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Hydrolysis of Toxic Natural Glucosides Catalyzed by Cyclodextrin Dicyanohydrins

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The hydrolysis of toxic 7-hydroxycoumarin glucosides and other aryl and alkyl glucosides, catalyzed by modified α- and β-cyclodextrin dicyanohydrins, was investigated using different UV, redox, or HPAEC detection assays. The catalyzed reactions all followed Michaelis–Menten kinetics, and an impressive rate increase of up to 7569 (k_{cat}/k_{uncat}) was found for the hydroxycoumarin glucoside substrate 4-MUGP. Good and moderate degrees of catalysis (k_{cat}/k_{uncat}) of up to 1259 were found for the natural glucosides phloridzin and skimmin. By using a newly developed catechol detection UV-assay, a weak degree of catalysis was also found for the toxic hydroxycoumarin esculin. A novel synthesized diaminomethyl β-cyclodextrin showed a weak catalysis of *p*-nitrophenyl β-D-glucopyranoside hydrolysis.

Introduction

In many parts of the world, ingestion of toxic glycosides causes death of humans and livestock. The digitalis (heart glycoside) family includes the potentially toxic digoxin (Figure 1), which is an inhibitor of the Na/K-ATPase, leading to a positive inotrope effect (i.e. increases heart contraction force).[1] Digoxin is one of the most prescribed of all cardiac drugs, and is used for heart failure and atrial fibrillation. However, the narrow therapeutic range of this agent increases the risk of intoxication, especially in the elderly population.[2] Also, atractyloside (Figure 1), a toxic component of the thistle plant *Atractylis gummifera* growing in North Africa, is a glycoside responsible for intoxication and children deaths every year.[3]

The glycoside esculin is the main toxic component of Horse Chestnuts (*Aesculus hippocastanum*), fraxin being another contributing factor.[4] Consumption of esculin can cause diarrhoea, vomiting, bleeding and other symptoms,[5,6] and can be potentially fatal if ingested in larger quantities, for humans or for livestock. However, hydrolysis of esculin affords glucose and the virtually non-toxic aglycon esculetin (Scheme 1).

In recent years, we have developed a number of artificial enzymes, based on the cyclodextrin skeleton, and we imagined that cyclodextrin glycosidases might be able to efficiently catalyze the hydrolysis of esculin and similar toxic natural glycosides to non-toxic components. The size and structure of the coumarin ring system makes it a potentially good fit for binding inside the hydrophobic cavity of the cyclodextrin,[7] whereafter cleavage of the glycosidic bond could take place (see below). If this was the case, cyclodex-

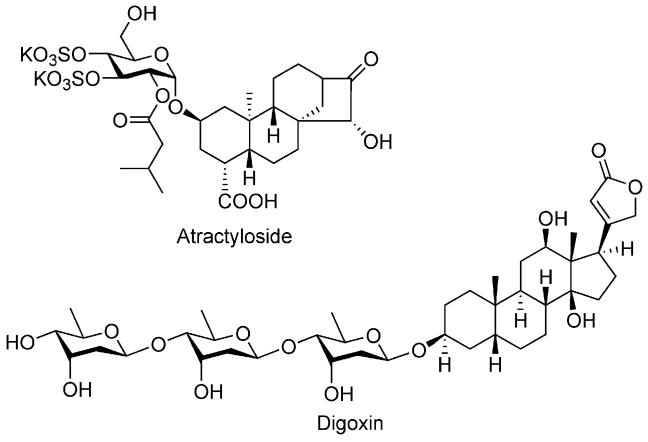
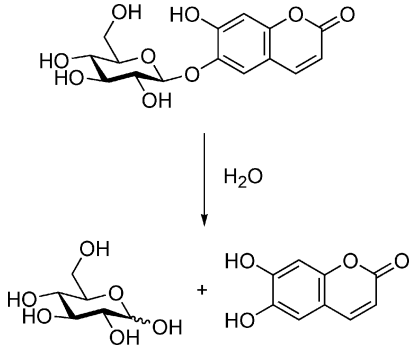


Figure 1. Toxic natural glycosides.



Scheme 1. Hydrolysis of esculin affording glucose and esculetin.

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trin glycosidases might find use as a potential starting point for the further development of a treatment for hydroxycoumarin glycoside poisoning.

Cyclodextrins are useful as artificial enzyme templates, since the hydrophobic interior can bind non-polar substrate parts, while the hydrophilic exterior makes the enzyme soluble in water. Native cyclodextrins are safe to ingest orally,[8] and are presently being used in medical agents,[9] as emulsifiers and food additives, to preserve flavor in dry food products[10] or prolong shelf life and aromaticity of fine oils.[11] From a green chemistry perspective, cyclodextrins are cheaply obtained directly from starch by enzymatic digestion and purification, and their use as artificial enzymes could include industrial, medical or environmental purposes. Through enzymatic catalysis of chemical processes, one can abolish the need for employed toxic reagents, optimize reaction yield and reduce by-product formation. Global warming and the greenhouse effect calls for renewable, efficient catalysts to make possible an environmentally sustainable production in chemistry and related sciences. We believe that cyclodextrins as artificial enzymes have many future applications as green chemistry catalysts,[12–14] and in the present work we direct our attention towards their use as artificial glycosidases.

Mimicking the catalytic power of natural enzymes that nature has spent thousands of years to perfect, is a work still in progress.[15–19] But since the cyclodextrins are much smaller than natural enzymes that contain several large structures and motifs in addition to the active site, the catalytic efficiency per weight unit for some of the cyclodextrin derivatives is now starting to approach that of some natural enzymes.[20–22] By selectively modifying perpendicular alcohol groups on the primary rim of the cyclodextrin,[23] we have achieved artificial oxidases, peroxidases and some of the first artificial glycosidases.[24–26] The cyclodextrin glycosidase activity relies on the cyanohydrin group, wherein the alcohol proton is made acidic by the electron withdrawing effect of the nitrile group, enabling acid-catalyzed hydrolysis of the glycosidic bond. The mechanism for the cyclodextrin-mediated cleavage of various aryl glycosides is assumed to take place as shown in Figure 2.

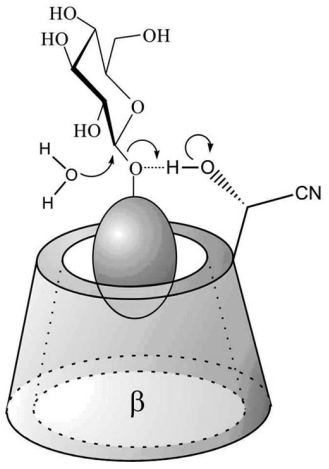


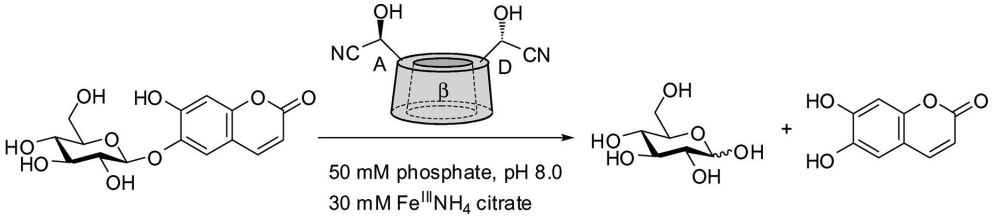
Figure 2. Mechanism of catalysis.

This mechanism is supported by the fact that when only one cyanohydrin group is present on the primary rim, this affords roughly half the catalysis rate of that achieved when having two cyanohydrin groups present. The reaction follows Michaelis–Menten kinetics and requires binding of the substrate in the cyclodextrin cavity, as proven by inhibition experiments with cyclopentanol.

Results and Discussion

We investigated the ability of cyclodextrin dicyanohydrins to catalyze the hydrolysis of various toxic natural glycosides of the 7-hydroxycoumarin type. In the case of esculin, we developed a quantitative catechol detection assay (described below), and used this for monitoring the esculinase effect of cyclodextrin. The hydrolysis of esculin, catalyzed by β-cyclodextrin dicyanohydrin, follows Michaelis–Menten kinetics, and results can be seen in Table 1. As the data show, esculin binds well to the cyclodextrin, affording a K_M value that is generally smaller than that of the previously tested aryl glycoside substrates.[13] However, catalysis of esculin hydrolysis was much weaker than that of the aryl glycoside substrates under similar conditions (esculin

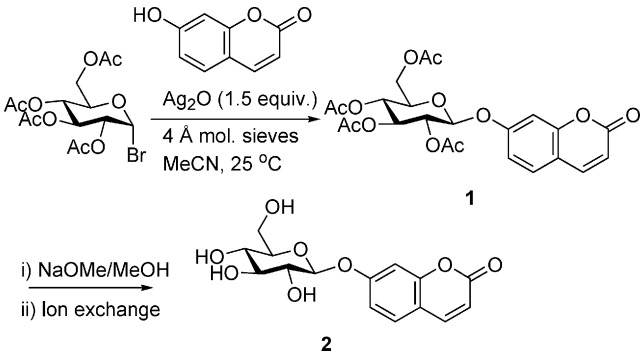
Table 1. The reactions were performed in 50 mM phosphate buffer, pH 8.0/30 mM Fe^{III} ammonium citrate, [β-cyclodextrin dicyanohydrin] = 1 mM. Esculin concentration was between 1–10 mM. The reactions were followed by measuring the absorption at 570 nm.



Temp. [°C]	k_{cat} [10^{-6} s^{-1}]	K_M [mM]	k_{cat}/k_{uncat}
25	2.60 ± 0.12	1.20 ± 0.73	22
40	2.50 ± 0.34	3.90 ± 1.05	60
50	3.19 ± 1.03	1.03 ± 1.38	26
60	3.19 ± 0.93	2.56 ± 1.87	10

k_{cat} is 20 times smaller at 50 and 60 °C).[14] Still, the data clearly show some catalysis, with a k_{cat}/k_{uncat} value of up to 60.

We also investigated as a substrate the toxic glucoside skimmin,[27] found amongst others in citrus fruits.[28] As skimmin is not readily commercially available, we synthesized it in two steps starting from tetraacetylated α-D-glucopyranosyl bromide (Scheme 2). Upon hydrolysis, skimmin affords glucose and umbelliferone (7-hydroxycoumarin), the latter of which was detected directly by measuring the absorption at 365 nm.



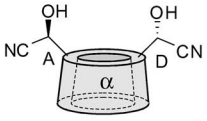
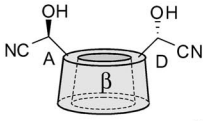
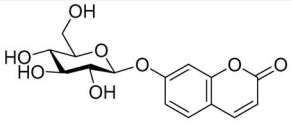
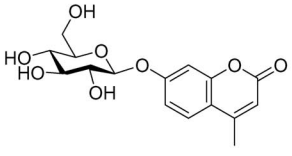
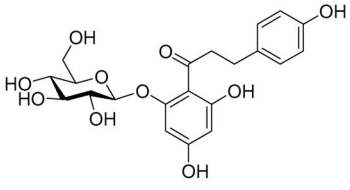
Scheme 2. Synthesis of skimmin.

Skimmin assay results are listed in Table 2. With β-cyclodextrin dicyanohydrin, a good rate of catalysis (k_{cat}/k_{uncat}) of 1259 was found for the reaction. A more moderate catalysis rate of 162 (k_{cat}/k_{uncat}) is obtained with α-cyclodextrin dicyanohydrin, which correlates well with the poorer ability of the α-cyclodextrin cavity to accommodate more bulky substrates, compared to that of β-cyclodextrin.

We also tested the artificial glycoside 4-MUGP (4-methylumbelliferyl β-D-glucopyranoside), which is used for detection of various bacterial strains,[29] and diagnostically in a test for Gaucher's disease.[30] The appearance of hydrolysis product, 4-methylumbelliferone, was monitored directly by measurement of the absorbance at 360 nm. An excellent degree of catalysis was found, with a k_{cat}/k_{uncat} value of 7569 for β-cyclodextrin dicyanohydrin, and aproximately half that value for α-cyclodextrin (Table 2). We assume that the high activity of this substrate, compared to skimmin, is due to the presence of the 4-methyl group. This can be explained in the following manner: skimmin, being less bulky, may have more binding modes to the catalyst and some of these binding modes may be unproductive. The substrate is effectively acting as its own inhibitor. 4-MUGP on the other hand may be limited to the productive binding modes by its structure. Therefore it is a much better substrate.

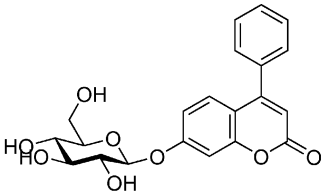
We also tested the dihydrochalcone phloridzin, a natural glucoside found in apple zest and pulp. Phloridzin acts as an antidiabetic agent, competitively inhibiting the intestinal uptake of glucose,[31] and the renal reabsorption of glucose in the proximal convoluted tubules. Phloridzin is a recognized toxic agent, albeit the toxicity is relatively low (200–400 mg/kg for induction of glycosuria).[32] Hydrolysis of phloridzin affords glucose and the flavanoid phloretin, and reaction progress was monitored by HPAEC glucose detec-

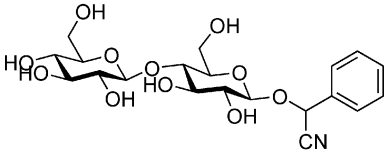
Table 2. The reactions were performed at 60 °C in 50 mM phosphate buffer, pH 8.0, [cyclodextrin] = 0.34–0.91 mM. Substrate concentration was between 0.5–13.2 mM; the reactions were followed by UV (‡) or HPAEC (‡‡).

Substrate	 k_{cat} [10^{-5} s^{-1}]	K_M [mM]	k_{cat}/k_{uncat}	 k_{cat} [10^{-5} s^{-1}]	K_M [mM]	k_{cat}/k_{uncat}
 Skimmin	0.62±0.04	7.49±0.77	162	4.83±0.03	3.93±0.06	1259 ‡
 4-MUGP	16.03±0.89	10.43±0.82	4471	20.71±3.27	13.71±2.89	7569 ‡
 Phloridzin		not tested		0.20	1.69	184 ‡‡

tion (Table 2). A moderate degree of catalysis is seen, with β-cyclodextrin dicyanohydrin (k_{cat} = 2×10^{-6} s^{-1}). Yet strong binding does indeed occur (K_M = 1.69 mM). This suggests that much of this binding does not lead to catalysis, which makes sense bearing in mind this substrate's extended aromatic moiety.

The skimmin analogue 4-phenylskimmin (Figure 3) was tested at 20 °C both with α- and β-cyclodextrin dicyanohydrin, but no catalysis was observed under these conditions.





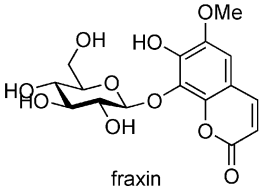


Figure 3. Compounds that were not substrates.

Amygdalin (Figure 3) is a phenylalanine-derived cyanogenic glucoside present in apricot kernels and bitter almonds (*Prunus amygdalus*).[33] Amygdalin was once suggested to possess anticancer activity, and in spite of lack of any evidence supporting this, it remains a popular anticancer agent used by people throughout the world, despite the risk of cyanide poisoning.[34] We tested this compound as a substrate at 60 °C (assay conditions as in Table 2), however, no catalysis was found.

The hydroxycoumarin glucoside fraxin (Figure 3) is the second largest contributant to Horse Chestnut toxicity. Using HPAEC glucose detection and the same assay conditions as for phloridzin, we tested fraxin for β-cyclodextrin dicyanohydrin-catalyzed hydrolysis, which affords fraxetin. The results revealed low activity with this substrate, though the binding appeared to be quite strong (small K_M value).

Using a reductive sugar detection assay (see below), we tested as substrates the compounds *n*-octyl β-D-glucopyranoside, *n*-pentyl β-D-glucopyranoside and benzyl β-D-glucopyranoside (Figure 4). We employed as catalysts both α- and β-cyclodextrin dicyanohydrin, testing at both 25 and 90 °C, but found no catalysis for any of these substrates. The most likely explanation for the lack of activity is the poorer leaving group capability of the aliphatic substituent in these compounds compared to the aromatic substituent in the good substrates.

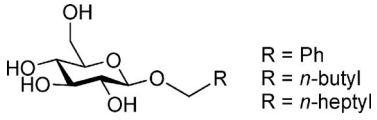
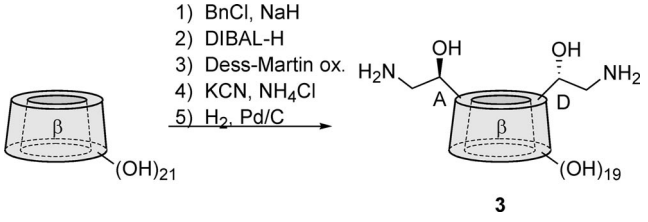


Figure 4. Simple glucoside substrates.

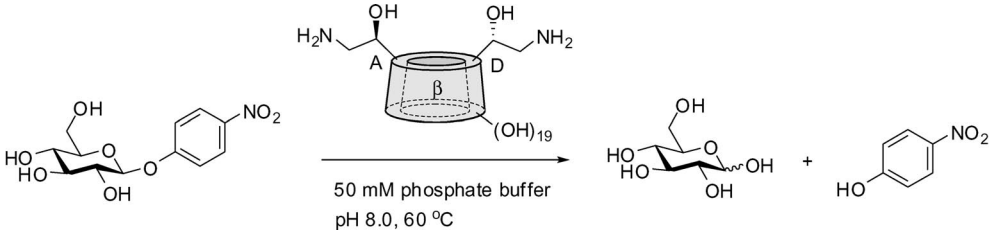
Diaminomethyl β-Cyclodextrin

In addition to the cyclodextrin dicyanohydrins employed in the above-mentioned assays, we also synthesized an amine analogue hereof. Using essentially the same route of



Scheme 3. Synthesis route for β-cyclodextrin dihydroxyamine (step 1–4 in ref.[32]).

Table 3. The assay was performed with [cyclodextrin] = 0.54 mM. Substrate concentration was between 1–26 mM. The reaction was followed by UV (400 nm).



k_{cat} [10^{-7} s^{-1}]	K_M [mM]	k_{cat}/k_{uncat}
2.24 ± 0.45	2.879 ± 1.83	8

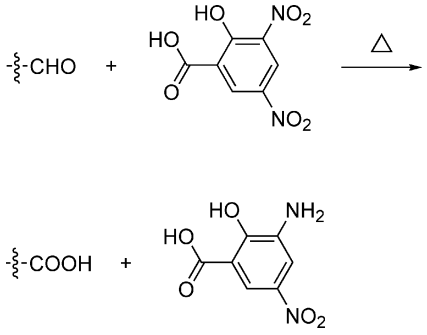
synthesis as for the β-cyclodextrin dicyanohydrin,[32] we achieved reduction of the nitrile group to the corresponding aminomethyl group by using an excess of palladium in the final de-*O*-benzylation step (Scheme 3).

The second step selectively de-*O*-benzylates only the positions 6^A and 6^D.[35]

The resulting cyclodextrin **3** was tested for catalysis of *p*-nitrophenyl β-D-glucopyranoside hydrolysis, but only little activity was found; however, the binding was fairly strong (Table 3). The small rate of catalysis is not too surprising, keeping in mind the poor electron-withdrawing ability of the aminomethyl group compared to the nitrile. This leads to decreased acidity of the alcohol proton and thus less acid catalysis potential.

Reductive Sugar Assay

In order to be able to investigate the hydrolysis of glycosides with non-UV detectable aglycon hydrolysis products, an alternative non-HPAEC assay for the detection of reducing sugars was implemented, which does not require expensive equipment and can even be performed on a large scale. For this purpose, the Bernfeld assay[36] was applied, which relies on a 3,5-dinitrosalicylic acid reagent which upon heating is reduced by the sugar aldehyde to 3-amino-5-nitrosalicylic acid,[37] which can be detected at 540 nm (Scheme 4).



Scheme 4. Bernfeld reducing sugar assay.

Using, for conversion purposes, a prepared standard curve of glucose concentration vs. absorption, the Bernfeld assay was used to measure the extent of hydrolysis of simple glycosides (see Exp. Sect. for details). As this assay is of general nature and easily conducted, the range of substrates that can be studied is very broad, allowing for various future experiments on a diverse selection of substrates.

Development of Quantitative Catechol Detection Assay

One of the most straightforward and convenient ways to monitor the kinetics of enzymatic reactions, is by continuous UV-measurements in a standard spectrophotometer. We wanted to create an assay methodology applicable for following the hydrolysis of esculin, a reaction which can not be monitored directly by UV since both esculin and esculetin have absorption maxima at 340–342 nm. The inspiration came from the field of molecular biology, wherein a ferric, bile-containing in vitro esculetin detection assay has been used for the purpose of classifying Lancefield group D streptococci into enterococci and non-enterococci, the latter of which posesses esculinase activity.[38,39] The original ferric assay, described by Meulen,[40,41] is a non-quantitative simple test with only two possible outputs, so from this, we developed a quantitative continous catechol assay.

The vicinal hydroxy groups in esculetin form together with the ferric ion a black-brownish complex, presumed to have the structure shown in Figure 5.[42] We measured the extinction coefficient for this complex to be ε = 1.85 $mM^{-1}\,cm^{-1}$.

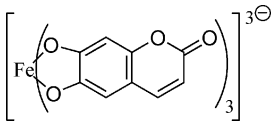


Figure 5. Structure of Fe^{III}-esculetin complex.

The development of the assay proceeded in a systematic order: First, we confirmed that dark coloration was a product only of esculetin in combination with Fe^{III}, and no different combination of these and other assay components gave rise to color change. Then, we performed a series of UV-measurements under a range of different conditions and concentrations, in order to determine the appropriate wavelength range for detection of the Fe^{III}-esculetin complex. From this, we saw that esculin and esculetin dominated the whole 200–400 nm range, but the 450–600 nm area showed some absorption of the ferric complex, unrelated to other components. In order to determine the precise optimal ferric complex detection wavelength, we performed an assay with β-glucosidase and esculin, with measurement of the increase of absorption at a number of different wavelengths in the 400–600 nm range (50 mM phosphate buffer, pH 8.0, 25 °C). From this, 570 nm proved to be the optimal detection wavelength. In the same assay, we experimented using different concentrations of Fe^{III} ion present, and found 30 mM to yield the best results.

We then validated the assay with β-glucosidase and esculin as a substrate. The resulting Hanes plot (Figure 6) is of good correlation and thus confirms the general validity of the assay.

We furthermore wanted to check the validity of the assay using cyclodextrin dicyanohydrins as enzymes. For this purpose, we synthesized the substrate *ortho*-hydroxyphenyl β-D-glucopyranoside **5** in two steps from β-D-glucose pentaacetate (Scheme 5). **5** is presumably cleaved by the cyclodextrin in the same fashion as the previously tested nitrophenyl glucopyranosides[14].

Using similar conditions as for the β-glucosidase assay (see Exp. Sect.), we found that the catechol assay was useful for monitoring the β-cyclodextrin dicyanohydrin-catalyzed hydrolysis of **5**, thus confirming the validity of the assay.

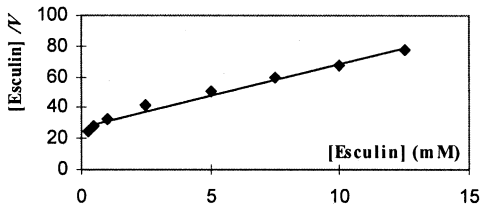
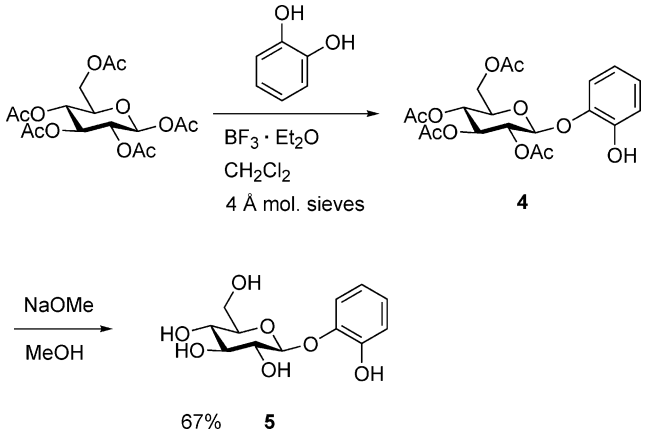


Figure 6. Hanes plot of β-glucosidase-catalyzed esculin hydrolysis, in 50 mM phosphate buffer at pH 8.0 and 25 °C, in the presence of 30 mM Fe^{III} ammonium citrate (R^2 = 0.98).



Scheme 5. Synthesis of substrate for catechol assay validation.

Conclusions

The breakdown and neutralization of toxic glycosides is an important task that could be performed enzymatically with great economical and environmental benefits. We have developed and implemented novel assay procedures and used these for performing hydrolysis assays of various toxic substrates with cyclodextrin dicyanohydrins as catalysts. As expected, little catalysis of *p*-nitrophenyl β-D-glucopyranoside hydrolysis was found for the analogue diaminomethyl β-cyclodextrin. Using a newly developed continuous catechol detection assay, a weak degree of catalysis was found for the toxic natural glycoside esculin with β-cyclodextrin dicyanohydrin. An equipment-light reductive sugar assay was used to asses the catalysis of simple benzyl or alkyl glucoside substrates, but none of the compounds were found active. A number of toxic hydroxycoumarin and related compounds were tested using HPAEC or UV detection. For skimmin and phloridzin, good to moderate degrees of catalysis were found with cyclodextrin dicyanohydrins, with k_{cat}/k_{uncat} values up to 1259. For the artificial glycoside 4-MUGP, β-cyclodextrin dicyanohydrin was able to efficiently catalyze the hydrolysis with an impressive k_{cat}/k_{uncat} value of up to 7569. Making artificial enzymes from scratch and using these successfully to achieve significant catalysis of chemical and biological reactions is challenging, but in several cases we observed good enzymatic ability of cyclodextrin dicyanohydrins. The results are encouraging, and open up the possibility for alternative uses of modified cyclodextrins as artificial enzymes. Through further chemical modification of the cyclodextrins, we hope in the future to accomplish improved rates of catalysis and to learn more about the factors governing catalysis.

Experimental Section

General: Solvents were distilled under anhydrous conditions. All reagents were used as purchased without further purification. Evaporation of organic solvents was performed on a rotary evaporator under reduced pressure and with the temperature kept below 40 °C. Columns for flash chromatography were packed with silica gel 60 (230–400 mesh) as the stationary phase. TLC silica plates (Merck 60, F_{254}) were visualized by addition of CMOL-visualizer [cerium sulfate (1%) and molybdic acid (1.5%) in 10% sulfuric acid] and heating until visible spots appeared, and by UV light when applicable. ^1H and ^{13}C NMR spectroscopic data were recorded on a Varian Mercury 400 MHz NMR spectrometer. Chemical shifts are given in ppm and referred to internal $SiMe_4$ (δ_H, δ_C = 0.00). Optical rotations were recorded at room temperature on a Perkin–Elmer 241 polarimeter. IR spectra were recorded on a Perkin–Elmer FT-IR Paragon 1000. Enzymatic UV-assays were performed using a Spectronic Genesys 5 spectrophotometer.

Procedure for Determining the Rate of Hydrolysis by Direct UV Measurement: Each assay was performed on 50 mM phosphate buffer samples (1 mL), prepared from aqueous solutions of the appropriate aryl glycoside at different concentrations (0.5 mL) mixed with phosphate buffer solution (0.5 mL) containing either cyclodextrin catalyst or nothing as control. Concentration of cyclodextrin dicyanohydrin catalyst was between 0.34–0.91 mM, and concentration of β-cyclodextrin diaminomethyl was 0.54 mM. The reactions were followed continuously at 60 °C using UV absorption at 365 nm for skimmin (ε = 15.02 $mM^{-1}cm^{-1}$), 360 nm for 4-MUGP (ε = 8.92 $mM^{-1}cm^{-1}$) and 400 nm for *p*-nitrophenyl β-D-glucopyranoside (ε = 17.59 $mM^{-1}cm^{-1}$). The reactions were monitored for 1–3 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 V were used to determine K_M and V_{max} from non-linear regression of V vs. [S] using the program Dataplot; k_{cat} was calculated as V_{max}/[cyclodextrin], k_{uncat} was determined as the slope from a plot of V_{uncat} vs. [S].

Procedure for Determining the Rate of Hydrolysis by Catechol Detection Assay: Each assay was performed as a direct UV-measurement assay, as described above. 30 mM Fe^{III} ammonium citrate was present in all cuvettes. UV-absorption was measured continuously at 570 nm for Fe^{III}-esculetin complex (ε = 1.85 $mM^{-1}cm^{-1}$).

For assay performed with esculin, substrate concentration was between 1–10 mM and β-cyclodextrin dicyanohydrin concentration was 1.0 mM.

For catechol-assay validation with glucoside **5**, the substrate concentration was between 3–15 mM and the β-cyclodextrin dicyanohydrin concentration was 2.5 mM. A linear correlation between the measured β-cyclodextrin dicyanohydrin-related increase in absorption and the *ortho*-hydroxyphenyl β-D-glucopyranoside concentration was found, as expected.

Procedure for Determining the Rate of Hydrolysis by HPAEC (High-Performance Anion-Exchange Chromatography): HPEAC was performed on a Dionex ICS 3000 system equipped with an

Electrochemical Detector with a gold working electrode and a silver/silver chloride reference electrode. The column used was a Dionex CarboPac PA1 (4 × 250 mm analytical column).

Each assay was performed on 50 mM phosphate buffer samples (3 mL), prepared from aqueous solutions of the appropriate aryl glycoside at 4 different concentrations (1.5 mL) mixed with phosphate buffer (1.5 mL) containing either cyclodextrin or nothing as control. The reactions were conducted at 60 °C and samples (200 μL) were taken out at specified times and cooled with ice. The sample was injected onto the HPAEC and then eluted at 25 °C at a flowrate of 1 mL/min. The following gradient system was used: gradient A (150 mM NaOH), gradient B (500 mM NaOAc in 150 mM NaOH). 0–5 min 100% A, 5.1–11.5 min 100% B and 12–15 min 100% A. The amount of glucose was determined from a standard curve which was produced from triple determinations of 6 different concentrations of glucose using the Dionex Chromeleon software.

Procedure for Determining the Rate of Hydrolysis by the Reductive Sugar Assay: Experiments were performed in 50 mM phosphate buffer at pH 8.0, with [cyclodextrin] = 0.42 mM. Each of the glycoside substrates was tested for hydrolysis at 3 different substrate concentrations, with each concentration tested for hydrolysis under 3 different conditions: in the presence of α-cyclodextrin dicyanohydrin, β-cyclodextrin dicyanohydrin and with buffer only, respectively. All of the above-mentioned measurements were furthermore performed at two different temperatures: 25 and 90 °C. For each experiment, a blank (water-containing) sample was included. A representative selection of the experiments were reconducted under identical conditions to assess validity and reproducability of the data. Reaction mixtures (with differering amounts of substrate and cyclodextrin) of 3 mL volume were left stirring in sealed glass vials in a heating block or at room temperature. At regular time intervals (hours) a small amount (0.35 mL) of each reaction mixture was added an equal volume (0.35 mL) of 3,5-dinitrosalicylic acid test solution. The assay mixture was then incubated at 100 °C for 5 min. The developed assay mixture was cooled to room temperature, diluted with water (3.5 mL), and the absorbance at 540 nm was measured. By comparing to a standardcurve of absorbance vs. glucose concentration, the hydrolysis progress was monitored.

7-[(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)oxy]-2*H*-1-benzopyran-2-one (1): To a solution of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (2.00 g, 4.86 mmol) and 7-hydroxycoumarin (790 mg, 4.87 mmol) in acetonitrile (15 mL) was added Ag_2O (1.35 g, 5.83 mmol) and the mixture was stirred overnight at room temperature in the absence of light. The mixture was then diluted with EtOAc (20 mL), filtered through a bed of Celite and washed with EtOAc (3 × 20 mL). The filtrate was washed with aq. KOH (0.25 M, 2 × 20 mL), brine (20 mL), and water (20 mL), dried ($MgSO_4$) and concentrated under reduced pressure. The crude product was recrystallized from methanol which afforded the desired product as white crystals (626 mg, 26%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.64 (d, J = 9.6 Hz, 1 H), 7.39 (d, J = 8.6 Hz, 1 H), 6.95 (d, 1 H), 6.90 (dd, J = 2.3, J = 8.5 Hz, 1 H), 6.31 (d, J = 9.5 Hz, 1 H), 5.36–5.26 (m, 2 H), 5.19–5.13 (m, 2 H), 4.28 (dd, J = 5.8, J = 2.3 Hz, 1 H), 4.18 (d, J = 12.3 Hz, 1 H), 3.96–3.88 (m, 1 H), 2.11 (s, 3 H), 2.06 (s, 6 H), 2.03 (s, 3 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.3, 169.9, 169.3, 169.1, 160.4, 159.2, 155.2, 143.0, 128.8, 114.3, 114.2, 114.0, 103.8, 98.1, 72.4, 72.1, 70.8, 68.0, 61.7, 20.5, 20.4 ppm. HR-MS (ES): *m*/*z* calcd. for $C_{23}H_{24}NaO_{12}$: 515.1166, found: 515.1166.

7-(β-D-Glucopyranosyloxy)-2*H*-1-benzopyran-2-one (Skimmin) (2): To a solution of **1** (0.30 g, 0.61 mmol) in dry methanol (40 mL) was added sodium methoxide (25 w/w% in 6.00 mL MeOH, 26.2 mmol) and the mixture was stirred at 5 °C for 2 h. The reaction was quenched by addition of dry ice (until pH 6–7). Methanol was removed under reduced pressure and the residue was subjected to ion exchange (IR-120 H). The resulting aqueous solution was concentrated in vacuo and subsequently dried using lyophilization, which afforded the desired product (175 mg, 89%). ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 8.00 (d, J = 9.6 Hz, 1 H), 7.65 (d, J = 8.6 Hz, 1 H), 7.05 (d, J = 2.3 Hz, 1 H), 7.01 (dd, J = 2.4, J = 8.6 Hz, 1 H), 6.33 (d, J = 9.6 Hz, 1 H), 5.38 (d, J = 4.8 Hz, 1 H, OH), 5.12 (d, J = 4.6 Hz, 1 H, OH), 5.05 (d, J = 5.3 Hz, 1 H, OH), 5.03 (d, J = 7.3 Hz, 1 H), 4.58 (t, J = 5.6 Hz, 1 H, OH), 3.71 (dd, J = 5.6, J = 10.4 Hz, 1 H), 3.44 (m, 2 H), 3.28 (m, 2 H), 3.17 (td, J = 7.1, J = 14.1 Hz, 1 H) ppm. ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 160.1, 154.9, 144.1, 129.3, 113.5, 113.1, 113.0, 103.0, 99.8, 77.0, 76.3, 73.0, 69.5, 60.5 ppm. HR-MS (ES): *m*/*z* calcd. for $C_{15}H_{16}NaO_8$: 347.0743, found: 347.0750.

(6^A*R*,6^D*R*)-6^A,6^D-Di-*C*-Aminomethyl-β-cyclodextrin (3): The product was obtained from de-*O*-benzylation of the corresponding perbenzylated dicyanohydrin β-cyclodextrin [(6^A*R*,6^D*R*)-6^A,6^D-Di-*C*-cyano-2$^{A–G}$,3$^{A–G}$,6$^{B,C,E–G}$-nonadecakis-*O*-benzyl-β-cyclodextrin][43] (1.0 g, 0.3 mmol), which was dissolved in MeOH/EtOAc (1:1, 150 mL). Then Pd/C (5%, 1.5 g) and TFA (7 drops) were added, and the reaction was left to stir at room temperature under hydrogen atmosphere for 2 days. The reaction progress was monitored by MALDI-TOF-MS. Filtration through a bed of Celite and evaporation of the solvent afforded **3** in quantitative yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.08–4.76 (m, 7 H, 1-H), 4.31–4.12, (m, 1 H), 4.12–3.90 (m, 1 H), 3.90–3.18 (m, 40 H), 3.18–2.96 (m, 2 H, 2-H) ppm. ^{13}C NMR (100 MHz, D_2O): δ = 101.9, 82.1, 81.4, 81.1, 80.7, 73.2, 72.8, 72.3, 71.8, 71.1, 68.9, 65.2, 65.1, 63.4, 60.7, 60.3, 42.8, 39.5 ppm. MALDI-TOF-MS *m*/*z* calcd. for $C_{44}H_{76}N_2NaO_{35}$: 1215.4121, found: 1215.6175.

***ortho*-Hydroxyphenyl 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranoside (4):** β-D-Glucose pentaacetate (2.00 g, 5.12 mmol) and pyrocatechol (621 mg, 5.64 mmol, 1.1 equiv.) were dissolved in dry dichloromethane (10 mL), in the presence of molecular sieves (4 Å, 5.0 g). Boron trifluoride–diethyl ether (0.64 mL, 5.12 mmol, 1 equiv.) was added dropwise. The reaction was stirred overnight at room temperature under nitrogen atmosphere. Reaction progress was monitored by TLC (silica, eluent diethyl ether/pentane 1:1). The reaction mixture was filtered and to the filtrate was added dichloromethane (100 mL). The organic layer was washed with sat. aq. $NaHCO_3$ (3 × 40 mL), 0.5 M NaOH (3 × 33 mL) and water, dried ($MgSO_4$), filtered and concentrated in vacuo, affording the crude product as a colorless foam. Partial purification of the crude product was performed by flash chromatography (eluent gradient, EtOAc/pentane 1:3 → EtOAc), affording primarely the desired product as a colorless solid (340 mg, 0.77 mmol), upon which the next step of the synthesis was based.

***ortho*-Hydroxyphenyl β-D-Glucopyranoside (5):** *ortho*-Hydroxyphenyl 2,3,4,6-tetra-*O*-acetyl β-D-glucopyranoside (**4**) (340 mg, 0.772 mmol) was dissolved in dry methanol (8 mL), sodium methoxide (3 mL, freshly made from sodium and methanol) was added to the solution. The reaction mixture was stirred at room temperature and the reaction progress was monitored by TLC (silica, eluent EtOAc/pentane 1:3). After 3.5 h the reaction mixture was made slightly acidic by addition of acetic acid to the mixture, after which all solvent was removed in vacuo. The product was purified by flash chromatography (eluent EtOAc/MeOH 5:1), which afforded the pure and desired product (141 mg, 0.518 mmol, 67%) as a colorless powder. IR (film): $\tilde{\nu}$ = 3419, 2925, 1600, 1501, 1465, 1375, 1268,

1204, 1109, 1069, 1015, 791, 731 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ = 7.09 (dd, ^3J = 8.0, ^4J = 1.6 Hz, 1 H, 3′-H/6′-H), 6.96–6.82 (m, 3 H, 3′-H/6′-H, 4′-H, 5′-H), 4.97 (d, $J_{1,2}$ = 7.6 Hz, 1 H, 1-H), 3.80 (dd, $J_{5,6a}$ = 2.4, $J_{6a,6b}$ = 12.4 Hz, 1 H, 6a-H), 3.65 (dd, $J_{5,6b}$ = 5.2, $J_{6a,6b}$ = 12.4 Hz, 1 H, 6b-H), 3.54–3.38 (m, 4 H, 2-H, 3-H, 4-H, 5-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 145.7, 144.7 (C-1′, C-2′), 124.3, 121.3, 117.0, 116.7 (C-3′, C-4′, C-5′, C-6′), 101.3 (C-1), 76.3, 75.6, 73.0, 69.5 (C-2, C-3, C-4, C-5), 60.6 (C-6) ppm. HR-MS (ES): *m*/*z* calcd. for $C_{12}H_{16}NaO_7$: 295.0794, found: 295.0802.

Supporting Information (see also the footnote on the first page of this article): NMR spectrum of compound **3**.

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[1] E. Weinhouse, J. Kaplanski, A. Danon, D. B. Nudel, *Life Sci.* **1989**, *44*, 441–450.
[2] C. G. Hanratty, P. McGlinchey, D. Johnston, A. P. Passmore, *Drug. Aging* **2000**, *17*, 353–362.
[3] C. Hamouda, A. Hedhili, N. Ben Salah, M. Zhioua, M. Amamou, *Vet. Hum. Toxicol.* **2004**, *46*, 144–146.
[4] G. Stanić, B. Jurišić, D. Brkić, *Croat. Chem. Acta* **1999**, *72*, 827–834.
[5] Z. Stefanova, H. Neychev, N. Ivanovska, I. Kostova, *J. Ethnopharmacol.* **1995**, *46*, 101–106.
[6] M. J. Martín, C. Alarcón, V. Motilva, *Ann. Pharm. Fr.* **1990**, *48*, 306–311.
[7] M. V. Rekharsky, Y. Inoue, *Chem. Rev.* **1998**, *98*, 1875–1917.
[8] J. Szejtli, *Comprehensive Supramolecular Chem.* **1996**, *3*, 5–40.
[9] V. J. Stella, R. A. Rajewski, *Pharm. Res.* **1997**, *14*, 556–567.
[10] J. Szejtli, *Chemical Intelligencer* **1999**, *5*, 38–45.
[11] H. J. Buschmann, D. Knittel, C. Jonas, E. Schollmeyer, *Lebensmittelchemie* **2001**, *55*, 54–56.
[12] B. Sueur, L. Leclercq, M. Sauthier, Y. Castanet, A. Mortreux, H. Bricout, S. Tilloy, E. Monflier, *Chem. Eur. J.* **2005**, *11*, 6228–6236.
[13] D. Kirschner, T. Green, F. Hapiot, S. Tilloy, L. Leclercq, H. Bricout, E. Monflier, *Adv. Synth. Catal.* **2006**, *348*, 379–386.
[14] A. Cassez, A. Ponchel, F. Hapiot, E. Monflier, *Org. Lett.* **2006**, *8*, 4823–4826.
[15] R. Breslow, *J. Phys. Org. Chem.* **2006**, *19*, 813–822.
[16] E. Kassianidis, D. Philp, *Angew. Chem. Int. Ed.* **2006**, *45*, 6344–6348.
[17] W. B. Motherwell, C. E. Atkinson, A. E. Aliev, Y. F. Stephanie, B. H. Wong, D. Warrington, *Angew. Chem. Int. Ed.* **2004**, *43*, 1225–1228.
[18] P. Travascio, D. Sen, A. J. Bennet, *Can. J. Chem.* **2006**, *84*, 613–619.
[19] K. Kikuchi, R. B. Hannak, M. J. Guo, A. J. Kirby, D. Hilvert, *Bioorg. Med. Chem.* **2006**, *14*, 6189–6196.
[20] Z. L. Fang, R. Breslow, *Org. Lett.* **2006**, *8*, 251–254.
[21] N. M. Milovic, J. D. Badjic, N. M. Kostic, *J. Am. Chem. Soc.* **2004**, *126*, 696–697.
[22] L. G. Marinescu, M. Bols, *Angew. Chem. Int. Ed.* **2006**, *45*, 4590–4593.
[23] For a new method of modifying the secondary rim, see: M. Fukudome, A. Matsushima, D. Q. Yuan, K. Fujita, *Tetrahedron Lett.* **2006**, *47*, 6599–6602.
[24] C. Rousseau, F. Ortega-Caballero, L. U. Nordstrøm, B. Christensen, T. E. Petersen, M. Bols, *Chem. Eur. J.* **2005**, *11*, 5094–5101.
[25] F. Ortega-Caballero, J. Bjerre, L. S. Laustsen, M. Bols, *J. Org. Chem.* **2005**, *70*, 7217–7226.
[26] J. Bjerre, T. H. Fenger, L. Marinescu, M. Bols, *Eur. J. Org. Chem.* **2007**, 704–710.
[27] K. Taeufel, *Nahrung* **1968**, *12*, 275–282.
[28] M. Runkel, M. Bourian, W. Legrum, *Fruit Processing* **1997**, *7*, 213–216.
[29] S. Oh, D. Kang, *Appl. Environ. Microbiol.* **2004**, *70*, 5692–5694.
[30] L. B. Daniels, P. J. Coyle, R. H. Glew, N. S. Radin, R. S. Labow, *Archives of Neurology* **1982**, *39*, 550–556.
[31] V. Crespy, O. Aprikian, C. Morand, C. Besson, C. Manach, C. Demigné, C. Remésy, *J. Nutr.* **2001**, 3227–3230.
[32] V. L. Singleton, F. H. J. Kratzer, *Agr. Food. Chem.* **1969**, *17*, 497–512.
[33] Y. P. Davol, *Indian J. Biochem.* **1967**, *4*, 54–55.
[34] G. W. Newton, E. S. Schmidt, J. P. Lewis, E. Conn, R. Lawrence, *Western J. Med.* **1981**, *134*, 97–103.
[35] A. J. Pearce, P. Sinaÿ, *Angew. Chem. Int. Ed.* **2000**, *39*, 3610–3612.
[36] P. Bernfeld, *Adv. Enzymol. Relat. Areas Mol. Biol.* **1951**, *12*, 379–428.
[37] L. D. Frost, *Chem. Educ.* **2004**, *9*, 239–241.
[38] S. M. H. Qadri, M. I. DeSilva, S. Zubairi, *J. Clin. Microbiol.* **1980**, *12*, 472–474.
[39] S. C. Edberg, R. W. Trepeta, C. M. Kontnick, A. R. Torres, *J. Clin. Microbiol.* **1985**, *21*, 363–365.
[40] F. C. Harrison, J. van der Leck, *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg.* **1909**, *22*, 547–551.
[41] A. Miskin, S. C. Edberg, *J. Clin. Microbiol.* **1978**, *7*, 251–254.
[42] B. D. Jain, H. B. Singh, *J. Indian Chem. Soc.* **1963**, *40*, 263–264.
[43] F. Ortega-Caballero, C. Rousseau, B. Christensen, T. E. Petersen, M. Bols, *J. Am. Chem. Soc.* **2005**, *127*, 3238–3239.

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