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Synthesis of Cycloheptaamylose 2-, 3-, and 6-Phosphoric Acids, and a Comparative Study of Their Effectiveness as General Acid or General Base Catalysts with Bound Substrates[1]

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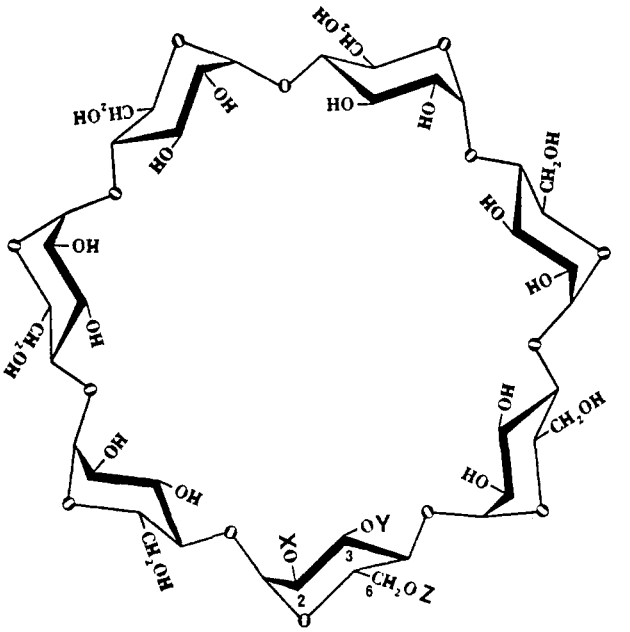
Abstract: The title compounds were prepared and examined as catalysts for the hydrolysis of *p*-nitrophenyl tetrahydropyranyl ether at low pH and the exchange of *p-tert*-butylphenacyl alcohol tritiated in the methylene group at high pH. All three isomers, as the phosphate dianions, were effective catalysts for the latter reaction, in which the functional group assists enolization of the bound ketone. Only the 3-phosphoric acid isomer showed net catalysis of the former reaction, bound substrate being hydrolyzed with the assistance of a monoanion phosphoric acid group.

The cyclodextrins (cycloamyloses) are of interest in the construction of enzyme models because they have a hydrophobic cavity of a size convenient to bind organic molecules and groups.[2] A number of reactions have been observed in which, in aqueous solutions, a cyclodextrin binds a substrate molecule into its hydrophobic interior and then catalyzes an interaction with one of the hydroxyl groups which rim the cyclodextrin cavity.[3] In addition, several kinds of functional group changes have been performed on cyclodextrin which make it even more interesting in the construction of artificial enzymes. For instance, the cavity has been modified in such a way as to provide it with a hydrophobic floor[4] to give improved binding and catalytic properties for some reactions. More generally, a multitude of functional groups have been attached to the secondary (carbons 2 and 3) and primary (carbon 6) edge of the cyclodextrin molecules.[5] In this way various nucleophilic catalysts, and catalytic metal ions, have been employed in the catalytic reactions of substrates which are also bound in the hydrophobic cavity. We now wish to report the synthesis and characterization of the three monophosphates (**1**, **2**, and **3**) of β-cyclodextrin (cycloheptaamylose)[6] in which the phosphoric acid group is attached respectively to carbons 2, 3, and 6.

Phosphate groups can act as either general base or general acid catalysts, depending on the pH. We wanted to see whether such catalysis could be demonstrated with a substrate bound in the cavity. We also wanted to explore the relative catalytic effectiveness of the three isomers[7,8] to learn more about the optimal placement of a catalytic group relative to the cavity.

As the substrate for general acid catalysis we used *p*-nitrophenyl tetrahydropyranyl ether (**4**), a model of a simple glycoside. Fife has shown[9] that **4** can be hydrolyzed with general acid catalysis in mixed aqueous or water solvents, with a much faster rate in water. Since the cyclodextrin molecule is quite large, its immediate vicinity might well be only partially aqueous in character even in water solution.[10] In fact, we did observe general acid catalysis of the hydrolysis of **4**, but only with isomer **2**. Although these compounds are of interest as catalysts in their own right regardless of their relationship to any natural system, it should be noted that the enzyme phosphorylase apparently also uses a phosphate group (attached to bound pyridoxal) as a catalyst in glycoside cleavage.[11]

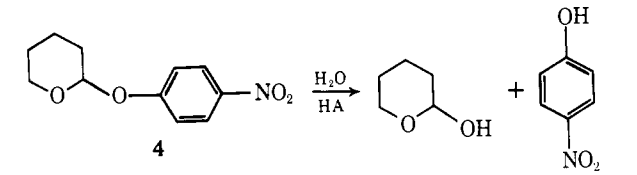


1, X = PO_3H_2, Y = Z = H
2, Y = PO_3H_2, X = Z = H
3, Z = PO_3H_2, X = Y = H
7, Z = $PO_3(C_6H_5)_2$, X = Y = H

Table I. Kinetic and Equilibrium Properties of **1**, **2**, and **3**

Measured value	β-Cyclodextrin	1	2	3	Footnote
pK_a's		2.51, 5.89	2.53, 5.85	2.58, 6.86	*a*
K_{diss} **4**, mM	1.0	2	3.0 ± 0.3	1.1	*b*
10^3k_{cat} **4**, min^{-1}	3.5	5.2	18.2	3.5	*c*
K_{diss} **5**, mM	1.2 ± 0.2	3 ± 2	2 ± 1	3 ± 2	*d*
10^3k_{cat} **5**, min^{-1}	0.004	0.33	0.53	0.50	*e*

[a] At 25.0 °C in H_2O. [b] Dissociation constants for the complexes of **4** with **1**, **2**, and **3** as their monoanions, in the pH range 4–5.5, at 25.0 °C. [c] Pseudo-first-order rate constants for the hydrolysis of **4** in a one-to-one complex with the cyclodextrins (i.e., at kinetic saturation). The pH was 4.00, and with buffer alone 10^3k_{cat} was 5.2 min^{-1}. [d] Dissociation constants for the complexes of **5** with **1**, **2**, and **3** as their dianions, at pH 9.0, at 37.0 °C. [e] Pseudo-first-order rate constants for the exchange reaction of **5** in a one-to-one complex with the cyclodextrins; see text for details.



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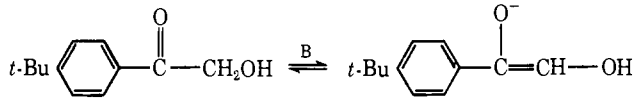

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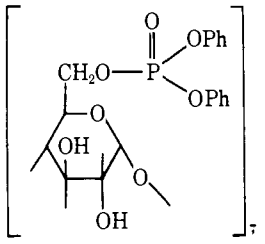
As the substrate for general base catalysis studies with **1**, **2**, and **3** we used *p-tert*-butylphenacyl alcohol[12] (**5**) tritiated in the methylene group. Base-catalyzed enolization of this compound was detected by loss of tritium. For solubility reasons the reaction was run with **1**, **2**, and **3** in 50% aqueous dimethyl sulfoxide–water, in which binding of hydrophobic substrates is almost as strong as in water.[13]

Synthesis and Characterization of the Cyclodextrin Phosphates. Reaction of cyclodextrins in organic solvents with bulky reagents generally occurs selectively on the unhindered primary hydroxyls of carbon 6. Thus reaction of cycloheptaamylose with an excess of diphenylphosphorochloridate in pyridine afforded heptakis(6-*O*-diphenylphosphoro)cycloheptaamylose (**6**). The monophosphorylated derivative (**7**) was



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prepared in a similar fashion using 0.4 equiv of diphenylphosphorochloridate to suppress further reaction. The phenyl groups were conveniently removed by catalytic hydrogenation, and the resulting cycloheptaamylose 6-monophosphate (**3**), as its ammonium salt, was isolated and purified by ion exchange chromatography. It was characterized as pure by analysis and neutralization equivalent and showed a C-13 downfield shift of 2.3 ppm for one methylene.

Reactions on the secondary side of cyclodextrin are best performed using intracomplex reactions of bound substrates.[14] Thus, a solution of cycloheptaamylose in aqueous sodium hydroxide was treated with 0.27 equiv (to suppress multiple reactions) of bis(*m*-nitrophenyl) phosphate over several days, and the products were then isolated by ion exchange chromatography. This affords a mixture of cycloheptaamylose 2-phosphate (**1**) and cycloheptaamylose 3-phosphate (**2**), undoubtedly formed by an initial phenylphosphate transfer to one of the hydroxyls from a complex of cycloheptaamylose with the substrate. This should be followed by intramolecular cyclization with elimination of the phenol to form the 2,3-cyclic phosphate, and finally hydrolysis of the cyclic phosphate to the mixture. Careful ion exchange chromatography separated **1** and **2**, which were both characterized as ammonium salts of cycloheptaamylose monophosphates by analysis and neutralization equivalent.

The structure assignments for **1** and **2** were made by proton and carbon NMR. The anomeric protons on carbons 1 of cycloheptaamylose are found at δ 4.87 in Me_2SO-d_6. In the cycloheptaamylose 2-phosphate (**1**) a new peak appears at δ 5.13 with an area approximately one-sixth that of the other anomeric protons. By contrast, the compound to which we assign the structure of cycloheptaamylose 3-phosphate (**2**) shows no new anomeric peak, just as expected since the electron-withdrawing group is further from the carbon-1 proton. Confirming this interpretation of the nature of the effect, we find that the ammonium salt of the 2-phosphate **1** shows a single anomeric proton shifted downfield by only 0.10 ppm from the others. The phosphate anion is less electron withdrawing than is the free phosphoric acid.

Further evidence for these structure assignments comes from ^{13}C NMR. Carbons 1 and 4 of the cycloheptaamylose can be clearly distinguished from the others, and in particular, the signal for carbon 1 is found at 100.8 ppm and that for carbon 4 at 81.8 ppm from Me_4Si in Me_2SO-d_6. In the compound to which we assign the structure **1** of the 2-phosphoric acid, we find that a signal from one of the C-1 carbons is shifted upfield by 1.1 ppm and furthermore is now split into a doublet (J = 5 Hz) by coupling with the phosphorus. This shift and splitting is as expected[15] for the separation of carbon and phosphorus by three bonds. In the compound to which we assign structure **2**, cycloheptaamylose-3-phosphoric acid, no shift or splitting is seen for C-1, but now one of the C-4 signals is moved 2.7 ppm upfield from the others and also is split into a doublet (J = 5 Hz) by the phosphorus.

The pK_a's of **1**, **2**, and **3** were determined by titration and are listed in Table I.

Results of Kinetics and Binding Studies. The rate of hydrolysis of substrate **4** was studied as a function of pH and concentration with the various cyclodextrin derivatives. A number of complicating factors are present. First of all, the cyclodextrin phosphates can exist in three different states of ionization. Secondly, the substrate hydrolysis is *inhibited* by binding of the substrate to cyclodextrin itself. The result of this is that only one of the cyclodextrin derivatives, cyclodextrin-3-phosphoric acid (**2**), showed net catalysis of the hydrolysis of **4**, and this only in a narrow pH region. This net catalysis was completely inhibited by the addition of cyclohexanol, which binds to the cyclodextrin and displaces the substrate from the cavity. With the cyclodextrin 2- and 6-phosphates, compounds **1** and **3**, the rate of hydrolysis of substrate **4** was *decreased* by adding the "catalyst" up to a saturating concentration, but the

effect was less than for cyclodextrin itself and was also pH dependent.

This suggests mild catalytic effects from the phosphoric acid groups on carbons 2 or 6 superimposed on a negative rate effect from the binding of the substrate to the cavity. This negative effect is almost certainly the result of the decreased polarity of the medium in which the substrate finds itself, since the hydrolysis of **4** is slower[9] in less polar media. The individual rate constants and binding constants for reactions of substrate **4** in its complex with each of the cyclodextrin phosphoric acids in the pH region in which it is the phosphate monoanion are listed in Table I.

Tritium–hydrogen exchange in **5** shows both specific base (hydroxide ion) and buffer catalysis (HCO_3^-/CO_3^{2-}). The hydroxide term was evaluated by extrapolation to zero buffer concentration and was linear (slope 1.08) in hydroxide concentration at pH 11.52, 12.03, and 12.53. Furthermore, in the presence of cycloheptaamylose (β-cyclodextrin), there is additional catalysis by complexing. The standard Hildebrand–Benesi treatment[16] of the rate at constant pH (12.03) with varying cycloheptaamylose concentration shows that the complex has a K_{diss} of 1.2 mM, with a rate constant 3.2 times that for the buffer solution alone.

Catalysis of tritium–hydrogen exchange in **5** was studied with solutions of **1, 2,** and **3** as the disodium salts. The pH's were 8.88, 9.13, and 8.95, respectively. All three compounds were good catalysts of exchange in **5**, and showed saturation kinetics. Dissociation constants for the complex of **1, 2,** and **3** with substrate **5** were comparable with that for β-cyclodextrin itself and are listed in Table I.

The catalytic rate constants within these complexes of **5** with **1, 2,** and **3** are listed in Table I. These rate constants can be compared with a k_{OH^-} extrapolated to pH 9.00 of 4.6×10^{-5} h^{-1}, so catalysis by the dianions of **1, 2,** and **3** is 430–700-fold compared with the medium alone. A kinetic titration with **2** as a function of added base revealed no detectable catalysis by the monoanion. As a control, the dianion of *trans*-cyclohexane-1,2-diol monophosphate was examined and showed no detectable catalysis of exchange in **5**.

Discussion

The results from these two studies are in striking contrast. Binding to β-cyclodextrin suppresses the acid-catalyzed hydrolysis of the tetrahydropyranyl ether **4**, and only one of the cyclodextrin phosphoric acids (**2**) showed net catalysis of the reaction. On the other hand, enolization of **5** is mildly catalyzed by binding to β-cyclodextrin and *strongly* catalyzed by the phosphate dianions of *all* three isomers, **1, 2,** and **3**. Nevertheless, several general points can be made.

One conclusion is that in water solution the immediate vicinity of the cyclodextrin cavity is only partially aqueous in character. This would explain the inhibition of acid-catalyzed hydrolysis of **4** by cycloextrin binding, since the hydrolysis of **4** is slower in less polar solvents. Thus the cyclodextrin molecule may imitate the often invoked ability of enzymes to exclude water from a reaction site.

A second conclusion is that for both reactions the most effective catalyst is that (**2**) with a catalytic group on carbon 3. That this should be so is not at first obvious, since carbon 3 has a hydrogen pointing toward the cavity, while the oxygen carrying the phosphate points somewhat away. Of course substrates such as **4** or **5** may bind so as to project from either the primary (C-6) or the secondary (C-2, C-3) side of the cavity, and the observed binding constants in Table I are simply composites of both possibilities. A substituent at C-2 could crowd the secondary side so as to shift the binding largely to the primary face. Since the observed catalytic rate constants in Table I are actually the rate constants for the correct binding geometry multiplied by the fraction of the complex which has that geometry, crowding at C-2 could show up as a decreased rate constant. In any case, our results suggest that C-3 is the best place to attach simple catalytic groups.[17]

A third conclusion is that for one of our reactions, base-catalyzed enolization of **5**, the primary side (C-6) is just as good a place for the catalytic group (although this partly reflects the fact that a phosphate on C-6 is more *basic*, as the pK_a's in Table I indicate). Since selective reactions at C-6 are easy to perform, these accessible derivatives of cyclodextrin should not be ignored.

Finally, our data confirm previous indications[8,18] that substrates such as **5** can bind to β-cyclodextrin so that the side chain projects from either the primary or the secondary face. Capping the cavity or incorporation of additional binding interactions would be needed to guarantee that binding occur only in the catalytically useful geometry.

Experimental Section

Materials. Cycloheptaamylose 6-Diphenylphosphate (7). Diphenylphosphorochloridate (2.7 g, 10 mmol) in 20 mL of pyridine was added to dried cycloheptaamylose (30.0 g, 26 mmol) dissolved in 250 mL of pyridine at room temperature. The solution was stirred for 48 h, then 10 mL of H_2O was added, and the mixture was evaporated to dryness at 35 °C (0.1 Torr). The solid was purified by five recrystallizations from 600 mL of boiling H_2O, then dried to afford 4.6 g (32%) of the product **7** as a white powder. The compound was pure by TLC, R_f 0.72,[19] and showed $[\alpha]^{25}$ 116° (dimethylformamide). In the NMR it showed aromatic hydrogens at δ 7.2, and the anomeric hydrogen on carbon 1 as a doublet at δ 4.7 (J = 2 Hz), in addition to other expected peaks. Integration of aromatic vs. anomeric hydrogens indicated 1.03 ± 0.06 diphenylphosphate per cycloheptaamylose. In a similar manner, using a large excess of diphenylphosphorochloridate, it is also possible to prepare[1] cycloheptaamylose hepta-6-diphenylphosphate (**6**).

Cycloheptaamylose 6-Phosphate (3) Ammonium Salt. The diphenylphosphate (**7**) was hydrogenated over Pt in ethanol at 50 lb pressure and room temperature for 1 week. The product was isolated by filtration, solvent evaporation, and chromatography on DEAE-cellulose and ion exchange columns (elution using 0.05 M NH_4HCO_3 buffer at ph 7.8 was monitored by continuous determination of optical rotation). The fractions containing the product were lyophilized to afford 65% of the ammonium salt of cycloheptaamylose 6-phosphate (**3**) as a white powder with R_f 0.32 and $[\alpha]^{25}_D$ 151° (H_2O). No UV absorptions could be detected, and analysis showed a carbon/phosphorus ratio of 17.3 (calcd for $C_{42}H_{80}NO_{38}P$: 16.3). The equivalent weight by titration was 1254 ± 25 (calcd 1231).

Cycloheptaamylose 2-Phosphate (1) and Cycloheptaamylose 3-Phosphate (2) Ammonium Salts. Bis(*m*-nitrophenyl)phosphate[20] (0.43 g, 1.3 mmol) was added to a solution of cycloheptaamylose (5.44 g, 4.8 mmol) in 200 mL of 0.1 M NaOH. The mixture was stirred at room temperature for 3 days, then heated at 50 °C for 2 h. The solution was cooled, the pH adjusted to 4, and the mixture of products precipitated by the addition of 1500 mL of acetone. The precipitate was collected, dried, and dissolved in 50 mL of H_2O. Careful chromatography on Bio-Rad AG-21K anion exchange resin with 0.5 M NH_4HCO_3 buffer at pH 7.8, after a preliminary elution with H_2O to remove unreacted cycloheptaamylose, afforded 1.2 g (76%) of the lyophilized mixture of **8** and **9**. This was rechromatographed on a QAE-A25 Sephadex anion exchange column, with 0.10 M NH_4HCO_3 pH 7.8 buffer, monitored by continuous recording of optical rotation, to afford a small fraction of cycloheptaamylose, a second fraction of the 3-phosphate **2**, and a third fraction of the 2-phosphate **1**. The products were separately lyophilized and rechromatographed to improve their isomeric purity, which in the final material was greater than 90% as estimated by TLC. The ammonium salt of cycloheptaamylose 2-phosphate (**1**) was obtained as a white powder, with $[\alpha]^{25}_D$ 142° (H_2O) and R_f 0.26. It had no UV absorption, and analysis showed 1.07 ± 0.06 phosphate per cycloheptaamylose. By titration, the equivalent weight was 1222 ± 25 (calcd 1231). The ammonium salt of the 3-phosphate (**2**) was the major component (67%) of the mixture, obtained as a white powder after lyophilization. It had $[\alpha]^{25}_D$ 112° (H_2O) and R_f 0.48. It also showed no UV absorption and analysis revealed 1.03 ± 0.06 phosphates per cycloheptaamylose. By titration, it had equivalent weight 1248 ± 25 (calcd 1231).

ω-^{3}H-*p-tert*-Butylphenacyl Alcohol (5). Following a related literature procedure,[12] solid *p-tert*-butylphenacyldiazomethane[21] (150 mg, 0.75 mmol) was added at room temperature to a mixture of 4 g of dioxane, 3.6 g of H_2O (specific activity 50 mC/mol), and 0.3 mL of 70% $HClO_4$. Nitrogen gas evolved for a period of 10 min as the pale-yellow solution turned clear. TLC on silica gel with ether solvent indicated complete reaction to a single product. The reaction mixture was extracted with 25 mL of ether and the aqueous layer discarded. The ether phase was washed with 4 × 25 mL of water, then dried with $MgSO_4$, and evaporated under vacuum at room temperature. This procedure yielded 115 mg (80%) of pure liquid **5** (specific activity 45 mC/mol): NMR ($CDCl_3$) δ 1.2 (s, 9 H, *t*-Bu), 4.3 (broad S, 1 H, OH), 4.7 (S, 2 H, CH_2), and 7.5 (q, 4 H, aromatics, J_{AB} = 8.5 Hz). When D_2O was used in place of H_2O or ^3H_2O in the above reaction, the NMR of the product was identical with that listed, except for the absence of a signal at δ 4.7, corresponding to the methylene hydrogens.

Kinetics. The hydrolysis of substrate **4** was followed spectrophotometrically by the appearance of *p*-nitrophenol at 346 nm. Pseudo-first-order rate constants were determined by standard computer techniques, in which all reactions were followed to greater than 90% completion and the infinity value was left as a variable. Correlation coefficients for individual determinations were 0.999 or greater, and the rate constants between individual determinations were reproducible within 10%. The temperature was controlled at 25.00 ± 0.02 °C. In every case the UV spectrum of the product mixture corresponded to that of a synthetically prepared mixture of products. Binding constants were determined kinetically by the Eadie method.[22]

Kinetics of ^{3}H Exchangs in 5. A 20 μL aliquot of a 50 mM stock solution of **5** in Me_2SO was added to 1.0 mL of 50% (v/v) aqueous Me_2SO, yielding a 1 mM solution containing a known concentration of catalyst, either buffer or a cyclodextrin derivative, at a measured pH. Reaction mixtures were thermostated at 37.00 ± 0.02 °C, and at appropriate time intervals 100 μL aliquots were withdrawn. Compound **5** was extracted with 2.00 mL of reagent grade toluene. Approximately 40 mg of $MgSO_4$ were added, and a 1.00 mL aliquot was withdrawn and added to 10 mL of toluene PPO/POPOP scintillation fluid. For a typical kinetic determination, at zero time, vials contained 1000 cpm and background was 25 cpm; seven data points were collected over 85% reaction. First-order rate constants were determined by standard computer techniques from the relative number of cpm per vial as measured on a Hewlett-Packard Tri-Carb Scintillation Counter. Reproducibility was within 5% between duplicate analysis, while typical correlation coefficients were 0.99 or greater.

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