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Artificial Glycosyl Phosphorylases

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Abstract: α- and β-Cyclodextrin $6^A,6^D$-diacids (**1** and **2**), β-cyclodextrin-6-monoacid (**14**), β-cyclodextrin $6^A,6^D$-di-*O*-sulfate (**16**) and β-cyclodextrin-6-heptasulfate (**19**) were synthesised. Acids **1**, **2** and **14** were made from perbenzylated α- or β-cyclodextrin, by diisobutylaluminum hydride (DIBAL)-promoted debenzylation, oxidation and deprotection. Addition of molecular sieves was found to improve the debenzylation reaction. Sulfates **16** and **19** were made by sulfation of the appropriately partially protected derivatives and deprotection. Catalysis of 4-nitrophenyl glycoside cleavage by these cyclodextrin derivatives was studied. Compounds **1**, **2** and **16** were found to catalyse the reaction, with the catalysis following Michaelis–Menten kinetics and depending first order on the phosphate concentration. In a phosphate buffer (0.5 M, 59 °C, pH 8.0), K_M varied from 2–10 mM and the k_{cat}/k_{uncat} ratio from 80–1000 depending on the stereochemistry of the substrate and the catalyst, with **2** being the best catalyst and with the sulfated **16** also displaying catalytic ability. The monoacid **14** and the heptasulfate **19** were not catalytic.

Keywords: artificial enzymes · cyclodextrins · glycosides · hydrolysis · kinetics

Introduction

Enzymatic catalysis of chemical reactions occurs with impressive selectivity and rate.[1] Thanks to enzymes' abilities to pick out specific molecules and to transform them selectively, biosynthesis greatly surpasses even modern chemical synthesis in its ability to make complex molecules. If chemists could learn how to create catalysts with the characteristics of enzymes—artificial enzymes (AEs)—chemical synthesis would in all likelihood be revolutionised, so research in artificial enzymes is an important frontier.[2–6]

Since enzymes achieve their astounding rate enhancements through proximity effects, artificial enzymes should be attainable through endowment of a host molecule with appropriate catalytic groups in order to mimic the active site of an enzyme. A very promising host molecule for artificial enzymes is the cyclodextrin (CD) molecule,[7–11] which is water-soluble and shows good binding constants to aromatic and minor aliphatic groups.[12] Its only drawback is that carrying out synthetic chemistry on CD derivatives is relatively difficult,[13] making the preparation of many analogues a slow process.

For this work we wished to create a CD-based AE that would be able to mimic the action of a glycosidase. It was anticipated that a CD containing appropriate catalytic groups at the secondary or primary rim should bind aromatic groups and, provided that these groups were correctly positioned, catalyse hydrolysis of an aryl glycoside (Figure 1). Glycosidases were the first enzymes to have their catalytic machinery unravelled; they each contain two carboxylate groups positioned 5.5 Å apart.[14] One carboxylate group acts as a general acid catalyst, whilst the other functions as a nucleophile. Presumably a CD containing two carboxylic acids at the rim with the appropriate separation and locked in optimal conformation should catalyse aryl glycoside hydrolysis at optimal pH.

The application of CDs as glycosidase mimics has been reported previously. Ohe et al. observed that α-CD (**3**) increased the rate of hydrolysis of 4-nitrophenyl α-mannopyranoside (**25**) by up to 7.6 times at pH 12, while the hydrolysis of the β-anomer was unaffected.[15] Conversion of other nitrophenyl glycosides was increased by **3** by factors of up to 8.6, and experiments with partially methylated α-CD derivatives indicated that the 2-OH group was necessary for the rate accelerations. β-CD (**4**) did not affect the hydrolysis.

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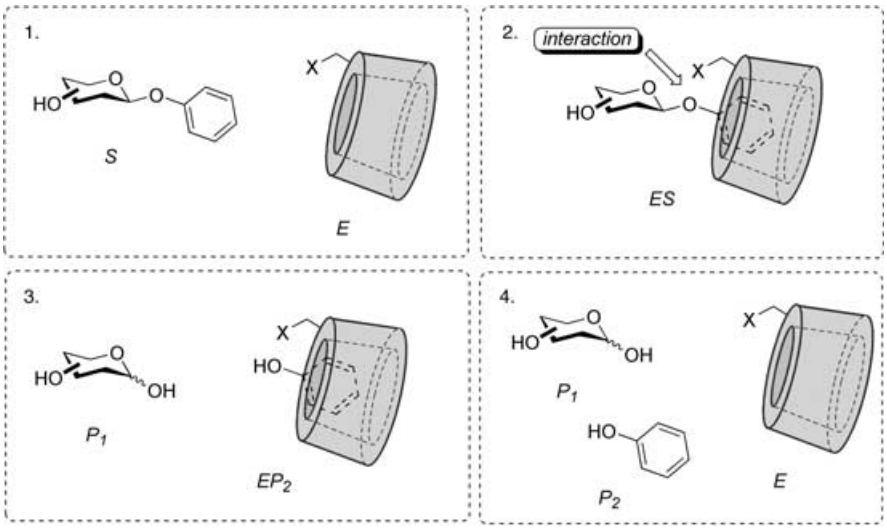


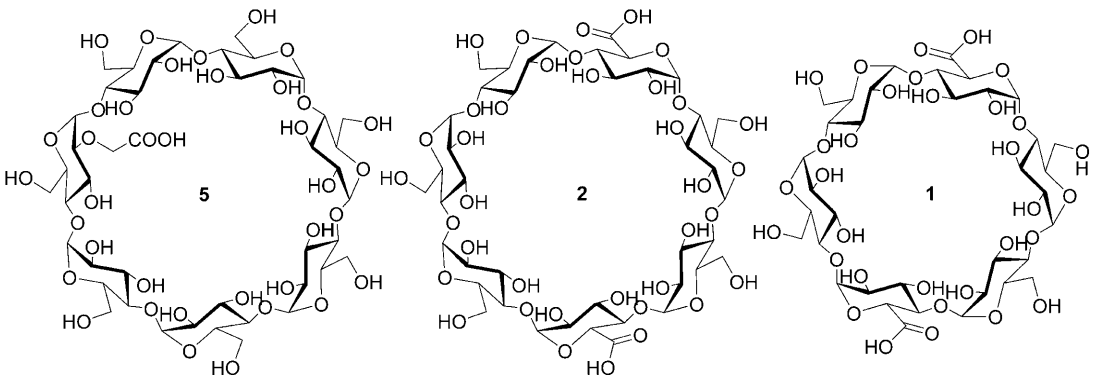
Figure 1. Catalytic action of a cyclodextrin-containing artificial glycosidase.

Since the 2-OH group of **3** is partially deprotonated at pH 12, it appears likely that an alcoholate may be associated with the observed acceleration. Nevertheless, it is also noteworthy that **4** had little effect on the hydrolysis of 4-nitrophenyl glycosides even under these relatively extreme pH conditions. Bennet and collaborators investigated the hydrolysis of 4-nitrophenyl 2-tetrahydropyranyl ether, a glycoside model, catalysed by **3** and **4**.[16] They found that **3** accelerated the hydrolysis about fourfold, while **4** decreased the rate of hydrolysis. A β-CD derivative with a single 2-O-carboxymethyl group (**5**) similarly decreased the hydrolysis rate. They also observed that **4** catalysed the hydrolysis of a 2-deoxyglucopyranosyl pyridinium salt with $k_{cat}/k_{uncat} = 7.5$.

The initial idea behind the present work was to mimic a glycosidase by incorporating two carboxylates into a CD. It was suggested that a derivative less conformationally flexible than **5** would be desirable, which suggested the idea of investigating diacids **1** and **2**. Inspection of models showed that the distances between the carboxylates were 5.0 Å in **1** and 6.5 Å in **2**, and thus relatively close to the distance found in a glycosidase. At a $pH \approx pK_a$ a significant part of the molecule should be in the monoprotonated form, similarly to the active form of a glycosidase. In a preliminary communication[17] we reported that **1** and **2** acted as artificial glycosidases of nitrophenyl glycosides at neutral pH. The catalysed conversion of 4-nitrophenyl glycosides was found to follow Michaelis–Menten kinetics. At pH 7.4 and 59 °C, k_{cat}/k_{uncat} varied from 12 to 35, depending on the stereochemistry of the substrate. We have now found that this reaction is phosphate-dependant and catalyses glycoside cleavage with k_{cat}/k_{uncat} ratios of up to 1000 at high phosphate concentrations. We also report the syntheses of, and catalysis studies with, the monoacid and sulfate analogues of **1** and **2**, which altogether suggest that the catalysis is caused by electrostatic effects.

Results and Discussion

Synthesis of 1, 2 and 14: Compounds **1** and **2**[18–20] were readily obtained from the diols **8** and **9**, made in turn by selective *O*-debenzylation of **6** or **7** by Pearce and Sinaÿ's procedure (Scheme 1).[21,22] This powerful method uses diisobutylaluminum hydride (DIBAL) in toluene to remove one or—more importantly—two benzyl groups from the perbenzylated CD in a selective fashion. Pearce and Sinaÿ obtained yields of 82–83 % of **8** or **9** through the use of DIBAL in toluene (0.5 M, 120–140 equiv) at 30–50 °C, and a 60 % yield of **12** (Scheme 2) with DIBAL (0.1 M, 35 equiv) at room temperature. In some experimentation to minimize the expenditure of DIBAL in this reaction we made the surprising and useful observation that 4 Å molecular sieves have a positive effect (Table 1). Treatment of **6** with 0.1 M DIBAL at room temperature normally provided mainly monool (Table 1, entry 1), but when 4 Å molecular sieves were added a good yield of **8** was obtained (Table 1, entry 2). Other types of



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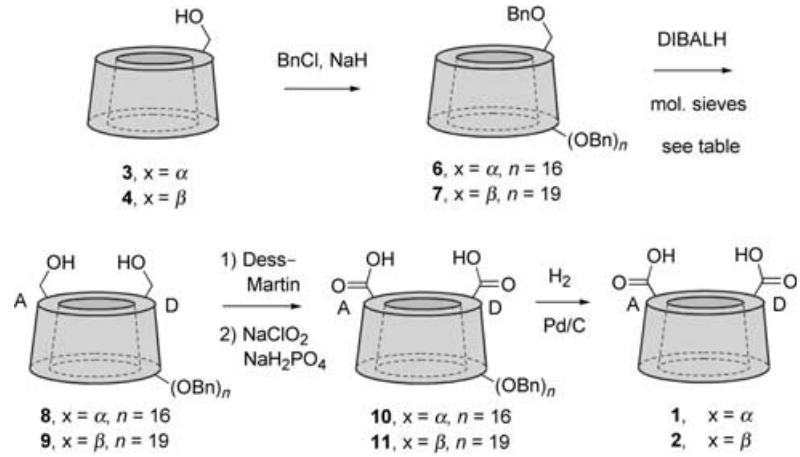
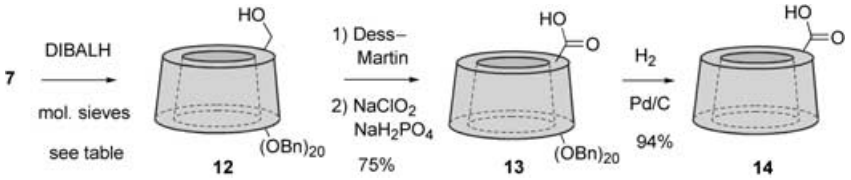


Scheme 1. Synthesis of diacids **1** and **2**.



Scheme 2. Synthesis of monoacid **14**.

Table 1. Isolated yields after chromatography of mono- and diol from the reduction of **6** or **7** with DIBAL with different additives at room temperature.[a]

Entry	Substrate	Additive [g mL^{-1}]	Monool [%]	Diol [%]	DIBAL conc. [M]
1	**6**	–	26	–	0.1
2	**6**	4 Å MS (0.08)	10	64	0.1
3	**6**	4 Å MS powder (0.08)	–	–	0.1
4	**6**	3 Å MS (0.08)	19	23	0.1
5	**6**	sodium aluminate (0.08)	–	–	0.1
6	**6**	sodium silicate (0.08)	31	–	0.1
7	**6**	–	7	–	0.03
8	**6**	4 Å MS (0.08)	47	10	0.03
9	**7**	4 Å MS (0.08)	–	81	0.3
10	**7**	4 Å MS (0.08)	55	-	0.03

[a] Reaction time was 21 h except at entry 8, which was 1.5 h. MS = molecular sieves.

molecular sieves such as 3 Å (Table 1, entry 4) had a smaller effect, as did sodium silicate and sodium aluminate (Table 1, entries 5 and 6). These experiments suggest that the effect is associated with the zeolite structure and not with the chemical constitution. With use of a lower DIBAL concentration (0.03 M), which otherwise had little effect (Table 1, entry 7), the monool could be obtained quite effectively (Table 1, entry 8). The conversion of **7** into **9** (Table 1, entry 9) or **12** (Table 1, entry 10) also worked better with the addition of molecular sieves to either more or less concentrated solutions of DIBAL. The diol **8** was oxidised to the dialdehyde by treatment with Dess–Martin reagent, and the dialdehyde was further oxidised to the diacid **10** (86%) by treatment with sodium chlorite[23] (Scheme 1). By similar methods **9** was converted to **11** in 84% yield. The two diacids **10** and **11** were hydrogenolysed to **1** and **2**, respectively, in quantitative yield.

The pK_a values of these two diacids were determined by titration. The individual pK_a values were too close to be determined individually, but an average pK_a of 3.5 was determined for **1** and a pK_a of 3.2 was determined for **2**. Simulation of the titration curves with plausible values of pK_a(1) and pK_a(2) showed that the pK_a values of **1** were definitely within in the 2.5–4.5 range, whilst those of **2** were between 2.2 and 4.2.

The β-CD monoacid **14** was made from **7** in a similar manner, with a DIBAL reaction with **7** (Table 1, entry 10) being employed to obtain **12** (Scheme 2). Oxidation first with Dess–Martin reagent and subsequently with $NaClO_2$ converted **12** into acid **13** in 75% yield. Hydrogenolysis of **13** gave the acid **14** in 94% yield.

Synthesis of sulfates 16 and 19: For comparison with the carboxylic acids it was also decided to synthesise *O*-sulfates. These molecules should be negatively charged, similarly to a carboxylate, but nonnucleophilic. Compound **9** was therefore converted into the disulfate **15** in 93% yield by treatment with sulfur trioxide·pyridine complex[24] (Scheme 3). Hydrogenolysis gave the CD disulfate **16**, isolated as its disodium salt, in 96% yield. This compound is essentially neutral in aqueous solution.

The desire to expand the negative charge at the primary rim prompted us to synthesise **19**, a β-CD with O-sulfates on all six OH groups. This was made as outlined in Scheme 3. The primary rim was silylated with *tert*-butyldimethylsilyl chloride (TBDMSCl), the secondary rim was acetylated, and the silyl groups were removed with BF_3 as described previously, to provide **17** (Scheme 3).[25] Sulfation with SO_3/pyridine[25] gave **18** in 92% yield, and this was deprotected with NaOMe to give the heptasulfate **19**, isolated as the heptasodium salt, in 88% yield.

Catalysis experiments: At pH 6–8, compounds **1** and **2**—and also disulfate **16**—catalysed the formation of 4-nitrophenol from 4-nitrophenyl β-D-glucopyranoside (**20**; Scheme 4) at catalyst concentrations of 1–3 mM in 50 mM phosphate

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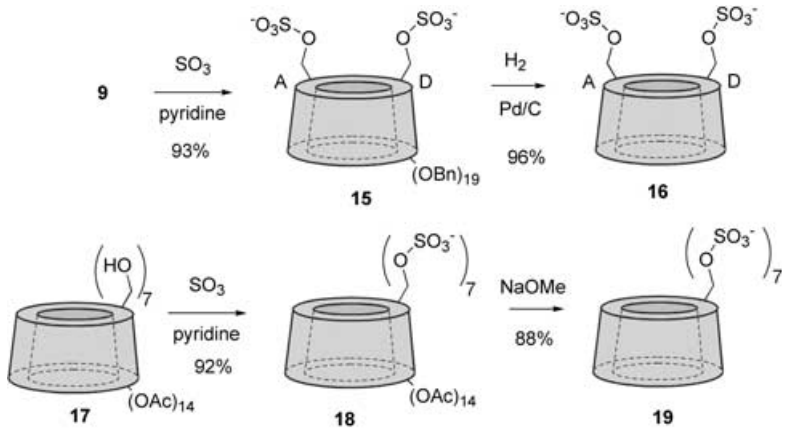
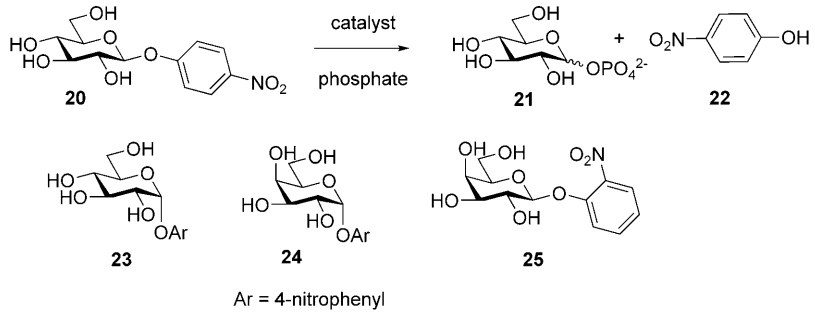


Scheme 3. Synthesis of disulfate **16** and heptasulfate **19**.



Scheme 4. Hydrolytic reaction and its substrates.

buffer. The stereoisomeric substrates **23**–**24** (Scheme 4) were also converted, whereas the 2-nitrophenyl galactoside **25** was not. β-CD (**4**), the monoacid **14** and the heptasulfates **18** and **19** were found not to be catalytic under these conditions.

The rate of catalysis (V_{cat}) was obtained from the determination of absorption at 400 nm in parallel experiments in the presence and in the absence of catalyst, giving a total rate (V_{tot}) and an uncatalysed rate (V_{uncat}), and $V_{cat} = V_{tot} - V_{uncat}$. With increasing substrate concentration V_{uncat} increases linearly while V_{cat} approaches a maximum value, a clear sign of Michealis–Menten-type kinetics (Figure 2a). A Haines plot of $[S]/V_{cat}$ against [S] gave an excellent linear relationship (Figure 2b), from which the K_M and k_{cat} values could be calculated. The k_{uncat} constant could be determined as the slope of the plot of V_{uncat} against substrate concentration.

The pH-dependence of the reaction of **20** in the presence of **2** in 50 mM phosphate buffer is shown in Figure 2c. Since **2** is in dianionic form over the entire pH range, the remarkable change in k_{cat} indicates dependence on phosphate, since the pH curve follows the ionisation of secondary phosphate ($pK_a = 7.2$). Indeed, no catalysis was observed when no phosphate was present, such as in a TRIS-sulfate buffer. When the phosphate buffer concentration was increased, k_{cat} also increased in a linear fashion (Figure 2d). This means that the data fit the rate law given in Equation (1), where P_i is phosphate and $k_{cat} = k'_{cat} \times P_i$. In other words, bound substrates undergo a second-order

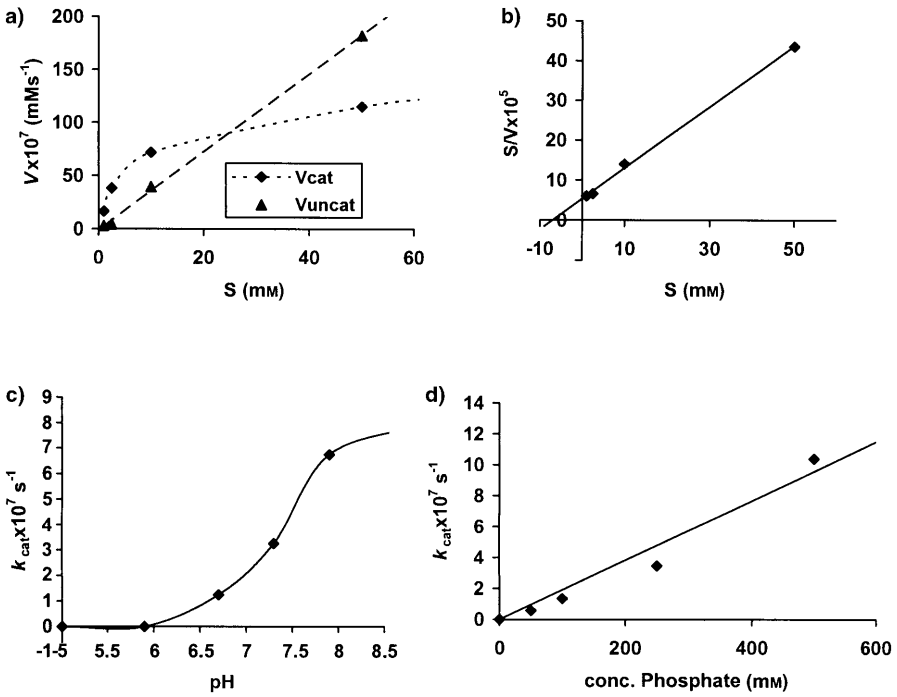


Figure 2. a) V_{cat} and V_{uncat} as a function of [S] (**20**) at pH 7.9, 59 °C in 50 mM phosphate buffer with 1.24 mM **2** as the catalyst. b) Haines plot for the conversion of **20** at pH 7.9, 59 °C in 50 mM phosphate buffer, with 1.24 mM **2** as the catalyst. c) pH-dependence of the formation of **22** from **20** catalysed by **2** at 59 °C. d) Phosphate-dependence of the formation of **22** from **20** catalysed by **1** at 35 °C, pH 8.0.

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reaction, which given the pH profile is likely to be substitution with monohydrogenphosphate.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES + P_i \xrightarrow{k'cat} E + P \quad (1)$$

The K_M value, which is defined as $K_M = \frac{k_{-1}+k_{cat}}{k_1}$, is essentially identical to the dissociation constant for the substrate–catalyst complex, because k_{cat} is likely to be much smaller than k_{-1} (i.e., $K_M \simeq \frac{k_{-1}}{k_1}$). The K_M values, which typically range from 3–10 mM, therefore reflect the efficiency of binding of the nitrophenyl group. This agrees well with the known dissociation constants of the complexes formed between **20** and α-cyclodextrin (**3**) and between **20** and β-cyclodextrin (**4**), which have been determined as 14 mM and 28 mM, respectively.

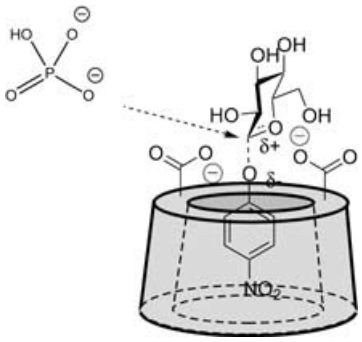
The kinetic constants for the reaction of **20** at 500 mM phosphate buffer at the optimum pH 8.0 are shown in Table 2. Under these conditions the catalytic effect is considerably more apparent than that observed in dilute phosphate,[17] with the catalytic efficiency over background reaction (k_{cat}/k_{uncat}) being up to a 989-fold increase in the best case (Table 2).

The best substrate in these experiments is the β-glycoside **20**, while the rate increases with α-glycoside substrates **23** and **24** are two to four times lower, so the configuration at the anomeric position is of significance. The α-glycosides also bind slightly more tightly (having lower K_M values) than **20**, but this is not important since the K_M value also reflects unproductive binding.

The stereochemistry of C4 in the substrate is, as one would expect, of no significance as the conversions of **23** and **24** are essentially very similar.

The best catalyst is **2**, with **1** being slightly inferior. The disulfate **16** is somewhat weaker. Remarkably, the monoacid **14** has no catalytic effect, and in this respect is identical to its precursor β-CD (**4**, Table 2). Interestingly, α-CD (**3**) has a weakly catalytic effect, but unlike **1**, **2** and **16** the catalysis does not follow Michaelis–Menten-type kinetics but is a simple substrate-dependant rate increase.

Since **1** and **2** are in carboxylate form in the catalytic pH range, the increased catalysis rate of bound substrate must be associated with a proximity effect from the negatively charged groups. Two possibilities can be imagined, either nucleophilic attack or an electrostatic stabilisation of a positively charged transition state. This was the rationale for investigating the sulfate **16**; nucleophilic catalysis is not possible in this compound and only electrostatic stabilisation can play a role. The observation that **16** is a catalyst strongly suggests that the catalysis is caused by electrostatic stabilisation of the transition state by the presence of the negative charge in its vicinity. The k_{cat} value of **16** is generally three times lower than that of **2**, which can be explained by the negative charge in the sulfate being more remote from the rim. The catalytic mechanism shown in Scheme 5 is proposed. The monoacid **14** displays no catalytic effect (Table 2), presumably because there is insufficient negative charge to have an appreciable effect. The heptasulfate **19** was investigated to increase the negative charge at the primary face. It was not catalytic, however, which is interpreted as due to there being too much crowding at the primary face to allow correctly aligned binding.



Scheme 5. Proposed mechanism for the catalytic ability of the diacids. The carboxylates stabilise positive charge in the transition state, facilitating substitution by secondary phosphate.

Table 2. Kinetic constants for the conversion of substrates in 500 mM phosphate buffer at pH 8.0. The catalyst concentration was from 0.1–2.0 mM.

Catalyst	S	Temperature	k_{cat} [$\times 10^7$ s^{-1}]	K_M [mM]	k_{cat}/k_{uncat}
1	**20**	35	10.4 ± 0.3	9.41 ± 0.80	706 ± 26
1	**20**	59	73.7 ± 10.9	11.9 ± 3.7	247 ± 38
1	**23**	59	183 ± 5	7.46 ± 0.72	301 ± 8
1	**24**	59	86.0 ± 4.2	4.89 ± 1.33	292 ± 15
2	**20**	59	188 ± 14	7.90 ± 1.92	989 ± 77
2[a]	**20**	59	9.23 ± 0.44	7.38 ± 0.94	42 ± 2
2	**23**	59	106 ± 4	4.77 ± 1.03	267 ± 10
2	**24**	59	141 ± 8	3.87 ± 1.57	407 ± 25
3	**20**	59	0.437 ± 0.077[b]	–[c]	1.40 ± 0.27[b]
16	**20**	59	97.6 ± 1.3	2.89 ± 0.14	309 ± 4
16	**23**	59	30.7 ± 3.6	0.96 ± 2.96	79.9 ± 9.6
16	**24**	59	23.7 ± 2.9	1.91 ± 3.07	64.5 ± 8.9
4, **14**, **19**	**20**	59	–[c]	–[c]	–[c]

[a] Carried out in 50 mM phosphate buffer. [b] Catalysis did not follow Michaelis–Menten kinetics, but the simple rate law $V = (k_{cat} + k_{uncat})[S]$ in the presence of 2.6 mM **3**. [c] No catalysis observed.

Conclusion

In summary, the cyclodextrin diacids have been found to catalyse phosphate-dependant glycoside cleavage with k_{cat}/k_{uncat} ratios of up to 1000. They form complexes with nitrophenyl glycosides with dissociation constants of 0.5–15 mM, thereby activating the nitrophenoxy groups towards being substituted by phosphate. The corresponding disulfate displays similar catalytic behaviour. It is therefore concluded

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that the catalysis is caused by electrostatic stabilisation of a positively charged transition state.

Experimental Section

General: Solvents were distilled under anhydrous conditions. All reagents were used as purchased without further purification. Evaporation was carried out on a rotary evaporator with the temperature kept below 40 °C. Glassware used for water-free reactions was dried (min. 2 h) at 130 °C before use. Columns were packed with silica gel 60 (230–400 mesh) as the stationary phase. TLC plates (Merck 60, F_{254}) were visualised by spraying with cerium sulfate (1 %) and molybdic acid (1.5 %) in 10 % H_2SO_4 and heating until coloured spots appeared. ^{1}H NMR, ^{13}C NMR and COSY were carried out on a Varian Mercury 400 instrument. Mass spectra (MALDI MS) were obtained on a Voyager DE PRO mass spectrometer (Applied Biosystems) with use of an α-cyanohydroxycinnamic acid (α-CHCA) matrix. Spectra were calibrated with angiotensin I (*m*/*z* 1296.69), adrenocorticotropic hormone (ACTH) (clip 1–17; *m*/*z* 2093.09), ACTH (clip 18–39; *m*/*z* 2465.20), and ACTH (clip 7–38; *m*/*z* 3657.93).

General procedure for de-*O*-benzylation of perbenzylated cyclodextrins

Synthesis of monool: Molecular sieves (4 Å, 1 g) were added at room temperature under nitrogen to a solution of per-*O*-benzyl-α-cyclodextrin (100 mg, 0.04 mmol) in anhydrous toluene (12 mL), and the system was stirred for 1 h. After that, DIBAL (1.17 mL, 1.17 mmol, 1.0 M in toluene) was added dropwise. The reaction mixture was stirred at room temperature for 1 h. The mixture was cooled to 0 °C, water (10 mL) was carefully added dropwise, and the mixture was stirred vigorously at room temperature for 15 min. It was diluted with EtOAc (10 mL) and filtered, with washing with EtOAc (3×10 mL). The organic layer was washed with brine (2×10 mL), dried ($MgSO_4$) and filtered, and the organic solvent was removed in vacuo. The residue was purified by chromatography (eluent gradient, EtOAc/pentane 1:5→1:2), to afford the monool (62 mg, 63 %), the diol (19 mg, 20 %) and starting material (16 mg, 16 %). The assignment is in agreement with the assignment in Ref. [20].

Synthesis of diol: Molecular sieves (4 Å, 50 g) were added at room temperature under nitrogen to a solution of per-*O*-benzyl-β-cyclodextrin (5 g, 1.65 mmol) in anhydrous toluene (270 mL), and the system was stirred for 1 h. After that, DIBAL (66 mL, 66.4 mmol, 1.0 M in toluene) was added dropwise. The reaction mixture was stirred for 21 h at room temperature and was then cooled to 0 °C, water (30 mL) was carefully added dropwise, and the mixture was stirred vigorously at room temperature for 15 min. The mixture was diluted with EtOAc (50 mL) and filtered, with washing with EtOAc (3×100 mL). The organic layer was washed with brine (2×75 mL), dried ($MgSO_4$) and filtered, and the organic solvent was removed in vacuo. The residue was purified by chromatography (eluent gradient, EtOAc/pentane 1:5→1:2), to afford diol (3.79 g, 81 %) as a white foam. The assignment is in agreement with the assignment in Ref. [20].

Hexadeca-*O*-benzyl-α-cyclodextrin-6^A,6^D-dicarboxylic acid (10): The Dess–Martin periodinane reagent (0.97 g, 2.30 mmol) was added to a solution of diol **8** (1.1 g, 0.46 mmol) in CH_2Cl_2 (50 mL), and the reaction mixture was stirred at room temperature for 4 h and then quenched by addition of Et_2O (50 mL) and saturated aqueous $NaHCO_3$ containing 3 g of $Na_2S_2O_3$ (75 mL). After having been stirred for an additional 2 h the solution was diluted with Et_2O (100 mL) and washed successively with saturated aqueous $NaHCO_3$ (50 mL) and water (50 mL). The organic phase was dried and concentrated.

$NaClO_2$ (0.78 g, 8.62 mmol) and NaH_2PO_4 (0.36 g) in water (11 mL) were added to a solution of the residue in *t*BuOH (27 mL), THF (11 mL) and 2-methylbut-2-ene (11 mL). The reaction mixture was stirred overnight and then quenched with aqueous HCl (1 M, 75 mL) and extracted with EtOAc (4×20 mL). The organic extracts were dried and concentrated, and the remaining oil was purified by column chromatography on silica gel (eluent, EtOAc/pentane 1:5, containing 1 % HCOOH), to provide compound **10** (0.92 g, 86 %) as a white foam: $[\alpha]_D$ = +21.5 (*c* = 1.1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.40–6.68 (m, 80 H; CH_{Ph}), 5.64 (d, *J* = 3.6 Hz, 2 H; H-1), 5.48 (d, J_{gem} = 10.4 Hz, 2 H; H–CH_{Ph}), 5.06 (d, J_{gem} = 10.4 Hz, 2 H; H–CH_{Ph}), 4.90 (d, J_{gem} = 10.4 Hz, 2 H; H–CH_{Ph}), 4.83–4.50 (m, 21 H), 4.48–4.33 (m, 8 H), 4.30–3.86 (m, 28 H), 3.57 (d, *J* = 11.6 Hz, 2 H), 3.52 (dd, *J* = 3.4 Hz, *J* = 9.8 Hz, 2 H), 3.43 (dd, *J* = 2.8 Hz, *J* = 8.8 Hz, 2 H), 3.23–3.03 (m, 5 H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.31 (C=O), 139.46, 139.18, 139.06, 138.41, 138.32, 138.28, 137.72, 136.29 (C_{ipso}), 128.63, 128.44, 128.43, 128.37, 128.34, 128.31, 128.16, 128.14, 128.08, 128.06, 127.95, 127.88, 127.71, 127.64, 127.28, 127.19, 127.07, 126.96, 126.28 (CH_{Ph}), 99.66, 98.22, 96.17 (C-1), 84.17, 81.16, 81.12, 80.49, 80.42, 79.25, 79.20, 77.91, 76.77, 76.34, 75.95, 74.16, 73.66, 73.37, 72.83, 72.56, 71.38, 70.63, 70.03, 68.73 (CH_2, CH) ppm; HRMS: calcd for $C_{148}H_{152}O_{32}Na$: 2464.0164; found 2464.0820.

α-Cyclodextrin-6^A,6^D-dicarboxylic acid (1): Compound **10** (2.34 g, 0.96 mmol) was dissolved in an AcOEt/MeOH mixture (1:1, 150 mL). Pd/C (250 mg) and TFA (cat.) were then added and the mixture was stirred overnight under hydrogen. Filtration through Celite and evaporation of the solvent gave compound **1** (0.95 g, 100 %) as a white foam: $[\alpha]_D$ = +103.6 (*c* = 1.0, H_2O); ^{1}H NMR (400 MHz, D_2O): δ = 4.99 (d, *J* = 3.6 Hz, 2 H; H-1), 4.96 (d, *J* = 3.6 Hz, 2 H; H-1), 4.90 (d, *J* = 3.6 Hz, 2 H; H-1), 4.02 (d, *J* = 9.2 Hz, 4 H), 3.92–3.66 (m, 18 H), 3.62–3.38 (m, 10 H) ppm; ^{13}C NMR (100 MHz, D_2O): δ = 172.36 (C=O), 101.97, 101.80, 100.34 (C-1), 81.75, 81.08, 81.03, 73.29, 72.93, 72.72, 72.03, 71.82, 71.75, 71.62, 71.33, 71.23, 60.36, 59.95 (CH_2, CH) ppm; HRMS: calcd for $C_{36}H_{56}O_{32}Na$: 1023.2652; found 1023.2448.

Nonadeca-*O*-benzyl-β-cyclodextrin-6^A,6^D-dicarboxylic acid (11): The Dess–Martin periodinane reagent (1.95 g, 4.60 mmol) was added to a solution of diol **9** (2.6 g, 0.92 mmol) in CH_2Cl_2 (100 mL), and the reaction mixture was stirred at room temperature for 4 h and then quenched by addition of Et_2O (100 mL) and saturated aqueous $NaHCO_3$ containing 3 g of $Na_2S_2O_3$ (100 mL). After having been stirred for an additional 2 h the solution was diluted with Et_2O (150 mL) and washed successively with saturated aqueous $NaHCO_3$ (50 mL) and water (50 mL). The organic phase was dried and concentrated.

$NaClO_2$ (1.39 g, 15.37 mmol) and NaH_2PO_4 (0.8 g) in water (22 mL) were added to a solution of the residue in *t*BuOH (53 mL), THF (22 mL) and 2-methylbut-2-ene (22 mL). The reaction mixture was stirred overnight and then quenched with aqueous HCl (1 M, 100 mL) and extracted with EtOAc (4×20 mL). The organic extracts were dried and concentrated. The remaining oil was purified by column chromatography on silica gel (eluent, EtOAc/pentane 3:10, containing 1 % HCOOH), to provide compound **11** (2.2 g, 84 %) as a white foam: $[\alpha]_D$ +30.7 (*c* = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.26–6.88 (m, 95 H; CH_{Ph}), 5.78 (d, *J* = 4.0 Hz, 1 H; H-1), 5.68 (d, *J* = 3.2 Hz, 1 H; H-1), 5.43 (d, J_{gem} = 10.4 Hz, 1 H; H–CH_{Ph}), 5.37 (d, J_{gem} = 10.0 Hz, 1 H; H–CH_{Ph}), 5.16 (d, J_{gem} = 11.2 Hz, 1 H; H–CH_{Ph}), 5.12 (d, J_{gem} = 10.8 Hz, 1 H; H–CH_{Ph}), 5.02 (d, J_{gem} = 11.2 Hz, 2 H; H–CH_{Ph}), 4.92–4.80 (m, 6 H), 4.78–4.46 (m, 20 H), 4.43–4.26 (m, 14 H), 4.22–3.78 (m, 22 H), 3.73 (d, *J* = 11.6 Hz, 1 H), 3.67 (d, *J* = 11.2 Hz, 1 H), 3.60–3.47 (m, 4 H), 3.43–3.15 (m, 8 H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.96, 171.89 (C=O), 139.66, 139.34, 139.28, 139.21, 139.15, 138.86, 138.65, 138.56, 138.47, 138.31, 138.28, 138.20, 138.13, 137.79, 137.73, 136.90, 136.67 (C_{ipso}), 128.69, 128.47, 128.40, 128.37, 128.33, 128.28, 128.22, 128.18, 128.08, 128.05, 127.98, 127.93, 127.86, 127.76, 127.69, 127.65, 127.60, 127.57, 127.37, 127.25, 127.10, 127.02, 127.00, 126.95, 126.81, 126.53, 126.47 (CH_{Ph}), 100.79, 100.18, 98.84, 97.90, 97.24, 96.17, 95.76 (C-1), 83.23, 82.37, 81.07, 80.44, 80.24, 79.74, 79.22, 78.16, 77.73, 76.53, 76.25, 75.61, 74.55, 74.15, 73.37, 73.13, 72.93, 72.73, 72.66, 72.59, 71.29, 70.74, 70.57, 70.44, 69.38, 68.95, 60.46 (CH_2, CH) ppm; HRMS: calcd for $C_{175}H_{180}O_{37}K$: 2912.1840; found 2912.9507.

β-Cyclodextrin-6^A,6^D-dicarboxylic acid (2):[18–20] Compound **9** (2.2 g, 0.76 mmol) was dissolved in an AcOEt/MeOH mixture (1:1, 150 mL). Pd/C (300 mg) and TFA (cat.) were then added and the mixture was stirred overnight under hydrogen. Filtration through Celite and evaporation of the solvent gave compound **1** (0.9 g, 100 %) as a white solid: $[\alpha]_D$ = +112.3 (*c* = 1.0, H_2O); ^{1}H NMR (400 MHz, D_2O): δ = 5.05 (d, *J* = 3.6 Hz, 1 H; H-1), 5.03 (d, *J* = 3.6 Hz, 1 H; H-1), 5.00–4.93 (m, 5 H; H-1), 4.18 (br d, *J* = 8.0 Hz, 2 H), 3.90–3.59 (m, 26 H), 3.57–3.42 (m,

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10H) ppm; ^{13}C NMR (100 MHz, D_2O): δ = 171.83 (C=O), 101.93, 101.86, 101.68, 101.58 (C-1), 81.87, 81.81, 81.39, 81.20, 81.01, 80.48, 73.24, 73.14, 72.95, 72.90, 72.25, 72.17, 71.79, 71.73, 71.62, 60.17, 59.51 (CH_2, CH) ppm; HRMS: calcd for $C_{42}H_{66}O_{37}Na$: 1185.3181; found 1185.3190.

Eicosa-*O*-benzyl-β-cyclodextrin-6-carboxylic acid (13): The Dess–Martin periodinane (255 mg, 0.60 mmol) was added to a solution of alcohol **12** (0.57 g, 0.20 mmol) in CH_2Cl_2 (20 mL), and the reaction mixture was stirred at room temperature for 1 h and then quenched by addition of Et_2O (20 mL) and saturated aqueous $NaHCO_3$ containing 0.6 g $Na_2S_2O_3$ (20 mL). After having been stirred for an additional 30 min, the solution was diluted with Et_2O (50 mL) and washed successively with saturated aqueous $NaHCO_3$ (20 mL) and water (20 mL). The organic phase was dried ($MgSO_4$) and concentrated.

The residue was dissolved in *t*BuOH (14 mL), THF (6 mL) and 2-methylbut-2-ene (5 mL), and a solution of $NaClO_2$ (0.36 mg, 4.0 mmol) and NaH_2PO_4 (0.4 g) in water (3 mL) was added. The reaction mixture was stirred overnight, and was then quenched with aqueous HCl (1 M, 10 mL) and extracted with EtOAc (3×10 mL). The organic extracts were dried ($MgSO_4$) and concentrated. The remaining oil was purified by column chromatography on silica gel (eluent gradient, EtOAc/pentane 1:5, then EtOAc/pentane 2:5, containing 1% HCOOH), to provide monoacid **13** (450 mg, 75%) as a white foam: $[\alpha]_D$ = +34.1 (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.28–6.90 (m, 100H; CH_{Ph}), 5.61 (d, J = 3.6 Hz, 1H; H-1), 5.55 (d, J = 3.6 Hz, 1H; H-1), 5.30–3.20 (m, 86H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.64 (C=O), 139.70, 139.56, 139.44, 139.22, 139.05, 138.78, 138.65, 138.48, 138.41, 138.35, 138.26, 137.93, 136.89 (C_{ipso}), 128.63, 128.59, 128.40, 128.30, 128.26, 128.15, 128.06, 127.89, 127.73, 127.63, 127.51, 127.46, 127.41, 127.22, 127.05, 126.90, 126.73 (CH_{Ph}), 99.60, 99.52, 98.89, 97.82, 97.57, 97.39 96.02 (C-1), 81.08, 80.92, 79.05, 73.56, 73.43, 73.26, 73.14, 72.93, 72.77, 72.61, 71.61, 69.10 (CH_2, CH) ppm; HRMS: calcd for $C_{182}H_{188}O_{36}Na$: 2972.2777; found 2973.4368.

β-Cyclodextrin-6-monocarboxylic acid (14): Compound **13** (0.36 g, 0.12 mmol) was dissolved in an EtOAc/MeOH mixture (1:1, 60 mL). Pd/C (35 mg) and TFA (cat,) were then added and the mixture was stirred overnight under hydrogen. Filtration through Celite and evaporation of the solvent gave compound **14** (0.13 g, 94%) as a white solid: $[\alpha]_D$ = +47.1 (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, H_2O): δ = 5.00–4.85 (m, 7H; H-1), 4.80–4.50 (m, 25H), 3.90–3.4 (m, 36H) ppm; ^{13}C NMR (100 MHz, H_2O): δ = 163.0 (C=O), 102.05, 102.01, 101.93, 101.74 (C-1), 82.24, 81.21, 80.20, 81.08, 73.28, 73.17, 72.51, 72.28, 72,17, 71.95, 71.89, 71.82, 71.68, 60.31 (CH_2, CH) ppm; HRMS: calcd for $C_{42}H_{68}O_{36}Na$: 1171.3388; found 1171.3064.

Nonadeca-*O*-benzyl-β-cyclodextrin-6^A,6^D-disulfate sodium salt (15): Sulfur trioxide pyridine complex (279 mg, 1.75 mmol) was added to a solution of diol **9** (500 mg, 0.17 mmol) in dry DMF (10 mL). After the system had been stirred for 3 h at 40°C the solvent was removed in vacuo. The remaining oil was purified by column chromatography on silica gel (eluent, CH_2Cl_2/MeOH 10:1) to give disulfate **15** (500 mg, 93%) as a white foam: $[\alpha]_D$ = +29.8 (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $(CD_3)_2SO$, 70°C): δ = 7.42–6.83 (m, 95H; CH_{Ph}), 5.57 (d, J = 2.8 Hz, 2H; H-1), 5.34 (d, J = 2.8 Hz, 1H; H-1), 5.23 (d, J = 2.8 Hz, 3H; H-1), 5.17 (d, J = 2.8 Hz, 1H; H-1), 5.09 (t, J = 12.0 Hz, 2H), 4.96–4.77 (m, 4H), 4.74–4.24 (m, 33H), 4.19–3.66 (m, 30H), 3.62–3.50 (m, 4H), 3.48–3.22 (m, 8H) ppm; ^{13}C NMR (100 MHz, $(CD_3)_2SO$): δ = 139.85, 139.79, 139.15, 139.68, 139.61, 139.52, 139.13, 139.07, 139.00, 138.95, 138.85 (C_{ipso}), 128.85, 128.71, 128.63, 128.56, 128.49, 128.44, 128.41, 128.07, 128.02, 127.97, 127.94, 127.85, 127.75, 127.64 (CH_{Ph}), 98.33, 97.85, 97.53, 96.63 (C-1), 81.51, 81.14, 80.68, 79.58, 78.85, 78.37, 77.51, 76.56, 75.70, 75.67, 75.48, 75.24, 74.97, 74.59, 73.14, 73.05, 72.96, 72.84, 72.72, 72.63, 72.41, 72.27, 71.96, 71.58, 69.61, 69.44, 65.75 (CH_2, CH) ppm; HRMS: calcd for $C_{175}H_{182}O_{41}S_2Na_3$: 3072.1291; found 3072.5337

β-Cyclodextrin-6^A,6^D-disulfate sodium salt (16): Compound **15** (500 mg, 0.16 mmol) was dissolved in an EtOH/H_2O mixture (1:1, 60 mL). $Pd(OH)_2$ (300 mg) and TFA (cat.) were then added and the mixture was stirred overnight under hydrogen. After filtration through Celite and evaporation of the solvent, the remaining oil was subjected to column chromatography on Amberlite IR-120 (sodium form) to give compound **16** (205 mg, 96%) as a white foam: $[\alpha]_D$ = +93.8 (c = 1.0, H_2O); ^{1}H NMR (400 MHz, D_2O): δ = 5.02–4.91 (m, 7H; H-1), 4.30–4.14 (m, 4H), 4.00–3.92 (m, 2H), 3.89–3.68 (m, 23H), 3.59–3.42 (m, 15H) ppm; ^{13}C NMR (100 MHz, D_2O): δ = 102.08, 101.97 (C-1), 81.35, 81.17, 80.97, 80.91, 80.86, 73.28, 73.15, 72.29, 72.11, 71.94, 71.85, 70.14, 67.22, 60.29, 60.14 ppm; HRMS: calcd for $C_{42}H_{68}O_{41}S_2Na_2$: 1338.247; found 1315.375 [M−Na], 1213.417 [M−(SO_3Na_2+H)].

Tetradeca-*O*-acetyl-β-cyclodextrin-6^A,6^B,6^C,6^D,6^E,6^F,6^G-heptasulfate sodium salt (18): Sulfur trioxide pyridine complex (1.62 g, 10.18 mmol) was added to a solution of **17** (500 mg, 0.29 mmol) in dry DMF (15 mL). After the system had been stirred overnight at 40°C the solvent was removed in vacuo. The remaining oil was purified by column chromatography on silica gel (eluent CH_2Cl_2/MeOH 10:3, containing 2% Et_3N) and column chromatography on IR-120 (sodium form) to give heptasulfate **18** (650 mg, 92%) as a white foam: $[\alpha]_D$ = +48.0 (c = 1.0, H_2O); ^{1}H NMR (400 MHz, D_2O): δ = 5.29 (t, $J_{2,3}$ = $J_{3,4}$ = 8.8 Hz, 7H; H-3), 5.20 (d, $J_{1,}$ = 3.6 Hz, 7H; H-1), 4.81 (dd, 7H; H-2), 4.41 (br d, J_{gem} = 14.4 Hz, 7H; H-6a), 4.26 (br d, 7H; H-6b), 4.11 (br d, 7H; H-5), 4.00 (t, $J_{4,5}$ = 8.8 Hz, 7H; H-4), 2.04 and 2.03 (s, 42H; CH_3CO) ppm; ^{13}C NMR (100 MHz, D_2O): δ = 173.24, 173.16 (C=O), 96.63 (C-1), 75.04, 71.32, 71.07, 70.26 (C-2, C-3, C-4, C-5), 66.82 (C-6), 20.78, 20.62 (CH_3CO) ppm; HRMS: calcd for $C_{70}H_{91}O_{70}S_7Na_7$: 2436.0889; found 2255.2012 [M−($SO_3Na)_2$+2H+Na].

β-Cyclodextrin-6^A,6^B,6^C,6^D,6^E,6^F,6^G-heptasulfate sodium salt (19): An aqueous solution of sodium hydroxide (4 M, 5 mL) was added to a solution of **18** (600 mg, 0.24 mmol) in MeOH (10 mL). After stirring overnight at room temperature, the reaction mixture was diluted with water (10 mL) and Amberlite IR-120 (H^+ form) until pH neutral, and the mixture was filtered and concentrated in vacuo. The remaining oil was purified by column chromatography on IR-120 (sodium form) to give heptasulfate **19** (400 mg, 88%) as a white foam: $[\alpha]_D$ = +88.3 (c = 1.0, H_2O); ^{1}H NMR (400 MHz, D_2O): δ = 5.09 (d, $J_{1,2}$ = 3.6 Hz, 7H; H-1), 4.28 (br d, J_{gem} = 11.2 Hz, 7H; H-6a), 4.20 (br d, 7H; H-6b), 4.03 (br d, 7H; H-5), 3.90 (t, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, 7H; H-3), 3.61 (t, $J_{4,5}$ = 9.5 Hz, 7H; H-4), 4.81 (dd, 7H; H-2) ppm; ^{13}C NMR (100 MHz, D_2O): δ = 101.05 (C-1), 79.40, 73.02, 72.06, 69.78 (C-2, C-3, C-4, C-5), 66.85 (C-6) ppm. HRMS: calcd for $C_{42}H_{63}O_{56}S_7$: 1687.012; found 1631.206 [M−SO_3+H+Na].

Procedure for determining the rate of hydrolysis: Each assay was performed on 2 mL samples prepared from aqueous solutions (1 mL) of the appropriate nitrophenyl glycoside at different concentrations mixed with phosphate buffer (0.1 M, 1 mL) containing **1**, **2**, **14, 16** or **19** (5 or 15 mg) or nothing (control). For pH 9.0 a 0.1 M borate buffer was used. The reactions were followed at 59°C by UV absorption at 400 nm and were typically monitored for 18 h. Velocities were determined as the slope of the progress curve of each reaction. Uncatalysed velocities were obtained directly from the control samples. Catalysed velocities were calculated by subtracting the uncatalysed velocity from the velocity of the appropriate cyclodextrin-containing sample. The catalysed velocities were used to construct Haines plots ([S]/V versus [S]) from which K_m and V_{max} were determined. k_{cat} was calculated as V_{max}/[cyclodextrin], k_{uncat} was determined as the slope from a plot of V_{uncat} versus [S]. The following extinction coefficients were determined for 4-nitrophenolate and used in the calculations: ε = 17.04 mM^{-1}cm^{-1} (pH 8.0, 59°C), 16.9 mM^{-1}cm^{-1} (pH 7.9, 59°C), 15.5 mM^{-1}cm^{-1} (pH 7.9, 25°C), 15.3 mM^{-1}cm^{-1} (pH 7.4, 59°C), 10.4 mM^{-1}cm^{-1} (pH 6.8, 59°C), 7.41 mM^{-1}cm^{-1} (pH 6.8, 25°C), 2.07 mM^{-1}cm^{-1} (pH 5.9, 59°C) and 0.96 mM^{-1}cm^{-1} (pH 5.9, 25°C). The inhibition experiments were made by adding cyclopentanol or aniline (15 μL) to a catalysed sample. Determination of glucose was made by use of the Sigma Glucose Assay Kit GAGO-20, based on glucose oxidase-catalysed oxidation of glucose to gluconic acid and hydrogen peroxide, and subsequent determination of the hydrogen peroxide by peroxidase-catalysed oxidation of *o*-dianisidine and absorption measurement at 540 nm.

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