Electronic Supplementary Material (ESI) for Chemical Communications

A fluorescence assay that detects long branches in the starch polysaccharide amylopectin

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Supporting Information

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S1 Materials

Amylopectin from potato starch was obtained from Sigma-Aldrich. It was dissolved in H_2O (10 mg/ml) by warming at 80 °C, then lyophilized to removed traces of 1-butanol and ethanol before use. D_2O was obtained from Cambridge Isotope Laboratories, Andover, MA, USA.

β-Amylase from barley type II-B (E.C. 3.2.1.2) (69 U/mg), isoamylase from *Pseudonomas sp.* (E.C. 3.2.1.68) (18500000 U/mg, 3.6 mg/ml and α-amylase from *Aspergillus oryzae* (E.C. 3.2.1.1) (35.7 U/mg) were purchased from Sigma Aldrich, and pullulanase from *Krebsiella planticola* (E.C. 3.2.1.41) (699 U/ml) was purchased from Megazyme.

HPTS-$C_{16}H_{33}$ was synthesized as described previously.[1,2] The maltooligosaccharide mixture with average DP6 was prepared by fractional precipitation with ethanol as described previously.[3] The maltooligosaccharide mixture with average DP14 was prepared by debranching of amylopectin from potato using isoamylase. The distribution of maltooligosaccharides in each mixture was determined by UPLC/MS analysis following fluorescence labelling at the reducing end with 2-aminobenzamide as described previously.[2]

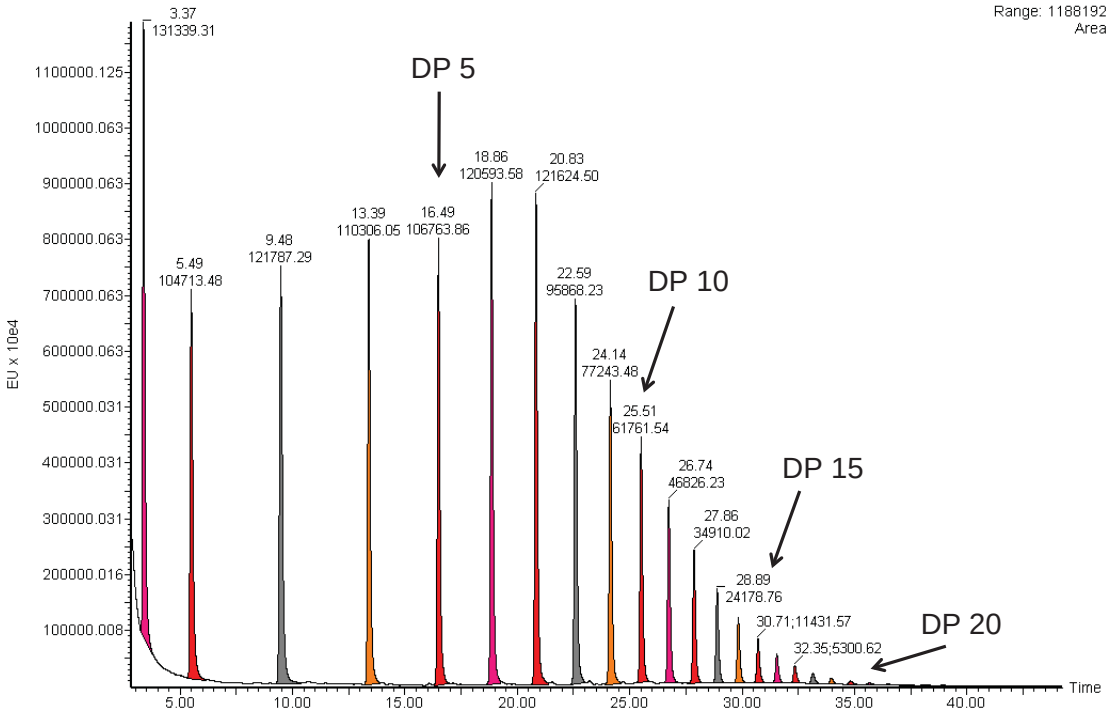

Figure S1. Fluorescence chromatogram showing UPLC analysis of the mixture of average DP 6 maltooligosaccharides used for binding studies.

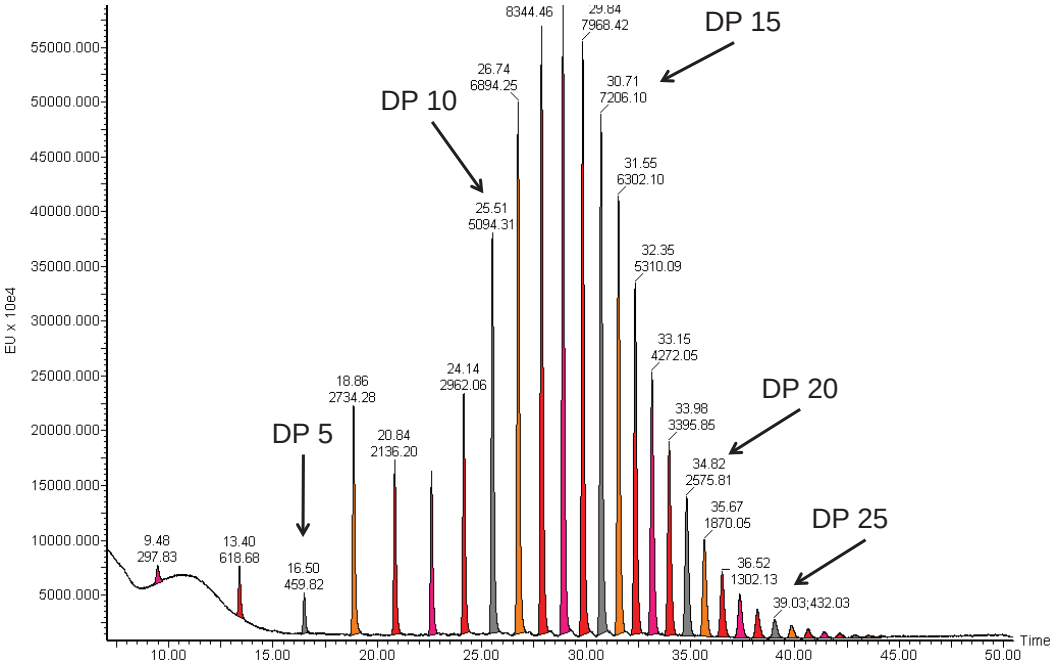

Figure S2. Fluorescence chromatogram showing UPLC analysis of the mixture of average DP 14 maltooligosaccharides used for binding studies.

S2 Instrumentation

Fluorescence spectra were recorded on a Molecular Devices SpectraMax Gemini EM fluorescence plate reader. Samples were excited at 300 nm and emission spectra were recorded over a range from 400 to 520 nm. Samples were loaded in 96 well standard black microplates obtained from Griener bio-one. Measurements were made using the top read settings and each well was read 30 times. The data was handled using SOFTmax pro.

^{1}H NMR spectra were recorded with water suppression by presaturation during the recycle delay. Spectra were either acquired as 16384 complex data points with an acquisition time of 3.4 s and a recycle delay of 2 s on a Bruker Avance 400 spectrometer or as 16384 complex data points with an acquisition time of 1.57 s and a recycle delay of 2 s on a Bruker Avance II 800 MHz spectrometer. Data were processed using Bruker Topspin 2.1

S3 Fluorescence Analysis

Samples for fluorescence analysis were prepared as 200 μl aqueous solutions in pH 6.5 NaH_2PO_4/Na_2PO_4 buffer in 96 well plates. In all cases the solutions were prepared by mixing a solution of HPTS-$C_{16}H_{33}$ with the appropriate volume of carbohydrate and/or buffer followed by addition of the polyamine (spermidine or spermine) and then mixing. The solutions were then left at room temperature for a minimum of 3 hours to equilibrate before analysis using a fluorescence plate reader. Note that it is best to add the polyamine last, as once formed the micelles may be kinetically-trapped and the mixture may require an impractical length of time to reach dynamic equilibrium.

S3.1 Fluorescence quenching with different polyamines

Solutions of HPTS-$C_{16}H_{33}$ (8 μM) and each of the amines, spermine, spermidine, 1,4-diaminobutane and 1,3-diaminopropane (40 μM), were prepared in 10 mM pH 6.5 sodium phosphate buffer. The HPTS-$C_{16}H_{33}$ solution (50 μl) was combined with

varying concentrations of polyamines and buffer in a 96-well plate format to give 200 μl final solutions. The mixtures were allowed to equilibrate for 4 hours, and then analysed.

S3.2 Job plots for spermine and spermidine interaction with HPTS-$C_{16}H_{33}$

Solutions of HPTS-$C_{16}H_{33}$ (2 μM) and either spermine (2 μM) or spermidine (2 μM) in 10 mM pH 6.5 sodium phosphate buffer were combined in varying ratios in a 96-well plate format to give total volumes in each well of 200 ul. The fluorescence emission at 440 nm was measured after 3 hours equilibration time. Job plots (continuous variation plots) were made by plotting:

on the x-axis [H]/([H]+[G]

on the y-axis $F_{(2\mu M\ H)} \times \{[H]/([H]+[G])\} - F_{obs}$,

where H = HPTS-$C_{16}H_{33}$ and G = spermine or spermidine

S3.3 Fluorescence analysis of HPTS-$C_{16}H_{33}$ binding to amylopectin, maltooligosaccharides DP3-8, maltooligosaccharide mixtures and α-cyclodextrin.

Aqueous solutions of the following were prepared in 0.5 mM pH 6.5 NaH_2PO_4/Na_2HPO_4 buffer: HPTS-$C_{16}H_{33}$ (4 μM); spermidine (8 μM); amylopectin (10 mg/ml and 2 mg/ml), maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose and maltooctaose (200 μM and 1 mM); average DP12 maltooligosaccharide mixture (20 μg/ml, 200 μg/ml, 500 μg/ml), average DP6 maltooligosaccharide mixture (20 μg/ml, 200 μg/ml, 1 mg/ml) and α-cyclodextrin (20 μg/ml, 200 μg/ml and 1 mg/ml).

HPTS-$C_{16}H_{33}$ solution (50 μl) was combined with spermidine solution (50 μl) and varying quantities of saccharide solutions and buffer to make a total of 200 μl. The solutions were left for 3-4 hours to equilibrate and then analysed for fluorescence at 440 nm upon irradiation at 300 nm.

S4 Detection of long branches in enzymatically-digested amylopectin

S4.1 Enzymatic degradation of amylopectin

A solution of amylopectin in D_2O (5 mg/ml) was prepared by heating at 90 °C for 10 minutes. 500 µl aliquots of the amylopectin solution were transferred to 5 nmr tubes. With the exception of one sample, which was retained untreated as a control, the samples of polysaccharides were digested with various hydrolytic enzymes at room temperature overnight: α-amylase (5 µl of a 1 mg/ml solution in D_2O), β-amylase (5 µl of a 1 mg/ml solution in D_2O), pullulanase (5 µl of the commercial suspension) and isoamylase (1 µl of the commercial suspension). The solutions were analysed by ^{1}H-NMR spectroscopy and hydrolysis was judged to be complete within 18 hours.

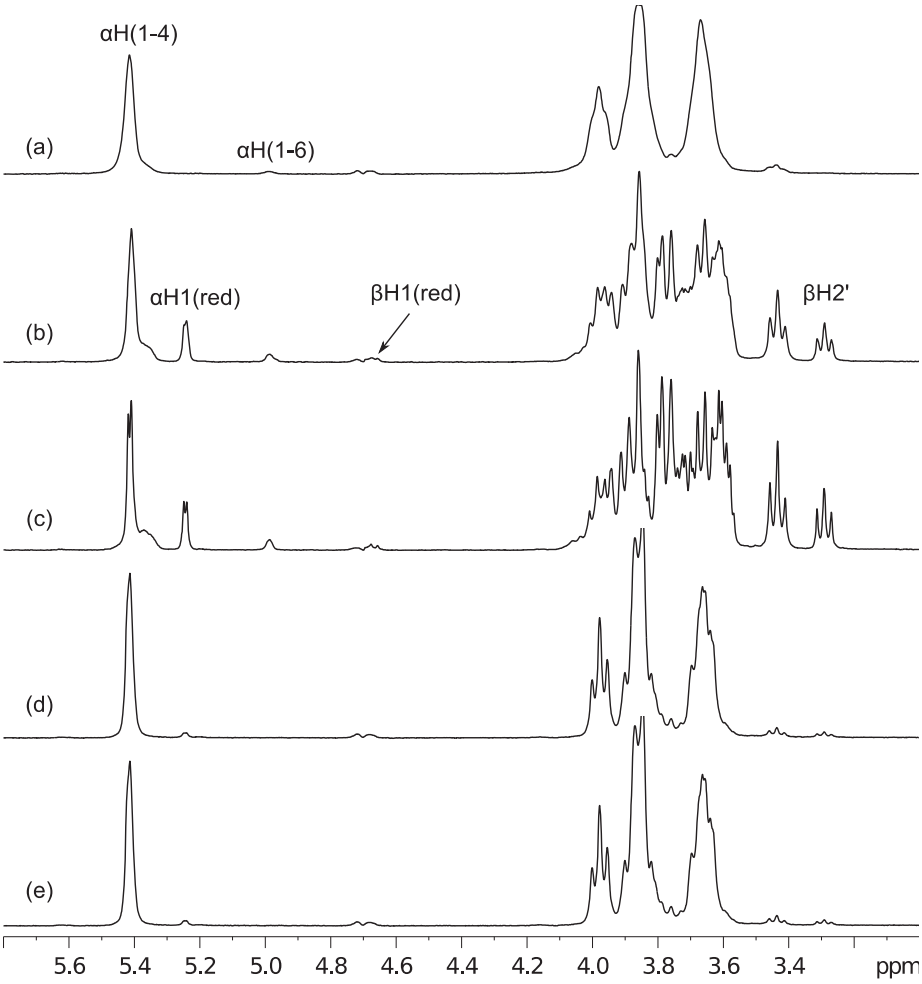

Figure S3: ^{1}H NMR spectra (400 MHz, 310 K, D_2O): amylopectin (5 mg/ml) alone (a), and amylopectin treated exhaustively with: (b) α-amylase (c), β-amylase (d), pullulanase and (e) isoamylase.

S4.2 Fluoreoscence analysis of enzyme-digested amylopectin

For fluorescence analysis, 50 μl aliquots of each of the 5 samples were diluted with 1200 μl of 0.5 mM pH 6.5 NaH_2PO_4/Na_2HPO_4 buffer to give 0.2 mg/ml solutions.

- For fluorescence plate analysis 100 μl of each of these solutions was combined with 50 μl of HPTS-$C_{16}H_{33}$ solution (4 μM) and 50 μl of spermidine solution (8 μM) (each in 0.5 mM pH 6.5 NaH_2PO_4/Na_2HPO_4 buffer), left to equilibrate for 3 hours and then analysed for fluorescence at 440 nm upon excitation at 300 nm.
- For analysis by naked-eye, 200 μl of each solution was mixed with 100 μl of a solution of HPTS-$C_{16}H_{33}$ (4 μM) and 100 μl of spermidine solution (8 μM) (all in 0.5 mM pH 6.5 NaH_2PO_4/Na_2HPO_4 buffer). The solutions were photographed after 3 hours while being irradiated with 365 nm light.

S5 NMR titrations

A solution of HPTS-$C_{16}H_{33}$ (0.5 mM) was prepared in D_2O. This solution was used to prepare solutions of maltohexaose (6 mM), maltoheptaose (6 mM) and maltooctaose (6 mM) and α-cyclodextrin in 0.5 mM HPTS-$C_{16}H_{33}$ in D_2O. The saccharide solutions were titrated into the HPTS-$C_{16}H_{33}$ solutions (500 μl) in NMR tubes and ^1H NMR spectra were acquired of the solutions following each addition after mixing. The titration with maltooctaose was monitored on an 800 MHz spectrometer while the other titrations were performed on a 400 MHz spectrometer. The binding isotherms were obtained by plotting the change in chemical shift of the methyl (H16) protons ($\Delta\delta_{obs}$) as described previously[1,2] (Figure S4, S5). NMR spectra from each titration are shown in Figures S6-S9. The association constants (K_a) were determined by fitting the 1-to-1 binding isotherms to *Equation 1*, where $[H]_o$ and $[G]_o$ are the total added concentrations of the saccharide and HPTS-$C_{16}H_{33}$, respectively, and $\Delta\delta_{max}$ is the difference between the chemical shift of H16 (CH_3) from the probe when unbound and when completely bound.

$$\Delta\delta_{obs} = \frac{\Delta\delta_{max}}{2[H_o]}\left[\left([G_o]+[H_o]+\frac{1}{K_a}\right) - \sqrt{\left([G_o]+[H_o]+\frac{1}{K_a}\right)^2 - 4[H_o][G_o]}\right] \qquad \textit{Equation 1}$$

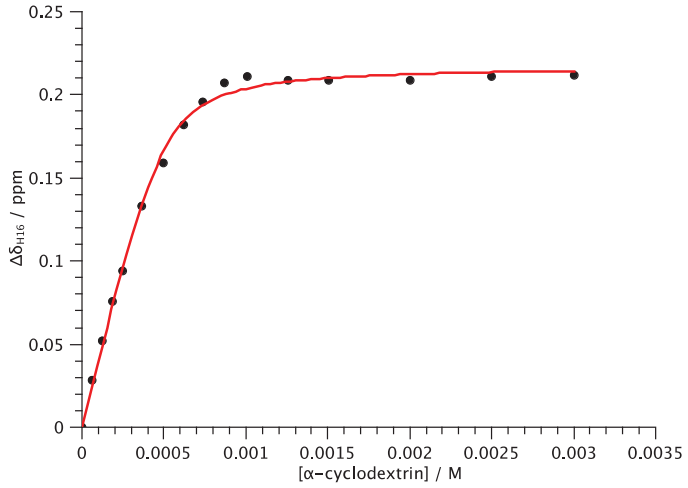

Figure S4 Fitted binding isotherms obtained from the change in chemical shift of H16 in HPTS-$C_{16}H_{33}$ in the presence of α-cyclodextrin

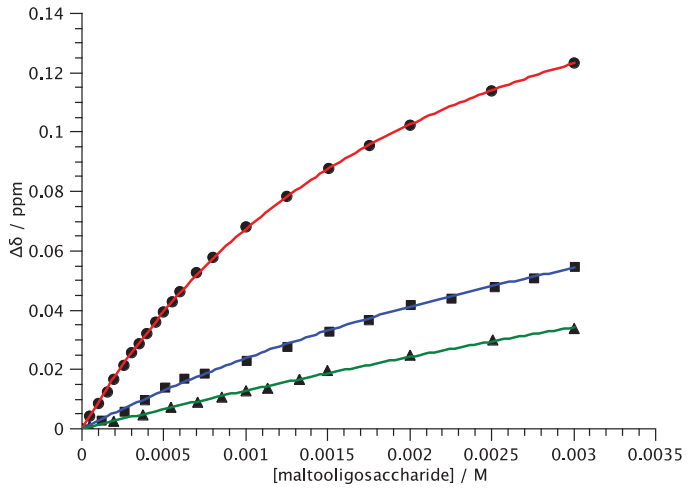

Figure S5 Fitted binding isotherms obtained from the change in chemical shift of H16 in HPTS-$C_{16}H_{33}$ in the presence of maltoligosaccharides: maltohexaose (trianges, green), maltoheptaose (squares, blue) and maltooctaose (circles, red).

Table S1 Association constants determined from ^{1}H NMR titrations.

Guest	K_a (M^{-1})	Δδ (ppm)
α-cyclodextrin	28 000 ± 4000	0.217 ± 0.002
maltooctaose	640 ±10	0.195 ± 0.001
maltoheptaose	220 ±20	0.142 ± 0.008
maltohexaose	70 ± 14	0.198 ±0.03

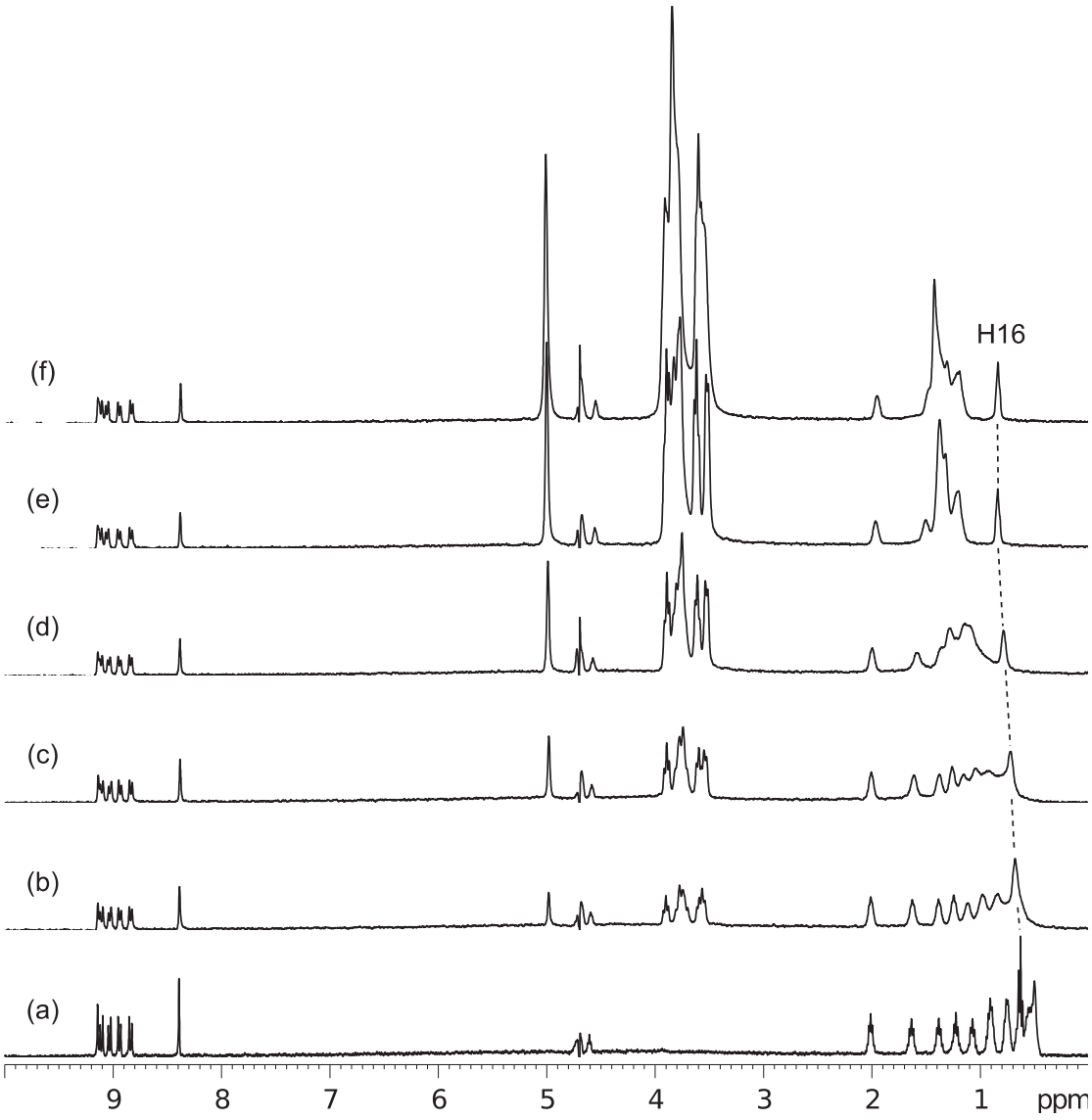

Figure S6 ^{1}H NMR spectra (400 MHz, 300 K, D_2O) of (a) HPTS-$C_{16}H_{33}$ (0.5 mM) and HPTS-$C_{16}H_{33}$ (0.5 mM) with α-cyclodextrin: (b) 0.25 mM, (c) 0.5 mM, (d) 1 mM, (e) 2 mM and (f) 3 mM.

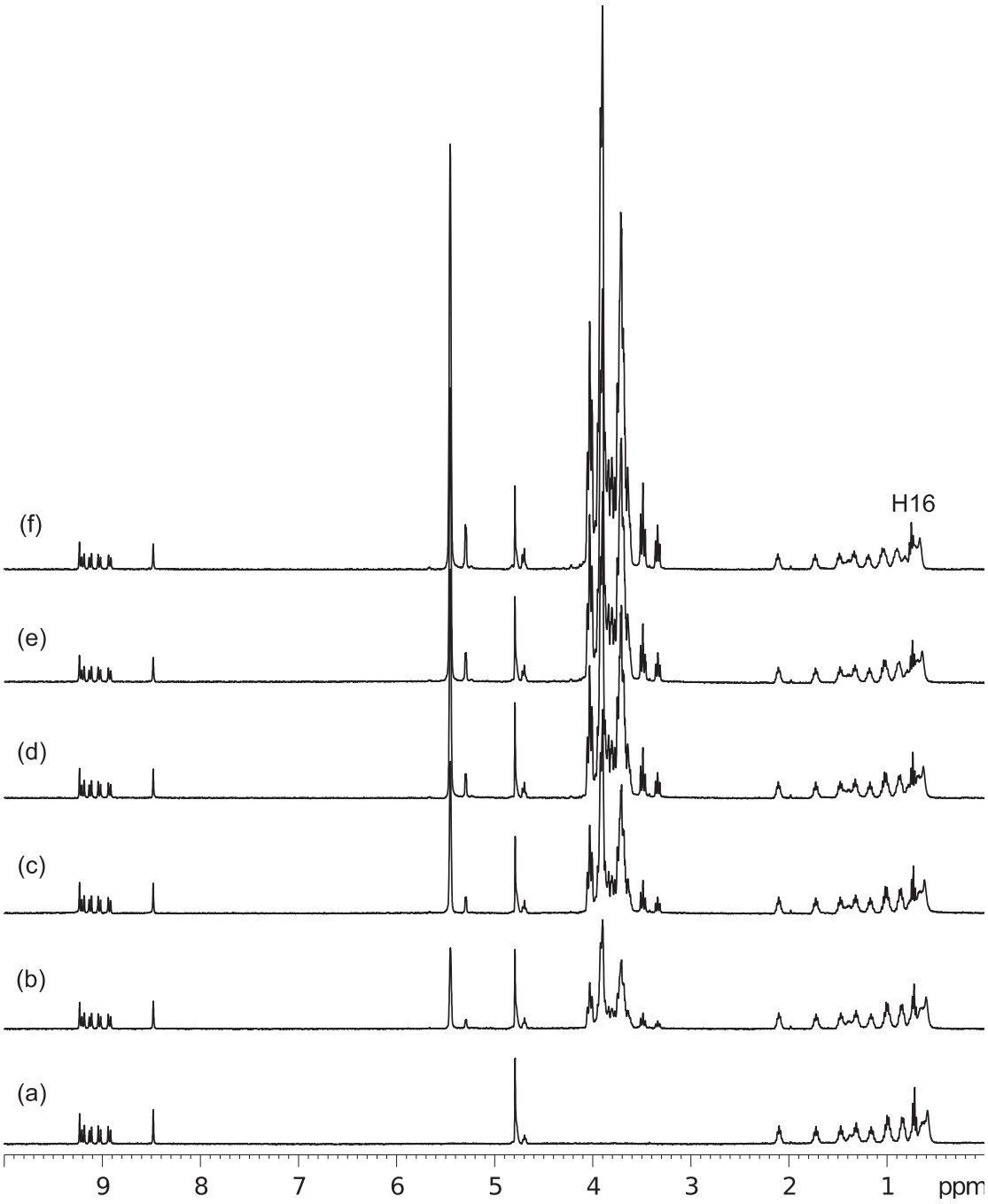

Figure S7 ^{1}H NMR spectra (400 MHz, 300 K, D_2O) of (a) HPTS-$C_{16}H_{33}$ (0.5 mM) and HPTS-$C_{16}H_{33}$ (0.5 mM) with maltohexaose: (b) 0.5 mM, (c) 1 mM, (d) 1.5 mM, (e) 2 mM and (f) 2.5 mM.

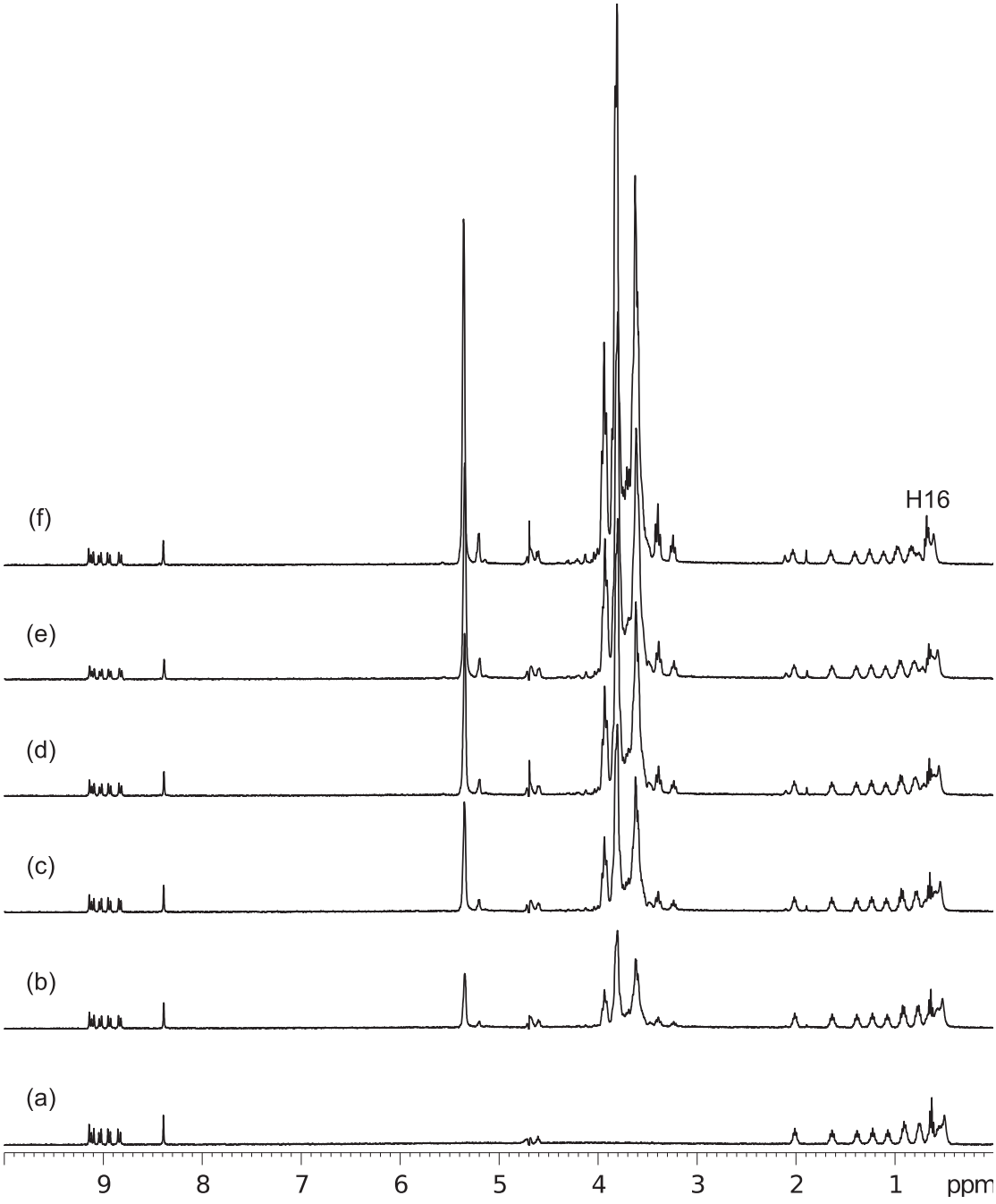

Figure S8 ^{1}H NMR spectra (400 MHz, 300 K, D_2O) of (a) HPTS-$C_{16}H_{33}$ (0.5 mM) and HPTS-$C_{16}H_{33}$ (0.5 mM) with maltoheptaose: (b) 0.5 mM, (c) 1 mM, (d) 1.5 mM, (e) 2 mM and (f) 2.5 mM.

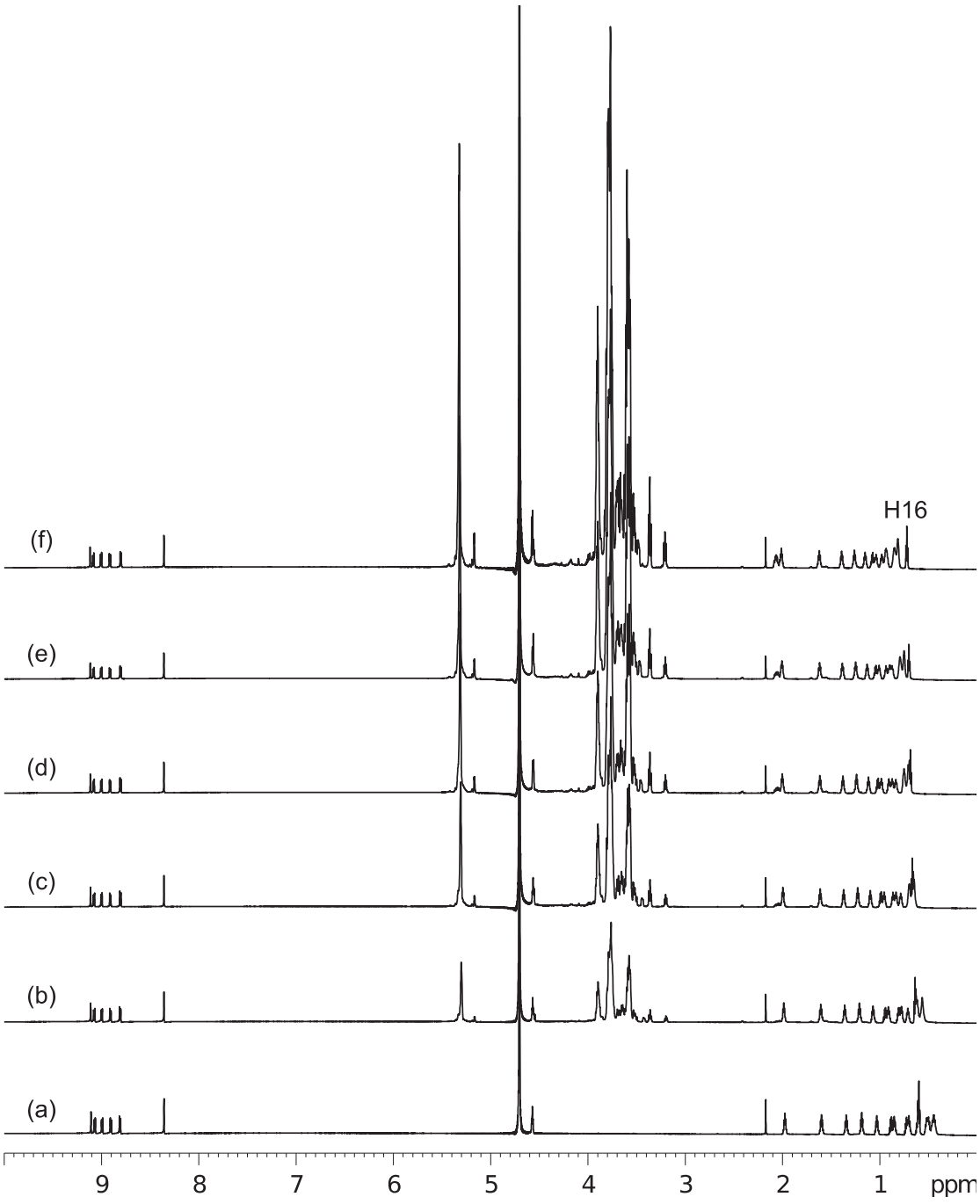

Figure S9 ^{1}H NMR spectra (800 MHz, 300 K, D_2O) of (a) HPTS-$C_{16}H_{33}$ (0.5 mM) and HPTS-$C_{16}H_{33}$ (0.5 mM) with maltooctaose: (b) 0.5 mM, (c) 1 mM, (d) 1.5 mM, (e) 2 mM and (f) 2.5 mM.

[1] S. R. Beeren and O. Hindsgaul, *Angew. Chem. Int. Ed.*, 2013, **52**, 11265-11268.
[2] S. R. Beeren, S. Meier and O. Hindsgaul, *Chem. Eur. J.*, 2013, **19**, 16314-16320.
[3] S. A. Johannesen, S. R. Beeren, D. Blank, B. Y. Yang, R. Geyer, O. Hindsgaul, *Carbohydr. Res.*, 2012, **352**, 94-100