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Artificial enzymes based on cyclodextrin with phenol as the catalytic group

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ABSTRACT

β-Cyclodextrin containing one or two N-linked *ortho*-*N*-acetylaminophenols has been synthesized and tested for their properties as artificial glycosidases. Four different *para*-nitrophenyl glycosides were used as substrates and k_{cat}/k_{uncat} values of up to 381 were found for the di-functionalized products, whereas the mono derivative showed up to 47 times rate enhancement. The optimum pH for the catalysis was found to be approximately pH 8 and the influence of phosphate concentration was investigated.

Enzymes are outstanding in their ability to catalyze reactions with rate enhancements[1] of up to 10^{19}. Significant interest has been devoted to understanding and copying nature's machines, and 'artificial enzymes' have become an important research area.[2] One of the model systems for artificial enzymes is based on cyclodextrins, which are cyclic oligomers consisting of 1,4-α-linked d-glucopyranosides, which results in an apolar cavity and a polar exterior, making it water soluble. The development in this specific area has moved from the unmodified (or randomly functionalized) cyclodextrin which can be used as an additive to dissolve organic compounds, to specifically modified artificial enzymes, chemzymes.[3] With improvements in methods for selective protecting group manipulations it has been possible to design structures which resemble the active site in an enzyme and to synthesize better and more sophisticated artificial enzymes. One group of enzymes of particular interest to us is the glycosidases, which are responsible for cleavage of the anomeric bond in various substrates. It is important for the improvements in biomass degradation as well as medicinal chemistry to understand these processes and the scope of chemzymes in this area should be investigated.

Our group has been interested in cyclodextrin-based glycosidases.[4] One advantage of these chemzymes is their broader substrate recognition compared with very specific natural enzymes.[5] It has been demonstrated that not only carboxylates, but also various other functional groups are able to function as glycosidases.[6] Bis-functionalized chemzymes normally exceed mono-functionalized chemzymes in rate acceleration, which can be explained by a higher probability that the substrate is bound in a manner where the functional group is close to the reaction site. The importance of the catalytical group orientation[7] for catalysis and pH tolerance[8] has earlier been investigated, and the need for a system which can be fine-tuned to a certain pK_a would greatly improve the future development of chemzymes. In this communication, we present the first cyclodextrin based artificial enzyme based on amido phenols.

2-Acetaminophenol was chosen as the catalytic group due to its ready introduction in the cyclodextrin scaffold by reductive amination of the known aldehyde **1**[9] to give the N-linked amino phenol, which upon acetylation would give the target chemzymes **4** and **8** in a comparatively few steps (Fig. 1). The perbenzylated β-cyclodextrin aldehyde **1** was therefore treated with 2-aminophenol in 1,2-dichloroethane (DCE) and sodium triacetoxyborane as the reductant and acetic acid as the catalyst (Scheme 1). The reaction mixture was degassed and kept in the dark to avoid oxidation of the aminophenol. When TLC showed complete conversion of the starting material, pyridine and acetic anhydride (Ac_2O) were added immediately, which gave **2** in 72% yield (2 steps). Selective *O*-deacetylation was conducted using LiOH in aqueous THF followed by neutralization using HCl (1 M in water) to give amide **3**. This product was hydrogenolyzed using Pd/C/H_2(g) in 2-methoxyethanol and TFA as the acid catalyst to give the desired mono-functionalized cyclodextrin **4** in 90% yield from **3** (Scheme 1).

The bis-functionalized cyclodextrin **8** was synthesized in a similar way using the known[8] perbenzylated β-cyclodextrin dialdehyde **5** as the starting material (Scheme 2). Reductive amination followed by acetylation gave **6** in 34% yield. Selective cleavage of the phenol acetates gave **7** in 86% yield, which was subjected to Pd/C-mediated hydrogenolysis of the benzyl protective groups to give the bis-functionalized product **8** in 91% yield.[10]

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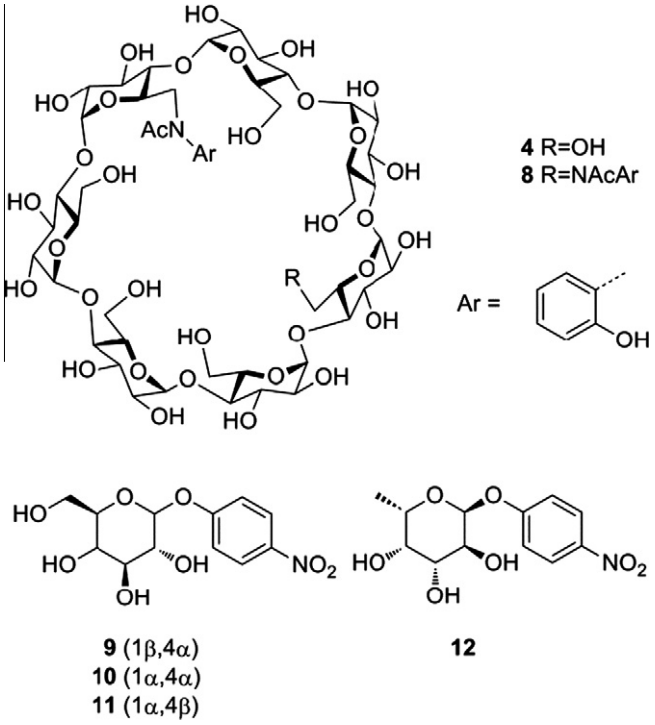
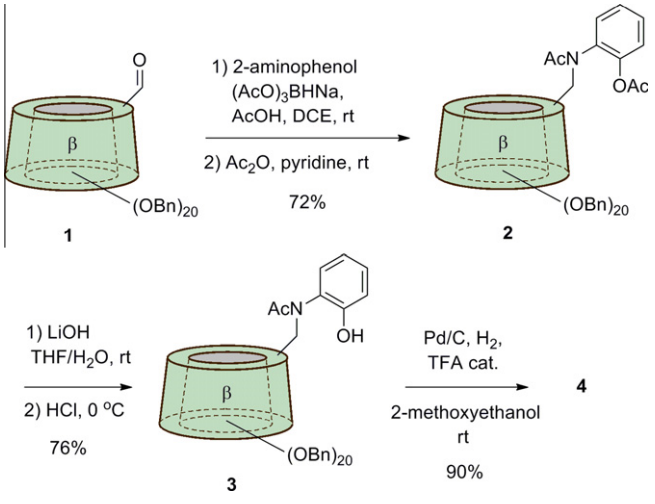


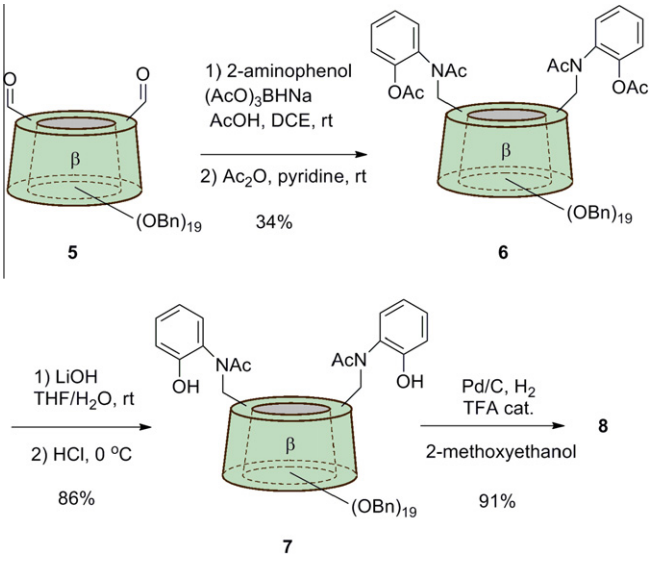
Figure 1. Artificial glycosidases **4** and **8** and substrates **9**–**12**.



Scheme 1. Synthesis of the artificial glycosidase **4**.

With the two model enzymes **4** and **8** in hand their catalytic properties were studied in the hydrolysis of 4-nitrophenyl glycosides **9**–**12** in phosphate buffer at 59 °C and pH 5.9–8.0. The experiments were based on monitoring the formation of 4-nitrophenol spectrophotometrically.[11]

Under these conditions, native β-cyclodextrin has no effect on the hydrolysis, but there is a spontaneous background reaction, which must be subtracted. Thus experiments were performed at different substrate concentrations (1–8 mM) with and without the presence of **4** or **8** (0.2–0.3 mM). After subtraction of the uncatalyzed (i.e., background) reactions from the catalyzed (i.e., cyclodextrin containing reactions), the net catalyzed rate was obtained for each substrate concentration. These data were used to create a Hanes plot (Fig. 2) from which K_M and V_{max} were determined.[12] The values shown in Table 1 are for many different conditions. We see that **4** and **8** displayed enzyme-like catalysis with substrates **9**–**12** with k_{cat} between 10^{-6} s^{-1} and 10^{-5} s^{-1}, and K_M from 0.5 to 6.6 mM. We also found that the rate acceleration (k_{cat}/k_{uncat}) performed by **4** or **8** varied from 29 to 381. Compared to previous artificial glycosidase catalysts this means that phenols **4** and **8** are less potent than cyanohydrins[8] have about the same effectiveness as the carboxylates[6a] and are more effective than aldehyde hydrates,[8] fluorinated alcohols,[6b] sulfates,[6a] and phosphates.[13]



Scheme 2. Synthesis of the artificial glycosidase **8.**

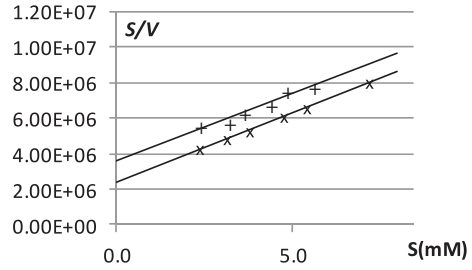


Figure 2. Hanes plot (S/V in mM/Unit vs substrate concentration in mM) for the hydrolysis of **10** catalyzed by **8** in the presence of no (×) or the competitive inhibitor *c*-pentanol (+). The K_i for *c*-pentanol is 14.3 mM.

Table 1
Kinetic constants for the conversion of substrates in phosphate buffer at 59 °C. The catalyst concentration was varied from 0.2 to 0.3 mM. *E* means enzyme and *S* means substrate

E	*S*	[PO_4^-] (mM)	pH	k_{cat} (10^7 s^{-1})	K_M(mM)	k_{cat}/k_{uncat}
8	9	500	5.9	28.9	0.59	29
8	9	500	6.8	25.8	2.86	90
8	9	500	7.1	30.7	2.53	45
8	9	500	7.4	88.0	6.57	268
8	9	50	8.0	18.0	2.25	96
8	9	100	8.0	23.2	3.08	76
8	9	250	8.0	46.5	3.86	202
8	9	500	8.0	83.4	5.91	381
8	9	500	8.9	47.7	4.30	112
8	10	500	8.0	64.2	3.04	358
8	11	500	8.0	78.5	2.04	290
8	12	500	8.0	69.6	3.86	121
4	9	500	7.4	10.3	4.58	31
4	9	500	8.0	10.4	1.62	47
4	10	500	8.0	10.1	4.42	56
4	11	500	8.0	12.6	0.93	47

Cyclopentanol was found to act as a competitive inhibitor of the reaction giving a K_i value of 14.3 mM (Fig. 2). This confirmed that binding to the cyclodextrin cavity was necessary for catalysis.

The k_{cat} was found to show linear dependence on the phosphate concentration (Fig. 3). Similar observations were earlier reported for cyclodextrin carboxylates where it was suggested that phosphate participated in the catalysis.[14] In this case the plot suggests that a minor portion of the catalysis is independent since the graph shows some catalysis at zero phosphate concentration (Fig. 3).

The dependence of pH of the catalysis (k_{cat}) by **8** on substrate **9** is shown in Figure 4. The data fit a crude bell-shaped pH curve following pK_as of 7.2 and 8.8 that is the catalysis increases as an acid with pK_a 7.2 is deprotonated and decreases as an acid with pK_a 8.8 is deprotonated. The low value is identical with the pK_a of the second deprotonation of phosphoric acid while the high value is reasonable for the phenol (2-hydroxyacetanilide pK_a = 9.3) meaning that the best catalysis is obtained when the phenol is protonated and HPO_4^{2-} is present.

A comparison of catalysts **4** and **8** (Table 1) revealed that monofunctionalized **4** is a 6 to 8 fold poorer catalyst than di-functionalized **8**. This could reflect that in **8** there is a higher probability that the bound nitrophenyl glycoside is close to the phenol: since the cyclodextrin conus is symmetrical the substrate can presumably bind in orientations having the oxygen lone pair pointing toward or away from the phenol.

There is no major difference in k_{cat} between the substrates **9**–**12** (Fig. 1) for either catalyst. There is a little larger variation in the K_M value between the substrate but nothing major. This all fits with the fact that the variation in the substrate structure has taken place outside the binding site and therefore does not significantly influence catalysis. The best rate enhancement was obtained with 4-nitrophenyl β-D-glucopyranoside **9** (381 times).

The above observations (Figs. 2–4) are in accordance with a major mechanism where (1) the phenol acts as a protonator that is, general acid catalysis, (2) monohydrogen phosphate (and possibly phosphate) acts as a nucleophile and (3) the nitrophenyl group of the substrate is bound in the cyclodextrin cavity prior to catalysis (Scheme 3). Figure 3 shows that a minor portion of the catalysis is

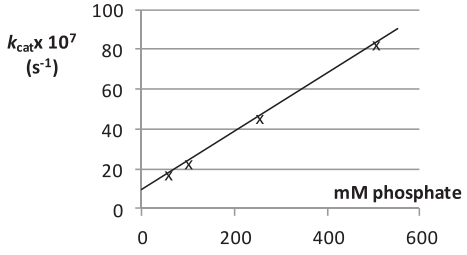


Figure 3. Dependancy of the hydrolysis of **9** catalyzed by **8** on the phosphate concentration.

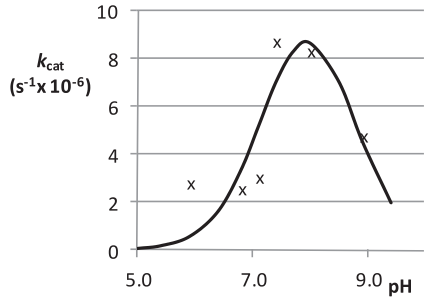
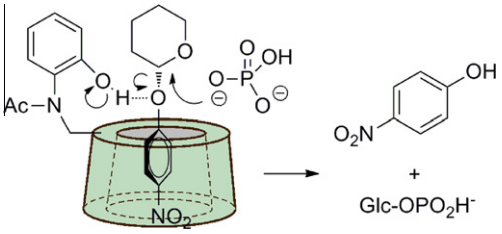


Figure 4. Dependence of k_{cat} on pH in a 500 mM phosphate buffer. The data fit to a curve constructed from two pK_a's of 7.2 and 8.8.



Scheme 3. Mechanism for the glycosidase activity of **4** or **8** that fits the data.

independent of the phosphate and a plausible mechanism for the phosphate independent catalysis is the mechanism of Scheme 3 but with water as the nucleophile. Though it was not specifically shown in this work that glucose or glucose phosphate was also formed this has been shown for other artificial glycosidases and it is a logical assumption that this is also the case here. In any case the reaction performs turnover without any visible drop in activity which indicates that glycosylation of the catalyst is not taking place.

In conclusion, we have shown that cyclodextrin functionalized with 2-acetamidophenols can function as artificial glycosidases. The presence of two catalytic groups enhances the rate acceleration with a factor of about 8 compared with the mono-functionalized derivative. k_{cat}/k_{uncat} values of up to 381 make this model system very promising for further development of artificial glycosidases.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2012.07.050.

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10. NMR spectroscopy of **7** and **8** was complicated by the appearance of two sets of rotamers due to the amides.
11. *Catalysis experiments*: The experiments were carried out on a spectrometer Spectronic Genesys 5 by Milton Roy and a Thermo Scientific Evolution 600. The artificial enzymes were dissolved in water (2.0 or 3.0 mM in stock solution). Substrates were dissolved in phosphate buffer with suitable concentration and pH. Each assay was performed on 8 or 14, 1 mL samples, with increasing substrate concentration, 2–8 mM. The chemzyme concentration in each sample was 0.2–0.3 mM. As a control, water was added instead of the artificial enzyme. The hydrolysis was monitored for 6–18 h, at 59 °C at 400 nm. Velocities were determined as the slope of the progress curve of each reaction. The velocities of uncatalyzed reactions were obtained directly from the control samples, those of catalyzed reactions were calculated by subtracting the uncatalyzed rate from the total rate of the appropriate CD-containing sample. The V_{cat} values were used to construct a Hanes-Plot ([S]/V v s [S]) from which

K_m and V_{max} were determined. k_{cat} were calculated as V_{max}/[CD]. k_{uncat} was determined as the slope of V_{uncat} versus [S]. The inhibition experiments were made by adding *c*-pentanol (22.04 mM) to a catalyzed sample.
12. See Supplementary data for kinetic details.
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