Rotaxane probes for protease detection by ^{129}Xe hyperCEST NMR

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SUPPORTING INFORMATION

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S1. General Methods

All solvents and reagents, including cucurbit[6]uril hydrate (CB6·XH_2O), and β-cyclodextrin hydrate (βCD·XH_2O), were purchased from commercial suppliers and used without further purification. *N*-propargylglycine,[S1] and **PyAA**$^{+}$ [S2] were prepared according to literature procedures. Thin layer chromatography (TLC) was performed on silica gel 60 F_{254} (E. Merck) and visualized under a UV lamp at 254 nm. Column chromatography was carried out on silica gel 60 (E. Merck, 230–400 mesh). A C-18 column was used for analytical and semi-preparative reverse phase high performance liquid chromatography (RP-HPLC) on an Agilent 1100 Series Capillary LC. Runs were eluted with H_2O/MeCN (0.1 % v/v TFA) and monitored using a UV-Vis detector. Nuclear magnetic resonance (NMR) spectra were recorded on Bruker Avance 600 or 500 with working frequencies of 600, or 500 MHz, respectively. Data for ^{1}H NMR spectra are reported as follows: chemical shift (δ ppm), multiplicity, coupling constant (Hz), and integration. Chemical shifts are referenced to the residual non-deuterated solvents for ^{1}H ($CDCl_3$: δ = 7.27 ppm, CD_3CN: δ = 1.94 ppm, $(CD_3)_2SO$: δ = 2.50 ppm) and ^{13}C ($CDCl_3$: δ = 77.0 ppm, CD_3CN: δ = 118.26 ppm, $(CD_3)_2SO$: δ = 39.52 ppm) nuclei. Matrix assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on a Voyager-DE system (PerSeptive Biosystems, USA) and data were analyzed using Data Explorer software.

S2. Synthetic Procedures

*N-propargylglycine-GPLGLAGRRK-Rhodamine (**1**).* Peptide **1** was synthesized using standard Fmoc solid phase peptide synthesis (SPPS) on Tentagel S-RAM resin (Rapp Polymere, Tuebingen, Germany). Side chain protecting groups used were Arg(pbf) and Lys(dde). Deprotection of the Fmoc groups was performed with two 10 min incubations in a 30% v/v piperidine in dimethylformamide (DMF) solution. Couplings were carried out using 10 equiv of amino acid with 10 equiv of 2-(6-chloro-1-*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU), and 20 equiv of *N*,*N*-diisopropylethylamine (DIPEA) in DMF for 20 min. The N-terminus of the peptide was modified on resin with *N*-propargylglycine using the same coupling conditions. After *N*-propargylglycine addition, dde side-chain deprotection was accomplished using three 5 min incubations with 2% v/v hydrazine-hydrate in DMF solution. After Lys(dde) deprotection, the peptide-resin was incubated overnight with 1.13 equiv of (5,6)-carboxy-tetramethylrhodamine *N*-succinimidyl ester (Sigma Aldrich. St. Louis, MO) and 2.26

equiv of DIPEA in DMF. Peptide **1** was cleaved from the resin after incubation with a solution of 95:2.5:2.5 TFA:TIPS:H_2O for 2 h. Excess TFA was removed under a stream of N_2 and the peptide was precipitated in cold diethyl ether. The precipitate was redissolved in H_2O and purified by RP-HPLC, eluting in an aqueous gradient 5% to 95% MeCN/0.1% TFA in H_2O/0.1% TFA over 45 min at a flow rate of 3.0 mL/min. After lyophilization, peptide **1** was obtained as a deep pink powder (3.8 mg). HRMS (MALDI-TOF-MS): *m/z* calcd for $C_{74}H_{108}N_{21}O_{15}$ $[M+H]^+$ 1530.83, observed 1529.86. ^{1}H NMR (600 MHz, D_2O, 293 K) δ = 8.20 (d, J = 1.9 Hz, 1H), 7.94 (dd, J = 7.9, 1.9 Hz, 1H), 7.42 (d, J = 7.9 Hz, 1H), 7.12 (dd, J = 9.5, 2.7 Hz, 2H), 6.85 (dd, J = 9.3, 2.4 Hz, 2H), 6.74 (s, 2H), 4.36 – 4.01 (m, 9H), 3.98 (s, 2H), 3.90 (d, J = 2.6 Hz, 2H), 3.76 (m, 4H), 3.50 (t, J = 6.9 Hz, 2H), 3.40 (m, 2H), 3.14 (s, 12H), 3.12 (s, 3H), 3.02 (m, 2H), 2.20 – 2.09 (m, 1H), 1.92-1.43 (m, 21H), 1.26 (d, J = 7.3 Hz, 4H), 0.80 (d, J = 6.3 Hz, 4H), 0.76 (dd, J = 10.7, 6.1 Hz, 6H), 0.72 (s, 3H).

*N-propargylglycine-GGGG (**S1**) and N-propargylglycine-GPLG (**S2**).* Peptides **S1** and **S2** were synthesized using standard Fmoc solid phase peptide synthesis (SPPS) on Gly-Wang resin (Anaspec, Fremont, CA). Deprotection of the Fmoc groups was performed with two 10 min incubations in a 30% v/v piperidine in dimethylformamide (DMF) solution. Couplings were carried out using 10 equiv of amino acid with 10 equiv of 2-(6-chloro-1-*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU), and 20 equiv of *N*,*N*-diisopropylethylamine (DIPEA) in DMF for 20 min. The N-terminus of the peptide was modified on resin with *N*-propargylglycine using the same coupling conditions. Peptides were cleaved from the resin after incubation with a solution of 95:2.5:2.5 TFA:TIPS:H_2O for 2-3 h. Excess TFA was removed under a stream of N_2 and the peptide was precipitated in cold diethyl ether. The precipitate was redissolved in H_2O and purified by RP-HPLC, eluting in an aqueous gradient 5% to 95% MeCN/0.1% TFA in H_2O/0.1% TFA over 45 min at a flow rate of 3.0 mL/min. After lyophilization, peptides were obtained as off-white powders (**S1,** 3.5 mg; **S2,** 3.8 mg). HRMS (MALDI-TOF-MS): *m/z* calcd for **S1** $C_{13}H_{20}N_5O_6$ $[M+H]^+$ 342.13, observed 342.4. *m/z* calcd for **S2** $C_{20}H_{32}N_5O_6$ $[M+H]^+$ 438.50, observed 438.66.

*PyAA-CB6-peptide pseudorotaxanes **3** and **5**, and rotaxane **2**.* Compounds $\mathbf{PyAA}^+$ (1 equiv, 2-10 μmol) and βCD·XH_2O (1.3 equiv) were mixed in H_2O and stirred at 60 °C to allow for

complexation. Once **PyAA**$^+$ dissolved, the solution was added to one of peptides **1, S1,** or **S2** (1 equiv), and the solution was then transferred to an Eppendorf charged with CB6·XH_2O (1 equiv). The solution was stirred at 60 °C for 6 h removed from light, and the reaction was monitored by MALDI-TOF MS. The solution was then filtered with a 0.2 μm spin filter to remove unreacted CB6, and the product was purified by semi-preparative RP-HPLC, eluting in an aqueous gradient 10% to 90% MeCN/0.1% TFA in H_2O/0.1% TFA over 35 min at a flow rate of 3.0 mL/min. RP-HPLC removed βCD from the pyrene stopper of the rotaxanes. After lyophilization, pseudorotaxanes **3** (3.1 mg (31% yield), and **5** (2.8 mg, 18% yield) were obtained as off-white powders and rotaxane **2** (4.9 mg, 84% yield) was obtained as a deep pink powder. HRMS (MALDI-TOF-MS): Pseudorotaxane **3.** *m/z* calcd for $C_{75}H_{84}N_{33}O_{18}$ $[M+H]^+$ 1734.64, observed 1734.04. Pseudorotaxane **5.** *m/z* calcd for $C_{68}H_{72}N_{33}O_{18}$ $[M+H]^+$ 1638.27, observed 1638.65. Rotaxane **2.** *m/z* calcd for $C_{129}H_{159}N_{49}O_{27}$ $[M+H]^+$ 2825.97, observed 2825.38. Rotaxane **2** ^{1}H NMR (600 MHz, D_2O, 293 K) δ = 8.54 (d, J = 9.2 Hz, pyrene, 1H), 8.19 (m, pyrene, 2H), 8.13 (m, pyrene, 2H), 8.08 (d, pyrene, J = 9.9 Hz, 2H), 8.01 (d, pyrene, J = 9.1 Hz, 1H), 7.98 (d, pyrene, J = 7.9 Hz, 1H), 7.93 (d, rhodamine, J = 7.6 Hz, 1H), 7.82 (t, rhodamine, J = 7.7 Hz, 1H), 7.44 (d, rhodamine, J = 7.8 Hz, 1H), 6.74 (d, rhodamine, J = 9.5 Hz, 2H), 6.55 (m, rhodamine, 2H), 6.41 (s, triazole, 1H), 5.82 (s, rhodamine, 2H), 5.61 (dd, CB6, J = 15.7, 9.4 Hz, 12H), 5.40 (s, CB6, 12H), 4.93 (s, 2H), 4.43 – 4.05 (m, CB6 and methylenes, 20H), 3.98 (m, 2H), 3.82 (m, 5H), 3.59 (s, rhodamine, 9H), 3.38 (s, 2H), 3.07-3.04 (m, 2H), 3.02 (s, 12H), 2.95 (s 2H), 2.23 (m, 1H), 2.03 – 1.90 (m, 2H), 1.88 – 1.39 (m, 18H), 1.31 (d, J = 7.4 Hz, 3H), 1.15 (t, J = 12 Hz, 1H), 0.92 – 0.67 (m, 13H).

S3. Protease-induced cleavage procedures

*MMP-2-induced cleavage of peptide **1**, monitored by RP-HPLC and MALDI-TOF MS.* To a solution of 100 μM **2** in 60 μL MMP-2 buffer (40 mM TRIS-Cl, 8 mM Zn^{2+}, 8 mM Ca^{2+}, 8 mM Na_2PO_4/$NaPO_4$, 0.04% Brij-35, pH 7) was added MMP-2 (active catalytic domain, Enzo Life Sciences, Farmingdale, NY) as a 0.1 mg/mL solution for a final concentration of 100 nM MMP-2, and the solution was incubated at 37 °C. Aliquots (15 μL) were taken at each timepoint of interest and analyzed by RP-HPLC eluting with an aqueous gradient of 10% to 90% MeCN/0.1% TFA in H_2O/0.1% TFA over 20 min at a flow rate of 1.0 mL/min, monitoring at the Abs_{560} of

rhodamine. Cleavage product **4** was confirmed by MALDI-TOF MS. *m/z* calcd for **4** $C_{54}H_{79}N_{16}O_{10}$ $[M+H]^+$ 1112.3, observed 1111.6.

*MMP-2-induced cleavage of rotaxane **2**, monitored by RP-HPLC, MALDI-TOF MS, and ^{129}Xe hyperCEST.* To a solution of 100 μM **2** in 150 μL MMP-2 buffer (40 mM TRIS-Cl, 8 mM Zn^{2+}, 8 mM Ca^{2+}, 8 mM $Na_2PO_4/NaPO_4$, 0.04% Brij-35, pH 7) was added MMP-2 (active catalytic domain, Enzo Life Sciences, Farmingdale, NY) as a 0.1 mg/mL solution for a final concentration of 100 nM MMP-2, and the solution was incubated at 37 °C. Aliquots (25 μL) were taken at each timepoint of interest and analyzed by RP-HPLC eluting with an aqueous gradient of 10% to 90% MeCN/0.1% TFA in H_2O/0.1% TFA over 20 min at a flow rate of 0.5 mL/min, monitoring at the Abs_{560} of rhodamine. Cleavage products **3** and **4** were confirmed by MALDI-TOF MS. At 24 h, the solution was diluted in ddH_2O to 5 μM **2** and 5 nM MMP-2, and a ^{129}Xe hyperCEST spectrum was obtained. HRMS (MALDI-TOF-MS): *m/z* calcd for **3** $C_{75}H_{84}N_{33}O_{18}$ $[M+H]^+$ 1734.64, observed 1732.0. HRMS (MALDI-TOF-MS): *m/z* calcd for **4** $C_{54}H_{79}N_{16}O_{10}$ $[M+H]^+$ 1112.3, observed 1110.8.

S4. Xenon hyperCEST NMR.

Xenon polarization was achieved using a home built spin-exchange optical-pumping setup resulting in a 10% polarization of a xenon gas mixture (2 % Xe, 10% N_2, 88% He).[S3] The hyperpolarized gas was bubbled directly into a 5 mm phantom containing the solution of interest for 20 s then left to settle for 2 s. The sample was held at 3.4 atm and 25 °C throughout. A 9.4 T (400 MHz) Varian VNMRS console was used for all hyperCEST experiments with optimized saturation power and duration for each sample. A standard hyperCEST pulse sequence was used sweeping the saturation frequency, at 20 dB, in 1000 Hz increments 7000 Hz to 29000 Hz, also including a measurement at 11250 and 11500 Hz where CB6 is typically observed, and at 19500 Hz, corresponding to the center of the $^{129}Xe@H_2O$ peak covering a 200 ppm range in total.[S4] The saturation pulse length was 4 s for three cycles at 20 dB. For % CEST effect data, an on/off saturation experiment was conducted saturating at the center of the CB6 peak then switching to 29000 Hz on the other side of the $^{129}Xe@H_2O$ peak alternating 8 times for a total of 4 on saturation and 4 off saturation values. Data processing was carried out using MATLAB. FIDs were zero-filled to 16384 points, baseline was corrected, apodized with an 11 Hz exponential,

and a Fourier transform was performed. Each $^{129}Xe@H_2O$ areas in the spectra were integrated and the contrast of each spectrum was compared between the maximum and minimum area in each data series. Each profile was fitted with Lorentz profile using ORIGINLAB.[S5]

The percent CEST effect is obtained by alternating the on and off resonance saturation frequencies and observing the integrated area of the dissolved xenon peak. For CB6 the on resonance saturation is around 11250 Hz so the off resonance saturation is placed equidistant on the other side of the water peak at 27750 Hz. The placement of the saturation peaks is such that if the saturation pulse for the on resonance peak has any overlap with the dissolved water peak, it will have the same overlap during the off resonance saturation. For this study the on/off cycle was repeated 4 times for each rotaxane and pseudorotaxane then the % CEST effect was determined bynormalizing the spectra and taking the signal from the on resonance and dividing it by the off resonance and subtracting from one, %CEST effect = $(1 - S_{on}/S_{off})$, for each cycle. The signal for xenon-NMR experiments is generated external to the sample of interest. This causes fluctuations in the amount of polarized xenon dissolved in water for each scan. By taking 25 off resonance scans the fluctuation is signal due to xenon polarization and bubbling was determined to be +/- 3.5% of the average $Xe@H_2O$ signal so a percent CEST effect must be greater than 7% for it to be considered significant. For the locked rotaxane the percent CEST effect was determined to be less then 4% and thus can be concluded that no measurable CEST is taking place.

S5. Supplementary Figures

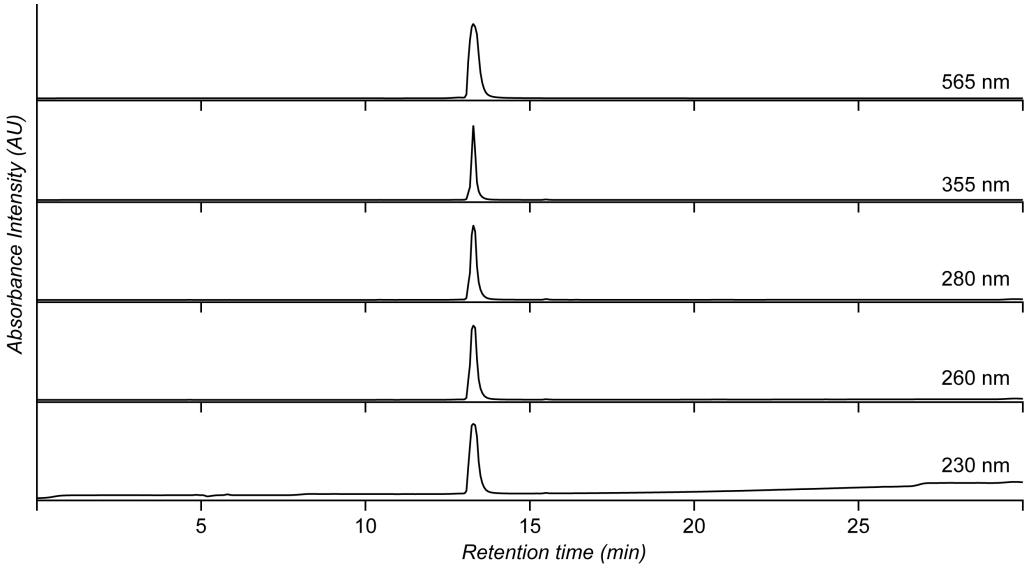

Figure S1. RP-HPLC chromatogram of purified rotaxane **2**. At all wavelengths measured, only one major peak is observed.

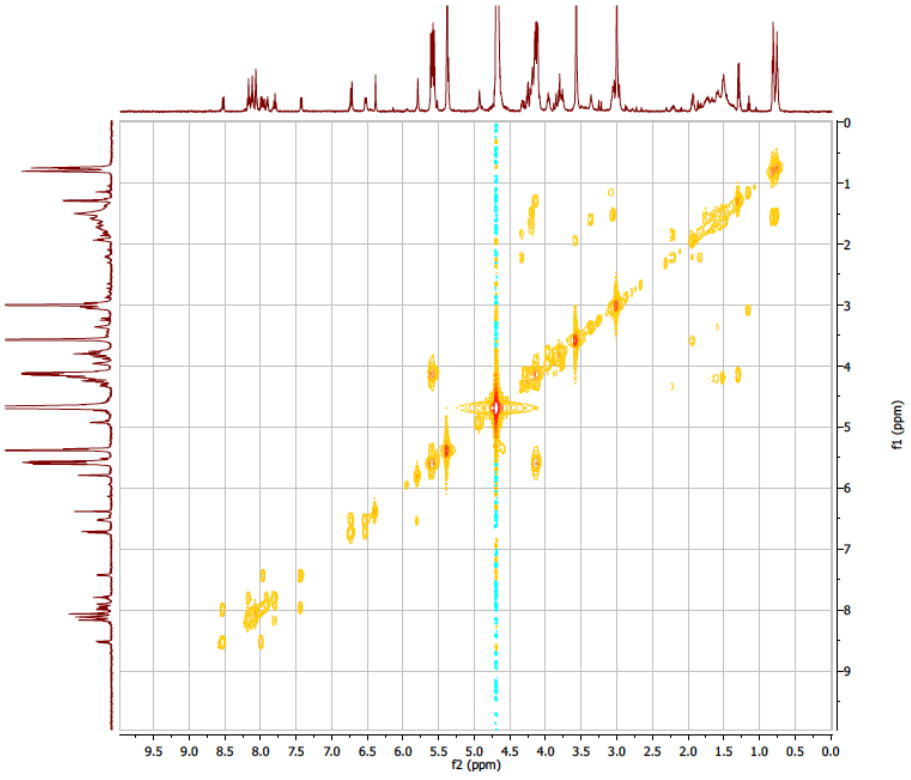

Figure S2. COSY NMR spectrum of rotaxane **2**.

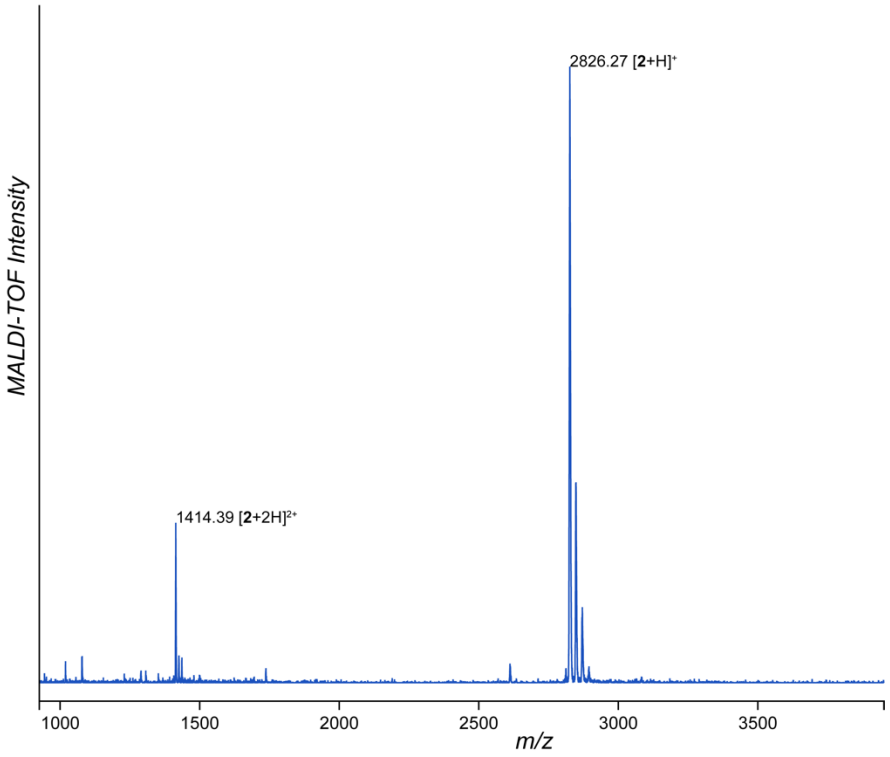

Figure S3. MALDI-TOF MS of rotaxane **2** after hyperCEST. MS confirmed that rotaxane **2** remained intact after hyperCEST spectra were obtained.

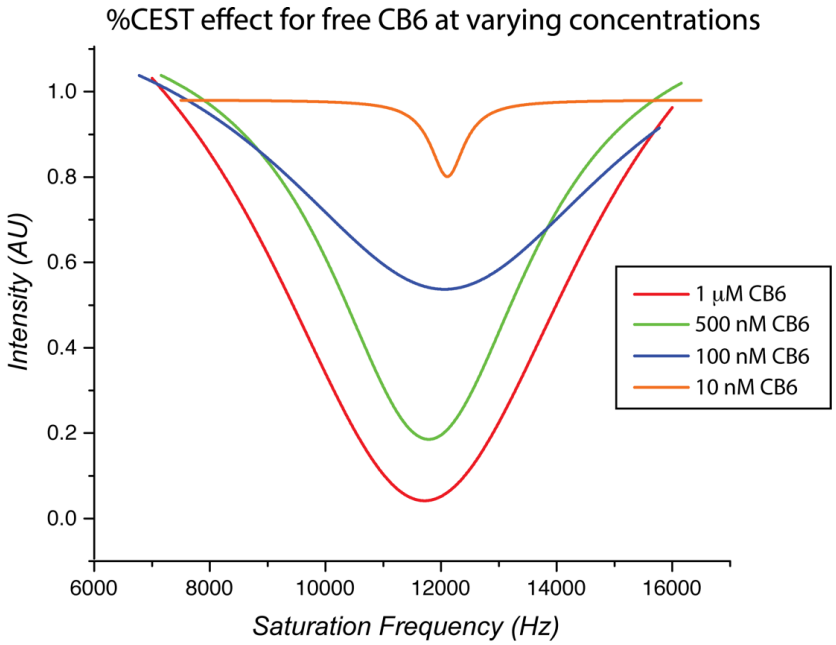

Figure S4. ^{129}Xe hyperCEST spectra of free CB6 at 1000, 500, 100, and 10 nM inNaPhos pH 7.

S6. References

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