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Supporting Information

pH-responsive templates modulate the dynamic enzymatic synthesis of cyclodextrins

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Contents

S1 Experimental details and instrumentation

S1.1 Materials

A stock solution of CGTase derived from *Bacillus macerans* was a kind gift to the group by Amano Enzyme, Inc., Nagoya, Japan. It was stored at 5˚C and used as received. All other chemicals were purchased from commercial suppliers and used as received. HPLC grade solvents or better were used for all reactions and chromatographic analysis. Colourless 0.65 mL centrifuge tubes were used for DCL reactions and centrifugation, and colourless 2 mL glass vials with 0.2 mL glass inserts were used for sample injection on chromatographic equipment.

S1.2 Instrumentation

Chromatographic analysis was performed on a Thermo Scientific Dionex UltiMate 3000 HPLC (ultra-high pressure) system with a Waters Acquity UPLC BEH Amide 1.7 µm 2.1 × 150 mm column maintained at 30 ˚C. The system was equipped with an auto-sampler, which was maintained at 20 ˚C. Injection volumes were 15 µL. The chromatographic system was connected to an Agilent Technologies 1260 Infinity ELSD operating with the evaporator at 90 ˚C, nebulizer at 70 ˚C, and a N_2 gas glow of 1.0 L/min. The eluents were water and acetonitrile, both containing 0.1 % formic acid. The gradient profile for HPLC runs was a linear gradient from 75 % acetonitrile in water to 55% acetonitrile in water over 8 minutes with a flow rate of 0.6 mL/min. Calibration curves from 0.01 mg/mL to 10 mg/mL for α-, β-, and γ-CD and linear α-1,4-glucans up to maltooctaose were used to correct for differences in the ELS detector response for different oligosaccharides. The calibration was based on masses injected (0.018 µg – 3.66 µg) and the resulting response curves were fitted to a simple power equation $M = kA^p$ (where M is the injected mass of compound, A is the area under the peak in the chromatogram and k and p are fitted parameters) using non-linear curve fitting (in OriginPro 2018b from OriginLab Corp.)[1]

S1.3 Preparation of buffers

A mixed buffer system consisting of both acetate and phosphate was used to cover the desired pH range 5-8. Previously, it was observed that changing the buffer might have a small effect on the DCL composition.[2] A total of 7 buffers were prepared (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0) from acetic acid and Na_2HPO_4. The buffers contained different concentrations of the two buffer components to reach the desired pH values, while avoiding the need for strong acid or base to adjust the pH value. This procedure was chosen to avoid changes in the ionic strength of the buffers due to adjustment with strong acid or base, as difference in ionic strength may affect the DCL composition. The ionic strength of the buffers was in all cases adjusted to 0.15 M by addition of NaCl. The ionic strength was calculated using the following equation:

$$\text{Ionic strength} = \tfrac{1}{2}(\Sigma C z^2)$$

The ionic strength depends on the concentration (C) and the sum of the charges (z) of the various ions in solution. Considering that the charge depends on the ionization degree of the buffer components and the pH, it was possible to calculate the ionic strength.

As an example, to prepare a buffer with pH 5, a solution of acetic acid (30 mM) and disodium hydrogenphosphate (20 mM) was prepared with milliQ water. The pH was measured, and the ionic strength was calculated to 0.04 M using the equation above, and the ionic strength was adjusted by addition of sodium chloride (0.11 M) to obtain an ionic strength of 0.15 M.

S1.4 Enzyme-mediated dynamic combinatorial libraries (DCLs)

All reactions were set-up and run at room temperature (approx. 22 ˚C). Starting material (α-CD) was dissolved in buffer at a concentration of 4 mM. Similarly, the templates were dissolved in buffer at a concentration of 4 mM. A reaction was set up by mixing equal volumes of starting material and buffer/template solutions in a centrifuge tube, reaching a total volume of 100-130 µL reaction mixture, and a final concentration of 2 mM starting material and either buffer or 2 mM template. All reactions were started and timed within ±1 second by adding CGTase stock. For sampling, 10 µL reaction mixture was diluted in 90 mL with 1% TFA in 3:1 acetonitrile/water in a 0.65 centrifuge tube. The sample was centrifuged for 4 min at 10,000 rpm, and the top fraction (80 µL) was transferred to 2 mL glass vials with glass inserts. Sample vials were closed with a screw cap, and 15 µL of each sample was injected on the chromatographic equipment within 24 hours.

S1.5 ^{1}H NMR titrations of 4-nitrophenol with α-, β-, and γ-CD in D_2O

Two buffers (50 mM acetate-phosphate pH 5.0 and 9.6) were prepared in D_2O, and adjusted with sodium chloride to ionic strength of 0.15 M. A stock solution of 4-nitrophenol (1 mM) in buffer was prepared. Solutions of α-, β-, and γ-CD were prepared by dissolving these compounds in the stock solution of 4-nitrophenol in buffer to reach the desired concentrations. The concentrations of CDs were 20-50 equivalent depending on the expected binding strength to ensure an optimal binding isotherm. A stock solution containing 4-nitrophenol and a stock solution containing 4-nitrophenol + CD were then mixed to a total volume of 500 µL in 12 NMR tubes, resulting in 12 tubes with constant concentration of 4-nitrophenol (1 mM) and different ratios of CD (0-50 mM). The ^{1}H NMR spectra were recorded at 298 K on a 400 MHz Bruker NMR spectrometer using a 5 mm probe. Standard pulse programs were used to obtain all spectra. Thirty two scans were recorded in deuterated water, which was used as internal reference at 4.7025 ppm for the first spectra containing only 4-nitrophenol and buffer. The subsequent spectra were referenced to the acetate peak in the first spectra, since deuterated water is a poor reference signal. The change in the chemical shifts of the signals coming from the protons in 4-nitrophenol was monitored, and the values were fitted to a non-linear 1:1 binding model to determine the binding affinity, K_a.

S2 Monitoring the CD distribution and evolution of DCLs at varying pH

S2.1 DCL at varying pH without template

The formation of DCLs by the action of CGTase on α-CD was tested in seven buffers across the pH range 5.0-8.0. The results showed that there was a difference in the reaction rate at pH 5.0-5.5 and 6.0-8.0, as the reaction rate was slower at low pH. Since the DCL of cyclodextrins forms transiently, and not strictly under thermodynamic control, i.e. background hydrolysis occurs during the reaction, the reaction rate becomes important for the assessment of when the *pseudo*-equilibrium of the DCL was reached. For pH 5.0-5.5, *pseudo*-equilibrium was reached after 60 min (without template), but for pH 6.0-8.0, *pseudo*-equilibrium was reached after 40 min (without template). The DCL composition between pH 5.0 and 8.0 was slightly different, although the difference was considered insignificant. Also, the total CD concentration varied slightly, but again it was taken to be insignificant.

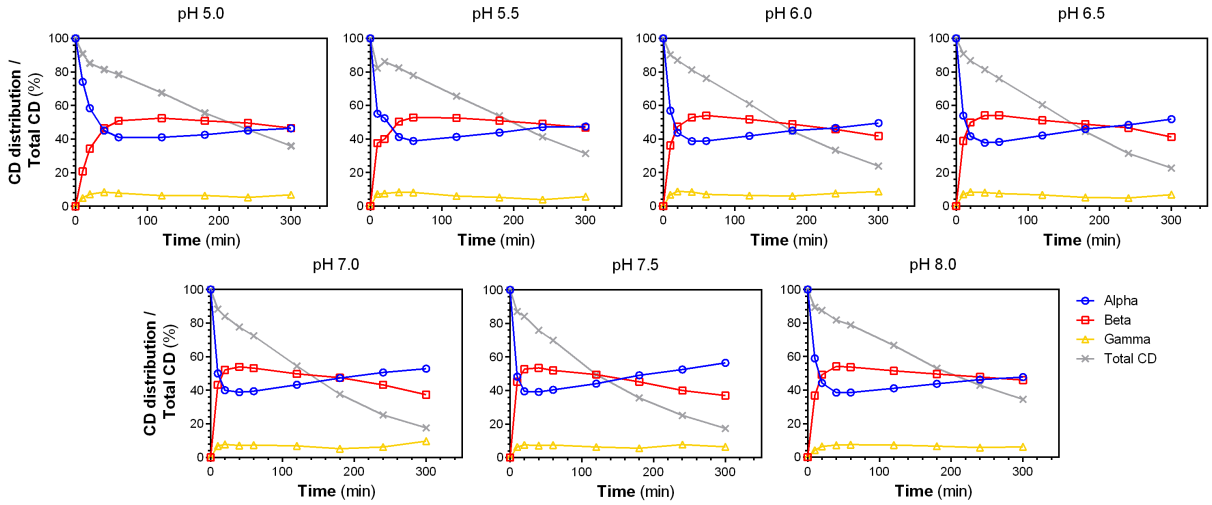


Figure S1. Distribution of the CD in the DCL as a function of time without template in the pH range 5.0-8.0. The total CD yield (grey) decreases as a function of time due to background hydrolysis. Hence, the system is under *pseudo*-thermodynamic control, and the equilibrium time point is taken to be after 60 min for pH 5.0 and 5.5, and after 40 min for pH 6.0-8.0.

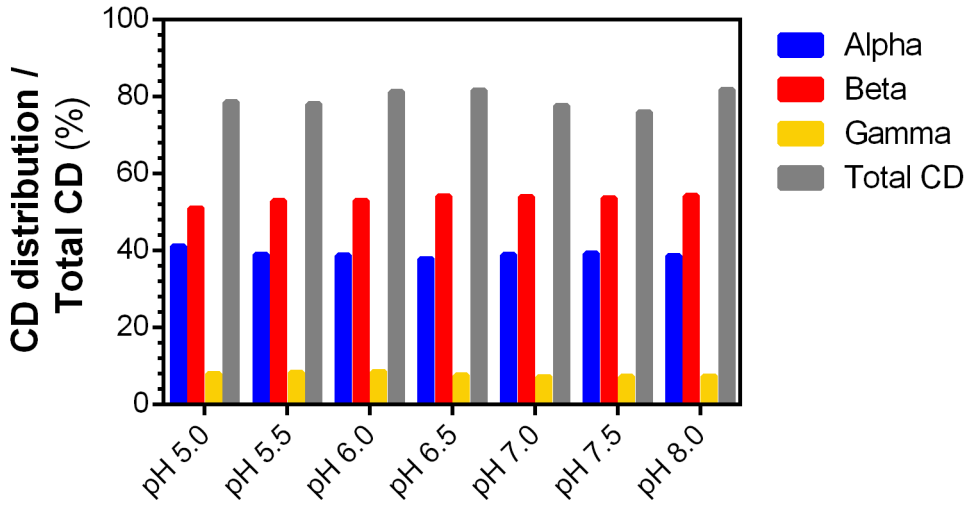


Figure S2. The figure shows the DCL distribution (without template) at seven pH values.

S2.2 DCLs with different ionisable templates

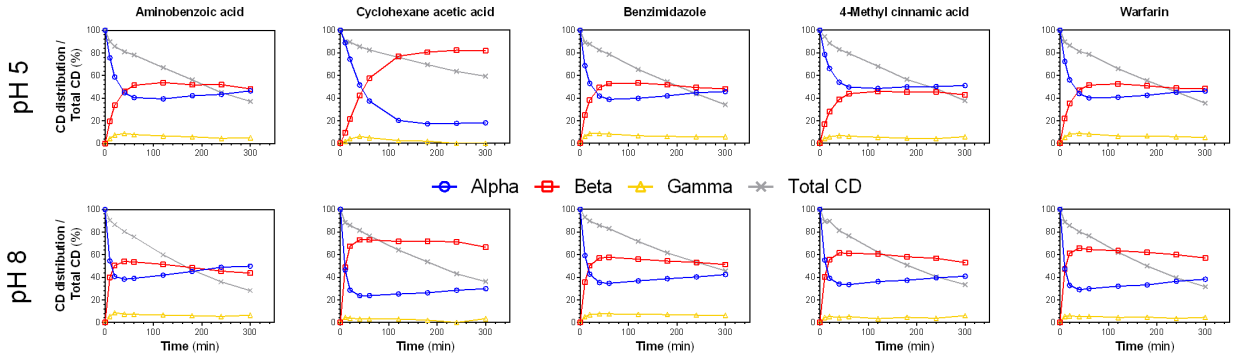
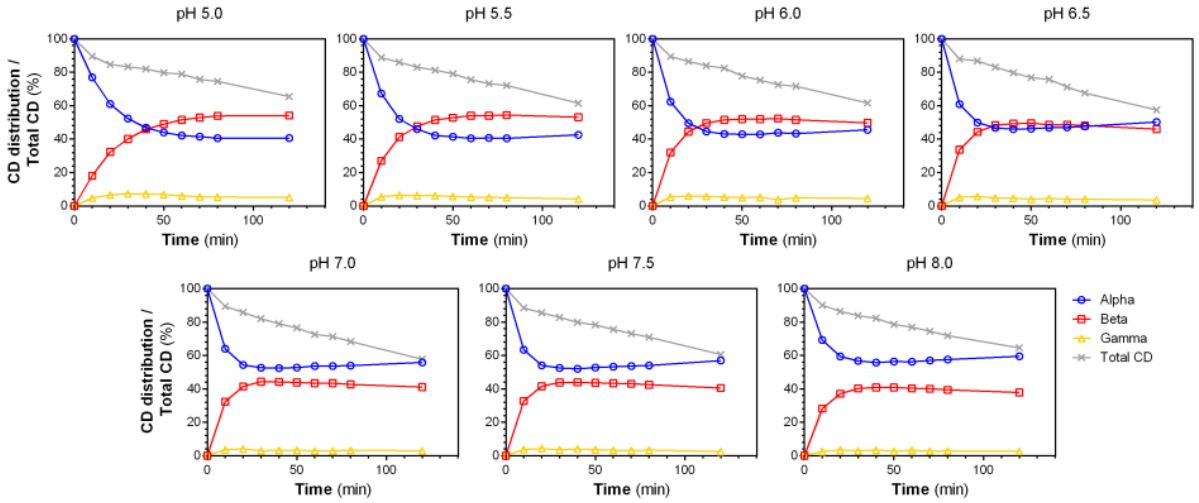


Figure S3. Distribution of CDs in DCLs as a function of time at pH 5.0 and pH 8.0 with five different templates (aminobenzoic acid (**1**), cyclohexane acetic acid (**5**), benzimidazole (**2**), 4-methyl cinnamic acid (**4**), warfarin(**6**)). The DCLs are under *pseudo*-equilibrium control, and the equilibrium time point differs between the different DCLs.

S2.3 DCLs with 4-nitrophenol (3) at varying pH

(a) 2 mM template concentration



(b) 10 mM template concentration

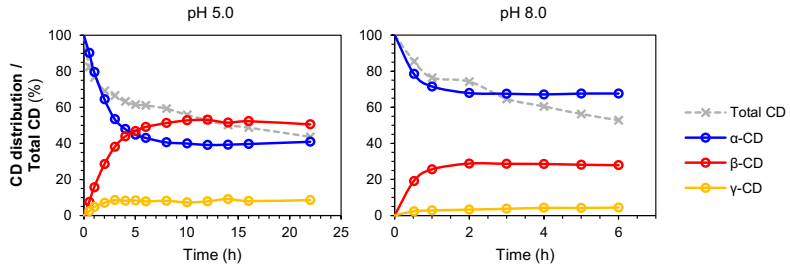


Figure S4. (a) Distribution of CDs in DCLs as a function of time in the pH range 5.0-8.0 with template 4-nitrophenol (**3**) at 2.0 mM concentration. The pseudo-equilibrium is reached after 80 min at pH 5.0, after 60 min at pH 5.5, after 50 min at pH 6.0, and after 30 min at pH 6.5-8.0. (b) Distribution of CDs as a function of time at pH 5.0 and pH 8.0 with template 4-nitrophenol (**3**) at 10.0 mM concentration. The pseudo-equilibrium is reached after 12 hours at pH 5.0 and after 2 hours at pH 8.0.

S2.4 Summary of CD distributions

In the following tables are listed the equilibrium CD distributions obtained for the DCLs presented in Figure 2 and 3 of the manuscript.

Table S1. Distribution of CDs in DCLs with various templates.

Template	Equilibrium CD distrbution α-CD/β-CD/γ-CD	
	pH 5	pH 8
No template	41 / 52 / 8	38 / 54 / 7
1	41 / 51 / 8	38 / 54 / 8
2	39 / 53 / 8	35 / 58 / 8
3	40 / 55 / 5	56 / 41 / 3
4	50 / 44 / 6	34 / 61 / 4
5	17 / 81 / 2	24 / 73 / 3
6	40 / 52 / 8	29 / 66 / 5

Table S2. Distribution of CDs in DCLs templated with 4-nitrophenol (**3**) at various pH.

pH	Equilibrium CD distribution α-CD/β-CD/γ-CD
5.0	41 / 54 / 5
5.5	41 / 54 / 5
6.0	43 / 52 / 5
6.5	47 / 48 / 5
7.0	53 / 44 / 3
7.5	53 / 44 / 4
8.0	57 / 40 / 3

S3 NMR Titrations

The systematic change in the chemical shift of the 4-nitrophenol protons as a function of CD concentration was monitored for the titrations of 4-nitrophenol (**3**) with α-, β-, and γ-CD, each at two different pH values (pH 5.0 and pH 9.6). Fitting the ^{1}H NMR data to a 1:1 binding model yielded the best fit values for the maximum change in the chemical shift, $\Delta\delta_{max}$, and the binding constant, K_a (Table 1 in the manuscript and Table S3). ^{1}H NMR spectra and fitted binding isotherms for each titration are shown in Figures S5 to S10.

Table S3. The table shows the maximum change in the chemical shift ($\Delta\delta_{max}$) and the binding constant (K_a) and their errors for the interactions between 4-nitrophenol and α-, β-, and γ-CD, respectively, at pH 5.0, and 9.6. The data are best fit values obtained from fitting ^{1}H NMR data for the proton that experiences the biggest chemical shift change to a 1:1 binding model.

	pH 5.0		pH 9.6	
	$\Delta\delta_{max}$ (ppm)	K_a (M^{-1})	$\Delta\delta_{max}$ (ppm)	K_a (M^{-1})
α-CD	0.31±0.003	165±5	0.31±0.002	1624±55
β-CD	0.05±0.001	265±19	0.07±0.003	412±52
γ-CD	0.15±0.010	42±5	0.16±0.010	93±21

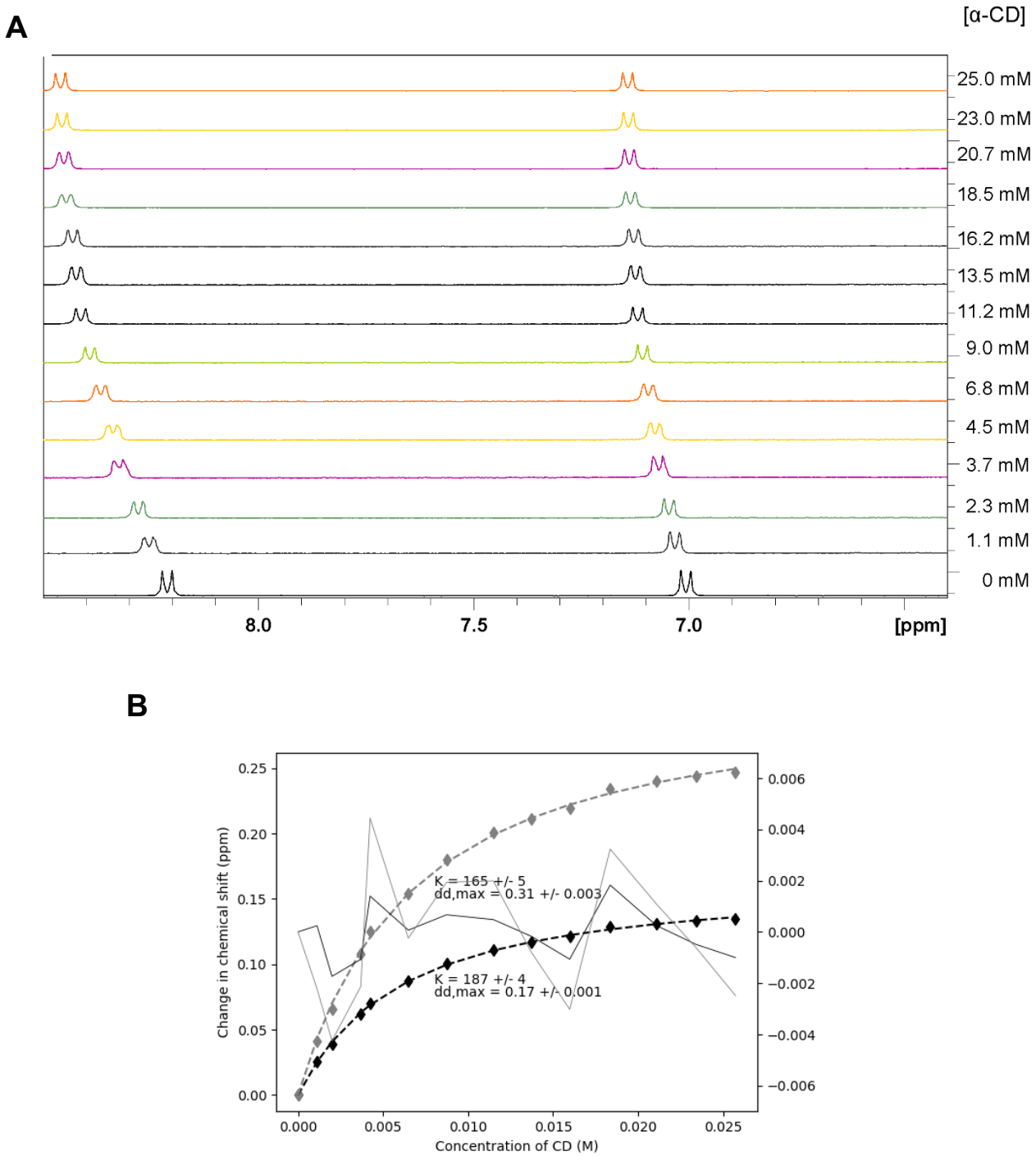


Figure S5. (a) Partial ^{1}H NMR spectra (aromatic region) of 4-nitrophenol in the presence of increasing amounts of α-CD in 50 mM acetate-phosphate buffer in D_2O, pH 5.0. (b) Fitted binding isotherm showing the change in the chemical shift (ppm) as a function of the CD concentration (M). The dashed lines represent the best fit to a 1:1 binding isotherm for each of the two signals. The residuals are plotted to show that there are no systematic deviations from the binding model.

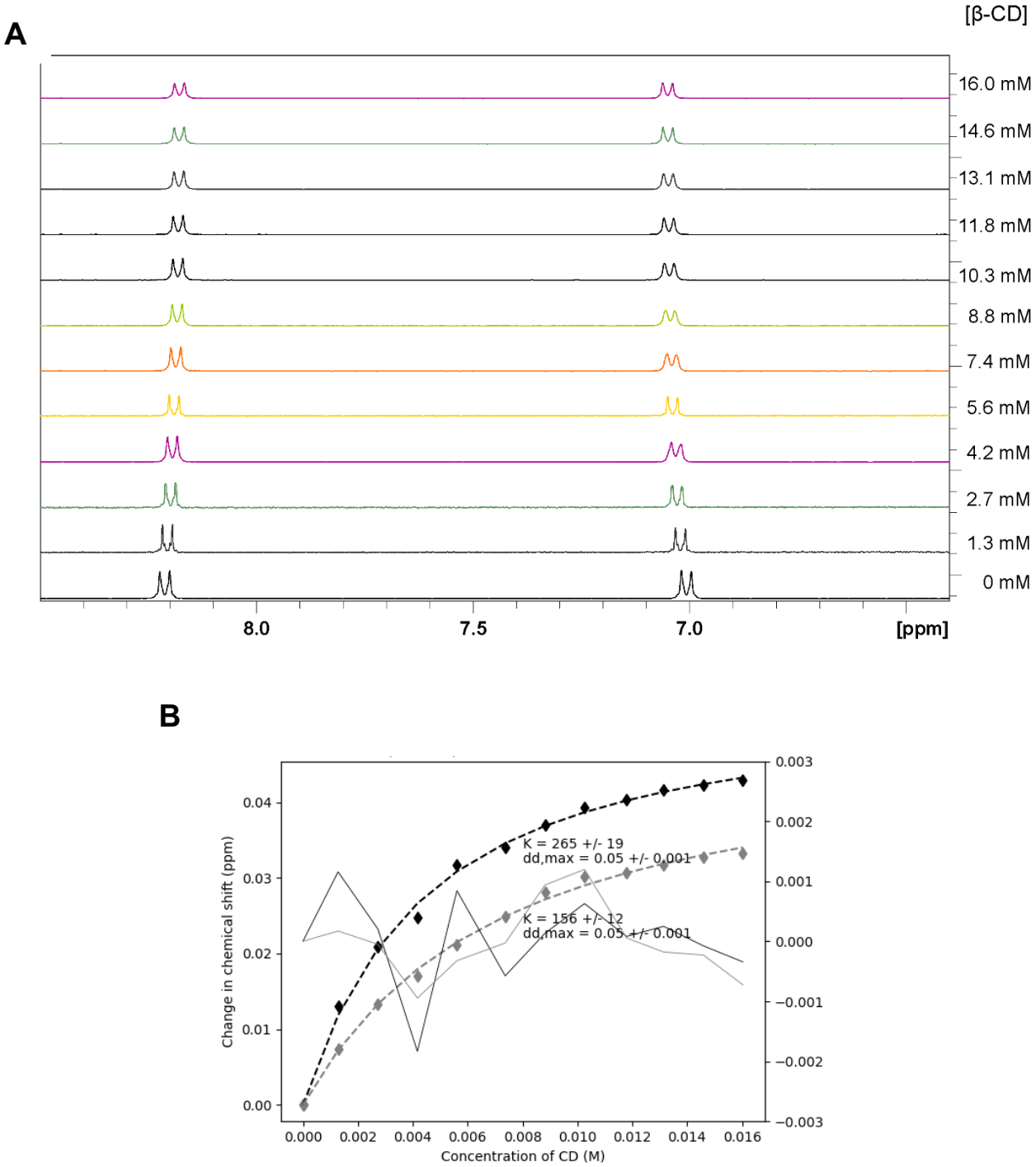


Figure S6. (a) Partial ^{1}H NMR spectra (aromatic region) of 4-nitrophenol in the presence of increasing amounts of β-CD in 50 mM acetate-phosphate buffer in D_2O, pH 5.0. (b) Fitted binding isotherm showing the change in the chemical shift (ppm) as a function of the CD concentration (M). The dashed lines represent the best fit to a 1:1 binding isotherm for each of the two signals. The residuals are plotted to show that there are no systematic deviations from the binding model.

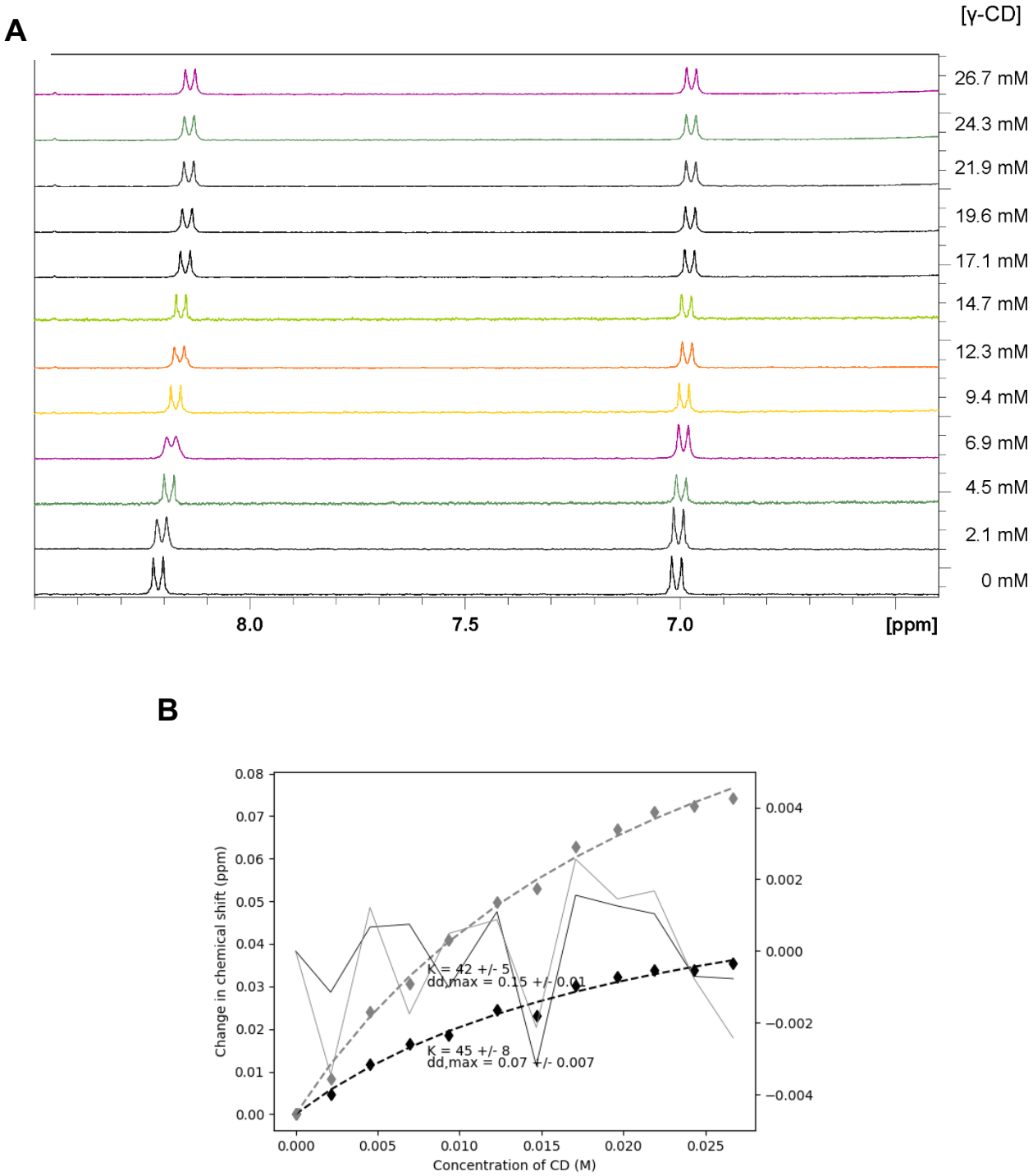


Figure S7. (a) Partial ^{1}H NMR spectra (aromatic region) of 4-nitrophenol in the presence of increasing amounts of γ-CD in 50 mM acetate-phosphate buffer in D_2O, pH 5.0. (b) Fitted binding isotherm showing the change in the chemical shift (ppm) as a function of the CD concentration (M). The dashed lines represent the best fit to a 1:1 binding isotherm for each of the two signals. The residuals are plotted to show that there are no systematic deviations from the binding model.

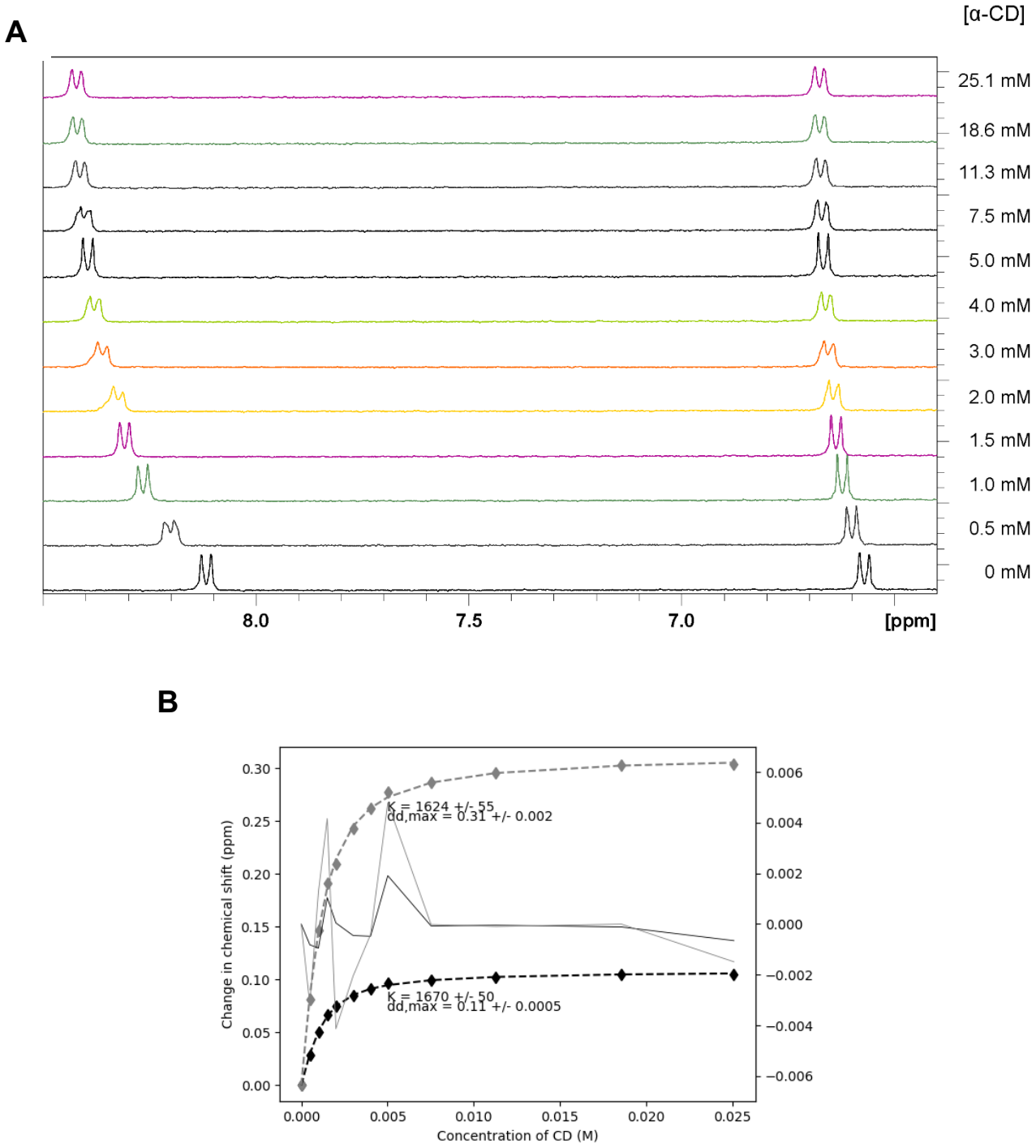


Figure S8. (a) Partial ^{1}H NMR spectra (aromatic region) of 4-nitrophenol in the presence of increasing amounts of α-CD in 50 mM acetate-phosphate buffer in D_2O, pH 9.6. (b) Fitted binding isotherm showing the change in the chemical shift (ppm) as a function of the CD concentration (M). The dashed lines represent the best fit to a 1:1 binding isotherm for each of the two signals. The residuals are plotted to show that there are no systematic deviations from the binding model.

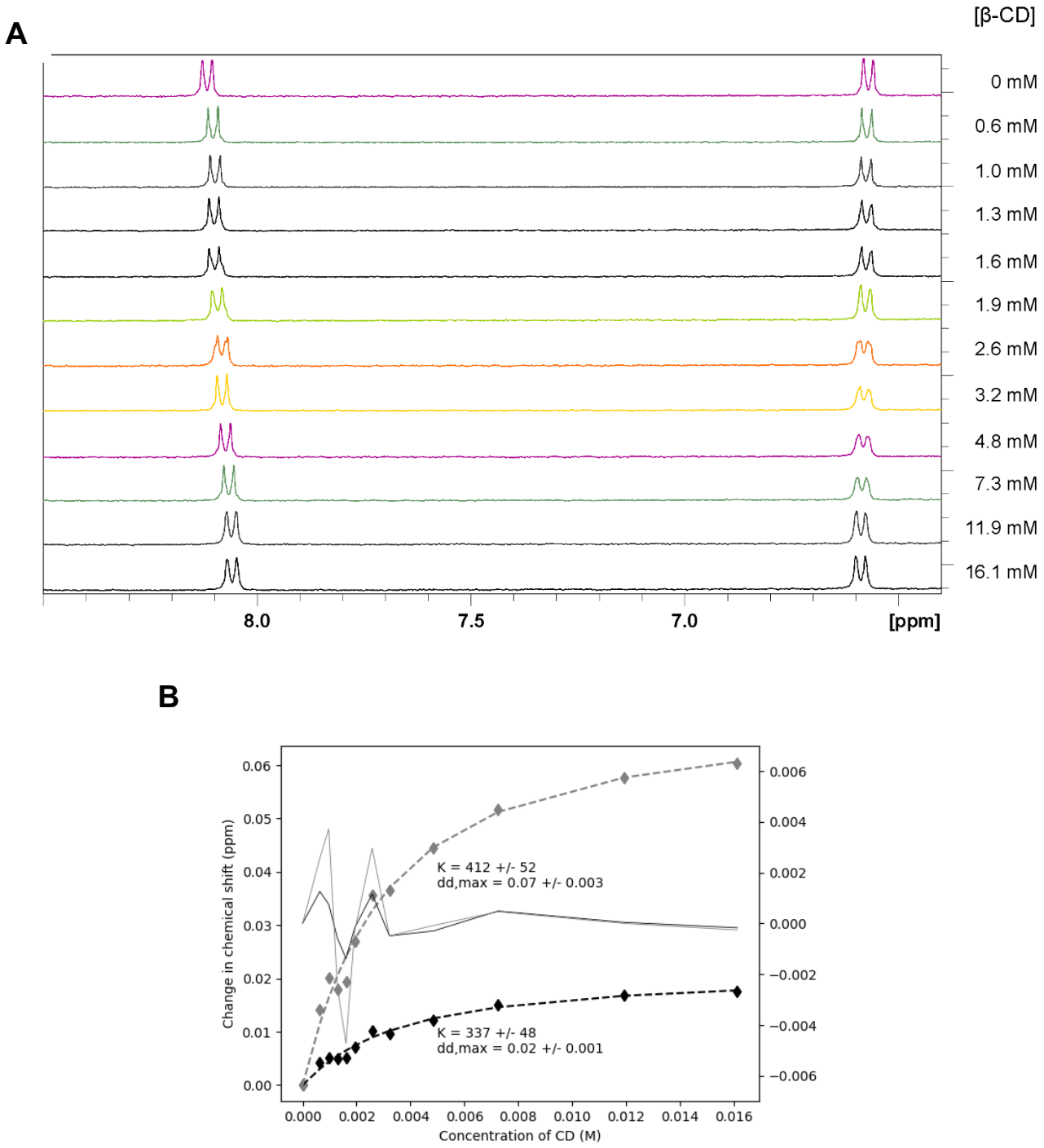


Figure S9. (a) Partial ^{1}H NMR spectra (aromatic region) of 4-nitrophenol in the presence of increasing amounts of β-CD in 50 mM acetate-phosphate buffer in D_2O, pH 9.6. (b) Fitted binding isotherm showing the change in the chemical shift (ppm) as a function of the CD concentration (M). The dashed lines represent the best fit to a 1:1 binding isotherm for each of the two signals. The residuals are plotted to show that there are no systematic deviations from the binding model.

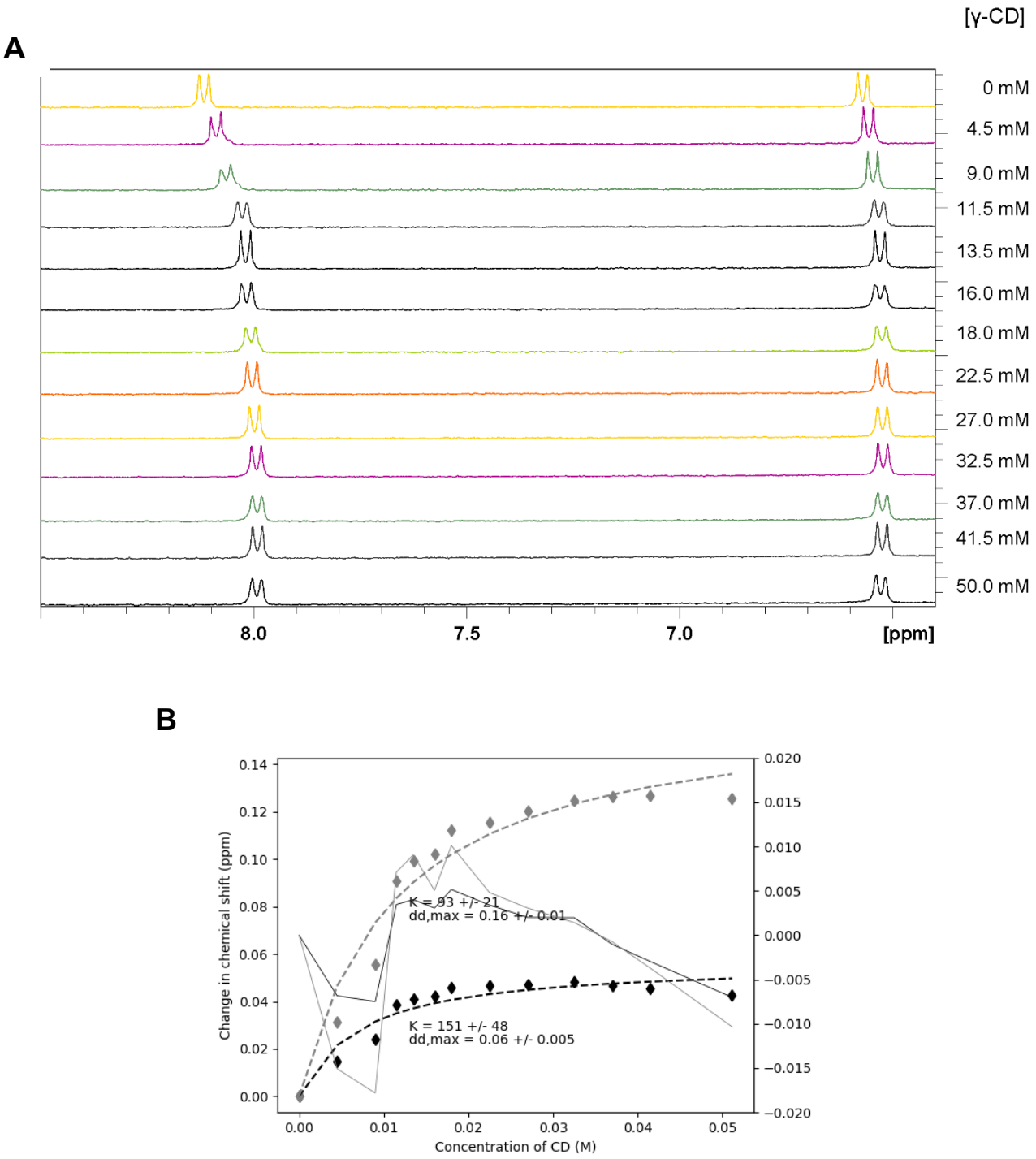


Figure S10. (a) Partial ^{1}H NMR spectra (aromatic region) of 4-nitrophenol in the presence of increasing amounts of γ-CD in 50 mM acetate-phosphate buffer in D_2O, pH 9.6. (b) Fitted binding isotherm showing the change in the chemical shift (ppm) as a function of the CD concentration (M). The dashed lines represent the best fit to a 1:1 binding isotherm for each of the two signals. The residuals are plotted to show that there are no systematic deviations from the binding model.

S4 DCL Simulations

S4.1 DCL simulation

Using the approach from the DCLSim software developed in the Otto group,[3] it is possible to simulate a DCL of CDs with and without template. DCLSim calculates the distribution of products in a DCL at equilibrium based on equilibrium constants and initial concentrations of building block and template. By combining a set of equations, which describe the mass balance in the system, and the equilibrium constant expressions, and solving these equations simultaneously, it is possible to calculate the relevant concentrations. Specifically, the calculations consider formation equilibria between starting material and products, and the binding equilibria between products and template.

Experimental binding constants (K_a) between products and template were available from ^{1}H NMR titrations. Relative formation constants (K_f) from DCLs without template were used for the calculations. K_f for α-, β-, and γ-CD were calculated according to the following equation:

$$K_f(CD_n) = \frac{[CD_n]_{eq}}{[G_0]^n}$$

where CD_n is a CD with n glucopyranose units, $K_f(CD_n)$ is the relative formation constant of the CD, $[CD_n]_{eq}$ is the concentration (M) of the CD at *pseudo*-equilibrium and $[G_0]$ is the total concentration of glucose units (M) in the transient CD DCL at *pseudo*-equilibrium. The calculated K_f values were 9.5×10^4 M^{-5}, 2.6×10^6 M^{-6}, and 7.6×10^6 M^{-7} at both pH 5.0 and pH 8.0, respectively.

S4.2 DCL simulations at using predicted binding constants for any pH

The approach from the DCLSim software were further developed by considering the effect of pH on the binding constants (K_a) between products and template. The weighted average binding constant (K_{app}) between CD and guest as a function of pH was previously published[4,5] (See Figure S11 for an example of binding between 4-nitrophenol and α-, β- and γ-CD). The average binding constant (K_{app}) was calculated according to the following equation:

$$K_{app} = (1 - \alpha)K_{neu} + \alpha K_{ion} \qquad \text{Eq. S1}$$

Where K_{neu} and K_{ion} are the binding constants for the complex formation between CD and neutral ionized templates, respectively. α is the dissociation degree of the template, (ionized template concentration/total template concentration), and it is linked to pH through the Henderson-Hasselbalch equation.

$$a = {}^{1}/_{(10^{pKa-pH} + 1)} \qquad \text{Eq. S2}$$

Hence, K_{app} is described as a function of pH based on the pK_a of the template, and the binding constants (K_{neu} and K_{ion}) of neutral and ionized template to CD.

The pK_a value of the template (4-nitrophenol) is 7.1, and the binding constants K_{neu} and K_{ion} for each CD were available from ^{1}H NMR titrations at pH 5.0 and 9.6 (See Table S1). At pH 5.0, 4-nitrophenol

is 99.3 % ionized, and at pH 9.6, it is 0.4 % ionized, hence, the two pH values represent ionized and neutral species of 4-nitrophenol. The weighted average binding constant (K_{app}) was then used as input for the DCLSim calculations, yielding the distribution of products in a DCL at *pseudo*-equilibrium as a function of pH. As seen in the manuscript, the agreement between DCL experiments and DCLSim calculations was excellent.

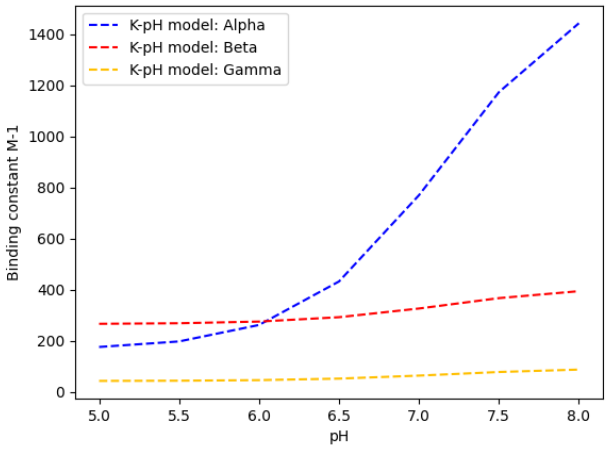


Figure S11. Weighted average binding constants of α-, β-, and γ-CD to 4-nitrophenol as a function of pH, in the pH range relevant for the enzymatic DCLs. The curves are based on the theoretical expression for the average binding constant (K_{app}), where the inputs are the pK_a of 4-nitrophenol and experimental binding constants determined by ^{1}H NMR.

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