

Supramolecular dimerisation of middle-chain Phe pentapeptides via CB[8] host-guest homoternary complex formation

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Equipment

High Pressure Liquid Chromatography (HPLC) was performed on a Varian 940-LC system. The reverse phase column used was an Agilent Eclipse Plus C18 5 μm 4.6x150 mm column. The gradient applied was from A:B 95:5 to 100% B in 30 min, where A was water (0.1% TFA) and B was acetonitrile (0.1% TFA). The UV-Vis trace was followed at 220 nm. Electrospray ionisation mass spectrometry (ESI-MS) was performed on a Thermo Scientific LTQ Orbitrap Velos mass spectrometer with an electrospray ion source in the positive ion mode. Peptide concentrations were established by absorbance at 214 nm using a Cary 400 spectrophotometer at room temperature. Fluorescence titration experiments were carried out using a Cary Eclipse 400 fluorimeter, with 257 nm excitation and 270–400 nm emission range of wavelengths, 5 nm excitation bandwidth, 10 nm emission bandwidth. Conditions of 800 V voltage, 0.5 s averaging time, 120 nm/min

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scanning rate and 1 nm data interval were applied. ^1H -NMR spectra were run on a Bruker Advance 500 TCI Cryoprobe spectrometer. Isothermal titration calorimetry (ITC) was performed on a MicroCal VP-ITC calorimeter at 25 °C.

Materials

The following compounds were bought from AGTC Bioproducts at peptide synthesis purity grade: (L)-Fmoc-Ala-OH, (L)-Fmoc-Arg-(Pbf)-OH, (L)-Fmoc-Glu(OtBu)-OH, (L)-Fmoc-His(Trt)-OH, (L)-Fmoc-Ile-OH, (L)-Fmoc-Leu-OH, (L)-Fmoc-Phe-OH, (L)-Fmoc-Val-OH, and O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), dichloromethane (DCM), dimethyl formamide (DMF). The following compounds were bought at analytical grade purity from Sigma–Aldrich: diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), phenol, thioanisole, monobasic potassium and dibasic sodium phosphate. HPLC-grade acetonitrile and N-methylpyrrolidone (NMP) and analytical grade purity piperidine, triisopropylsilane (TIS) and ethanedithiol (EDT) were bought by Fisher Scientific. Cucurbit[8]uril and cucurbit[7]uril (CB[8] and CB[7]) were synthesised according to a published procedure.¹ Water was obtained from a Synergy UV Ultrapure water system (18.2 M Ω -cm at 25 °C). A stock solution of 0.5 M phosphate buffer was adjusted to pH 7.4 and sterile filtered. 10 mM phosphate buffer was prepared as needed by diluting the 0.5 M stock and adjusting to pH 7.4. The concentration of CB[8] was standardised by calorimetric titration with methyl viologen, using binding constants reported in literature.¹

Methodologies

Peptide synthesis

Fmoc solid-phase peptide synthesis (SPPS) was carried out on the CEM Liberty Automated Microwave Peptide Synthesiser using NovaPEG Rink amide resin 0.49 mmol/g as solid support for a 0.1 mmol scale. Fmoc deprotection was accomplished by a solution of 20% piperidine in DMF

using a power of 45 W, at a temperature of 75 °C over 180 s. The peptide coupling was performed using a double coupling strategy and 5 equivalents of amino acids and HBTU, both dissolved in DMF, and 10 equivalents of base (DIPEA solution 2 M in NMP). The coupling was run with a 25 W power, at 75 °C over 600 s. Microwave irradiation cannot be used with Arg and His, thus Fmoc deprotection and coupling were run at room temperature, for 900 s and 1 h, respectively. Cleavage cocktails used were based on a high percentage of TFA. A mixture of TFA/water/TIS 95:2.5:2.5 was used for sequence **2** and **3** and it was allowed to shake for 1.5 h before filtering from the resin. In sequence **1**, on account of the Arg presence, TFA/water/thioanisole/phenol/EDT 82.5:5:5:5:2.5 mixture was used for cleavage over 3 h at room temperature. After the cleavage procedure, peptides were precipitated in diethyl ether and centrifuged for 4 up to 6 times at 4 °C, using 4000 rpm over 5 min before being lyophilised and stored as a white powder at -20 °C. Peptide purity was verified by HPLC analysis and ESI-MS. Fresh peptide solutions were prepared for each experiment and their concentration was established by UV-Vis absorbance. The peptides used are low in strongly absorbing residues, therefore the use of the molar extinction coefficients (ϵ) at 214 nm was preferred over ϵ at 280 nm. The coefficient were calculated following the instructions of Gruppen's paper.²

Fluorescence Titrations

The titrations were accomplished by measuring the fluorescence emission of filtered solutions in 10 mM phosphate buffer at pH 7.4 of the sequences **1-3** with increasing concentration of CB[8] or CB[7]. The peptide concentrations were held constant at 40 μ M, while the concentration of CB[8] or CB[7] was varied over the range 0-35 μ M and 0-55 μ M, respectively. The ratio between the fluorescence intensity at 284 and 303 nm at each addition of CB[8] (or CB[7]) over the initial intensity was plotted against the ratio of CB[8] (or CB[7]):peptide concentration to obtain an indicative stoichiometry of the complex. All the plots are an average of at least three independent analysis repetition.

¹H-NMR Titrations

¹H-NMR titrations were performed in pure D₂O, collecting 90 acquisitions at 2 s relaxation time for each spectrum. Sequences **1** and **3** were prepared at a 0.5 mM concentration, sequence **2** was prepared at a 0.25 mM concentration. For each sample CB[8] was exactly weighed in a vial and dissolved in the peptide solution at the right concentration ratio.

ITC Titrations

The conditions applied were 25 °C temperature, 307 rpm stirring speed, 250 s initial delay and 25 injections of 10 μl spaced by 300 s. ITC titrations were carried out using sequences **1** and **3** at 1 mM and **2** at 0.3 mM in a 10 mM phosphate buffer solution at pH 7.4. CB[8] was dissolved in water at 50 μM and used at this concentration in the titrations with all of the sequences. The filtered solutions were kept at 23 °C and degassed for 10 min prior to the experiments. All the titrations have been repeated three times. Data were analysed with MicroCal Origin software. The stoichiometry was checked with the “one binding site” model and then the data were fitted with the “sequential binding site” model. In addition to sequences **1-3**, an ITC titration of a sequence without any Phe residues (LVIIAE) was run as negative control (Figure S18). LVIIAE was at 0.3 mM in a 10 mM phosphate buffer solution at pH 7.4, while CB[8] was dissolved in water at 25 μM.

Fluorescence titration upon CB[7] addition

The fluorescence titrations conducted upon increasing CB[8]/peptide ratio resulted in a different behaviour among the three sequences. The data at both 284 nm and 303 nm for all of the sequences are reported in Figure S1. The same titrations were, therefore, repeated using CB[7] instead of CB[8] to evaluate the three sequences behaviour in the presence of a smaller macrocycle, able to include just one Phe residue at time. As shown in Figure S2, sequence **1** does not exhibit any changes in emission upon increasing in CB[7] concentration. Whereas, both sequence **2** and **3**,

which are more hydrophobic and thus already aggregated in aqueous solution, show quenched emission at 303 nm; the fluorescence reaches a minimum at CB[7]/peptide ratio equal to 1 in both cases. These data support the hypothesis of a binding phenomenon able to at least partially disrupt the π - π stacking characteristic of such hydrophobic peptide chains.

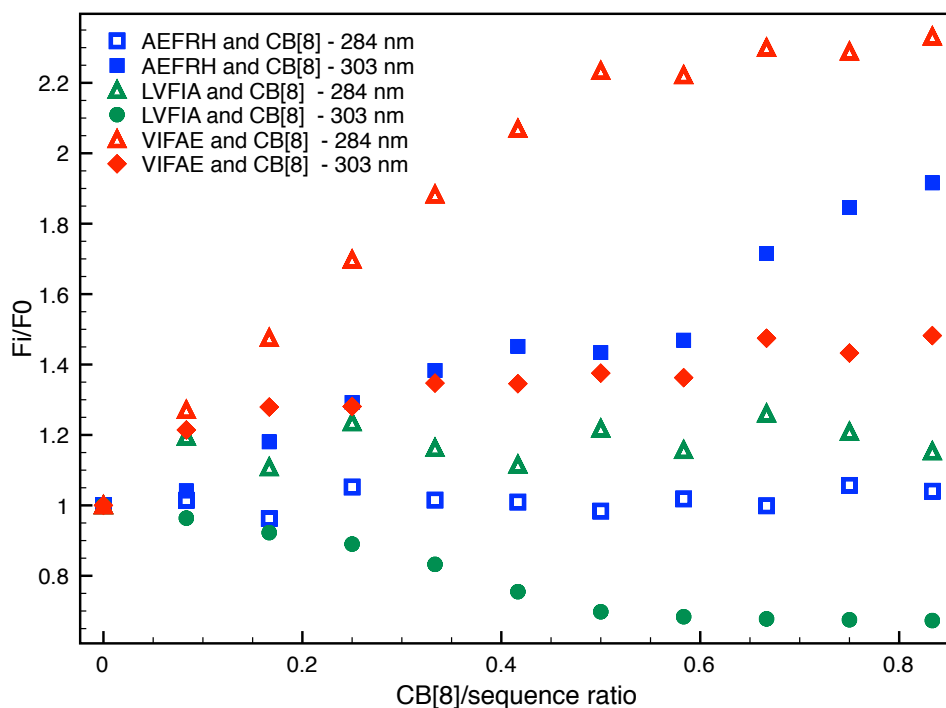


Figure S1: Plot of relative emission at 284 nm and 303 nm vs CB[8]/peptide ratio. The concentration of the sequences was 40 μ M and CB[8] was added up to 35 μ M. All the analyses have been repeated three times.

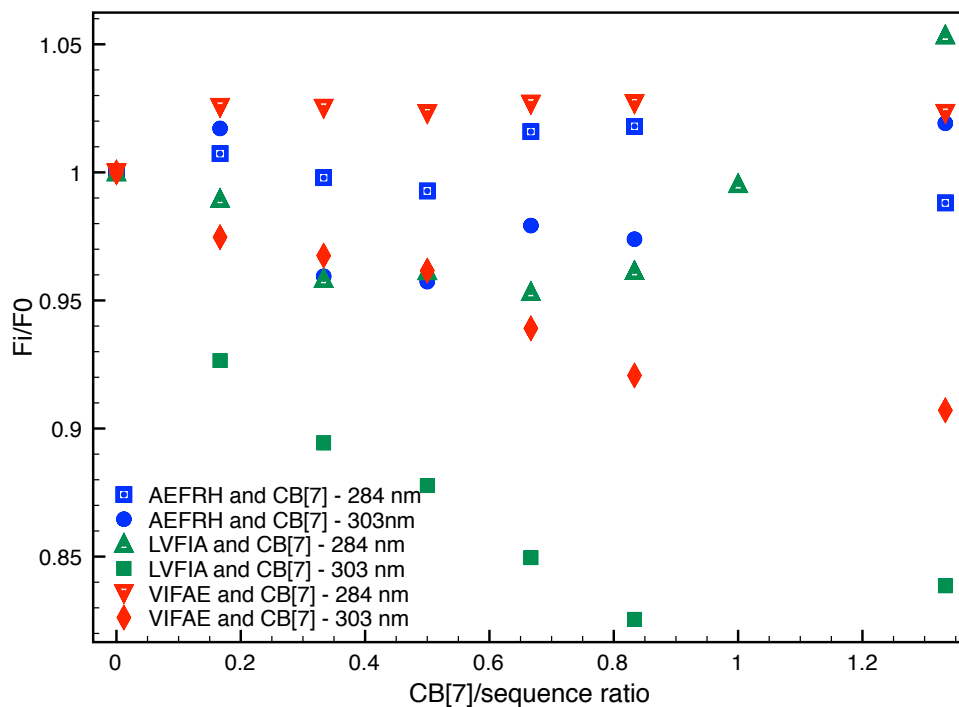


Figure S2: Plot of relative emission at 284 nm and 303 nm vs CB[7]/peptide ratio. The concentration of the sequences was 40 μM and CB[7] was added up to 55 μM . All the analyses have been repeated three times.

Energy minimisation

Sequence **3** exhibits particularly high binding constants towards CB[8], therefore we ran a short minimisation experiment to reveal possible peptide conformations in water and gain more insights concerning the reasons of such a strong interaction.

We employed the software ChemBio3D Ultra, running an *ab initio* minimisation HF 6-21G, at 298.15 K and applying a polarisable continuum model (PCM) with water as the solvent. The conformation thus obtained was expressed using PyMOL.

The calculation shows a propensity for the positively charged N-terminus to collapse close to the negatively charged C-terminus side chain (Glu side chain), almost creating a circular structure that pushes the Phe phenyl ring towards the solvent (Figure S3). This particular conformation is most likely the cause of higher binding constants of this sequence, compared to the others, and CB[8]. These observations led to further considerations concerning the importance of the guest

environment in CB[8] ternary complex formation.

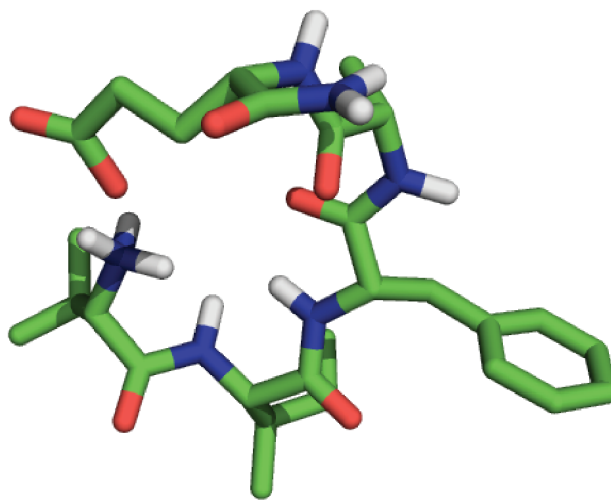


Figure S3: Sequence 3, VIFAE, minimised conformation.

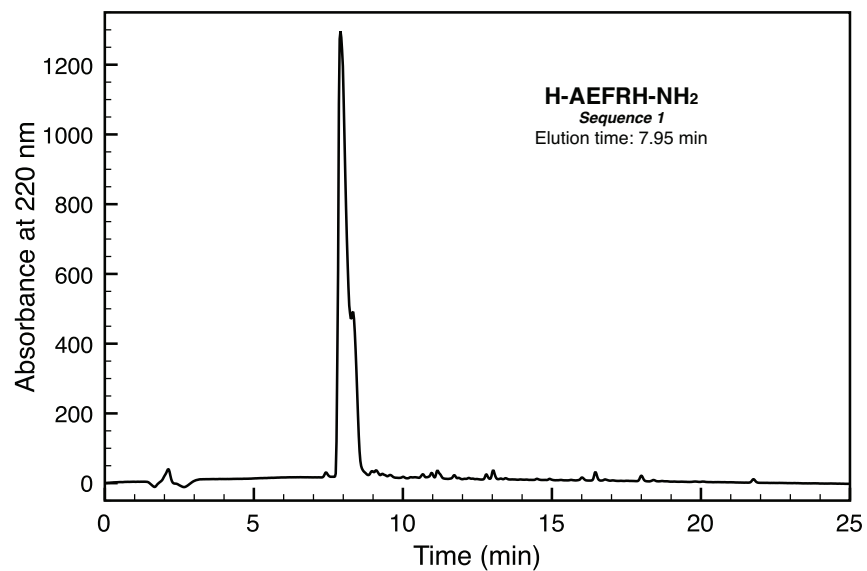


Figure S4: Sequence 1 HPLC.

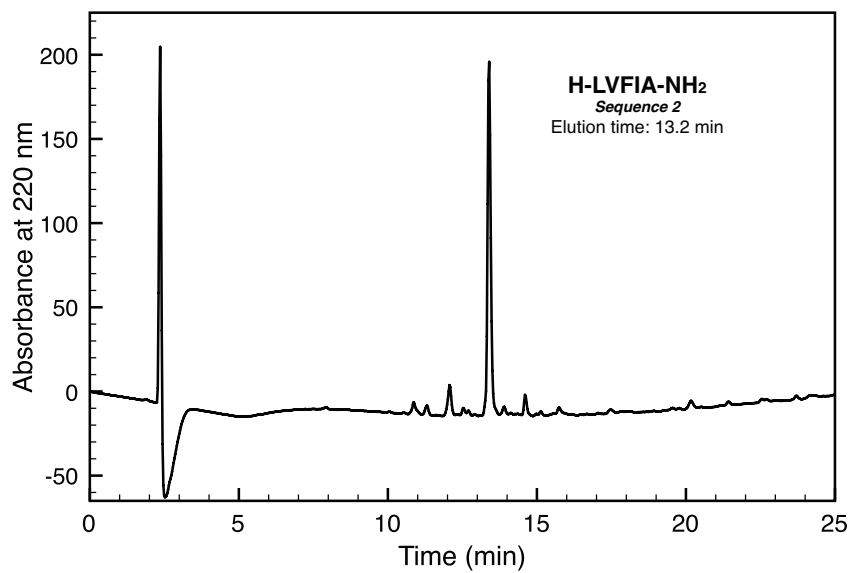


Figure S5: Sequence 2 HPLC.

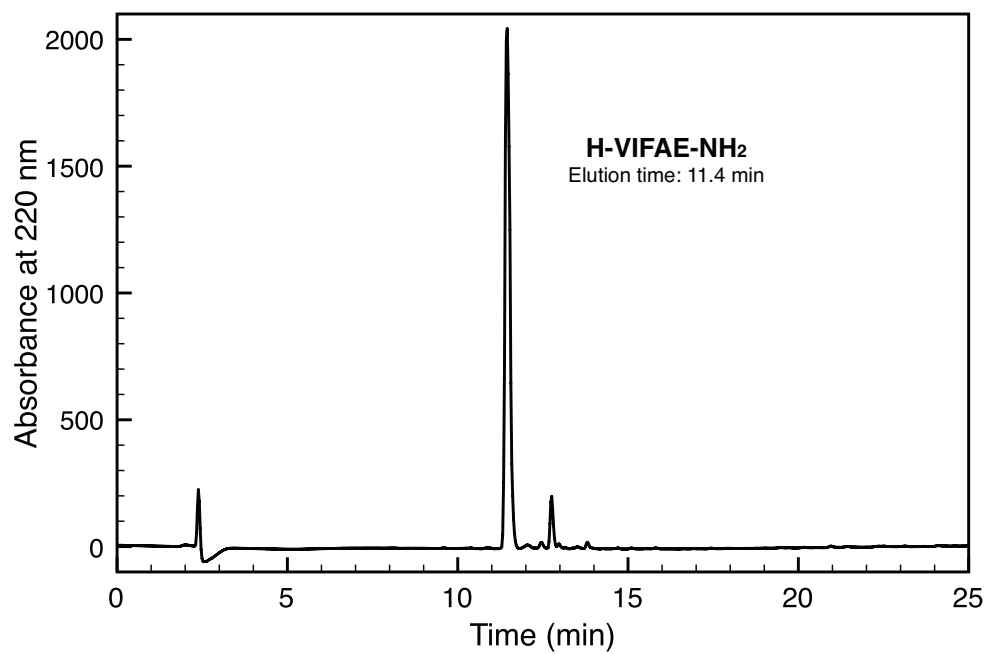


Figure S6: Sequence 3 HPLC.

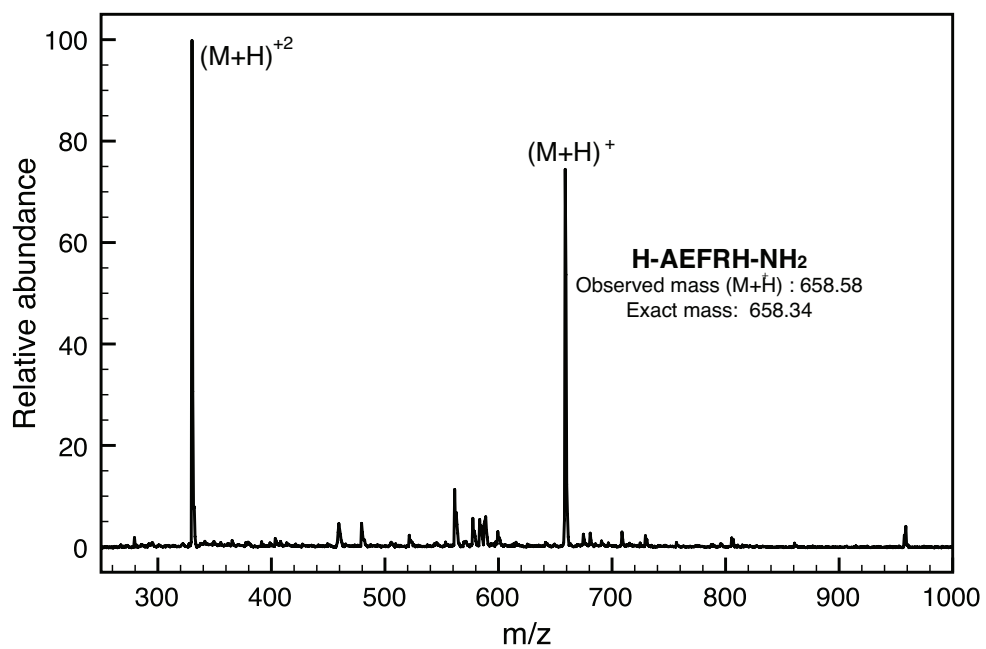


Figure S7: Sequence 1 ESI-MS.

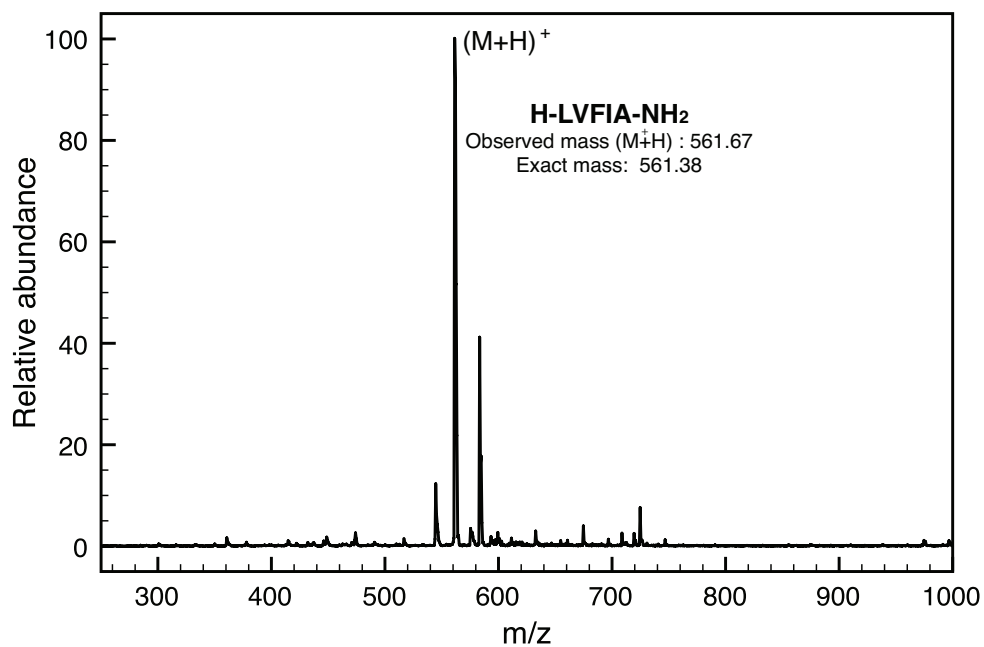


Figure S8: Sequence 2 ESI-MS.

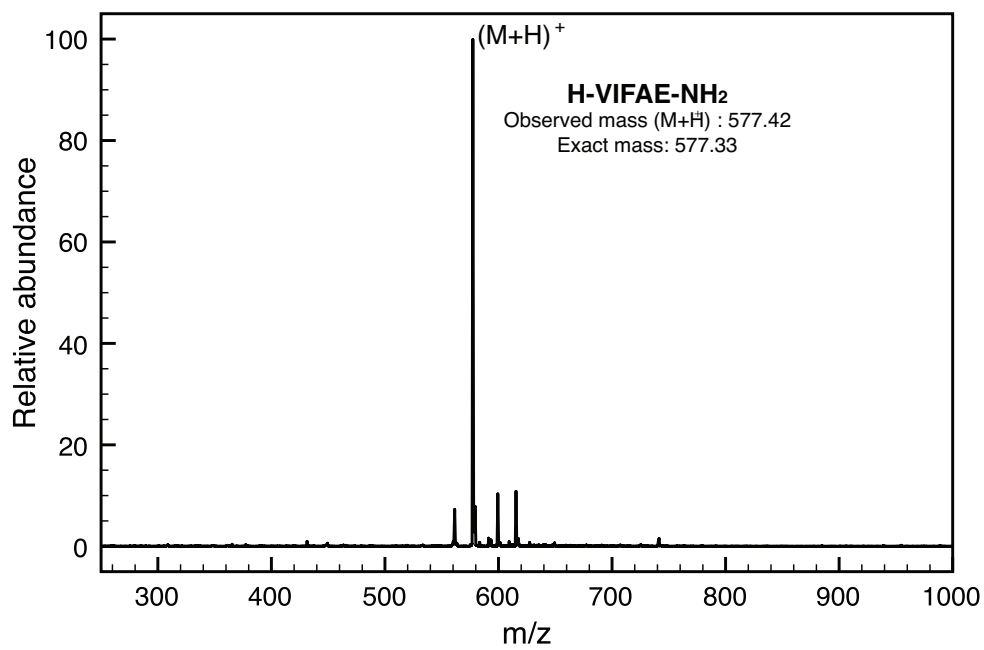


Figure S9: Sequence 3 ESI-MS.

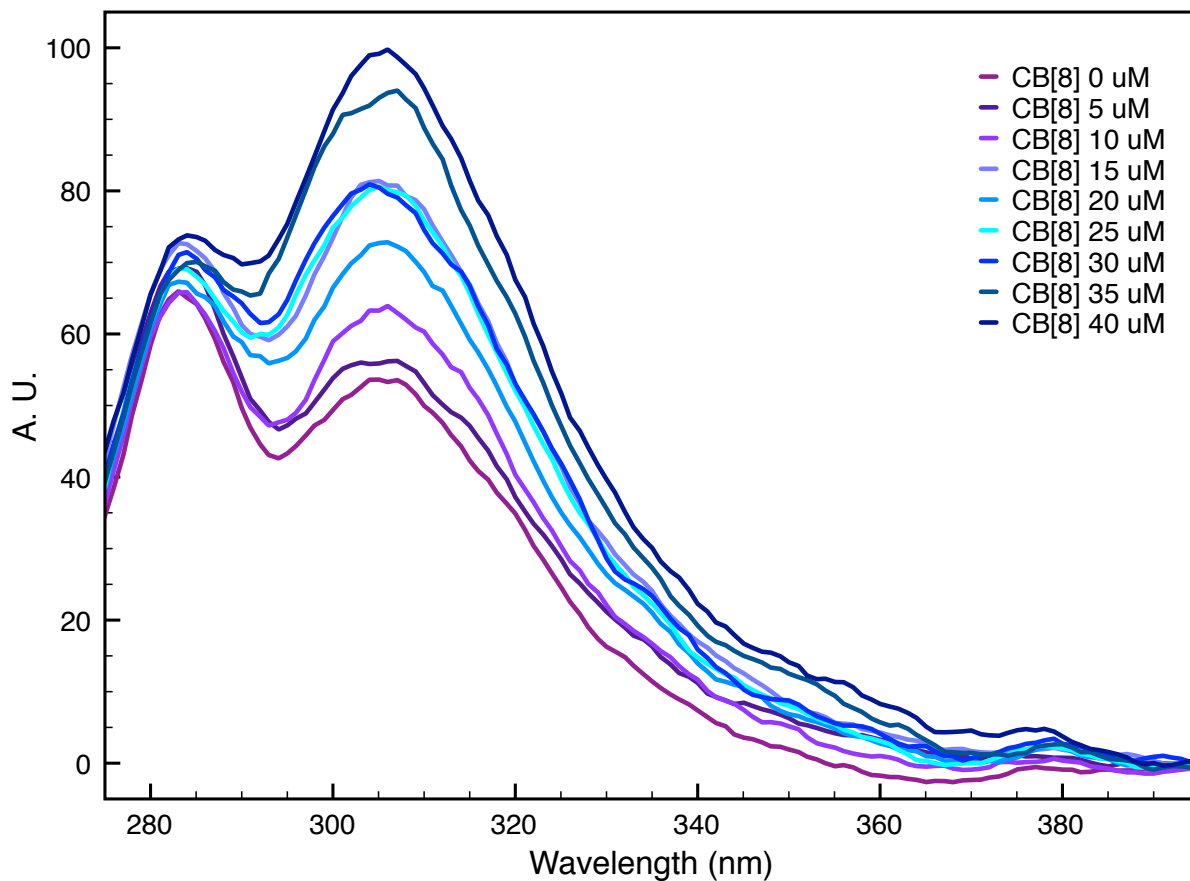


Figure S 10: Fluorescence emission in 10 mM phosphate buffer of sequence **1** with increasing concentrations of CB[8]. The concentration of the sequence was 40 μM and CB[8] was added up to 40 μM . All the analyses have been repeated three times.

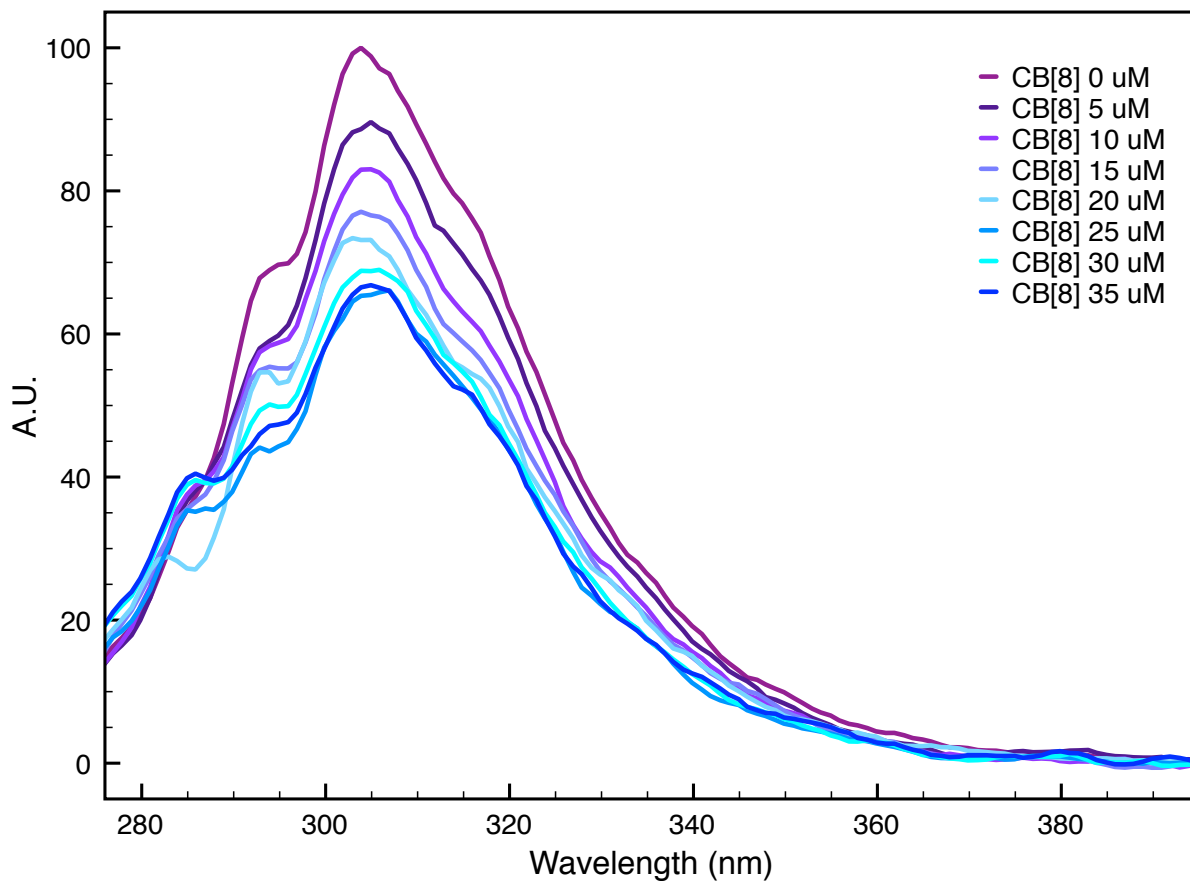


Figure S 11: Fluorescence emission in 10 mM phosphate buffer of sequence **2** with increasing concentrations of CB[8]. The concentration of the sequence was 40 μM and CB[8] was added up to 35 μM . All the analyses have been repeated three times.

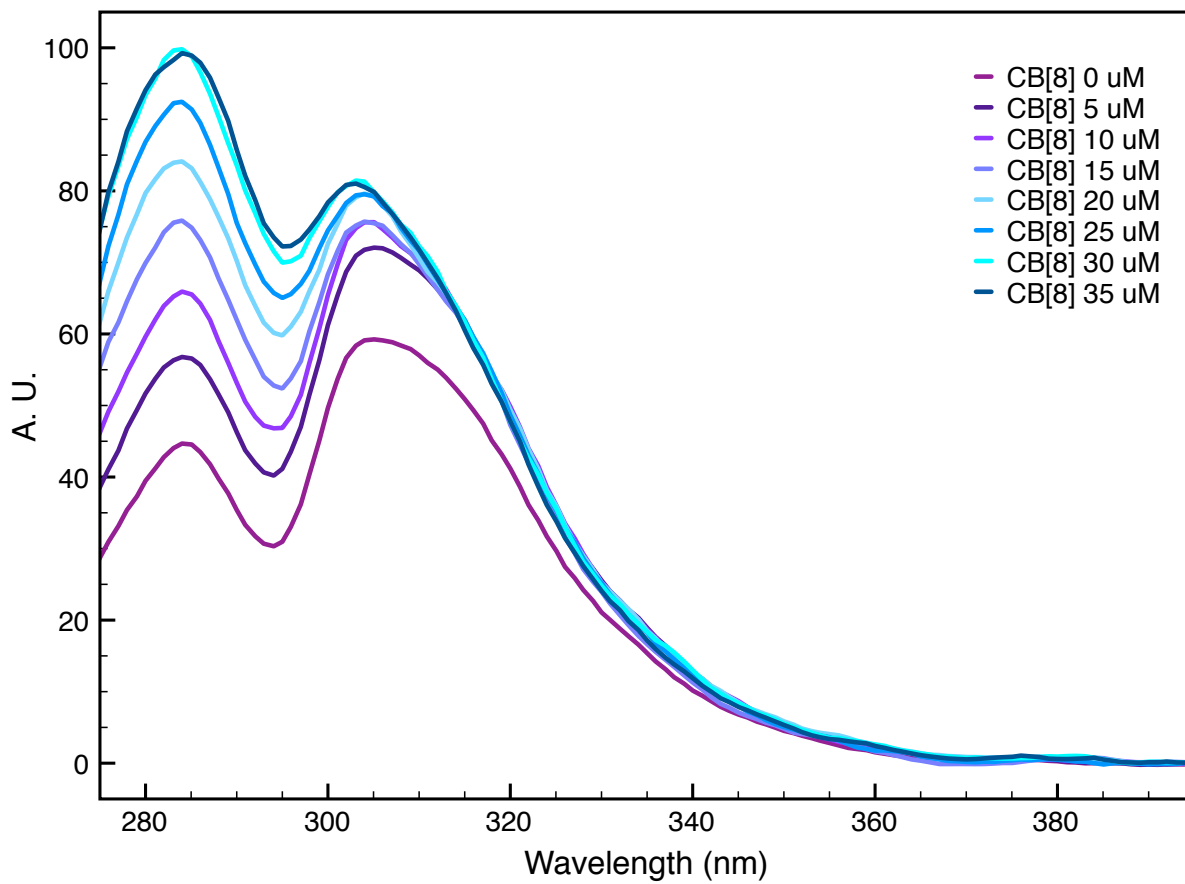


Figure S 12: Fluorescence emission in 10 mM phosphate buffer of sequence **3** with increasing concentrations of CB[8]. The concentration of the sequence was 40 μM and CB[8] was added up to 35 μM . All the analyses have been repeated three times.

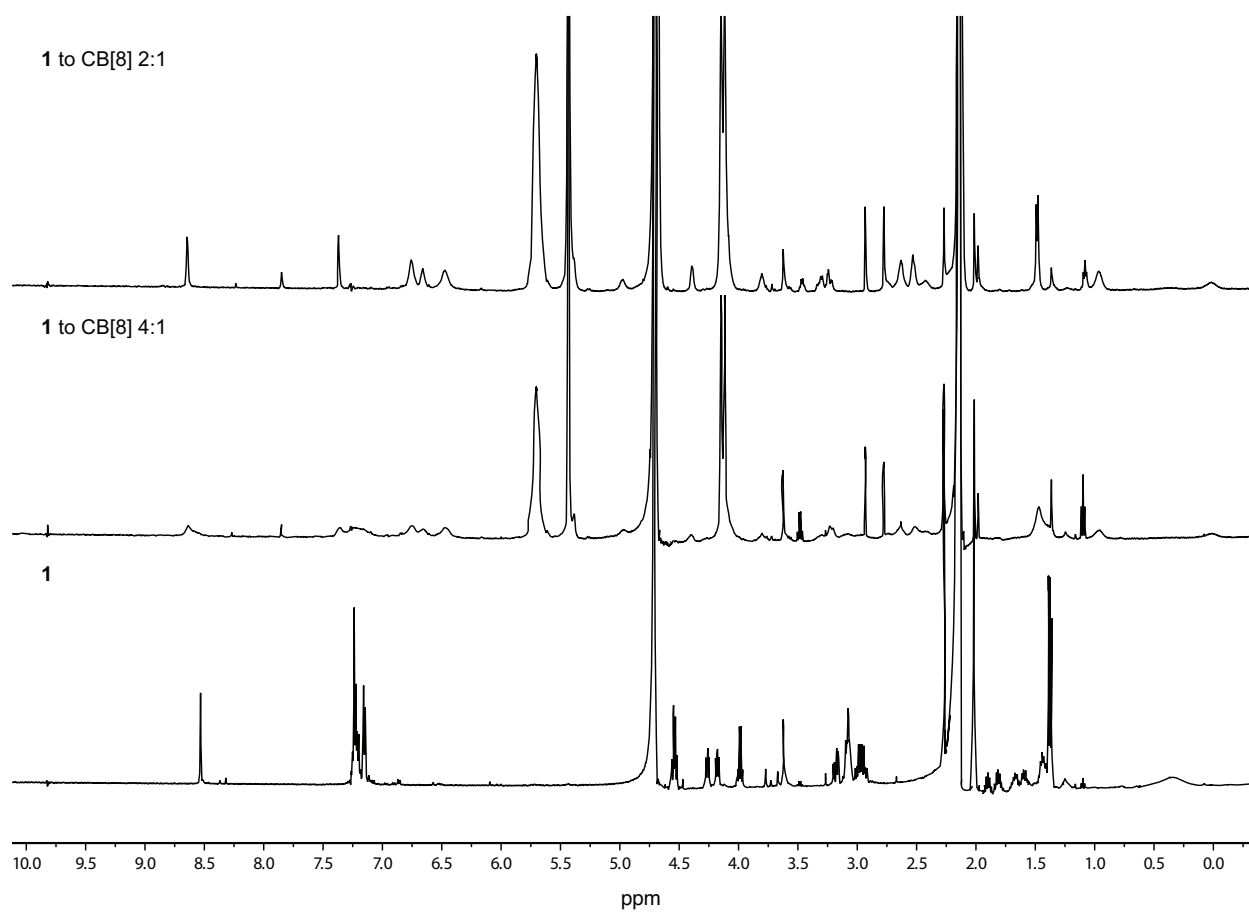


Figure S 13: $^1\text{H-NMR}$ in D_2O of sequence **1** with an increasing amount of CB[8]. From the bottom: only sequence (**1**), (**1**)/CB[8] 4:1 and (**1**)/CB[8] 2:1. Sequence **1** was prepared at a 0.5 mM concentration. In each sample the exact amount of CB[8] was dissolved in the peptide solution to obtain the right concentration ratio.

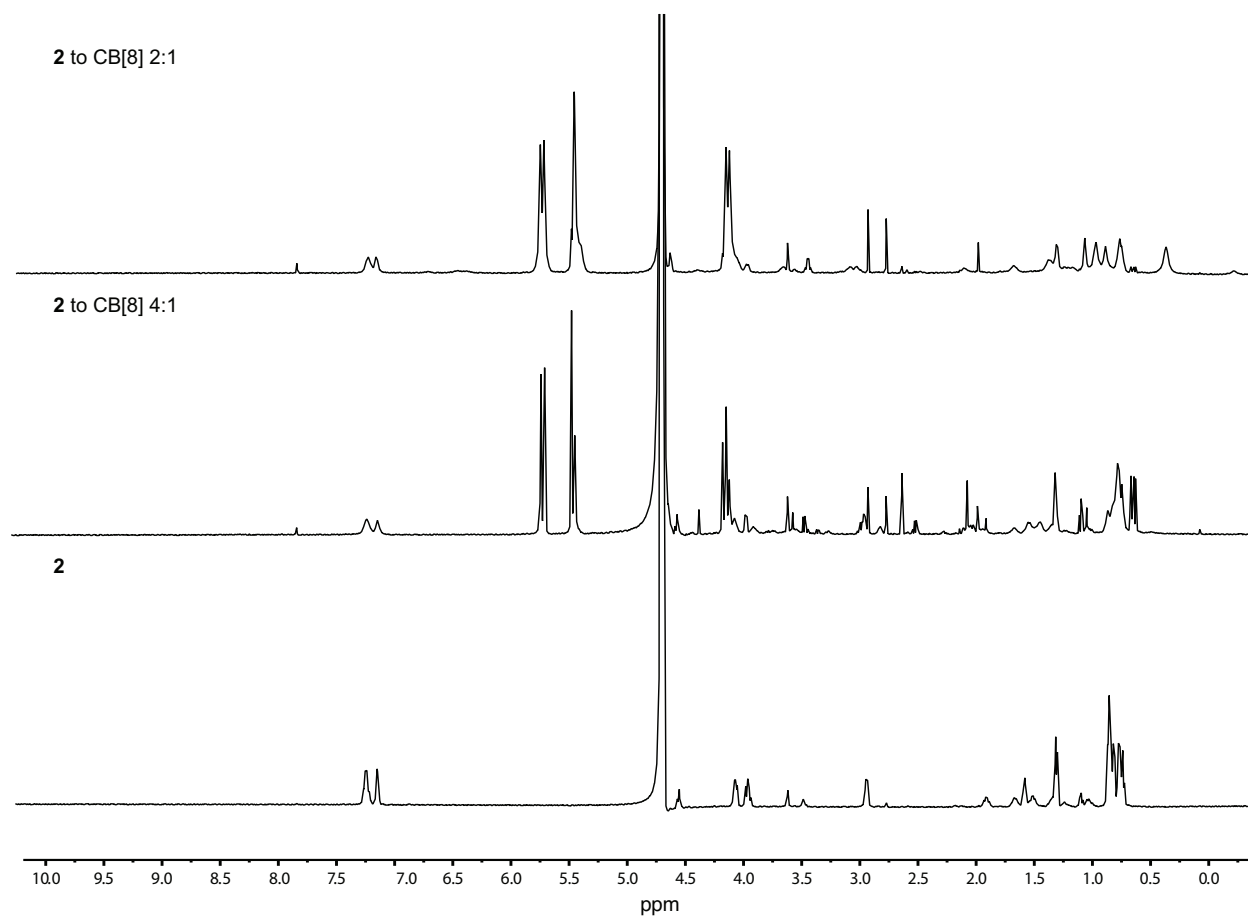


Figure S14: ¹H-NMR in D₂O of sequence **2** with an increasing amount of CB[8]. From the bottom: only sequence (**2**), (**2**)/CB[8] 4:1 and (**2**)/CB[8] 2:1. Sequence **2** was prepared at a 0.25 mM concentration. In each sample the exact amount of CB[8] was dissolved in the peptide solution to obtain the right concentration ratio.

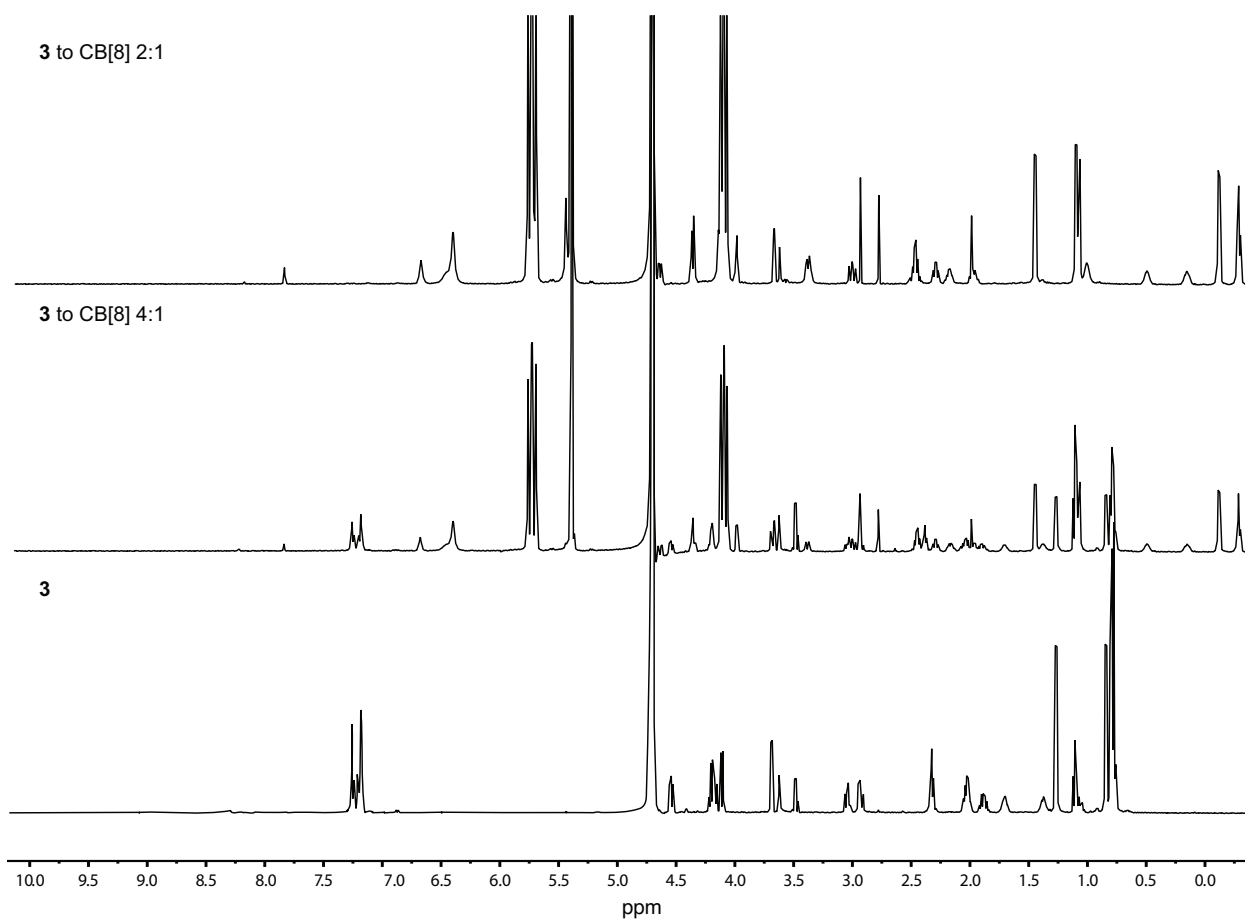


Figure S 15: ¹H-NMR in D₂O of sequence **3** with an increasing amount of CB[8]. From the bottom: only sequence (**3**), (**3**)/CB[8] 4:1 and (**3**)/CB[8] 2:1. Sequence **3** was prepared at a 0.5 mM concentration. In each sample the exact amount of CB[8] was dissolved in the peptide solution to obtain the right concentration ratio.

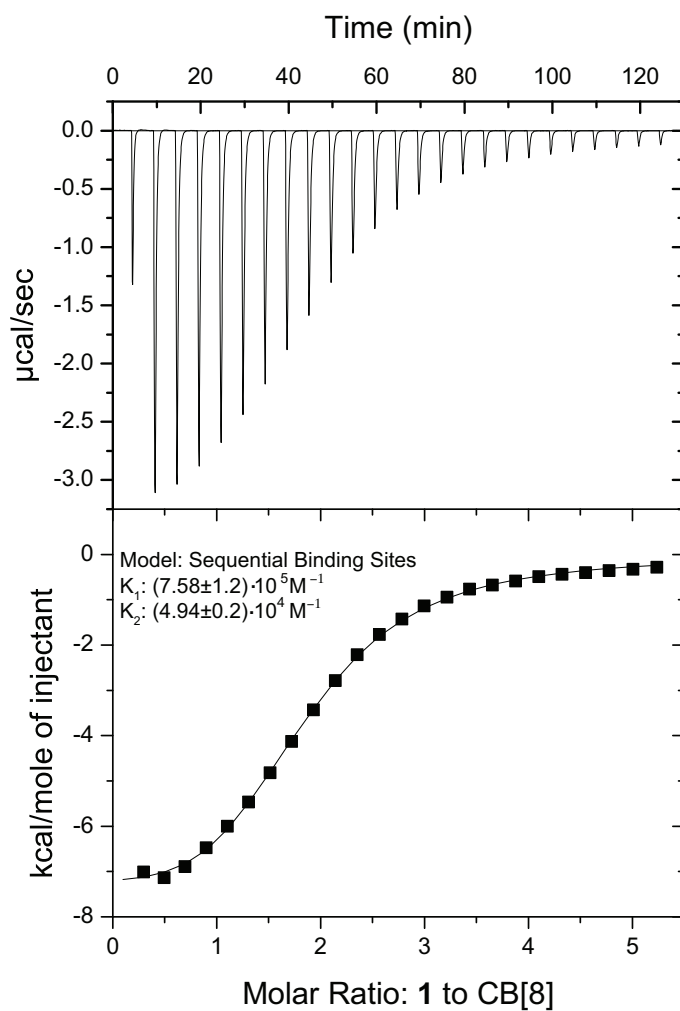


Figure S16: ITC titration of sequence 1 into a CB[8] solution. Sequence 1 was at a 1 mM concentration in 10 mM phosphate buffer and CB[8] was at a 50 μM concentration in water. The analyses were run at 25 °C.

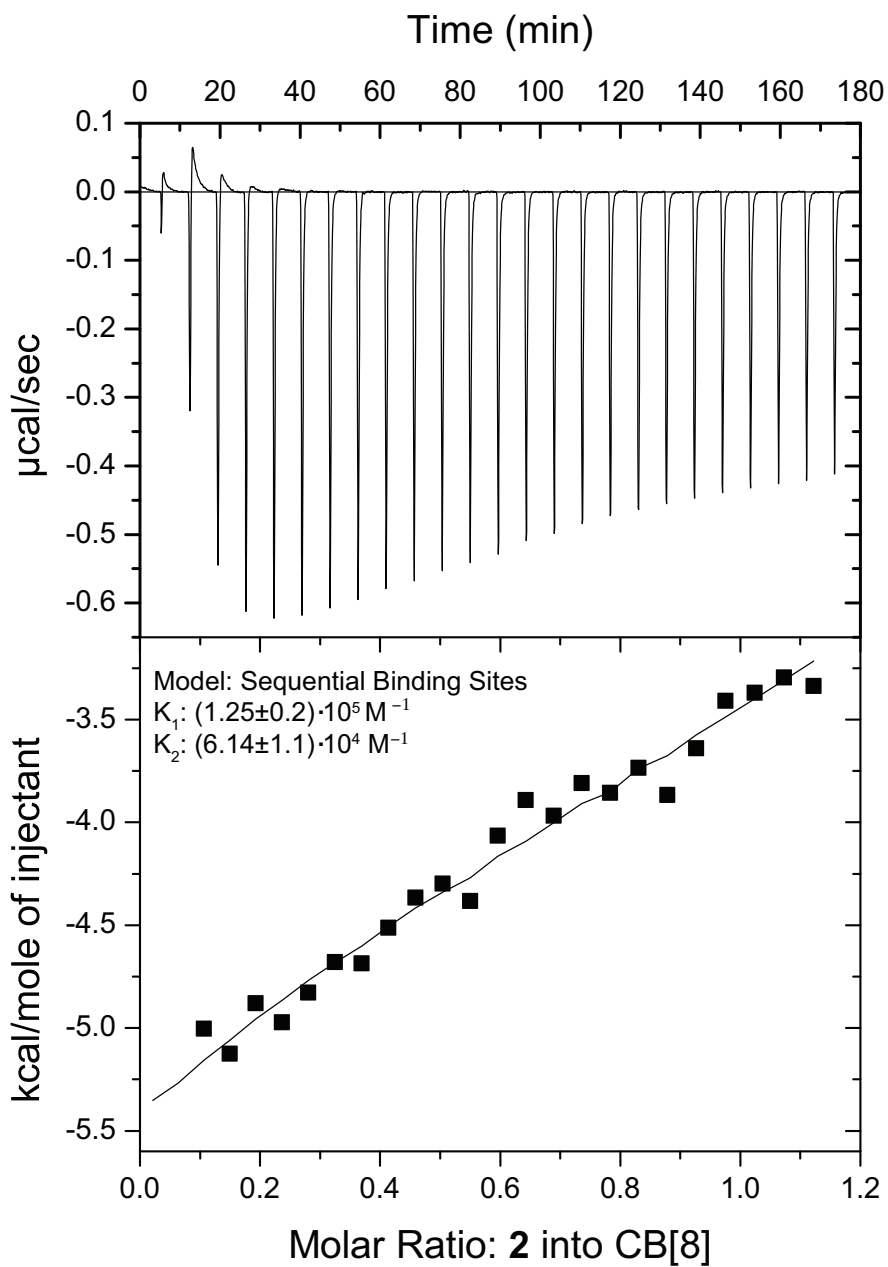


Figure S 17: ITC titration of sequence **2** into a CB[8] solution. Sequence **2** was at a 0.3 mM concentration in 10 mM phosphate buffer and CB[8] was at a 50 μM concentration in water. The analyses were run at 25 $^\circ\text{C}$.

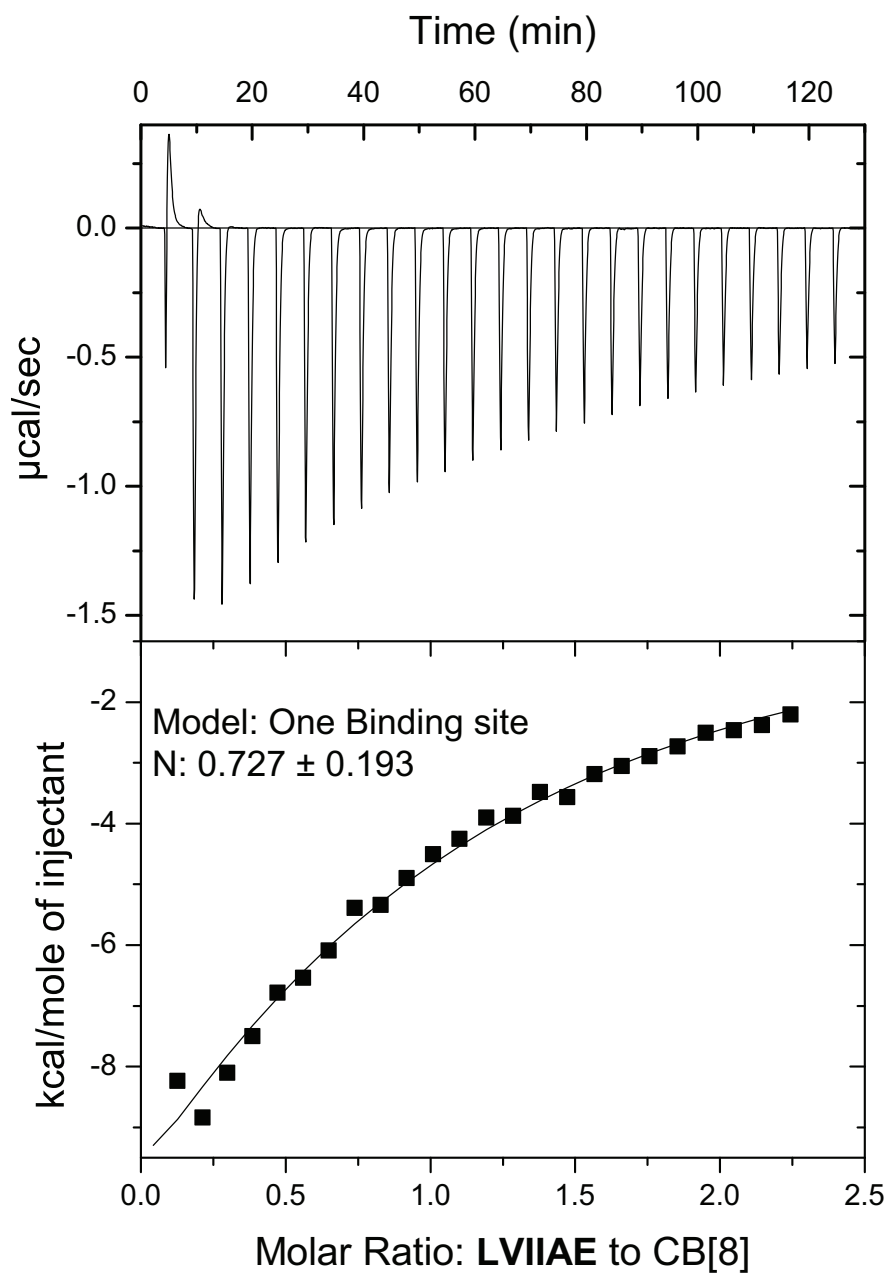


Figure S18: ITC titration of sequence LVIIE into a CB[8] solution. Sequence LVIIE was at a 0.3 mM concentration in 10 mM phosphate buffer and CB[8] was at a 25 μ M concentration in water. The analyses were run at 25 $^{\circ}$ C.

References

- (1) Lee, J. W.; Samal, S.; Selvapalam, N.; Kim, H.-J.; Kim, K. *Acc. Chem. Res.* **2003**, *36*, 621–30.
- (2) Kuipers, B. J.; Gruppen, H. *J. Agric. Food Chem.* **2007**, *55*, 5445–5451.