Electronic Supplementary Material (ESI) for ChemComm.

Cucurbit[8]uril templated supramolecular ring structure formation and protein assembly modulation

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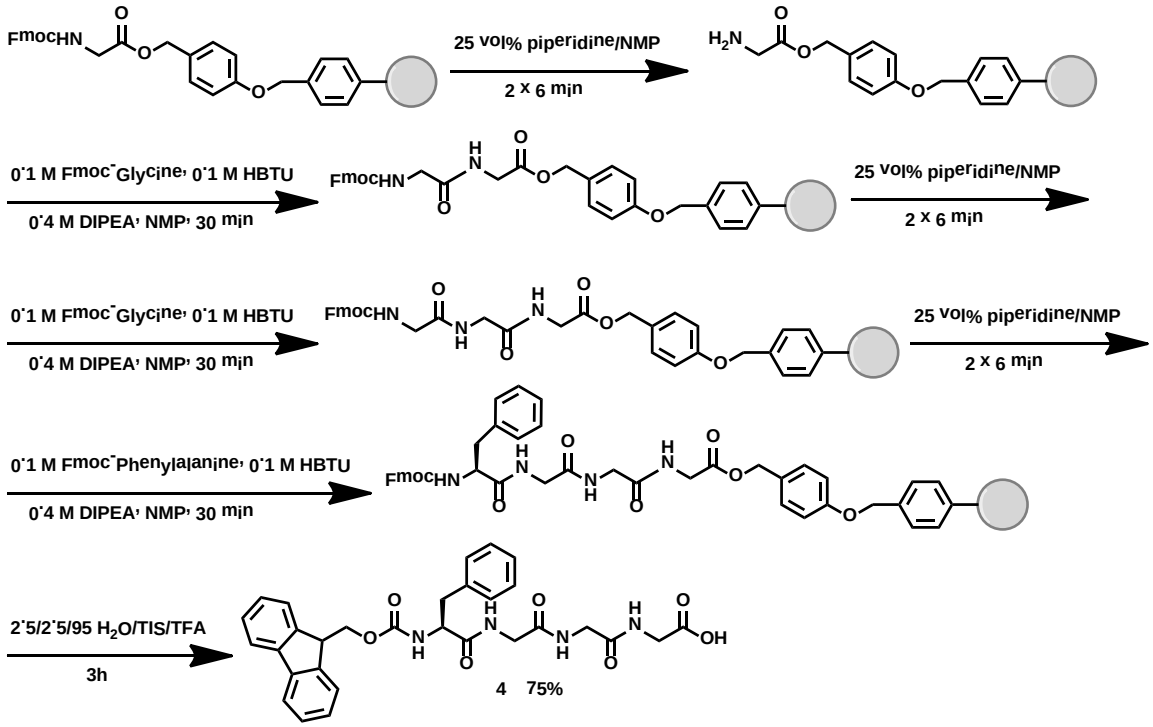
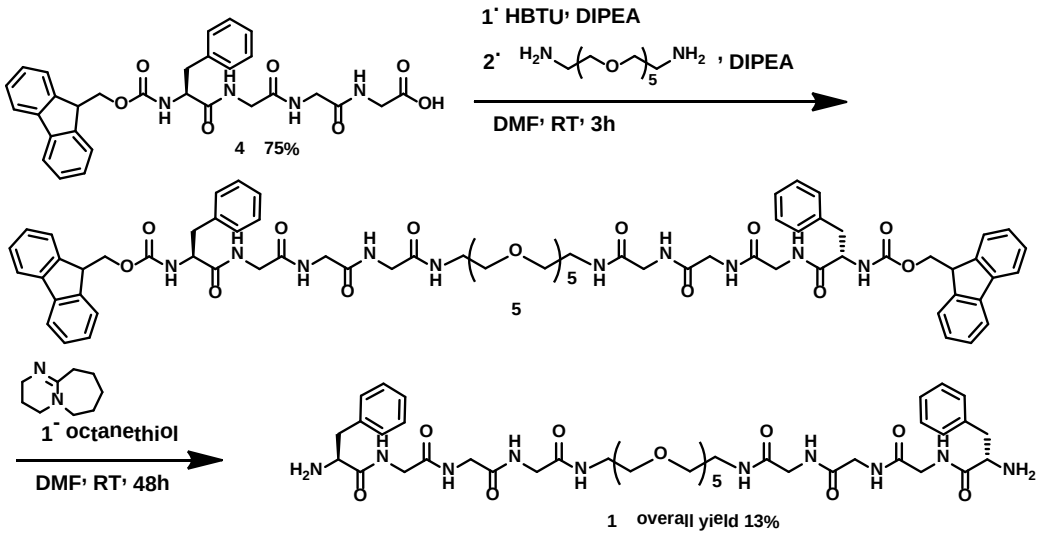
Supporting info

1. Experimental section

Synthesis



SI Scheme 1. Solid phase peptide synthesis of **4**. The first step is the removal of the Fmoc group of the glycine on the Gly-Wang resin with piperidine. The second step is the coupling of the HBTU activated Fmoc-glycine. This is repeated for the second Fmoc-glycine after which the Fmoc-phenylalanine is coupled. The Fmoc-FGGG peptide is then cleaved from the resin with a H_2O/TIS/TFA mixture.



SI Scheme 2. Synthesis route to compound **1**, starting with the coupling of Fmoc-FGGG (**1**), obtained from solid phase peptide synthesis, to a penta(ethylene glycol) diamine spacer (**5**) and as a final step deprotection of the phenylalanine with DBU.

Solid Phase Peptide Synthesis of 4 (Fmoc-Phenylalanine-Glycine-Glycine-Glycine-COOH)

Custom-made glassware was used for the manual solid phase peptide synthesis (SPPS). The synthesis was performed from C- to N-terminus on a 1.6 mmol scale (2.286 g of Fmoc-Gly-Wang resin with a loading of 0.70 mmol/g). The resin was swollen with N-methyl-2-pyrrolidone (NMP) for 30 min, the Fmoc group was removed by treatment with piperidine in NMP (20 vol%, 16 mL, 2 x 6 min) and washed with NMP (10 mL, 5 x 30 sec).

The coupling of the amino acids (glycine, glycine and phenylalanine) was performed in cycles containing the following steps: First, the amino acid was coupled by adding a cocktail containing the appropriate amino acid (200 mM in NMP, 32 mL), activator *O*-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU, 0.4 M in NMP, 16 mL) and base N,N- diisopropylethylamine (DIPEA, 1.6 M in NMP, 16 mL) in the concentration ratio of 1/1/4 (30 min). Then the resin was washed with NMP (10 mL, 5 x 30 sec), the Fmoc group was removed by treatment with piperidine in NMP (25 vol%, 16 mL, 2 x 6 min) and again washed with NMP (10 mL, 5 x 30 sec). These steps were repeated for the following amino acids with exception of the last (phenylalanine). Here the deprotection step was not performed. Instead, the resin was washed with NMP (10 mL, 5 x 30 sec), DCM (10 mL, 4 x 30 sec), and finally with diethyl ether (10 mL, 3 x 30 sec).

The peptide was cleaved from the resin with a cleavage solution of trifluoracetic acid (TFA), triisopropylsilane (TIS) and water (95/2.5/2.5 vol%, 15 mL, 3 hr shaking). The solution was concentrated by evaporation and precipitated in 3 x 40 mL 20 vol% hexane in diethyl ether for 30 minutes at -30 °C, centrifuged (2000 rpm, 10 min) and decanted. The solid pellet was dissolved in an acetonitrile water mixture and lyophilized to gain a white powder (673 mg, 1.2 mmol, 75% yield).

^{1}H-NMR (400 MHz, DMF-d_7): δ = 8.49 (t, 1H, -N*H*-), 8.17 (m, 2H, -N*H*-), 7.94 (d, 2H, Fmoc, -C*H*=), 7.76 (d, 1H, -N*H*-), 7.70 (d, 2H, Fmoc, -C*H*=), 7.46-7.20 (m, 9H, Phe and Fmoc, -C*H*=), 4.49 (m, 1H, Phe, -C*H*-), 4.25-4.18 (m, 3H, Fmoc, -CH- and –CH_2-), 3.97-3.93 (m, 6H, -CH_2), 3.28 and 3.00 (m, 2H, Phe, -CH_2-) ppm

^{13}C-NMR (100 MHz, DMF-d_7): δ = 173.40, 172.17, 170.49, 170.44, 145.27, 145.23, 142.18, 139.51, 130.49, 129.27, 128.77, 128.21, 127.43, 126.57, 126,45, 121,12, 67.45, 57.95, 48,05, 43.82, 43.25, 41.75, 38.73 ppm.

LC-MS (linear gradient: 2-100% acetonitrile): m/z $[M+H]^+$ calculated = 559.60 g/mol, m/z observed $[M+H]^+$ = 559.33 g/mol.

Synthesis of 5 ([Fmoc-FGGG]$_2$-OEG$_5$)

4 eq. **4** (248.0 mg, 0.44 mmol) was dissolved in DMF. 3.8 eq. HBTU (158.5 mg, 0.418 mmol) and 4 eq. DIPEA (77.5 µL, 0.44 mmol) were also dissolved in DMF and added to the peptide. This was then added to 1 eq. OEG_5-bisamine (30.0 mg, 0.107 mmol) and 4 eq. DIPEA (77.5 µL, 0.44 mmol) in DMF. After 80 min the mixture was evaporated by airflow and after a total of 190 min the product was precipitated in ether, cooled at -20 °C for 30 min, centrifuged (2000 rpm, 10 min), and decanted. Yield not determined but immediately proceeded with the next step.

^{1}H NMR (400 MHz, DMF-d_7): δ = 8.50 (t, 1H, -N*H*-), 8.21 (t, 1H, -N*H*-), 8.12 (t, 1H, -N*H*-), 7.93 (d, 2H, Fmoc, -C*H*=), 7.80 (t, 1H, -N*H*-PEG), 7.76 (d, 1H, Fmoc-N*H*-), 7.70 (d, 2H, 2H, Fmoc, -C*H*=), 7.46-7.19 (m, 9H, Phe and Fmoc, -C*H*=),4.48 (m, 1H, Phe, -C*H*-), 4.28-4.18 , (m, 3H, Fmoc, -CH- and –CH_2-), 3.95 (t, 2H), 3.91 (d, 2H, -CH_2-), 3.85 (d, 2H, -CH_2), 3.56-3.54 (m, 4H, OEG, -CH_2), 3.48 (t, 2H, -CH_2-), 3.33 (t, 2H, -CH_2-) 3.28 and 2.99 (m, 2H, Phe, -CH_2-) ppm.

^{13}C-NMR (100 MHz, DMF-d_7): δ = 173.48, 170.89, 170.46, 170.12, 157.51, 145.27, 145.24, 130.50, 129.28, 128.78, 128.19, 127.45, 126.58, 126.51, 121.14, 71.40, 71.34, 71.27, 71.12, 70.39, 67.46, 57.92, 48.10, 43.88, 43.74, 43.44, 40.01, 38.76 ppm.

LC-MS: m/z $[M+H]^+$ calculated = 1362.5 g/mol, m/z $[M+H]^+$ observed = 1361.50 g/mol.

Synthesis of 1 ([FGGG]$_2$-OEG$_5$)

For the deprotection, the pellet of **5** was dissolved in DMF and 0.7 eq. DBU (47 µL, 0.311 mmol) and 10 eq. 1-octanethiol (771 µL, 4.44 mmol) were added. The reaction was followed by LC-MS and after a total of 48 h, the product was purified by precipitation in a cooled mixture of hexane (20 vol%) in ether, centrifuged (2000 rpm, 10 min), decanted, dissolved in water and lyophilized. The product was further purified by preparative LC-MS to obtain a 13% overall yield (13.0 mg, 0.014 mmol). Two preparative LC-MS runs were needed, linear gradients: 1) 12-17% acetonitrile in water 2) 16-20% acetonitrile in water.

^{1}H-NMR (400 MHz, DMF-d_7): δ = 8.43 (t, 1H, -N*H*-), 8.30 (t, 1H, -N*H*-), 8.17 (t, 1H, -N*H*-), 7.81 (t, 1H, -N*H*-PEG), 7.34-7.22 (m, 5H, -C*H*=), 3.93 (t, 2H), 3.89 (d, 2H, -CH_2-), 3.84 (d, 2H, -CH_2-), 3.62-3.56 (m, OEG, CH_2), 3.49 (t, 2H, -CH_2-), 3.33 (t, 2H, -CH_2-) 3.17 and 2.72 (m, 2H, Phe, -CH_2-) ppm.

^{13}C-NMR (100 MHz, DMF-d_7): δ = 176.44, 171.15, 170.53, 170.17, 140.22, 130.50, 129.34, 127.30, 80.28, 71.39, 71.35, 71.28, 71.12, 70.38, 66.34, 57.87, 43.78, 43.74, 43.43, 42.05, 40.01 ppm.

LC-MS: m/z $[M+H]^+$ calculated = 917.6 g/mol, m/z $[M+H]^+$ observed = 917.42 g/mol.

Experimental methods

Unless stated otherwise, all reagents and chemicals were obtained from commercial sources and used without further purification.

NMR. ^{1}H-NMR and ^{13}C-NMR spectra were recorded at room temperature using a 400 MHz Varian Mercury NMR spectrometer or a 500 MHz Varian Unity Inova NMR spectrometer. Proton and carbon chemical shifts are reported in parts per million downfield from TMS (in DMF-d7) or TMSP (in D_2O). Spectrum analysis was done with MestReNova v6.0.2-5475, Mestrelab Research S.L. Temperature-dependent and pH-dependent experiments were performed on the 500 MHz NMR spectrometer. A 1:1 mixture of Q8 and compound **1** (100 µM) in D_2O was measured at 10, 20, 30, 40, 50, 60 and 70 °C. Additionally, 1:1 mixture of Q8 and compound **1** (100 µM) in D_2O was measured at neutral, basic and acidic conditions (all at 20 °C). To obtain a basic solution, 50 µL 1 M NaOH was added to obtain a solution with pH > 9; subsequently 50 µL 1 M HCl was added to return to neutral conditions, followed by the addition of 50 µL 1 M HCl to obtain a solution with pH < 3. For DOSY-NMR, spectra were recorded with a 500 MHz Varian Unity Inova NMR spectrometer in D_2O at room temperature. Samples were not spinning during measurements. The DOSY bipolar pulse pair stimulated echo with convection compensation (Dbppste_cc) sequence was used for the determination of the diffusion of Q8. Experiments of 256 to 1024 transients for 15 to 30 steps were recorded with initial gradient strengths ranging from 1.085 G/cm to 32.55 G/cm. For each measurement, the gradient pulse duration, diffusion delay and maximum gradient strength were adjusted in order to obtain an 80% reduction of the signal at the highest gradient strength. Solvent suppression was performed using presaturation (PRESAT) with a delay of 2 sec, a saturation frequency of -175.2 Hz at 4.65 ppm, and a power of 2 dB.

IR. Infrared spectra were recorded using a Perking Elmer Spectrum One FT-IR spectrometer.

LCMS. Liquid chromatography – mass spectrometry (LC-MS) data was obtained using a Thermoscientific LCQ fleet spectrometer. Separation was obtained by using a GRACE reverse phase C18 column with gradients of acetonitrile in water (both containing 0.1% formic acid).

RP-HPLC. Preparative reverse phase high pressure liquid chromatography (RP-HPLC) was performed on a Varian Pro Star HPLC system coupled using using a Vydac protein & peptide C18 column. Gradients of acetonitrile in water (both containing 0.1 % TFA) were used to elute products.

MALDI. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on a PerSeptive Biosystems Voyager-DE PRO spectrometer with an UV nitrogen laser using α-cyano-4-hydroxycinnamic acid (CHCA) and 2-[(2E)-3-(4-tert-Butylphenyl)-2-methylprop-2-enylidene]malononitrile (DCTB) matrices.

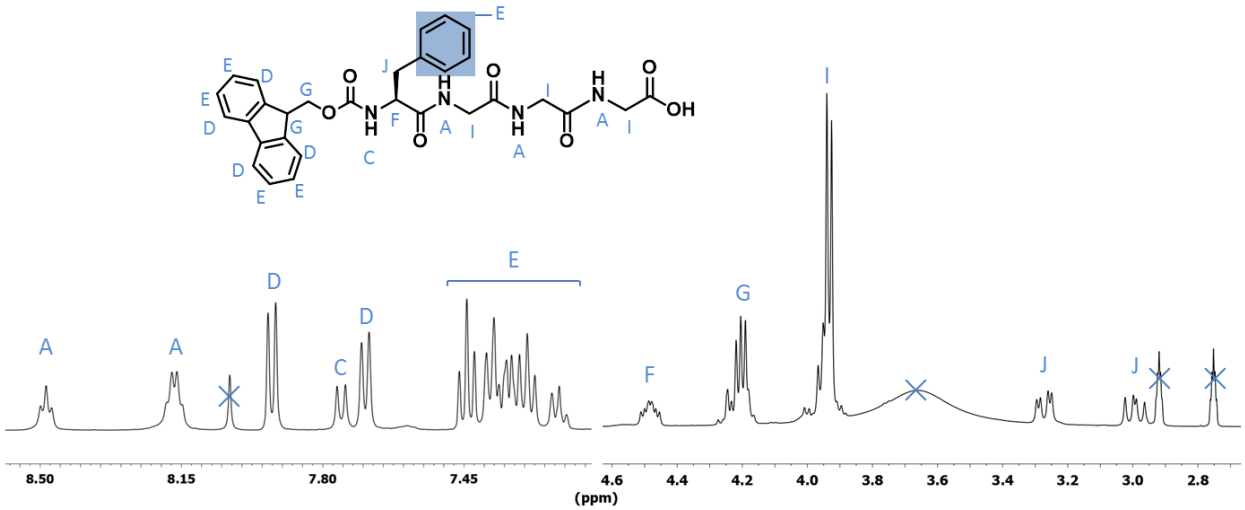
ITC. All isothermal titration calorimetry (ITC) experiments were performed with a General Electronics MicroCal ITC200 system in a 10 mM pH 7.0 sodium phosphate buffer. Prior to the measurements all samples were degassed using a General Electronics MicroCal ThermoVac. Measurements were performed with 40 times 1 µL titrations of $[FGGG]_2$-OEG_5 (500 µM) with a spacing of 200 sec in ~200 µL, 50 µM Q8 at 27 °C. Data analysis was performed with Origin 7.0 of which the one set of sites binding model was applied. The data point of the first titration was removed and the mean generated heat after saturation (the data of approximately the 10 last titrations) was subtracted from the complete data set before fitting the model.

SEC. Size exclusion chromatography (SEC) experiments were performed on a general electronics Superdex 75 column (General Electronics) equilibrated with a 10 mM sodium phosphate pH 7.0, coupled to a Shimadzu SPD-10AV UV-Vis measuring the absorption at 260 nm. In each experiment, 2.5 µL of the samples was manually injected onto the column using a 30 min run with a flow rate of 0.1 mL/min.

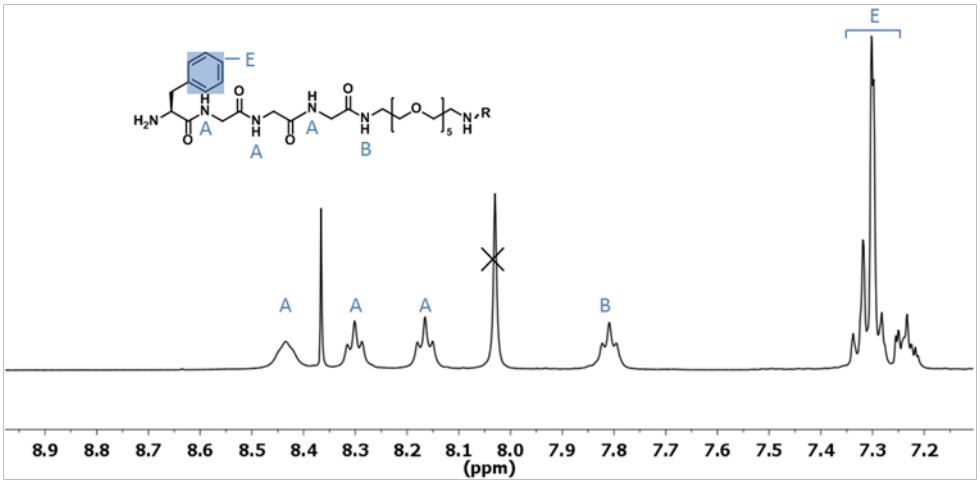
QTOF-MS. QTOF mass spectrometry was performed with a Waters Xevo G2 Quadrupole time of flight spectrometer. Samples were prepared in ammonium acetate buffer (pH 6.8) and measurements were performed with 1 µL injections in the positive electrospray (ESI) mode. MaxEnt 1 software was used for deconvolution.

Fluorescence anisotropy. The anisotropy measurements were performed in a 10 mM, pH 7.0, sodium phosphate buffer in quartz cuvettes of 10 mm light path with Cary Eclipse fluorescence spectrophotometer (Varian) at 20 °C with an excitation wavelength of 500 nm. Emission was measured from 520 to 700 nm. The FGG-YFP protein was kindly provided by Dung T. Dang.[1] The concentration was determined using a NanoDrop ND1000 (Nanodrop technologies) and adjusted using the absorbance at 514 nm and a molar extinction coefficient of 83400 M^{-1} cm^{-1}. Titrations were performed with spacings of 10-30 min except for experiment 3 (Figure 5c) where equilibrium times up to two days were applied.

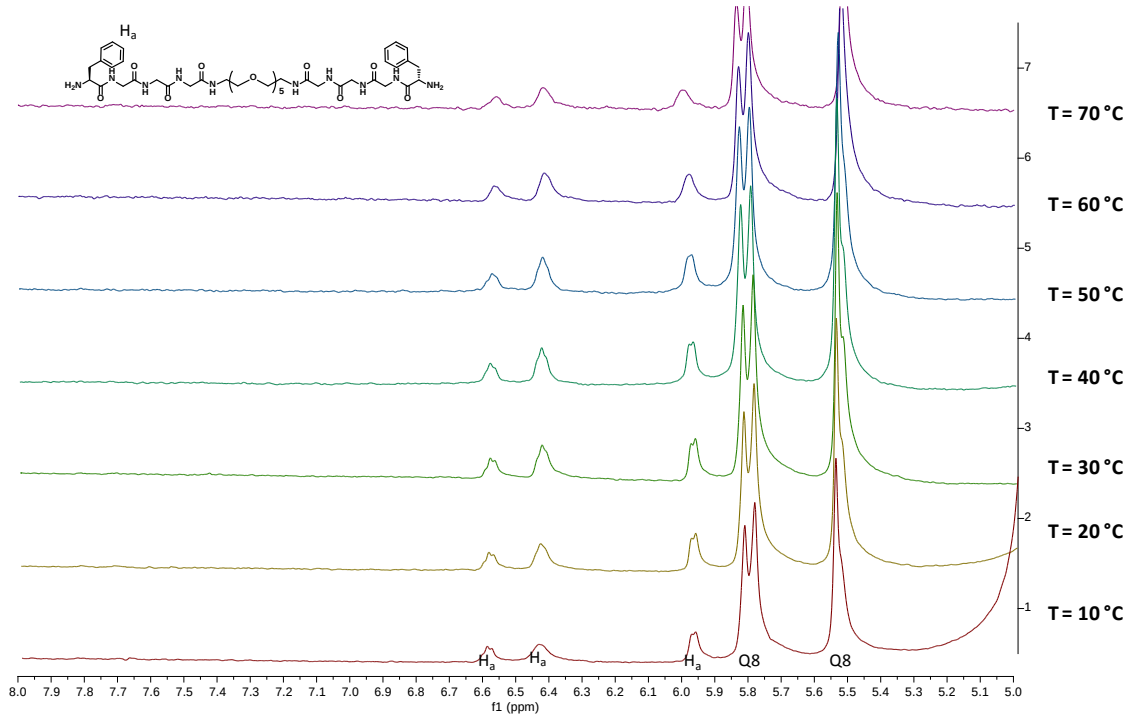
2. ^{1}H-NMR spectra



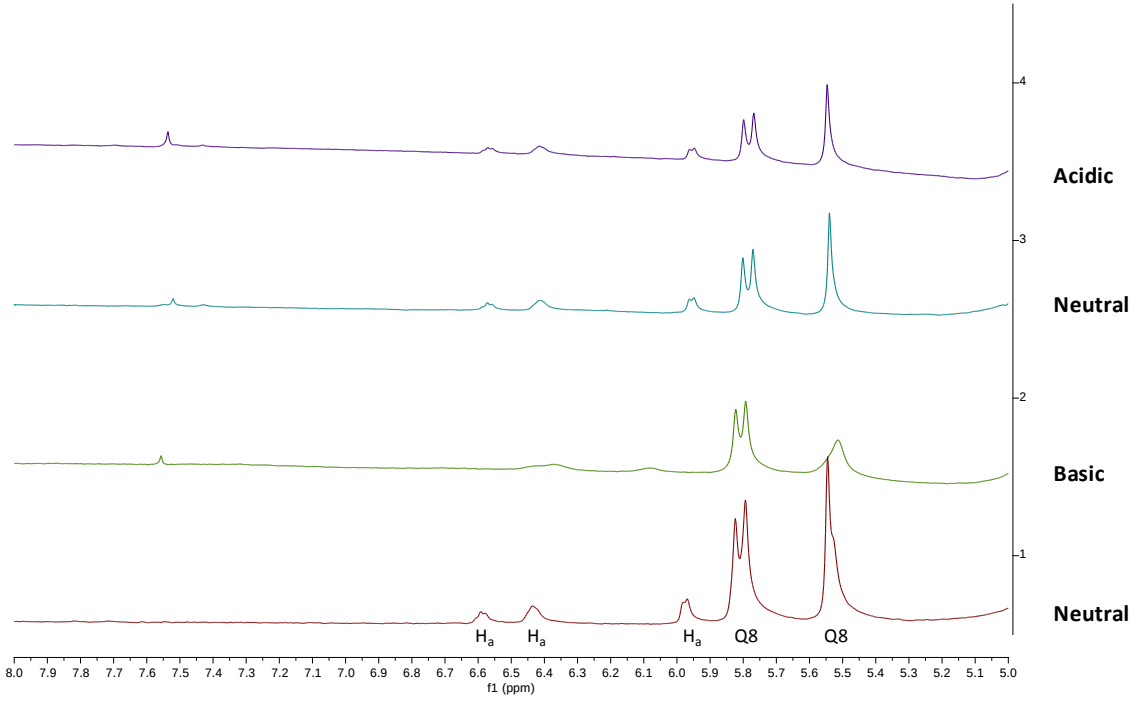
SI Fig 1. ^{1}H-NMR spectrum of **4** in DMF-d7.



SI Fig 2. ^{1}H-NMR of compound **1** in DMF-d7.

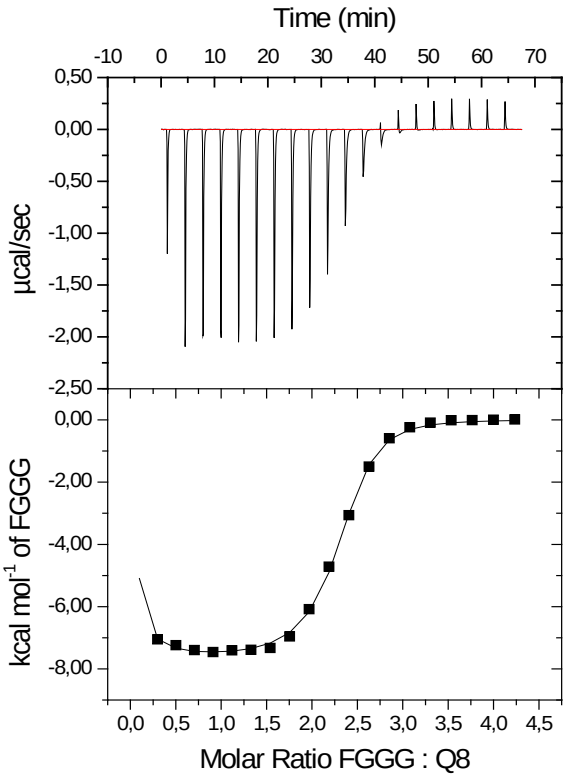


SI Fig 3. [1]H-NMR spectra of compound **1** and Q8 in a 1:1 mixture of 100 µM in D_2O heated from 10 °C (red) to 70 °C (violet). No change in complexation was observed.



SI Fig 4. [1]H-NMR spectra of compound **1** and Q8 in a 1:1 mixture of 100 µM in D_2O at neutral pH (red spectrum; this is the same spectrum in SI Fig 3 T=20 °C), basic pH (green spectrum, pH > 9 by addition of 50 µL 1 M NaOH), neutral pH (blue spectrum, by addition of 50 µL 1 M NaOH and 50 µL 1 M HCl) and acidic pH (purple spectrum, pH < 3 by addition of 50 µL 1 M NaOH and 100 µL 1 M HCl). The protons of the free phenylalanine were not observed at any of the conditions measured.

3. ITC experiments



SI Fig 5. ITC data of FGGG titrated to Q8.

SI Table 1. The thermodynamic parameters of the binding of **1** to Q8 determined by ITC compared to these parameters of the formation of Q8·$FGGG_2$ and Q8·FGG_2 as determined by Urbach *et al.*.[2]

Complex	$\Delta H_{ter/ring}$ [a] (kcal/mol)	$K_{ter/ring}$ [b]	$\Delta G_{ter/ring}$ [c] (kcal/mol)	$\Delta S_{ter/ring}$ [d] (cal/mol/deg)
Q8·FGG_2	-29.6 ± 0.2	$1.5 \pm 0.2 \cdot 10^{11}$ M^{-2}	-15.4	-47.3
Q8·$FGGG_2$	-16.2 ± 1.0	$1.2 \pm 0.1 \cdot 10^{10}$ M^{-2}	-13.8	-8.00
Q8·**1**	-16.6 ± 0.1	$9.0 \pm 1.0 \cdot 10^{6}$ M^{-1}	-9.54	-23.5

[a] *Enthalpy measured by ITC experiments at 27 °C.* [b] *Ternary (FGG and FGGG) and ring (**1**) binding constants determined by ITC.* [c] *Gibbs free energy values calculated from K_{ter} and K_{ring}.* [d] *Entropy values calculated from $\Delta H_{ter/ring}$ and $\Delta G_{ter/ring}$.*

4. Reaction equilibria and equations

$Q8 + \mathbf{1} \leftrightarrow Q8 \cdot \mathbf{1}$	$\mathrm{K}_1 = \frac{[Q8 \cdot \mathbf{1}]}{[Q8][\mathbf{1}]}$	intermolecular
$Q8 \cdot \mathbf{1} \leftrightarrow Q8 \cdot \mathbf{1}_2$	$\mathrm{K}_2 = \frac{[Q8 \cdot \mathbf{1}_2]}{[Q8 \cdot \mathbf{1}]}$	intramolecular

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$Q8 + \mathbf{1} \leftrightarrow Q8 \cdot \mathbf{1}_2 \qquad K_{ring} = \frac{[Q8 \cdot \mathbf{1}_2]}{[Q8][\mathbf{1}]}$

$C_{eff} = \frac{K_2}{K_1} \qquad K_2 = K_1 C_{eff}$

$K_{ring} = K_1 K_2 = K_1^2 C_{eff}$

$Q8 + FGGG \leftrightarrow Q8 \cdot FGGG$	$\mathrm{K}_1' = \frac{[Q8 \cdot FGGG]}{[Q8][FGGG]} \approx K_1$	intermolecular
$Q8 \cdot FGGG + FGGG \leftrightarrow Q8 \cdot FGGG_2$	$\mathrm{K}_2' = \frac{[Q8 \cdot FGGG_2]}{[Q8 \cdot FGGG][FGGG]} \approx K_1$	intermolecular

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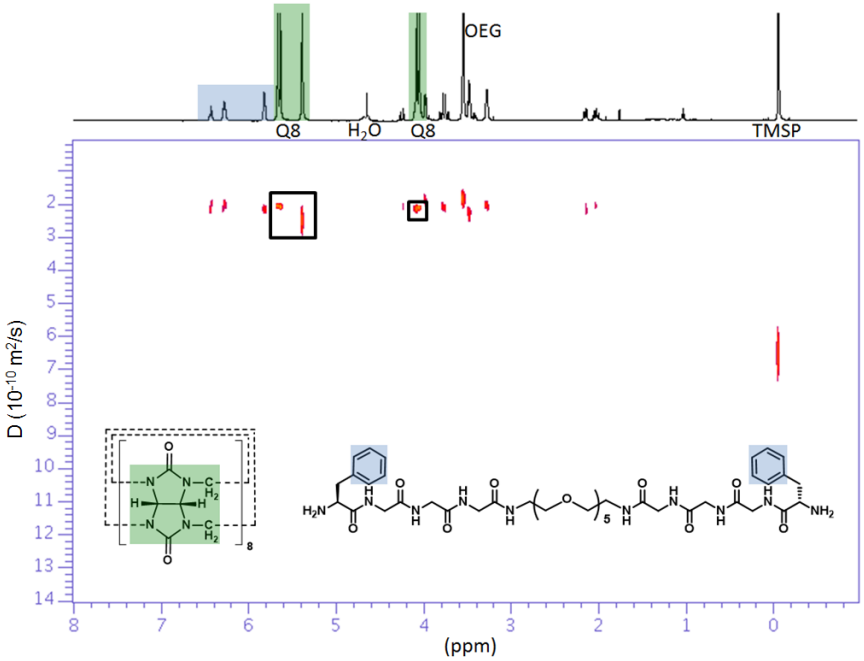
$Q8 + 2\,FGGG \leftrightarrow Q8 \cdot FGGG_2 \qquad \mathrm{K}_{\mathrm{ter}} = \frac{[Q8 \cdot FGGG_2]}{[Q8][FGGG]^2}$

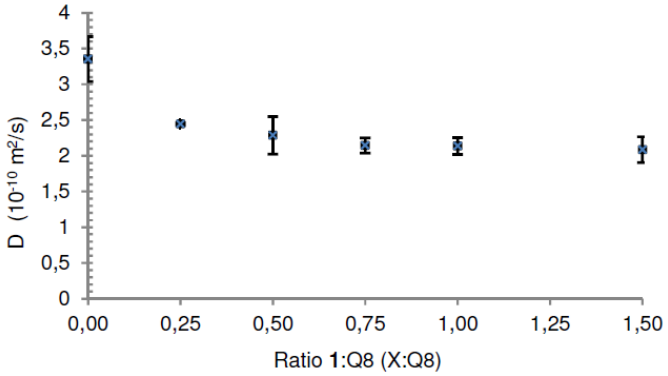
$K_{ter} = K_1' K_2' \approx K_1^2$

$K_{ring} = K_1^2 C_{eff} \approx K_{ter} C_{eff}$

5. DOSY-NMR experiments

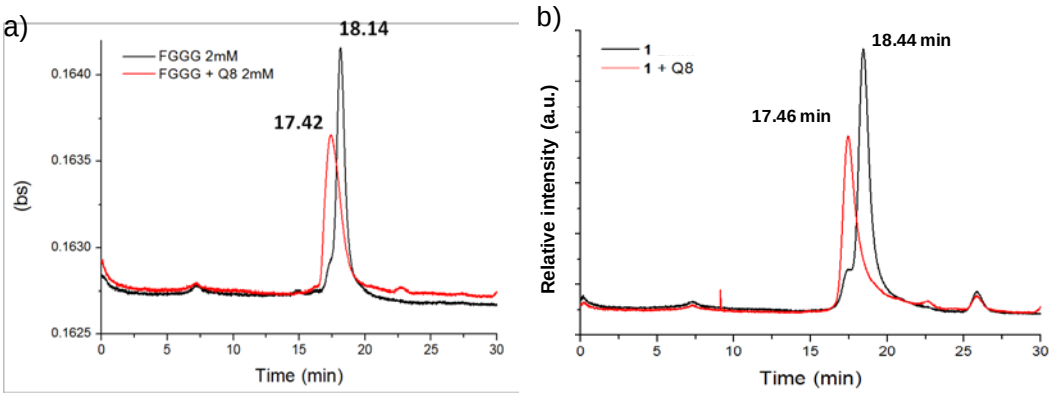


SI Fig 6. A 2D spectrum of a DOSY-NMR measurement on a 1:1 mixture of Q8 and **1** displaying the diffusion coefficients of Q8,**1** and TMSP. The diffusion coefficients of Q8 (signals in squares) of all mixtures were used to compare the different mixtures.



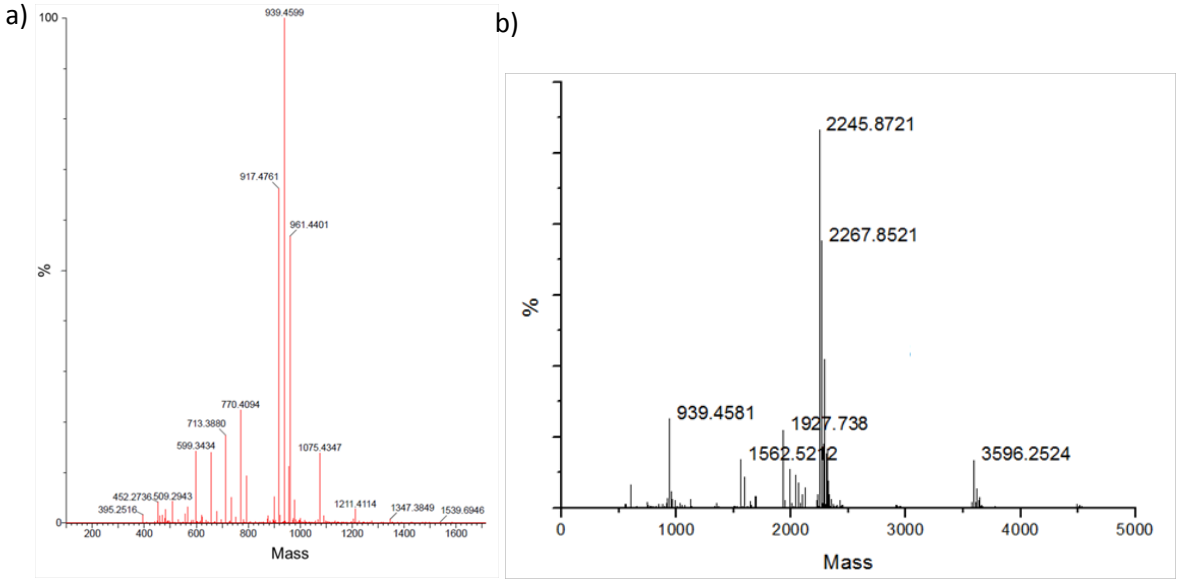
SI Fig 7. Diffusion coefficients of Q8 measured over a range of added equivalents of **1** determined by DOSY-NMR.

6. SEC spectra



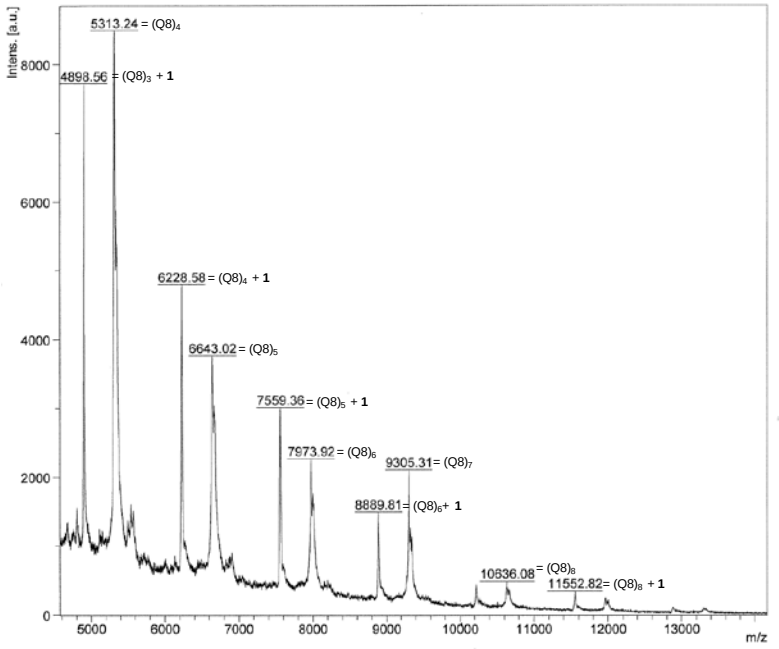
SI Fig 8. (a) GPC trace of FGGG (black) and a 1:1 mixture of FGGG and Q8 (red) at 2 mM (filtered). The traces show a slight difference in retention time indicating complexation. (b) GPC trace of **1** (black) and a 1:1 mixture of **1** and Q8 (red) at 2 mM (filtered). The traces show a slight difference in retention time indicating complexation.

7. QTOF-MS spectra



SI Fig 9. (a) Deconvoluted QTOF-MS spectrum of 50 µM **1** in a 10 mM pH 6.8 ammonium acetate buffer with a calculated $[M+H]^+$ of 917.5 g/mol. Calculated $[M+H]^+$ for the fragmentations are respectively; -phenylalanine: 770.9 g/mol and for each missing glycine: 713.8, 656.8 and 599.7 g/mol, (b) Deconvoluted QTOF-MS spectrum of a 50 µM 1:1 mixture of **1** and Q8. In addition to the Q8·**1** complex with a calculated $[M+H]^+$ of 2246.5 g/mol an excess of free **1** with a calculated $[M+Na]^+$ of 939.4 g/mol and the $Q8_2$·**1** with a calculated $[M+Na]^+$ of 3597.6 g/mol were detected.

8. MALDI-TOF MS spectrum



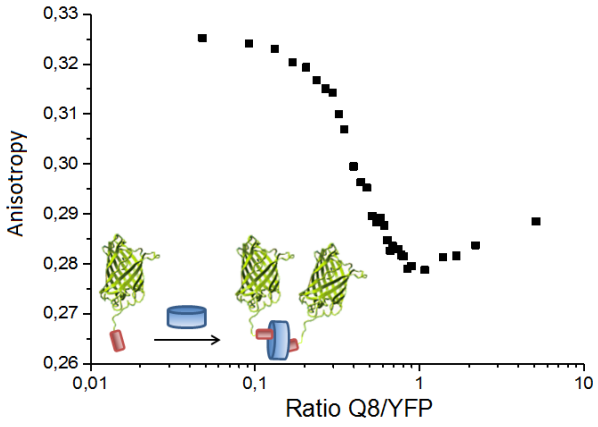
SI Fig 10. MALDI-TOF-MS measurement of a 1:1 Q8:**1** 50 µM solution in water.

9. FGG-m-YFP sequence

The amino acid sequence of the FGG modified yellow fluorescent protein (YFP):

FGGASWHPQFEKSAMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTF
GYGLQCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLE
YNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSYQSKLSKDPNEKRDHMVL
LEFVTAAGITLGMDELYK.[1]

10. Fluorescence Anisotropy graph



SI Fig 11. Fluorescence anisotropy measurement: titration of Q8 to 2 µM FGG-mYFP.

11. References

1 Dang, D.T., Supramolecular cucurbit[8]uril induced protein dimerization; Thesis, Eindhoven University of Technology, 2012.

2 L. M. Heitmann, A. B. Taylor, P. J. Hart, A. R. Urbach, *J. Am. Chem. Soc.* 2006, **128**, 12574-12581.