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An artificial enzyme that catalyzes hydrolysis of aryl glycosides

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Abstract—Two cyclodextrin A,D-diacids were synthesized and found to catalyze the hydrolysis of nitrophenyl glycosides with k_{cat}/k_{uncat} of up to 35. The hydrolysis followed Michaelis–Menten kinetics, could be inhibited by compounds binding the cavity, and occurred optimally at pH 7.4.

The mildness, selectivity and speed of biochemical processes greatly exceed ordinary chemical reactions. This efficiency is achieved exclusively by enzyme catalysis. Enzymes use binding and proximity effects to achieve astounding rate enhancements (up to 10^{23})[1] for specific reactions and substrates. It would be highly desirable if chemists could mimic the enzyme catalytic process and learn how to create catalysts for otherwise impossible processes. This worthwhile but difficult objective is aggressively pursued in the ongoing pioneering work on creating artificial enzymes or enzyme mimics.[2] Thus artificial enzymes that mimic enzymes such as ribonuclease,[3a] esterases,[3b,c] phosphatases[3d] and proteases have been recently reported.[3e] However relatively little attention has been given to the design of enzyme mimics catalyzing the very widespread and important biological process of glycosyl transfer. It has been shown that α- or β-cyclodextrin can accelerate the hydrolysis of 2-deoxyglycosyl pyridinium salts,[4] and that α-cyclodextrin can accelerate the hydrolysis of 4-nitrophenyl glycosides up to 6-fold at pH 12.[5] However, an enzyme mimic that catalyzes the hydrolysis of a true glycoside at neutral pH with significant rate enhancement has not been previously reported.

We report here that the cyclodextrin diacids **1** and **2** (Fig. 1), catalyze the hydrolysis of aryl glycosides. These compounds were intended to mimic a retaining or inverting glycosidase, which contain two carboxylic acid groups that are 5.5 or 10.5 Å apart, respectively.[6] In the resting enzyme one carboxylate is protonated, while one is anionic. In the glycosidase mimic **1** the two carboxylates are 6.5 Å apart, while they are 5.0 Å apart in **2** and the desired carboxylate ionization can be achieved

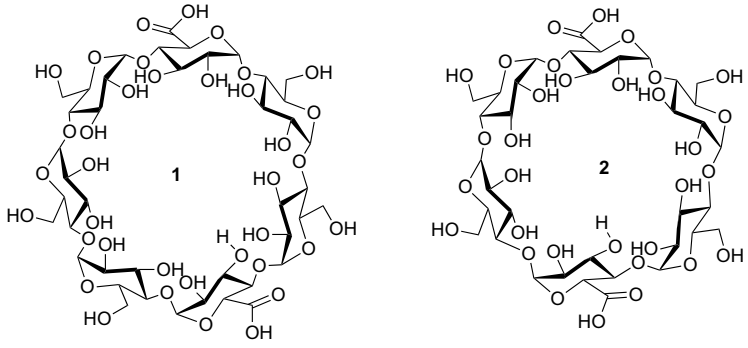

Figure 1. The artificial glycosidases **1** and **2**.

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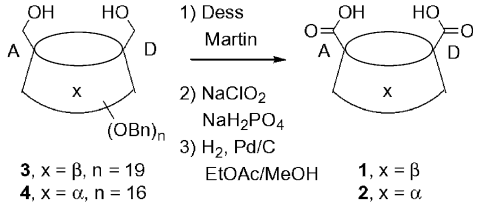
by adjusting the pH. An important difference between these mimics and a glycosidase is that **1** and **2** recognize the aromatic part of an aryl glycoside whilst the glycosidase recognizes mainly the saccharide portion.

The synthesis of **1**[7] and **2** was carried out as shown in Scheme 1. The known diols **3** and **4**[8] were oxidized first with Dess Martin reagent to give the crude dialdehydes, and subsequently with $NaClO_2$ to the protected diacids in 84–86% yield. Hydrogenolysis of the diacids gave **1** and **2** in quantitative yield.[9]

Both **1** and **2** were found to catalyze the hydrolysis of 4-nitrophenyl β-glucopyranoside at room (25 °C) or elevated temperature (59 °C). A significant increase in the initial velocity for formation of 4-nitrophenol was observed when 2.2 mM of **1** was present at pH 7.4 (Fig. 2).[10] By subtraction of the uncatalyzed rate, a catalytic rate was obtained, which was measured at different concentrations of substrate and used to create a Hanes plot (Fig. 3).[11] This clearly shows that the reaction follows enzyme kinetics. From the plot, K_M and V_{max} were obtained and from the latter value k_{cat} (from $k_{cat} = V_{max}/[\mathbf{1}]$). From a plot of V_{uncat} versus substrate concentration, k_{uncat} was obtained as the slope.

This gave a K_M of 13.3 mM, a k_{cat} of $3.34 \times 10^{-7}\,s^{-1}$ and a k_{cat}/k_{uncat} of 35. Since k_{cat} is small, K_M is essentially identical to the dissociation constant for the enzyme–substrate complex (K_s), and this value is close to the reported dissociation constant of 28 mM for this substrate to β-cyclodextrin.[12] While k_{cat} is much smaller than that of natural glycosidases,[13] the increase over the uncatalyzed reaction is nevertheless significant. No significant catalysis was observed with β-cyclodextrin itself. That a binding step is involved in the catalysis was supported by inhibition experiments: addition of cyclopentanol or aniline to the experiment outlined on Figure 2 inhibited the catalysis by 42% at [S] = 10 mM, [cyclopentanol] = 82 mM and by 67% with [aniline] = 82 mM (pH 7.4, 59 °C).



Scheme 1. Synthesis of **1** and **2** from known diols **3** and **4**.[7]

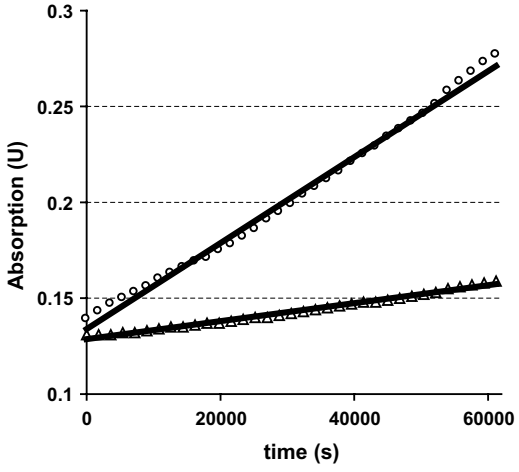


Figure 2. Progress curve for the hydrolysis of 2.5 mM 4-nitrophenyl β-D-glucopyranoside at pH 7.4 (50 mM phosphate), 59 °C in the presence (○) and absence (△) of 2.2 mM of **1**. Absorption was measured at 400 nm.

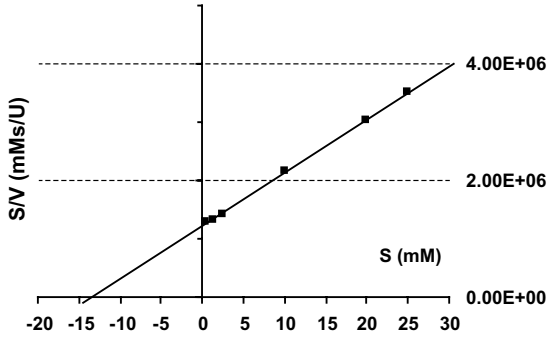


Figure 3. Hanes plot for the hydrolysis of 4-nitrophenyl β-D-glucopyranoside catalyzed by **1** at pH 7.4, 59 °C. The concentration of **1** was 2.2 mM.

The dependence of pH on the catalytic power of **1** on the hydrolysis of 4-nitrophenyl glucoside is shown in Figure 4. No catalysis was found at and below pH 6.4. Titration of **1** and **2** did not allow determination of individual pK_a's for **1** and **2** as they were too close, but the average pK_a's of the carboxylates $\frac{1}{2}pK_{a1} + \frac{1}{2}pK_{a2}$ were found to be 3.2 for **1** and 3.5 for **2**. This nevertheless means that for catalysis, both carboxylates must be deprotonated. Interestingly K_M is 12–13 mM when **1** is in dianionic form, but drops reflecting increased substrate binding, when one of the carboxylates starts to become protonated.

Since the reactions above were followed in their initial stages (substrate conversion < 5%) the formation of 4-nitrophenol might not be the result of turnover but a glycosyl transfer reaction and irreversible glycosylation of the enzyme. The glucose formed was therefore deter-

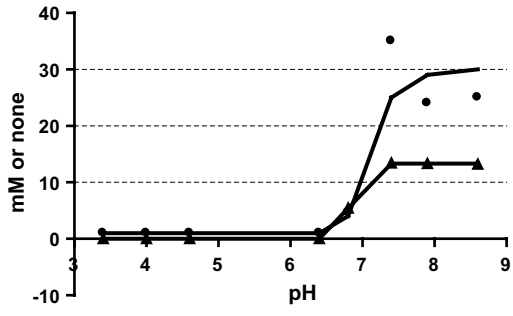


Figure 4. k_{cat}/k_{uncat} (●) and K_M (▲ in mM) as a function of pH for the hydrolysis of 4-nitrophenyl β-D-glucoside catalyzed by **1** (2.2 or 6.5 mM) at 59 °C in 50 mM phosphate buffer. No net catalysis was observed below pH 6.8.

Table 1. Kinetic parameters for various substrates at pH 7.4 (50 mM phosphate) and 59 °C

Substrate	Catalyst	$k_{cat} \times 10^7$ (s^{-1})	K_M (mM)	k_{cat}/k_{uncat}
4-Nitrophenyl β-D-glucoside	**1**	3.34	13.4	35
4-Nitrophenyl α-D-glucoside	**1**	2.82	12.2	24
4-Nitrophenyl α-D-mannoside	**1**	1.16	0.65	12
4-Nitrophenyl α-D-galactoside	**1**	2.6	0.7	23
2-Nitrophenyl β-D-galactoside	**1**	No catalysis		
4-Nitrophenyl β-D-glucoside	**2**	1.46	2.4	18

The catalyst concentration was 2.2 mM for **1** and 2.8 mM for **2**.

mined by use of a glucose oxidase kit and found to be proportional to the formation of 4-nitrophenol. The reaction is therefore true catalysis. This is not completely surprising as the carboxylate should be a better leaving group than 4-nitrophenolate.

A number of other nitrophenyl glycosides were also tested as substrates at the optimum pH (Table 1). The α-glucoside and α-galactoside were almost as good substrates as the β-glucoside while the α-mannoside was slightly worse. Interestingly the mannoside and the galactoside bind considerably better to **1** as seen from their low K_M values. Overall it is seen that variation of the sugar moiety has a minor effect on catalysis. A variation in the aromatic group, on the other hand to a 2-nitrophenyl, had a dramatic effect and removed all catalysis.

The catalytic power of **2** is somewhat smaller to that of **1** (Table 1), but is compensated for by a smaller K_M value.

From the above data it is evident that catalysis by **1** and **2** involves binding of the aryl group of the substrate. The pH data as well as the insignificant catalysis by β-cyclodextrin shows that both carboxylates are necessary for catalysis. The catalysis may occur by nucleophilic catalysis or electrostatic stabilization of a developing positive charge. Further work with more analogues is in progress to investigate this point.

Acknowledgements

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9. Compounds **1** and **2** gave satisfactory NMR and HRMS (MALDITOF) spectra.
10. Each assay was performed on 2 mL samples prepared from 1 mL aqueous solutions of the appropriate nitrophenylglycoside at different concentrations mixed with 1 mL of 0.1 M phosphate buffer containing either **1** or **2** (5 or 15 mg) or nothing as control. For pH 9.0 a 0.1 M borate buffer was used. The reactions were followed at 59 °C using UV absorption at 400 nm and typically monitored for 18 h. Velocities were determined as the slope of the progress curve of each reaction. Uncatalyzed velocities were obtained directly from the control samples. Catalyzed velocities were calculated by subtracting the uncatalyzed velocity from the velocity of the appropriate cyclodextrin-containing sample. The catalyzed velocities were used to construct a Hanes plot ([S]/V vs [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 inhibition experiments were conducted by adding cyclopentanol or aniline (15 μL) to a catalyzed sample. Determination of glucose was made using a Sigma Glucose Assay Kit GAGO-20, which relies on glucose oxidase catalyzed oxidation of glucose to gluconic acid and hydrogen peroxide, and subsequent determination of the hydrogen peroxide by peroxidase catalyzed oxidation of *o*-dianisidine and absorption measurements at 540 nm.
11. Normally an enzyme concentration that is significantly lower than substrate concentration is assumed for the Michaelis–Menten equation to be valid. However, the equation is also valid when $K_M \approx K_s$ (K_s is the substrate dissociation constant) which is the case here. Then $d[ES]/dt \approx 0$ is also fulfilled (steady state conditions) over shorter time periods.
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