Electronic Supplementary Material (ESI) for Chemical Science.

Supporting information

Light-driven release of Cucurbit[8]uril from a bivalent cage

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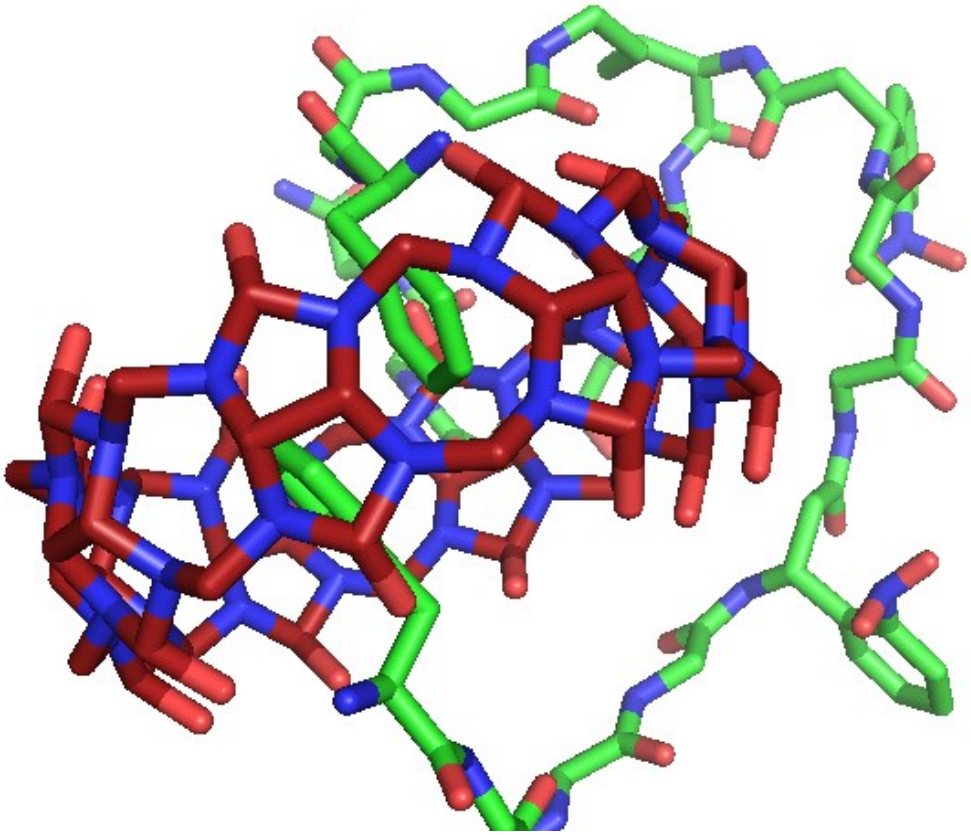
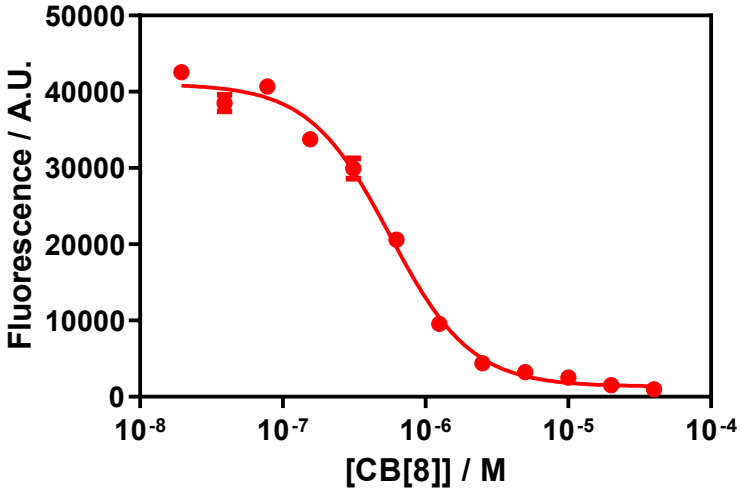
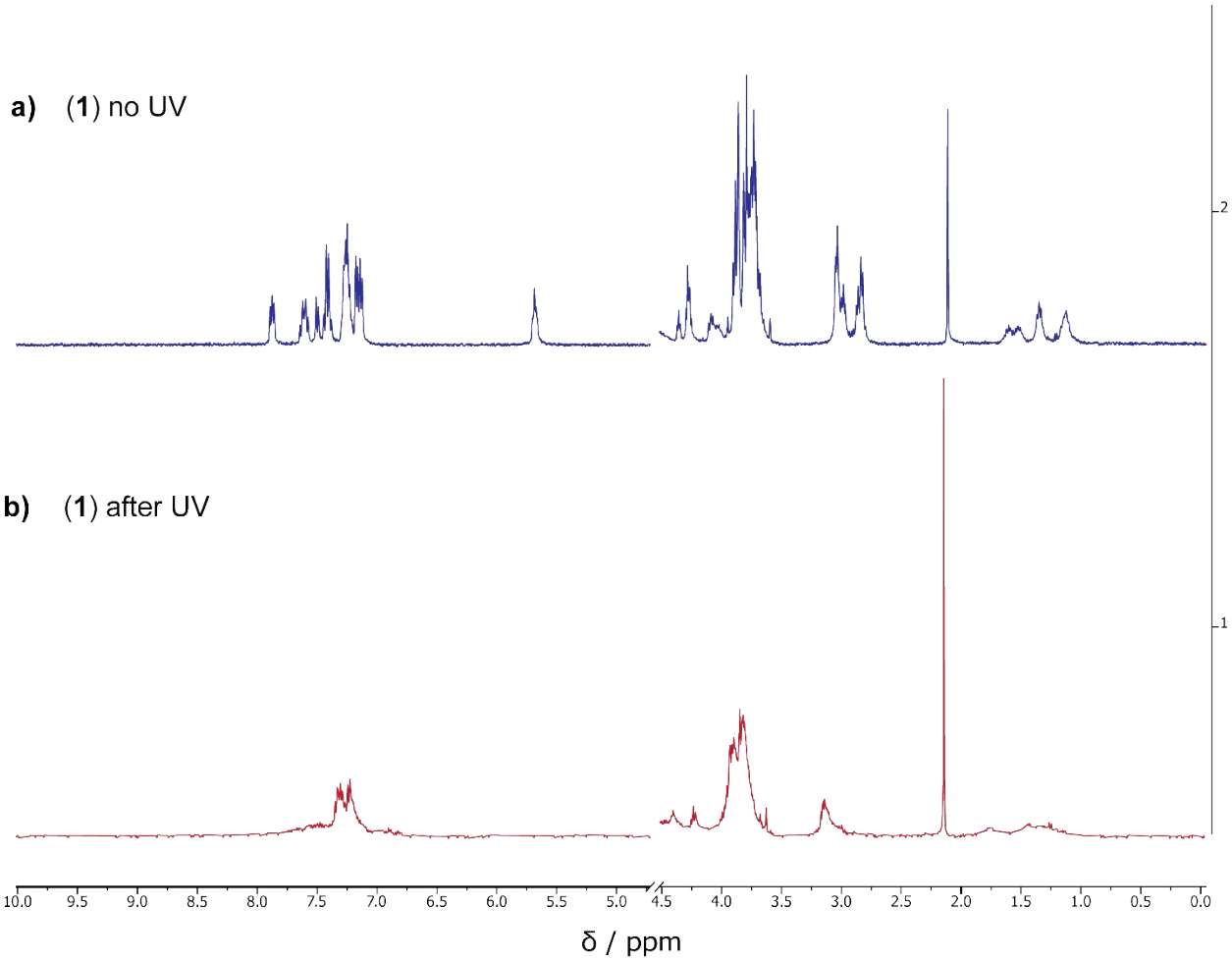
Supporting Figures



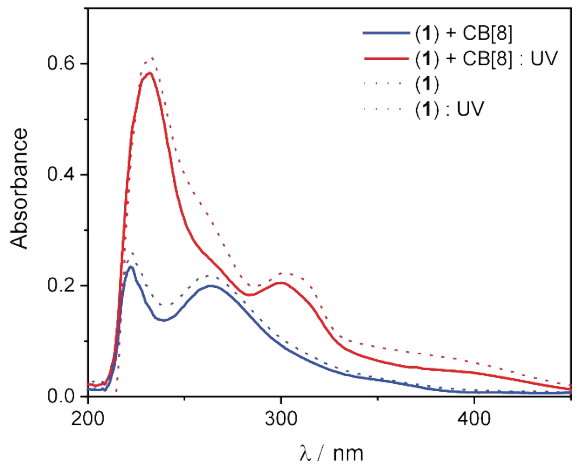
Supporting Figure S1: Visualization of structure of bivalent cage (**1**) (Sequence: *H_2N*-FGGSG(Anp)SG(Anp)K(GGF)SGGSGG-*$CONH_2$*) in complex with CB[8]. The peptide backbone is sufficiently flexible to allow ditopic binding without causing significant strain. Structure is based on $(FGG)_2$•CBB[8] protein crystal structure (PDB code: 5N10[1]) and modified with PyMOL (Schödinger) and ChemBio3D Ultra 14.0 (Perkin Elmer). Serine sidechains are omitted for clarity.



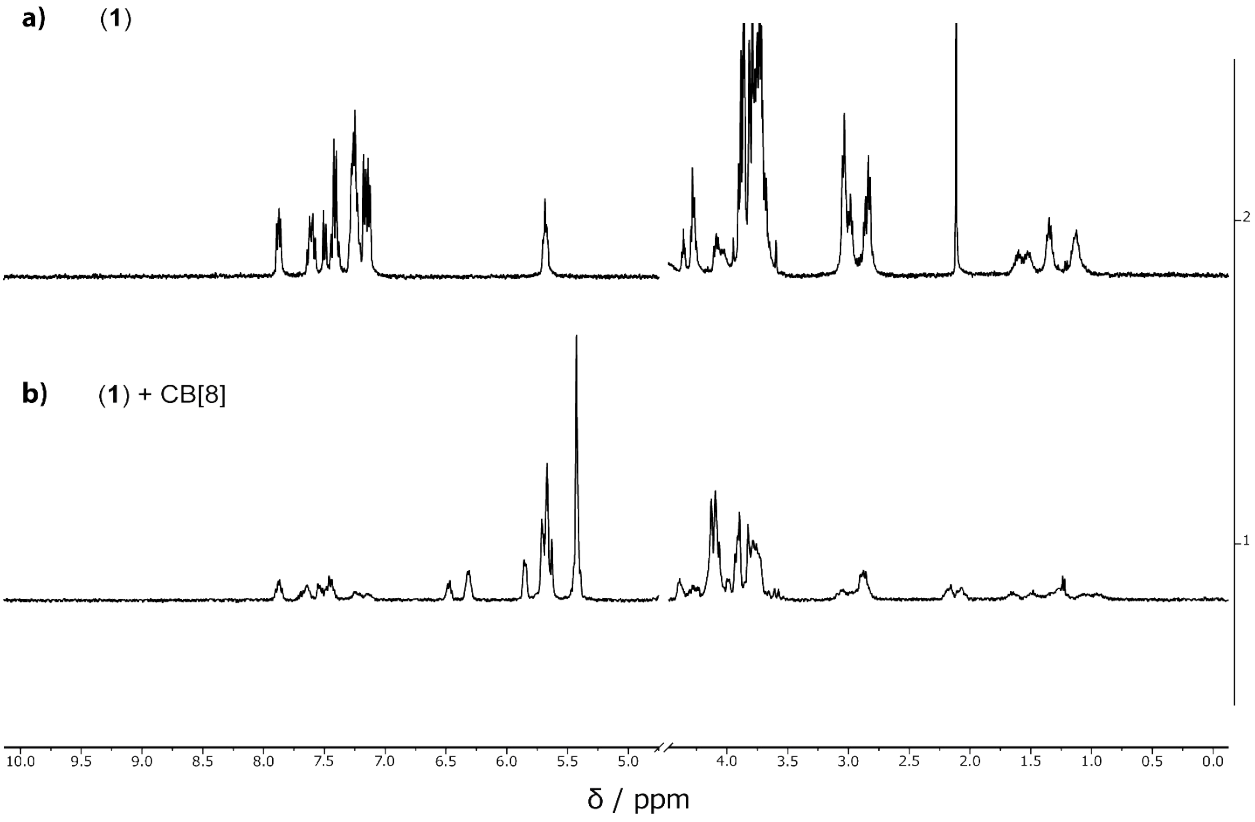
Supporting Figure S2. Direct fluorometric titration of CB[8] to 100 nM Acridine Orange (AO) in HBS (10 mM HEPES, 150 mM NaCl, 0.01% TWEEN-20, pH 7.4; Ex:485 nm, Em 535 nm). Binding of AO inside the CB[8] cavity results in a decrease of fluorescence due to auto quenching. At around 1μM of CB[8] most fluorescence in quenched. This CB[8] concentration is used in further displacement assays. Experiments are performed in triplicate, error bars represent the standard deviation.



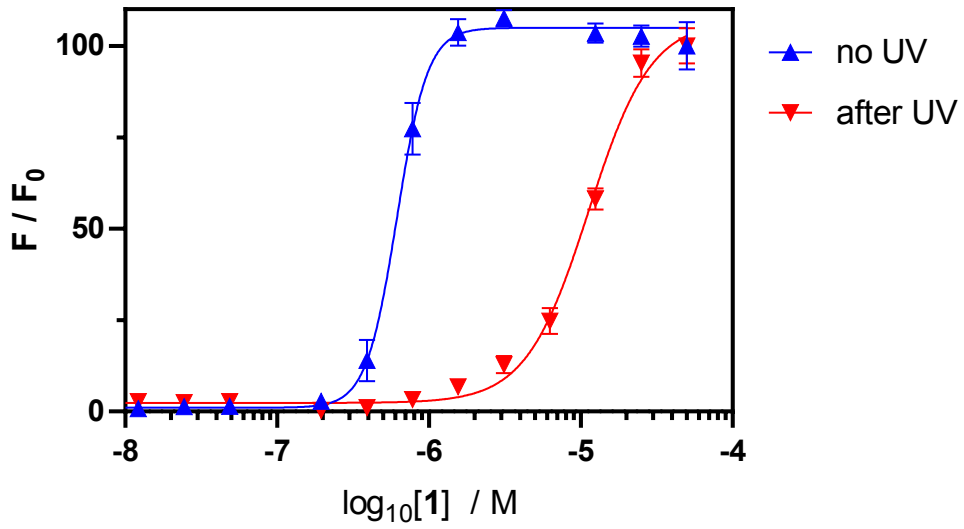
Supporting Figure S3. ^{1}H-NMR of **1** (500 μM) a) before and b) after UV exposure in D_2O. Spectrum surrounding the water suppression (4.6 ppm) is omitted.



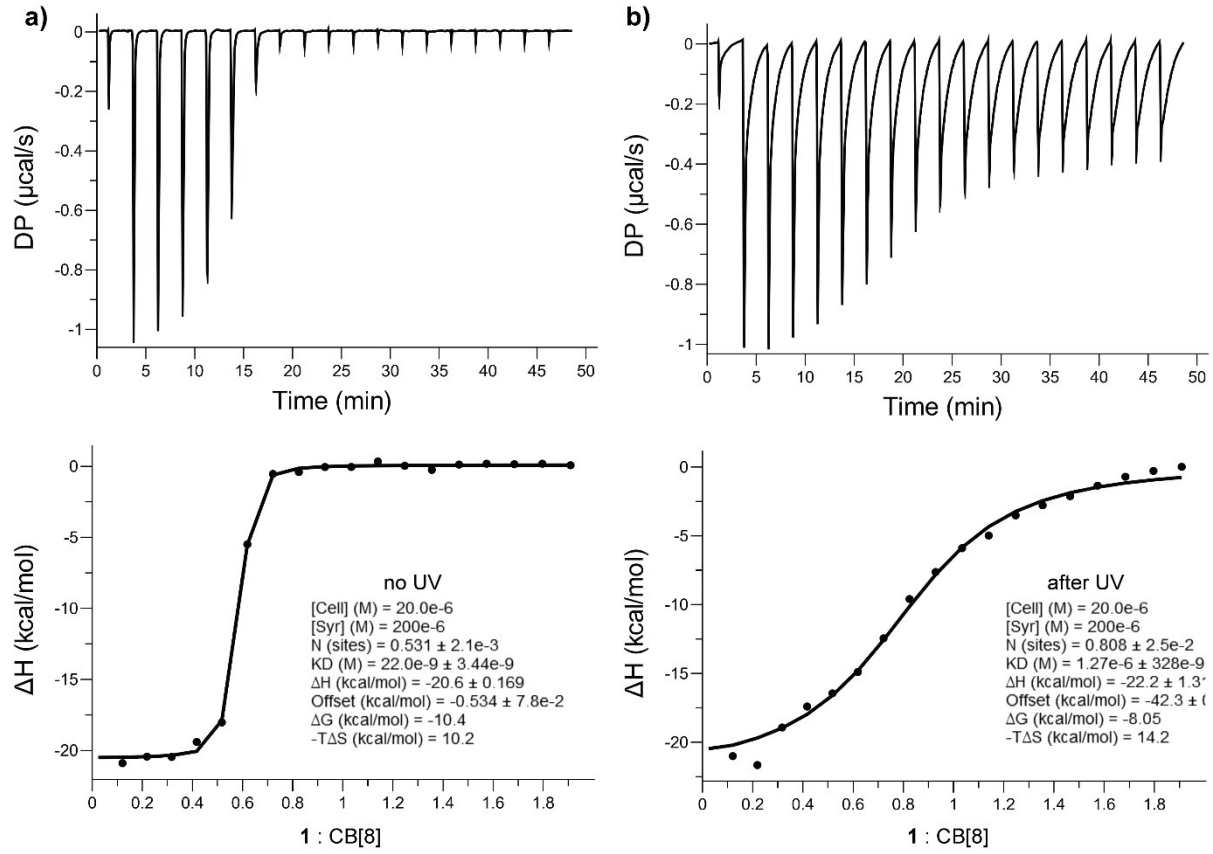
Supporting Figure S4. UV-Vis absorption spectra of compound **1** (30 μM) and compound **1** with CB[8] (30 μM:30 μM) before and after UV-radiation in Activity Assay Buffer.



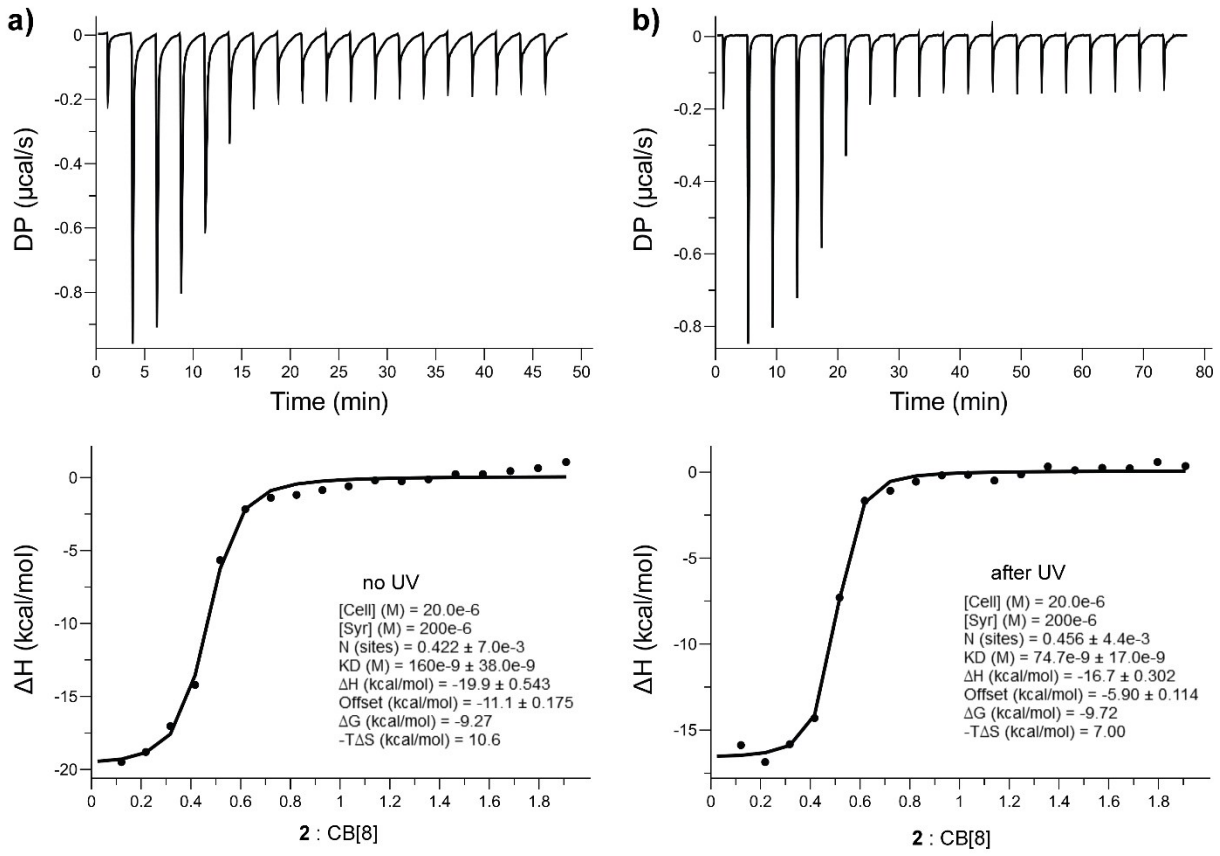
Supporting Figure S5. ^{1}H-NMR complex study of a) **1** (500 μM) and b) **1** (500 μM) with CB[8] (450) μM in D_2O. Spectrum surrounding the water suppression (4.6 ppm) is omitted.



Supporting Figure S6. Fluorescent displacement assay of **1** to pre-formed AO•CB[8] complex (1 μM : 1 μM) and **1** after 20 minutes of UV-exposure in HBS (10 mM HEPES, 150 mM NaCl, 0.01% TWEEN-20, pH 7.4; Ex:485 nm, Em 535 nm). Experiments are performed in triplicate, error bars represent the standard deviation.



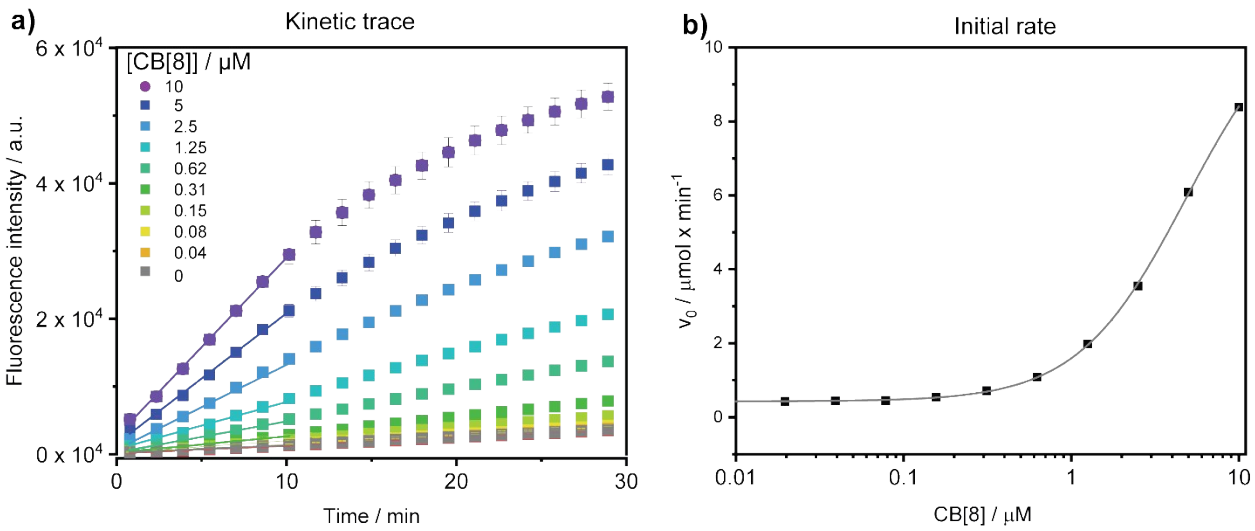
Supporting Figure S7. a) Isothermal titration calorimetry (ITC) experiment of 200 *µ*M **1** to 20 *µ*M CB[8] in activity assay buffer (20 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, 2mM TCEP, pH 7.0) at 25ºC. Top: raw data of power versus time. Bottom: integrated enthalpy versus the molar ratio. b) ITC titration of 200 *µ*M **1** to 20 *µ*M CB[8] after 20 minutes of UV exposure in activity assay buffer (20 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, 2mM TCEP, pH 7.0) at 25ºC. Top: raw data of power versus time. Bottom: integrated enthalpy versus the molar ratio.



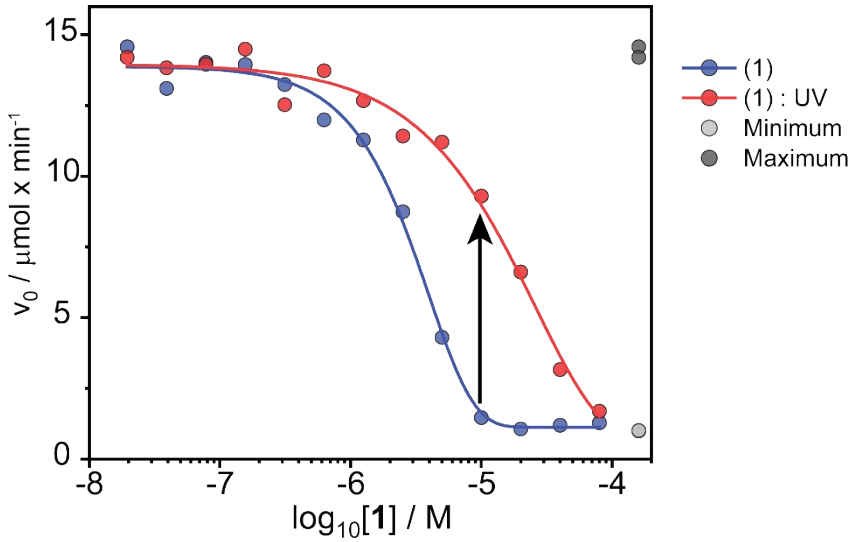
Supporting Figure S8. a) Isothermal titration calorimetry (ITC) experiment of 200 *µ*M **2** to 20 *µ*M CB[8] in activity assay buffer (20 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, 2mM TCEP, pH 7.0) at 25ºC. Top: raw data of power versus time. Bottom: integrated enthalpy versus the molar ratio. b) ITC titration of 200 µM **2** to 20 µM CB[8] after 20 minutes of UV exposure in activity assay buffer (20 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, 2mM TCEP, pH 7.0) at 25ºC. Top: raw data of power versus time. Bottom: integrated enthalpy versus the molar ratio.

Supporting Table S1. Overview of isothermal titration calorimetry characteristics.

	K_D / nM	**n**	**ΔG / kcal·mol^{-1}**	**ΔH / kcal·mol^{-1}**	**-TΔS/ kcal·mol^{-1}**
1	22 ± 3.4	0.53	-10.4	-20.6 ± 0.2	10.2
1 after UV	1300 ± 330	0.83	-8.05	-22.2 ± 1.3	14.2
2	160 ± 38	0.42	-9.27	-19.9 ± 0.5	10.6
2 after UV	75 ± 19	0.46	-9.72	-16.3 ± 0.3	7.0



Supporting Figure S9. CB[8] dependence of caspase-9 activity. a) Kinetic trace of *Ac*-LEHD-AFC cleavage by 1 μM caspase-9 as followed by fluorescence (Ex: 400 nm, Em: 520 nm) at various concentrations of CB[8] in activity buffer (20 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, 2 mM TCEP, pH 7.0). Experiments are done in duplo, error bars represent the standard deviation. b) Substrate cleavage rate as a function of concentration CB[8] determined by the initial slope.



Supporting Figure S10. Optimization screen of **(1)** concentration for light-triggered dimerization of caspase-9 by CB[8]. (**1**) is titrated to 1 μM casp-9, 10 μM CB[8] and 200 μM *Ac*-LEHD-AFC in 100 μL activity buffer (20 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, 2 mM TCEP, pH 7.0). 'minimum' contains only casp-9 the 'maximum' casp-9 and CB[8]. Rate is determined by the initial slope of fluorescence. fluorescence (Ex: 400 nm, Em: 520 nm).

Experimental

Materials

Cucurbit[8]uril (CB[8]), and acridine orange (AO) were obtained via Sigma-Aldrich. The exact purity of CB[8] was determined by UV-VIS titration as described by Kaifer et al[2]. The synthetic substrate Ac-LEHD-AFC was obtained via Enzo Life Sciences.

Water was purified by a Millipore purification train. Deuterated solvents were obtained from Cambridge Isotope Laboratories. Special amino acids (Fmoc-Lys(Mmt)-OH, Fmoc-Anp-OH and Fmoc-β-Ala-OH) were ordered from Iris Biotech, all other Fmoc protected amino acids were acquired from Novabiochem. Resins are obtained from Rapp Polymer. All solvents were obtained from Biosolve, Actu-All chemicals or Carlo Ebra Reagents at peptide synthesis or analytical grade.

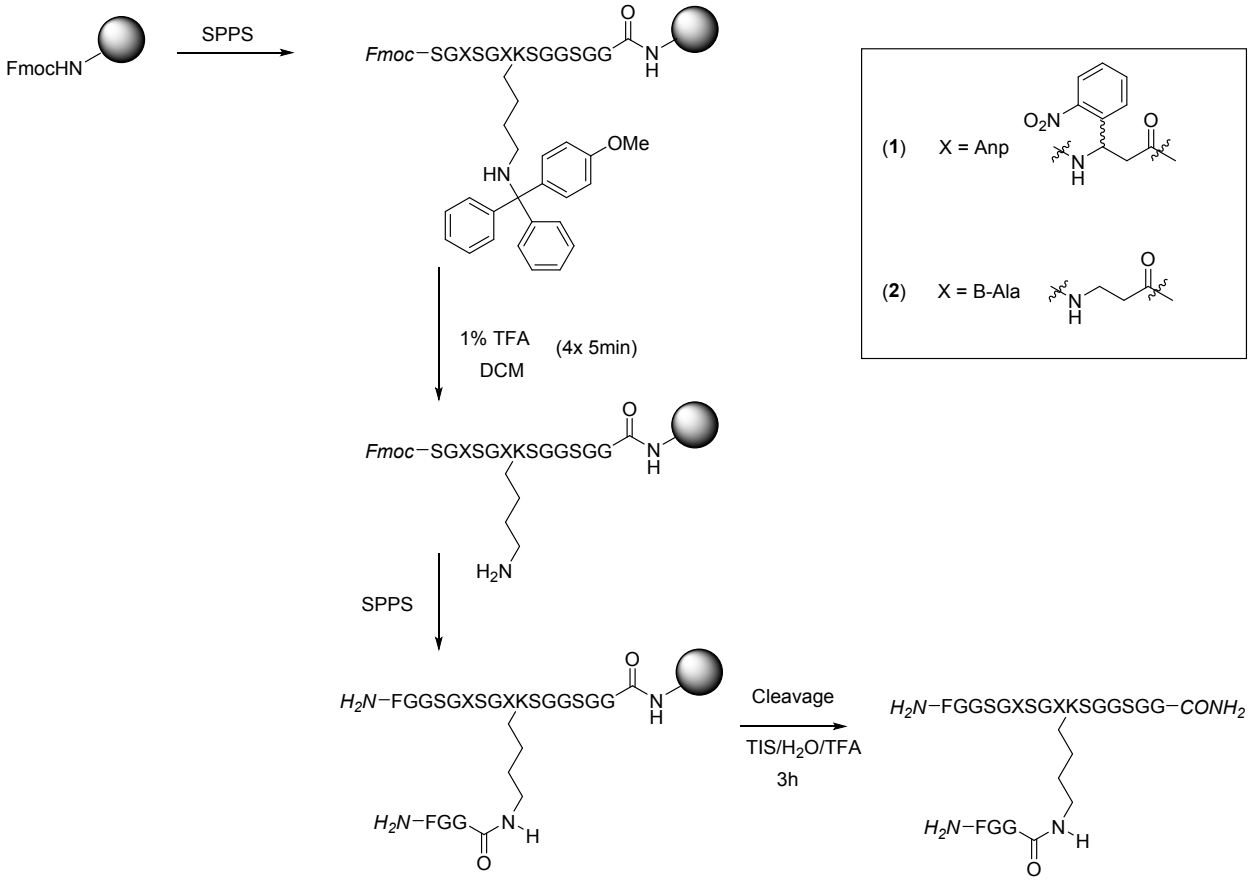
CB[8] stock preparation

CB[8] can be challenging to dissolve. Fresh stocks were made, on each day of experiments. CB[8] is weighed on a microbalance and dissolved in the desired buffer or milliQ at around 40 μM. The stock is sonicated and heated to 40˚C for around 1 hour, and vortexed at repeated intervals. Concentrations up to 80 μM could be reached via this method.

(Automated) Solid Phase Peptide Synthesis

All peptides were synthesized via Fmoc solid-phase peptide synthesis (SPPS) using an automated Intavis MultiPep RSi peptide synthesizer. TentaGel R-RAM resin (Rapp Polymere; 0.18 mmol/g loading) was used for the synthesis of the peptides. Fmoc-protected amino acid building blocks (Novabiochem) were dissolved in dimethylformamide (DMF) and coupled sequentially by double coupling to the resin using 1.2/0.5 M N,N-diisopropylethylamine(DIPEA)/(2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (HBTU) with a reaction time of 30min. The Fmoc-amino-3-(2-nitrophenyl)propanoic acid (Anp) residues were coupled using single coupling, but with reaction times of 60 min. Capping was performed using 1:1:3 Ac_2O/pyridine/DMF. Fmoc-deprotection was achieved using 20% piperidine in DMF (3 x 5 minutes). The deprotection of the Lys_{mmt} residue was performed manually with short reaction cycles of 1% trifluoracetic acid (TFA) in dichloromethane (DCM) for 4x5 min, with repeated washing with DCM in between until no more yellow color appeared[3]. After the lysine deprotection resins were placed back in the machine, and two FGG motifs are simultaneously synthesized onto the resin assuming now a 200 *μ*mol scale. Resin cleavage of the protected peptide was performed using 2.5%/2.5%/95% triisopropylsilane (TIS)/ H_2O/ trifluoroacetic acid (TFA) for 3 hours and followed by precipitation in cold diethyl ether

(-30 ˚C). The resultant crude was then re-dissolved in acetonitrile/water/0.1%TFA and lyophilized.



Supporting Figure S11. General synthesis scheme of bivalent FGG photocleavable cage **1** and control **2** by solid phase peptide synthesis (SPPS). The backbone of the cage was made via automated solid phase peptide synthesis incorporating either two Anp or β-Ala residues. The second FGG-tag was introduced from a branching lysine side chain, protected with a 4-*methoxytrityl* (Mmt) that was selectively deprotected manually before adding the last three residues again via automated synthesis.

Preparative HPLC-MS

All peptides were purified by preparative HPLC using shallow linear gradients of H_2O/acetonitrile + 0.1% trifluoracetic acid (21 - 22% acetonitrile for **1**; 12 - 18% acetonitrile for **2**) over a reverse phase C18 (Alltima HP 125 x 20 mm, Alltech) at a flowrate of 20 mL / min. The system is composed of a LCQ Deca XP Max (Thermo Finnigan) ion-trap mass spectrometer equipped with a Surveyor auto sampler, a Surveyor photodiode detector array (PDA) detector (Thermo Finnigan), and a PrepFC fraction collector (Gilson Inc). Solvents were pumped with a high-pressure gradient system using two LC-8A pumps (Shimadzu). Spectra were analysed using the

ThermoFischer Xcalibur MS software. Afterwards, the purity of all peptides was confirmed by analytical LCMS.

Analytical LC-MS

Analytical Liquid Chromatography coupled with Mass Spectrometry (LC-MS) was performed on a C4 Jupiter SuC4300A 150 x 2.0 mm column using ultrapure water with 0.1% formic acid (FA) and acetonitrile with 0.1% FA, in general with a gradient of 5% to 95% acetonitrile in 10 minutes, connected to a Thermo Fischer LCQ Fleet Ion Trap Mass Spectrometer. Spectra were analysed using the ThermoFischer Xcalibur MS software.

Protein expression and purificatin

Caspase-9 expression and purification was preformed according to Dang et al[4]:

A glycerol stock was available containing E. coli BL21 (DE3) (NovaBlue) encoded with a pET28a-HIS-SUMO-FGG-C9-STREP plasmid. Under sterile conditions three small cultures of LB medium with supplemented kanamycin (15 *µ*g / mL) were inoculated with the glycerol stock and incubated overnight. Subsequently three 2L cultures of LB medium with supplemented kanamycin (15 *µ*g / mL) in 5L baffled culture flasks were inoculated with the 8mL overnight cultures. The large cultures were incubate at 37°C and 160 RPM until an optical density OD_{600} around 0.5 - 0.7 was reached. Hereafter, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the flask to a final concentration of 1mM to induce expression. The cultures were incubated overnight for expression at 18°C and 160 RPM. The next day the cells were harvested suing centrifugation (10 min, 10.000 RCF, 4°C) in large 400mL tubes. After centrifugation the supernatant was discarded and cell pellet was stored at -80°C. Subsequently the pellet was resuspended in lysis buffer (10mL per gram of cell pellet; 1x PBS + 370 mM Na_2HPO_4, 10% (v/v) glycerol, 20 mM imidazole, 0.1 mM TCEP, pH 7.4) containing 1µL of Benzonase Nuclease per 10mL of basic buffer (with addition of 2mM $MgCl_2$), and cells were lysed using a EmulsiFlexC3 High Pressure homogenizer from Avestin at 15.000-20.000 psi. Cell lysate was removed via centrifugation for 30 min at 40.000 RCF and 4°C. The cell lysis supernatant was applied to a loaded Ni-column (Ni-loaded, 5mL HisTrap from GE healthcare at 4°C) and was washed subsequently with basic buffer (1x PBS + 370 mM NaCl, 10% (v/v) glycerol, 20 mM imidazole, 0.1 mM TCEP, pH 7.4) and wash buffer (basic buffer + 0.1% Triton X-100 v/v) alternately. Then the protein was eluded off the column with elution buffer (basic buffer + 230 mM imidazole) and was collected in the flow through. Cleavage of the His_6-SUMO tag was performed in a dialysis tube (SnakeSkin™ Dialysis Tubing, 3.5K MWCO, 16 mm, Thermo Fischer Scientific) using dtUD1 SUMO hydrolase (1:500) and is

dialyzed against 4L of dialysis buffer (25 mM Tris, 75 mM NaCl pH 7.8) by stirring overnight at 4°C.The protein was purified through removing the His_6-SUMO tag by using a second Ni-column and collecting the flow through and a subsequent washing step using the wash buffer. Both fractions were combined and subsequently the protein was buffer exchanged and concentrated using Amicon Ultra-15mL Centrifugal Filter (10kDa MWCO, Millipore) against activity assay buffer (20 mM Na_2HPO_4, 150 mM,NaCl, 1 mM EDTA, 2 mM TCEP, pH 7.0). Concentration of the protein was determined using a Nanodrop. The purity of the protein was assessed using SDS-page and Q-Tof.

^{1}H-NMR

Proton (1H) NMR (400 MHz) spectra were recorded on a Bruker Avance 400 MHz spectrometer. All spectra are water suppression scans. The spectra were analysed using MestReNova software.

UV-A intensity measurement

A Opsytec RM12 Radiometer equipped with a Opsytec RM12 UV-A sensor (0-200 mW / cm^2) has been used to determine the UV-A (320-400nm) intensity in mW / cm^2. This flat sensor could only record collimated/parallel light.

UV radiation – UV lamp

Promed UVL-36 UV lamp, equipped with four 9W UV bulbs. (50 Hz, 230 V, 36 W). Intensity of UV light was determined with above mentioned sensor around 6 mW / cm^2.

Radiation with the UV lamp was performed by placing a small aluminium-covered container filled with ice-water under the UV lamp. Samples were loaded in 100 – 500 µL Eppendorf tubes and placed into the container.

LC-MS cleavage experiment

For all peptides, a reaction vessel was created. Around 1 mg/mL per peptide (approximately 500 *µ*M) from the original stock was dissolved in UP-mQ in a 500 *µ*L Eppendorf tube. Subsequently, around 0.5 mg/mL of a reference peptide (*Ac*-AEGFPApTV-*COOH* obtained from Genscript) was added to establish an internal standard in the LC-MS for normalization. The reaction is placed under the UV lamp for certain periods of time. After UV radiation at a certain time point, the reaction vessel was vortexed and a sample was taken from the vessel. This was added to a LC-MS vial and 5-8x diluted with UP-mQ (0.1% FA). All samples were measured on the analytical LC-MS. The first 3 minutes were diverted from the MS to bypass residual salts in the samples

from the MS system. After measurement, all data was analysed on the ThermoFischer Xcalibur MS software. With this software all ion counts of the possible peptide fragments after cleavage were integrated. All area's under the curve (AUC) were exported to excel, and normalized to the ion count AUC of the reference peptide. Subsequently a plot representing the decrease of cleavable peptide over time could be acquired. The results were analysed and visualized using Origin 2020 (OriginLab). Fitting was done with an exponential decay formula $y = y_0 + A_1*\exp(-(x-x_0)/t_1)$.

UV-VIS measurements

All UV-Vis measurements were performed on a Varian Cary 50 UV-Vis spectrophotometer using a 10 mm quarts cuvette (Hellma). Data was analysed and visualized using Origin 2020 (OriginLab). Raw spectra were smoothened using 10 point Stavitzsky-Golay filter. The intensities at 230 nm were fitted using an exponential association formula $y = y_0 + A_1*(1 - \exp(-x/t_1))$.

Fluorometric assays

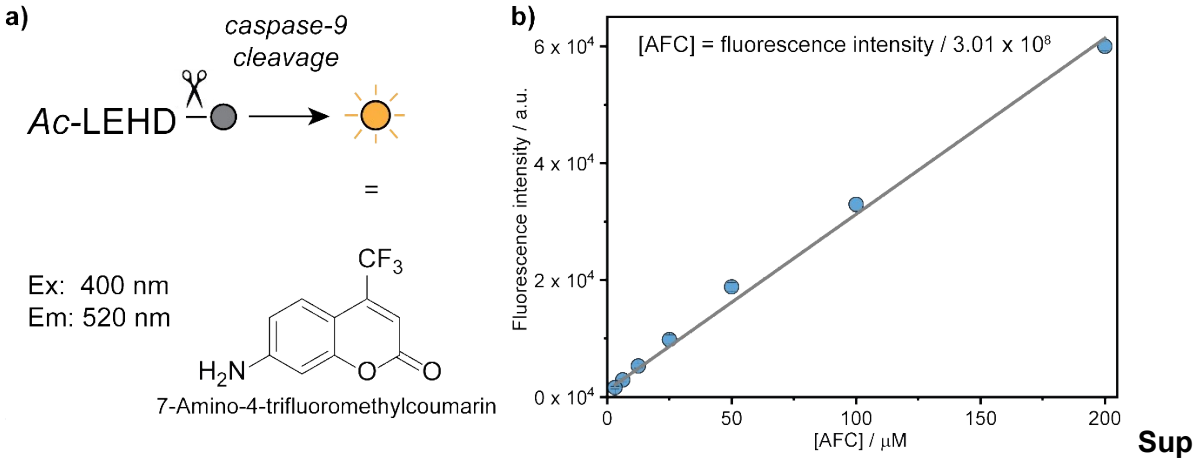
All fluorometric assays were performed using a filter-based microplate reader (Tecan Infinite F500) using a filterset (λex:485 nm/20 nm, λem:535 nm/25 nm) and an integration time of 50 µs in black, round-bottom 384 microwell plates with wells of a volume of 10µL (Corning, #25916024) in triplicate at RT. Direct fluorometric titrations were performed for determining acridine orange (AO) affinity for CB[8] using 100nM acridine orange hydrochloride hydrate (AO) (Sigma Aldrich) in HBS-buffer containing 10mM HEPES (pH 7.4), 150mM NaCl, by a dilution series varying the concentration of CB[8]. For the fluorometric displacement assay (Figure 3 main manuscript), solutions of acridine orange hydrochloride hydrate (1 μM) and CB[8] (1 μM) in HBS Buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.01% TWEEN-20) was using varying the concentrations of the bivalent FGG-cage (**1**) and control (**2**) peptides. The whole plate was placed under the UV-lamp for UV irradiation for certain periods of time. In-between time points, the plate was shortly incubated (shaker) and fluorescence was recorded using the plate reader. The data was fitted with four-parameter logistic model (4PL) using Origin 2020 (OriginLab). In addition the titrations were performed where the peptides are irradiation beforehand (Supporting Figure S6).

Isothermal titration calorimetry

ITC experiments were carried out with a MicroCal PEAQ-ITC isothermal titration calorimeter (Malvern Panalytical) in activity assay buffer (20 mM Na_2HPO_4, 150 mM NaCl, 1 mM EDTA, 2mM TCEP, pH 7.0). Affinities were measured with 200 μM bivalent FGG-cage **1** or 200 μM control **2** in the syringe and 20 μM CB[8] in the cell (3 μL injections,150s interval, 125rpm). UV-exposure was done for 20 minutes. All samples were degassed prior to use. Data was analysed with the corresponding software MicroCal PEAQ-ITC Analysis Software (Malvern).

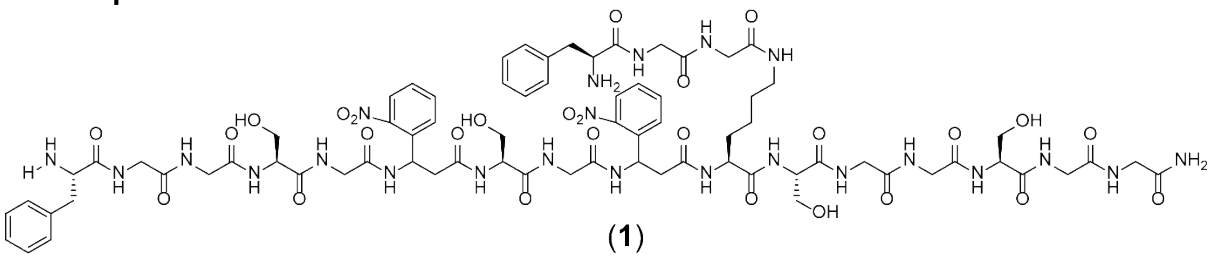
Enzyme activity assays

Enzyme activity was measured using the synthetic tetrapeptide caspase-9 substrate Ac-LEHD-AFC (dissolved in dry DMSO at 10 mM), which is cleaved by 1 μM caspase-9 after the aspartic acid residue releasing and uncaging the fluorescent dye AFC. Substrate was added to a final concentration of 200 μM and proteolytic cleavage was monitored over time in 96-well plates (Greiner) (100 μl reaction volume) at 20 °C by measuring fluorescence (excitation: 400/20 nm, emission: 520/25 nm) using a filter-based Tecan infinite plate reader. Fluorescence units were converted to concentration using a calibration curve (Supplementary Figure S12). To this end, varying concentrations of free AFC (dissolved in dry DMSO at 10 mM) were added to activity buffer (20 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, 2mM TCEP, pH 7.0), and fluorescence was measured as described. Raw data of all activity assays were extracted and converted. Enzyme activity (in μmol min^{-1}) was determined by fitting the initial slope of the kinetic trace to a linear curve.

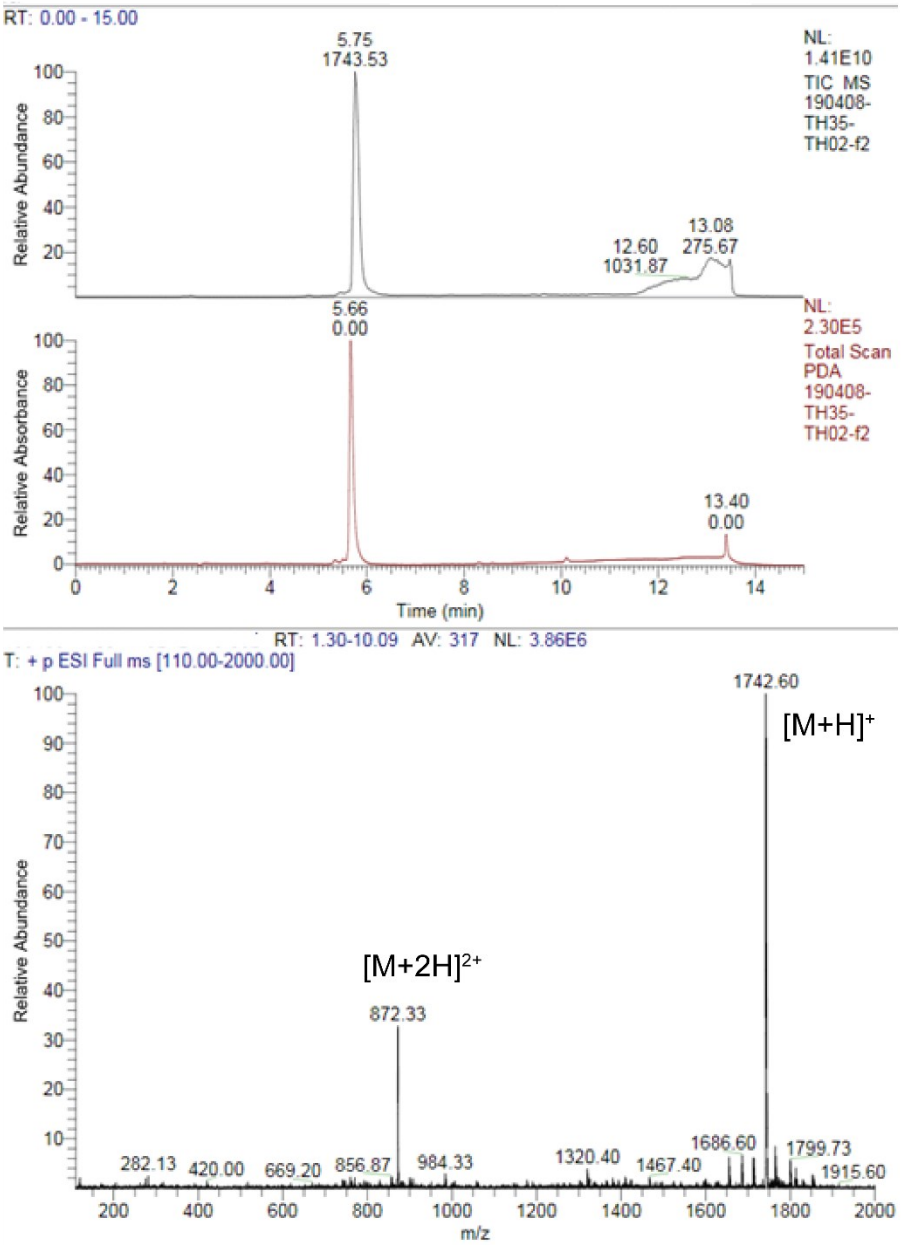


Supplementary Figure S12. Concentration calibration of the synthetic caspase-9 substrate *Ac*-LEHD-AFC. a) Schematic depicting caspase-9 cleavage of the synthetic tetrapeptide *Ac*-LEHD-AFC. Proteolytic activity of caspase-9 leads to cleavage behind the aspartic acid residue, releasing and unquenching the coumarin derivative AFC. The fluorescence increase over time can be measured in a platereader by excitation (ex.) at 400 nm and measuring the emission (em.) at 520 nm. b) Graph depicting the mean fluorescence intensity of free AFC at various concentrations (two-fold dilution from a 60 μL 200 μmol stock solution in activity buffer), and fitting the data to a linear curve. Calibrations were performed in triplicate with three different stock solutions of AFC. The concentrations were chosen such that the fluorescence intensities of all experiments fall within the range of the calibration curve.

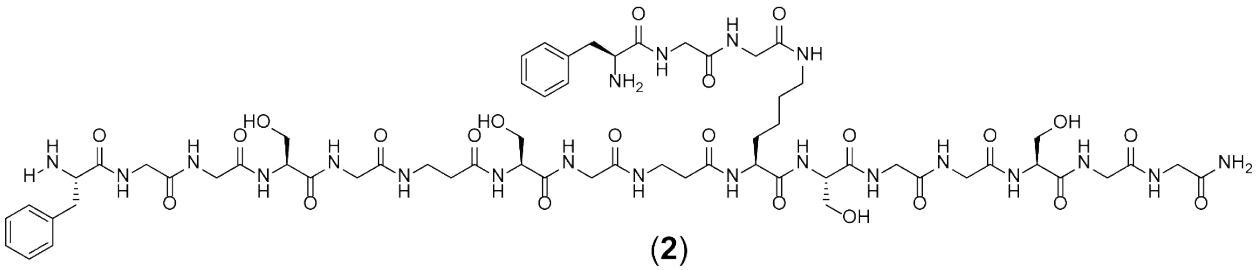
LCMS spectra



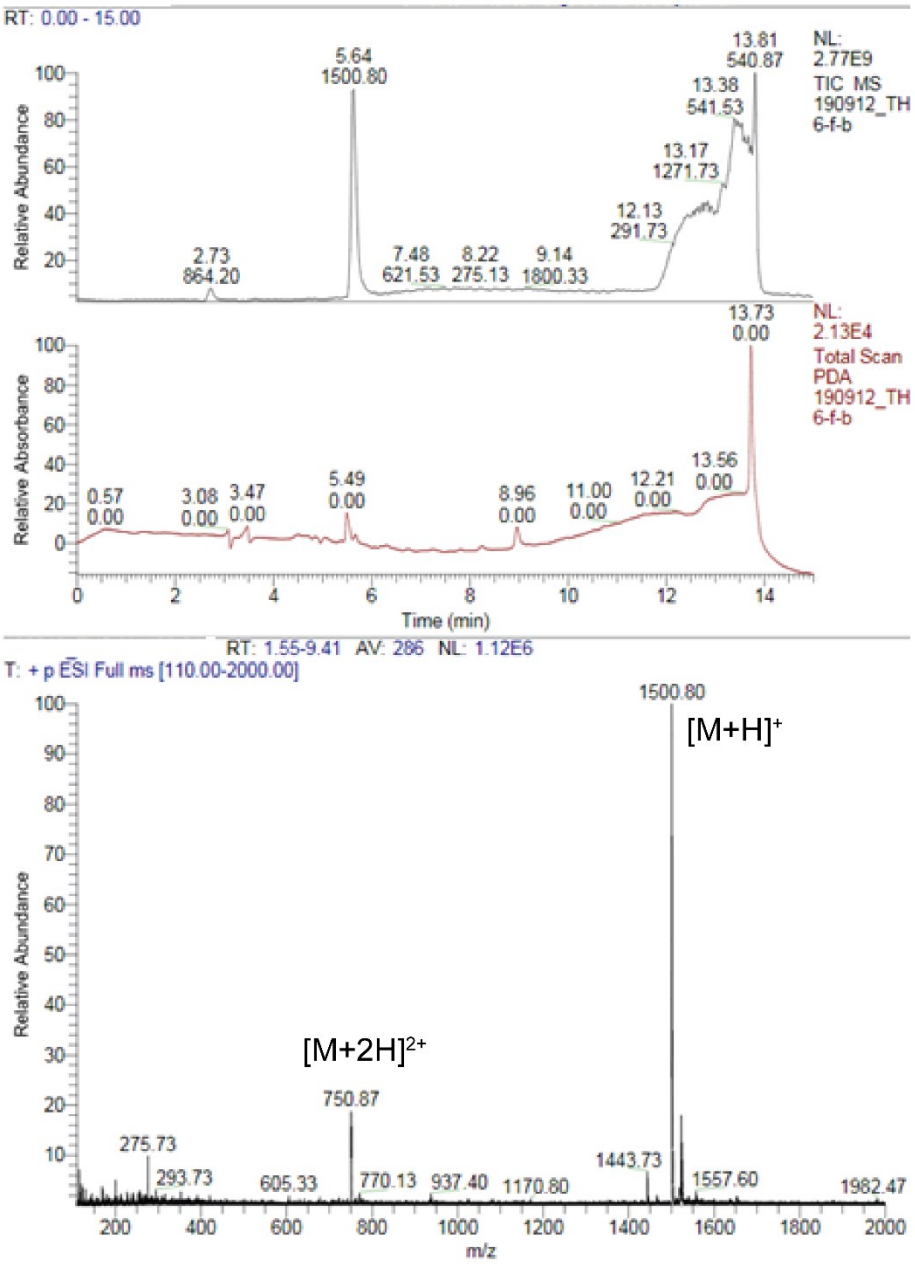
(**1**)



Supplementary Figure S13. Analytical LCMS data of **1** (sequence: *H_2N*-FGGSGAnpSGAnpK(GGF)SGGSGG-*CONH2*). Rt=5.7; ESI-MS: $[M+H]^{1+}$, Calculated mass: 1742.7, Observed: 1742.6; $[M+2H]^{2+}$, Calculated mass: 872.4, Observed: 872.3.



(**2**)



Supplementary Figure S14. Analytical LCMS data of **2** (sequence: H_2N-FGGSG(β -Ala)SG(β-Ala)K(GGF)SGGSGG-$CONH_2$). Rt=5.6; ESI-MS: $[M+H]^{1+}$, Calculated mass: 1500.4, Observed: 1500.8; $[M+2H]^{2+}$, Calculated mass: 750.7, Observed: 750.9.

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