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Larsen, Dennis; Beeren, Sophie

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COMMUNICATION

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

Dennis Larsen[a] and Sophie R. Beeren*[a]

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Cyclodextrins (CDs) are commercially produced via enzymatic *breakdown* of starch or amylose. In contrast, we show that cyclodextrins can be synthesised directly from the disaccharide maltose in good yields by exploiting the use of templates to favour the enzymatic *build-up* of cyclodextrins. Using cyclodextrin glucanotransferase to catalyse reversible transglycosylation, and 1-adamantane carboxylic acid as the template, we can synthesise β-CD from maltose in approximately 70% yield. This work represents a step towards supramolecular control over enzymatic production of complex oligosaccharides from simple building blocks.

The efficient production of oligosaccharides continues to be a challenge for the synthetic chemistry community.[1] Stepwise syntheses, involving sequential protection, glycosylation and deprotection, can be impressive, but require highly-skilled synthetic chemists and are rarely scalable, and thus only a few therapeutically important oligosaccharides are produced synthetically on an industrial scale.[2] In contrast, other biopolymers, oligonucleotides and proteins, are readily assembled using a combination of automation (e.g. DNA synthesis) and biotechnologies that employ template-controlled enzymatic synthesis (e.g. PCR and protein expression).[3] Chemoenzymatic and automated approaches to oligosaccharide synthesis that harness the unique capacity of enzymes to control chemoselectivity and stereoselectivity have shown recent success.[4] We are seeking to develop an approach that exploits enzymes to generate dynamic mixtures of oligosaccharides that interconvert via the reversible formation of glycosidic linkages, and employs synthetic templates to bind, select and amplify specific oligosaccharide products.

Cyclodextrins (CDs) are macrocyclic oligosaccharides that exhibit truncated cone-like structures made up of typically 6, 7 or 8 (α, β, or γ-CD) D-glucopyranose units linked by α-1,4-glycosidic bonds. Due to the relatively hydrophobic nature of the CD cavities, and their concomitant ability to bind and the CD cavities, and their concomitant ability to bind and increase the aqueous solubility of poorly soluble compounds, they have found wide applicability within many fields, such as in the cosmetics, pharmaceutics, and food industries.[5] Commercially available CDs are produced by treating biopolymers made up of D-glucopyranose units linked by α-1,4-glycosidic bonds (α-1,4-glucans), such as starch or amylose, with cyclodextrin glucanotransferase (CGTase), which breaks down the biopolymer into primarily CDs and small amounts of linear oligosaccharides.[6] The build-up of cyclodextrins from monosaccharides using synthetic organic chemistry has been described but these are complex multistep-syntheses that are impressive, but inevitably low yielding.[7]

We have recently shown how the process by which CGTase makes CDs from an α-1,4-glucan source is dynamic and responsive.[8] The enzyme scrambles the glycosidic linkages in linear and cyclic α-1,4-glucans enabling the interconversion of CDs via a series of transglycosylation steps. The CDs in this dynamic system form as a kinetically-trapped subsystem that exists transiently before the eventual conversion of all glucan material to glucose (**G1**), the thermodynamic product.[8a] The CDs exist in what is essentially a transient Dynamic Combinatorial Library (DCL), and can interconvert via enzyme-mediated dynamic covalent chemistry. Using template molecules, we can control the selective interconversion of CDs. For example, we could successfully turn α-CD into β-CD by treating α-CD with CGTase in the presence of 1-adamantane carboxylic acid, a compound that binds strongly and selectively to β-CD.[8a]

In the present communication we show that by exploiting template effects, CDs can be synthesised directly from maltose (**G2**) (Fig. 1). Our approach of building up CDs from small building blocks is the reverse process of the polymer breakdown used in current CD production, and represents a step towards supramolecular control over enzymatic production of complex oligosaccharides from simple carbohydrate building blocks.

[a] *Department of Chemistry, Technical University of Denmark, DK-2800 Kongens Lyngby, Denmark.*
* To whom correspondence should be addressed: sopbee@kemi.dtu.dk.
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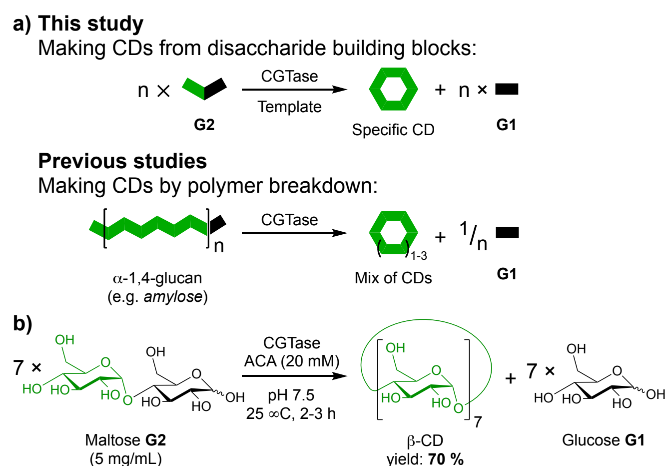

Fig. 1. a) Concept: synthesis of specific CDs by CGTase-catalysed build-up from the shortest α-1,4-glucan oligomer, maltose (**G2**) in the presence of a template in contrast to the commercial production of CDs by CGTase-catalysed breakdown of α-1,4-glucan polymers. b) β-CD can be enzymatically synthesised from **G2** in 70% yield by using adamantane carboxylic acid (ACA) as a template.

Maltose (**G2**), and not glucose (**G1**), is the simplest building block from which CDs can be assembled by CGTase. CGTase scrambles α-1,4-glycosidic linkages in a two-step process.[9] Firstly, a covalent enzyme-substrate glycosyl donor complex is formed by cleaving one α-1,4-glycosidic linkage in the substrate, and if the substrate is non-macrocyclic, releasing D-glucose (**G1**) or a longer α-1,4-glucan. Secondly, a new α-1,4-glycosidic linkage is formed when the enzyme is displaced by a glycosyl acceptor. If this second step involves an intramolecular reaction, a CD is formed, and if it is intermolecular a new linear α-1,4-glucan is formed. In the case of **G2** as a substrate, the reducing end (hemiacetal) D-glucopyranose unit (illustrated with black rectangles in Fig. 1a can thus be considered a "leaving group." To form 1 mole of α-CD, 6 moles of **G2** would thus be required and 6 moles of **G1** are generated as a by-product (see section S2, ESI).

To investigate whether or not the inexpensive, readily available, and highly soluble maltose (**G2**) could be utilised to synthesise CDs, we used NMR spectroscopy. CGTase (0.57 mg/mL) was added to a solution of **G2** (10 mg/mL) in D_2O buffered with sodium phosphate (50 mM, pH 7.5) at 25 °C. The relative total concentration of CDs was quantified by comparing the integrals for anomeric (H1) signals of the CDs ($H1_{CDs}$, highlighted in blue in Fig. 2a, 5.06 – 4.96 ppm), the reducing-end (hemiacetal) α-anomer of the linear oligomers (α-$H1_{red.ends}$, highlighted in yellow, 5.18 – 5.13 ppm) and the α-1→4 linked internal (acetal) units of the linear oligomers ($H1_{internal}$, highlighted in red, 5.36 – 5.28 ppm) (Fig. 2a). Some CD formation was observed, but the conversion was poor, and a maximum molar yield of 10% was obtained after 1.5-2 hours. We report the molar CD yield taking into consideration that only acetal glucopyranose units can be incorporated into CDs. Therefore, molar yield = 100% × (6[α-CD] + 7[β-CD] + 8[γ-CD])/((n-1)$[G_n]_o$) where $[G_n]_o$ is the concentration of the linear starting material used in the reaction and n is the number of D-glucopyranose units in the starting material (i.e. n = 2 for **G2**).

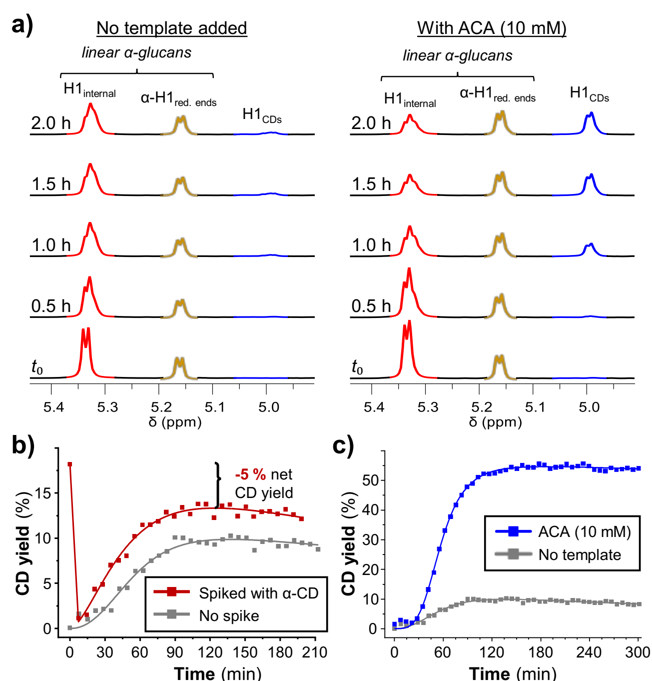

Fig. 2. CD yields obtained from **G2** in presence of different templates as quantified by ^1H NMR spectroscopy. a) Partial ^1H NMR spectra during the reaction of **G2** (10 mg/mL) in sodium phosphate buffer (50 mM, pH 7.5) with CGTase (0.57 mg/mL) at 25 °C in the absence and presence of ACA (10 mM). The coloured regions of the spectra correspond to the peak for the similarly coloured protons highlighted in the general structures for α-glucans and CDs (box). b) Plot of calculated CD yields (^1H NMR) vs. time with and without α-CD spike and c) in presence and absence of ACA.

The relative concentrations of α, β, and γ-CD were not determined at this point, as their signals are not sufficiently well resolved using ^1H NMR, but from previous experiments we expected all three would form.[8a,c]

CD production from **G2** did not start immediately (Fig. 2b, grey trace). We observed an initial lag-phase, which can be explained by the fact that a number of intermolecular transglycosylation steps are required in order to build up linear α-glucans that are long enough to produce CDs via an intramolecular transglycosylation. At least 6 transglycosylation steps are required to convert **G2** to α-CD (see section S2, ESI). The build-up of longer α-glucans during the first 30 mins is seen in the broadening of the H1 signal from the internal α-1-4 linked D-glucopyranose units (5.36 – 5.28 ppm), due to the presence of several overlapping signals (Fig. 2a, left).

We initially thought that a more efficient conversion of **G2** to CDs could perhaps be achieved by 'spiking' our reactions with small amounts of longer linear α-glucans or CDs in order to reduce the number of transglycosylation steps required to convert the **G2** to CDs. **G2** (9 mg/mL) was thus treated with CGTase in the presence of α-CD (1 mg/mL) (Fig. 2b and section S3, ESI). Although the lag-phase was indeed shortened by 'spiking' the reaction with α-CD, the large excess of short glycosyl acceptors rapidly converted the α-CD to short linear oligosaccharides resulting in a CD yield increase that was lower than the amount of CD spike (section S3, ESI).

Having previously shown that templates can promote the selective synthesis of CDs from maltohexaose (**G6**) by forming

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specific stabilising host-guest complexes,[8a] we hypothesised that higher conversion of **G2** to CDs might be promoted by addition of a template. **G2** (10 mg/ml) was therefore treated with CGTase (0.57 mg/mL) in the presence of sodium dodecyl sulfate (SDS) (5 mM), 1-adamantane carboxylic acid (ACA) (10 mM) or sodium tetraphenylborate ($NaBPh_4$) (10 mM), known to bind α-CD, β-CD and γ-CD, respectively. In addition to reaction monitoring by ^1H NMR spectroscopy, high performance liquid chromatography (HPLC) coupled to an evaporative light scattering (ELS) detector was utilised in order to ascertain the specific CD composition formed. Calibration curves from 0.01 mg/mL to 10 mg/mL for all three CDs as well as the linear α-1,4-glucans **G1** to **G8** were made in order to correct for differences in the response of ELS detector to different oligosaccharides (S1, ESI).

In the presence of templates, we saw remarkably high levels of CD synthesis from **G2** (Fig. 2a,b and Figs. S12, S13, ESI). The yields and distribution of CD products are shown in Figure 3a. Most impressively, β-CD was synthesised from **G2** in 58 ± 4 % yield in 3 hours with > 95% selectivity using ACA as a template.[10] To optimise the selective synthesis of β-CD from **G2**, we examined the influence of template concentration and starting **G2** concentration. By increasing the concentration of ACA template from 10 mM to 20 mM, the yield increased to 67 ± 2 % (Fig. 3b). By decreasing the starting concentration of **G2** to 5 mg/mL we obtained a further improved yield of 70 ± 3 % in the presence of 20 mM ACA (Figs 1b, 3c and Fig. S14, ESI). We propose that the increased conversion of **G2** to CDs in the presence of templates is due to a kinetically controlled "capture" mechanism. As soon as CDs are formed, they are bound by the templates, thus stabilising them and preventing their conversion back to short linear α-1,4-glucans, and ultimately glucose.

In a true dynamic combinatorial library operating under thermodynamic control, the product distribution at equilibrium should be dependent only on the available building blocks and not on the specific starting material. In contrast, since this dynamic system forms CDs out-of-equilibrium in a kinetic trap, the choice of starting material is significant. It is noteworthy that the highest yield of CDs from **G2** in the absence of templates is 10% while we have previously observed yields of over 60% for the same reaction started from **G6**. To better understand this effect, each of the linear α-1,4-glucans ranging from maltose (**G2**) up to maltooctaose (**G8**) was treated with CGTase in the absence of template and the reactions monitored over time using HPLC-ELS (Fig. 4a,b and Figs. S15 - S17, ESI).

The molar CD yield was found to increase, and the time to reach maximum CD yield to decrease, as the length of the oligosaccharide starting material was increase (Fig. 4a). There appears to be an inverse correlation between the CD yield and the number of transglycosylation steps required to form CDs from the starting material, making **G2** the most challenging substrate. In all cases it was observed that once formed, a steady concentration of medium length (**G4-G6**) and long (**G7** and **G8**) α-1,4-glucans is observed, while CDs are first built up and then slowly converted into the short linear α-1,4-glucans (**G1**-**G3**) (Fig. 4b). For **G2-G6**, which must first undergo at least

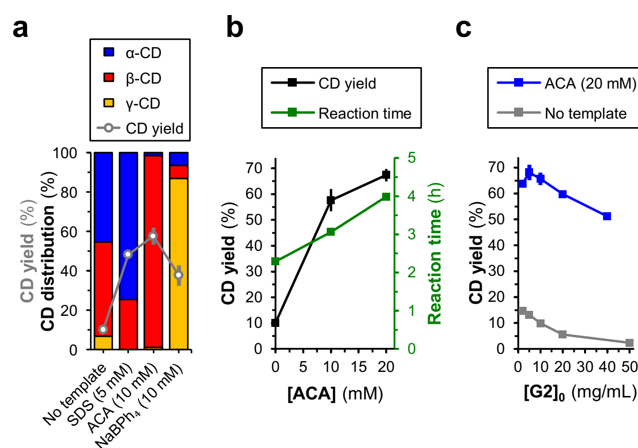

Fig. 3. a) Molar CD yields and CD composition (HPLC-ELS) obtained in reaction of **G2** (10 mg/mL) with CGTase in the presence and absence of templates. b) Dependence of molar CD yield and reaction time on ACA concentration. c) Plot showing the influence of **G2** starting concentration on molar CD yield with and without ACA (20 mM). Conditions otherwise as in Fig. 2.[10]

one intermolecular transglycosylation to form a longer linear α-1,4-glucan before cyclisation to form CDs is possible, we expect a lag-phase before CDs begin to form. This is not immediately evident from Fig. 4b for **G4** and **G6**, but can in fact be observed for **G6** when the reaction is analysed with very high time resolution (Fig. S18, ESI). In contrast, when the reaction is started from **G7** and **G8**, CDs can form directly, resulting in higher quantities of α-glucan material being kinetically trapped as CDs and therefore the higher CD yields.

To summarise the workings of the system we propose the generalised energy diagram depicted in Fig. 4c. The short linear α-glucans (**G1**-**G3**) represent the thermodynamically more stable products (with **G1** being the final product upon reaction *ad infinitum*),[8a] while the CDs exist in a kinetically trapped subsystem. Exchange of D-glucopyranose building blocks between these two extremes must go via the medium length (**G4**-**G6**) and long (**G>6**) linear α-glucans, which therefore represent higher energy intermediates. Long linear α-glucans are only ever observed in very low concentrations, as they can readily convert to CDs (Fig 4b). Upon addition of templates an additional, deeper kinetic trap is formed, which significantly boosts the CD yields, even when starting from **G2**. Meanwhile addition of a template to the reaction starting from **G2** does not affect the lag-phase since longer α-1,4-glucans must first assemble before they can cyclise. The HPLC-ELSD data provide direct evidence of this, since the accumulation of higher quantities of kinetically-trapped CDs in the presence of template is first seen after the longer α-glucans (**G7** and **G8**) are detected in the system, i.e. after approximately 30 minutes of reaction (Fig. 4d).

In conclusion, we have developed a method to enzymatically build up cyclodextrins from maltose, in contrast to the conventional method which relies upon the breakdown of polysaccharides. In the absence of templates, this route is inefficient and low yielding. In combination with templates, dramatically improved CD yields can be obtained (up to ca. 70% in the case of the ACA template). The overall CGTase- mediated

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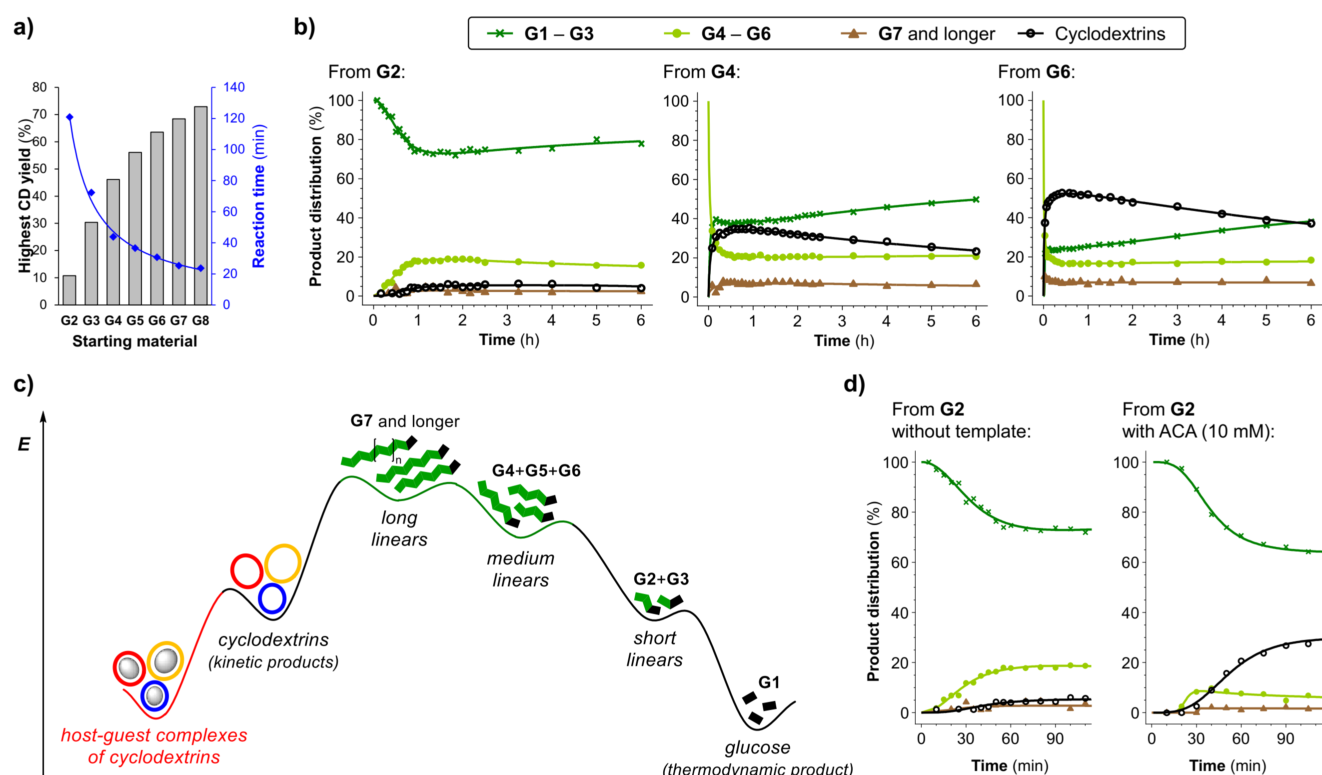

Fig. 4. a) Highest achieved molar CD yield and the reaction time needed to reach this yield as a function of starting material. b) Composition of reactions started from either **G2**, **G4** or **G6** (HPLC-ELS) showing higher CD yields and diminished lag-phases as the length of the starting α-glucan increases (for from all starting materials **G2** – **G8**, please see Fig. S15-17, ESI). c) Proposed energy diagram for the dynamic system afforded upon treating α-glucans with CGTase in the presence of templates for cyclodextrins. d) Compositions of reactions from **G2** in the absence and presence of ACA template (HPLC-ELS) over the first 100 minutes.

system of α-glucans is dynamic but CDs are formed out-of-equilibrium, which means that the choice of starting material has an influence on the CD yield. Exploiting knowledge of this dynamic system allows us to use templates to kinetically trap CD products that are otherwise formed transiently and sparingly, in order to favour their production profoundly. This system represents a first step towards the use of dynamic oligosaccharide systems to produce higher value and more complex oligosaccharides from simple building blocks by stabilising them with supramolecular interactions.

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Conflicts of interest

There are no conflicts to declare.

Notes and references

1 (a) L. Krasnova and C.-H. Wong, *J. Am. Chem. Soc.*, 2019, **141**, 3735. (b) T. J. Boltje, T. Buskas and G.-J. Boons, *Nat. Chem.*, 2009, **1**, 611. (c) P. H. Seeberger and D. B. Werz, *Nature*, 2007, **446**, 1046.

2 (a) X. Zhu and R. R. Schmidt, *Angew. Chemie*, 2009, **48**, 1900. (b) O. J. Plante, E. R. Palmacci and P. H. Seeberger, *Science*, 2001, **291**, 1523. (c) P.-A. Driguez, P. Potier and P. Trouilleux, *Nat. Prod. Rep.*, 2014, **34**, 980.

3 L. Wen, G. Garrett, C. Gibbons, J. Zhang, M. R. Gadi, H. Zhu, J. Fang, X. Liu, Y. Kong, R. G. Wang, *Chem. Rev.*, 2018, **118**, 8151.

4 (a) A. A. Joseph, A Pardo-Vargas and P. H. Seeberger, *J. Am. Chem. Soc.*, 2020, **142**, 8561. (b) T. Li, L. Liu, N. Wei, J.-Y. Yang, D. G. Chapla, K. W. Moremen, G.-J. Boons, *Nat. Chem.*, 2019, **11**, 229. (c) J. Zhang, C. Chen, M. R. Gadi, C. Gibbons, Y. Guo, X. Cao, G. Edmunds, S. Wang, D. Liu, J. Yu, L. Wen and P. G. Wang, *Angew. Chemie Int. Ed.*, 2018, **57**, 16638.

5 N. Sharma and A. Baldi, *Drug. Deliv*. 2016**, 23**, 729-747. E. M. M. Del Valle, *Proc. Biochem.*, 2004, **39**, 1033.

6 A. Biwer, G. Antranikian and E. Heinzle, 2002, **59**, 609.

7 (a) D. Ikuta, Y. Hirata, S. Wakamori, H. Shimada, Y. Tomabechi, Y. Kawasaki, K. Ikeuchi, T. Hagimori, S. Matsumoto and H. Yamada, *Science*, 2019, **364**, 674. (b) T. Nakagawa, K. Ueno, M. Kashiva and J. Watanabe, *Tetrahedr. Lett.*, 1994, **35**, 1921. (c) K. Maiti, G. C. Samantha, G. C. Daskhan and N. Jayaraman, *Carbohydr. Chem.*, 2017, **42**, 165.

8 (a) D. Larsen and S. R. Beeren, *Chem. Sci.*, 2019, **10**, 9981. (b) D. Larsen, P. M. Bjerre and S. R. Beeren, *Chem. Commun.*, 2019, **55**, 15037. (c) D. Larsen and S. R. Beeren, *Chem. Eur. J.*, 2020, **26**, 11032.

9 J. C. M. Uitdehaag, B. A. van der Veen, L. Dijkhuizen and B. W. Dijkstra, *Enz. Microbiol. Techn.*, 2002, **30**, 295.

10 Where the CD yield was determined by both NMR spectroscopy and HPLC-ELSD, it is reported as the average of the two determinations ± one standard deviation.

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