Supporting information

For

Mixed non-covalent/covalent assemblies and equilibrating molecular networks that survive 5 molar urea

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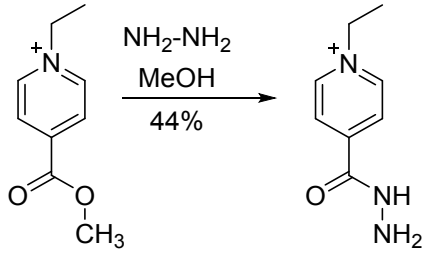
1. General information and materials

^{1}H NMR were recorded on a Bruker Avance Neo 500 MHz spectrometer unless otherwise indicated, and processed with MestReNova by Mestrelab Research S.L and TopSpin 3.5. All reported chemical shifts were reported in ppm with respect to an internal standard: either maleic acid at 6.2 ppm or 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP) at 0.0 ppm. Deuterated solvents were purchased from Sigma Aldrich. Sodium citrate buffer ($C_6H_8O_7$/$Na_3C_6H_5O_7$, 50 mM, pD 5) in D_2O was prepared with pD adjustments using NaOD/DCl solutions. Urea was dissolved in D_2O before lyophilizing to a powder to afford d_4-urea, which was used as isolated in all NMR experiments in order to minimize signals from exchangeable NH protons. NaCl, 4-methoxybenzhydrazide (**H-MeO**), **4** and hydrazine hydrate were purchased from Sigma-Aldrich. 4-ethylbenzhydrazide (**H-Bz**) was purchased from Enamine. **3** was synthesized following literature protocol.[1]



4 **H-Py+**

The following protocol was adapted from previous literature.[2] **4** (0.290 g, 1 mmol) was dissolved in MeOH (25 mL), 50% hydrazine hydrate (186 uL, 3 mmol) was added and the reaction was heated to reflux for 6 h. The orange reaction mixture was concentrated under vacuum and the crude was re-crystallized with EtOH to afford yellow crystals of **H-Py+** (44% yield). Characterization data matched that of reported literature.[2]

2. qNMR protocol, solution prep and sample calculations

General protocol for producing hydrazone dimers
Stock solutions were made in citrate buffer (50 mM, pD 5) containing maleic acid (3 mM) of **3** and respective hydrazides were measured by NMR, with sufficient D1 relaxation time (25 s) to determine accurate concentrations. Appropriate volumes of each were mixed in an Eppendorf tube to make a 1:1 (5 mM) solution for non-competition experiments or 1:1:1 (4 mM) solutions for competitive experiments with a final volume of 500 µL. The order of addition was as follows: buffer, hydrazide(s), then **3**. The hydrazone solutions were allowed to sit at room temperature for 2 h before NMR spectra were taken.

General protocol for the addition of solute to hydrazone dimers
Stock solutions of NaCl (5.8 M) and d_4-urea (14 M) were made in D_2O citrate buffer (50 mM, pD 5). Appropriate volumes were added to hydrazone solutions and the samples were allowed to sit at room temperature for 1 h before NMR spectra were taken. For experiments with both NaCl and urea, the order of addition was as follows: buffer, hydrazide(s), **3**, urea then NaCl.

The known concentration of the internal standard was used to calculate the concentration of each species in solution from the average of all visible non-overlapped resonances assigned to that species.
The percent dimer was calculated as follows: % dimer=[Dimer]/[Dimer]+[**3**]
The percent dimer left in solution was calculated as follows: % dimer in sol.=[Dimer]/[Dimer]$_{0mM\text{-}NaCl}$

3. ^{1}H NMR spectra of **D-Bz**, **D-MeO**, **D-Py+**

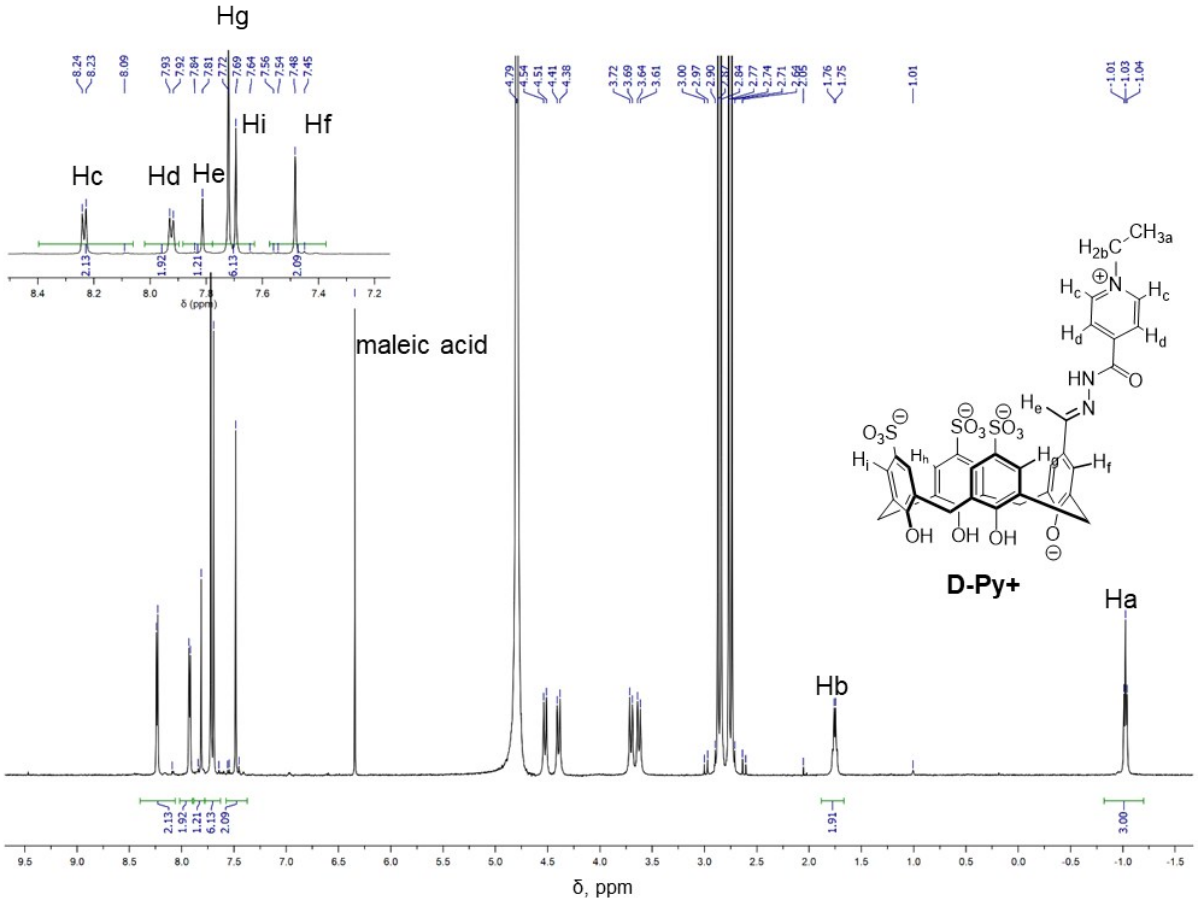


Figure S1. ^{1}H spectrum of **D-Py+** (5 mM, 1:1 **3**:**H-Py+**) in citrate buffer (50 mM, pD 5.0).

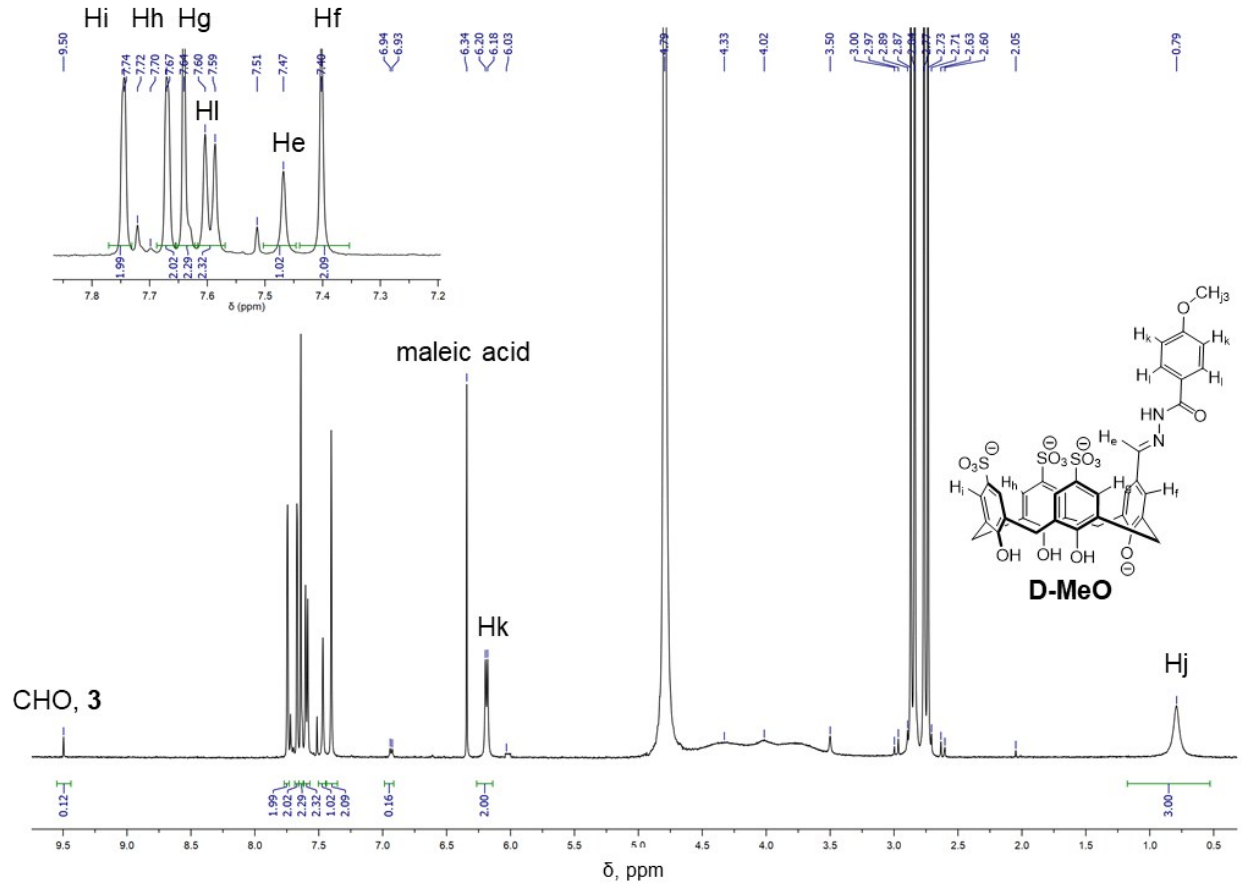


Figure S2. ^{1}H spectrum of **D-MeO** (5 mM, 1:1 **3**:**H-MeO**) in citrate buffer (50 mM, pD 5.0).

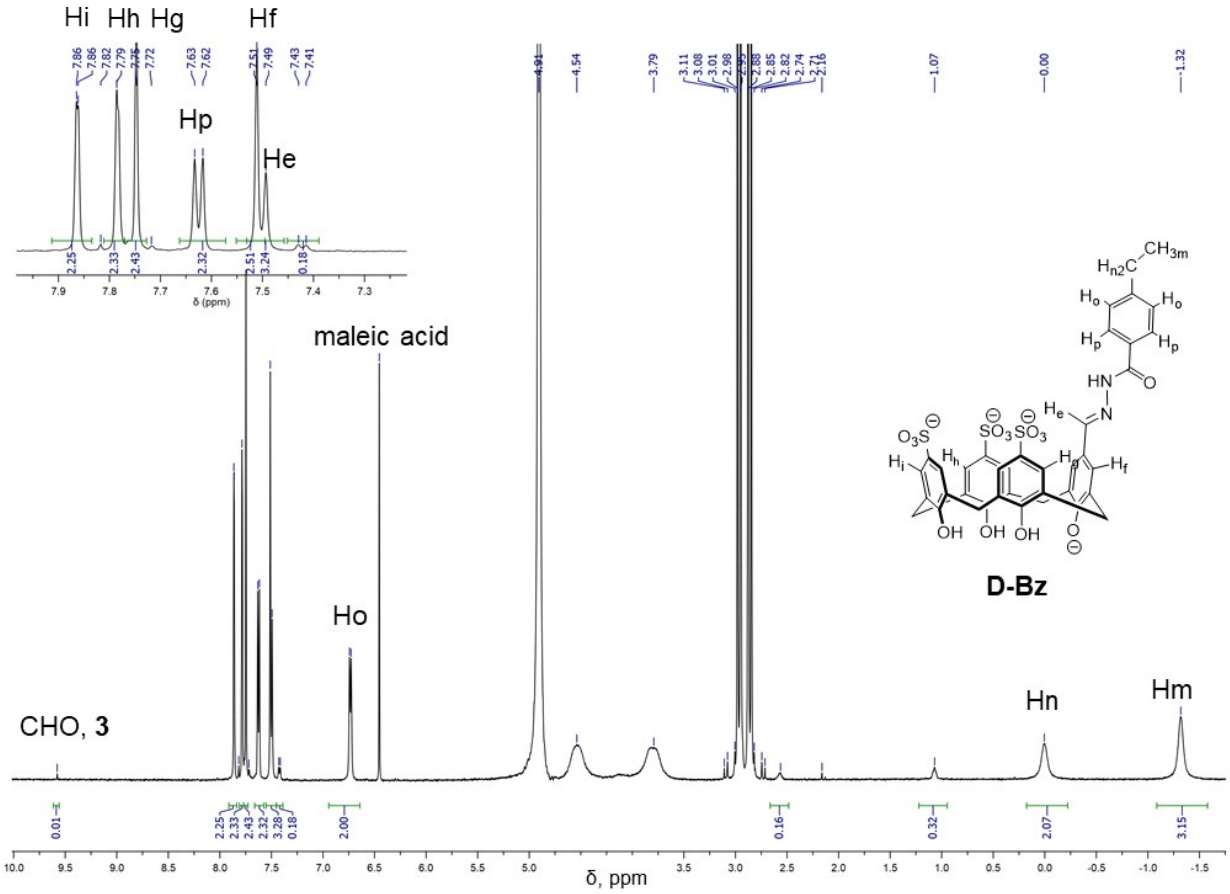


Figure S3. ^{1}H spectrum of **D-Bz** (5 mM, 1:1 **3**:**H-Bz**) in citrate buffer (50 mM, pD 5.0).

Table S1. HSQC-assigned carbon and proton hydrazone resonances in **DBz**, **DPy+**, and **DMeO**

Compound	^{13}C chemical shift, ppm	^{1}H chemical shift, ppm
DBz[a)]	149	7.26
DPy+[b)]	150	7.88
DMeO[b)]	148	8.30

a) dimer (30 mM) in citrate buffer (50 mM, pD 5.0) with 15% MeOD. b) dimer (30 mM) in d_6-DMSO

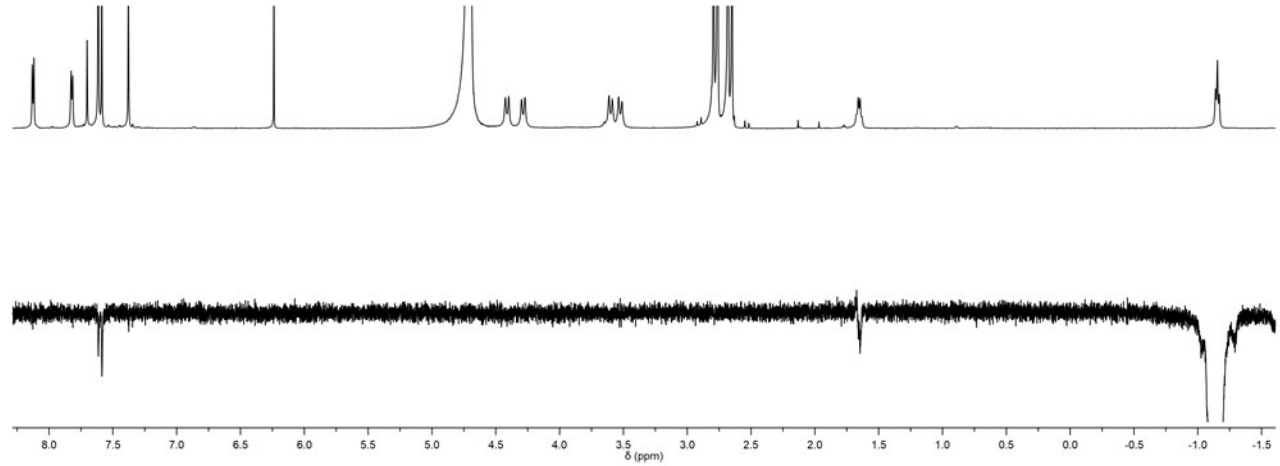


Figure S4. 1-D selective NOE shows intermolecular interactions of **D-Py+** between upper rim protons, H_i and $H_{h/g}$, and irradiated CH_3 protons on the pendant group, only possible if two of the same copy arrange so that the pendant group of one copy is encapsulated by the pocket of the second copy.

4. DOSY analysis on D-Py+, D-MeO, D-Bz

Table S2. DOSY measured diffusion coefficients and calculated hydrodynamic radii corresponding to each selected resonance associated to **D-Bz**

Resonances[i)]	D (m2/s)	rH (Å)[ii)]
Hi	2.17E-10	11.63243
Hp	2.19E-10	11.52619
Ho	2.16E-10	11.66468
Hn	2.53E-10	9.989064
Hm	2.29E-10	11.04699

i) atoms labeled in section 2 ii) calculated from Stokes-Einstein equation where the viscosity of water is 8.70E-4 Pa·s at 300 K

The average diffusion coefficient was 2.27E-10 m^2/s and the average hydrodynamic radius was 11.2 ± 0.7 Å.

Table S3. DOSY measured diffusion coefficients and calculated hydrodynamic radii corresponding to each selected resonance associated to **D-OMe**

Resonances[i)]	D (m2/s)	r (Å)[ii)]
Hj	2.19E-10	11.52093
Hg	2.22E-10	11.37556
Hl	2.31E-10	10.92270
Hk	2.20E-10	11.45818

i) atoms labeled in section 2 ii) calculated from Stokes-Einstein equation where the viscosity of water is 8.70E-4 Pa·s at 300 K

The average diffusion coefficient was 2.23E-10 m^2/s and the average hydrodynamic radius was 11.4 ± 0.3 Å.

Table S4. DOSY measured diffusion coefficients and calculated hydrodynamic radii corresponding to each selected resonance associated to **D-Py+**

resonances	D (m2/s)	r (Å)
Hc	2.38E-10	10.62389
Hd	2.03E-10	12.44693
Hi	2.25E-10	11.21385
Hf	2.27E-10	11.11509
Hb	2.23E-10	11.34488
Ha	2.25E-10	11.21883

i) atoms labeled in section 2 ii) calculated from Stokes-Einstein equation where the viscosity of water is 8.70E-4 Pa·s at 300 K

The average diffusion coefficient was 2.23E-10 m^2/s and the average hydrodynamic radius was 11.3 ± 0.6 Å.

5. ^{1}H NMR of competition experiments: **D-Py+**/**D-MeO**, **D-Py+**/**D-Bz** and **D-Bz**/**D-MeO**

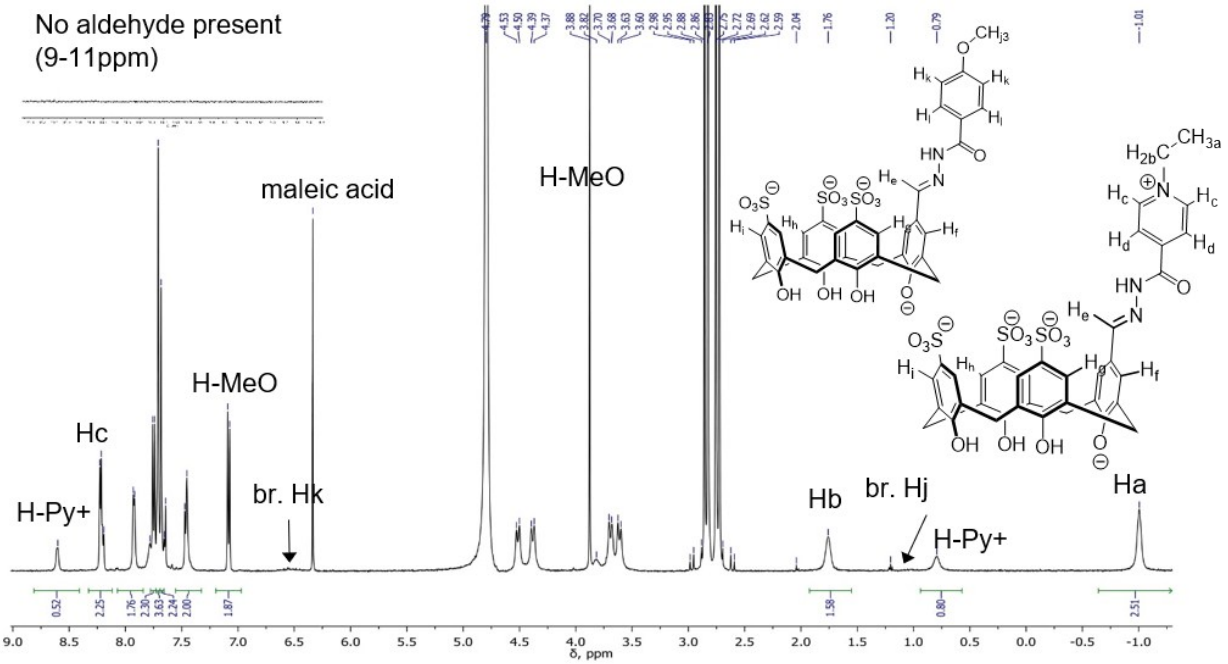


Figure S5. ^{1}H NMR spectrum of 1:1:1 mixture of **3**:**H-Py+**:**H-MeO** (4 mM) in citrate buffer (50 mM, pD 5.0)

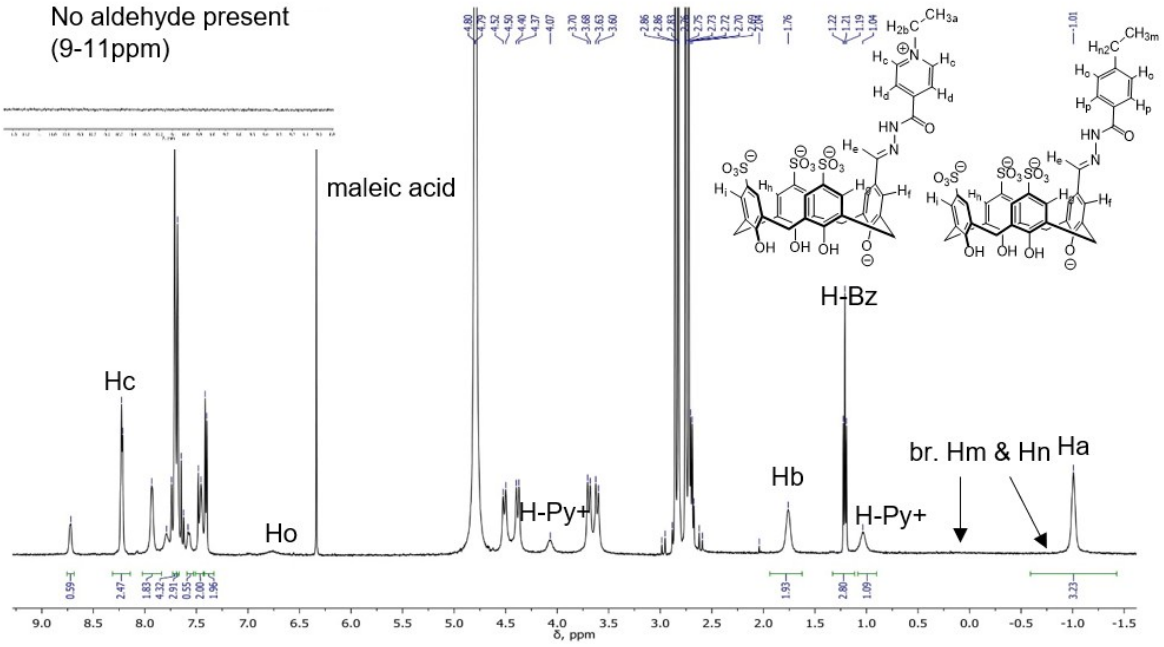


Figure S6. ^{1}H NMR spectrum of 1:1:1 mixture of **3**:**H-Py+**:**H-Bz** (4 mM) in citrate buffer (50 mM, pD 5.0)

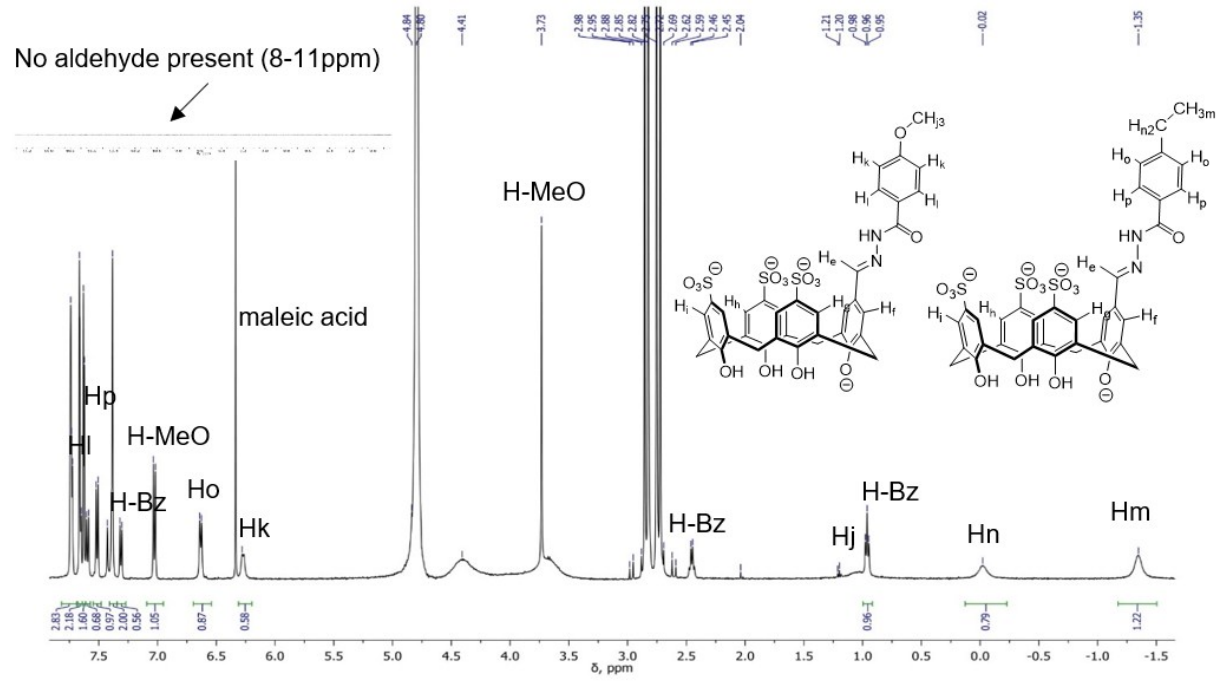


Figure S7. ^{1}H NMR spectrum of 1:1:1 mixture of **3**:**H-Bz**:**H-MeO** (4 mM) in citrate buffer (50 mM, pD 5.0)

6. ^{1}H NMR titrations and calculations with **D-Bz**, **D-MeO**, **D-Py+**
   a. Urea (200 mM, 600 mM, 1 M, 2.5 M, 5 M)

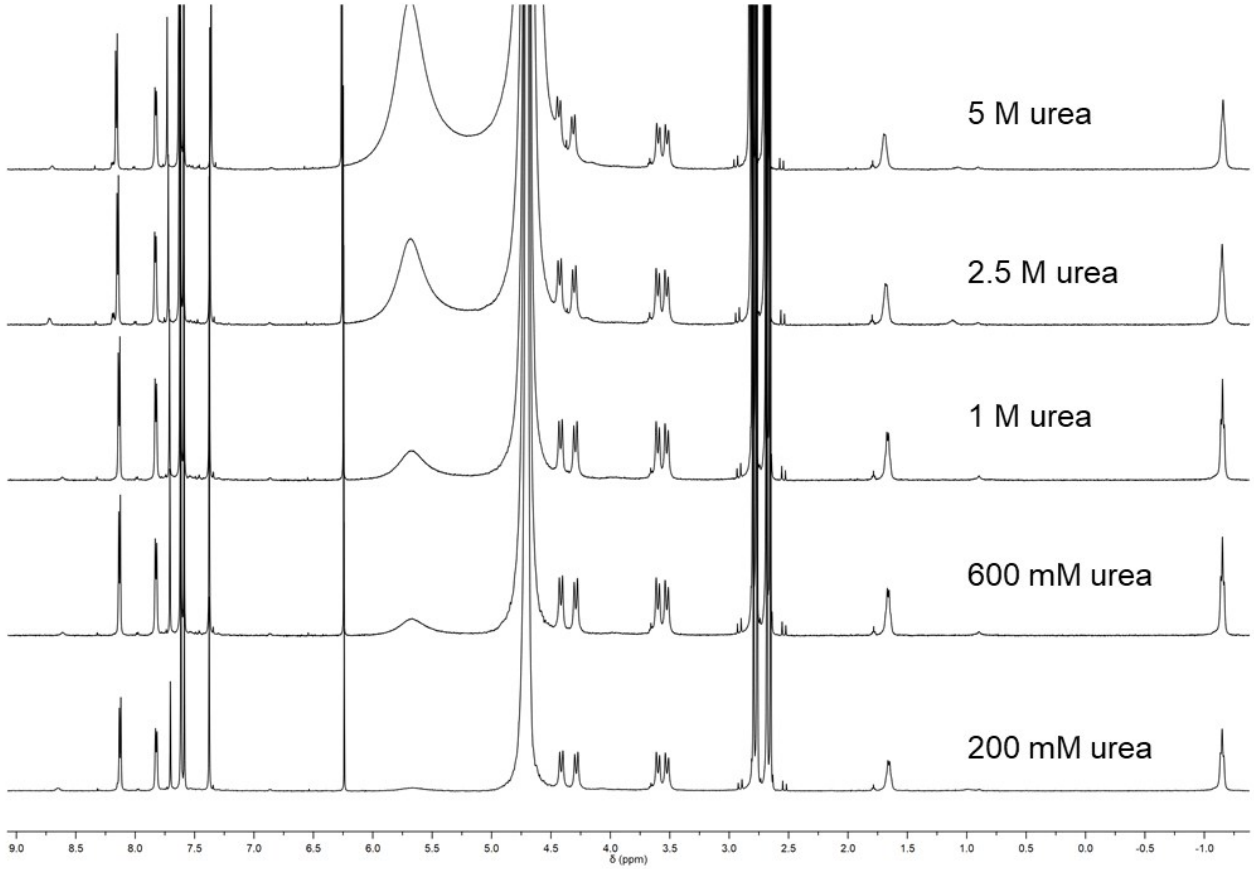


Figure S8: ^{1}H NMR titrations of urea (14 M stock) into **D-Py+** (5 mM) in citrate buffer (50 mM, pD 5) shows very little change in the resonances associated to the dimer (CH_3 –1.03 ppm, CH_2 1.74 ppm, *ortho*-

protons 8.25 ppm), indicating at high concentrations of urea the dimer remains assembled. Solutions contain 1:1 **H-Py+**:**3** (5 mM, ea.) and maleic acid as the internal standard (3 mM).

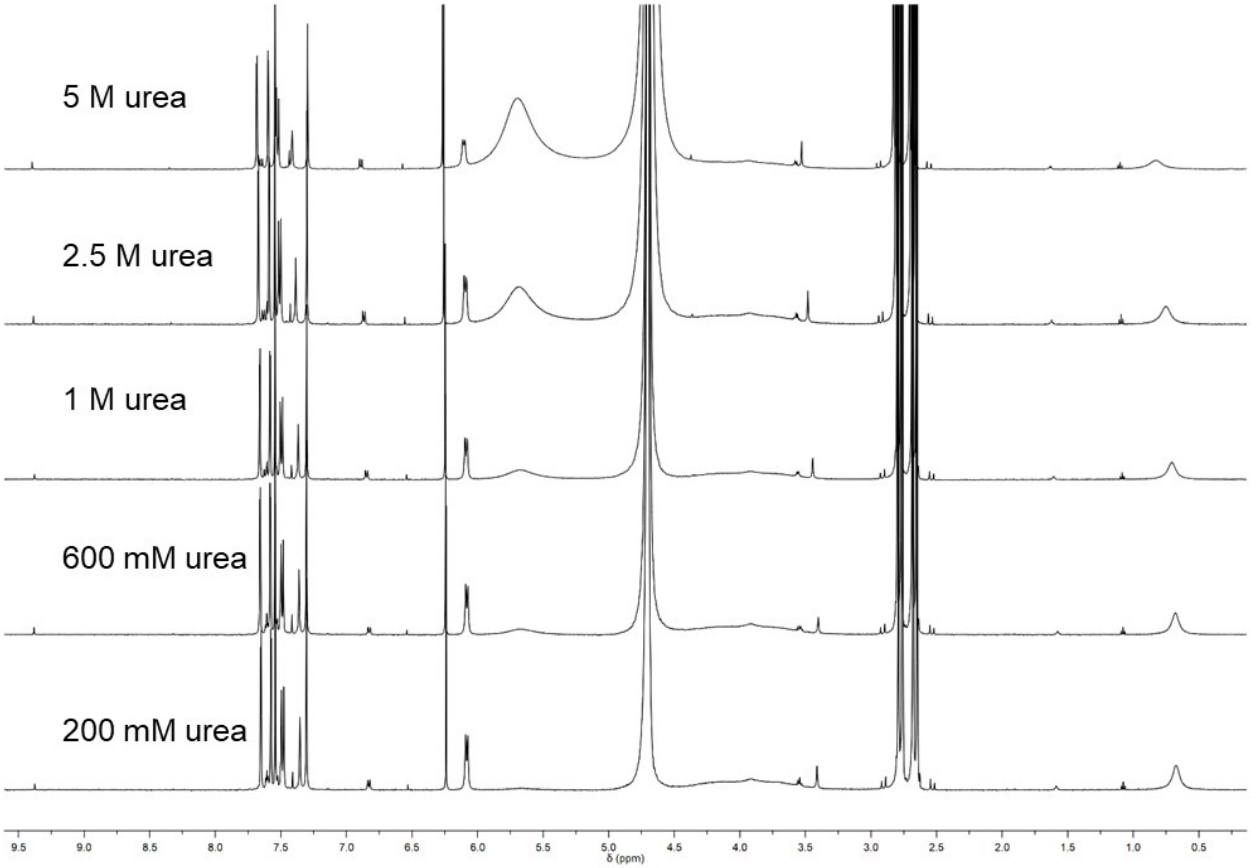


Figure S9: ^{1}H NMR titrations of urea (14 M stock) into **D-MeO** (5 mM) in citrate buffer (50 mM, pD 5) shows very little change in the resonances associated to the dimer (OMe 0.78 ppm, *ortho*-protons 6.20 ppm), indicating at high concentrations of urea the dimer remains assembled. Solutions contain 1:1 **H-MeO**:**3** (5 mM, ea.) and maleic acid as the internal standard (3 mM).

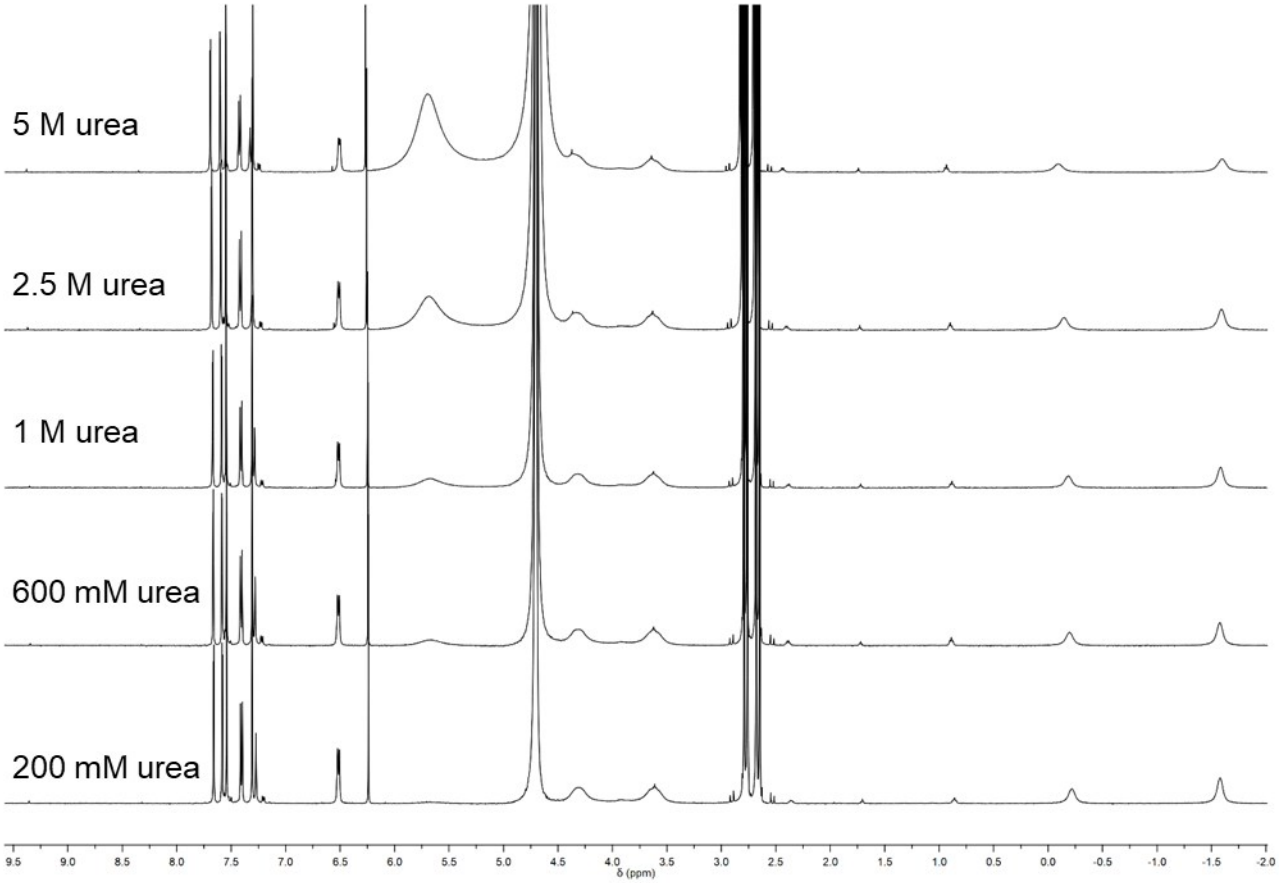


Figure S10: ^{1}H NMR titrations of urea (14 M stock) into **D-Bz** (5 mM) in citrate buffer (50 mM, pD 5) shows very little change in the resonances associated to the dimer (CH_3 –1.33 ppm, CH_2 –0.21 ppm, *ortho*-protons 6.51 ppm), indicating at high concentrations of urea the dimer remains assembled. Solutions contain 1:1 **H-Bz**:**3** (5 mM, ea.) and maleic acid as the internal standard (3 mM).

b. NaCl (200, 400, 600 mM)

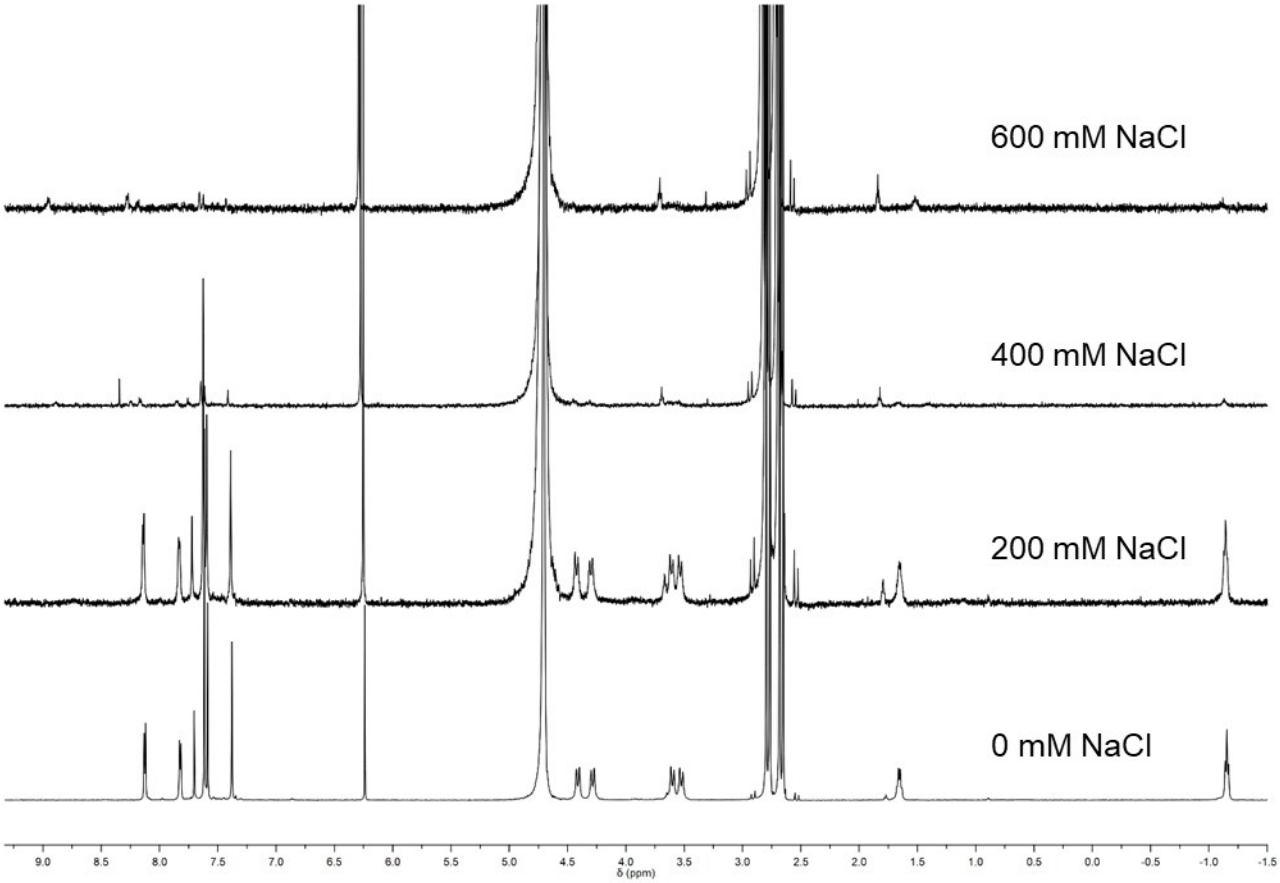


Figure S11: ^{1}H NMR titrations of NaCl (5.8 M) into **D-Py+** (5 mM) in citrate buffer (50 mM, pD 5) shows decreasing signal due to precipitation starting at 200 mM NaCl and nearly all material is lost at 600 mM NaCl. Solutions contain 1:1 **H-Py+**:**3** (5 mM, ea.) and maleic acid as the internal standard (3 mM).

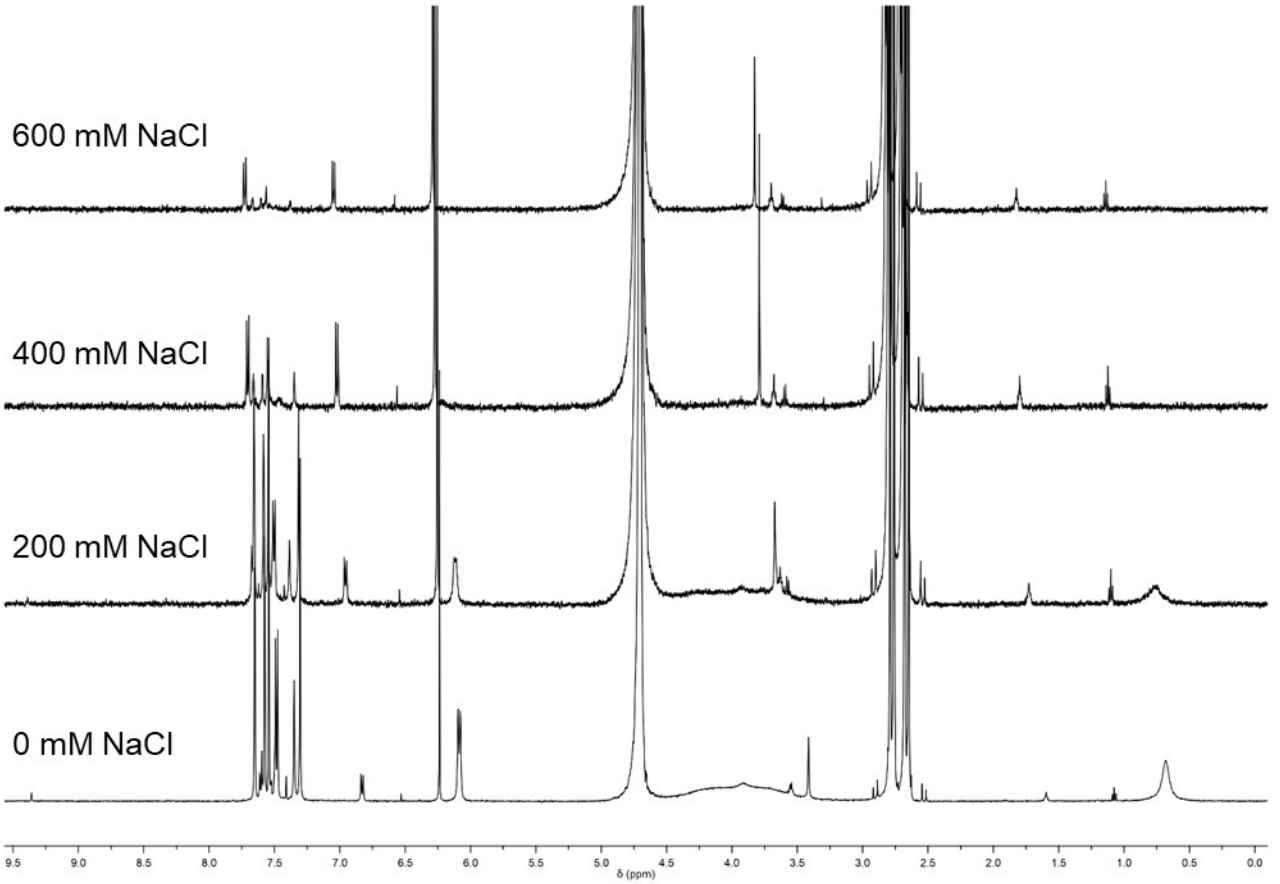


Figure S12: ^{1}H NMR titrations of NaCl (5.8 M) into **D-MeO** (5 mM) in citrate buffer (50 mM, pD 5) shows decrease dimer due to precipitation starting at 200 mM NaCl and nearly all material is lost at 600 mM NaCl, leaving behind **H-MeO**. Solutions contain 1:1 **H-MeO**:**3** (5 mM, ea.) and maleic acid as the internal standard (3 mM).

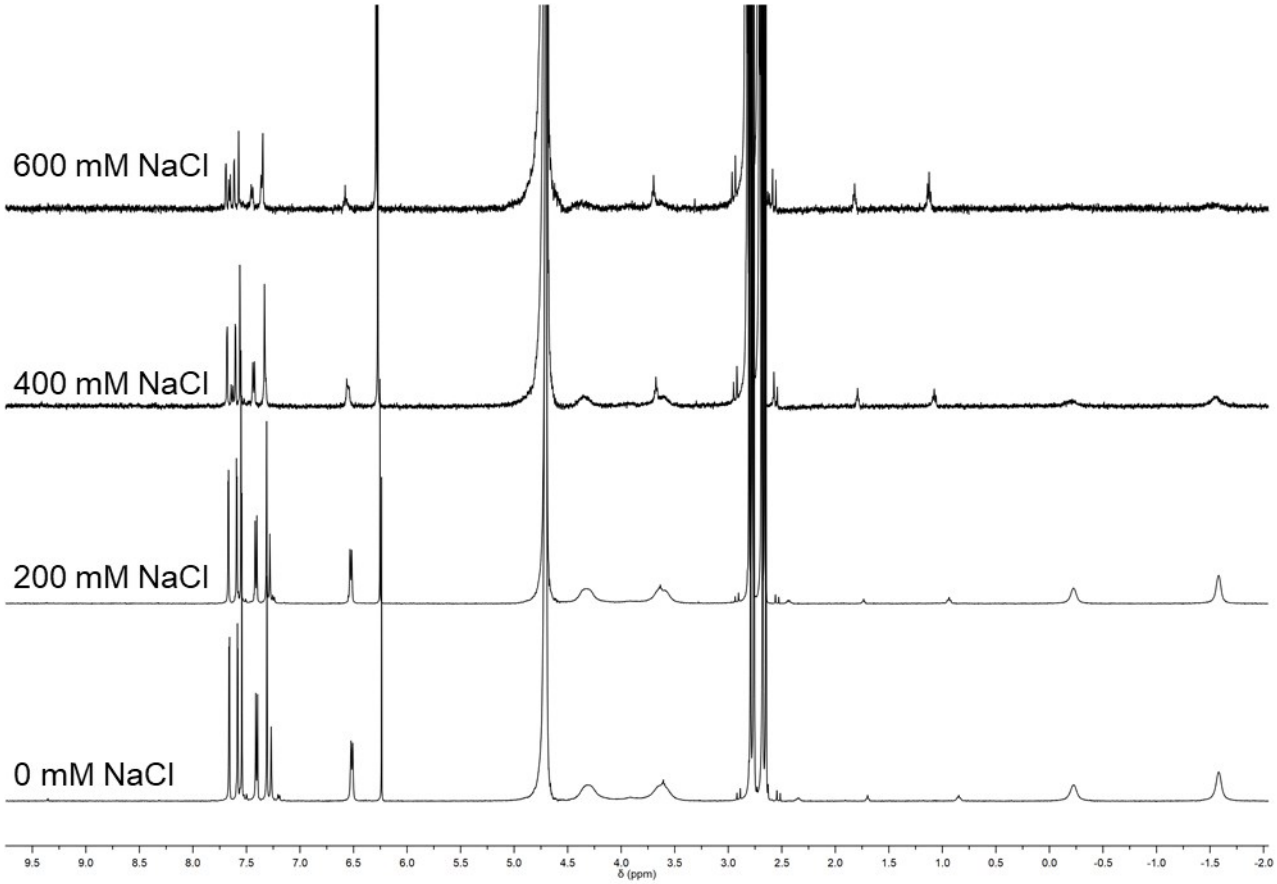


Figure S13: ^{1}H NMR titrations of NaCl (5.8 M) into **D-Bz** (5 mM) in citrate buffer (50 mM, pD 5) shows decrease dimer due to precipitation starting at 200 mM NaCl and nearly all material is lost at 600 mM NaCl, leaving behind **H-Bz**. Solutions contain 1:1 **H-Bz**:**3** (5 mM, ea.) and maleic acid as the internal standard (3 mM).

c. 5 M urea and NaCl (200, 400, 600 mM, 1 M)

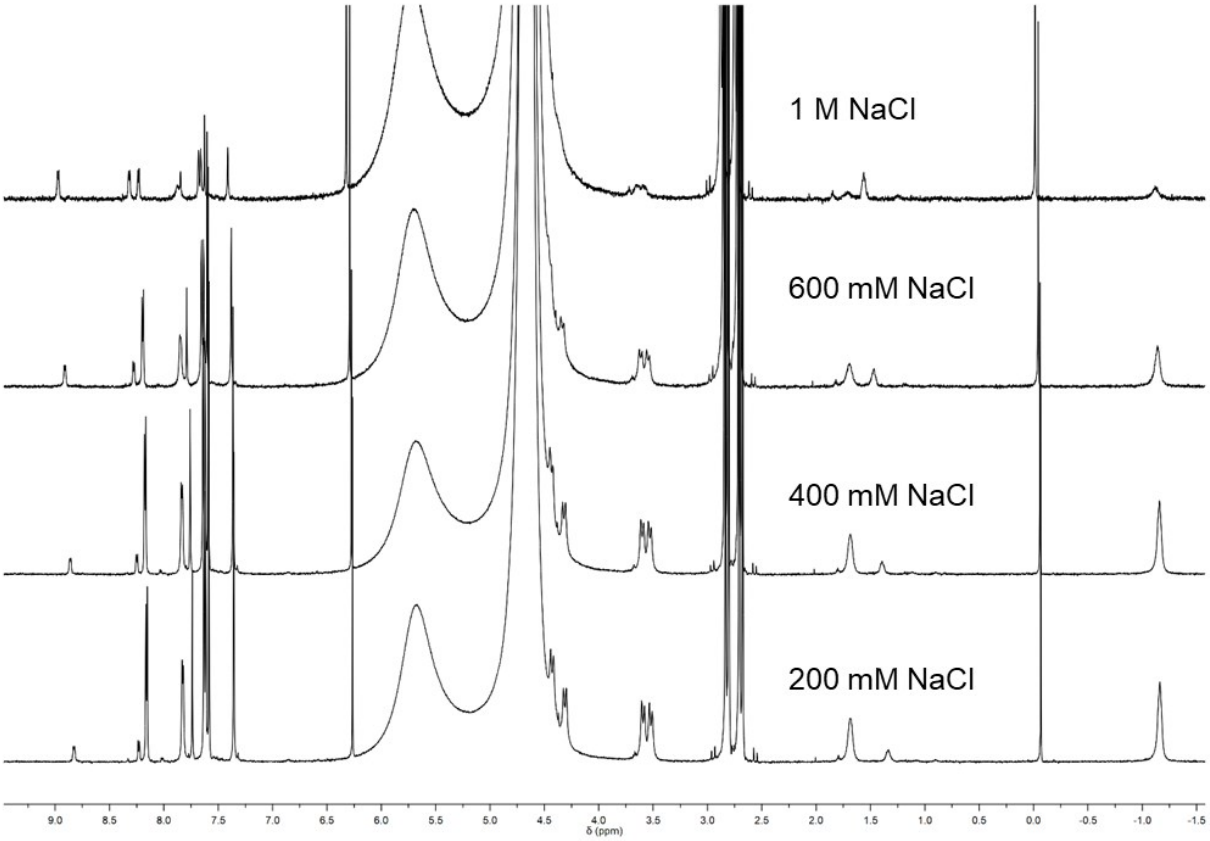


Figure S14: ^{1}H NMR titrations of NaCl (5.8 M) into **D-Py+** (5 mM) with 5 M urea in citrate buffer (50 mM, pD 5) shows the presence of **H-Py+** resonances at 200 mM NaCl, a decrease in dimer intensity due to precipitation starting at 600 mM NaCl yet resonances of dimer remain alongside **H-Py+** at 1 M NaCl. 1:1 **H-Py+**:**3** (5 mM, ea.) and TSP as the internal standard (1 mM).

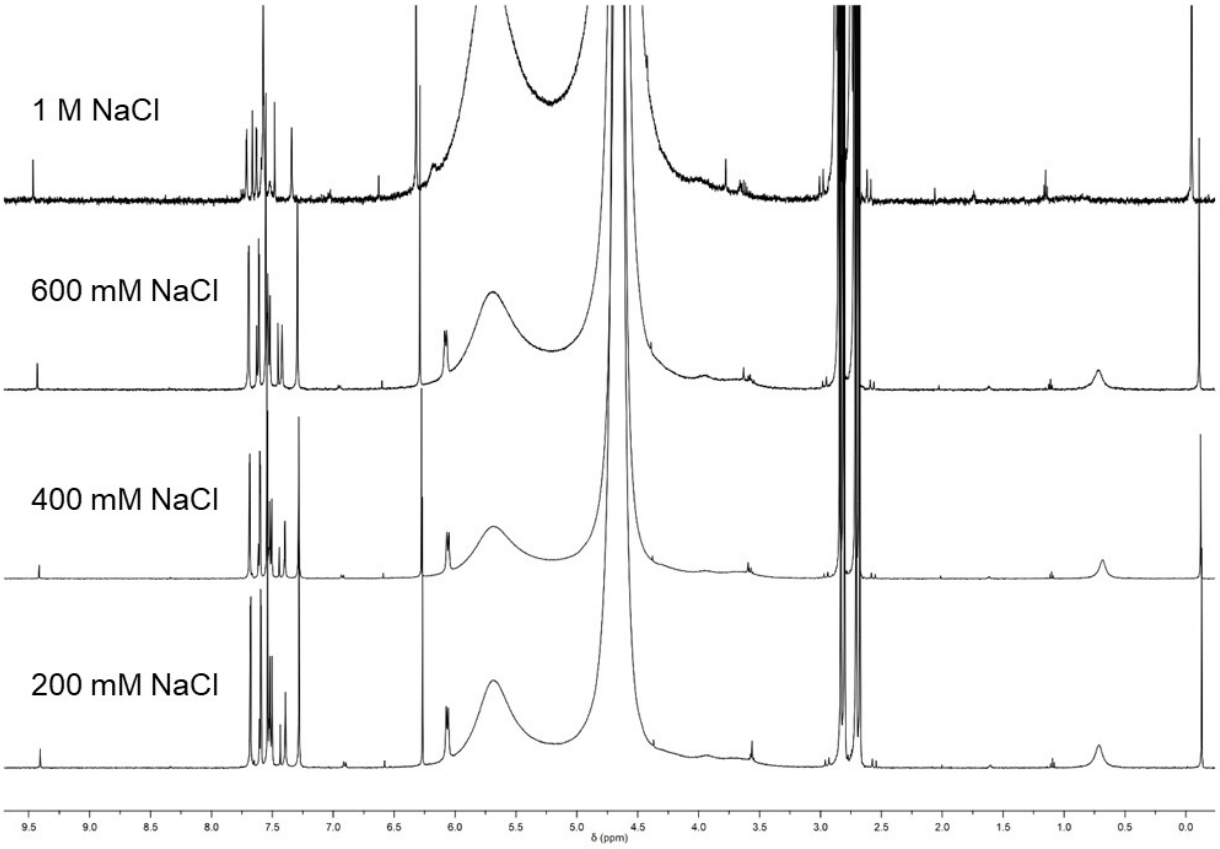


Figure S15: ^{1}H NMR titrations of NaCl (5.8 M) into **D-MeO** (5 mM) with 5 M urea in citrate buffer (50 mM, pD 5) shows the presence of **H-MeO** resonances at 200 mM NaCl, a decrease in dimer intensity due to precipitation starting at 400 mM NaCl yet resonances of dimer remain alongside **H-MeO** at 1 M NaCl. 1:1 **H-MeO**:**3** (5 mM, ea.) and TSP as the internal standard (1 mM).

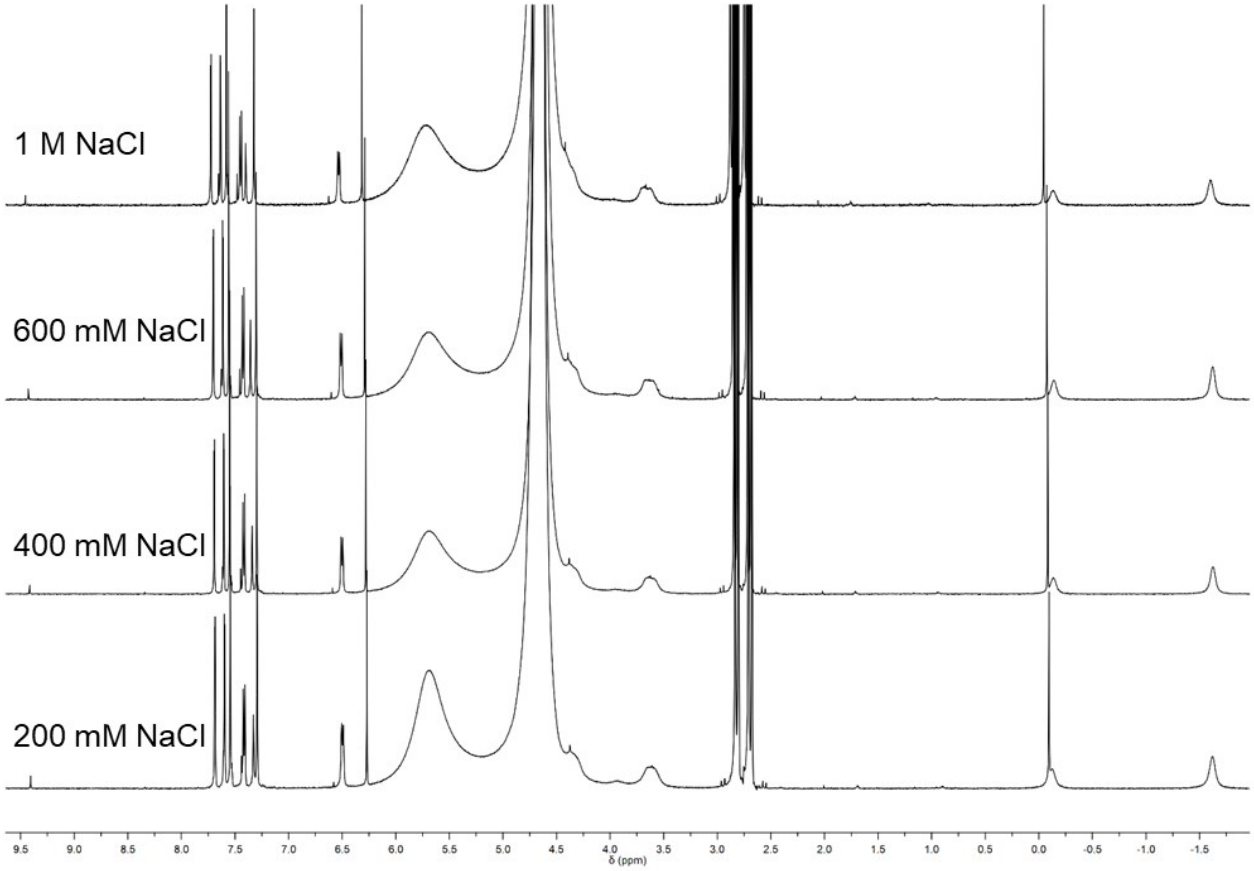


Figure S16: ^{1}H NMR titrations of NaCl (5.8 M) into **D-Bz** (5 mM) with 5 M urea in citrate buffer (50 mM, pD 5) shows a slight decrease in dimer intensity due to precipitation starting at 1 M NaCl yet no new resonances from **H-Bz** become apparent during the titration. 1:1 **H-Bz**:**3** (5 mM, ea.) and TSP as the internal standard (1 mM).

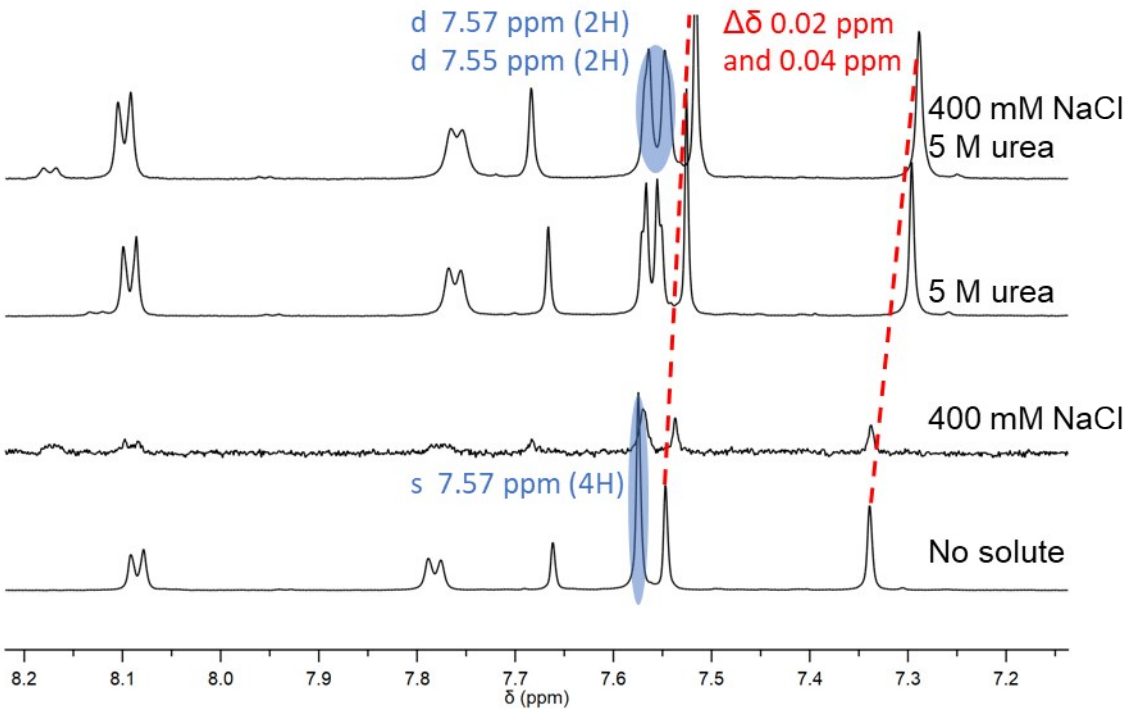


Figure S17. ^{1}H NMR stack plot of **DPy+** (5 mM) with three different co-solute conditions. In the presence of urea (5 M), singlet upper rim protons at 7.57 ppm (highlighted in blue) split into two sets of doublets and the other two sets of upper rim singlets shift slightly. This indicates direct urea interaction along the upper rim of the calixarene. This is not observed in the resonances that remain in solution with NaCl (400 mM). When urea (5 M) is present with NaCl (400 mM), the upper rim resonances look similar as in the urea only condition suggesting urea is again directly interacting with calixarene instead of the Na+.

7. ^{1}H NMR stack plots of competition experiments with added solutes (300 mM NaCl and 300 mM NaCl, 4.4 M urea)

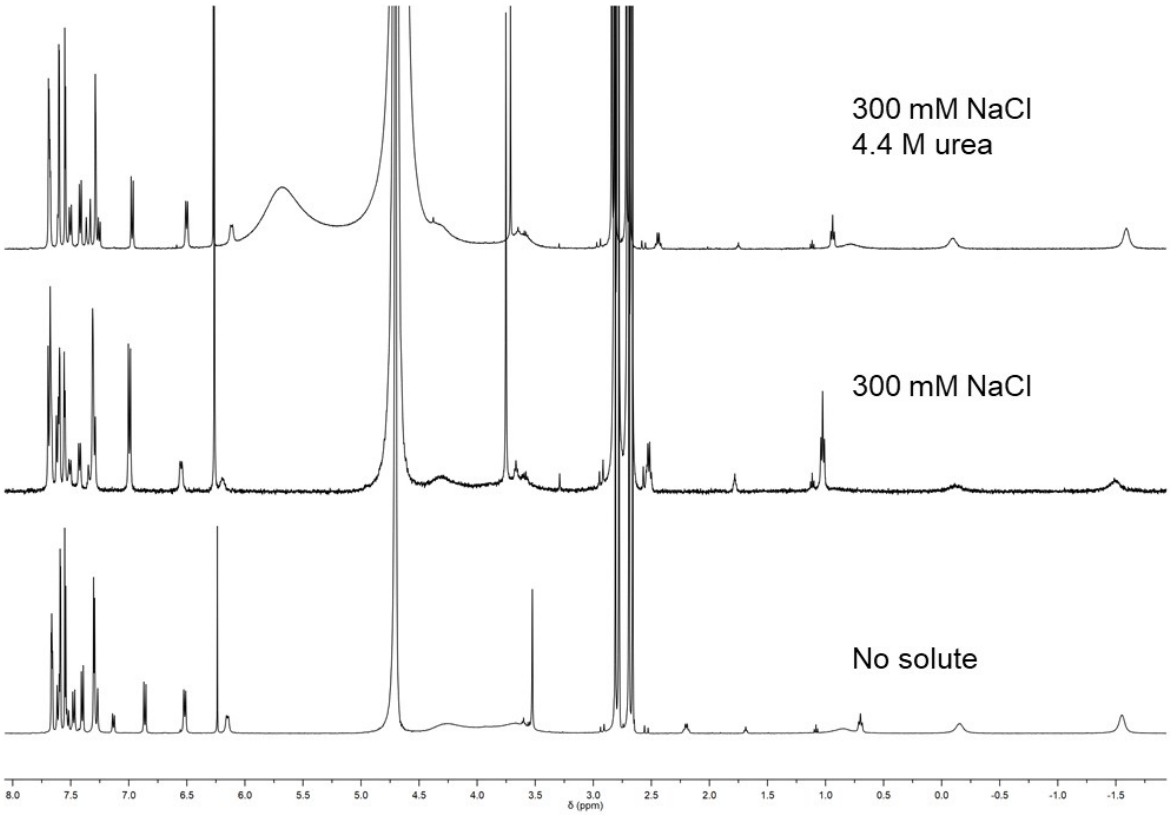


Figure S18. ^{1}H spectra show that neutral hydrophobic, **D-Bz** and neutral polar**, D-MeO** are both equally capable of existing in strongly denaturing conditions. Without solute, **D-Bz** is favoured 60:40, while 300 mM NaCl induces precipitation shown by increased **H-Bz** resonances and decreased **D-MeO** resonances. When both 4.4 M urea and 300 mM NaCl is present the spectra resemble that of no solute present.

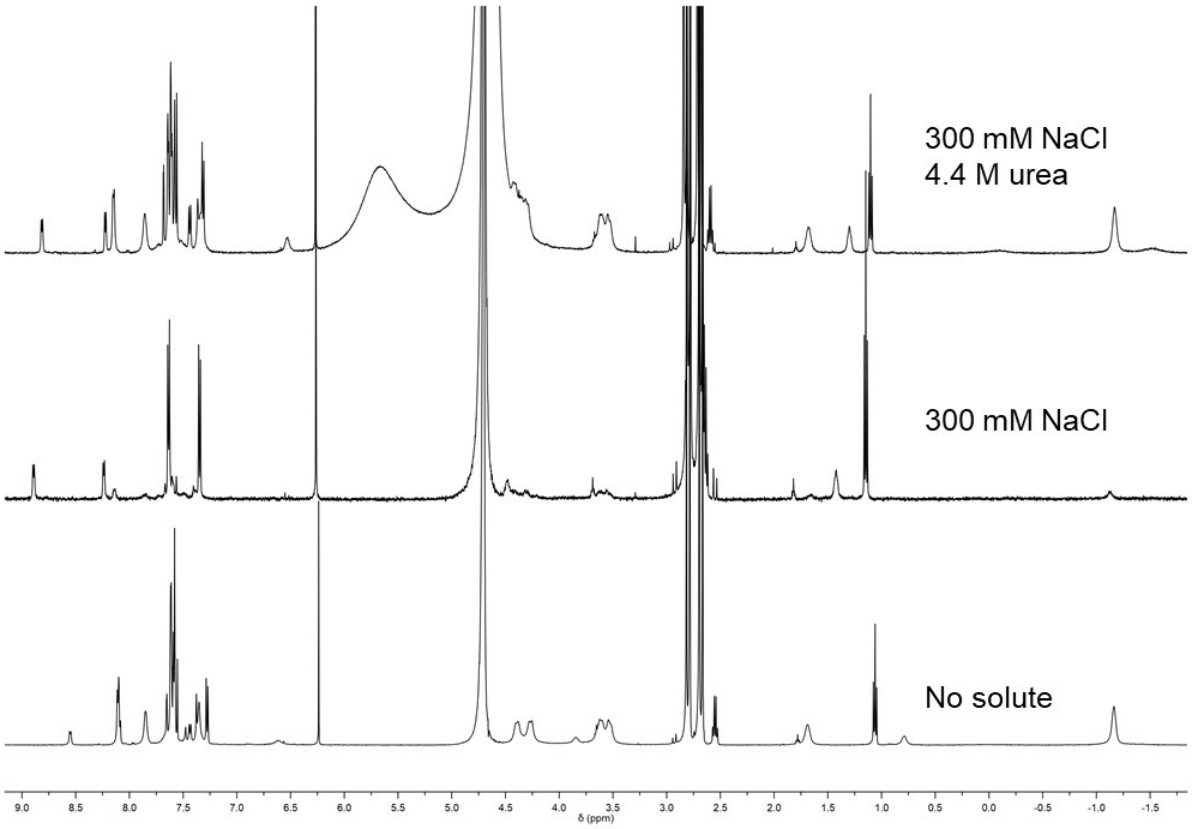


Figure S19. ^{1}H spectra show that neutral hydrophobic, **D-Bz** is more resilient to extreme solute conditions than the charged hydrophobic, **D-Py+** derivative. Without solute, **D-Py+** is favoured 71:29, while 300 mM NaCl induces precipitation of both dimers. When 4.4 M urea and 300 mM NaCl is added, the spectra shows an increase in **D-Bz** resonances (*ortho*-protons, 6.54 ppm) and a proportional decrease in **D-Py+** resonances (*ortho*-protons, 8.13 ppm), shifting the ratio to 64:36.

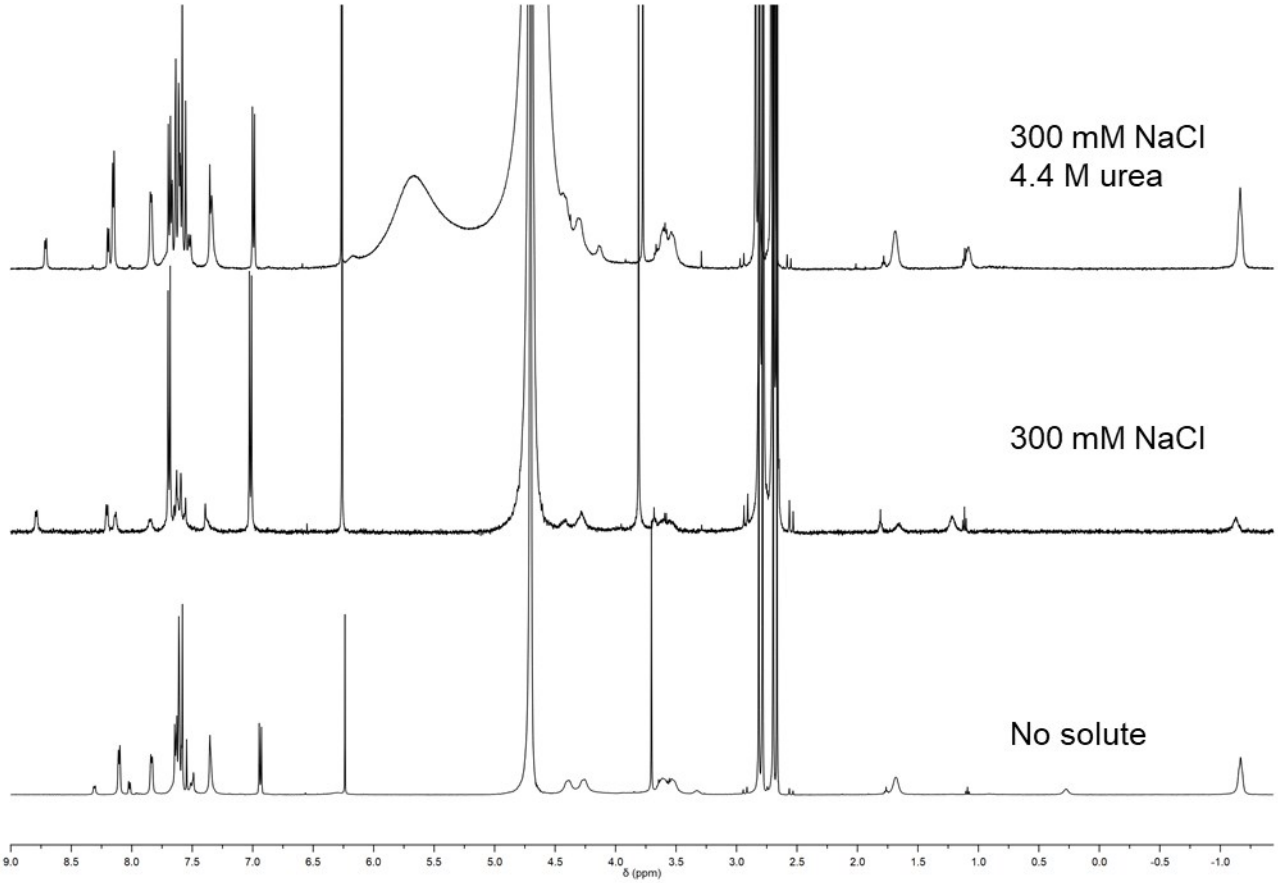


Figure S20. ^{1}H spectra show that neutral polar, **D-OMe** is more resilient to extreme solute conditions than the charged hydrophobic, **D-Py+** derivative. Without solute, **D-Py+** is favoured 84:16, while 300 mM NaCl induces precipitation of both dimers. When 4.4 M urea and 300 mM NaCl is added, the spectra shows an increase in **D-Bz** resonances (*ortho-protons*, 6.2 ppm) and a proportional increase in **H-Py+** resonances (*ortho*-protons, 8.71 ppm), shifting the ratio to 68:32.

References

1 Beatty, M. A.; Borges-González, J.; Sinclair, N. J.; Pye, A. T.; Hof, F., *J. Am. Chem. Soc.* 2018**, 140**, 3500-3504.

2 Ahmad, Y.; Habib, M. S.; Iqbal, M.; Qureshi, M. I.; Craig, J. C.; Garnett, J. L., . . . Suddens, A. J., *J. Am. Chem. Soc.* 1964, 4053-4088.