Electronic Supplementary Material (ESI) for Chemical Science.

Discovery of a Cyclic 6+6 Hexamer of D-Biotin and Formaldehyde

Micke Lisbjerg, Bo Michael Jessen, Brian Rasmussen, Bjarne Enrico Nielsen, Anders Østergaard Madsen and Michael Pittelkow*

Department of Chemistry, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen Ø, Denmark. E-mail: pittel@kiku.dk. Homepage: www.pittelkow.kiku.dk

Contents

1 Apparatus

1.1 LC-MS

HPLC analyses were performed on a Dionex UltiMate 3000 system coupled to an UltiMate 3000 diode array UV/Vis detector. Separations were achieved using a Dionex Acclaim RSLC 120 C18 2.2 μm 120 Å 2.1 × 100 mm column maintained at 20 °C. The mobile phase solutions were 0.1 % formic acid in H_2O and 0.1 % formic acid in MeCN. The water used as eluent was purified by a Millipore system. LC/MS analysis was carried out on a Bruker MicrOTOF-QII-system with an ESI-source with the following settings: nebulizer 1.2 bar, dry gas 8.0 L min^{-1}, dry temperature 200 °C, capillary 4500 V, end plate offset −500 V, funnel 1 RF 300.0 Vpp, ISCID energy 0.0 eV, funnel 2 RF 400.0 Vpp, hexapole RF 400.0 Vpp, quadrupole ion energy 5.0 eV, low mass 300.00 *m/z*, collision energy 10.0 eV, collision RF 630.0 Vpp, transfer time 120.0 μs, and pre puls storage 1.0 μs. The LC/MS data were processed using DataAnalysis v. 4.0 SP 5.

In the processing of HRMS measurements a sodium formate calibrant solution eluting in the first part of the LC-run was used to calibrate the system in each measurement.

1.2 NMR

^{1}H-NMR and ^{13}C-NMR spectra were recorded at 500 MHz and 126 MHz, respectively, on a Bruker Ultrashield Plus 500 spectrometer using residual non-deuterated solvent as the internal standard.

All NMR samples in D_2O were equipped with a DMSO-d_6 lock tube and the spectra were calibrated according to the non-deuterated DMSO signal (marked with * in the spectra). All D_2O samples contains 200 mM Na_2CO_3 unless otherwise stated.

The ^{1}H-NMR titrations and the 1D-TOCSY experiments were recorded on a 500 MHz varian spectrometer, using a standard pentaprobe, except for the NaCl and NaBr titrations which were carried out on a Bruker Ultrashield Plus 500. The 1D-TOCSY was performed to confirm the spin system of the aliphatic sidechain on the Biotin[6]uril (**2**) and the experiment was carried out with different mixing times.

1.3 Isothermal Titration Calorimeter

A Microcal VP-ITC microcalorimeter was used for all titrations. The temperature was set at 30°C and a stirring speed of 307 rpm was used. 56 injections of 5 μl of halide solution, with 240 s between each injection and an initial delay of 60 s were used for all titrations.

1.4 Elemental analysis

Elemental analysis for C, H, N and Cl was performed with a CE Instrument: FLASH 1112 series EA, at the microanalytical laboratory, University of Copenhagen.

1.5 Polarimeter

Optical rotation data were obtained on a Perkin Elmer 341 Polarimeter.

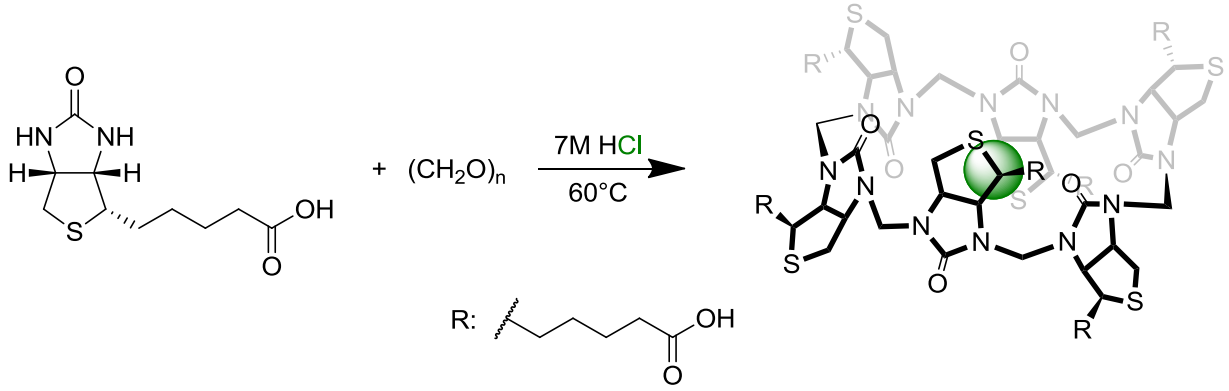
1.6 Diffractometer

All single-crystal X-ray diffraction data were collected at 122(1) K either on a Nonius KappaCCD area-detector diffractometer, equipped with an Oxford Cryostreams low-temperature device, using graphite-monochromated MoKα radiation, or a Bruker D8 Venture equipped with a IμS microfocus source, a KAPPA goniometer, a nitrogen cryostream cooling device and a PHOTON 100 detector, using MoKα radiation. The structures were solved using direct methods (SHELXS97) and refined using the SHELXL2013 software package.

1.7 Chemicals

Unless otherwise stated, all chemicals were purchased from commercial suppliers and used as received. Solvents were HPLC grade and used as received.

2 **Biotin[6]uril-HCl:**



Biotin (1.03 g ; 4.2 mmol) and para-formaldehyde (588 mg ; 19.5 mmol) were mixed in 7 M HCl (50 ml) and the heterogeneous solution was heated to 60 °C for 2 days. The solution was allowed to cool to 25°C and water (80 ml) was added, followed by filtration. The filtrate was washed with water (4 × 20 ml). The product was dried in vacuum. Yield: 520 mg, 48 %.
M.p. > 150°C (decomp.). $[\alpha]_D^{20}$ = -81.9 (c = 1, 0.1 M NaOH and 15 eq. NaCl).
The product was analysed by NMR in methanol-d_4 and under these conditions the carboxylic acids in Biotin[6]uril are esterfied.
^{1}H NMR (500 MHz, methanol-d_4) δ = 4.75 (s, 6H), 4.43 (s, 6H), 4.22 (dd, *J* = 9.3, 5.5 Hz, 6H), 3.40 (ddd, *J* = 11.5, 5.5, 3.1 Hz, 6H), 3.09 (dd, *J* = 12.9, 4.0 Hz, 6H), 2.98 (dd, *J* = 12.9, 6.2 Hz, 6H), 2.42 – 2.31 (m, 12H), 1.88 – 1.77 (m, 6H), 1.77 – 1.53 (m, 18H), 1.53 – 1.33 (m, 12H).
^{13}C NMR (126 MHz, MeOD) δ = 175.84, 162.40, 63.88, 61.28, 55.18, 52.00, 51.33 (h, *J* = 22.1 Hz), 35.10, 34.63, 29.61, 29.05, 25.61.
HRMS (ESI$^+$) of the D_{18} ester: *m/z* $[M+H]^+$ calc. for $C_{72}H_{91}D_{18}N_{12}O_{18}S_6$: 1639.7496; found:1639.7431.
HRMS (ESI$^+$) of the acid: *m/z* $[M+H]^+$ calc. for $C_{66}H_{97}N_{12}O_{18}S_6$: 1537.5363; found:1537.5382.
Elemental Analysis for $C_{66}H_{97}N_{12}O_{18}S_6Cl$: [calc.] (found); C [50.35] (50.48), H [6.21] (6.09), N [10.68] (10.63), Cl [2.25] (1.90).

When Biotin[6]uril was dissolved in MeOD-d_4 it was converted into the hexa-D_{18} ester in two days, probably because of the inclusion of HCl which catalyse the esterification. Figures S1 to S10 corresponds to the fully esterified Biotn[6]uril-D_{18} ester.

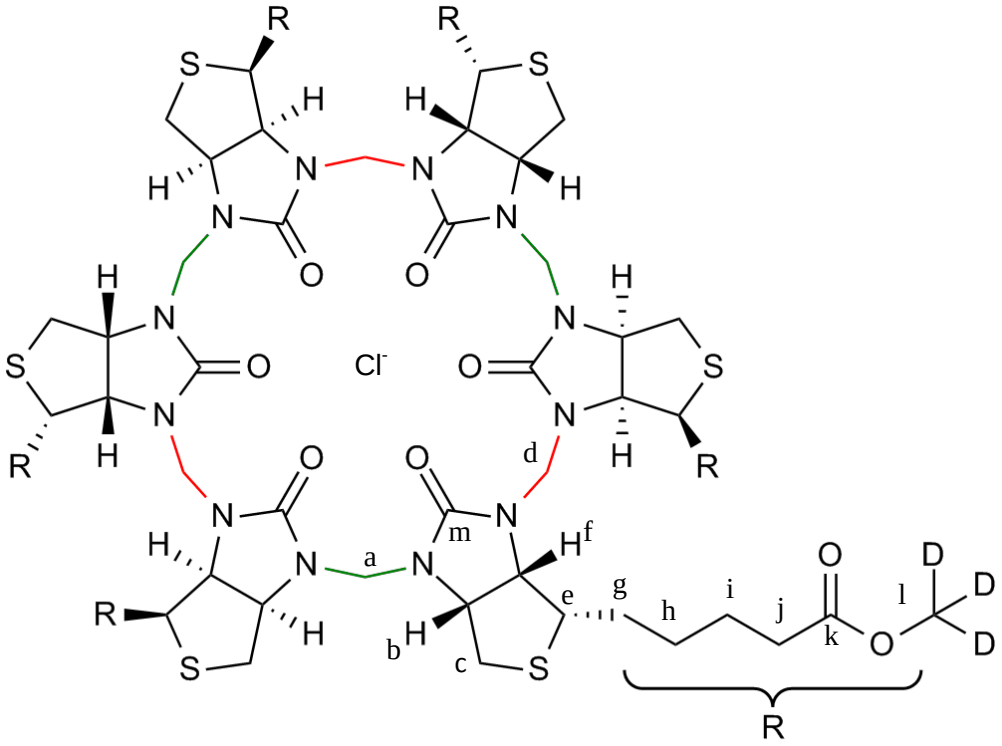


Figure S1: Schematic representation of Biotin[6]uril.

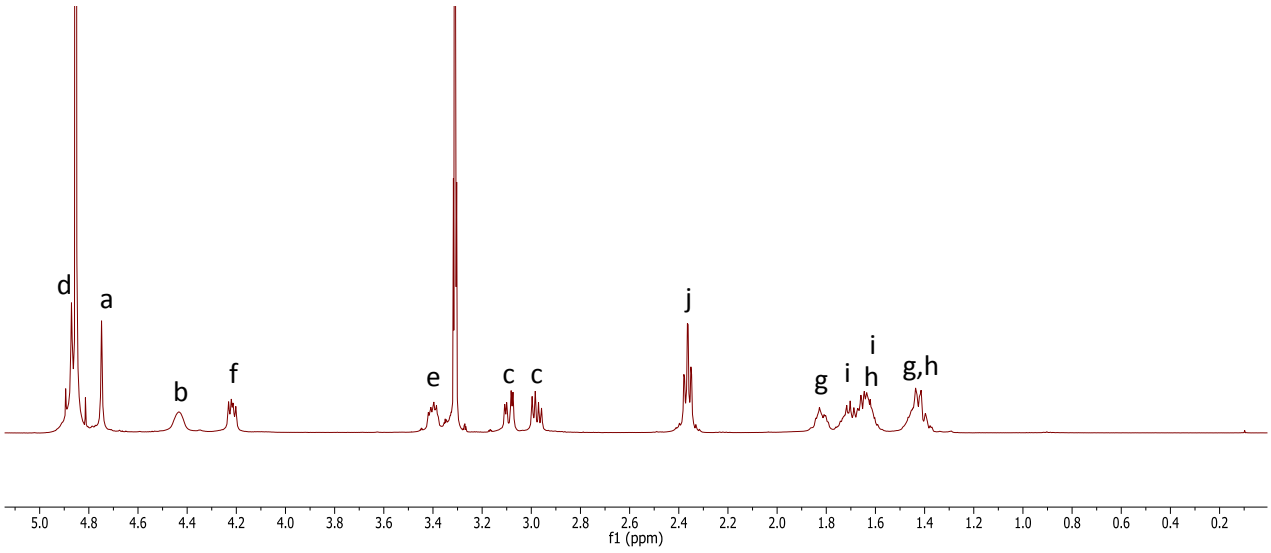


Figure S2: ^{1}H-NMR (500 MHz, MeOD-d_4): Biotin[6]uril-hexamethylester-D_{18}.

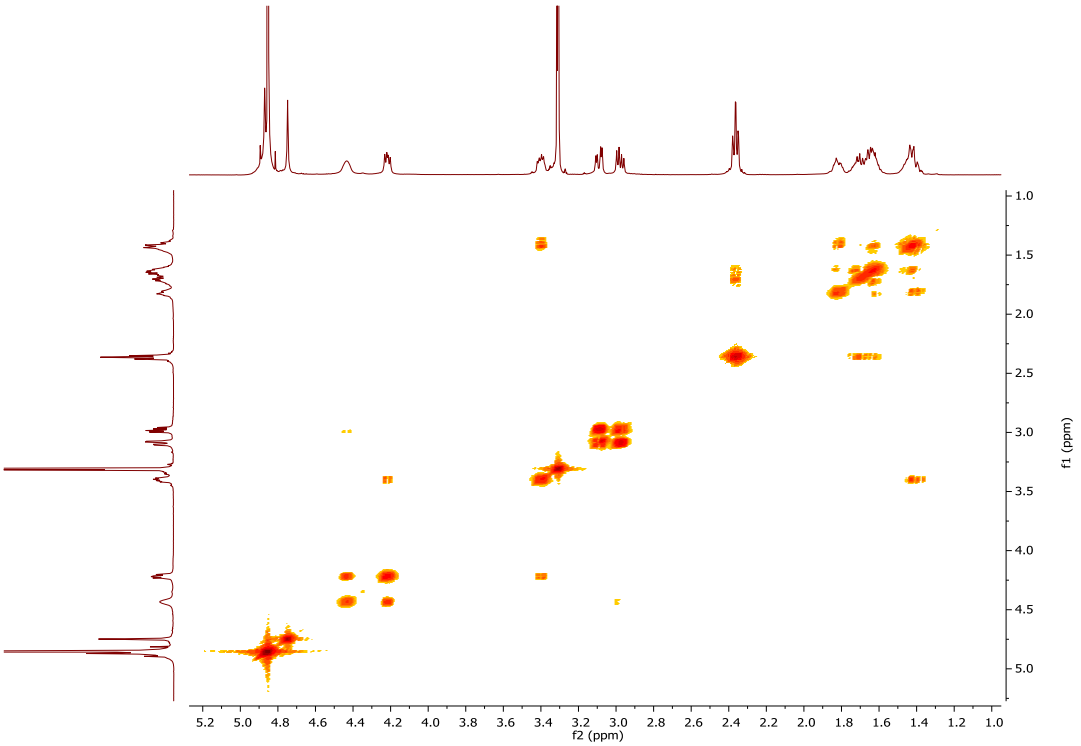


Figure S3: 2D-NMR (500 MHz, MeOD-d_4) COSY: Biotin[6]uril-hexamethylester-D_{18}.

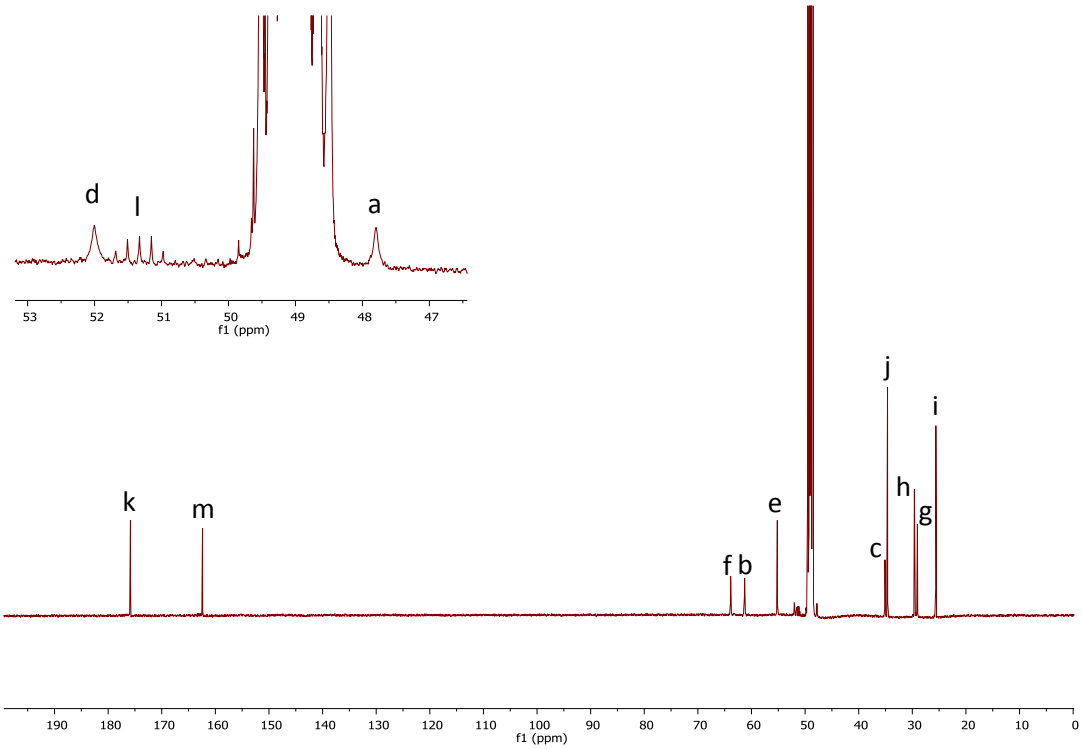


Figure S4: ^{13}C-NMR (126 MHz, MeOD-d_4): Biotin[6]uril-hexamethylester-D_{18}, with a zoom of 46 ppm to 53 ppm showing carbon l which is spilt due to the coupling to three deuteriums.

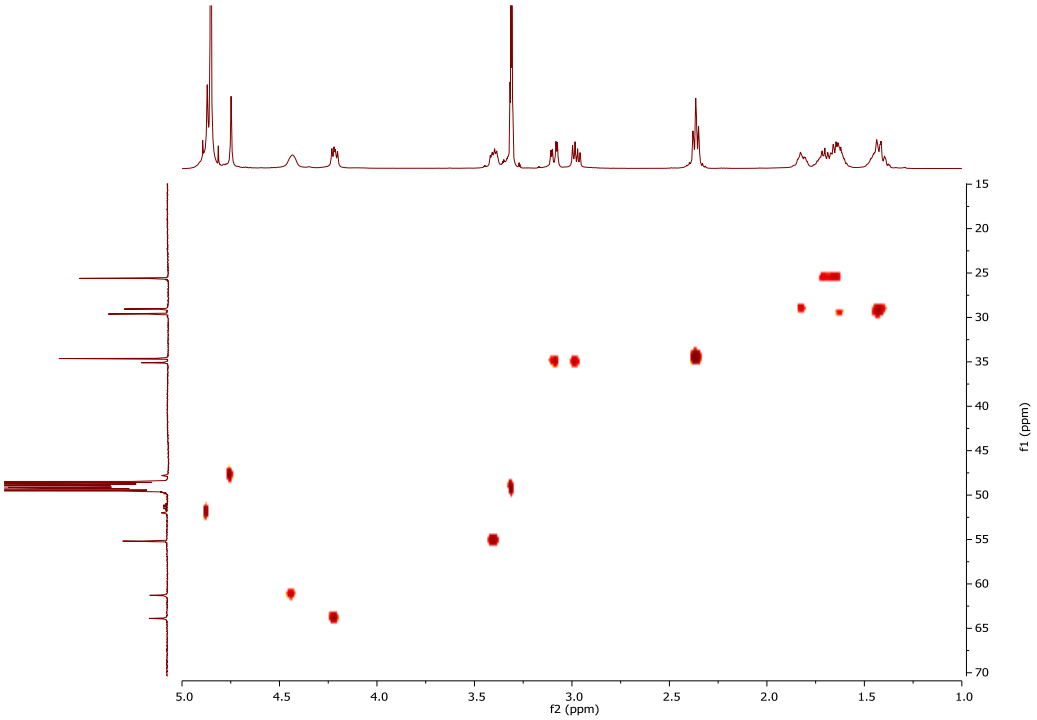


Figure S5: 2D-NMR (500MHz, MeOD-d_4) HSQC: Biotin[6]uril-hexamethylester-D_{18}.

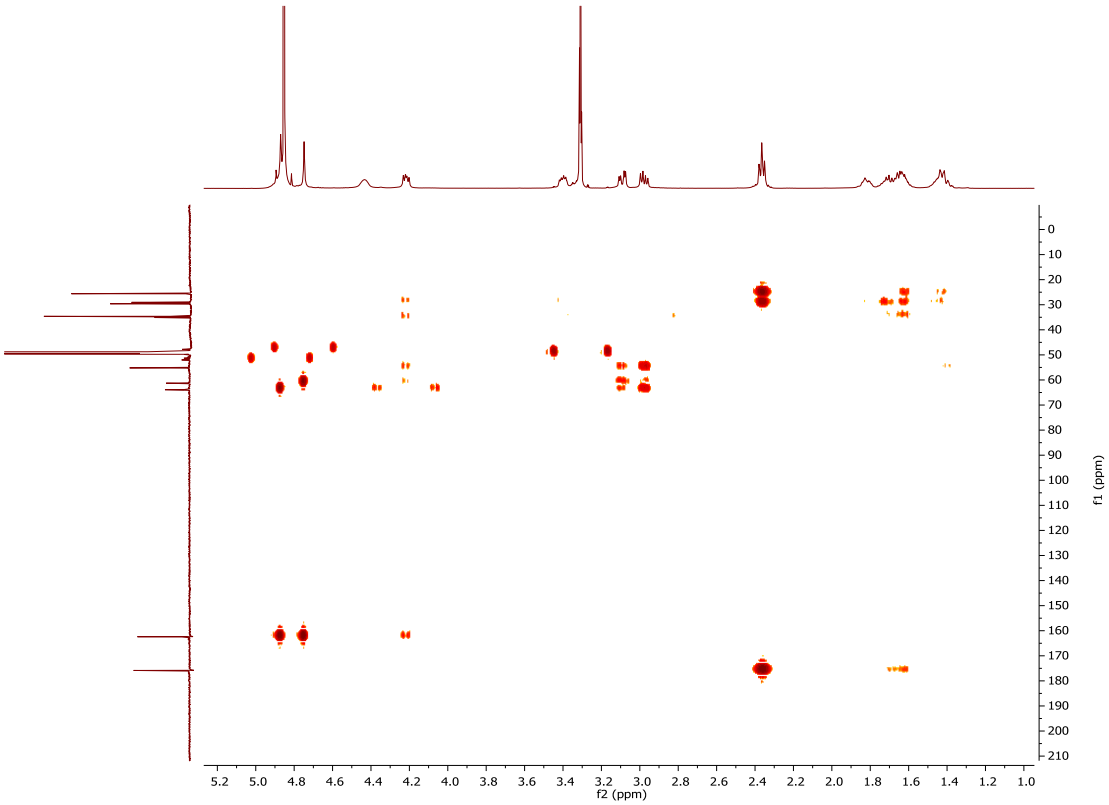


Figure S6: 2D-NMR (500MHz, MeOD-d_4) HMBC: Biotin[6]uril-hexamethylester-D_{18}.

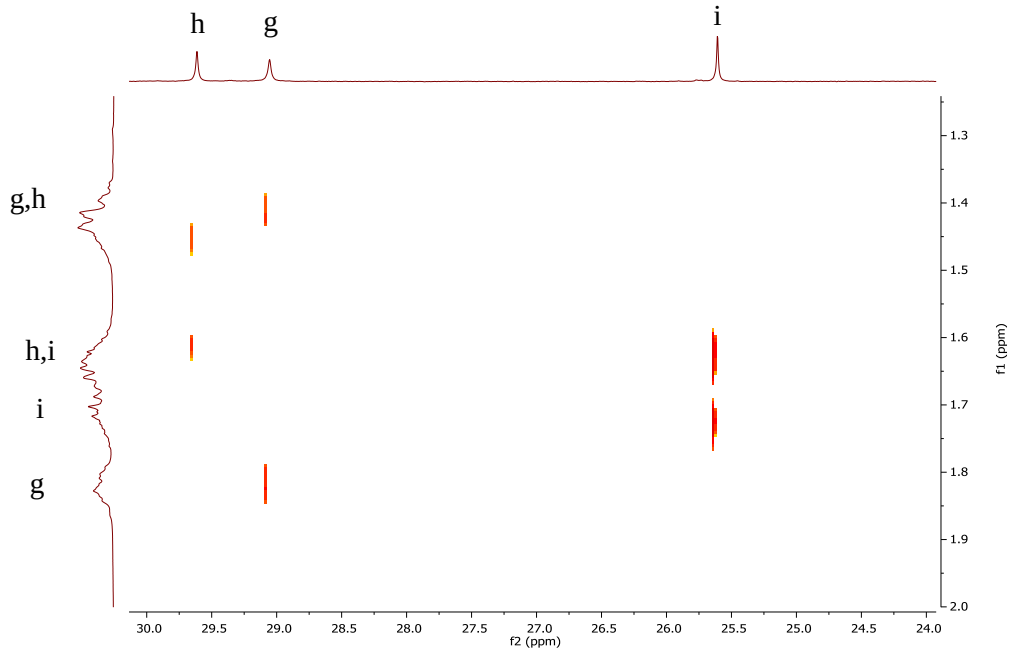


Figure S7: 2D-NMR (500 MHz, MeOD-d_4) HCCOSW: Biotin[6]uril-Hexamethylester-D_{18} showing the overlapping signals form the aliphatic side chain.

To fully characterize the aliphatic side chain of Biotin[6]uril an 1D-TOCSY was conducted from each side of the aliphatic side chain (the j and e proton).

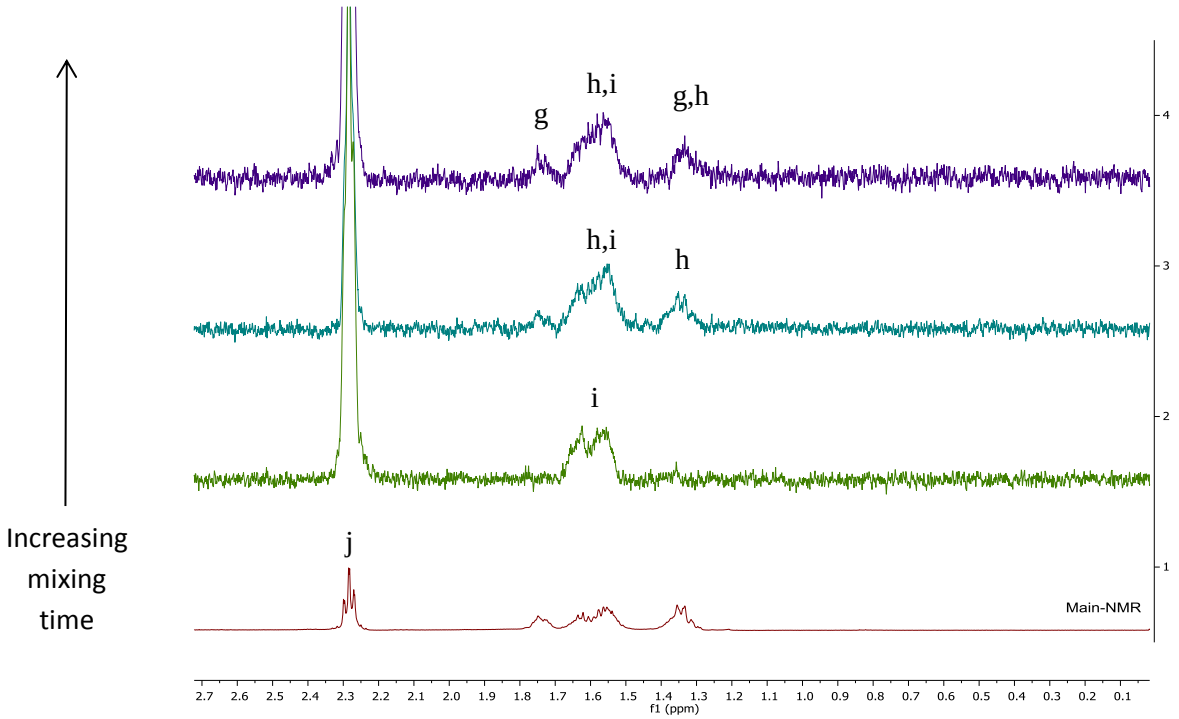


Figure S8: 1D-TOCSY (500 MHz, MeOD-d_4) for the 2.3 ppm signal (j): Biotin[6]uril-Hexamethylester-D_{18}. The closest neighbor to the j proton is the split i proton (spectrum 2), increasing the mixing time further shows the h and g proton (spectra 3 and 4).

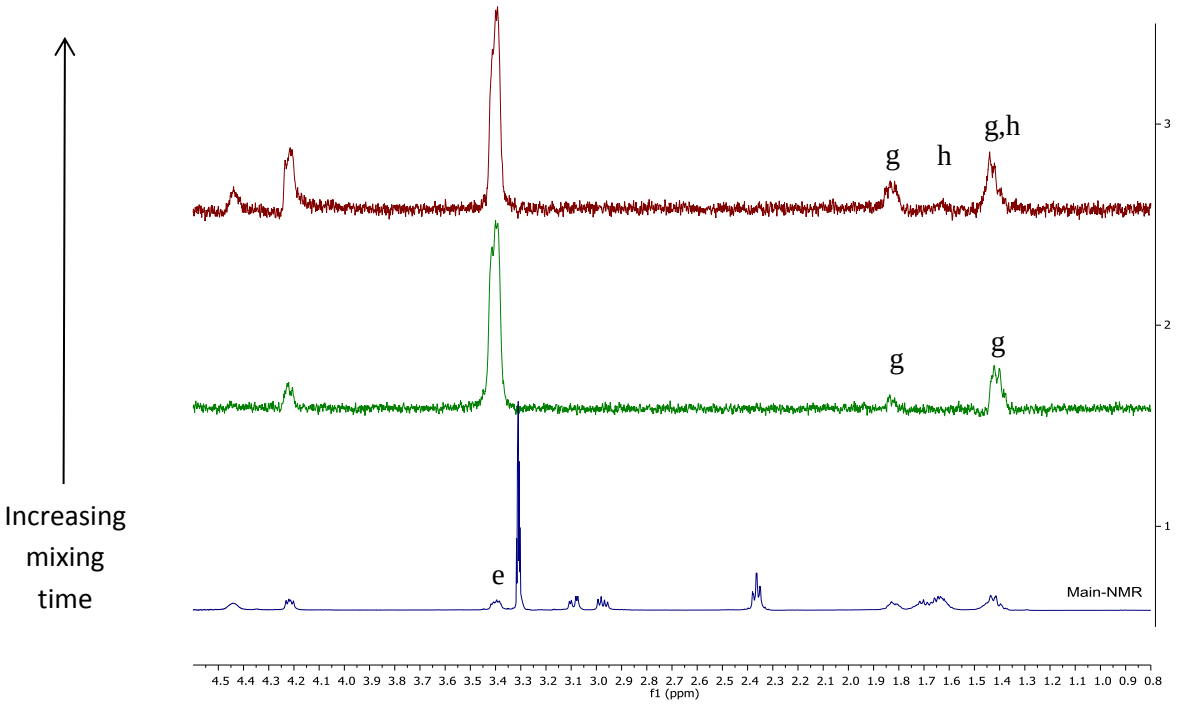


Figure S9: 1D-TOCSY (500 MHz, MeOD-d_4) for the 3.4 ppm signal (e): Biotin[6]uril-Hexamethylester-D_{18}. The closest neighbor to the e proton is the split g proton (spectrum 2), increasing the mixing time further shows the h proton (spectra 3).

To evaluate if there were aggregation or binding to the chloride dilution experiments of the Biotin[6]uril (**2**) at various concentration in water at and added NaCl was carried out (Figure S10).

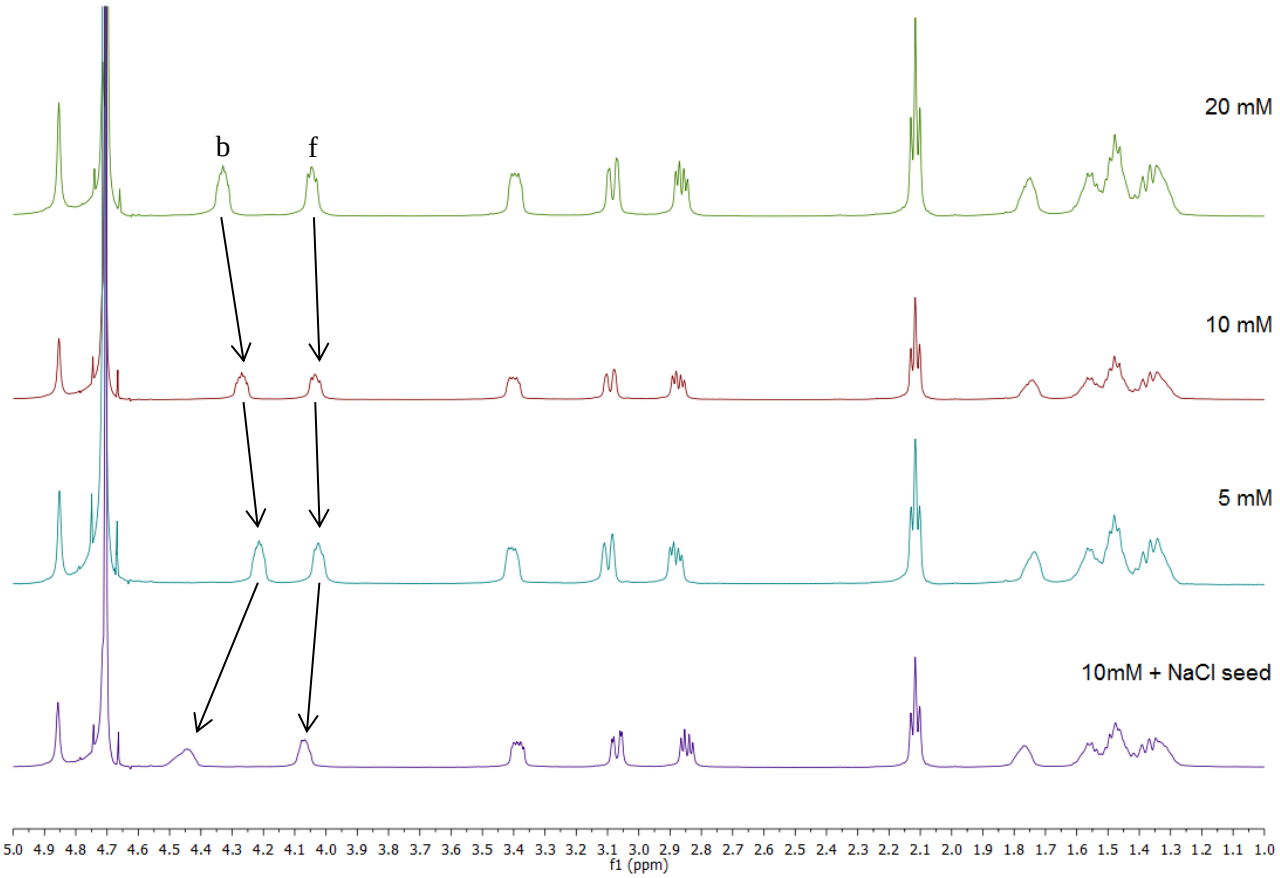


Figure S10: ^{1}H-NMR (500 MHz, D_2O) of Biotin[6]uril-HCl at different concentrations. The signals (b and f) move due to binding of chloride. The signals are set according the triplet at 2.15 ppm.

3 Possible regioisomers of Biotin[6]uril

The side chain on biotin makes the two urea nitrogen atoms non-equivalent and when the biotin units are converted to the Biotin[6]uril this gives rise to a number of isomers. There are nine regioisomers as shown in Figure S11.

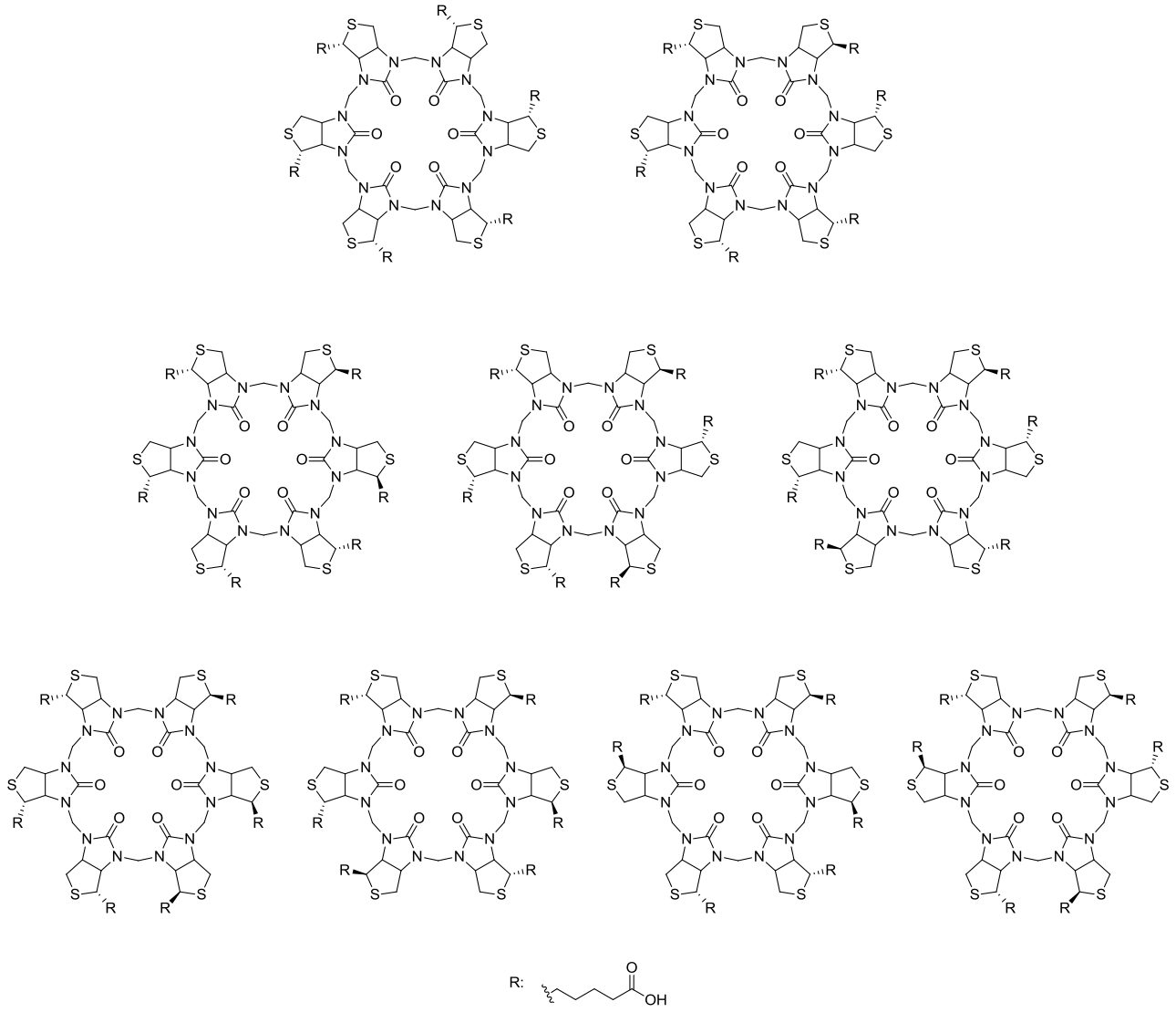


Figure S11: Structure of the nine possible regioisomers of Biotin[6]uril.

4 Chloride free Biotin[6]uril

Biotin[6]uril-HCl (116 mg ; 73.7µmol) was dissolved in 1 M NaOH (15 ml MilliQ), and $TlNO_3$ (29.7 mg ; 111.5 µmol) was added. The solution was stirred overnight at room temperature. The reaction mixture was filtered and acidified with conc. H_2SO_4. The product was filtered off and washed with water (3 × 10 ml).
Yield: 76 mg 67.1%.
M.p. > 140°C (decomp.). $[\alpha]_D^{20}$ = -85.6 (c = 1, 0.1 M NaOH).
^{1}H NMR (500 MHz, D_2O): δ = 4.37 (s, 6H), 4.23 (s, 6H), 3.72 – 3.62 (m, 6H), 3.57 – 3.48 (m, 6H), 2.98 – 2.89 (m, 6H), 2.67 – 2.59 (m, 6H), 2.41 (dd, *J* = 13.0, 5.7 Hz, 6H), 1.64 (t, *J* = 7.0 Hz, 12H), 1.24 (s, 6H), 1.15 – 0.76 (m, 30H).
^{13}C NMR (126 MHz, D_2O): δ = 182.75, 160.15, 60.96, 58.74, 53.07, 50.21, 45.80, 36.47, 32.62, 27.46, 26.47, 24.62.
Elemental Analysis for $C_{66}H_{96}N_{12}O_{18}S_6$: [calc+$H_2O$] (found+$H_2O$); C [50.95] (50.85), H [6.35] (6.22), N [10.80] (10.54).

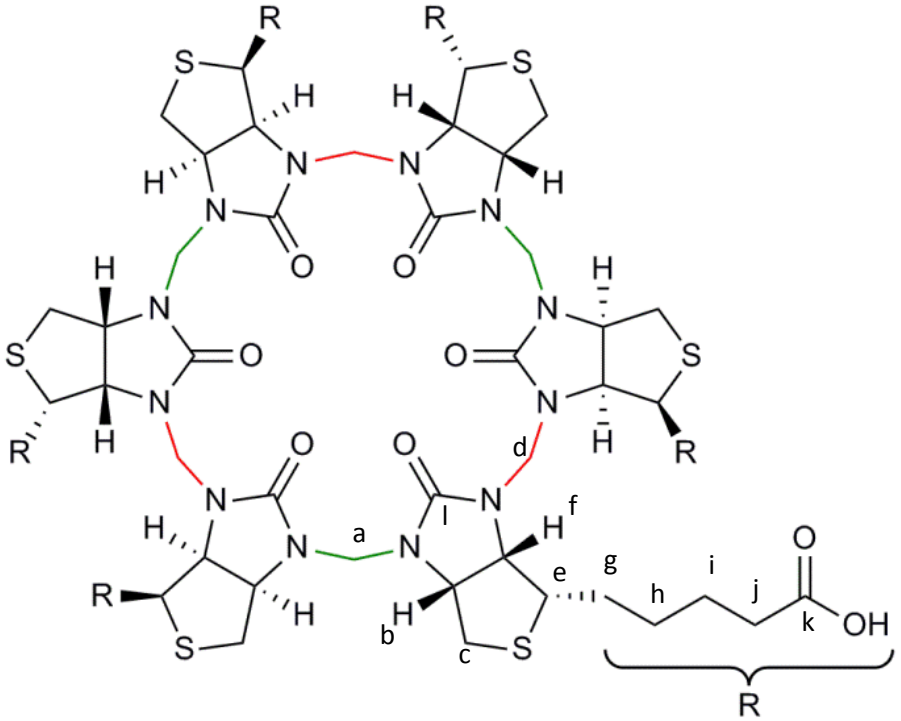


Figure S12: Schematic representation of Biotin[6]uril.

The chloride free Biotin[6]uril was analysed in water with an external standard of DMSO-d_6 (Figure S13-S18).

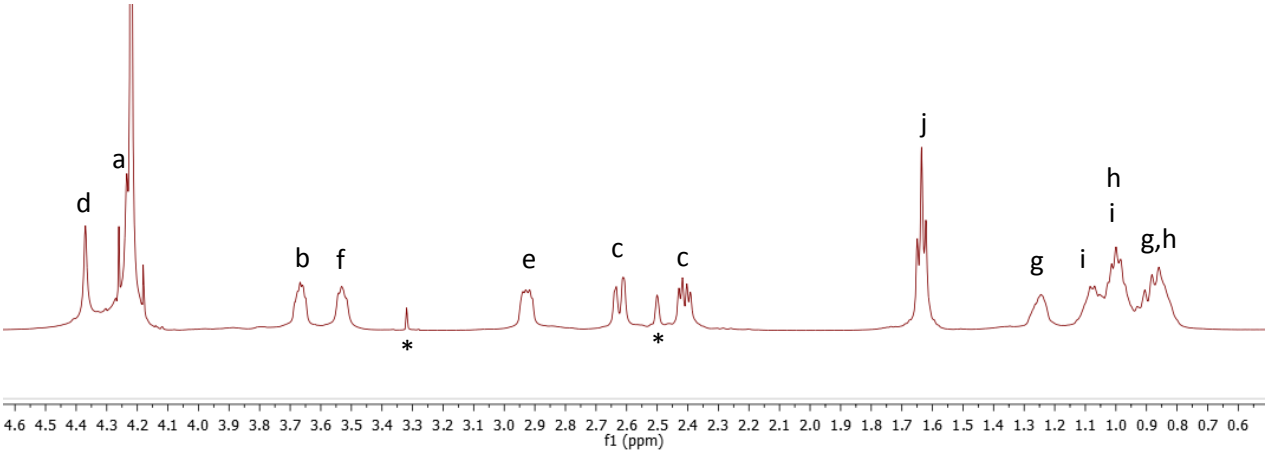


Figure S13: ^{1}H-NMR (500 MHz, D_2O): Biotin[6]uril. *External reference of DMSO-d_6.

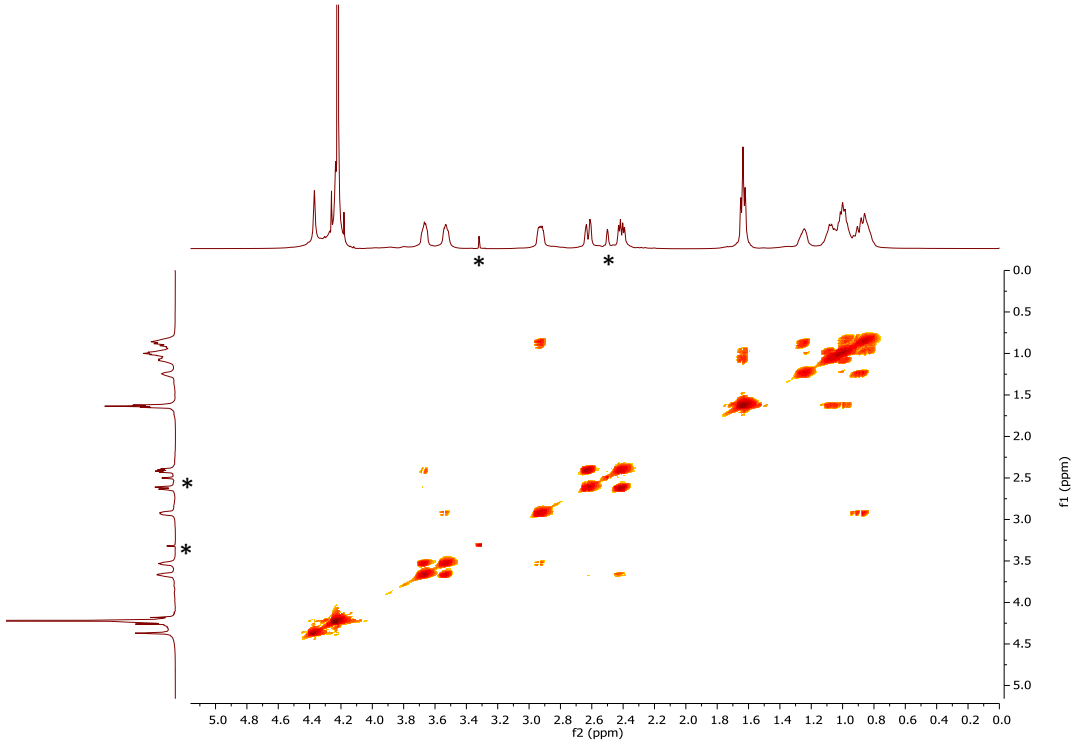


Figure S14: 2D-NMR (500 MHz, D_2O) COSY: Biotin[6]uril. *External reference of DMSO-d_6.

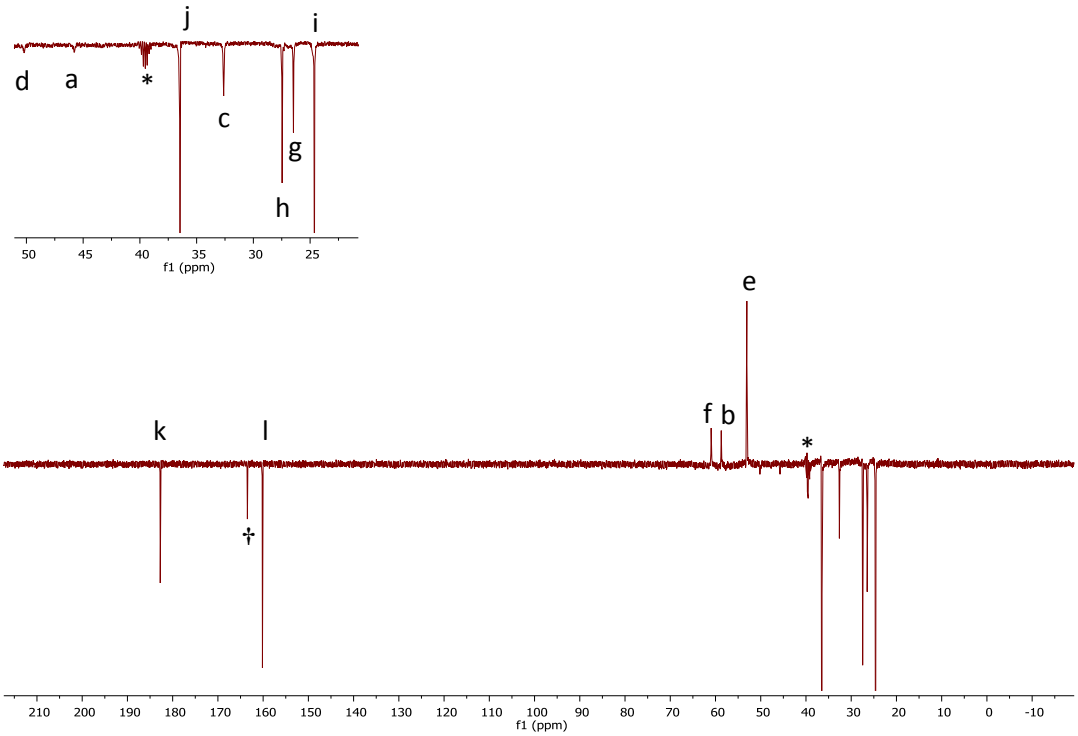


Figure S15: ^{13}C-NMR (126 MHz, D_2O) APT: Biotin[6]uril with a zoom of 22.5 ppm to 52.5 ppm. *External reference of DMSO-d_6 † Na_2CO_3.

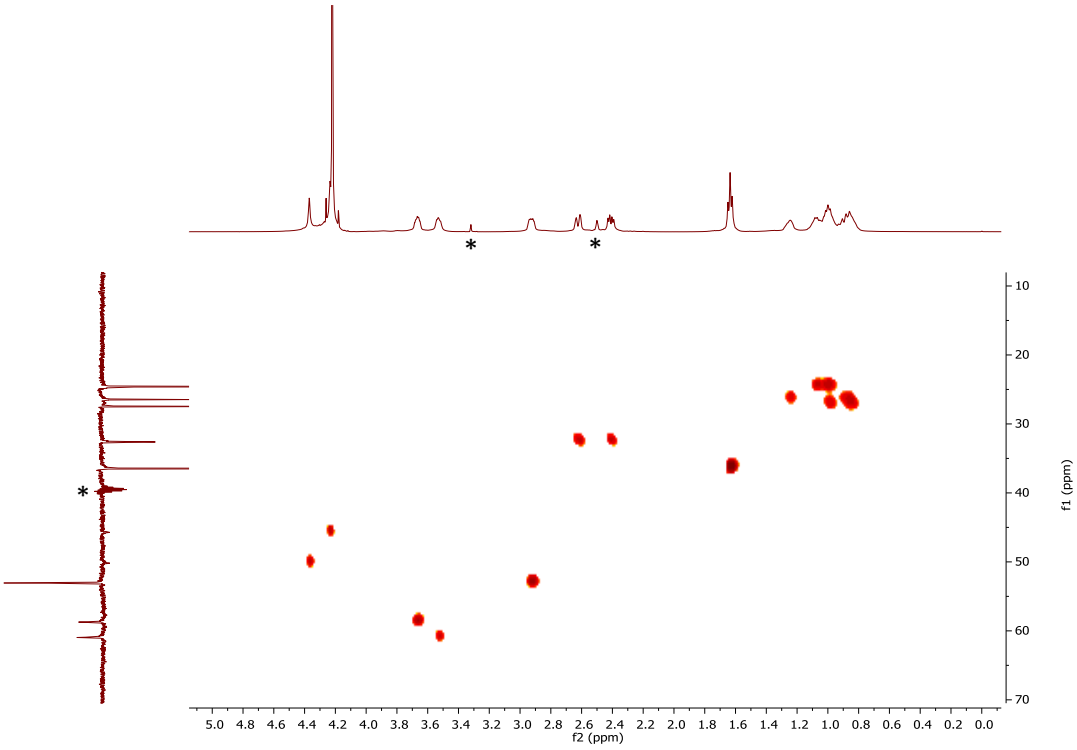


Figure S16: 2D-NMR (500 MHz, D_2O) HSQC: Biotin[6]uril. *External reference of DMSO-d_6.

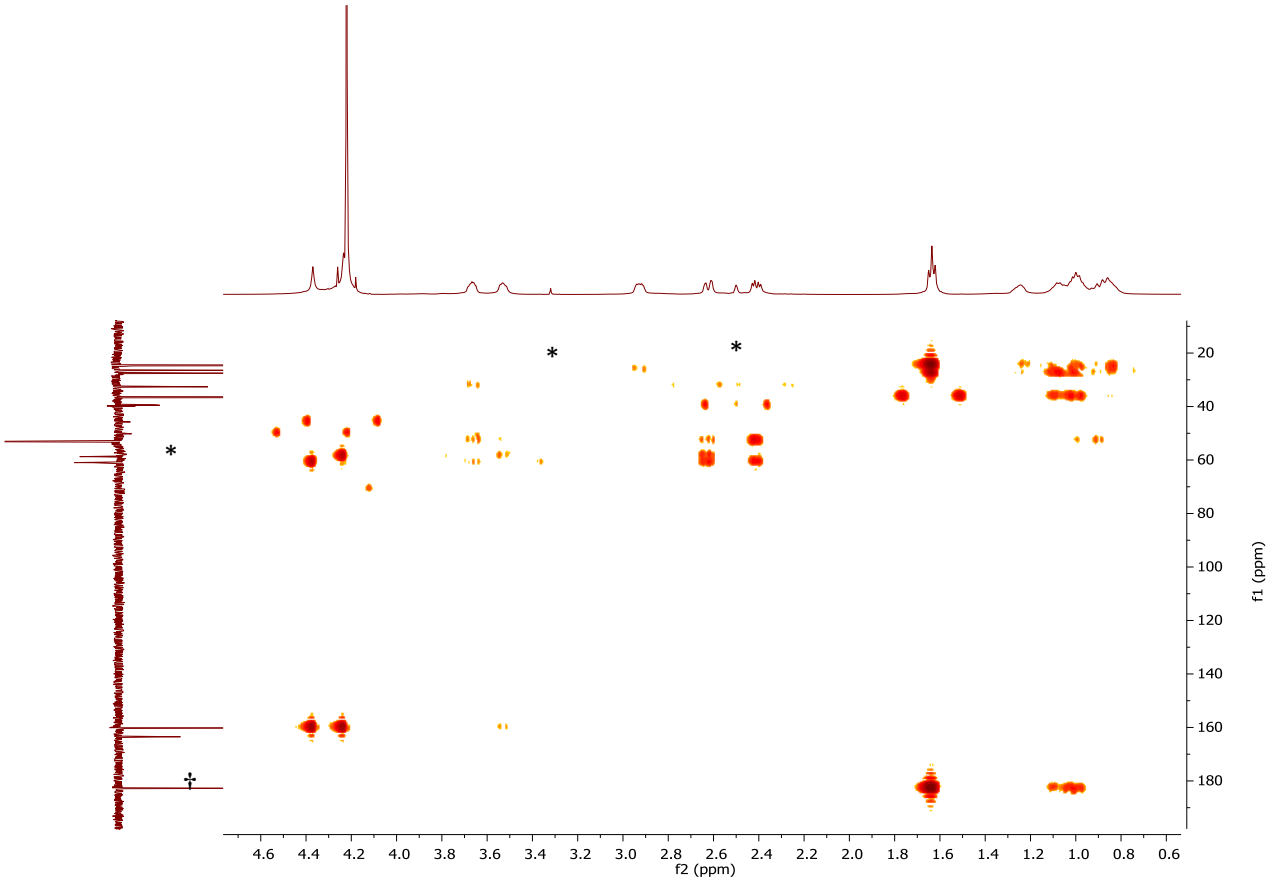


Figure S17: 2D-NMR (500MHz, D_2O) HMBC: Biotin[6]uril. *External reference of DMSO-d_6 †Na_2CO_3.

To evaluate the removal of the chloride and to investigate aggregation dilution experiments was carried out in water with different concentrations of Biotin[6]uril with an external standard of DMSO-d_6.

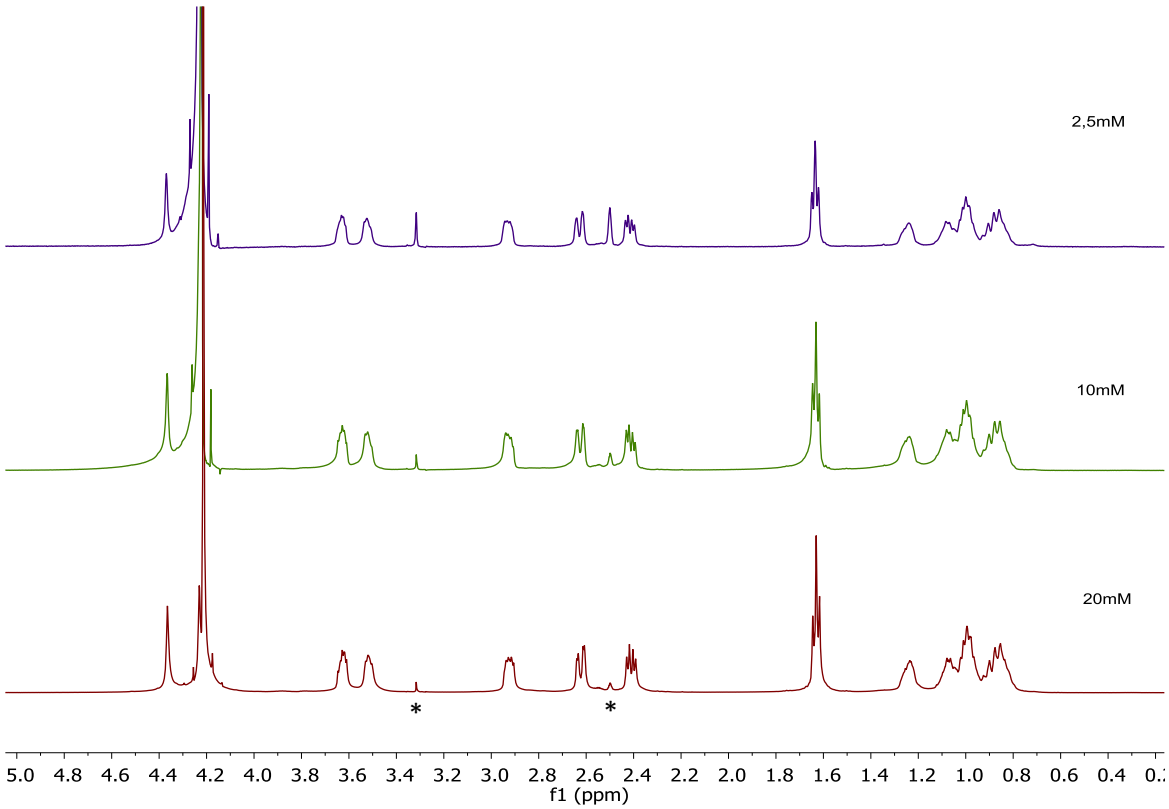


Figure S18: ^{1}H-NMR (500 MHz, D_2O) Biotin[6]uril at different concentrations. No signals move which confirms that the chloride is removed, and that there is no aggregation. *External reference of DMSO-d_6.

5 Screening of HCl and H_2SO_4 concentrations

All the screening experiments were setup with 100 mg of Biotin and 1. equivalents of para-formaldehyde in 5 ml of either HCl or H_2SO_4 at varied acid concentrations. The reactions were left overnight at 50 °C. The solids were separated by centrifugation. The reaction run in concentrated HCl and H_2SO_4 did not precipitate, and water was added, this made the reaction run in conc. HCl precipitate but not the reaction with conc. H_2SO_4. The solids were washed with water (3 × 5 ml), followed by dissolving in Na_2CO_3 and analysis by LC-MS (Figure S19 and Figure S20). The conc. H_2SO_4 was analysed directly by LC-MS.

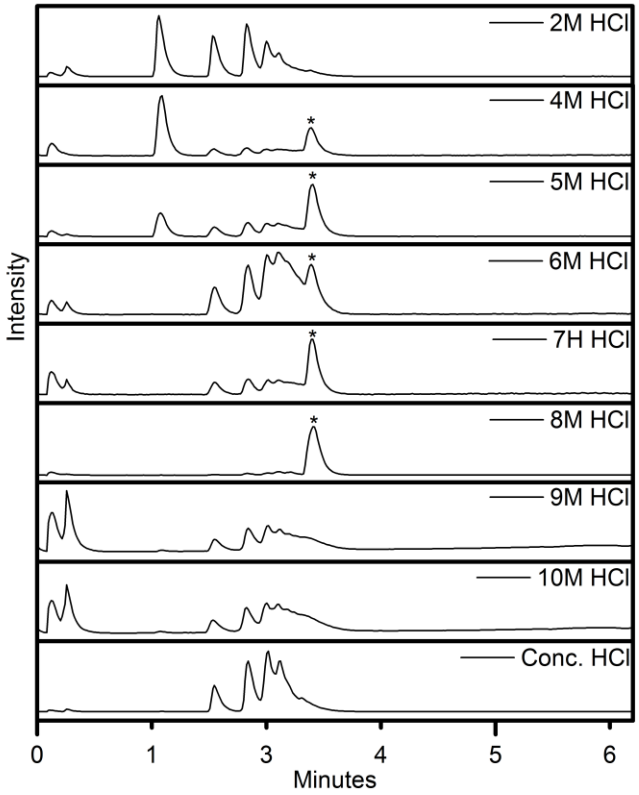


Figure S19: Total Ion Chromatogram of the solids from the reaction between biotin and para-formaldehyde at different concentration of HCl. The asterisk (*) indicates Biotin[6]uril all other peaks are linear or cyclic oligomers. LC-MS method B was used.

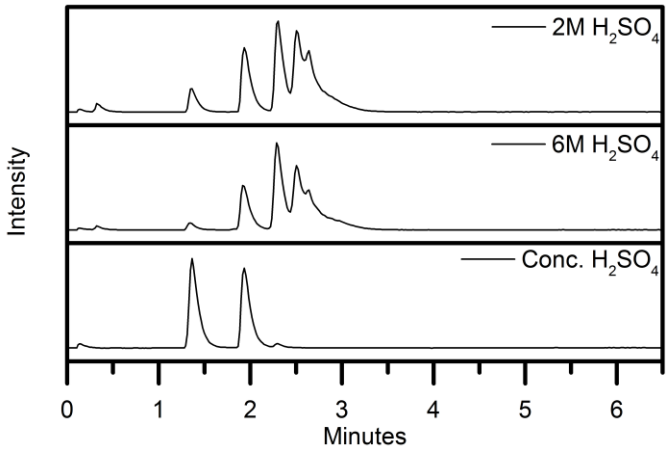


Figure S20: Total Ion Chromatogram of the solids from the reaction between biotin and para-formaldehyde at different concentration of H_2SO_4. All peaks are linear or cyclic oligomers, no Biotin[6]uril was detected for any of the samples. LC-MS method B was used.

6 Biotin[6]uril from H_2SO_4 and NaBr

Biotin (204 mg ; 0.84 mmol), para-formaldehyde (112 mg ; 3.7 mmol) and NaBr (5.19 g ; 50.4 mmol) was dissolved in 2.5 M H_2SO_4 (10 ml) and the heterogeneous solution was heated to 60 °C for 2 days. The mixture was cooled to room temperature and water (20 ml) was added. The solids were filtered and washed with water (3 × 5 ml). The solids were dissolved in 2 M NaOH, filtrated and precipitated with 5 M HBr followed by filtration. The filtrate was washed with water (3× 5 ml) and dried in vacuum.
Yield: 135 mg 63 %.
M.p. > 150°C (decomp.).
^{1}H NMR (500 MHz, D_2O): δ = 4.36 (s, 12H), 3.72 (m, 6H), 2.96 – 2.84 (m, 6H), 2.63 – 2.50 (m, 6H), 2.35 (dd, *J* = 13.0, 6.2 Hz, 6H), 1.64 (t, *J* = 7.0 Hz, 12H), 1.38 – 1.27 (m, 6H), 1.13 – 0.79 (m, 30H).
^{13}C NMR (126 MHz, D_2O): δ = 182.83, 160.57, 60.98, 59.04, 53.15, 49.86, 45.66, 36.51, 32.35, 27.64, 26.70, 24.72.
HRMS (ESI$^+$) of the acid: *m*/*z* $[M+H]^+$ calc. for $C_{66}H_{97}N_{12}O_{18}S_6$: 1537.5363; found: 1537.5320.
Elemental Analysis for $C_{66}H_{97}N_{12}O_{18}S_6Br$: [calc] (found); C [48.97] (49.29), H [6.04] (6.01), N [10.38] (10.36).

The Biotin[6]uril synthesised form H_2SO_4 and NaBr containg HBr was analysed in water with an external standard of DMSO-d_6 again Biotin[6]uril was isolated as a single isomer (Figure S21-S24).

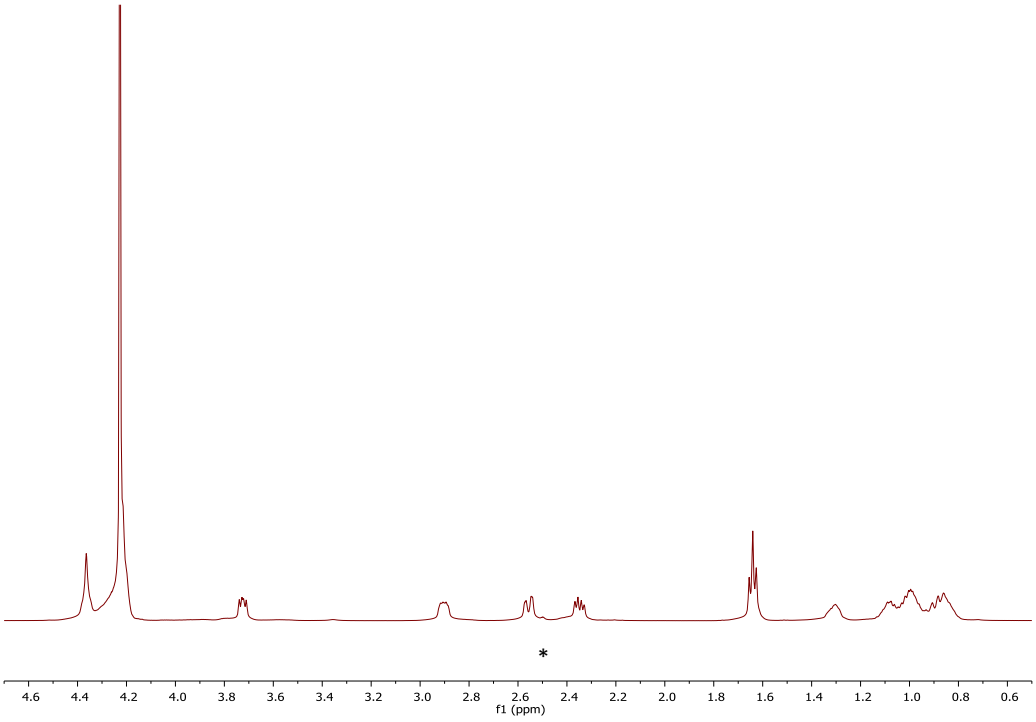


Figure S21: ^{1}H-NMR (500 MHz, D_2O): Biotin[6]uril-HBr. *External reference of DMSO-d_6. The b and f proton has moved due to bindning of HBr.

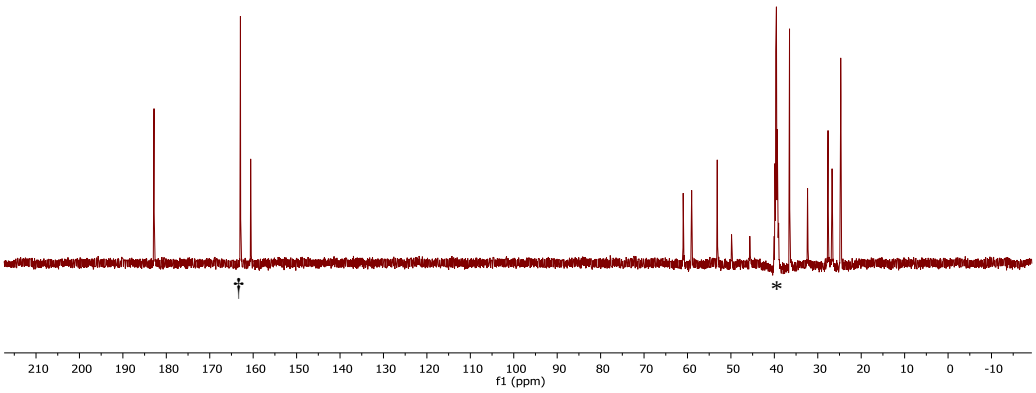


Figure S22: ^{13}C-NMR (126 MHz, D_2O): Biotin[6]uril-HBr with a zoom of 22 ppm to 51 ppm. *External reference of DMSO-d_6 †Na_2CO_3.

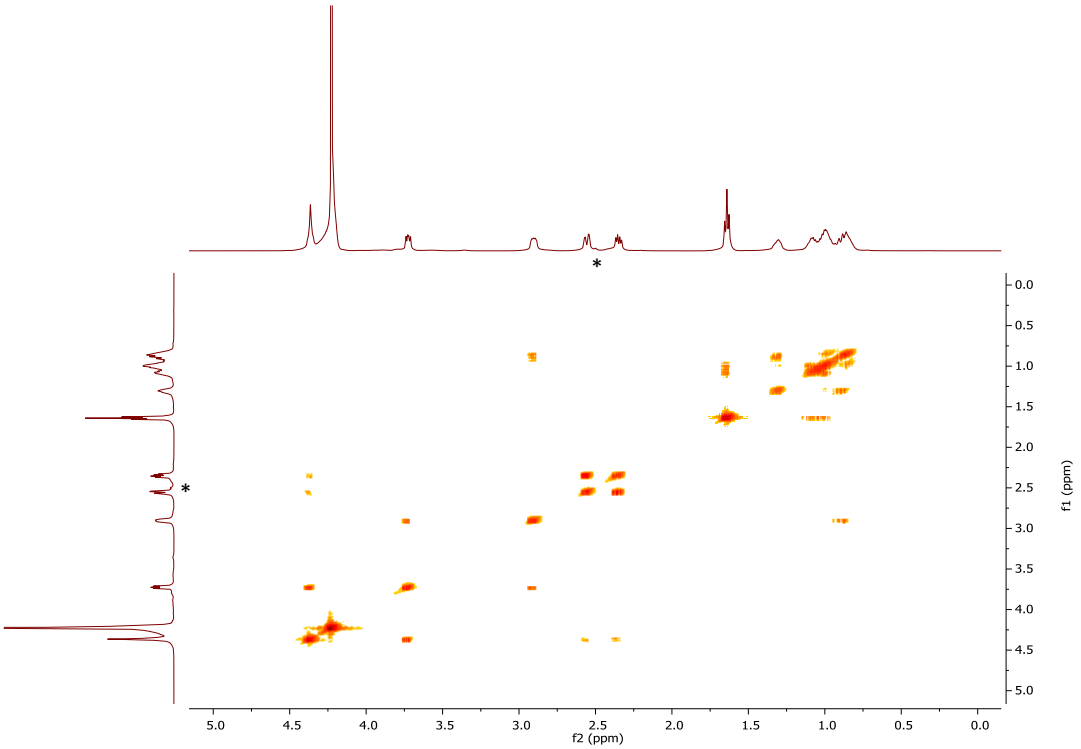


Figure S23: 2D-NMR (500MHz, D_2O) COSY: Biotin[6]uril-HBr. The b and f proton has moved due to bindning of HBr. *External reference of DMSO-d_6.

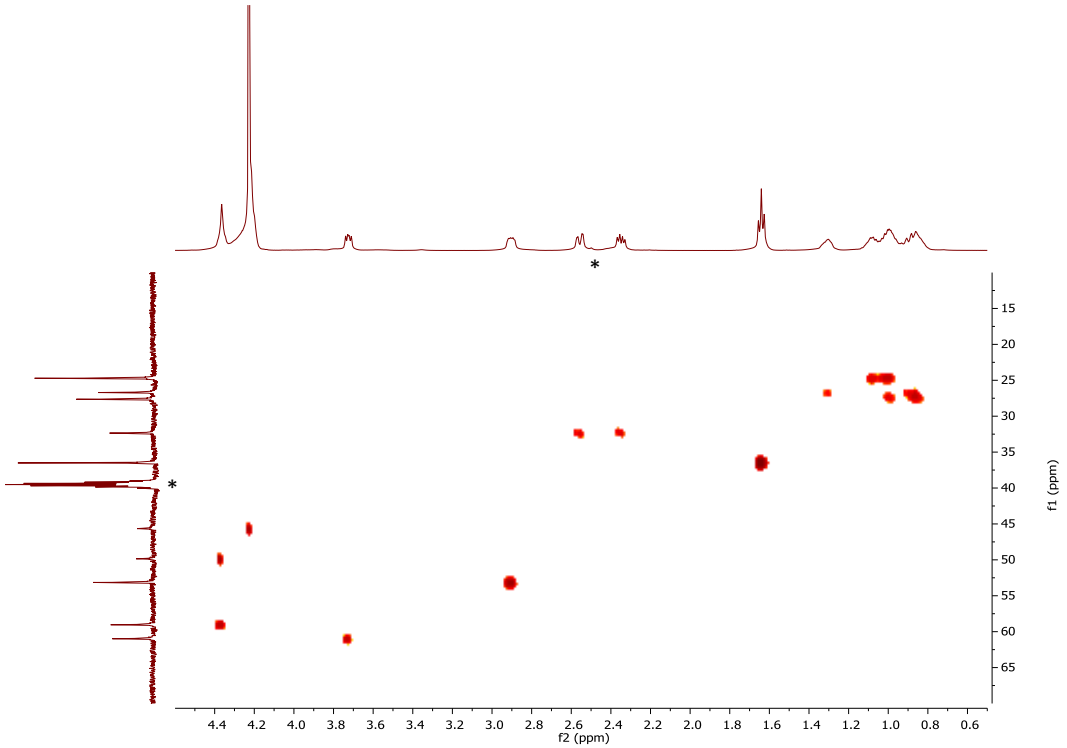


Figure S24: 2D-NMR (500MHz, D_2O) HSQC: Biotin[6]uril-HBr. The b and f proton has moved due to bindning of HBr. *External reference of DMSO-d_6.

7 Biotin[6]uril from HBr and HI

All the screening experiments were setup with 100 mg of Biotin and 5. equivalents of para-formaldehyde in 5 ml of either HBr (Figure S25) or HI (Figure S26) at varied concentration of acid. The reactions were left for two days at 60 °C. The solids were separated by centrifugation. In the reaction with 7 M HBr and 8 M HBr precipitation was observed after addition of water. In the HI experiments precipitation was only observed for the reaction with 3 M HI which was analysed by LC-MS. All other HI experiments were analysed directly from the reaction mixture. The solids were washed with water (3 × 10 ml), followed by dissolving in Na_3PO_4 and analysis by LC-MS using method B.

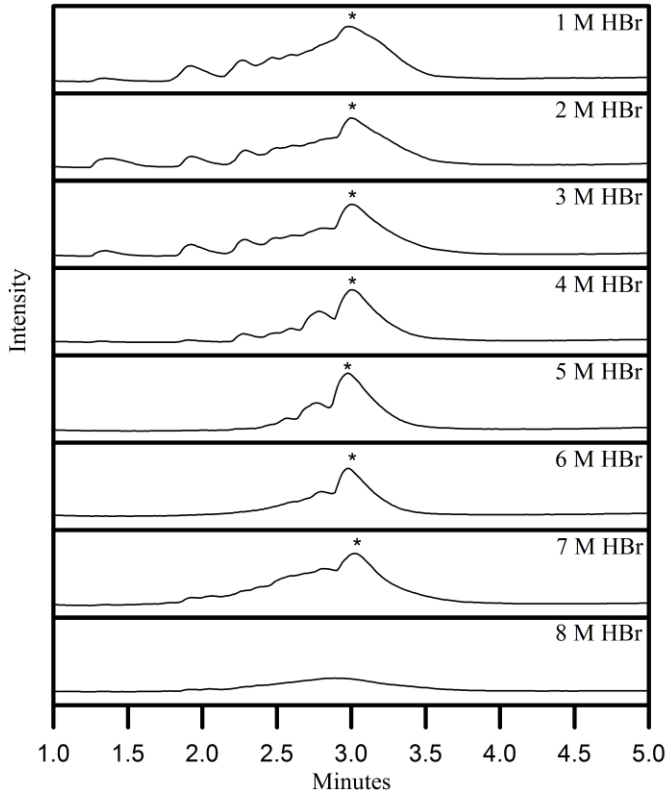


Figure S25: Total Ion Chromatogram of the solids from the reaction between biotin and para-formaldehyde at different concentration of HBr. The asterisk (*) indicates Biotin[6]uril all other peaks are linear or cyclic oligomers. LC-MS method B was used.

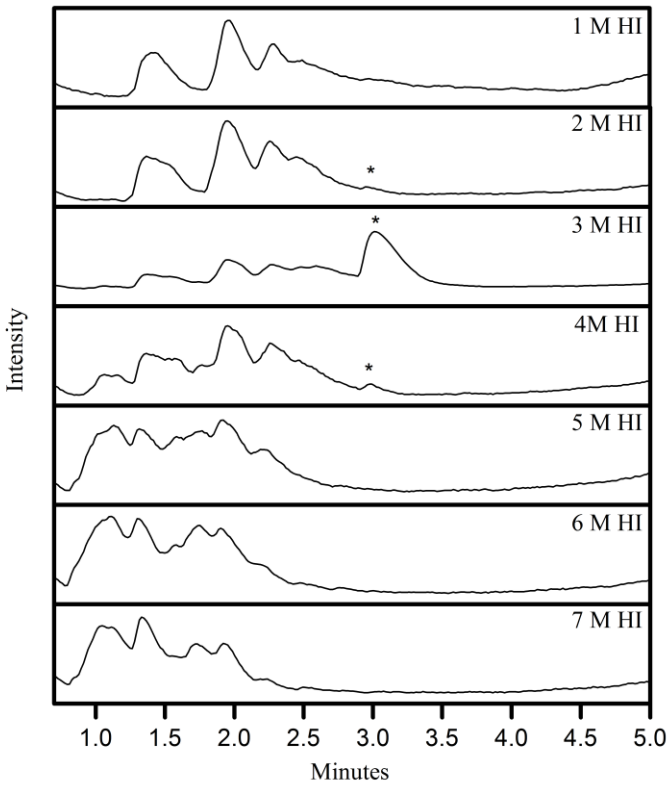


Figure S26: Total Ion Chromatogram of the solids from the reaction between biotin and para-formaldehyde at different concentration of HI. The asterisk (*) indicates Biotin[6]uril all other peaks are linear or cyclic oligomers. LC-MS method B was used.

8 General Job Plot Method

A 20 mM solution of NaX (Cl^-, Br^- or I^-) in 200 mM Na_2CO_3 (D_2O) was mixed with a solution of 20 mM Biotin[6]uril in 200 mM Na_2CO_3 (D_2O) in the ratios shown in Table 1 and each sample was analyzed by ^1H-NMR. For KX a 2 mM solution of KX (X = Cl^-, Br^- or I^-) in 20 mM Na_2CO_3 was mixed with 2 mM of Biotin[6]uril in 20 mM Na_2CO_3 in the same amounts as in Table 1.

Table 1: Sample composition of Job plot.

Sample no.	µl of Biotin[6]uril	µl of NaCl solution
1	50	450
2	100	400
3	150	350
4	200	300
5	225	275
6	250	250
7	275	225
8	300	200
9	350	150
10	400	100
11	450	50
12	500	0

Table 2: Titration data for proton b.

Sample no.	χ(Biotin[6]uril)	δ(Biotin[6]uril) (Hz)	$\Delta\delta$(Biotin[6]uril) (Hz)	χ(Biotin[6]uril) $\Delta\delta$
1	0.1	1805	46	4.6
2	0.2	1801	42	8.4
3	0.3	1797	38	11.4
4	0.4	1791	32	12.8
5	0.45	1791	32	14.4
6	0.5	1785	26	13
7	0.55	1781	22	12.1
8	0.6	1777	18	10.8
9	0.7	1772	13	9.1
10	0.8	1768	9	7.2
11	0.9	1563	4	3.6
12	1.0	1759	0	0

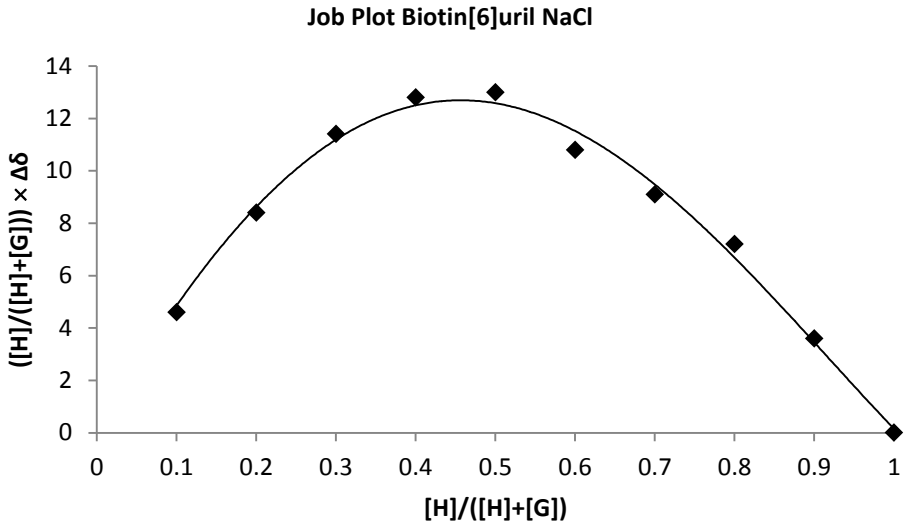


Figure S27: Job plot of NaCl and biotin[6]uril, it is the b proton which is followed.

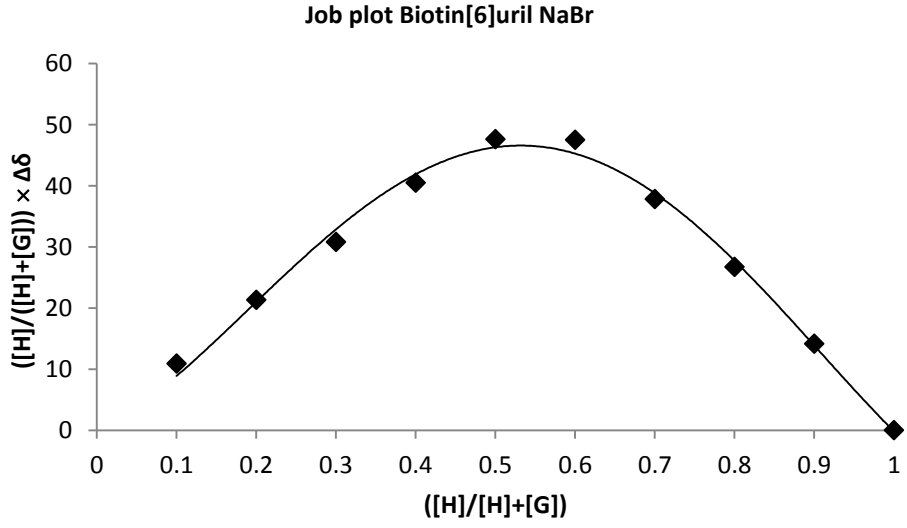


Figure S28: Job plot of NaBr and biotin[6]uril, where proton f s followed.

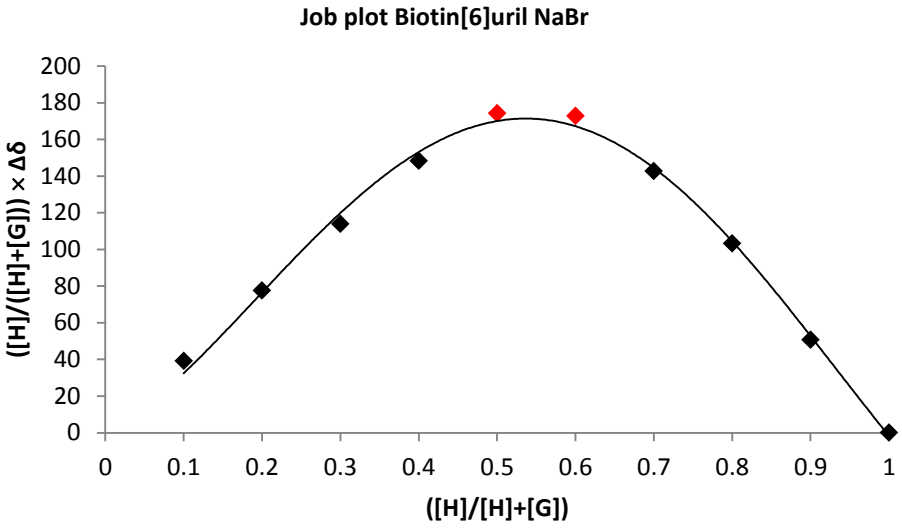


Figure S29: Job plot of NaBr and biotin[6]uril, where the proton b is followed. The red dots are estimated results, because the proton signal is under another signal.

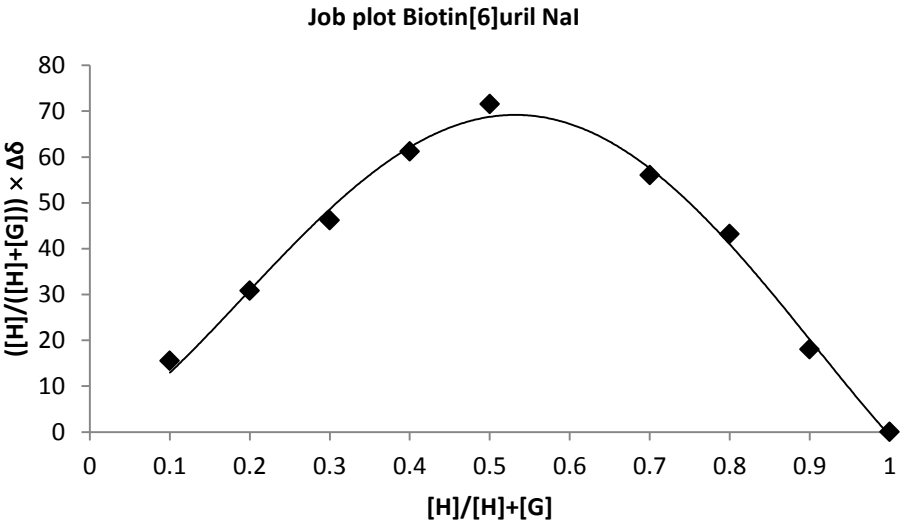


Figure S30: Job plot of NaI and biotin[6]uril, where the proton f is followed. The 0.6 is omitted as the signal is under the water signal.

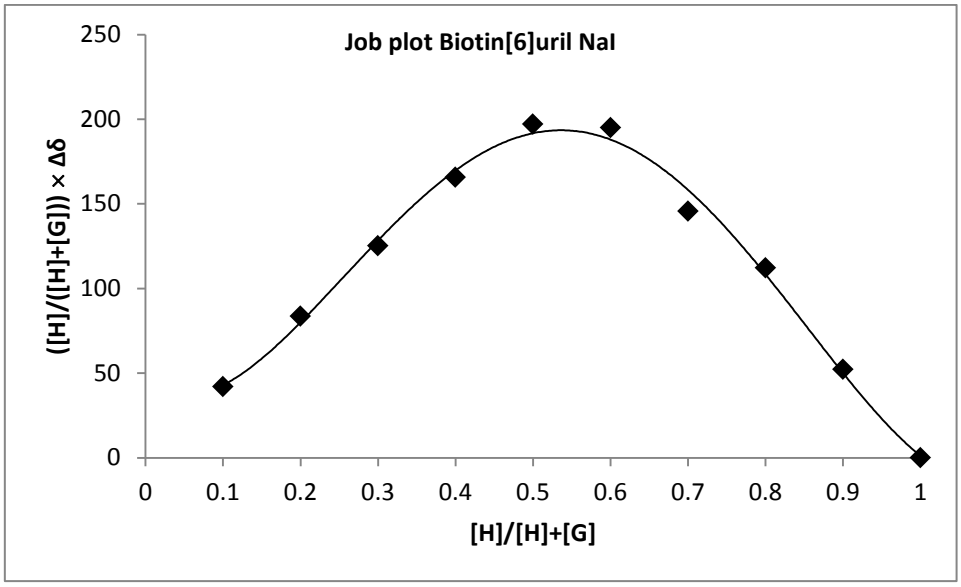


Figure S31: Job plot of NaI and Biotin[6]uril, were the proton b is followed.

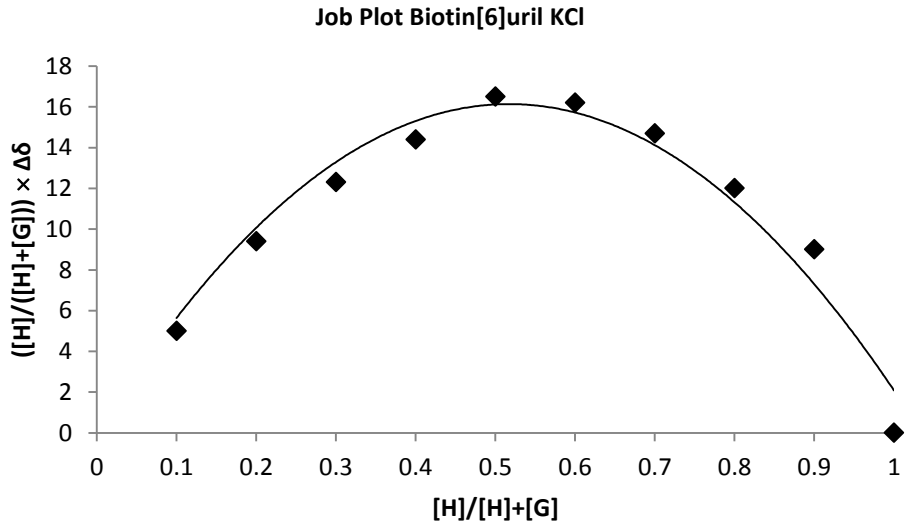


Figure S32: Job plot of KCl and Biotin[6]uril, were the proton f is followed.

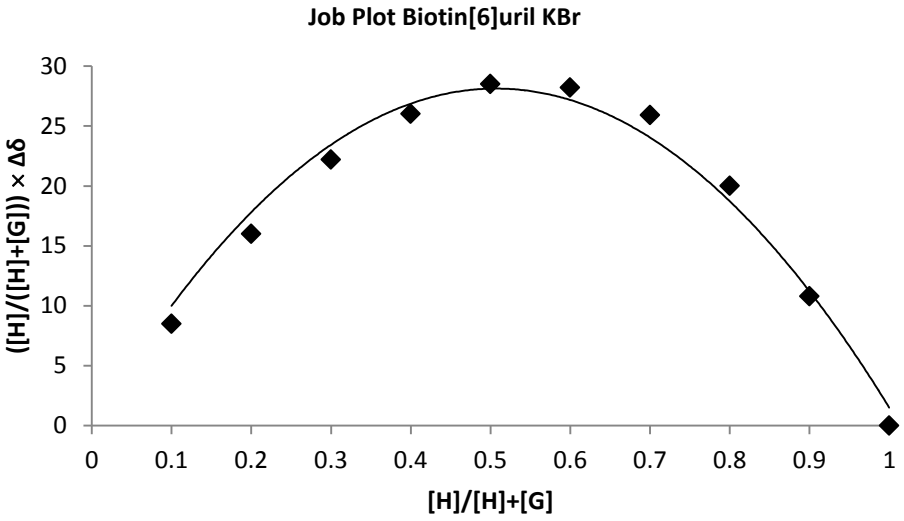


Figure S33: Job plot of KBr and Biotin[6]uril, were the proton f is followed.

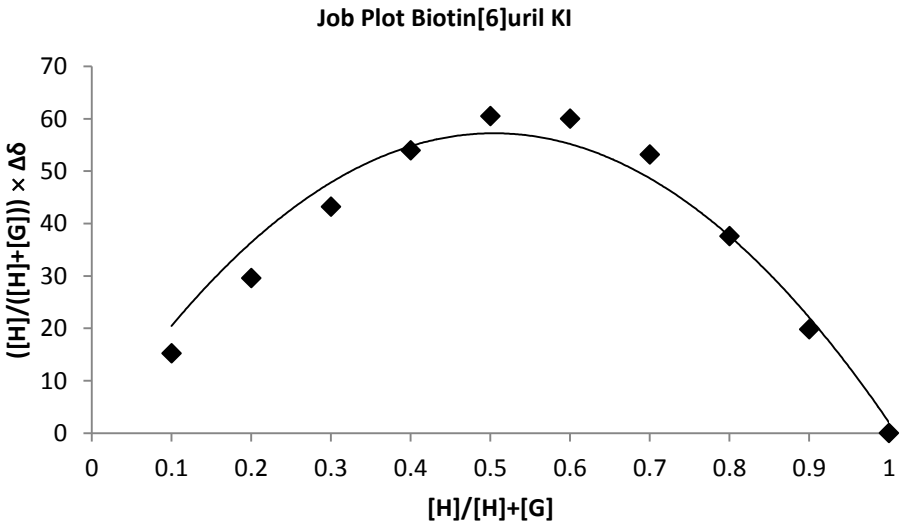


Figure S34: Job plot of KI and Biotin[6]uril, were the proton f is followed.

9 Binding Constant Determination from NMR Titrations

From the Job plots a 1:1 stoichiometry between host and guest was found. Hence, the equilibrium constant for the host-guest complexation is given by Eq. 1. In this expression, the denominator is expanded by substitution with $[H] = [H]_0 - [HG]$ and $[G] = [G]_0 - [HG]$ giving Eq. 2.

$$K = \frac{[HG]}{[H][G]} \quad \text{(Eq. 1)}$$

$$K = \frac{[HG]}{([H]_0 - [HG])([G]_0 - [HG])} = \frac{[HG]}{[H]_0[G]_0 - [HG]([H]_0 + [G]_0) + [HG]^2} \quad \text{(Eq. 2)}$$

Eq. 2 can be rearranged to the second order equation (Eq. 3) with $[HG]$ as the unknown, and the general solution is given in Eq. 4.

$$0 = [HG]^2 - [HG]\left([G]_0 + [H]_0 + \frac{1}{K}\right) + [H]_0[G]_0 \quad \text{(Eq. 3)}$$

$$[HG] = \frac{1}{2}\left(\left([G]_0 + [H]_0 + \frac{1}{K}\right) \pm \sqrt{\left([G]_0 + [H]_0 + \frac{1}{K}\right)^2 - 4[G]_0[H]_0}\right) \quad \text{(Eq. 4)}$$

Only the solution where the last term is subtracted is chemically meaningful because the solution with a plus sign results in a concentration of complex that is higher than the smallest of the numbers $[G]_0$ and $[H]_0$.

Eq. 4 gives an expression where the unknowns are $[HG]$ and K. The purpose is to find K and ^{1}H-NMR was used to provide a measure of $[HG]$. Various amounts of ionic guests were titrated into a solution of the biotin[6]uril under conditions where the total concentration of host was constant and the movement of a host signal (denoted δ) was followed.

Under the used conditions the complexation was fast on the chemical shift time scale, and therefore the observed signal δ is as a weighted average of the signals δ_H (chemical shift of the proton in pure host) and δ_{HG} (chemical shift of the proton in pure complex) with the mole fractions x_H and x_{HG} as the weighting factors. This is expressed in Eq. 5 which via standard manipulations can be written as Eq. 6

$$\delta = \delta_H x_H + \delta_{HG} x_{HG} \quad \text{(Eq. 5)}$$

$$= \delta_H \frac{[H]_o - [HG]}{[H]_o} + \delta_{HG} \frac{[HG]}{[H]_o}$$

$$= \delta_H + (\delta_{HG} - \delta_H) \frac{[HG]}{[H]_o} \quad \text{(Eq. 6)}$$

For each measurement in the titration, the change from δ_H to the observed δ was calculated and denoted $\Delta\delta = \delta - \delta_H$. The unknown quantity, $\delta_{HG} - \delta_H$, indicates the maximal obtainable change in the titration and is denoted $\delta_{\Delta HG}$. With these notations, Eq. 6 can be rewritten to Eq. 7 and by substitution of Eq. 4 into Eq. 7, the final fitting equation Eq. 8 is obtained.

$$\Delta\delta = \delta_{\Delta HG} \frac{[HG]}{[H]_o} \quad \text{(Eq. 7)}$$

$$= \frac{\delta_{\Delta HG}}{2[H]_0}\left(\left([G]_0 + [H]_0 + \frac{1}{K}\right) - \sqrt{\left([G]_0 + [H]_0 + \frac{1}{K}\right)^2 - 4[G]_0[H]_0}\right) \quad \text{(Eq. 8)}$$

In Eq. 8 the quantities $\delta_{\Delta HG}$ and K are unknown but linked to the measurable quantity $\Delta\delta$ and the known $[H]_0$ and $[G]_0$. In Origin 8.6, $\delta_{\Delta HG}$ and K were determined by fitting Eq. 8 to the titration data.

10 NMR Titrations

The ^{1}H-NMR (500 MHz, D_2O) titrations were carried out by the following method.
Two solutions were used for the ^{1}H-NMR titrations one containing 5.01 mM of Biotin[6]uril in 200 mM Na_2CO_3 (pH = 10.8) used for NaCl, and one with 0.505 mM Biotin[6]uril in 20 mM Na_2CO_3 (pH = 10.8) used for NaBr, NaI and KX. The sodium or potassium halides were added while keeping the Biotin[6]uril concentration constant.

10.1 NaCl Titration

Table 3: NMR titration of Biotin[6]uril and NaCl.

Sample no.	NaCl (mM)	δ(Biotin[6]uril) (Hz)	$\Delta\delta$(Biotin[6]uril) (Hz)
1	0	2912	0
2	3.50	2924	13
3	6.79	2926	14
4	9.91	2933	21
5	12.86	2939	27
6	15.65	2943	31
7	18.31	2946	34
8	20.83	2948	37
9	23.23	2951	39
10	25.51	2953	41
11	27.69	2954	43
12	29.77	2956	44
13	31.76	2957	46
14	37.81	2960	48
15	43.08	2963	51
16	46.83	2964	52
17	51.03	2964	53
18	54.78	2965	53
19	58.12	2966	55
20	61.22	2967	55
21	120	2971	59

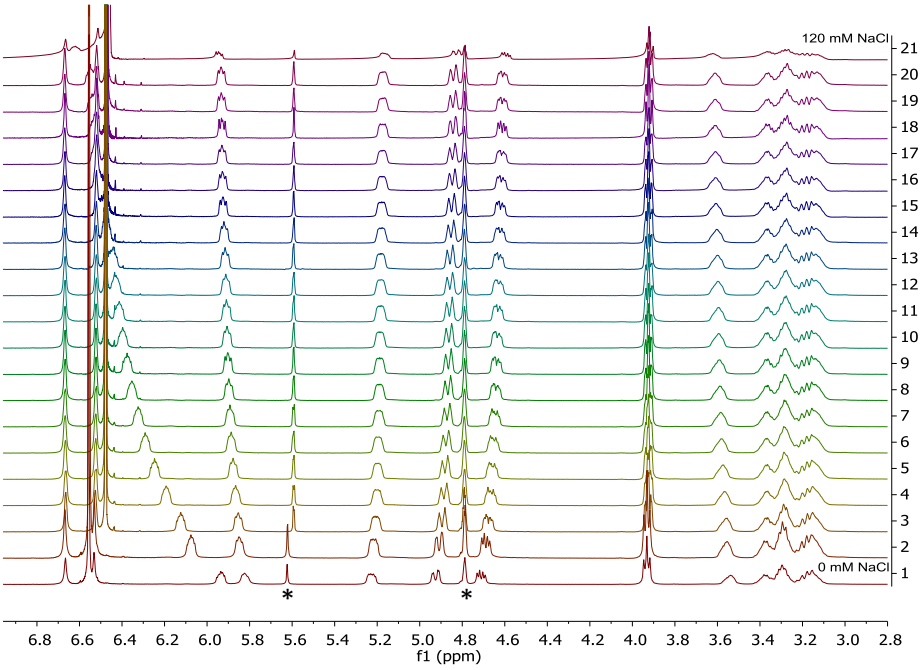


Figure S35: ^{1}H-NMR (500 Mhz, D_2O) titration series for Biotin[6]uril and NaCl. *External reference of DMSO.

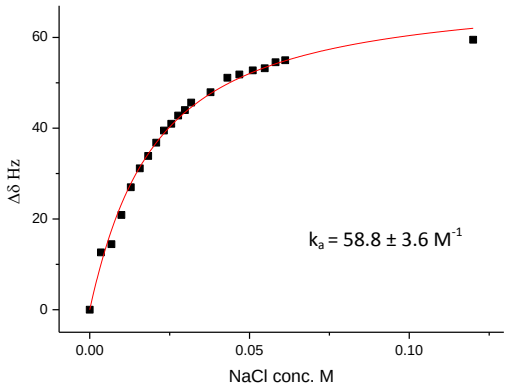


Figure S36: Plot showing experimental data for ^{1}H-NMR titration of NaCl following proton f together with the fitted curve (red).

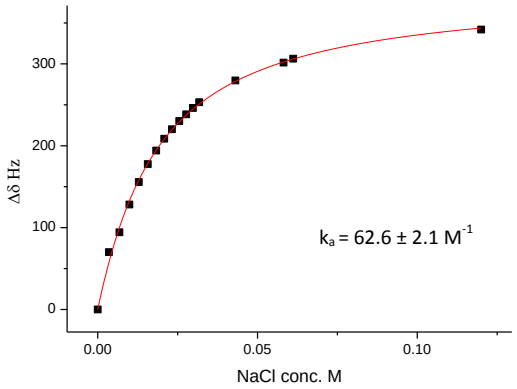


Figure S37: Plot showing experimental data for ^{1}H-NMR titration of NaCl following proton b together with the fitted curve (red).

10.2 NaBr Titration

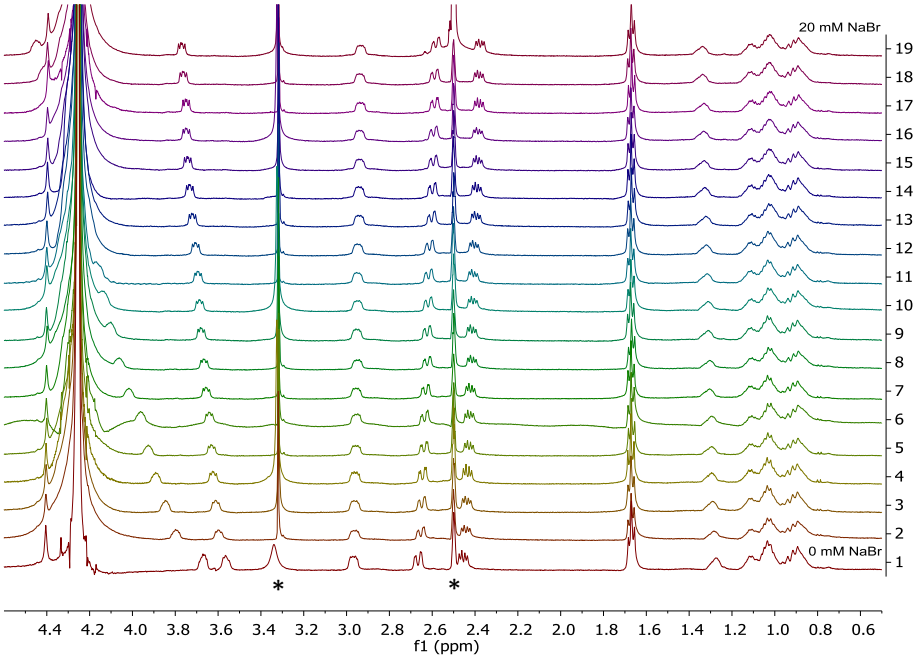


Figure S38: ^{1}H-NMR (500 Mhz, D_2O) titration series for Biotin[6]uril and NaBr. *External reference of DMSO.

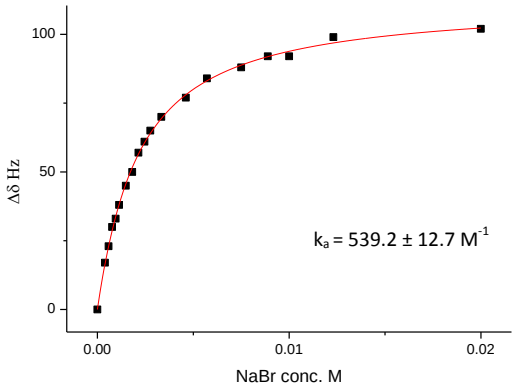


Figure S39: Plot showing experimental data for ^{1}H-NMR titration of NaBr following proton f together with the fitted curve (red).

10.3 NaI Titration

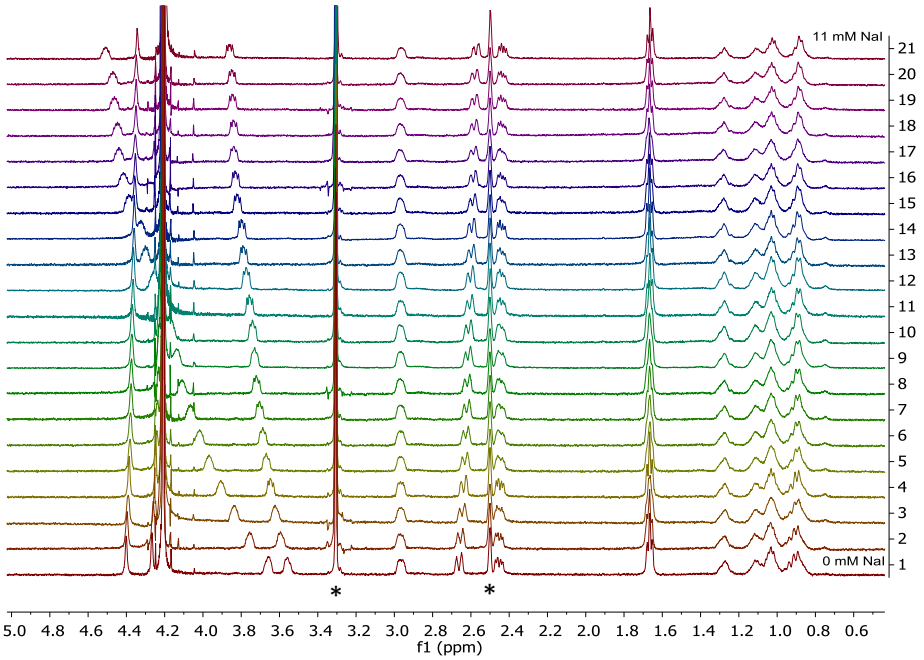


Figure S40: ^{1}H-NMR (500 Mhz, D_2O) titration series for Biotin[6]uril and NaI. *External reference of DMSO.

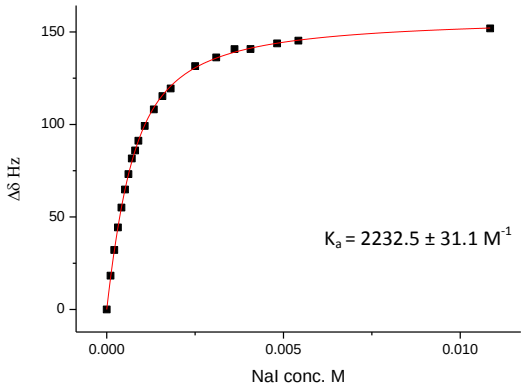


Figure S41: Plot showing experimental data for ^{1}H-NMR titration of NaI following proton f together with the fitted curve (red).

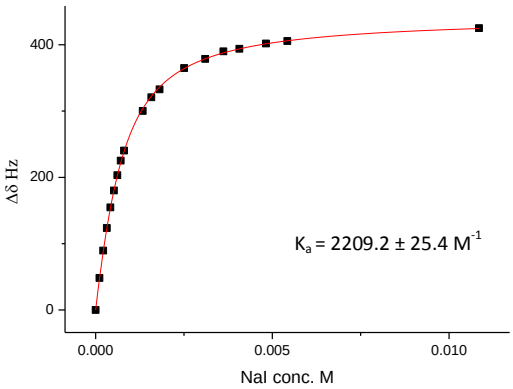


Figure S42: Plot showing experimental data for ^{1}H-NMR titration of NaI following proton b together with the fitted curve (red).

10.4 KCl Titration

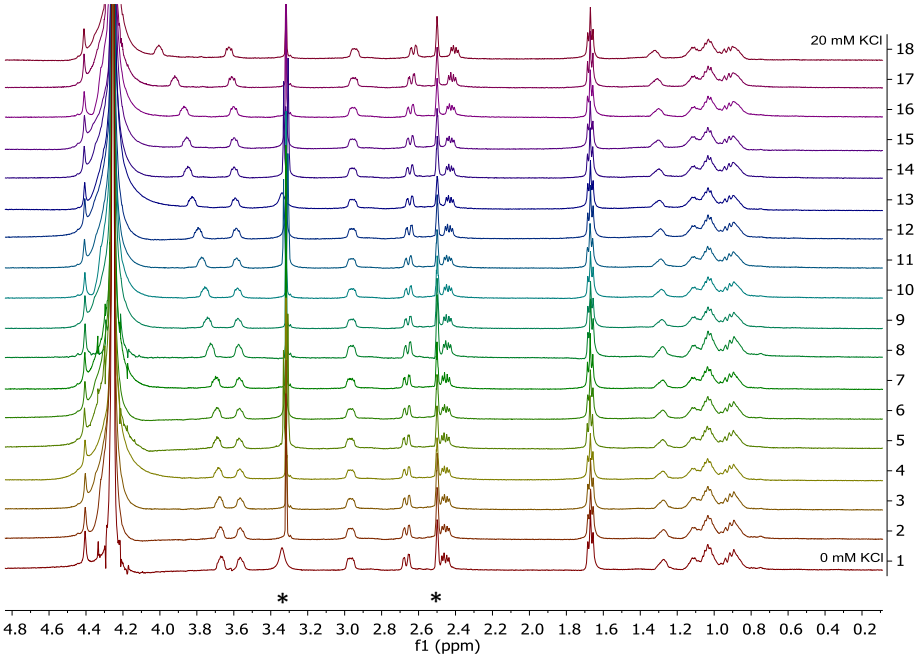


Figure S43: ^{1}H-NMR (500 Mhz, D_2O) titration series for Biotin[6]uril and KCl. *External reference of DMSO.

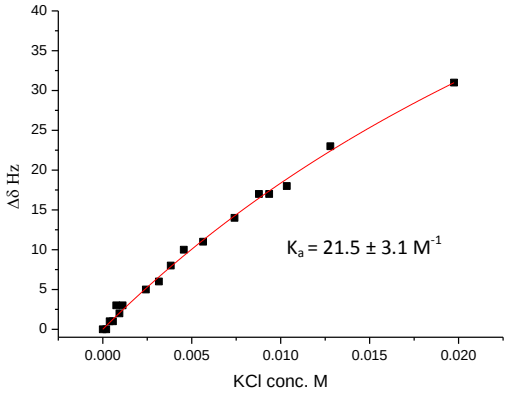


Figure S44: Plot showing experimental data for ^{1}H-NMR titration of KCl following proton f together with the fitted curve (red).

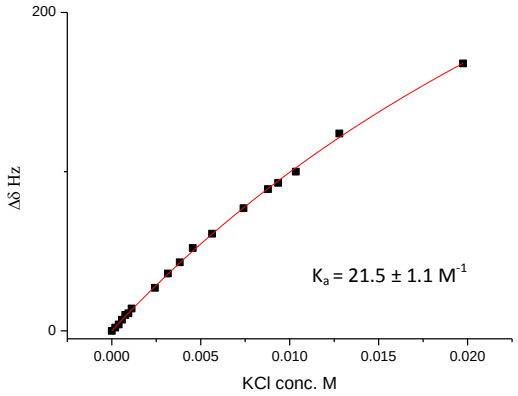


Figure S45: Plot showing experimental data for ^{1}H-NMR titration of KCl following proton b together with the fitted curve (red).

10.5 KBr Titration

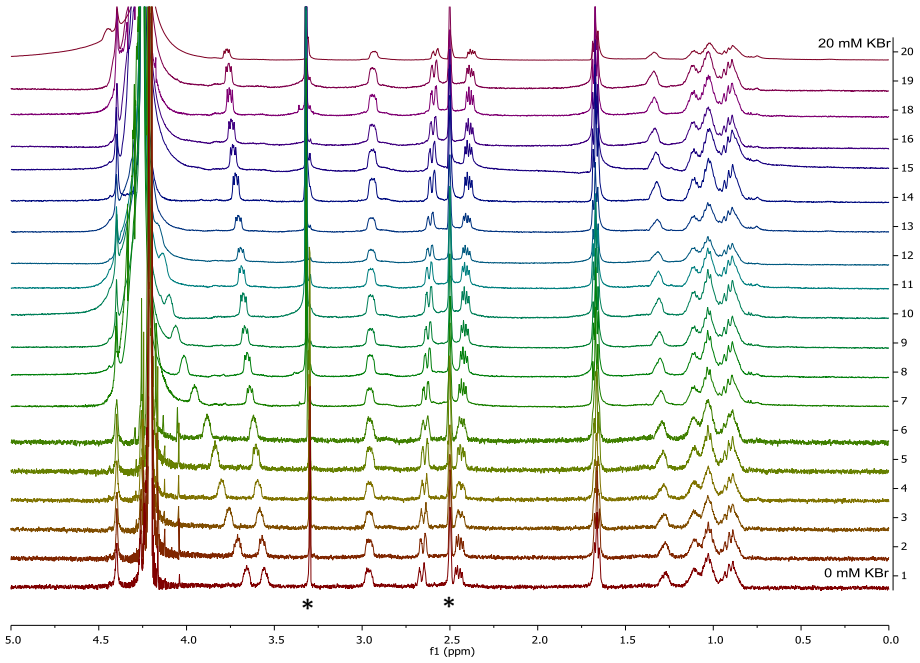


Figure S46: ^{1}H-NMR (500 Mhz, D_2O) titration series for Biotin[6]uril and KBr. *External reference of DMSO.

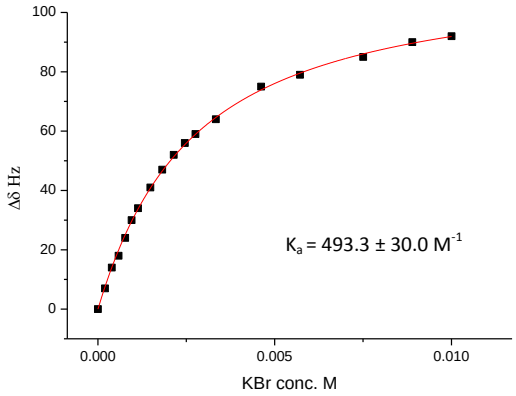


Figure S47: Plot showing experimental data for ^{1}H-NMR titration of KBr following proton f together with the fitted curve (red).

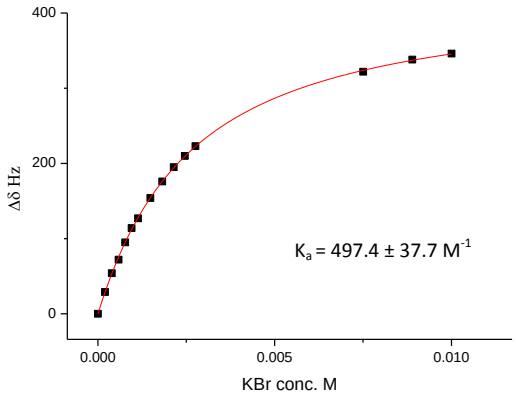


Figure S48: Plot showing experimental data for ^{1}H-NMR titration of KBr following proton b together with the fitted curve (red).

10.6 KI Titration

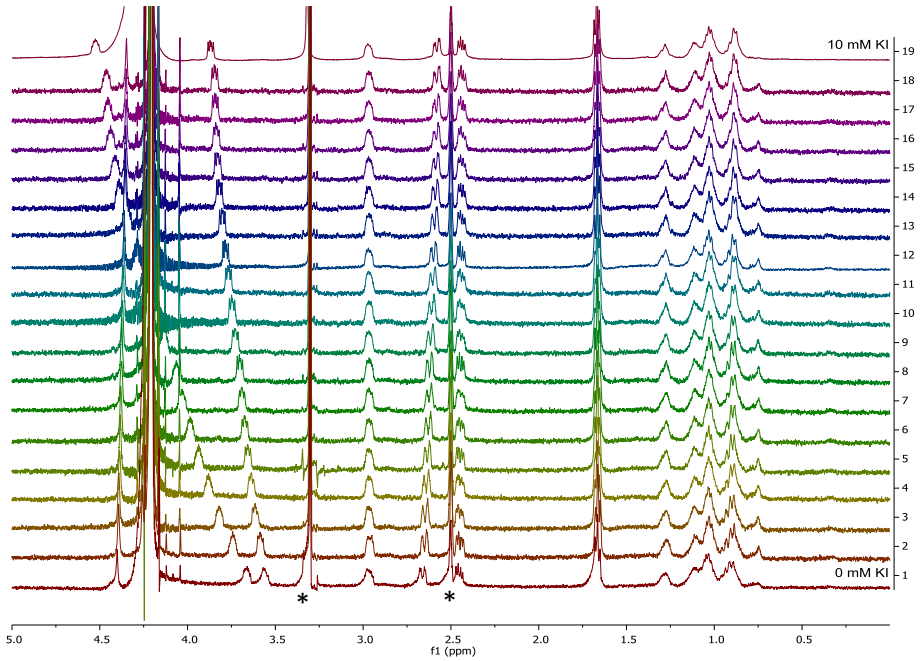


Figure S49: ^{1}H-NMR (500 Mhz, D_2O) titration series for Biotin[6]uril and KI. *External reference of DMSO.

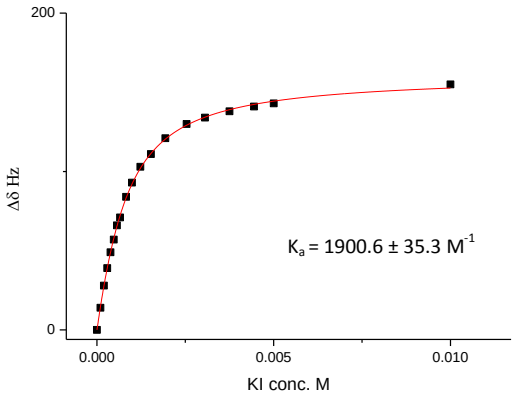


Figure S50: Plot showing experimental data for ^{1}H-NMR titration of KI following proton f together with the fitted curve (red).

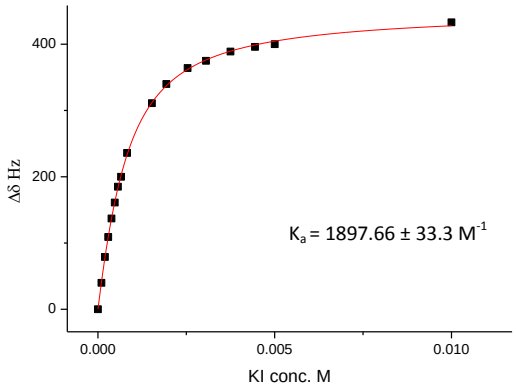


Figure S51: Plot showing experimental data for ^{1}H-NMR titration of KI following proton b together with the fitted curve (red).

11 ITC Titrations

Biotin[6]uril was dissolved in Na_2CO_3 buffer (Table 4: Samples for ITC), and the pH was adjusted to 10.8 with NaOH. The sodium halides were dissolved in $NaHCO_3$ (Table 4: Samples for ITC) and the pH was adjusted to 10.8 with NaOH. Dilution experiments for the Biotin[6]uril and the sodium halides were run under the same parameters, and subtracted from the raw data. The raw data were analyzed using Origin and fitted by the routines provided by MICROCAL. A low c fitting procedure with reduced χ^2 was used to fit the data.[1]

Table 4: Samples for ITC

NaCl ITC			NaBr ITC			NaI ITC		
Biotin[6]uril (mM)	NaCl (mM)	Na_2CO_3 (mM)	Biotin[6]uril (mM)	NaBr mM	Na_2CO_3 (mM)	Biotin[6]uril (mM)	NaI (mM)	Na_2CO_3 (mM)
0.501	409	20	1.02	81.91	40	0.689	29.06	30

[1] *J. Am. Chem. Soc.*, **2003**, *125* (48), pp 14859–14866

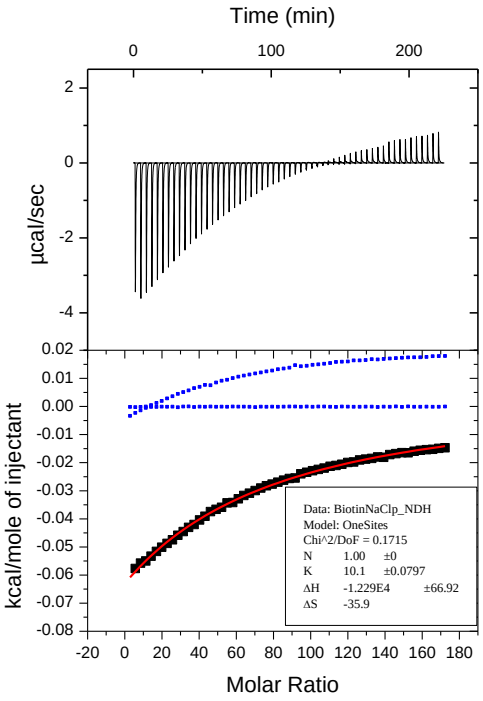


Figure S52: Data for the titration of Biotin[6]uril with NaCl at pH 10.8. Fitted curve (red), dilution subtracted (blue).

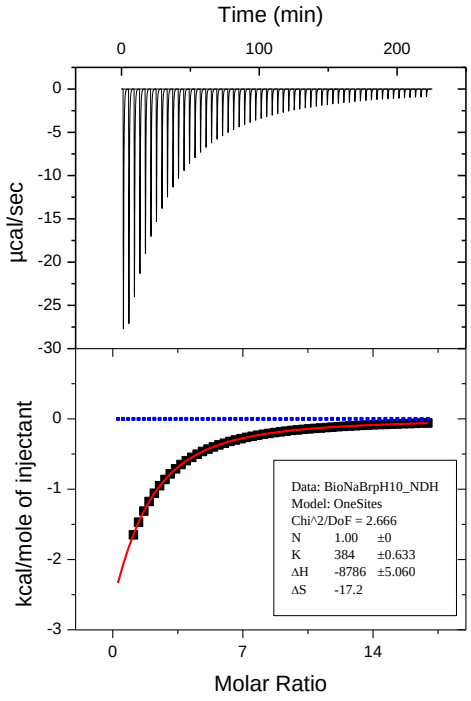


Figure S53: Data for the titration of Biotin[6]uril with NaBr at pH 10.8. Fitted curve (red), dilution subtracted (blue).

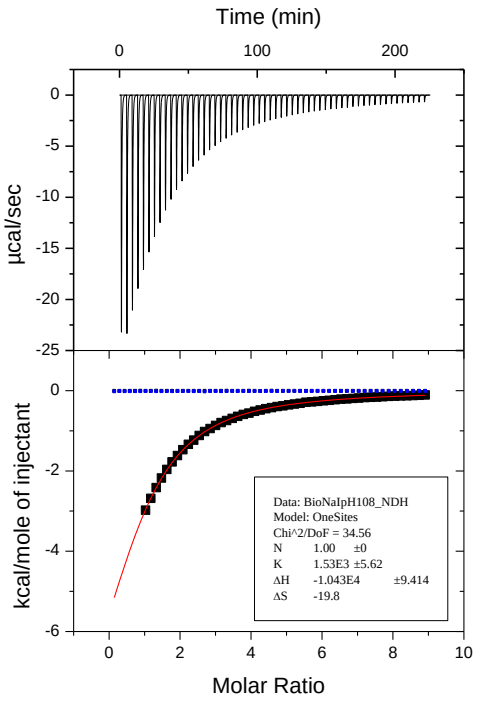


Figure S54: Data for the titration of Biotin[6]uril with NaI at pH 10.8. Fitted curve (red), dilution subtracted (blue).

12 Crystal Structure

Biotin[6]uril containing NaI:

The crystal of Biotin[6]uril containing NaI, for single crystal x-ray diffraction was produced by vapor diffusion of ether into an ethanol solution containing Biotin[6]uril and NaI.

$C_{66}H_{96}N_{12}O_{18}S_6$ 3(H_2O) 2(C_2H_6O) NaI; M = 1833.98; Monoclinic; a = 12.468(12)Å, b = 16.713(16)Å, c = 21.06(2)Å, α = 90°, β = 103.28(5), γ = 90°; V = 4272(7)Å^3; T = 122 K; space group $P2_1$; Z = 2; μ(Mo-K_α) = 0.07 mm^{-1}; 72612 reflections measured, 20893 independent reflections (R_{int} = 0.091). The final R_1 value was 0.064 [$F^2 > 2\sigma(F^2)$]. The final R_1 value was 0.113 (all data). The final $wR(F^2)$ (all data) value was 0.159, The goodness of fit on F^2 was 1.03

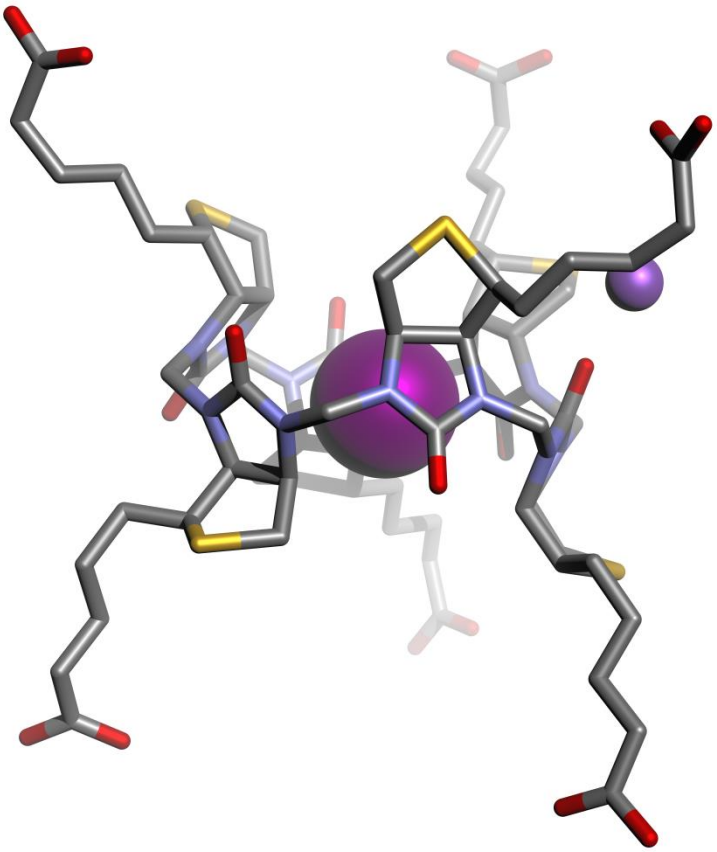


Figure 55: Structure of Biotin[6]uril- NaI complex; hydrogen atoms, disordered atoms and lattice solvent omitted for clarity. Yellow: sulphur; Blue: nitrogen; Gray: carbon; Red: oxygen; Dark purple: iodide; Light purple: sodium.

The structure can be obtained free of charge at
http://www.ccdc.cam.ac.uk/Community/Requestastructure/Pages/DataRequest.aspx?
CCDC nr: 988132

Biotin[6]uril:

The crystal of Biotin[6]uril, for single crystal x-ray diffraction, was produced by slow evaporation of ethanol.

The structure contained heavily disordered solvent molecules, these were removed using the solvent masking procedure implemented in the olex2 software; It was found the unit cell contained two solvent accessible voids each with a volume of 169 $Å^3$ and each with an electron count corresponding to one water and one ethanol molecule. These solvent molecules have been omitted in the formula.

$C_{66}H_{96}N_{12}O_{18}S_6$ $2(C_2H_6O)$; M = 1722.17; Monoclinic; a = 12.1810(11) Å, b = 16.841(4) Å, c = 20.8050(14) Å, α = 90°, β = 102.322°(14), γ = 90°; V = 4169.6(12) $Å^3$; T = 122 K; space group $P2_1$; Z = 2; μ(Mo-K_α) = 0.07 mm^{-1}; 30136 reflections measured, 15339 independent reflections (R_{int} = 0.068). The final R_1 value was 0.076 [$F^2 > 2\sigma(F^2)$]. The final R_1 value was 0.114 (all data). The final $wR(F^2)$ (all data) value was 0.232. The goodness of fit on F^2 was 1.09

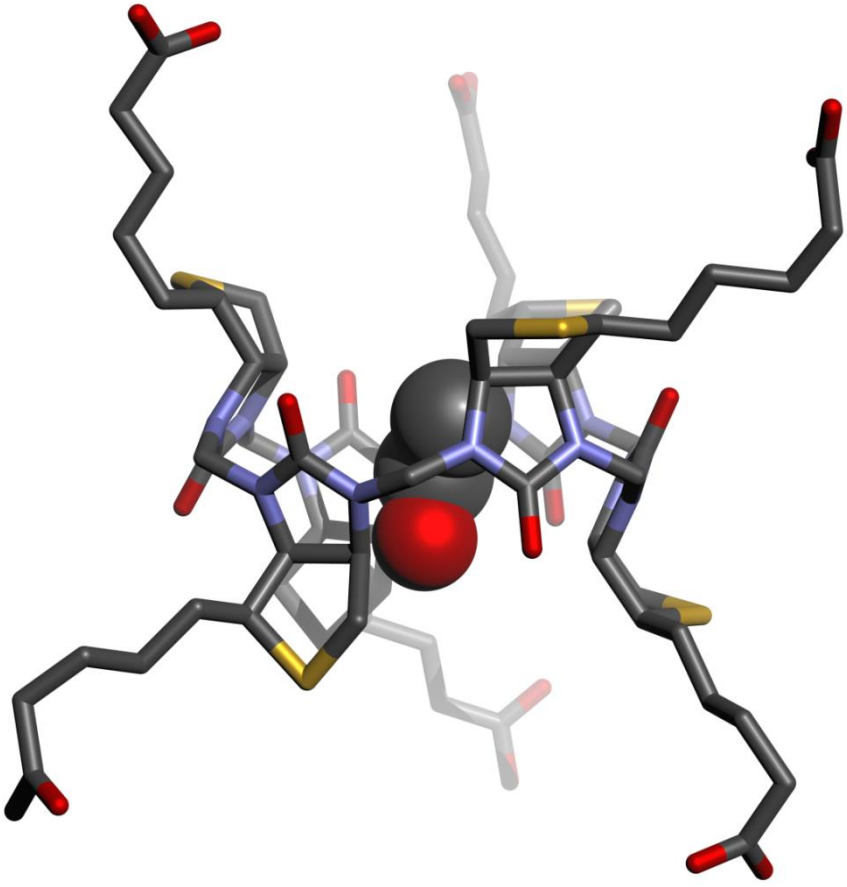
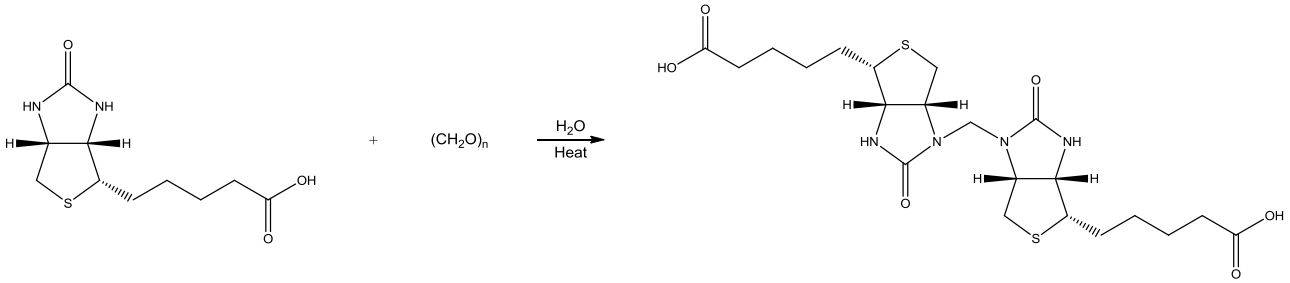


Figure 56: Structure of Biotin[6]uril with a ethanol molecule in the cavity, other solvent molecules, disordered atoms and hydrogen atoms is omitted for clarity. Yellow: sulphur; Blue: nitrogen; Gray: carbon; Red: oxygen;.

The structure can be obtained free of charge at
http://www.ccdc.cam.ac.uk/Community/Requestastructure/Pages/DataRequest.aspx?
CCDC nr: not yet recieved

13 Biotin-Dimer



Biotin (448 mg ; 1.8 mmol) and para-formaldehyde (30.7 mg ; 1.0 mmol) were dissolved in 40 ml water with 4 drops of conc. H_2SO_4. The mixture was heated to reflux with a heat gun until everything was dissolved. The reaction mixture was cooled to room temperature, and centrifuged. The white solid was dissolved in 2 M NaOH (10 ml) and filtered. The solution was acidified with conc. H_2SO_4 and the product was filtered off, and washed with water (3 × 10 ml).
Yield: 104 mg, 11 %.
M.p. 215-217°C. $[\alpha]_D^{20}$ = +40.3 (c = 0.25, 0.1 M NaOH).
^{1}H NMR (500 MHz, D_2O) δ = 4.16 (s, 2H), 3.98 (dd, *J* = 8.0, 4.7 Hz, 2H), 3.83 (dd, *J* = 8.1, 4.6 Hz, 2H), 2.83 (ddd, *J* = 8.9, 6.1, 4.5 Hz, 2H), 2.57 (d, *J* = 13.4 Hz, 2H), 2.40 (dd, *J* = 13.4, 4.8 Hz, 2H), 1.66 (td, *J* = 7.3, 2.2 Hz, 4H), 1.27 – 1.15 (m, 2H), 1.13 – 0.97 (m, 6H), 0.88 (p, *J* = 7.6 Hz, 4H).
^{13}C NMR (126 MHz, D_2O) δ = 182.86, 162.26, 62.86, 58.41, 54.12, 48.33, 36.36, 35.93, 27.29, 26.59, 24.72.
HRMS (ESI^+) of the acid: *m/z* $[M+H]^+$ calc. for $C_{21}H_{32}N_4O_6S_2$: 501.1836 ; found: 501.1828.
Elemental Analysis for $C_{21}H_{32}N_4O_6S_2$: [calc] (found); C [50.38] (50.14), H [6,44] (6.15), N [11.19] (10.95).

The Biotin-Dimer was analysed in water with an external standard of DMSO-d_6 and was isolated as a single isomer (Figures S57-S61).

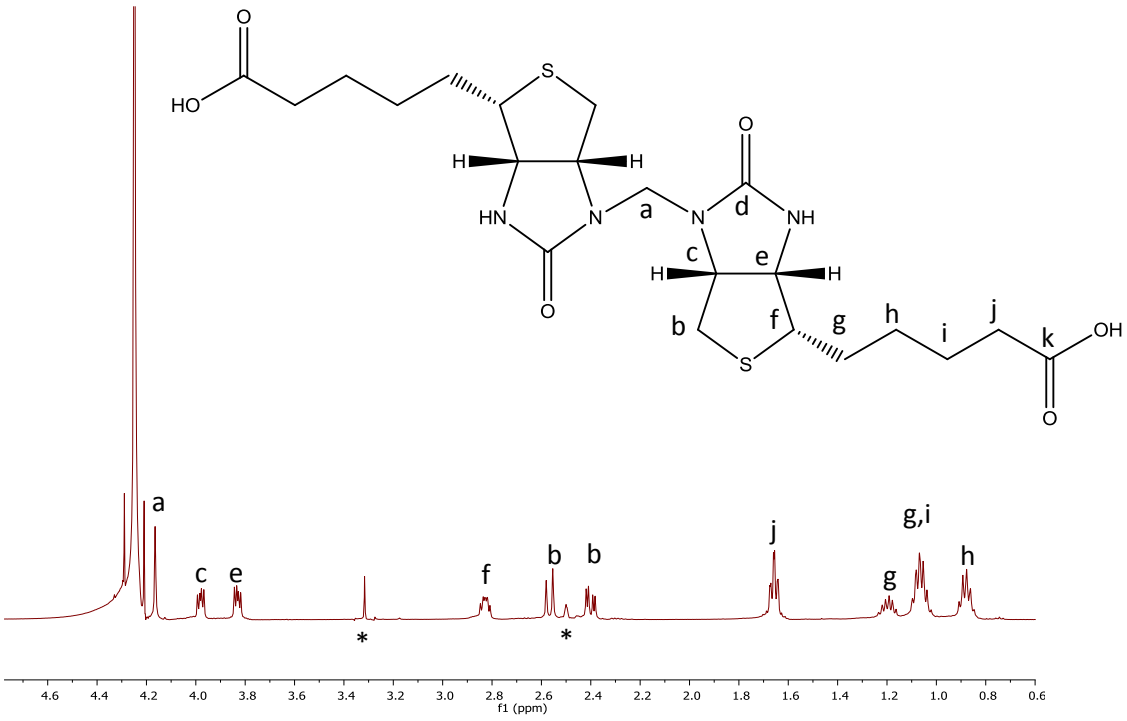


Figure S57: ^{1}H-NMR (500 MHz, D_2O) of the Biotin-dimer. *External reference of DMSO-d_6.

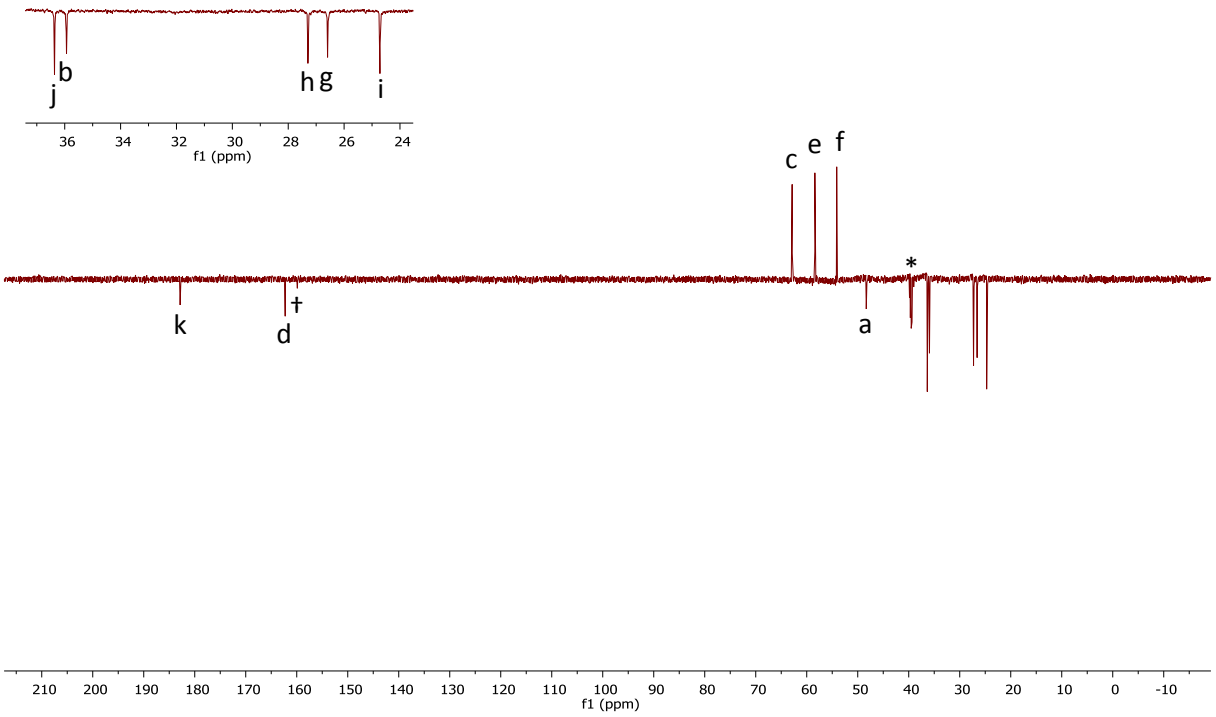


Figure S58: ^{13}C-NMR (126 MHz, D_2O) APT of the Biotin-dimer, with a zoom of 24 to 37 ppm showing the aliphatic sidechain and carbon b. *External reference of DMSO-d_6 † Na_2CO_3.

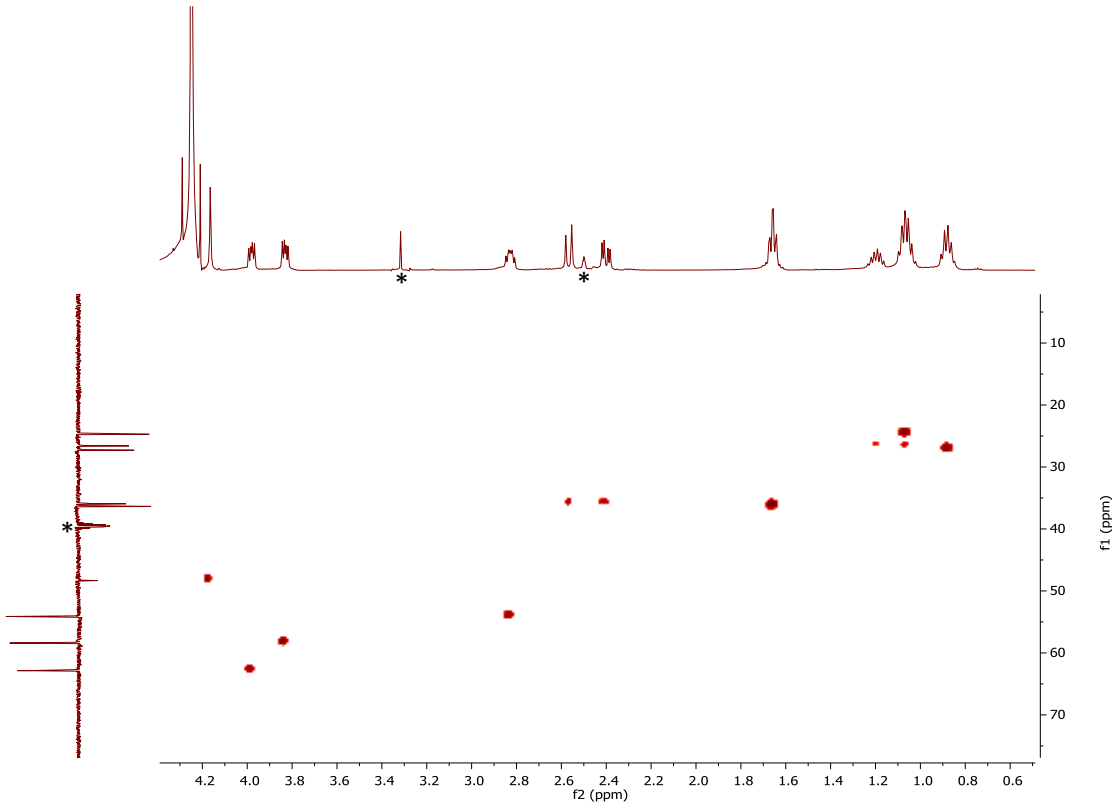


Figure S59: 2D-NMR (500MHz, D_2O) HSQC of the Biotin-dimer. *External reference of DMSO-d_6.

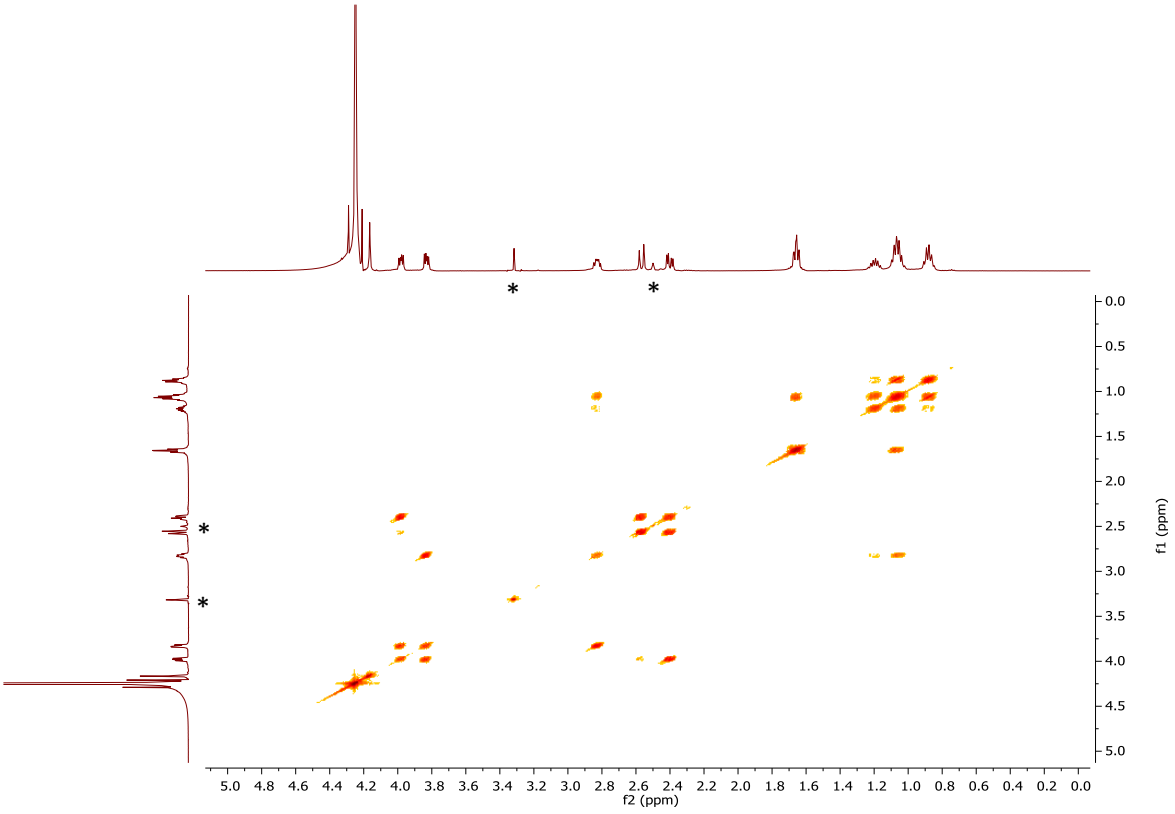


Figure S60: 2D-NMR (500MHz, D_2O) COSY of the Biotin-Dimer. *External reference of DMSO-d_6.

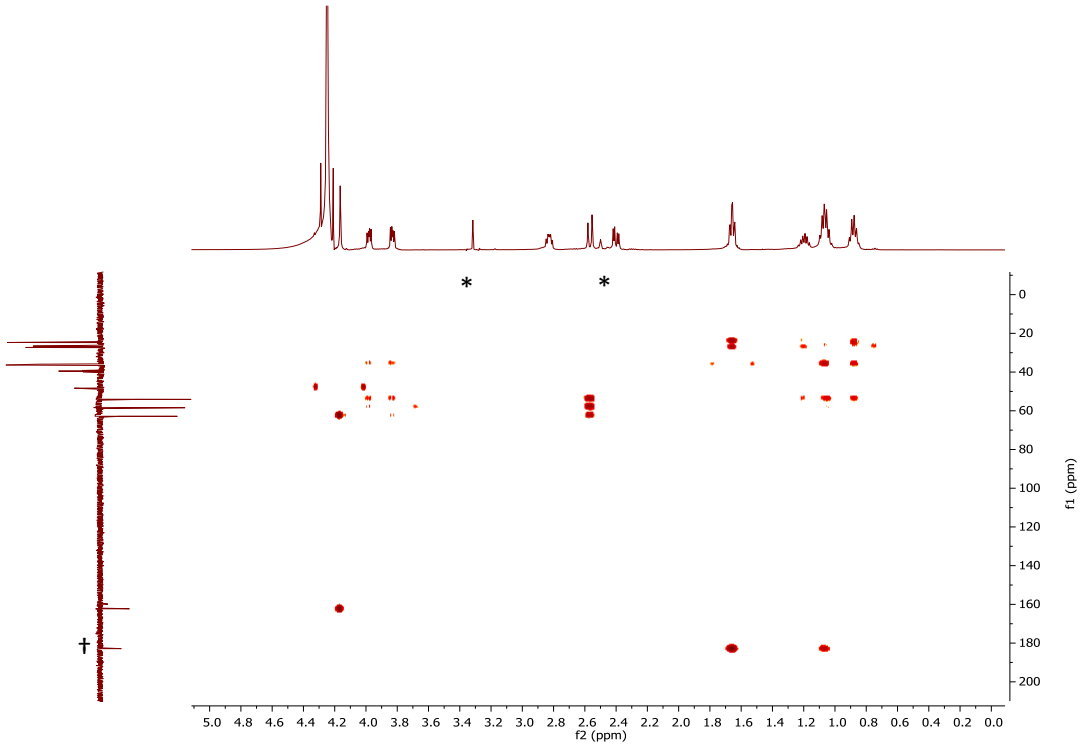


Figure S61: 2D-NMR (500MHz, D_2O) HMBC of the Biotin-dimer. *External reference of DMSO-d_6 † Na_2CO_3.

14 Reaction mixture composition for the Biotin[6]uril followed by LC-MS

In order to get a better understanding of when different smaller oligomers were formed in the reaction sequence from biotin to Biotin[6]uril the composition in the reaction mixture was followed by LC-MS. LC-MS method A was used for all experiments (Figure S62, and Figure 6A in the article).

14.1 Standard Curves

In order to follow the reaction on LC-MS, a set of standard curves of Biotin, Biotin-dimer and Biotin[6]uril was set up. The standard curves were made at 209 nm.

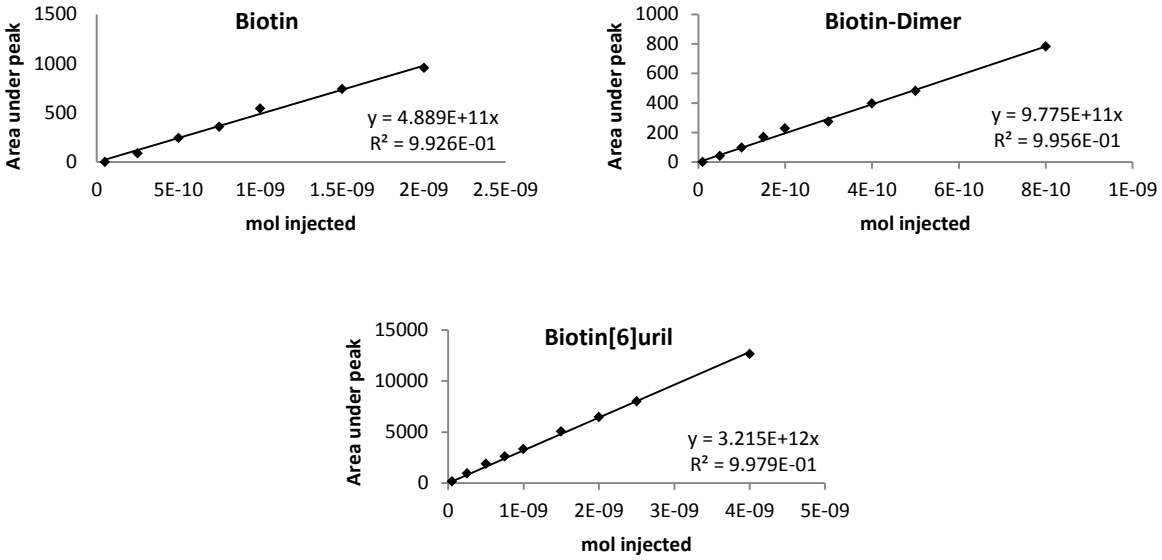


Figure S62: Standard curves at 209 nm of Biotin, Biotin-dimer, and Biotin[6]uril.

General example of a standard curve: Different volumes of a solution of 0.50 mM Biotin[6]uril in 12.5% ammonia solution, was injected into the LC-MS. The area under the peak at 209 nm in the UV-chromatogram corresponding to Biotin[6]uril was extracted and plotted against the injected amount of Biotin[6]uril. The plot was set to intercept at 0.0.

The slope of the Biotin[6]uril is approximately six times the slope of the Biotin-monomer, while the slope of the Biotin-dimer is approximately two times the slope of the Biotin-monomer. It is assumed that the trimer, tetramer, and the pentamer of Biotin have the same slope dependencies (Table 6).

All samples from supporting 14.2 stayed within the linear range from Figure S62.

14.2 Evolutuion of the Reaction from D-Biotin to Biotin[6]uril

Biotin (510 mg ; 2.08 mmol) and para-formaldehyde (317 mg; 10.56 mmol) were mixed with shaking and ultra-sonication. From the obtained solid mixture 10 samples were prepared. 7 M HCl was added to all the samples in amounts giving 82 mM concentration of biotin. The samples were closed and heated in an iron vessel to 59-60°C with stirring. After the indicated time in Table 5 the reaction mixture was quenched with 25% ammonia. 0.1 µl of each sample was injected into the LC-MS, and the area under the peaks from a UV-trace at 209 nm was recorded (Table 5).

Table 5: Area of corresponding Biotin oligomers at 209 nm.

	Monomer	Dimer	Trimer	Tetramer	Pentamer	Hexamer
10 min	1302	594	211	79	21	0
20 min	1186	690	258	59	27	129
50 min	1289	713	203	66	21	209
2 hour	1304	732	265	83	31	297
4 hours	1224	767	236	55	37	525
8 hours	1092	474	90	36	18	786
1 day	840	237	59	24	39	2042
2 days	323	135	46	14	27	2121
3 days	337	159	32	30	44	2147
4 days	333	128	53	53	81	2414

Table 6: Relative area of Biotin oligomers. The area of each peak has been divided by the number of biotin untis in the corresponding oligomer. For example the pentamer has been divided by five.

	Monomer	Dimer	Trimer	Tetramer	Pentamer	Hexamer
10 min	1302	297	70	20	4	0
20 min	1186	345	86	15	5	21
50 min	1289	357	68	17	4	35
2 hour	13049	366	88	21	6	49
4 hours	1224	384	79	14	7	88
8 hours	1092	237	30	9	4	131
1 day	840	119	20	6	8	340
2 days	323	68	15	4	5	353
3 days	337	80	11	8	9	358
4 days	333	64	18	13	16	402

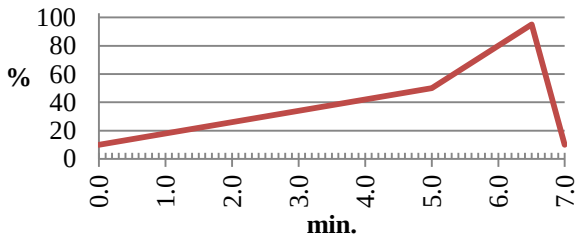
Table 7: Relative fraction of oligomers (%)

	Monomer	Dimer	Trimer	Tetramer	Pentamer	Hexamer
10 min	76.89	17.53	4.16	1.17	0.25	0
20 min	71.52	20.80	5.18	0.89	0.32	1.29
50 min	72.87	20.16	3.82	0.94	0.24	1.97
2 hour	71.08	19.95	4.81	1.13	0.34	2.70
4 hours	68.20	21.37	4.38	0.77	0.41	4.88
8 hours	72.68	15.77	2.00	0.60	0.24	8.71
1 day	63.06	8.89	1.47	0.45	0.59	25.54
2 days	42.04	8.80	1.98	0.47	0.69	46.02
3 days	42.04	9.94	1.33	0.95	1.10	44.65
4 days	39.34	7.58	2.09	1.56	1.91	47.52

Figure 6a from the article shows that in the early stages D-Biotin and a linear dimer are the most abundant species, and after 2 days Biotin[6]uril is the main product.

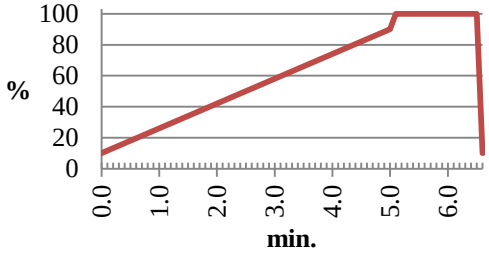
14.3 HPLC Specifications

Separation of the oligomers in the LC-MS was achieved by HPLC using a Dionex Acclaim RSLC 120 C18 2.2 µm 120 Å 2.1 × 100 mm column maintained at 20 °C, and the solvent profile shown in Figure S63 and S64. Solvent A is H_2O with 0.1% formic acid and solvent B is acetonitrile with 0.1% formic acid.



Time (min)	%A	%B
0.00	90	10
5.00	50	50
6.50	5	95
7.00	90	10
Flow-rate	0.600 mL/min	

Figure S63: Solvent profile used in LC-MS run A.



Time (min)	%A	%B
0.00	90	10
5.00	10	90
5.10	0	100
6.50	0	100
6.60	90	10
Flow-rate	0.600 mL/min	

Figure S64: Solvent profile used in LC-MS run B.

15 Formation of Biotin[6]uril with other acids

To evaluate if another acid than HCl could be used in the preparation of Bioitn[6]uril, four experiments were set up and analyzed by LC-MS (Table 8). From the four experiments it was established that sulfuric acid did not produce the Biotin[6]uril by itself. When combining sulfuric acid and a template like bromide or chloride the Biotin[6]uril formed.

Biotin (510 mg ; 2.08 mmol) and para-formaldehyde (317 mg; 10.56 mmol) were mixed with shaking and ultra-sonication. Four samples were made as seen in Table 8.

Table 8: Amount of solid biotin mixture, acid and template for the four samples**.**

	A	B	C	D
Biotin	25.0 mg	25.8 mg	26.6 mg	25.0 mg
7 M HCl	-	-	-	771 µl
3.5 H_2SO_4	771 µl	796 µl	820 µl	-
NaCl	Saturated	-	-	-
NaBr	-	-	7 M	-

The samples were stirred for 2 days at 60°C after which they were quenched with 25% ammonia. 0.1 µl was injected into the LC-MS and anlysed using the LC-MS method A.

Data evaluation was carried out as in the above reaction mixtures.