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ORIGINAL ARTICLE

Simple cyclodextrin aldehydes as excellent artificial oxidases

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Abstract Cyclodextrin based oxidases, with a ketone as functional group are well known as good artificial enzyme mimics (Fenger et al. Org Biomol Chem 7:933–943; Marinescu and Bols Angew Chem Int Ed 45:4590–4593; Bjerre et al. Eur J Org Chem 704–710; Marinescu et al. J Am Chem Soc 127:17578–17579). We here report a series of modified cyclodextrins, having aldehydes as functional groups. The aldehyde based artificial enzymes have, in most cases, better catalysis than the ketones, because of their powerful covalent binding of hydrogen peroxide. Among the modified cyclodextrins studied are mono and di aldehydes on the 6 positions, with or without methylated hydroxyl groups. The aldehyde functionality was also introduced close to the secondary side, by attaching ethoxy-2-al or propoxy-3-al to the 2 position. The modified cyclodextrins showed excellent enzymatic activity towards oxidation of different aminophenols, and 4-methoxy benzyl alcohol with hydrogen peroxide as a stoichiometric oxidant. Rate enhancements up to 4,600 were achieved for oxidation of 4-methoxy benzyl alcohol, where as oxidation of amines gave rate enhancements up to 3,400. The artificial oxidases catalyses oxidations under enzymatic conditions (water, pH 7, 25 °C), following Michaelis–Menten kinetics. To confirm the enzyme activity, inhibition studies with sodium naphthalene-2-sulfonate were carried out. These studies showed competitive inhibition of the enzymes, verifying the cyclodextrins enzyme like character.

Keywords Cyclodextrin · Artificial enzyme · Oxidation · Inhibition · Catalysis · Inclusion

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Introduction

Enzymes are very fascinating, catalysing reactions in biological systems that would take millions of years, in only seconds. One of the highest rate enhancements achieved is 10^{17} for OMP decarboxylase [5]. To mimic enzymes with the same rate enhancements would have a great impact on economy as well as the environment.

Catalysis of chemical reactions by artificial enzymes are indeed helpful, in the sense that they can catalyse under native enzyme conditions, leading a way into green chemistry, and being environmentally friendly by avoiding various organic solvents and chemicals.

To synthesise an artificial enzyme one needs a template capable of binding a given class of compounds. Here the cyclodextrins abilities of forming inclusion complexes and ease of chemical modification, makes the cyclodextrin an obvious template choice [6] (Fig. 1).

Materials and methods

Chemicals

All chemicals were provided by Sigma–Aldrich and used without further purification. Phosphate buffer solutions with different pH were prepared and used for kinetic studies. Solvents were distilled under anhydrous conditions.

Enzyme kinetics

All enzyme kinetics were measured by UV–Vis on a Thermo Scientific Evolution 600. The reaction velocities (V) were measured from seven samples with a fixed enzyme concentration (0.17 mM) and increasing substrate

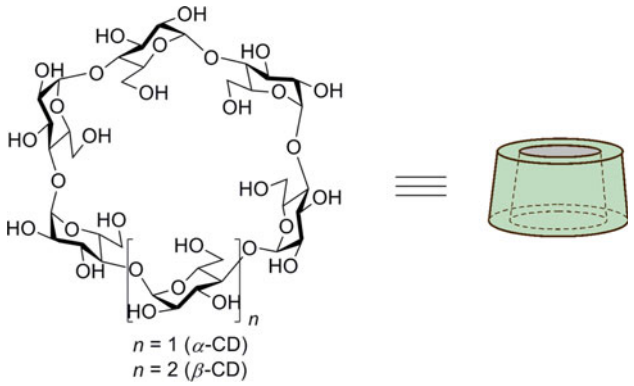


Fig. 1 α and β cyclodextrin, represented as a cone structure, capable of binding small lipophilic molecules

concentrations ([S] = 1–10 mM) in a phosphate buffer solution (0.9 mM, pH 7) containing hydrogen peroxide (64 mM) at 25 °C. The uncatalysed reactions (k_{uncat}) were measured in the same way, but having buffer instead of enzyme. To be sure that the reactions followed Michaelis–Menten kinetics, Wolf-Hanes plots were made ([S]/V vs. [S]). Enzyme rate calculations were done by using least-square nonlinear regression using Data plot, fitting to the Michaelis–Menten equation determinating V_{max} and K_M. k_{cat} can be calculated from V_{max} and the cyclodextrin concentration [7].

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{cat}}{\longrightarrow} E + P \qquad K_M = (k_2 + k_{cat})/k_1$$

Inhibition studies were done by having a fixed enzyme concentration and varying substrate and inhibitor concentration, giving a Dixon plot, from which the inhibition constant K_i easily could be determined [8].

Results and discussion

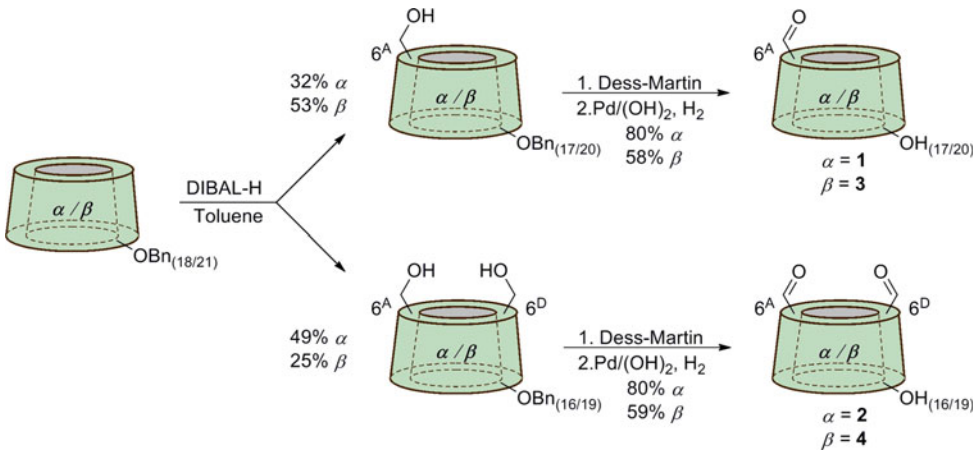
Various simple cyclodextrin aldehydes were synthesised and tested for their ability to oxidize aromatic amines, benzylic alcohols, hydroquinone and catechol. Furthermore a series of inhibition studies were carried out to verify their properties as artificial enzymes.

Synthesis

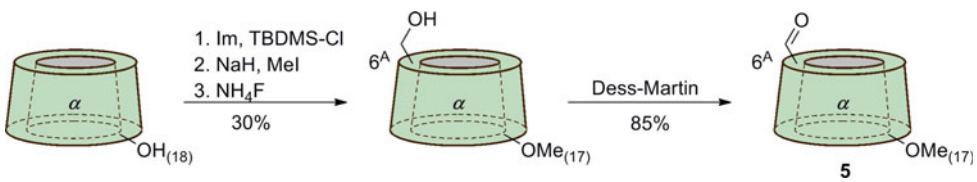
Among the aldehydes synthesised, were the series of four α and β cyclodextrin mono (6^A) and dialdehydes ($6^{A,\,D}$), as shown in Scheme 1 (**1**–**4**). The monool and diol perbenzylated α/β cyclodextrins were synthesised by the selective debenzylation by DIBAL-H as reported earlier by Pearce and Sinaÿ [9] followed by oxidation and subsequent debenzylation as reported by Fenger et al. [10]. The series of the four permethylated α/β cyclodextrin mono- and dialdehydes has also been synthesised [10].

The 6^A-oxo-α-permethylcyclodextrin **5** was synthesised analogously to a procedure by Chen et al. [10, 11]. Starting by mono protection of α-cyclodextrin on the 6 position, then subsequent methylation, removal of the protecting group and finally oxidation to compound **5** (Scheme 2).

To study the effect of aldehydes on the secondary side of the cyclodextrins, chemically modification on the 2^A position was done by allylation, methylation, hydroboration/



Scheme 1 Synthesis of the four primary side cyclodextrin aldehydes



Scheme 2 Synthesis of permethylated primary side aldehyde **5**

oxidation [10, 12], to give the two methylated β-cyclodextrins with either an 2-acetaldehyde or a 3-propanal group as shown in Scheme 3.

Substrate oxidation with carbonyl and hydrogen peroxide, a mechanistic insight

Perhydrate oxidations of amines and alcohols are well known [1–4]. The believed mechanism for the oxidation is shown in Scheme 4. (a) Hydrogen peroxide attacks the carbonyl forming the active perhydrate. (b) An inclusion complex forms from the activated artificial enzyme and substrate, and reaction takes place. (c–d) The bound product leaves the cavity, and the unactivated enzyme is ready for yet another catalytic cycle.

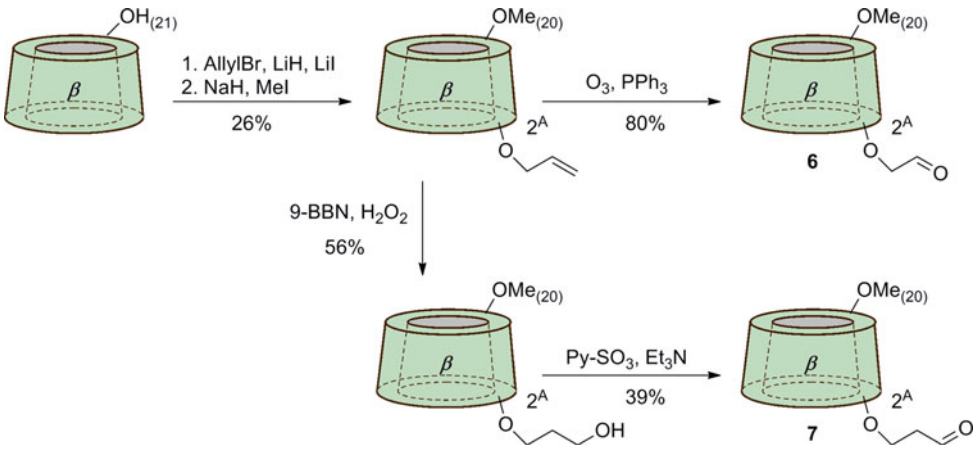
Catalysis

As mentioned earlier the reactions were studied by UV–Vis by increase in absorption of a given product, depending on the substrate. Among the substrates tested were 2-aminophenol, 4-aminophenol, hydroquinone, catechol and 4-methoxybenzyl alcohol.

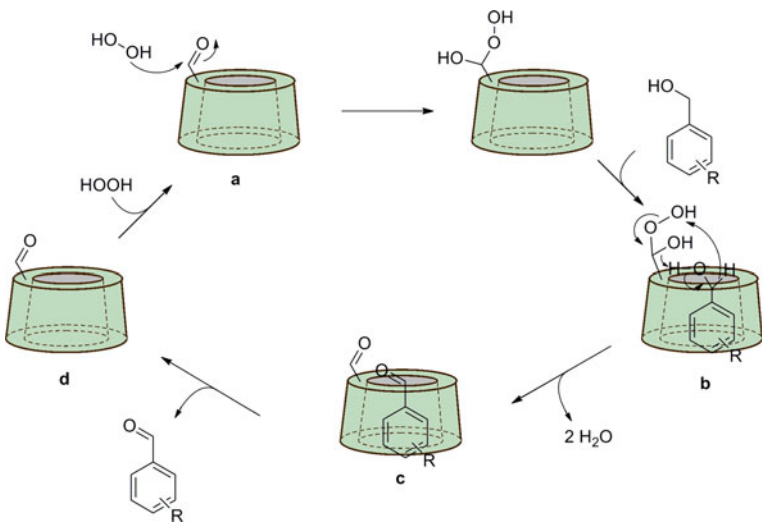
For oxidation of 2-aminophenol the observed product is compound **8** [13] and for oxidation of 4-aminophenol the observed product is compound **9** [14] (Scheme 5). The 4-methoxybenzyl alcohol is oxidized to the corresponding aldehyde.

The oxidation of hydroquinone to *p*-benzoquinone was more complex than firstly anticipated. Following the reaction for *p*-benzoquinone at 246 or 425 nm, did not work, since the hydroquinone and *p*-benzoquinone can join to make a quinhydrone charge transfer complex. Following the reaction of the quinhydrone charge transfer complex, did not work either, possibly due to subsequent decomposition of *p*-benzoquinone and their by the quinhydrone charge transfer complex [15].

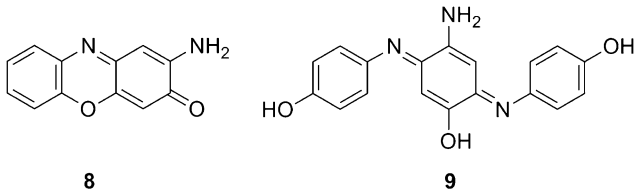
Instead the reaction was followed at 289 nm, measuring conversion of hydroquinone. By following the reaction at this wavelength, the enzyme concentration should be kept low due to the n → π* absorbance of the carbonyl group on the enzyme. Also the hydrogen peroxide concentration



Scheme 3 Synthesis of the secondary side aldehydes **6** and **7**



Scheme 4 Proposed enzymatic catalytic cycle for oxidation, with cyclodextrin aldehydes and hydrogen peroxide



Scheme 5 Oxidation products of 2-aminophenol and 4-aminophenol

should be low, because of a possible further oxidation of *p*-benzoquinone to 2,5-dihydroxy-*p*-benzoquinone [16] (Scheme 6).

The highest rate enhancement (k_{cat}/k_{uncat}) achieved for oxidation of hydroquinone was 1,030, with a K_M of 1.04 mM, by compound **2**. The experiment was done in a phosphate buffered solution at pH 7, 25 °C, 1 h, with a hydrogen peroxide concentration of 6.4 mM. The enzyme concentration was 5.27E−5 M, and the substrate concentration between 0.1 and 0.9 mM.

Oxidation of catechol leads to the unstable *o*-quinone.

A scan of the background reaction was done, at a catechol concentration of 0.94 mM, and hydrogen peroxide of 6.40 mM. No conversion of catechol was observed at 276 nm. This was not expected as the catechol should be oxidized to *o*-quinone in the presence of hydrogen peroxide [17]. An enzyme screening of compounds **2–7** under the same conditions, for 2½ h did not show any noticeable change at 276 nm, but a rise at 245 nm. Hence the catechol oxidation was abandoned.

The kinetic amine oxidation results of compound **1–7** are shown in Table 1 and the kinetic results for oxidation of 4-methoxy benzyl alcohol in Table 2.

For oxidation of 2-aminophenol a linear relationship is observed between the number of aldehyde groups present and the rate observed, as seen by the pairs **1–2** and **3–4**. Between compound **1** and **3** (α and β cyclodextrin respectively) there is not a difference, the same is also seen for compound **2** and **4**. Compound **6** is quite interesting, it has only got one aldehyde group, but is just as effective as the primary dialdehydes, this pattern is almost the same for compound **5**. The K_M value gives an indication of the binding of the substrate, for compound **7** the K_M value is quite high and the k_{cat} is quite low, possibly indicating catalysis outside the cavity of the cyclodextrin, because of the long tethered aldehyde.

Oxidation of 4-aminophenol shows almost the same trend as for 2-aminophenol oxidation with compound **1–4,** the more aldehyde groups the higher the activity. The permethylated 6^A α cyclodextrin **5** showed a drop in intensity compared to the non methylated analog. Compound **6** was again, surprisingly, the best artificial enzyme, with a very high k_{cat} compared to the other compounds.

For oxidation of 4-methoxy benzyl alcohol, compound **5** was the best, with an impressive k_{cat}/k_{uncat} of 4,600.

Scheme 6 Oxidation of hydroquinone

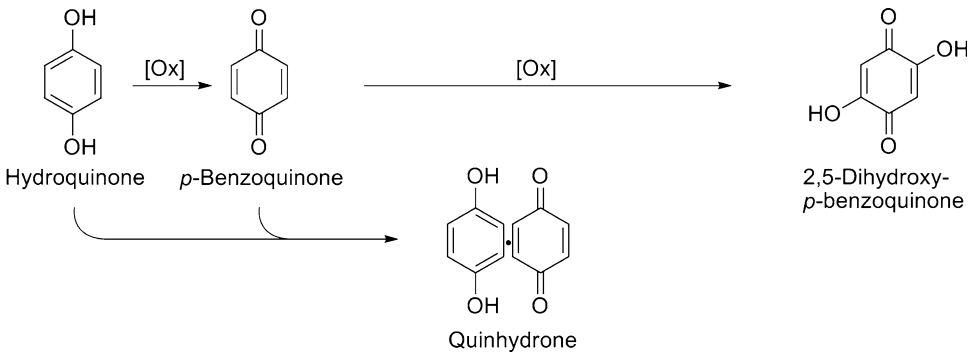


Table 1 Oxidation of 2-aminophenol and 4-aminophenol

Enzyme	2-Aminophenol			4-Aminophenol		
	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}
1	597 ± 86	19.6 ± 3.0	1426 ± 229	102 ± 59	19.2 ± 11.1	142 84
2	1131 ± 98	26.2 ± 2.3	2700 ± 303	283 ± 67	42.8 ± 6.6	395 ± 66
3	684 ± 97	18.4 ± 2.7	1632 ± 258	390 ± 37	54.7 ± 9.4	544 ± 109
4	1438 ± 147	28.9 ± 3.0	3432 ± 427	442 ± 33	48.3 ± 4.2	616 ± 79
5	1013 ± 25	23.9 ± 0.8	2419 ± 182	47.3 ± 4.7	15.3 ± 2.1	66 ± 10
6	1223 ± 52	23.7 ± 2.0	2919 ± 242	2438 ± 464	93.8 ± 19.3	3401 ± 740
7	605 ± 395	51.5 ± 33.6	1443 ± 947	632 ± 803	103 ± 131	881 ± 1123

Surprisingly the secondary side aldehyde **6** was not better at oxidizing benzylic alcohols than the primary side aldehydes.

Inhibition studies

Even though the kinetic studies confirmed that the cyclodextrins had enzyme like character, by following the Michaelis–Menten kinetics, a simple inhibition study would either reconfirm or reject the statement. To verify the enzymatic activity, a series of inhibitors were tried.

Table 2 4-Methoxy benzyl alcohol oxidation

Enzyme	4-Methoxy benzyl alcohol		
	k_{cat} (10^{-5} s^{-1})	K_M (mM)	k_{cat}/k_{uncat}
5	0.66 ± 0.25	36.7 ± 16.2	4642 ± 1737
6	0.13 ± 0.02	0.24 ± 0.75	882 ± 82

The inhibitors tried were: cyclopentanol, sodium *p*-toluenesulfonate and sodium naphthalene-2-sulfonate. The inhibition experiments were tried for both oxidation of 2-aminophenol and 4-aminophenol. For inhibition experiments with cyclopentanol there was a problem with solubility, a drop in the rate of 4-aminophenol oxidation with compound **6** was thus observed.

Competitive inhibition was observed for oxidation of 2-aminophenol with sodium naphthalene-2-sulfonate as inhibitor. The K_i values of the cyclodextrins were all around 100 mM, and shown in Table 3 [18] (Figs. 2, 3).

Inhibition with sodium *p*-toluenesulfonate gave higher K_i values than with sodium naphthalene-2-sulfonate.

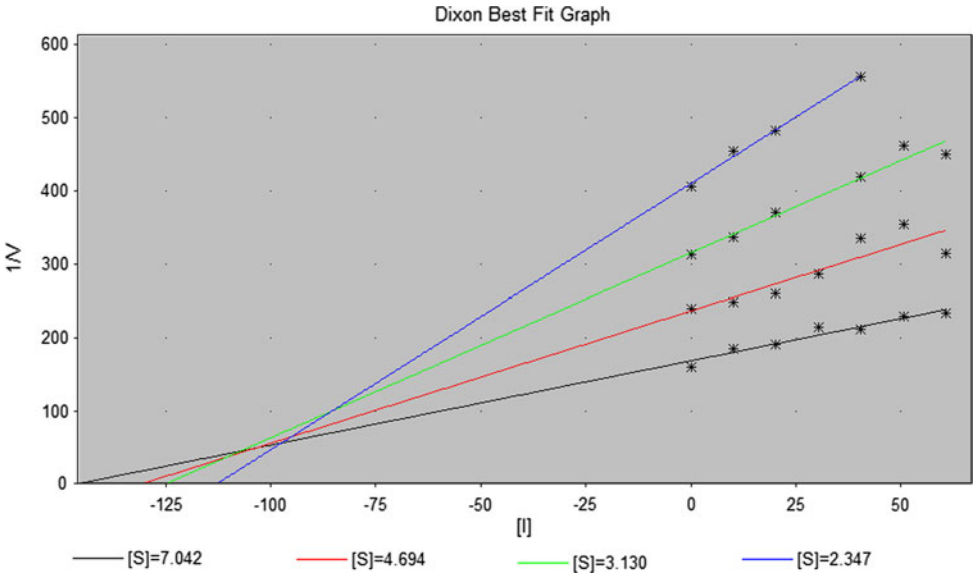


Fig. 2 Dixon plot of compound **1**

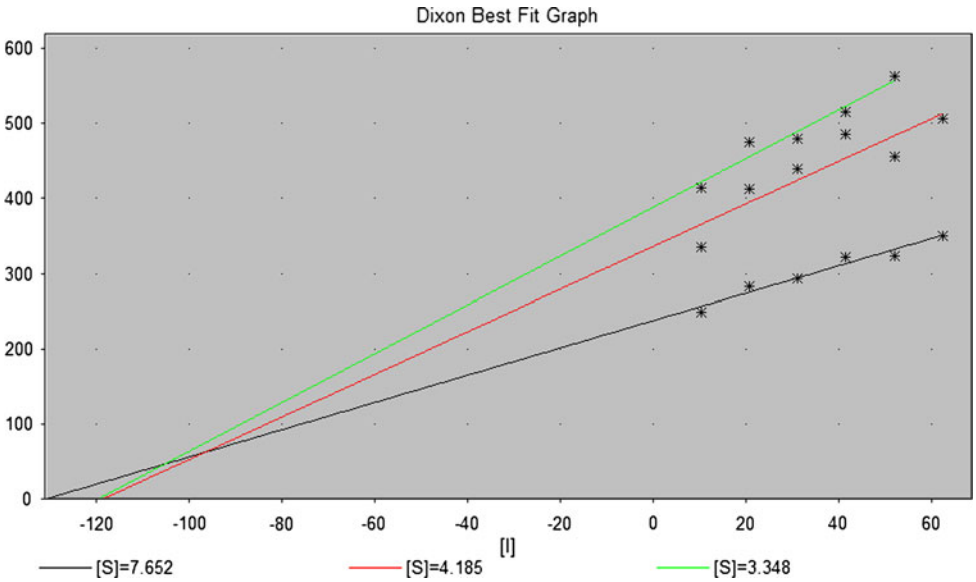


Fig. 3 Dixon plot of compound **4**

Table 3 K_i values determined from Dixon plots, with sodium naphthalene-2-sulfonate as inhibitor

Enzyme	**1**	**3**	**4**	**5**
K_i (mM)	107	105	115	140

Conclusion

Cyclodextrin aldehydes are indeed simple and effective artificial oxidases. Compound **6** was the most versatile enzyme capable of catalysing both 2-and 4-aminophenol with great rate enhancements, higher than previously reported [1–4]. Oxidation of 4-methoxy benzyl alcohol was quite good for compound **5**, but not that impressive for compound **6**. The enzyme like properties of the cyclodextrin aldehydes were further proven by inhibition studies with various inhibitors.

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