Supporting Information

Building up cyclodextrins from scratch – templated enzymatic synthesis of cyclodextrins directly from maltose

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S1. EXPERIMENTAL DETAILS

S1.1 MATERIALS

Buffer salts, D_2O, α-glucan starting materials and templates were purchased from commercial suppliers and used as received unless stated otherwise. HPLC grade solvents or better as supplied from commercial vendors were used for chromatographic experiments. High purity water was obtained by filtering deionized water through a commercially available water purifications system (Merck Millipore Synergy UV). Colorless Corning CoStar 0.65 mL centrifuge tubes were used for sample preparation (for HPLC, see details below), while colorless 2 mL glass vials with PTFE-lined screw cap septa with a 0.2 mL glass insert were used for reactions (for HPLC monitoring) as well as for short term sample storage and injection on chromatographic equipment. NMR samples were measured in capped standard 5 mm borosilicate glass NMR tubes. A stock solution of CGTase derived from *Bacillus macerans* was kindly gifted to our group by Amano Enzyme, Inc., Nagoya, Japan. It was stored at 5 °C and used as received. The concentration of CGTase in this stock solution was assessed by nanodrop (further details below).

S1.2 INSTRUMENTS

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 autosampler which was maintained at 20 °C. Injection volumes were typically in the 10.0 – 15.0 µL range, depending on analyte concentrations. For detection, the chromatographic system was connected to an Agilent Technologies 1260 Infinity ELSD, operating at evaporator and nebulizer temperatures of 90 and 70 °C, respectively, and an N_2 gas flow of 1.0 L/min (see full calibration details below). NMR spectroscopy was performed on a Bruker 400 MHz instrument with a broadband (multiple nuclei) probe equipped with a heating coil to maintain sample temperature.

S1.3 MONITORING REACTIONS USING ^{1}H NMR SPECTROSCOPY

A stock solution (500 µL) containing **G2** and possibly template at the desired concentrations was put into an NMR tube and maintained in the instruments for at least five minutes to ensure it had heated up to the desired temperature. A reference spectrum was measured before starting the reaction. But for a few exceptions (mentioned below), all spectra were acquired with 32 scans and were recorded at 298 K with a delay time of 10 seconds between scans to ensure complete relaxation before the next pulse. The reaction was started by addition of CGTase (25 µL of a 12 mg/mL stock resulting in 0.57 mg/mL in the reaction) and immediately turning the capped NMR tube upside down at least three times to ensure proper mixing. Vigorous shaking was avoided to prevent formation of emulsions, which can be quite prevalent with several of the amphiphilic templates applied in these reactions. The exact time of CGTase addition according to the instrument computer was logged, and the exact time point of all subsequent spectra were correlated to this based on the acquisition date/time logged by the computer, properly corrected so the time point for each spectrum correlates to the midpoint of the acquisition period. In most cases, the instrument was simply set to keep measuring consecutive spectra until stopped manually, resulting in roughly 6 minutes and 49 seconds between each data point. For reactions starting from **G2** at 2 mg/mL in the presence of 20 mM ACA, the number of scans was doubled to 64 in order to obtain higher quality data, with an acceptable concomitant loss of time-resolution (ca. 11 minutes and 30 seconds between each data point).

All spectra were processed in MestreNova applying batch processing to allow analysis of the large number of spectra. The spectra were autophased, then multiple point baseline correction was applied before the three regions of interest (CDs at 4.96 – 5.06 ppm, reducing end α-H1 at 5.18 – 5.13 ppm and the H1 from the glucopyranose units in linears that are not the reducing ends at 5.36 – 5.28 ppm) were integrated. Due to being much closer to the suppression frequency of the residual HDO signal at 25 °C, the cyclodextrin region was found to be under-reported in spectra measured at 25 °C. This was accounted for by calculating a conversion factor from a spectrum measured at 60 °C, where the HDO signal (and therefore the suppression frequency) is out of the way of the sugar H1 signals due to the temperature dependency of the HDO peak's ppm value. It was assumed that the integral for the H1 peak of the glucopyranose units that are not reducing ends is unaffected by the water suppression. The relative integrals observed in this reference spectrum at 60/25 °C were 1.425/1.085 = 1.31 for the CD signal region, 0.786/0.783 = 1.00 for the reducing end α-H1 signal, indicating that also the α-H1 signal is essentially unaffected by the water suppression. Therefore, the absolute CD integral was adjusted by a factor of 1.31. Only the α-H1 signal of the reducing end is integrated (the β-H1 signal is too close to the suppressed HDO peak for meaningful integration). Thus, to calculate the total reducing end integral, the α-H1 absolute integral was multiplied by (1+1.63), since the α:β ratio of D-glucose is reported to be 1:1.63 (38:62) at 25 °C.[S1] The molar CD yield was calculated from these adjusted absolute integrals.

S1.4 Monitoring reactions using HPLC-ELSD

Reactions were started by adding CGTase (50 µL of a 12 mg/mL stock solution per mL reaction resulting in 0.57 mg/mL in the reaction) into a pre-tempered (at ambient temperature (22 °C) or 30 °C) stock solution of **G2** (5 – 10 mg/mL) with or without template (10 – 20 mM). The temperature in reactions at 30 °C was maintained by keeping the reaction vessels in a specially fitted thermostatically heated aluminium block. The exact time of CGTase addition was logged, and aliquots (usually 2.0 µL) were transferred to a quenching mixture (usually 80.0 µL, i.e. a dilution factor of 41) in a centrifuge tube within ± 5 seconds of the desired sample time. The quenching mixture was made up of 1 % TFA (to stop the enzyme reaction by acidification) and 10 mM NH_4Cl (to alter the retention of phosphate ions, which would otherwise overlap with **G1** during the chromatographic analysis) in 3:1 MeCN/water (to ensure proper mixing with the eluent during HPLC injection by matching the solvent composition in the sample). The sample was centrifuged at ca. 10,000 G for 4 minutes and the top fraction (usually 75 µL) was transferred to a 2 mL clear glass vial with a 0.2 mL glass insert and closed airtight using a screw cap with a PTFE lined rubber septum for subsequent analysis on the chromatographic equipment. This centrifugation was performed only to limit the amount of enzyme being injected onto the column (for longer column life time), and it had no influence on the carbohydrate composition in the sample, as assessed by comparison to non-centrifuged samples.

For chromatographic analysis, a HILIC-type column (Waters Acquity UPLC BEH Amide 1.7 µm 2.1 × 150 mm) maintained at 30 °C was used and typical injection volumes were 15.00 µL. The eluents were water (A) and acetonitrile (B), both with 0.1 % formic acid. For separation, a linear gradient program running from 25 to 45 % eluent A over 8.00 minutes with a flow rate of 0.60 mL/min was used, after which the column was washed for three minutes with 100 % eluent A. Before each injection, at least five column volumes of the starting eluent was applied to condition the column.

The resulting chromatograms were analysed and the peak area was converted to a concentration (by weight) through calculations based on calibrations performed individually with **G1** – **G8** as well as α-CD, β-CD and γ-CD by analyzing stock solutions using the above-mentioned chromatographic method. The calibrations were based on µg injected in the 0.018 – 3.66 µg range, and the resulting response

curves were fitted non-linearly (using OriginPro 2018b from OriginLab Corp.) to a simple power equation:

$$M = kA^p$$

where M is the injected mass of the compound, A is the area under the peak in the chromatogram, while the fitted parameters k and p are referred to as the coefficient and the exponent, respectively. By knowing the exact dilution factor of the sampled aliquot and the injection volume used on the chromatographic equipment, the calculated injected mass of a compound can easily be converted to a concentration by weight in the actual reaction mixture.

All injections were performed in triplicate and good fits were obtained for all the analytes in this study, as is evidenced by the calibration curves in Fig. S1 below.

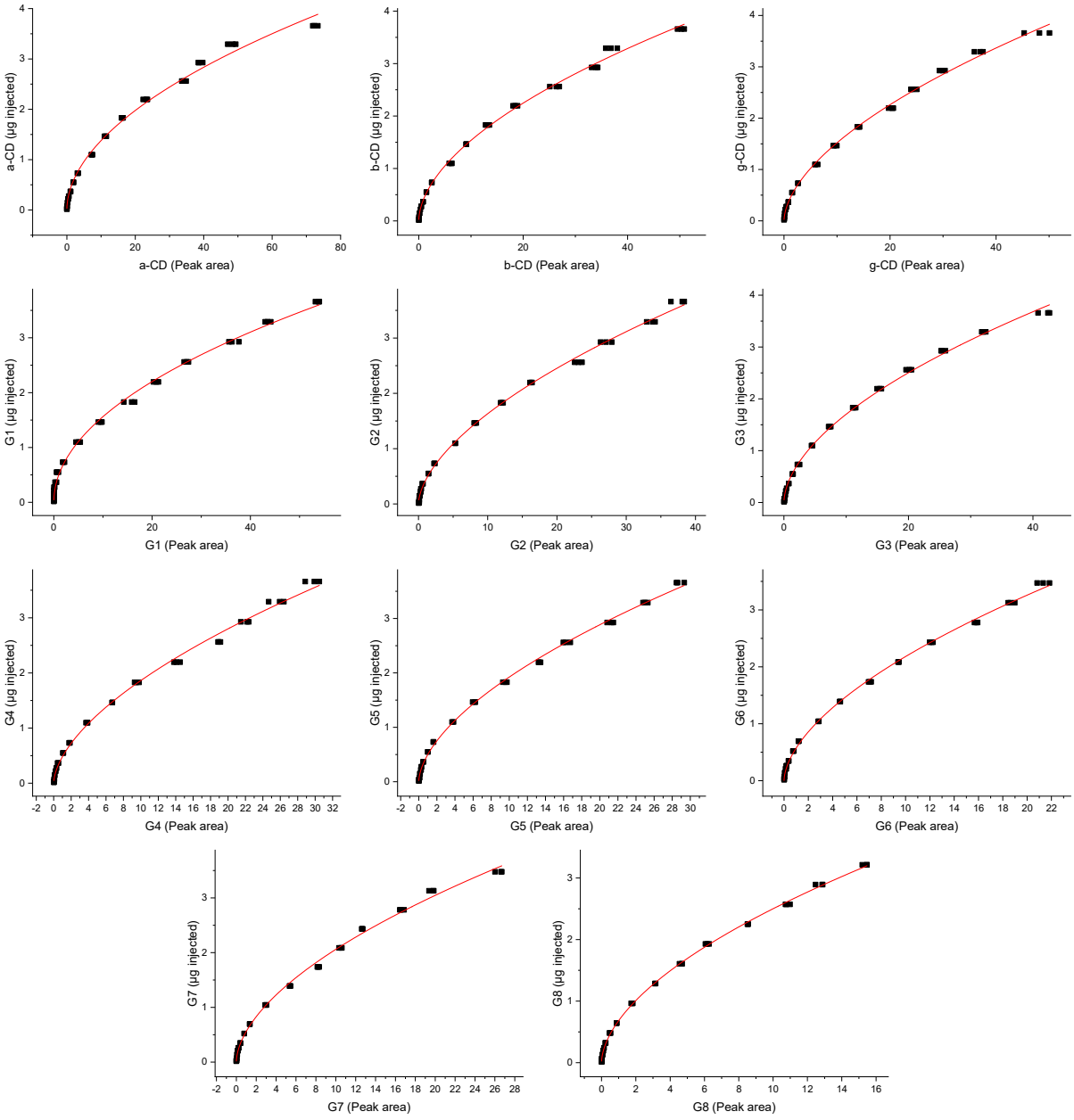

Fig. S1. Calibration curves obtained for α-CD, β-CD, γ-CD and **G1** – **G8**. Data points represented by black squares and best fit to the power equation mentioned above shown as a red line. Values from the best fits are shown in Table S1 below.

The values of k and p obtained from these fits, along with the errors on the fits and the resulting adjusted R^2-values, are listed in Table S1 below.

Table S1. Values obtained from fit for the calibration of the HPLC-ELS method.

Species	*k*	error on *k*	*p*	error on *p*	adjusted R^2
α-CD	0.416	0.014	0.520	0.009	0.995
β-CD	0.434	0.009	0.549	0.006	0.998
γ-CD	0.406	0.009	0.574	0.006	0.998
G1	0.499	0.016	0.495	0.009	0.996
G2	0.421	0.009	0.588	0.007	0.998
G3	0.473	0.009	0.556	0.006	0.998
G4	0.481	0.012	0.588	0.009	0.997
G5	0.501	0.009	0.584	0.006	0.999
G6	0.574	0.008	0.580	0.005	0.999
G7	0.561	0.011	0.565	0.007	0.998
G8	0.684	0.006	0.563	0.004	0.999

Due to impurities of **G5** in commercial grade **G6**, **G6** in commercial grade **G7**, and **G7** in commercial **G8**, the obtained calibration method for **G5** was first used to assess the amount of **G5** in the commercial source of **G6** used for this calibration. It was found to contain 5.1 % **G5** by mass. Since the two species are separated during the chromatographic analysis, this could easily be accounted for by adjusting the mass of **G6** accordingly during the subsequent data analysis. Having thus obtained a valid calibration for **G6**, the same procedure was used to account for the **G6** impurities (4.9 % by mass) in the commercial source of **G7**, and using the valid calibration thus obtained for **G7**, the **G7** impurities (12.2 % by mass) in the commercially sourced **G8** could be assessed in order to obtain valid calibration curves for all compounds of interest.

S2. PATHWAYS TO CDS FROM **G2**

In order to turn **G2** into e.g. α-CD using transglycosylation reactions catalyzed by CGTase, one can imagine a sequential reaction process taking place, in which one **G2** is elongated with the D-glucopyranose unit that is not the reducing end from another **G2** to form a **G3** (and a **G1**). This **G3** can then be elongated in the same manner using another **G2** (and again producing one **G1**), and so on and so forth until a **G7** has been formed. **G7** is long enough to form one α-CD in a macrocyclisation (and one **G1**), as illustrated in Fig. S2.

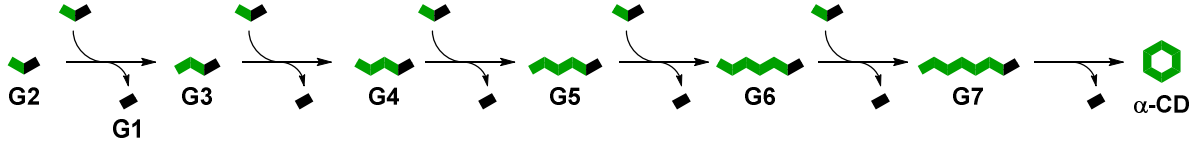

Fig. S2. Formation of α-CD from **G2** in a sequential pathway. The transglycosylations are catalyzed by CGTase.

Due to the high **G2** concentration present in the early stages of CGTase-catalysed conversion of **G2**, the parallel reaction pathway seen in Fig. S2 is not completely unrealistic, but in a real life mixture, more complex pathways are also very likely to be followed. For instance, once reasonable amounts of **G4** molecules have been formed, two of these could combine to make a **G7** (and a **G1**) which could macrocyclise to from α-CD, as in Fig. S3.

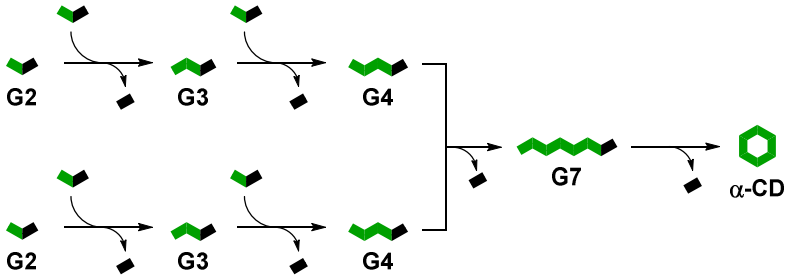

Fig. S3. Formation of α-CD from **G2** by parallel **G4** production followed by combination to **G7** and then macrocyclisation.

Note that the reaction pathways in Figs. S2 and S3, though different, require the same number of **G2** molecules (six in both cases) and have the same number of individual reaction steps (also six in both cases). They also produce the same number of **G1** molecules (six in both cases). Thus, no matter the pathway taken, one can summarise the formation of α-CD from **G2** as illustrated in Fig. S4.

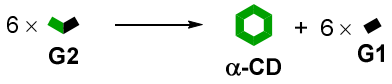

Fig. S4. Generalised reaction scheme for formation of one α-CD molecule from six **G2** molecules (with concomitant production of six **G1** molecules).

And Fig. S5 illustrates the reaction in even more general terms for formation of any **CDn** consisting of *n* D-glucopyranose units (valid for $n > 6$) from **G2**:

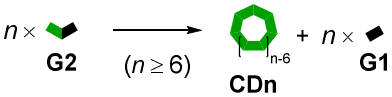

Fig. S5. Generalised reaction scheme for formation of one **CDn**, corresponding to a cyclodextrin with *n* D-glucopyranose units, from **G2** (with concomitant production of *n* **G1** molecules). *n* is a positive integer that must be six or above in order to make any CD.

In order to understand the time-lag for CD production observed in reactions from **G2**, one has to consider the pathways shown in Figs. S2 and S3, as they highlight the lengthy pathways that must be

followed, which the generalized reaction schemes in Figs. S4 and S5 fail to capture. For reactions starting from **G4**, a significantly shorter pathway that only requires one intermolecular transglycosylation between two **G4** molecules to make one **G7** molecule (compared to the five steps needed from **G2**) can be imagined, as illustrated in Fig. S6.

Fig. S6. Formation of α-CD from **G4** in a sequential pathway. Only one intermolecular transglycosylation (compared to five when starting from **G2**) followed by one macrocyclisation is needed.

As is also evident from Fig. S6, starting a library directly from **G7** would mean no intermolecular transglycosylations are needed in order to make a CD in a macrocyclisation.

Looking at the available pathways for all the α-glucans from **G2** up to **G8**, one can reasonable divide them into three main categories. One category of *long α-glucans*, consists of **G7** and longer α-glucans, that can make CDs directly without the need for preceding intermolecular transglycosylations. The next category consists of the *medium length α-glucans* **G4**, **G5** and **G6**, which all have to go via a pathway including at least one intermolecular transglycosylation before being able to make CDs. The last group, consisting of the *short α-glucans* **G2** and **G3**, need to go via pathways that have more than one intermolecular transglycosylation step before being able to make CDs.

S3. CD YIELDS OVER TIME IN CD-SPIKED REACTIONS FROM **G2**

S3.1 SPIKING WITH α-CD

The reactions were started from **G2** with 0, 5, 10 or 20 % by weight of α-CD, keeping a constant total α-glucan concentration of 10 mg/mL, and the CD yield over time was monitored by ^{1}H NMR spectroscopy. Changes in relative peak intensities (as described in the experimental section above) were used to determine the CD yield over time. Representative examples of the obtained spectra are shown in Fig. S7.

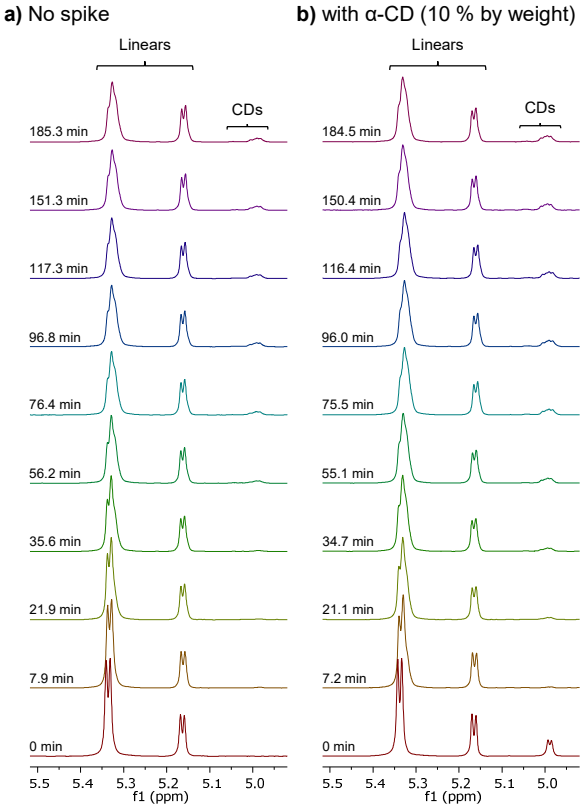

Fig. S7. ^{1}H NMR spectra obtained during untemplated reactions started from **G2** with (a) no other α-glucan spike or (b) with 10 % by weight of α-CD spike (total α-glucan concentration of 10 mg/mL) in phosphate buffered (50 mM, pH 7.5) D_2O maintained at 25 °C.

It is noteworthy that the CD signal almost completely disappears as soon as the enzyme is added to the spiked reactions, a behavior that was observed for all spiked reactions (example in Fig. S7b, further examples below). When comparing the spiked reaction above (Fig. S7b) to the unspiked reaction (Fig. S7a), a slightly broader peak is observed for the internal α-H1 of the linear species (at 5.3 – 5.4 ppm) in the first spectrum after enzyme addition (i.e. after ca. 7 – 8 minutes of reaction), indicating that the spiking material is immediately converted into linear α-glucans which then aid in the build-up of CDs.

Over time, this results in slightly higher CD yields obtained after ca. 1.5 to 2 hours with higher α-CD content present from the beginning (Fig. S8 below). However, it is also evident, that the increase in CD yields are significantly lower than the amount of CDs put in from the beginning, demonstrating that spiking reactions started from **G2** leads to rapid conversion of the added CD into linear α-glucans rather than leading to a net increase in CD production.

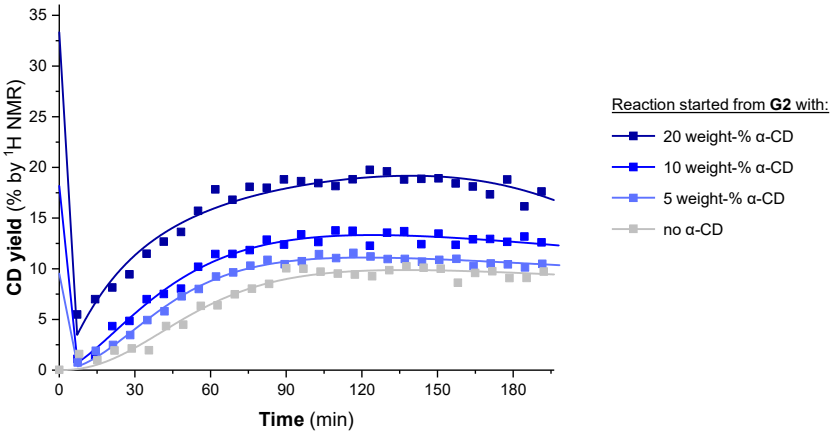

Fig. S8. CD yields over time in reactions from **G2** with 0 – 20 % by weight of α-CD (total α-glucan concentration of 10 mg/mL) based on ^{1}H NMR spectroscopy.

Note that starting from 10 % by weight of α-CD and 90 % by weight of **G2** is equivalent to starting the reaction at a molar CD yield of ca. 18.2 %. This is because only 55 % of the sugar material by weight can be converted into CDs. All the 10 % by weight of α-CD consists of hydrolysable glucopyranose units that can become part of a CD, but only half of the **G2** material are hydrolysable glucopyranose units, and thus the 90 % by weight of **G2** only contributes 45 % by weight of hydrolysable units. Thus, the starting concentration of 10 % by weight of α-CD out of a total of 55 % by weight of hydrolysable glucopyranose units correspond to ca. 18.2 % of the hydrolysable glucopyranose units present that can be used to form CDs, are already part of a CD (10/55 ≈ 0.182). Similar calculations leads one to the conclusion that starting reactions from **G2** in the presence of 5 % or 20 % by weight of α-CD (or any α-glucan material that consists only of hydrolysable glucopyranose units) corresponds to starting the reaction at molar CD yields of ca. 8.7 % or 33.3 %, respectively, as is illustrated by where the blue lines start in Fig. S8 above.

S3.2 SPIKING WITH OTHER CDS

To investigate if longer length α-glucan spiking material could achieve better CD yields from **G2**, reactions were also started from **G2** in presence of 10 weight-% β-CD, γ-CD or cycloamylose, which is a commercial material made up of large ring cyclodextrins with degrees of polymerisation of ca. 25 – 50. The reactions were monitored by ^{1}H NMR spectroscopy as above, but the results showed no appreciable difference in the CD yields over time with respect to the spiking material (Fig. S9).

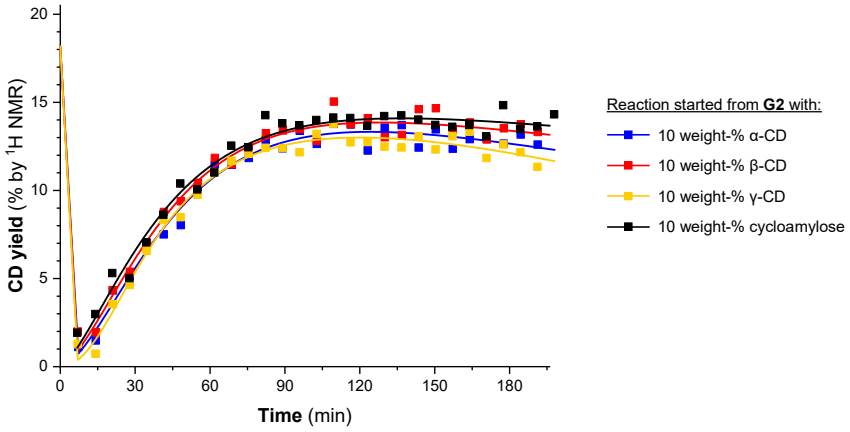

Fig. S9. CD yields over time in reactions from **G2** with 10 % by weight of different α-glucans (total α-glucan concentration of 10 mg/mL) based on ^{1}H NMR spectroscopy.

S3.3 COMBINING SPIKING AND TEMPLATING

While the spiking experiments did not increase net CD production, we were encouraged by the high CD yield increases seen with addition of templates. Therefore we also tested whether spiking the templated reactions could lead to an improved CD yield. Reactions from **G2** in presence of either ACA or $NaBPh_4$ (10 mM) were started with 10 % α-CD (total α-glucan concentration of 10 mg/mL) and monitored by ^{1}H NMR spectroscopy according to the procedure described above (Fig. S10).

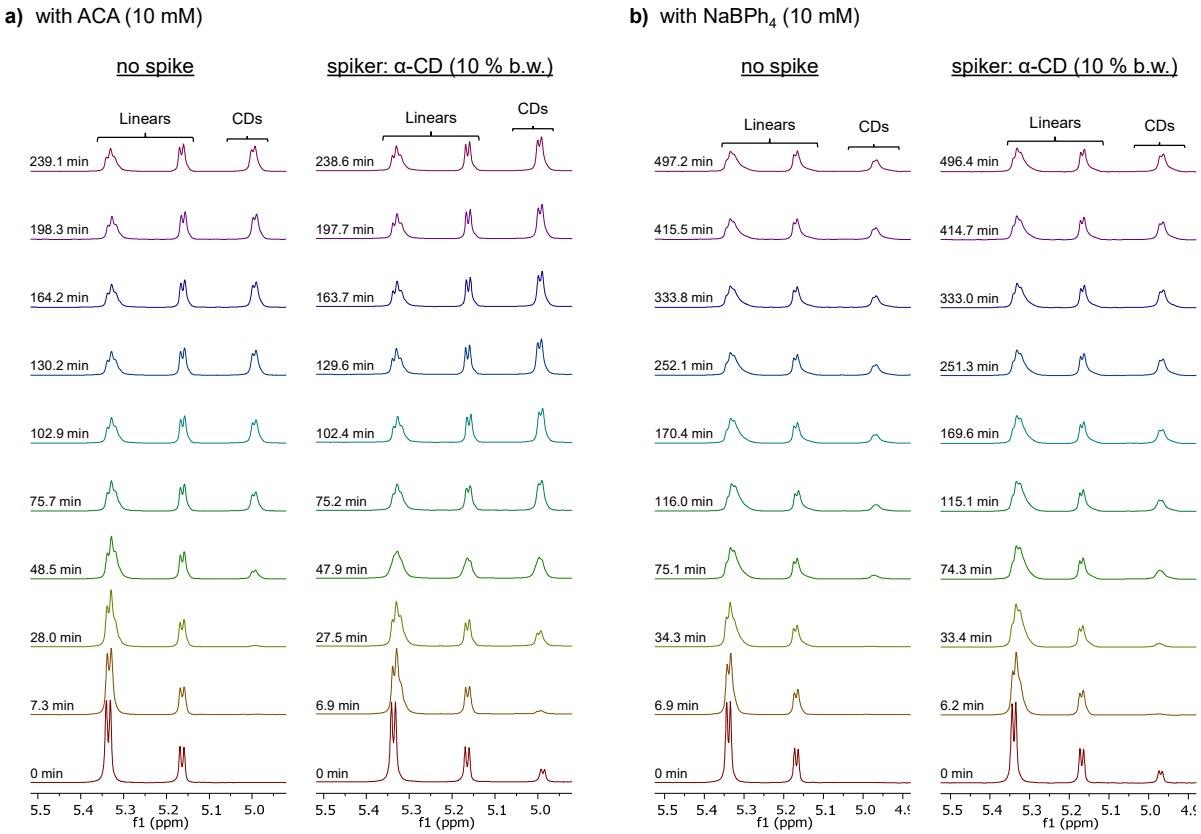

Fig. S10. ^{1}H NMR spectra obtained during templated reactions from **G2** with and without α-CD spike (10 % by weight, total α-glucan concentration 10 mg/mL) in sodium phosphate buffered (50 mM, pH 7.5) D_2O maintained at 25 °C. (a) With ACA (10 mM) as template. (b) With $NaBPh_4$ (10 mM) as template.

The results showed that spiking with α-CD in presence of templates did drastically reduce the lag-phase and also led to slightly higher observed CD yields as seen in Fig. S11 below. However, the increases in CD yields were, in both cases, lower than the amount of CDs put in from the beginning. This is in accordance with what was observed for spiking untemplated reactions (Fig. 2c of the manuscript and Figs. S8 and S9 above).

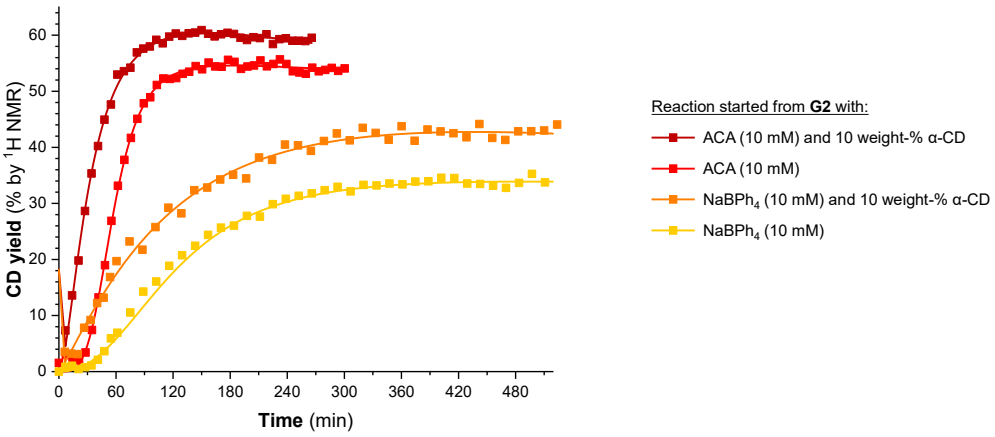

Fig. S11. CD yields over time in reactions from **G2** in presence of templates and with 0 or 10 % by weight of α-CD (total α-glucan concentration of 10 mg/mL) based on ^{1}H NMR spectroscopy.

S4. CD YIELDS OVER TIME IN UNTEMPLATED AND TEMPLATED REACTIONS FROM **G2**

S4.1 TEMPLATE SCREENING

Reactions started from **G2** (10 mg/mL) with CGTase (0.57 g/mL) in the presence of various templates were set up in phosphate buffered (50 mM, pH 7.5) D_2O maintained at 25 °C (^{1}H NMR spectroscopy) or water maintained at room temperature (ca. 22 °C) (HPLC-ELSD) and monitored using both ^{1}H NMR spectroscopy and HPLC-ELSD. Representative results from the NMR studies are illustrated in Figs. S7 and S10 above, while representative chromatograms obtained using the HPLC-ELSD method are shown in Fig. S12 below.

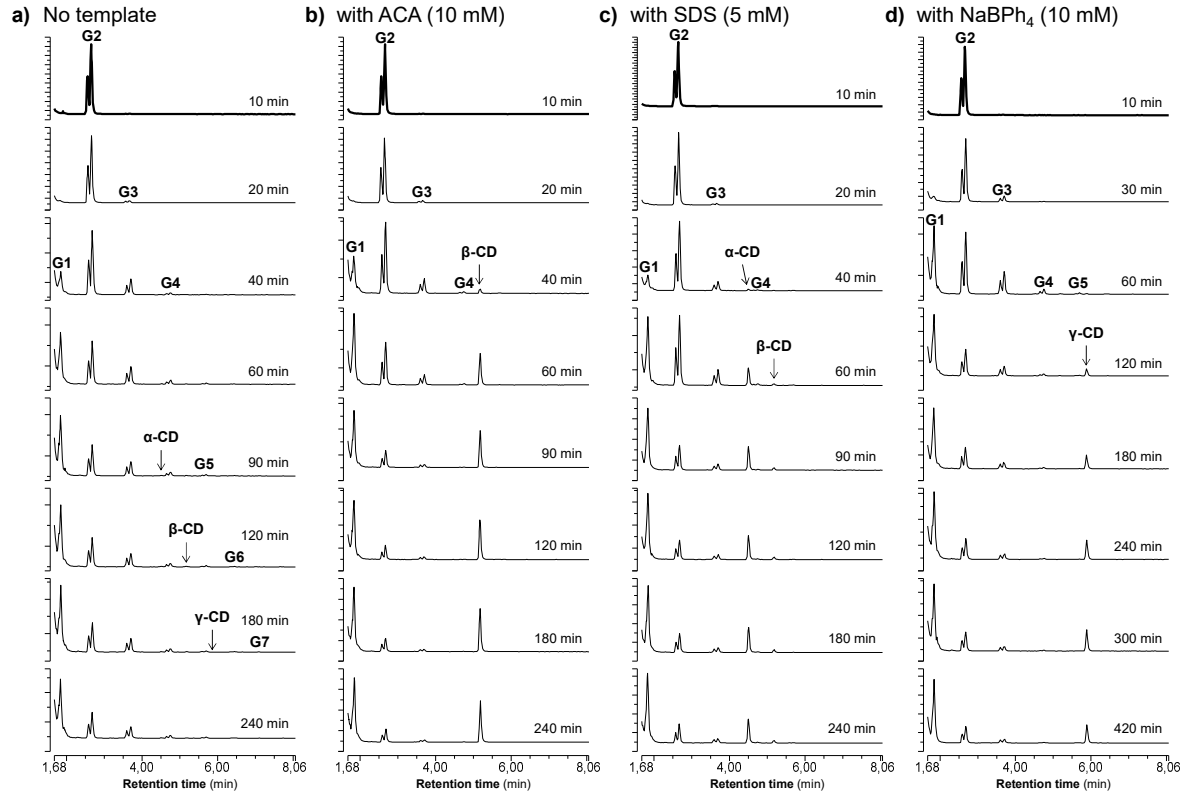

Fig. S12. Chromatograms (ELSD) obtained upon taking aliquots at the noted time of reactions from **G2** with various templates in sodium phosphate buffered (50 mM, pH 7.5) water maintained at room temperature (ca. 22 °C). Prominent peaks are labelled as they appear during the reaction. The chromatograms are normalized to the highest peak due to the large range of signal intensities caused by the non-linearity of the ELSD. Signal intensities are in units of mV, and tick marks are spaced 25 mV apart.

All peaks were assigned based on their retention time by comparison to commercially available genuine samples of the analytes **G1** – **G8** and α-, β- and γ-CD. The results with the two methods (^{1}H NMR and HPLC-ELSD) are generally in good agreement and demonstrate the large increase in CD yields obtained using templates (Fig. S13).

Highest CD yields were determined by fitting the data to a biphasic Hill equation (solid lines in Fig. S13) and deriving the maximum yield from the obtained equation parameters. This was deemed more accurate than simply using the highest measured CD yield, since the fitting procedure aids in determining a weighted data-based midpoint between the random measurement errors that are evident from the obtained data. The biphasic Hill equation was chosen only for its ability to accurately

fit to the data, containing a sigmoidal increasing phase followed by a sigmoidal decrease, and the parameters obtained from the fits were not interpreted to have any chemical or biochemical relevance beyond allowing the determination of a maximum yield with less experimental error.

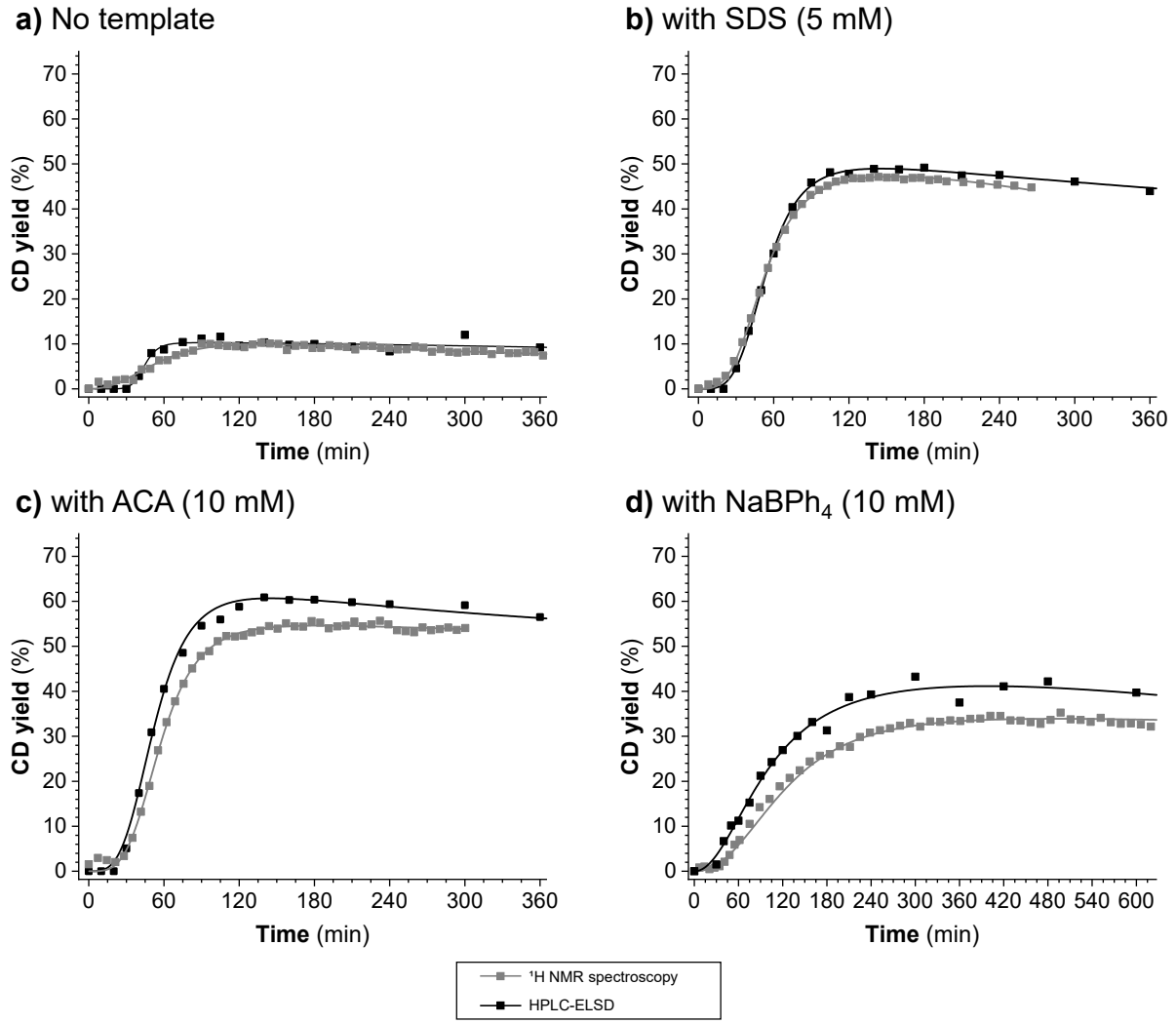

Fig. S13. CD yields over time in reactions from **G2** (10 mg/mL) in presence of various templates (5 – 10 mM) as measured by both ^{1}H NMR spectroscopy and HPLC-ELSD.

S4.2 Optimising CD yields with ACA template

Since templating with ACA gave the highest CD yield in the template screening above, a reaction with higher concentration of ACA (20 mM) was also monitored by both techniques. The result indicated that higher concentration of ACA led to slightly higher CD yields (compare Fig. S13c above to Fig. S14a below), and therefore a 20 mM concentration of ACA was chosen for the subsequent screening of the starting concentration of **G2**. A set of reactions started from **G2** (2 – 40 mg/mL) in the presence of 20 mM ACA, but otherwise identical conditions as above, were monitored by ^{1}H NMR spectroscopy and the results showed that the highest CD yield was obtained when starting from 5 mg/mL of **G2** (Fig. S14b). Therefore this reaction was also performed using the HPLC-ELSD method to support the obtained result. For comparison, a range of reactions started from **G2** (2 – 50 mg/mL) without any template were also monitored by ^{1}H NMR spectroscopy, showing that in absence of templates, the highest CD yields (albeit much lower than for templated reactions) were achieved at the lowest **G2** starting concentration (Fig. S14c).

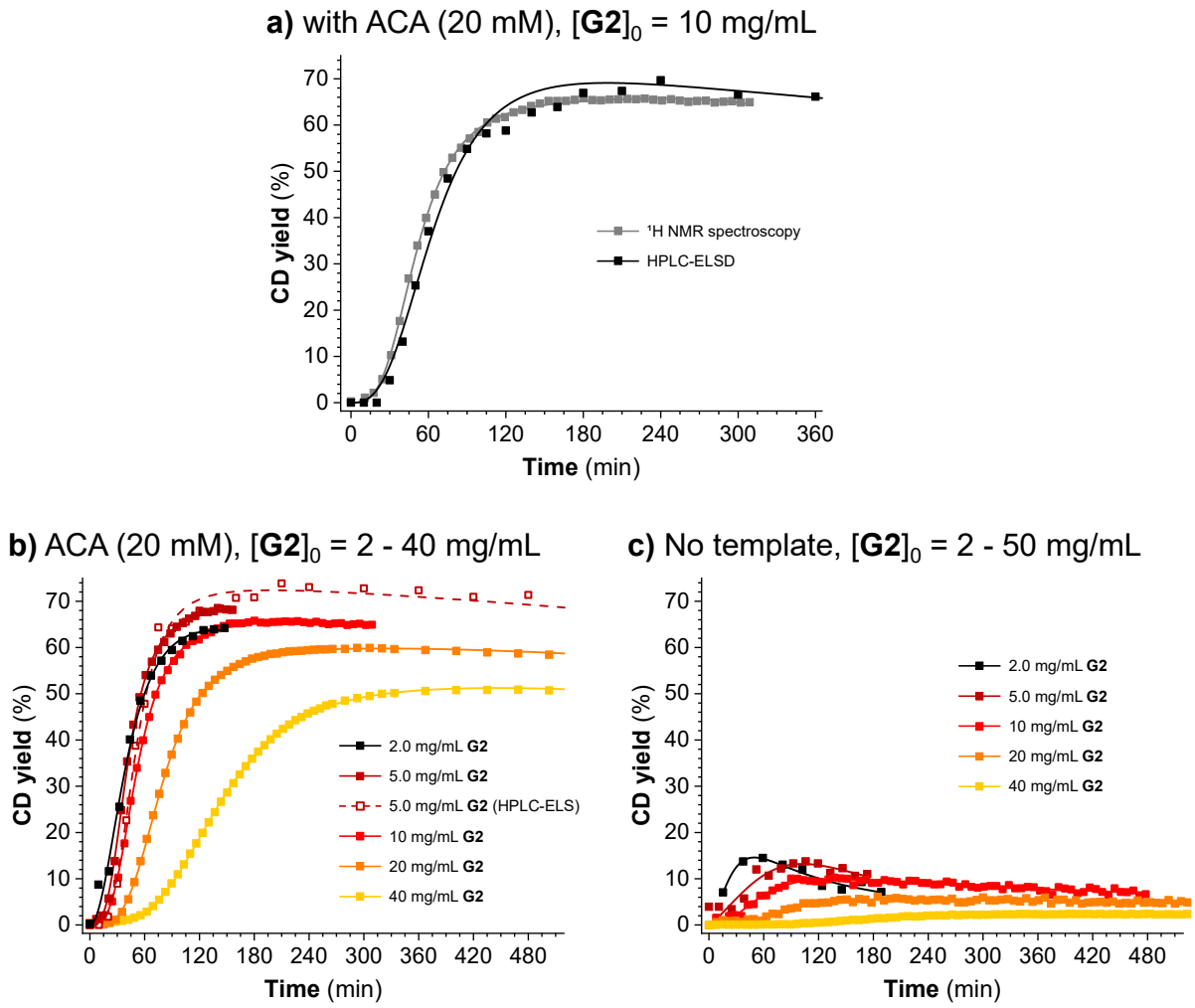

Fig. S14. CD yields over time in reactions with varying starting concentrations of **G2** and varying concentrations of ACA according to ^{1}H NMR spectroscopy unless stated otherwise. a) With 20 mM ACA using the regular 10 mg/mL starting concentration of **G2**. b) With 20 mM ACA at a range of starting concentrations of **G2**. c) With no ACA at a range of starting concentrations of **G2**.

S5. CD YIELDS OVER TIME IN UNTEMPLATED REACTIONS FROM **G1 – G8**

Reactions under identical conditions as above, except maintained at 30 °C, were set up started from either of the linear α-glucans **G1** – **G8** and monitored using HPLC-ELSD as described above. Representative chromatograms are shown in Figs. S15 and S16 below.

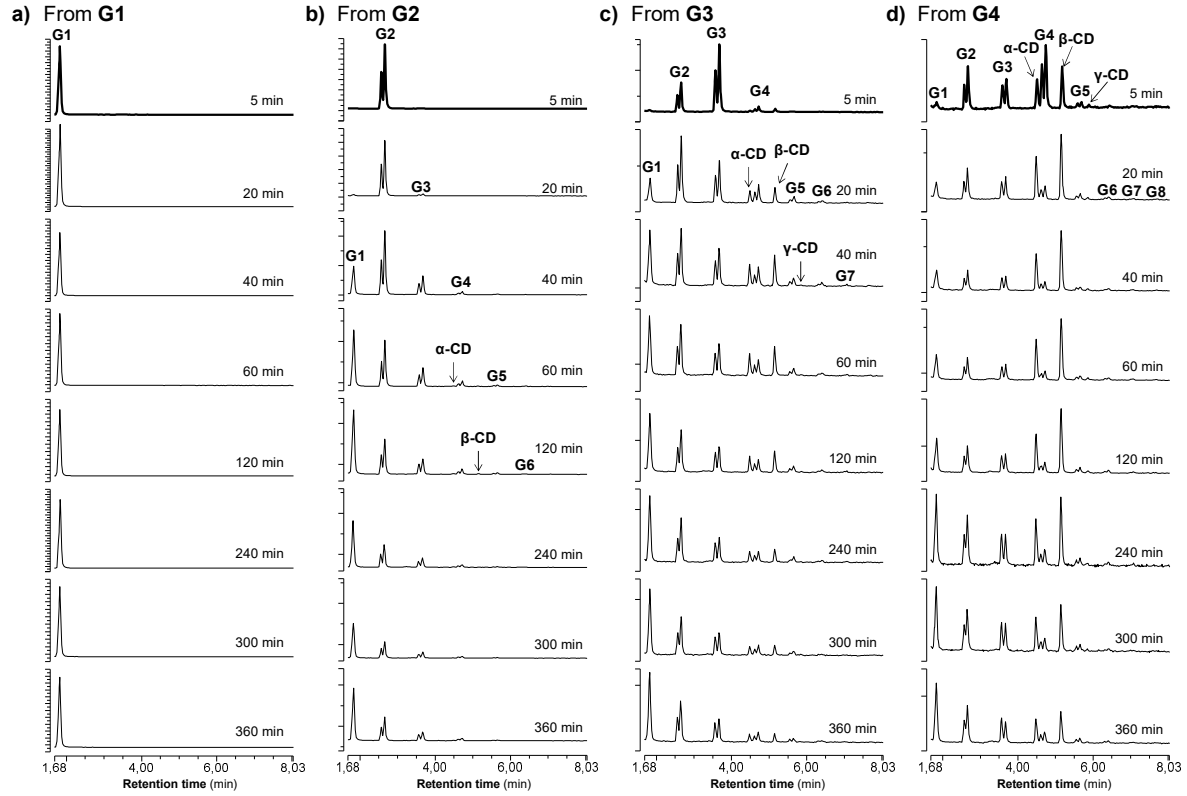

Fig. S15. HPLC chromatograms obtained (ELSD) from aliquots taken at the indicated time in reactions started from **G1**, **G2**, **G3** or **G4** (10 mg/mL) in sodium phosphate buffered (50 mM, pH 7.5) water at 30 °C. Prominent peaks are labelled as they appear during the reaction. The chromatograms are normalized to the highest peak due to the large range of signal intensities caused by the non-linearity of the ELSD. Signal intensities are in units of mV, and tick marks are spaced 25 mV apart.

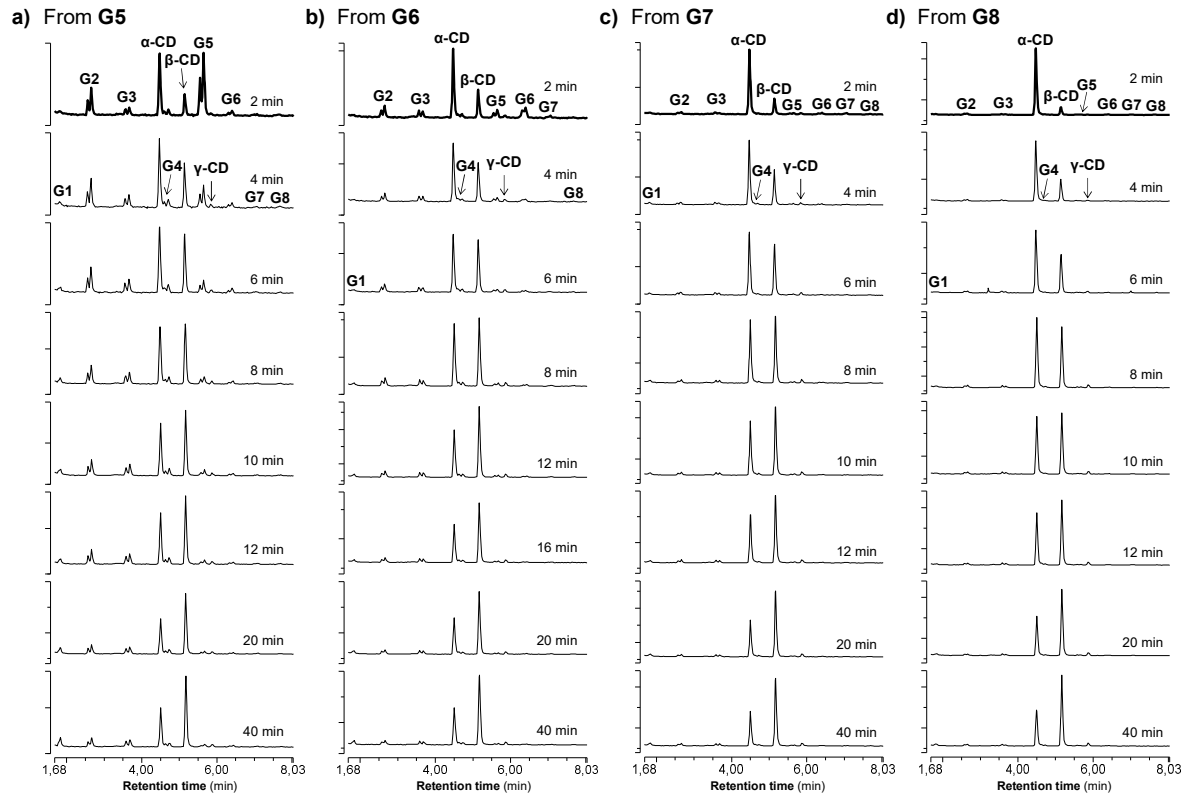

Fig. S16. HPLC chromatograms obtained (ELSD) from aliquots taken at the indicated time in reactions started from **G5**, **G6**, **G7** or **G8** (10 mg/mL) in sodium phosphate buffered (50 mM, pH 7.5) water at 30 °C. Prominent peaks are labeled as they appear during the reaction. The chromatograms are normalized to the highest peak due to the large range of signal intensities caused by the non-linearity of the ELSD. Signal intensities are in units of mV, and tick marks are spaced 25 mV apart.

No conversion was seen for **G1** (Fig. S15a), but all the other α-glucans gave CD yields that peaked within the first six hours of the experiment (Fig. S17).

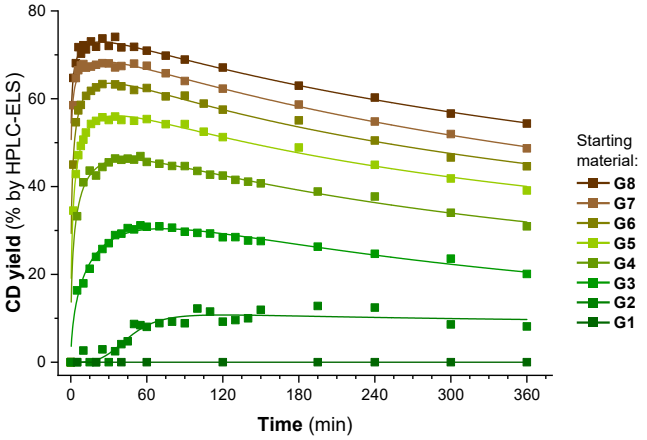

Fig. S17. CD yields over time in reactions started from either of **G1** – **G8** (10 mg/mL) in sodium phosphate buffered (50 mM, pH 7.5) water maintained at 30 °C by use of CGTase (0.57 mg/mL) according to HPLC-ELSD.

Maximum CD yields were determined by fitting the data to a biphasic Hill equation, as described above. Note that the molar CD yields are calculated based on the amount of hydrolysable sugar units in each starting material. For instance, only half of the D-glucopyranose units in **G2** can be converted into CDs, while 7 out of the 8 D-glucopyranose units in **G8** can in principle be converted into CDs.

S6. HIGH TIME-RESOLVED MONITORING OF REACTION FROM **G6**

A reaction starting from **G6** (10 mg/mL) in sodium phosphate buffered (50 mM, pH 7.5) water at room temperature was monitored with high time-resolution by taking aliquots of the reaction every 10 seconds into pre-prepared vials with quenching mixture. The samples were analysed by HPLC with evaporative light scattering detection. Subsequent plotting of the combined CD peak areas as a percentage of all detected sugars revealed a sigmoidal curve (Fig. S18), which could be caused by the fact that **G6** in itself is not long enough to form CDs, and therefore the system must first build up a steady concentration of longer linear intermediates. This is in accordance with the proposed energy diagram in Fig. 4c of the manuscript, where only **G7** and longer is ascribed to the highest energy intermediates that can form CDs directly.

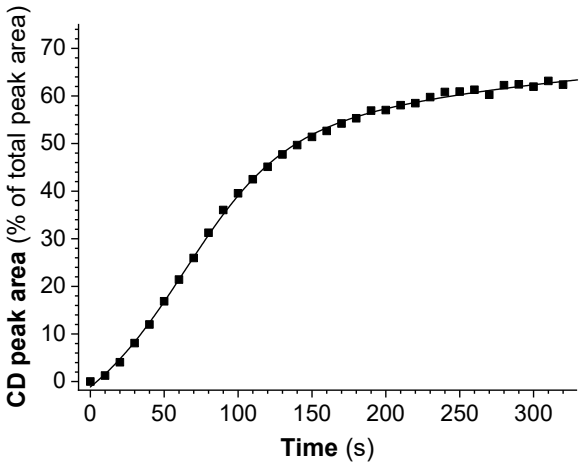

Fig. S18. High time-resolved monitoring of a reaction started from **G6** reveals a sigmoidal build-up of CDs in the first 100 seconds of reaction.

SUPPORTING REFERENCES

S1. G. R. Periyannan, B. A. Lawrence and A. E. Egan, *J. Chem. Ed.*, 2015, **92**, 1244.