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Supporting Information

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Assessment of cooperativity in ternary peptide-cucurbit[8]uril complexes

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General methods. Chemicals were purchased from Sigma Aldrich unless differently specified and used as received. UV-Vis spectroscopy was performed using a Perkin Elmer UV-Vis spectrophotometer (Lambda 850). High performance liquid chromatography (HPLC) was performed on Water (2535) setup equipped with analytical and preparative XBridge C_{18} columns. Mass spectrometry analysis was performed using a Waters ESI(+)-ToF spectrometer (Micromass LCT). Calorimetry measurements were conducted on a microcalorimeter (Microcal). ^{1}H-NMR spectra were recorded in D_2O on an Ultrashield 600 (Bruker, ^{1}H-NMR 600 MHz) spectrometer. Residual solvent protons were used as an internal standard, and chemical shifts are given in units of parts per million (ppm) relative to tetramethylsilane (TMS).

Synthesis. Peptide PheGly$_6$ was synthesized using an automatic solid phase peptide synthetic robot (Multisyntech), following standard Fmoc protocols.[S1] Purification of the peptide was done by reversed phase HPLC in a gradient of H_2O/acetonitrile (10/90% to 0/100% in 70 min), and characterization by analytical HPLC and mass spectrometry. ESI-MS $[M+H]^+$ = 507.26 (calc. 507.22); $[M+Na]^+$ = 529.24 (calc. 529.21).

Concentration assessment of CB[8] solutions. Solutions of CB[8] for calorimetry studies were freshly prepared in PBS buffer (Dulbecco's Phosphate Buffered Saline, Sigma Aldrich, 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25 ºC). Solutions of CB[8] for ^{1}H-NMR spectroscopy experiments were freshly prepared in D2O. In both cases, the solid CB[8] was solubilized always for more than 2 h under ultrasonication at approximately 80 ºC. The obtained solutions were then filtered through a 200 nm pore diameter membrane filter and the concentration of CB[8] was accurately determined by titrating an aliquot of each solution, according to a UV-Vis spectroscopy procedure reported by Kaifer and coworkers.[S2] The resulting titer of the solutions of the batch of CB[8] used in this work was 82±3%.

Working range of concentrations. To find a suitable working range of concentrations, two main conditions were taken into account. Firstly, the solubility of CB[8] in aqueous media, around 100 μM,[S3] sets the upper limit of the working range. Secondly, the concentrations must be high enough to provide complexation, which defines the lower limit. Considering that in the course of a titration all species must be present at about equal concentrations, say *x*, it follows that:

$$K_{ter} = \frac{[HG_2]}{[H][G]^2}$$

$$[H] \cong [G] \cong [HG_2] = x$$

Thus:

$$K_{ter} = \frac{1}{x^2}$$

For an expected[S4] overall ternary binding constant K_{ter} on the order of 10^{11} M^{-2}, this yields approximately $x = 3$ μM. Hence the proper working range of concentrations was set between 1 and 50 μM.

Isothermal titration microcalorimetry. Calorimetry measurements were conducted at 298 K. Solutions were freshly prepared in PBS buffer and degassed prior to use. ITC measurements were made in triplicate and at three different sets of concentrations as described in the main text, with solutions of CB[8] between 10 and 50 μM in the cell, and of the peptide $PheGly_2$ or $PheGly_6$ in the syringe at a concentration equal to 20 times the one of the host in the cell (Figure S1). As a reference, the peptide $PheGly_6$ was titrated in PBS (in the absence of CB[8]). This blank curve has not been subtracted from the enthalpograms of complexation in the presence of CB[8], but a constant dilution heat is assumed and co-optimized in the fitting procedure. The measured heat values matched the heat of dilution considered in the fitting (Figure S2).

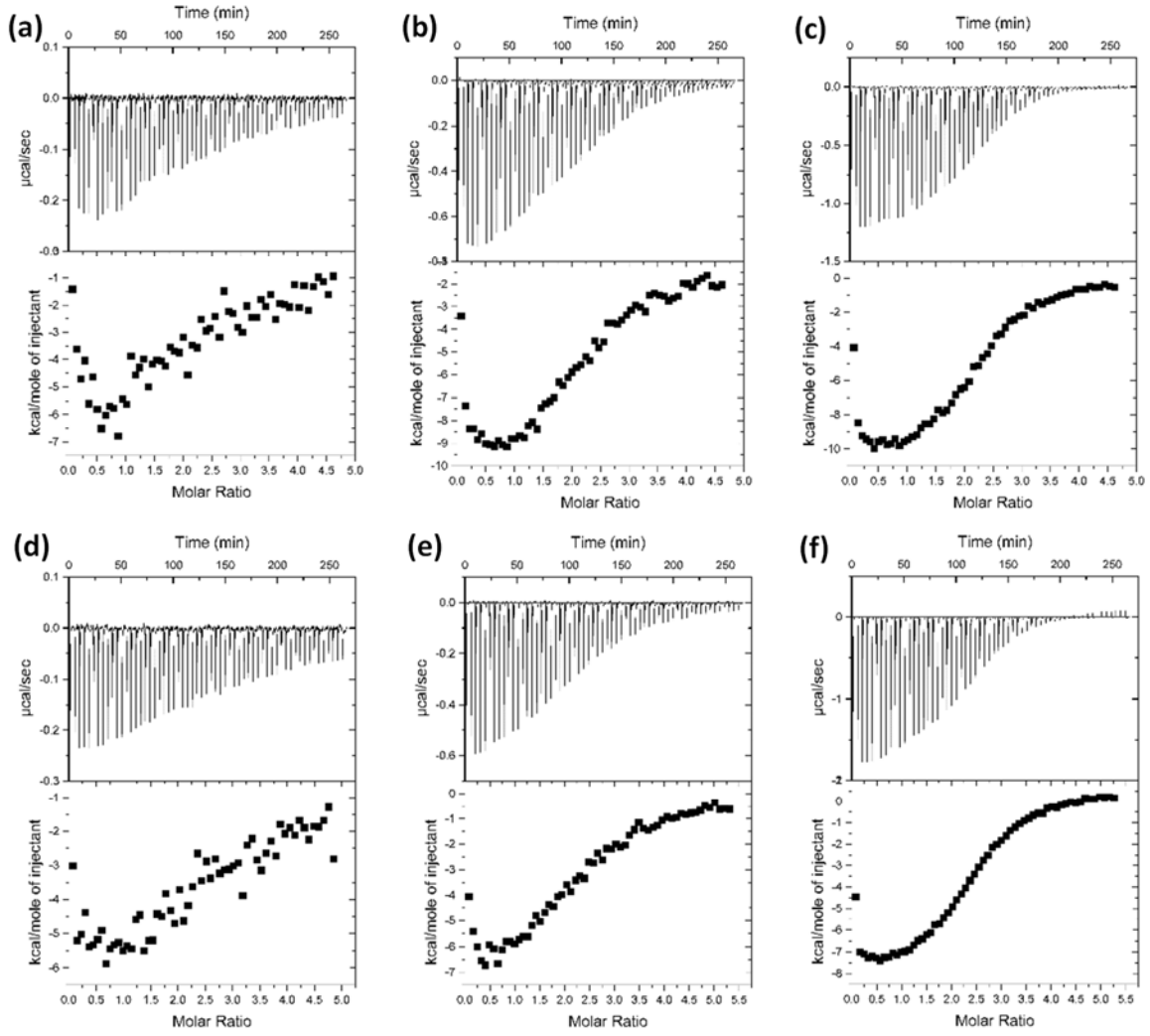

Figure S1. Enthalpograms for solutions 10, 20 and 30 µM CB[8] respectively titrated with 0.2, 0.4 and 0.6 mM $PheGly_2$. (d-f) Enthalpograms for solutions 10, 20 and 50 µM CB[8] respectively titrated with 0.2, 0.4 and 1 mM $PheGly_6$.

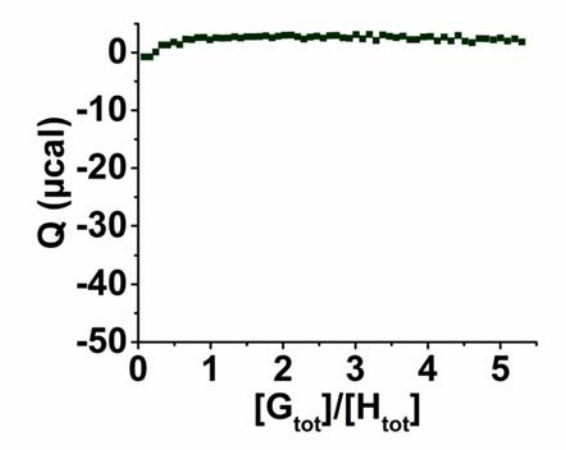

Figure S2. Enthalpograms for a PBS solution at 0 μM CB[8] titrated with 1 mM $PheGly_6$.

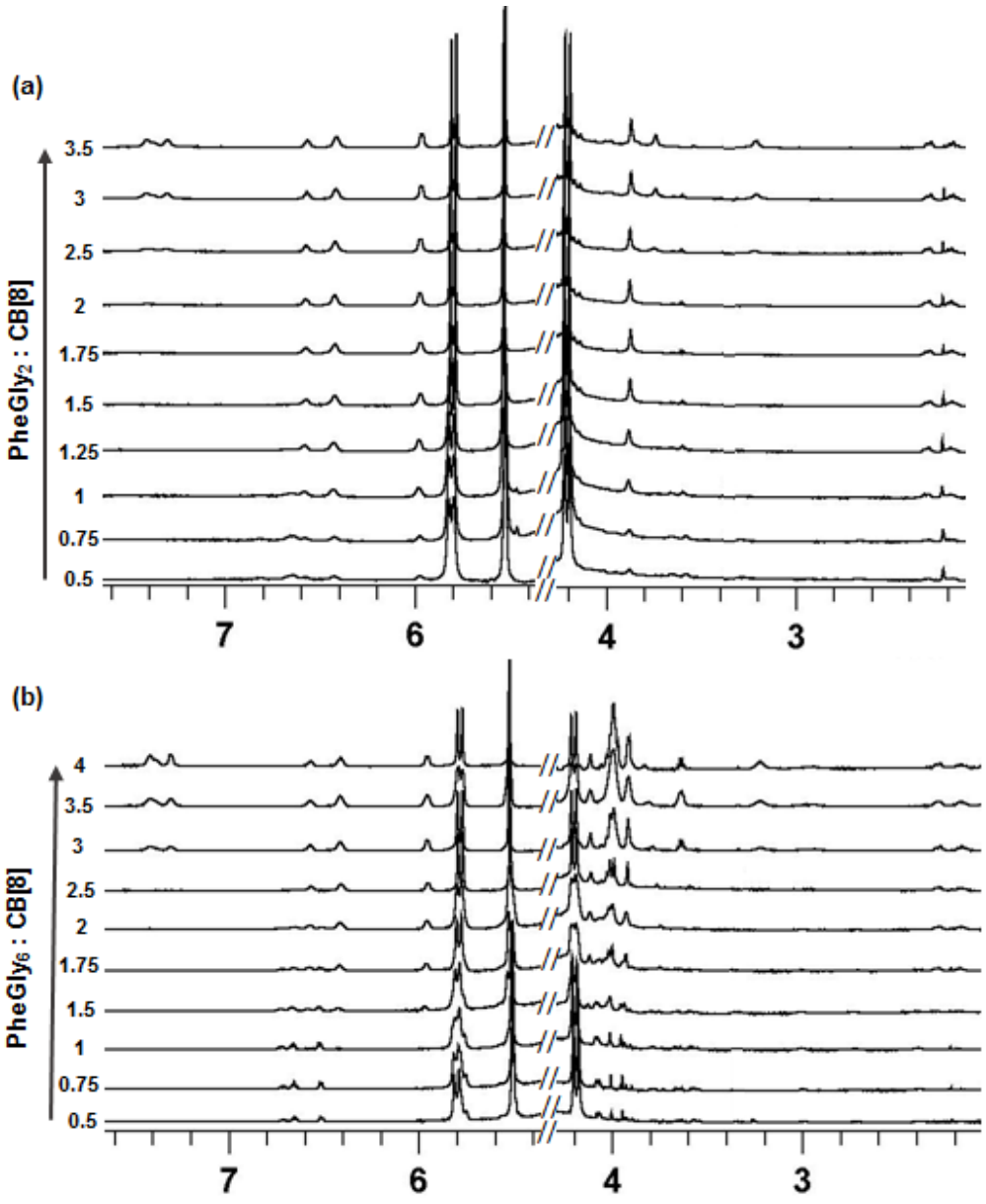

Figure S3. ^{1}H-NMR titrations of CB[8] (50 μM) with $PheGly_2$ (a) and $PheGly_6$ (b) in D_2O at 25ºC.

Fitting model for ITC and ^{1}H-NMR data.

Considering the equilibria described in the main text in Figure 1**Error! Reference source not found.**, the distributions of all species can be derived from the following:

$$\begin{cases} K_1 = \frac{[HG]}{[H][G]} \\ K_2 = \frac{[HG_2]}{[HG][G]} \\ H_{tot} = [\mathrm{H}] + [\mathrm{HG}] + [\mathrm{HG_2}] \\ G_{tot} = [\mathrm{G}] + [\mathrm{HG}] + 2[\mathrm{HG_2}] \end{cases}$$

By substitution,

$$\begin{cases} [HG] = K_1[H][G] \\ [HG_2] = K_2[HG][G] \\ H_{tot} = [\mathrm{H}](1 + K_1[\mathrm{G}] + K_1K_2[\mathrm{G}]^2) \\ G_{tot} = [\mathrm{G}](1 + K_1[\mathrm{G}] + 2K_1K_2[\mathrm{H}][\mathrm{G}]) \end{cases}$$

Thus,

$$\begin{cases} [HG] = K_1[H][G] \\ [HG_2] = K_2[HG][G] \\ [\mathrm{H}] = H_{tot}/(1 + K_1[\mathrm{G}] + K_1K_2[\mathrm{G}]^2) \\ [\mathrm{G}] = G_{tot}/(1 + K_1[\mathrm{G}] + 2K_1K_2[\mathrm{H}][\mathrm{G}]) \end{cases} \quad \text{(I)}$$

For the fitting of calorimetric isotherms, the experimental data Q_{exp}were fitted according to the equation:[S5]

$$Q_{calc} = Q_{dil} + V(\Delta H_1[HG] + \Delta H_2[HG_2])$$

Where Q_{calc} is the calculated heat, Q_{dil} is the heat of dilution and V is the volume.

For the fitting of the species distributions as quantified by ^{1}H-NMR measurements, the system (I) was used directly to express the calculated distribution of species in function of the parameters

K_1 and K_2. Given the low sensitivity of the technique and the limitations described in the text in terms of concentrations, we performed the titration experiments at the maximum CB[8] concentration (50 μM), on a 600 MHz instrument, accumulating 10,000 scans and carefully optimizing the baseline and phase in the area of interests (around 5-7.5 ppm). The interference of the residual water at 4.8 ppm was limited by using freshly opened 100% D_2O to prepare the solutions. Solutions were prepared and stored under argon. However, the conditions (1-2 hours sonication at 80˚C) to solubilize CB[8] always created a substantial water signal. Water suppression sequences showed to cancel also most of the significant peaks and have been avoided.

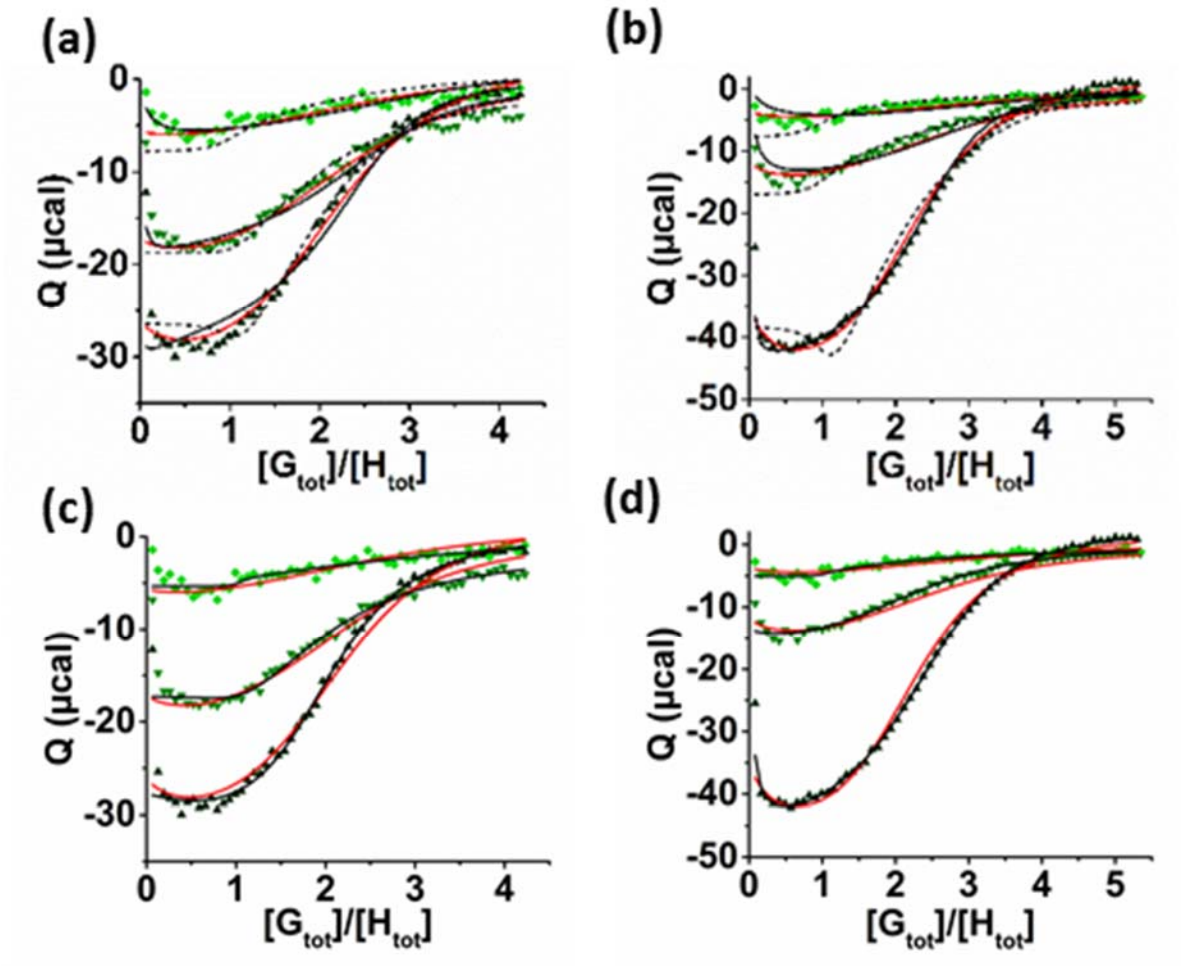

Figure S4. ITC data (markers) of binding CB[8] (H, three initial concentrations) with $PheGly_2$ (G) (a, c) and $PheGly_6$ (G) (b, d) in PBS (10 mM phosphate buffer, 2.7 mM KCl and 137 mM NaCl, pH 7.4). ITC data were simultaneously fitted (solid red lines) to a model with K_1, K_2, ΔH^0_1, and ΔH^0_2 as fit parameters. For comparison, the fitting obtained at fixed values of the K_1/K_2 ratio are reported for K_1/K_2 equal 10^{-3} (solid black lines, a) and 10^2 (dashed black lines, b) as well as the independent fitting of each titration experiment (solid black lines, c and d).

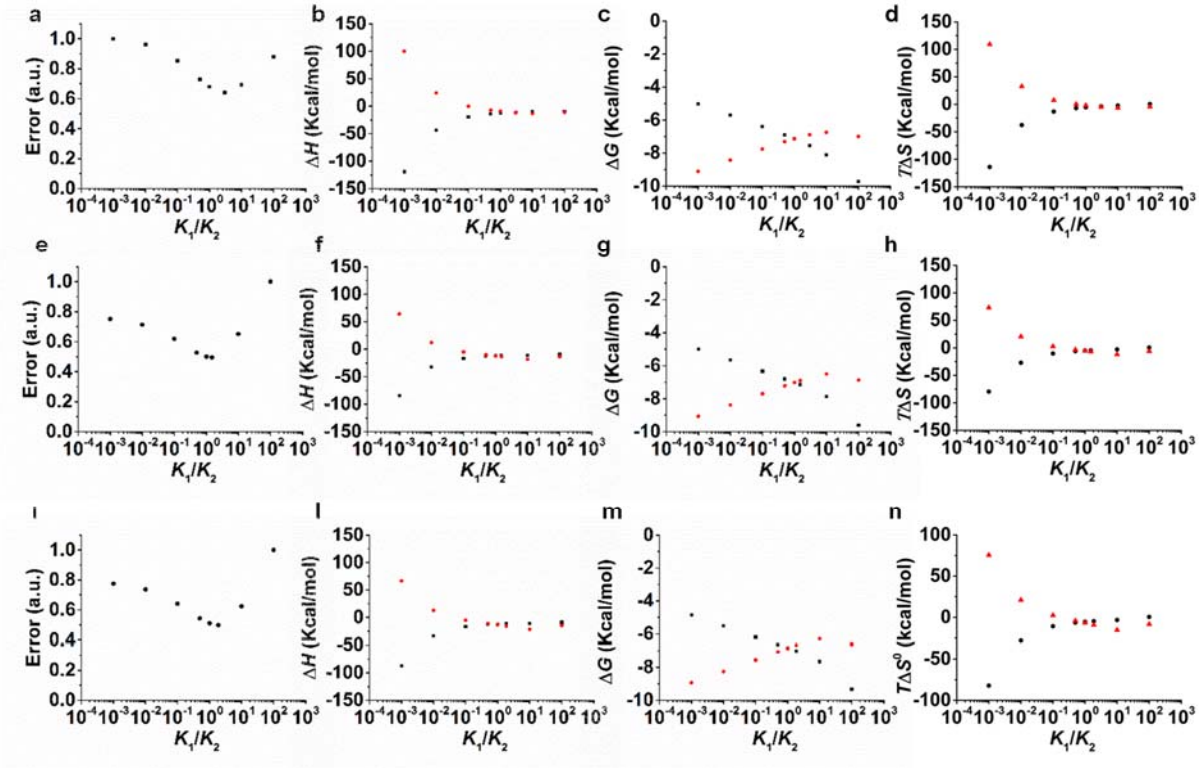

Figure S5. ITC study relative to PheGly$_2$. Plots of the normalized fitting error, ΔH^0, ΔG^0 and -TΔS^0 calculated at fixed values of the ratio K_1/K_2 over three set of calorimetric experiments are reported respectively in a-d, e-h and i-n. Black and red data points are for the binding of the first and second guest, respectively.

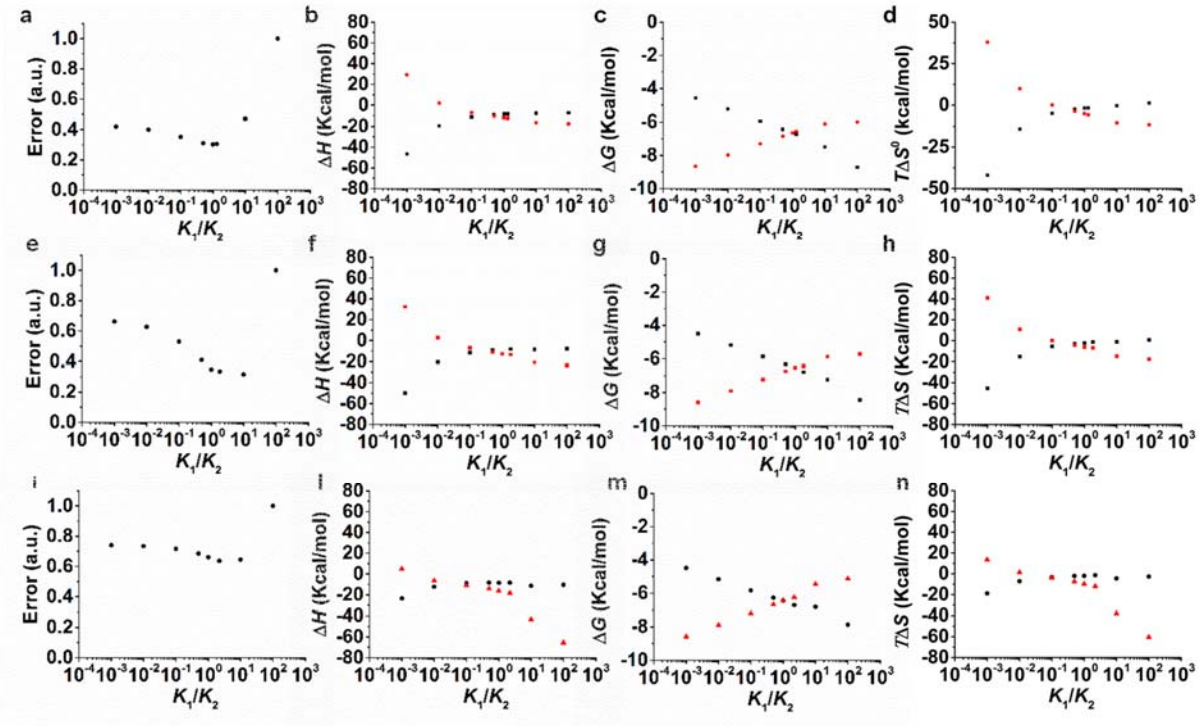

Figure S6. ITC study relative to $PheGly_6$. Plots of the normalized fitting error, ΔH^0, ΔG^0 and $-T\Delta S^0$ calculated at fixed values of the ratio K_1/K_2 over three set of calorimetric experiments are reported respectively in a-d, e-h and i-n. Black and red data points are for the binding of the first and second guest, respectively.

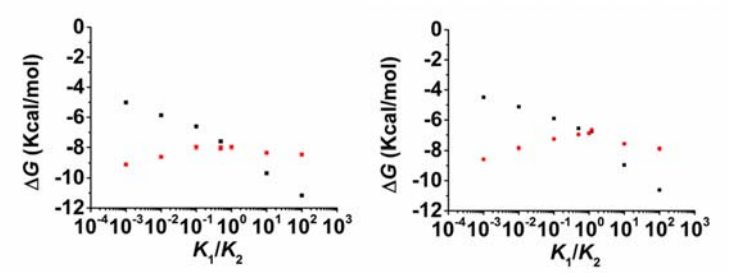

Figure S7. ^{1}H-NMR study. Plots of the difference of Gibbs free energy calculated at fixed values of the ratio K_1/K_2 are reported respectively for (left) $PheGly_2$ and (right) $PheGly_6$. Black and red data points are for the binding of the first and second guest, respectively.

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S2. Yi, S.; Kaifer, A. E. Determination of the purity of cucurbit[n]uril (n = 7, 8) host samples. *J. Org. Chem.* **2011,** *76* (24), 10275-10278.
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S4. Heitmann, L. M.; Taylor, A. B.; Hart, P. J.; Urbach, A. R. Sequence-specific recognition and cooperative dimerization of N-terminal aromatic peptides in aqueous solution by a synthetic host. *J. Am. Chem. Soc.* **2006,** *128* (38), 12574-12581.
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