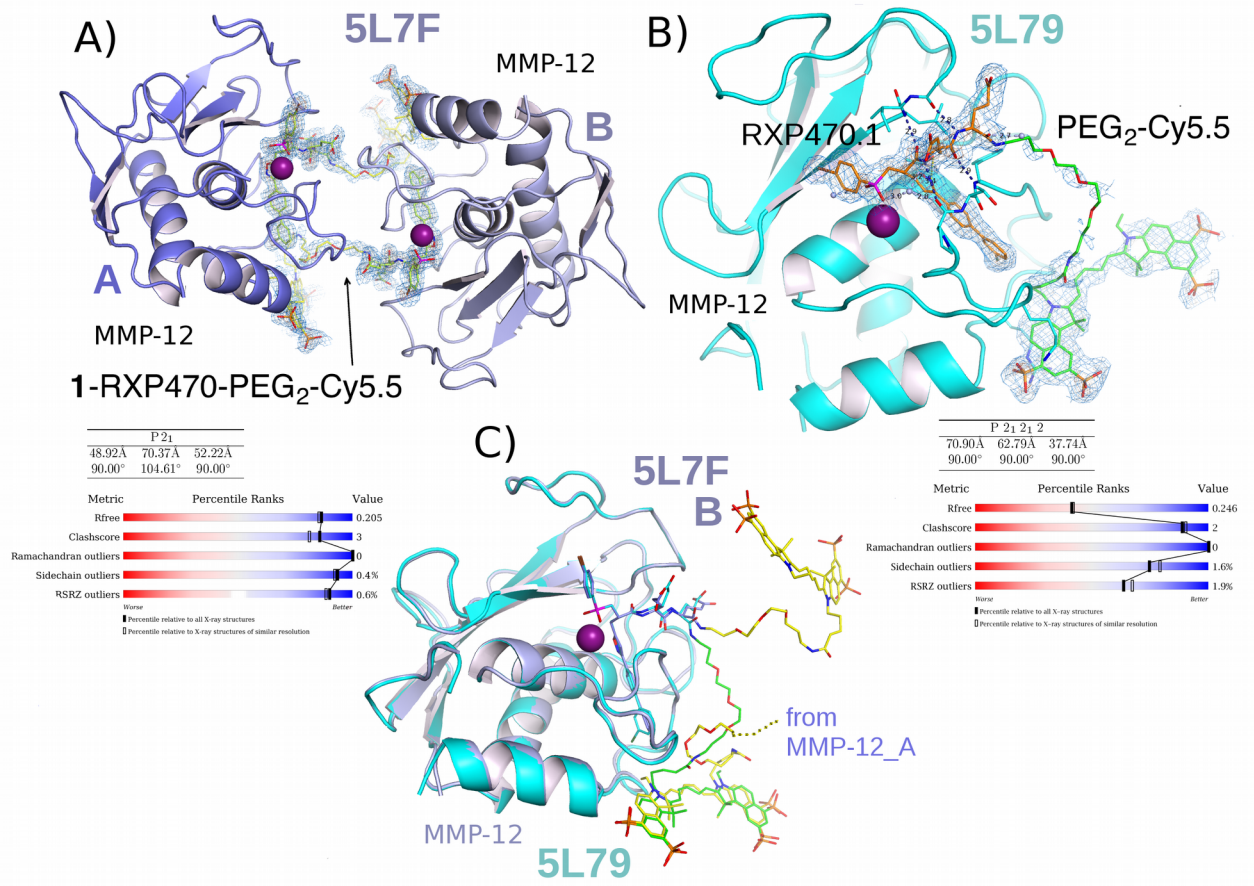


Figure S1. Comparison of the positioning of the fluorophore in the $P2_1$ (PDB code 5L7F, in blue) and $P2_12_12$ (PDB code 5L79, in cyan) crystal forms of the complex between MMP-12 and RXP470.1-PEG_2-Cy5.5. **A)** The asymmetric unit of the $P2_1$ crystal forms contains two complexes where the PEG_2-Cy5.5 adduct to RXP470.1 (ligand code R47) bound to monomer A contacts MMP-12 of monomer B. The PEG_2-Cy5.5 linked to RXP470.1 bound to MMP-12 from monomer B is positioned between Arg^{249} and Lys^{233} of monomer A. **B)** In the $P2_12_12$ crystal form the electron density for the PEG2 linker is weaker than that in the $P2_1$ crystal form. The Cy5.5 group might contact a symmetry-related molecule as in the monoclinic polymorph, but the best fit is obtained with the cyanine in contact with the same MMP-12 molecule. **C)** Superimposition of MMP-12 in the $P2_1$ and $P2_12_12$ crystal forms showing the alternative positioning of the PEG_2-Cy5.5 in the two polymorphs.

Interactions between RXP470.1-PEG_2-Cy5.5 and MMP-12. The three MMP-12 structures that bind RXP470.1 (4GQL[15]), with the phosphinic inhibitor and the fluorescent probes conjugated to the same inhibitor show only minor differences which are typical of the variability found when analysing polymorphs (see Table S2). The interactions made by the PEG_2-Cy5.5 (PDB ligand code 6PJ) moiety may

have minor significance *in vivo*. Under crystallization conditions, two distinct assemblies can be distinguished. The dominant one consists of the fluorophore-stabilized dimer where the Cy5.5 moiety from MMP-12 molecule A interacts with molecule B and *vice versa* (Figure S1-A; Table S2) that gives rise to the $P2_1$ polymorph.

Table S2: Ligand-MMP-12 distances

PDB code	**5L7F**		**5L79**	**4GQL**
Ligand PDB code	R47-6PJ		R47-6PJ	R47
Ligand	**RXP470.1-PEG2-Cy5.5**		**RXP470.1-PEG2-Cy5.5**	**RXP470.1**
Space group	$P2_1$		$P2_12_12$	$P2_12_12$
Unit-cell (Å/°)	48.92 70.37 52.22 / 90.0 104.6 90.0		70.90 62.79 37.74	69.58 63.39 36.90
Resolution	1.8		2.07	1.15
RXP470.1 direct interactions				
Molecule	A	B	A	A
R47-N1 – Thr 215-Oγ1	3.6	3.7	3.5	3.8
R47-N1 – Pro 238-O	3.0	3.2	3.4	3.7
R47-N3 – Gly 179-O	3.0	3.0	2.8	3.0
R47-O2 – Glu 219-Oε2	2.7	2.6	2.6	2.5
R47-O3 - Zn^{++}	2.0	2.0	2.0	1.9
R47-O4 – Leu 181-N	2.9	2.8	2.9	2.8
R47-O7 – Tyr 240-N	2.8	2.9	2.9	2.9
Cy5.5 interactions				
Molecule	A	B	A	
6PJ-O2(B) - Arg 249-NH2	3.1			
6PJ-O2(A) - Arg 249-NH2		3.1		
6PJ-O11 - Arg 249-NH2			2.8	
6PJ-O2(B) - Ala 252-N	2.7			
6PJ-O2(A) - Ala 252-N		2.8		
6PJ-O11- Ala 252-N			3.2	
6PJ-O3(A) – Lys 233-Nζ		2.9		
6PJ-O3(B) – Lys 233-Nζ	3.1-HOH-2.4			
6PJ-O12 – Lys 233-Nζ			3.6	
RXP470.1 water mediated interactions				
Molecule	A	B	A	A
R47-N4...HOH...Tyr 240-O	3.0-HOH-3.1	2.8-HOH-2.8	-	-
R47-O3...HOH...Pro 238-O			3.0-HOH-2.8	2.8-HOH-2.7
R47-O6...$(HOH)_x$...Pro 238-O	3.0-HOH-2.7	3.0-HOH-2.7	-	2.7-HOH-2.7-HOH-2.7
R47-O8...HOH...Gly 179-N	2.8-HOH-2.8	3.4-HOH-2.7	-	-
R47-O9...HOH...Glu 201-Oε1	2.6-HOH-2.5	3.5-HOH-2.7	-	-
R47-O9...HOH...Lys 177-Nζ	-	3.5-HOH-2.8	-	-

The alternative assembly, found in the $P2_12_12$ polymorph, that may have some relevance to the situation in a more dilute environment than that found in the crystallization drop, is the monomeric case, where the Cy5.5 moiety folds back onto the same MMP-12 molecule so that one of the two benzo-indole disulfonate moieties establishes salt bridges with Lys^{233} and Arg^{249} and a hydrogen bond with the amide of Ala^{252}. In the crystal lattice this interaction is strengthened by an interaction between a sulfonate on the second benzo-indole disulfonate moiety with Arg^{127}, an interaction that has no significance in solution.

Affinity and selectivity profile. Human MMP-8, -9, -12 and -13 were produced at the CEA in Saclay according to a procedure previously reported[17]. Other MMPs were purchased from R&D Systems

(Minneapolis, MN, USA). MMP inhibition assays were carried out in 50 mM Tris-HCl buffer, pH = 6.8, 10 mM $CaCl_2$ at 25°C as described[18]. Pro-MMPs were pre-activated by p-aminophenylmercuric acetate following the method described by R&D Systems. Titration experiments were carried out to determine the active enzyme concentration for each MMP prior to the assay[15]. Continuous kinetic assays were performed by recording the fluorescence increase induced by the cleavage of fluorogenic substrates (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 for MMP-1, -2, -7, -8, -9, -10, -12, -13, and -14 and Mca-Arg-Pro-Lys-Pro-Val-Glu- Nva-Trp-Arg-LysDNP-NH_2 for MMP-3). A typical experiment was performed in 200 µL of Tris buffer containing 0.2–0.5 nM of MMP, and a 4.5 µM concentration of fluorogenic substrates. ADAMTS-4 inhibition assays were carried out in 50 mM Tris-HCl buffer, 100 mM NaCl, 10 mM $CaCl_2$, pH 6.8, at 25°C, as described[19]. These assays were performed using 5-FAM-Ala-Glu-Lys-Gln-Gly-Arg-Pro-Ile-Ser-Ile-Ala-Lys-TAMRA-NH_2 as substrate (0.9 µM, Enzo Life Sciences, New York, NY) and human ADAMTS-4 (1 nM, R&D systems). TACE inhibition assays were carried out in 20 mM Tris-HCl buffer (pH 6.8) at 25°C, using MCA-Lys-Pro-Leu-Gly-Leu-Dpa- Ala-Arg-NH_2 substrate (4.5 µM, R&D systems) and TACE (0.6 nM, R&D systems). Human MMP-12 inhibition assays in the presence of mouse serum albumin (Sigma Aldrich, Saint Louis, MO, product number: A3559, batch SLBB2600V) or human serum albumin (Sigma Aldrich, Saint Louis, MO, product number: A3782, batch 090M7001V) were performed following the procedure described above. Inhibition assays in diluted mouse plasma were conducted in the presence of human MMP-12 (6 nM) using a selective MMP-12 substrate developed in our laboratory (Mca-Pro-Leu-Gly- Cys(Mob)-Glu-Glu-Dpa-NH2, 18 µM). Black, flat-bottomed, 96-well non-binding surface plates (Corning-Costar, Schiphol-RijK, Netherlands) were used for these tests. Fluorescence signals were monitored using a Fluoroskan Ascent photon counter spectrophotometer (Thermo-Labsystems, Courtaboeuf, France) equipped with a temperature control device and a plate shaker. For each compound evaluated, the percentage inhibition was determined at five concentrations in triplicate, within the range between 20-80. Ki values were determined using the method proposed by Horovitz and Leviski[20].

Table S3: K_i values (nM) for RXP470.1 and compounds **1**-**5** towards a set of metalloproteases.

Enzyme K_i	hMMP-1	hMMP-2	hMMP-3	hMMP-7	hMMP-8	hMMP-9	hMMP-10	hMMP-12	hMMP-13	hMMP-14	TACE	ADAMTS-5
RXP470.1	>10000	52±4	80±2	518±40	120±40	170±50	17±2	**0.26±0.05**	11±1	150±21	>10000	6100±100
1	>10000	973±63	31±2	1623±13	2065±22	4167±41	71±5	**0.90±0.07**	74±7	1133±61	>10000	1490±129
2	4658±39	862±16	187±15	1417±76	1501±35	2172±53	95±11	**6.7±0.5**	122±15	511±96	>10000	>10000
3	>10000	1231±21	155±10	821±83	2098±21	3179±30	62±4	**5.2±0.5**	181±3	1566±16	>10000	>10000
4	6387±50	2128±22	526±69	944±96	1775±15	3760±44	256±15	**5.9±0.8**	261±21	1606±19	>10000	>10000
5	5777±70	860±16	18±5	1273±14	1272±50	2229±22	10±1	**0.34±0.04**	80±4	445±11	4272±17	1915±13

Pharmacokinetics, *ex vivo* tissue assessment of biodistribution and urine analysis. Pharmacokinetics and biodistribution studies were performed in compliance with the National Animal Welfare Regulations at the CEA in Saclay. The experiments were approved by the local ethics committee for animal experimentation. C57BL/6 mice (Charles River Laboratories, L'Abrésie, France) were individually housed in polycarbonate cages in a conventional animal facility and had access *ad libitum* to food and drink. 1 nmole of probes **1**, **3**, **4** or **5** was injected intravenously in the tail vein (n=3 for each group) under anaesthesia (2% isoflurane). At various time points (0, 1 min, 5 min, 10 min, 15 min, 30 min, 45 min, 60 min, 90min, 120 min, 180 min and 240 min), blood samples (whole blood) were collected from the retro orbital sinus under anaesthesia with 2% isoflurane. Animals were sacrificed by carbon dioxide asphyxiation after 4 hours and the different organs (liver, kidneys, spleen and lungs) were harvested *post-mortem*. Blood and organs were imaged by fluorescence reflectance using FMT 1500's planar imaging capability (Perkin Elmer, Waltham, MA). Imaging was conducted with the appropriate excitation (Ex) and emission (Em) filter sets: Ex/Em = 675/720 nm for probes **1**, **3** and **5,** and Ex/Em = 745/800 nm for probe **4**. Blood fluorescence was converted to % injected dose per gram (%ID/g) using a standard curve derived from serial dilutions of each probe in mouse blood. Tissue fluorescence was quantified as mean efficiency per pixel and was presented as an arbitrary unit (AU). In the case of mice injected with probes **1**, **3** and **4**, urine was collected and analysed by liquid chromatography (Agilent XDB C18 column) and mass spectrometry (Electrospray in a positive mode of ionization). For each probe UV detection was perfoemed at the wavelength corresponding to the maximal absorbance of its conjugated fluorochrome.

Table S4: IC_{50} values (nM) for compounds **1** and **5** towards hMMP-12 in presence of mouse serum albumin (mSA) and in mouse plasma.

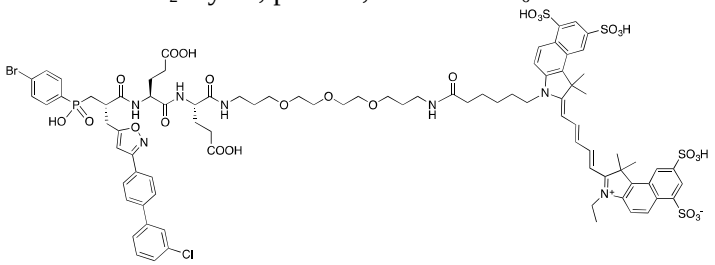
	IC_{50}/hMMP-12 (mSA = 5µM)	IC_{50}/hMMP-12 Mouse plasma, (d_{10} mSA ≈ 50µM)
1	47±1	1630±50
5	3.7±0.3	132±11

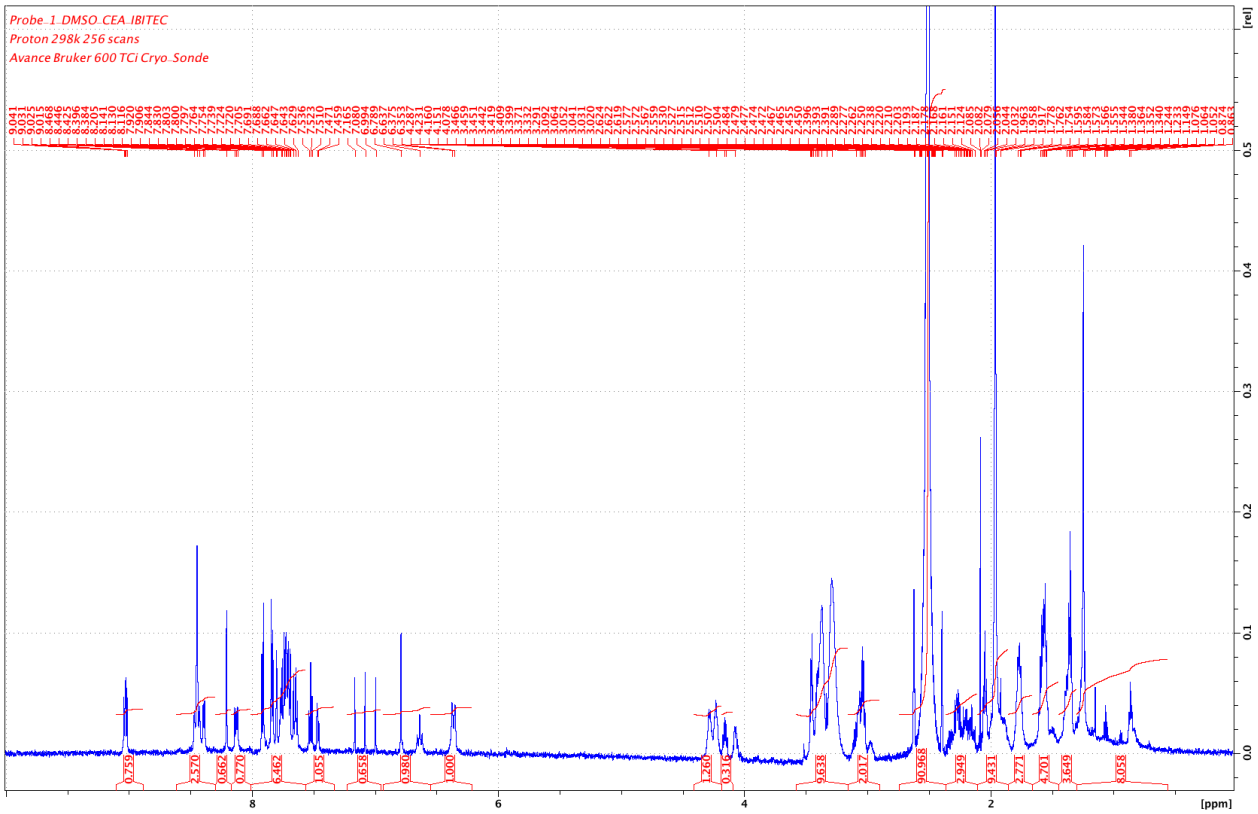
Production of MMP-12-FITC. The synthetic gene encoding the catalytic domain of the human MMP-12 (Residues 106-273, UNIPROT entry number P39900) was obtained from Geneart (Geneart-AG, Germany). The gene was cloned with 4 extra amino acids (LCTP) at its C-terminal into the pET11a vector between the NdeI and BamHI sites, for expression under the T7 promoter. The plasmid was propagated in the *Escherichia coli* strain XL1-Blue at 37 °C, and the construct was verified by DNA sequencing. The recombinant protein was expressed in E. coli BL21 (DE3 Star) cells carrying the MMP-12 catalytic domain-encoding plasmid. A Fernbach flask containing 1 L of ZYP-5052 auto-induction medium[21] supplemented with Ampicillin (100 µg/ml) was inoculated with an overnight culture to reach 0.05 O.D. at 600 nm. Cultures were performed using a two-step protocol (4h at 37°C then 18h at 20°C). At the end of culture, cells were harvested by centrifugation (5000 × g, 30 min, 4 °C) and the pellets were suspended in buffer A (100 mM Tris-HCl, pH 8.5, 5 mM benzamidinochloride, 5 mM 2-mercaptoethanol). Cell lysis was performed using two passages through a cell disruption system at 4 °C (AVESTIN-EmulsiFlex-C3) and the lysate was then centrifuged at 4 °C for 30 min (8000 × g). The pellets were washed three times with buffer B (100 mM Tris-HCl, pH 8.5, 2 M urea, 5 mM 2-mercaptoethanol) and dissolved in buffer C (100 mM Tris-HCl, pH 8.5, 8 M urea, 2 mM 2-mercaptoethanol). Refolding and purification steps were carried out as previously described[22] in the presence of 2 mM 2-mercaptoethanol in order to maintain the free cysteine residue in a reduced state. The refolded protein was analysed by SDS-PAGE and found to migrate as a single band. The concentrated MMP-12 LCTP solution (35 µM) was desalted using a Hi-Trap column equilibrated with a degassed conjugation buffer (20 mM HEPES, pH 7.5, 5 mM $CaCl_2$, 200 mM NaCl, 0.1 mM $ZnCl_2$) to remove 2-mercaptoethanol. A 10 mg/mL stock solution of 5-iodoacetamido fluorescein (5-IAF) was freshly prepared in DMF and 5 molar excess amount was added to the protein solution. The conjugation reaction was performed overnight at 4 °C, protected from light. Unreacted 5-IAF was removed through dialysis against fresh conjugation buffer. The molecular weight of the resulting hMMP-12 conjugated to a FITC tag (hMMP-12-FITC) was confirmed by electrospray ionization mass spectrometry. Its ability to degrade fluorogenic substrate was confirmed through a standard activity assay described above. hMMP-12-FITC was stored at -80 °C in HEPES, 20 mM pH 7.5, 5 mM $CaCl_2$, 200 mM NaCl, 0.1 mM $ZnCl_2$.

Culture of HeLa cell lines, inhibition assay and fluorescent imaging of MMP-12-FITC/probe 3 associated to cells. Human HeLA cells were kindly provided by Institut Curie (Paris, France). Cells were cultured at 37°C in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) without phenol red, supplemented with 20% fetal bovine serum and 1% penicillin/streptomycin. HeLa cells were grown overnight to 90% confluence on sterile glass cover slips and were harvest with TrypLE (Gibco) at 37 °C. DMEM media was then added to the cell suspension and the cells were centrifuged. Packed cells were re-suspended in DMEM and counted, then adjusted to the appropriate concentration. For inhibition assays in

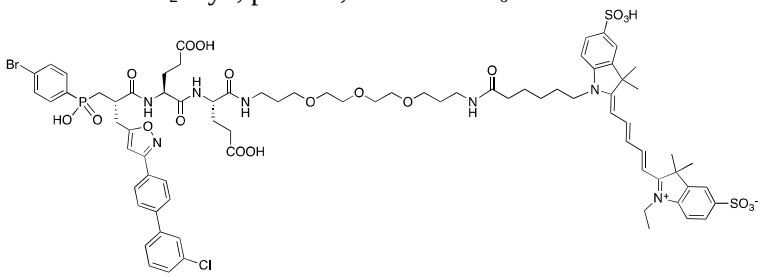
the presence of probe **3**, 3×10^5 HeLa cells were deposited on a chambered coverglass (Lab-Tek) and incubated with 200 nM hMMP12-FITC for 15 min at 4°C. The cell suspension was then washed three times with 20 mM HEPES, pH 7.0, 5 mM $CaCl_2$, 0.1 mM $ZnCl_2$ to remove the unbound MMP-12. Metalloproteinase activity was evaluated for 30 min at 25° C in the presence of a MMP12-selective substrate developed by our group (Mca-GluProGluLeuGluGluDpa, 4 µM). For inhibition assays, probe **3** at 1 nM, 10 nM and 100 nM was incubated for 15 min at 4 °C and the response fluorescence was monitored as described above. Based on an estimated quantity of MMP-12 at 2 nM, an affinity constant of 11 ± 2 nM can be determined. For cell imaging, HeLa cells grown overnight to 90% confluence on sterile glass cover slips were placed on ice to impede membrane trafficking. After two washing steps with 20 mM HEPES, pH 7.0, 5 mM $CaCl_2$, 0.1 mM $ZnCl_2$, the cells were incubated with 200 nM hMMP-12-FITC for 15 min at 4°C and washed thrice with 20 mM HEPES, pH 7.0, 5 mM $CaCl_2$, 0.1mM $ZnCl_2$. Probe **3** at 200 nM was added to the medium and left for 15 min at 4 °C. Three additional washing steps with same buffer were carried out before imaging. Images were acquired with a Nipkow Spinning Disk confocal system (Yokogawa CSU-X1-A1) mounted on a Nikon Eclipse Ti E inverted microscope, equipped with a 60x Apochromat TIRF oil-immersion objective (NA:1.49). Blue (491 nm, Cobolt Calypso, 100mW) and red (642 nm, Toptica iBeam Smart 640-S, 150mW) lasers were used for excitation of hMMP-12-FITC and probe **3** with a 6SIDCC dye respectively. A quad band dichroic mirror (405/491/561/641 nm, Semrock) was used with bandpass filters of 525/45 nm and 692/40 nm (Semrock) to detect FITC and Cy5.5-6SIDCC, respectively. Images were recorded with an EMCCD Evolve camera (Photometrics).

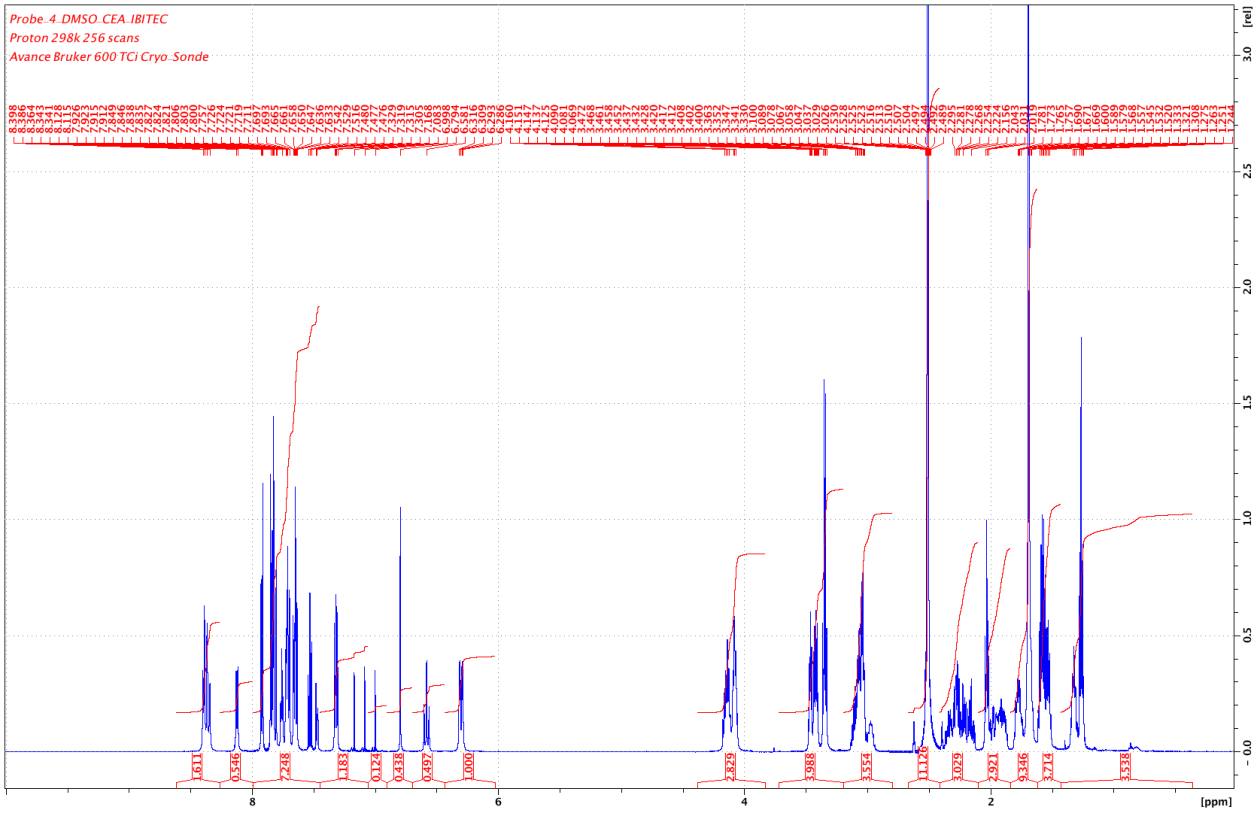
RXP470-PEG$_2$-Cy5.5, probe **1**, ^{1}H/DMSO d$_6$



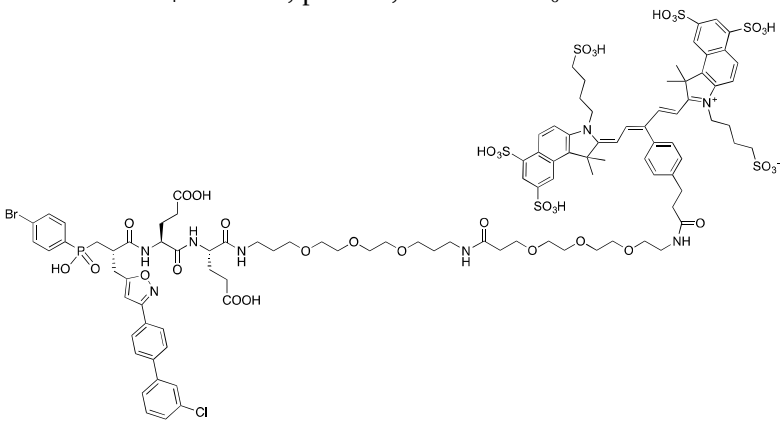


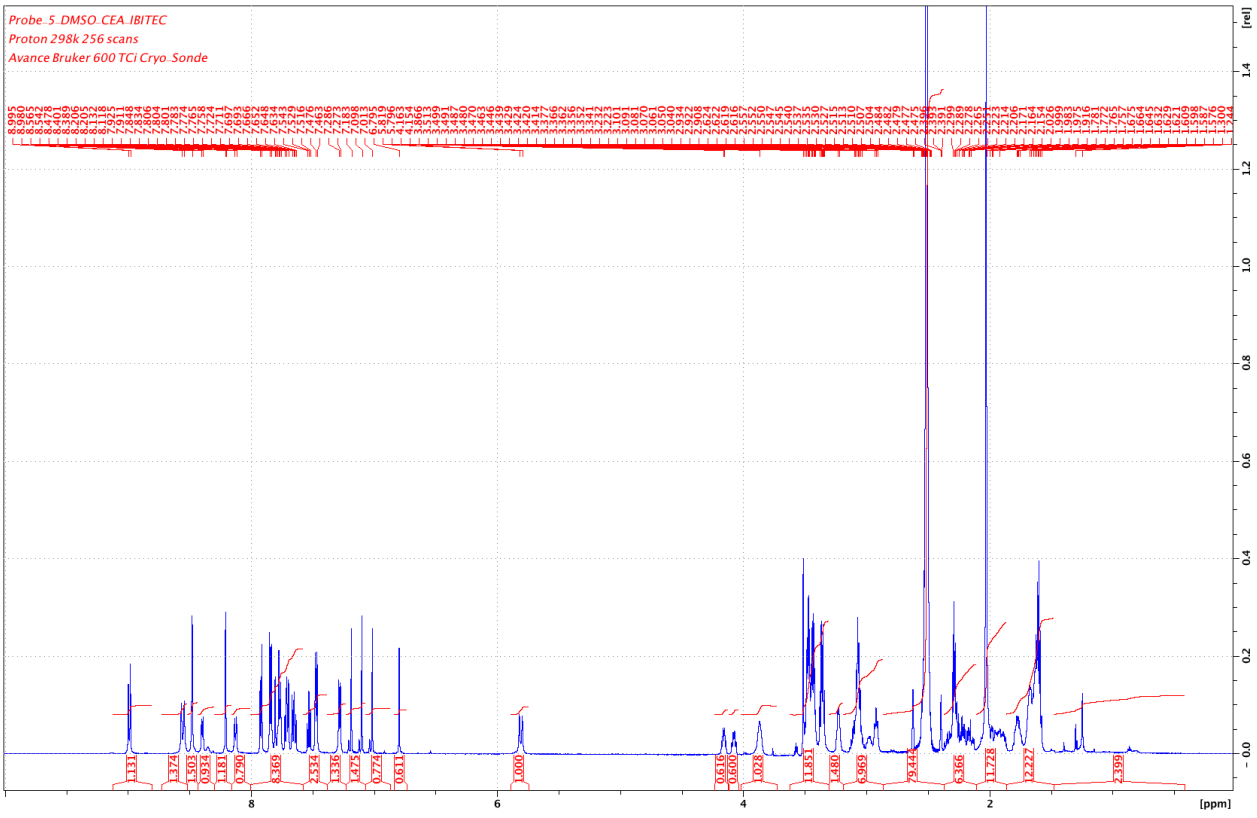
RXP470-PEG$_2$-Cy5, probe **2**, ^{1}H/DMSO d$_6$



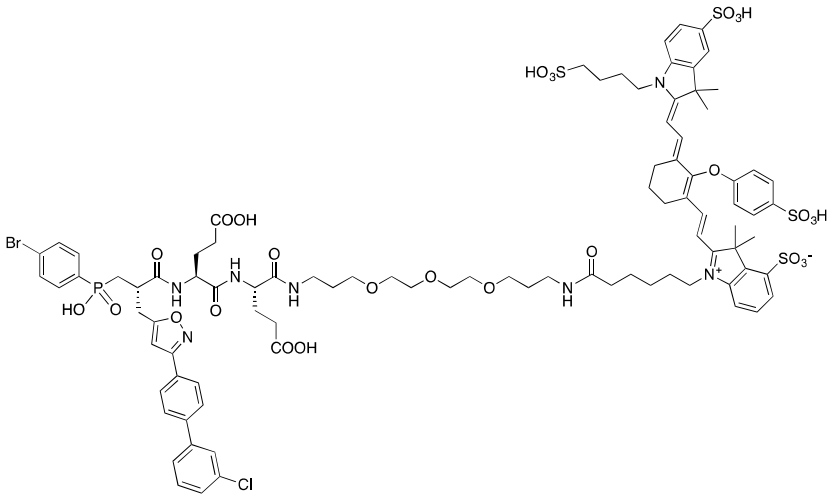


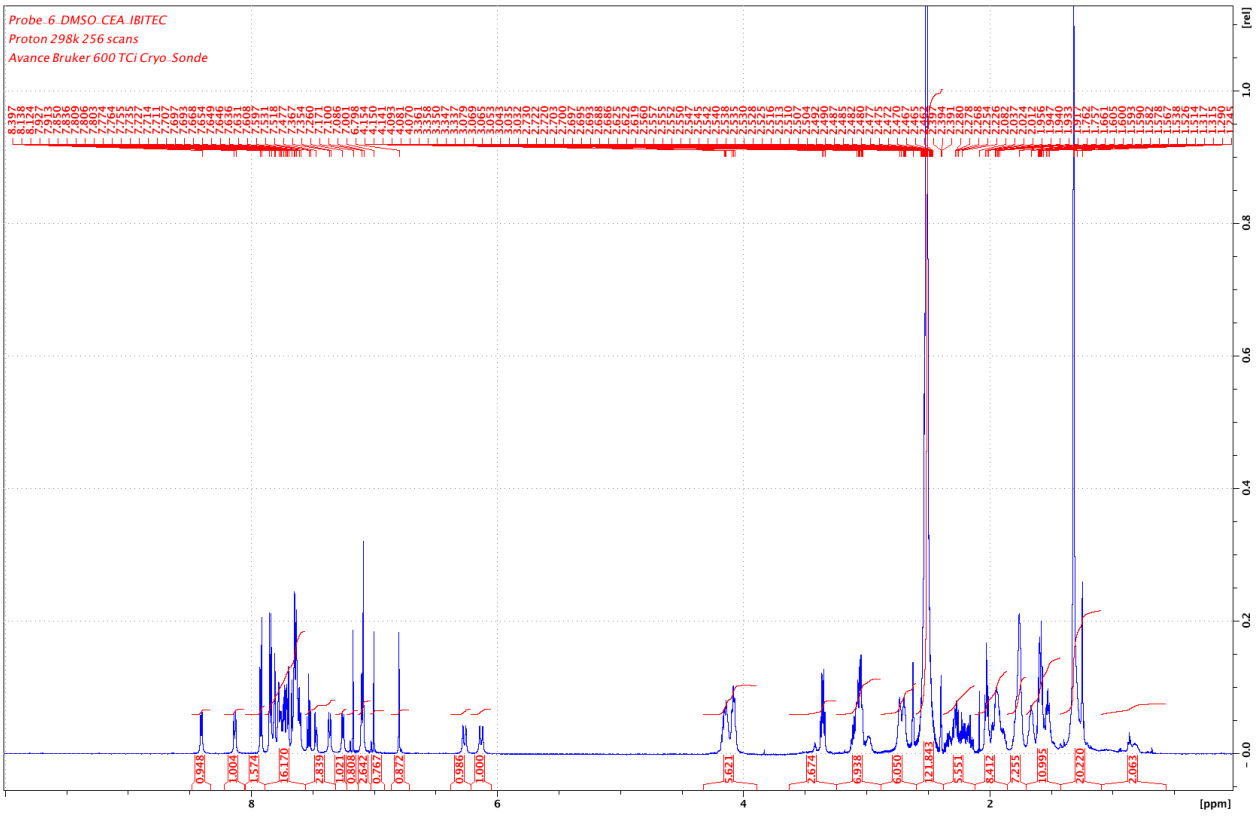
RXP470-PEG$_4$-6SIDCC, probe **3**, ^{1}H/DMSO d$_6$





RXP470-PEG$_2$-CW800, probe **4**, ^{1}H/DMSO d$_6$





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