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Inhibition of Dextransucrase by α-D-Glucose Derivatives

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ABSTRACT

α-D-Glucopyranosyl fluoride was modified at positions 2, 3, or 5 and these analogs were tested as substrates and inhibitors of dextransucrase from *Leuconostoc mesenteroides* B-512F. The analogs studied were 2-deoxy-2-fluoro-α-D-glucopyranosyl fluoride, 3-deoxy-3-fluoro-α-D-glucopyranosyl fluoride, 3-deoxy-3-thio-α-D-glucopyranosyl fluoride, and 5-thio-α-D-glucopyranosyl fluoride. Kinetic constants for α-D-glucopyranosyl fluoride were also determined.

None of the α-D-glucopyranosyl fluorides were accepted as substrates for dextransucrase. 2-Deoxy-2-fluoro-α-D-glucopyranosyl fluoride, 3-deoxy-3-fgluoro-α-D-glucopyranosyl fluoride, and 3-deoxy-3-thio-α-D-glucopyranosyl fluoride were competitive inhibitors with K_i values of 63, 93, and 53 m*M*, respectively. The K_m for α-D-glucopyranosyl fluoride was found to be 26 m*M*. The data indicate that the hydroxyl groups at C_2 and C_3 are important for proper binding of α-D-glucopyranosyl fluoride for the active site of dextransucrase and that the C_3-hydroxyl probably acts as a hydrogen-bond donor.

Index Entries: Dextransucrase; α-D-glucopyranosyl fluoride; dextran; *Leuconostoc mesenteroides;* inhibition.

INTRODUCTION

Dextransucrases from several species and strains of *Leuconostoc* and *Streptococcus* utilize sucrose as an α-D-glucosyl donor for the synthesis of

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D-glucans. Dextran from *L. mesenteroides* NRRL B-512F consists of 95% α-(1,6)-linked D-glucosyl residues and about 5% α-(1,3)-linked branches. It has been shown that dextransucrases will also polymerize the D-glucosyl groups from α-D-glucopyranosyl α-L-sorbofuranoside (*1*), *O*-β-D-galactopyranosyl-(1,4)-β-D-fructofuranosyl α-D-glucopyranoside (lactulosucrose) (*2*), *p*-nitrophenyl α-D-glucopyranoside (*3*), and α-D-glucopyranosyl fluoride (*4*). These substrates differ from sucrose only in that the leaving group, fructose is changed. The α-D-glucosyl moiety, which is most important for binding and subsequent polymerization, is unchanged. Grier and Mayer (*5*) synthesized a series of α-1-fluorosugars that were used to further examine substrate specificity. The configurations of single hydroxyl groups at specific positions of the glucosyl moiety were changed. These analogs of α-D-glucopyranosyl fluoride were found to be inhibitors of dextransucrase but did not act as glycosyl donors. Epimerizations at C2, C3, and C4 caused competitive inhibition. Similarly, 4- and 6-deoxy-glucopyranosyl fluoride were also competitive inhibitors. Binder and Robyt found that 6-deoxysucrose (*6*), 6-thiosucrose (*6*), 3-deoxysucrose (*7*), and 3-fluorosucrose (*7*) are competitive inhibitors for dextransucrase from *S. mutans*.

It is likely that the hydroxyl groups are important in binding the substrate to the active site via hydrogen-bond formation. New substrates might be found for dextransucrase that have hydroxyls on the glucose ring replaced with other groups that can also participate in hydrogen bonding. These would lead to dextran derivatives with interesting properties. Thiol (SH) groups could serve as hydrogen-bond donors and fluorine atoms might act as hydrogen-bond acceptors. We have synthesized stereospecific thiol and fluorine containing analogs of α-D-glycopyranosyl fluoride to determine whether new substrates for the enzyme might exist. Four derivatives of α-D-glucopyranosyl fluoride were synthesized: 2-deoxy-2-fluoro-α-D-glucopyranosyl fluoride, 3-deoxy-3-fluoro-α-D-glucopyranosyl fluoride, 3-deoxy-3-thio-α-D-glucopyranosyl fluoride, and 5-thio-α-D-glucopyranosyl fluoride. Reported here are the results of kinetic studies of the analogs and α-D-glucopyranosyl fluoride with *Leuconostoc mesenteroides* NRRL B-512F dextransucrase.

MATERIALS AND METHODS

General Methods

The structures of all compounds were determined by ^{1}H NMR spectroscopy at 200 MHz. A large coupling of $J \approx 53$ Hz at $\delta \approx 6$ ppm was indicative of fluorination at the anomeric carbon. Silica thin-layer chromatography plates (TLC) were visualized with molybdate stain. Evaporations were carried out on a rotary evaporator under reduced pressure. Column chromatography was performed using silica gel. *Dextransucrase* from *Leuconostoc mesenteroides* B-512F dextransucrase was obtained from Sigma Chemical Co. and used without further purification.

Enzyme Assays

Assays were performed at 30°C in 0.05*M* sodium acetate buffer, pH 5.2. Dextransucrase activity was measured by monitoring the appearance of fructose. One unit of enzyme is defined as the amount that will liberate 1.0 μmol of fructose/min from 292 m*M* sucrose under these conditions. All reactions contained 0.2 U of dextransucrase with appropriately diluted sucrose and/or novel substate in a total vol of 1.0 mL. From each reaction mixture, 100-μL aliquots were taken periodically, diluted with 10 mL water and chromatographed. Activity of dextransucrase toward the novel substrates was monitored by measuring any decrease in the concentration of the substrate as a function of time.

Carbohydrate concentrations were measured by high-pressure liquid chromatography using a Dionex BioLC liquid chromatograph, with a Dionex IonPac AS-6 anion exchange column (*8*). Eluent was 100 m*M* sodium hydroxide, and detection was achieved using the Dionex PAD2 pulsed amperometric detection system.

Preparation of Substrates

α-D-Glucopyranosyl fluoride was prepared as described by Hall and Manville (*9*). 2-Deoxy-2-fluoro-α-D-Glucopyranosyl fluoride was prepared by the procedure of Korytnyck et al. (*10*). 3-Deoxy-3-fluoro-α-D-glucopyranosyl fluoride was prepared by directly dissolving 4.3 g of 3-deoxy-3-fluoro-glucose (*11*) in neat acetic anhydride (85 mL), adding anhydrous sodium acetate (5.4 g), and refluxing for 20–30 min. Dilution of the reaction mixture with methylene chloride (CH_2Cl_2), extracting with water, drying the organic layer with anhydrous $MgSO_4$, and solvent evaporation followed by recrystallization from ethanol, yielded 3-deoxy-3-fluoro-glucose tetraacetate (4.4 g, 87%). Fluorination of the anomeric carbon was performed in the same manner as previously described for glucopyranosyl fluoride (*9*) with the exception that a silica gel column was used to purify the 3-deoxy-fluoro-α-D-glucopyranosyl fluoride triacetate (2.2 g, 60%). The triacetate was deprotected by dissolving in it methanol, than adding sodium methoxide until the solution became basic. Thin-layer chromatography showed that the reaction was complete in about 1 h. Neutralization, accomplished by the addition of H^+-cation exchange resin, followed by filtration and evaporation, yielded 1.3 g of pure 3-deoxy-3-fluoro-α-D-glucopyranosyl fluoride (35% based on the triacetate). ^1H NMR showed the large anomeric fluorine coupling of $J = 53$ Hz at $\delta = 5.55$ ppm.

3-Deoxy-3-thio-α-D-glucopyranosyl fluoride was prepared as follows: 3-Trifluoromethanesulfonyl-1,2:5,6-diisopropylidene allose (*12*) (12.9 g) was dissolved in 225 mL of absolute ethanol; 3.7 g of sodium hydrosulfide was added and the mixture was refluxed (70–80°C) until TLC showed the disappearance of all starting material. The ethanol was then evaporated *in vacuo* and the residue redissolved in methylene chloride. The CH_2Cl_2 layer was washed several times with water and dried over anhydrous $MgSO_4$.

Removal of the CH_2Cl_2 left a yellow oil. Column chromatography using 10:1 hexane:ethyl acetate, followed by recrystallization from 2-propanol, gave 3-thio-1,2:5,6-diisopropylidene glucose as white crystals (3.1 g, 35%). Removal of the isopropylidene-protecting groups was performed as described for 3-deoxy-3-fluoro-glucose (*11*). 4.9 g of 3-thio-1,2:5,6-diisopropylidene glucose yielded 2.0 g (58%) of pure 3-thio-glucose after two recrystallizations from methanol.

Anal. Calc. for $C_6H_{12}O_5S$: C, 36.73; H, 6.12. Found: C, 36.47; H, 6.15.

The crystals of 3-thio-glucose were dissolved in 100 mL of pyridine and 10 mL of acetic anhydride. To this solution a catalytic amount (approx. 0.1 g) of dimethylaminopyridine was added and stirred for 2 h. Methylene chloride was added and the organic phase was washed once with 0.1*N* HCl and then twice with water. After drying over anhydrous $MgSO_4$, the solvent was evaporated *in vacuo* leaving 3-thio-glucose pentaacetate (3.6 g, 87%). 3-Thio-glucopyranosyl fluoride tetraacetate (1.3 g, 37%) was recovered after fluorination of the pentaacetate at the anomeric carbon as previously described for glucopyranosyl fluoride (*9*). 3-Deoxy-3-thio-glucopyranosyl fluoride (0.33 g, 47%) was obtained after deprotection of the tetraacetate by the method described for glucopyranosyl fluoride (*9*).

Anal. Calc. for $C_6H_{11}FO_4S$: C, 36.36; H, 5.56. Found: C, 35.75; H, 5.61.

^{1}H NMR δ, ppm: 2.91 (t, J=10.70 Hz), 5.49 (dd, J=2.53, 53.62 HZ).

5-Thio-α-D-glucopyranosyl fluoride was prepared starting with 5-thio-D-glucose obtained from the eight-step procedure of Driguez and Henrissat (*13*). The 5-thio-D-glucose was acetylated as described above for 3-deoxy-3-thio-glucose pentaacetate and then fluorinated and deprotected in the same manner as described for glucopyranosyl fluoride (*9*). Unfortunately, the fluorination step did not proceed cleanly; the yield was very poor and many side products were present. An NMR spectrum of the crude material showed a doublet at $\delta=6$ ppm, indicating fluorination at the anomeric carbon. This crude material was deprotected without further purification. NMR showed the doublet and disappearance of the acetates at $\delta=2$ ppm. HPLC indicated that the compound was ~85% pure. Owing to insufficient material, elemental analysis was not performed.

RESULTS AND DISCUSSION

The activity of dextransucrase with sucrose was determined by measuring the increase of fructose concentration with time in order to verify the accuracy of HPLC for monitoring the reaction. HPLC gave base-line separation between sucrose, fructose, and glucose. A double reciprocal plot gave a K_m value of 11.2 m*M* (data now shown), which agrees with the literature

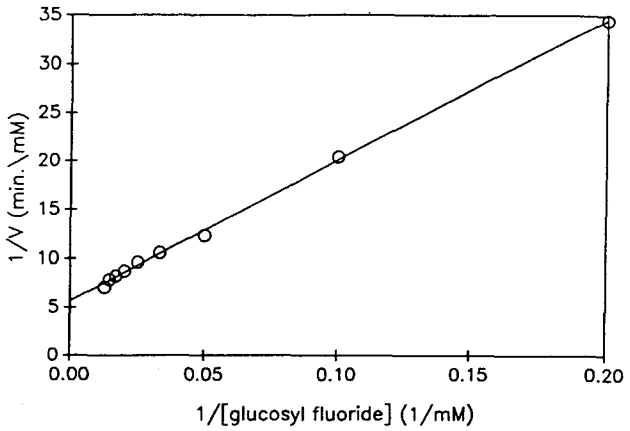

Fig. 1. Double-reciprocal plot for α-D-glucopyranosyl fluoride as a substrate for dextransucrase.

value of about 12 m*M* (*14*). The use of HPLC provided simultaneous determination of fructose, sucrose, inhibitor, and substrate concentrations.

Genghoff and Hehre (*4*) and Figures and Edwards (*15*) demonstrated that α-D-glucopyranosyl fluoride could serve as a substrate for dextransucrase. Though glucopyranosyl fluoride was originally observed to be a substrate for B-512F dextransucrase (*4*), no kinetic constants for this enzyme have been previously reported. Jung and Mayer (*16*) demonstrated that glucopyranosyl fluoride is an excellent analog of sucrose for dextransucrase from *S. sanguis* ATCC 10558. The kinetic constants for the two compounds are comparable and the substrates were found to be competitive with each other.

The K_m for α-D-glucopyranosyl fluoride was found by determining the rate of decrease of glucopyranosyl fluoride concentration using HPLC, without any of the problems usually found when the reaction is monitored by the release of fluorine. From a double-reciprocal plot, the K_m for glucopyranosyl fluoride was found to be 26 m*M* (Fig. 1).

The concentration of any of the four glucopyranosyl fluoride derivatives that could be accepted as substrates for dextransucrase was expected to decrease on incubation with the enzyme. Reactions were run in parallel with an enzyme-free control that was identical to the reaction mixture but with the enzyme solution replaced by an identical volume of buffer. No concentration decrease was observed with any of the derivatives, indicating that none were accepted as substrates. An indication of the compound's ability to bind to the enzyme's active site is whether or not it is a competitive inhibitor of the enzyme. Since none of the synthesized compounds were accepted as substrates for dextransucrase, studies were performed to determine if they act as inhibitors. In these studies, sucrose was the substrate for a series of reactions with the novel glucopyranosyl

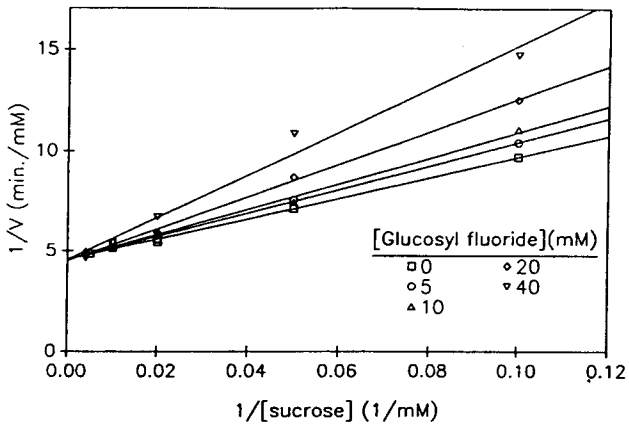

Fig. 2. Double-reciprocal plot for sucrose with α-D-glycopyranosyl fluoride as a competitive inhibitor.

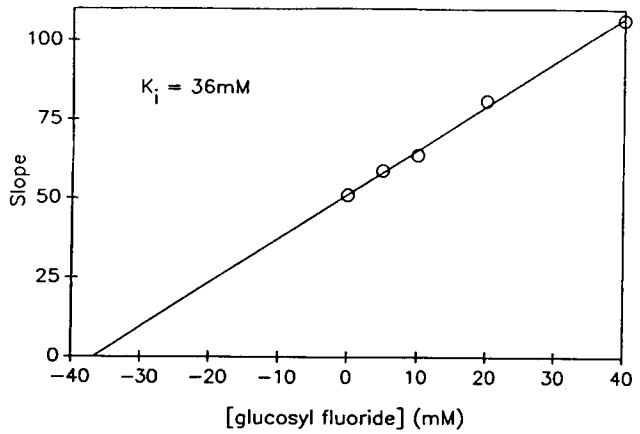

Fig. 3. Slopes from Fig. 2 vs α-D-glucopyranosyl fluoride concentration. X-intercept = $-K_i$.

fluoride derivatives present at varying concentrations. The values for the inhibition constants were obtained by plotting the slopes of Lineweaver-Burke plots of rate data vs inhibitor concentration.

The results of the kinetic studies for glucopyranosyl fluoride are shown in Fig. 2. As was expected, glucopyranosyl fluoride is a competitive substrate. Figure 3 shows the slopes of the Lineweaver-Burke plots of Fig. 2 vs glucopyranosyl fluoride concentration. This plot gave a K_i of 36 m*M*. Since glucopyranosyl fluoride is a competitive substrate of sucrose, this K_i correlates reasonably well with the K_m value obtained for glucopyranosyl fluoride.

Figure 4 shows the results of the kinetic studies of the glucopyranosyl fluoride derivatives. As can be seen, they are all competitive inhibitors of

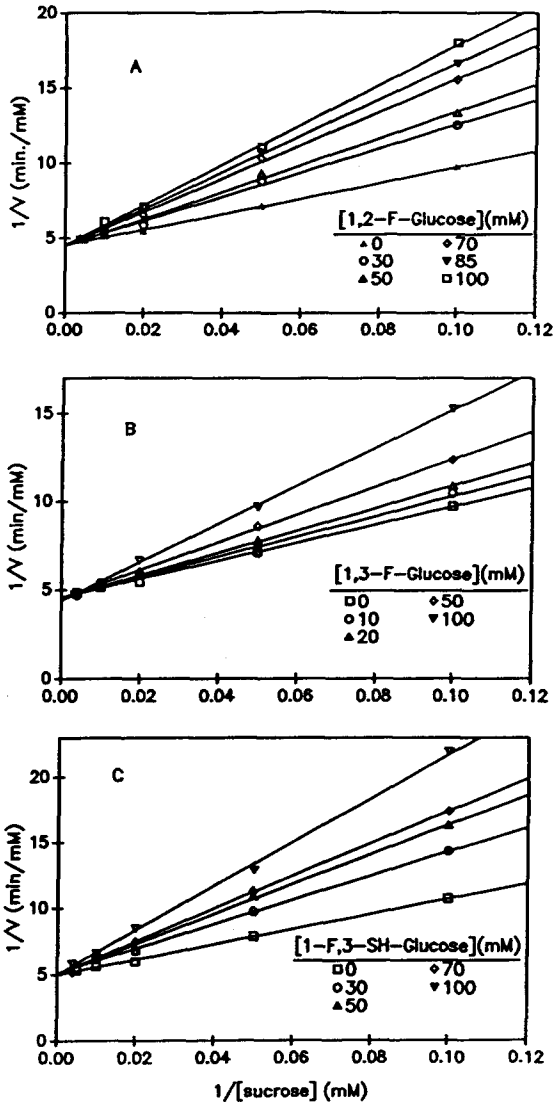

Fig. 4. Double-reciprocal plots for sucrose with α-D-glucopyranosyl fluoride derivatives as competitive inhibitors. **A** 2-deoxy-2-fluoro-α-D-glucopyranosyl fluoride; **B** 3-deoxy-3-fluoro-α-D-glucopyranosyl fluoride; **C** 3-deoxy-3-thio-α-D-glucopyranosyl fluoride.

dextransucrase. The least-squares-fit values of the inhibition constants are summarized in Table 1 along with K_m and K_i values for sucrose and glucopyranosyl fluoride.

Binder and Robyt (7) assayed 3-deoxy-3-fluorosucrose with dextransucrase from *S. mutans* 6715 and found that the fluorine modification at C_3 considerably decreased the binding at the active site of the enzyme. The

Table 1
Behavior of Novel Monomers with Dextransucrase

Sugar	K_m, mM	K_i, mM	Type of inhibition	Accepted as substrate
Sucrose	11.2	-	-	Yes
α-D-Glucopyranosyl fluoride	26	36	competitive	Yes
2-Deoxy-2-fluoro-glucopyranosyl fluoride	-	63	competitive	No
3-Deoxy-3-fluoro-glucopyranosyl fluoride	-	93	competitive	No
3-Deoxy-3-thio-glucopyranosyl fluoride	-	35	competitive	No
5-Thio-glucopyranosyl fluoride	-	-	-	No

K_i of 3-deoxy-3-fluoro-glucopyranosyl fluoride similarly reflects a large decrease in binding as a result of replacement of the 3-hydroxyl by fluorine. The results of Grier and Mayer (*5*) for allosyl fluoride show that an epimerization of the C_3-hydroxyl also decreases the binding affinity. These results indicate that the 3-hydroxyl group plays an important role in the binding of substrate to the active site of dextransucrase. Grier and Mayer also assayed mannosyl fluoride, the C_2 epimer of glucopyranosyl fluoride, with dextransucrase (*5*). They found that this epimerization caused an increase in the K_i, indicating reduced binding to the active site. Replacing the 2-hydroxyl with fluorine in 2-deoxy-2-fluoro-glucopyranosyl fluoride similarly increased the inhibition constant. Both these results demonstrate the importance of the 2-hydroxyl in binding.

The C_3-fluorine of 3-deoxy-3-fluoro-α-D-glucopyranosyl fluoride can contribute to binding by acting as a hydrogen bond acceptor. In contrast, the thiol group in 3-deoxy-3-thiol-α-D-glucopyranosyl fluoride can participate in binding by donating a hydrogen bond. Since the 3-thiol derivative binds to dextransucrase with a K_i approximately one half that of the 3-fluoro derivative, it is likely that the 3-hydroxyl acts as a hydrogen-bond donor rather than acceptor.

2-Deoxy-2-fluoro-α-D-glucopyranosyl fluoride can also act as a hydrogen bond acceptor in the same manner as the 3-fluoro derivative. The 2-fluoro derivative has a lower binding affinity similar to that of the 2-hydroxyl fluoride, which cannot hydrogen-bond to the C_2 site. The lower binding may be an indication that the 2-hydroxyl acts as a hydrogen-bond donor. A possible reason that the 2-fluoro derivative does not react as a substrate for glucosyl transfer is the higher electronegativity of fluorine compared to oxygen. The higher electronegativity could affect the ability of the anomeric fluorine to act as a leaving group, thus inhibiting the reaction.

Finally, 5-thio-α-D-glucopyranosyl fluoride did not react with dextransucrase. Replacing the ring oxygen with sulfur causes the ring to alter its conformation slightly because of the larger atomic radius of sulfur. Even though the conformation change is small, the derivative was not accepted as a substrate by the enzyme. Unfortunately, there was not enough 5-thioglucopyranosyl fluoride to do a complete series of reactions for an inhibition study. It was surprising that none of these derivatives were substrates for the enzyme. They are activated in an appropriate manner and since they are competitive inhibitors, they must bind in a manner similar to sucrose. It is possible that even though these analogs bind to the active site, they do not orient properly for reaction to occur.

Robyt and coworkers (*17–19*) have probed the active sites of dextransucrases from three different strains of microorganisms with a number of sucrose derivatives. They found that derivatizations at C_6 of sucrose (6-deoxy, 6-thio, 6-fluoro, and 6-bromo) resulted in competitive inhibition. Derivations at C_4 (4-deoxy, 4-chloro-galacto) gave noncompetitive inhibition and derivatizations of C_3 produced noncompetitive (3-deoxy), competitive, and/or mixed (3-fluoro) inhibition. None of the derivatives were found to be accepted as substrates by any of the enzymes.

The results of Robyt, Grier, and Mayer, and those reported here show that all of the hydroxyls of the glucose moiety of sucrose or glucopyranosyl fluoride are necessary for proper binding and subsequent polymerization. From all indications, dextransucrase is extremely selective toward the glucose moiety and it is unlikely that other analogs involving derivatizations of hydroxyls on the glucose moiety will be accepted as substrates.

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