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INHIBITION- AND ACCEPTOR-REACTION STUDIES OF *Streptococcus mutans* 6715 GLUCOSYLTRANSFERASES WITH 3-DEOXYSUCROSE, 3-DEOXY-3-FLUOROSUCROSE, AND α-D-ALLOPYRANOSYL β-D-FRUCTOFURANOSIDE*

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ABSTRACT

Three new sucrose analogs modified at C-3 have been studied as inhibitors and substrates for the glucosyltransferases (glucansucrases) of *Streptococcus mutans* 6715. Although none of the analogs were found to be substrates for polymer synthesis with either the soluble-polysaccharide producing enzyme, GTF-S, or the insoluble-polysaccharide producing enzyme, GTF-I, 3-deoxysucrose and 3-deoxy-3-fluorosucrose were able to donate glycosyl residues for acceptor reactions with both enzymes. Modification at C-3 considerably decreased the binding at the active site of both enzymes, since all of the analogs had inhibition constants at least one order of magnitude greater than the K_m value for sucrose.

INTRODUCTION

We have been synthesizing sucrose analogs[1–3] to investigate the effect that modifications have on the ability of the analogs to inhibit or act as substrates for the glucosyltransferases produced by *Streptococcus mutans*[4]. Much of the early work in this area was done with naturally occurring oligosaccharides based on sucrose, such as planteose[5] or enzymically synthesized molecules such as α-D-galactopyranosyl β-D-fructofuranoside[6] or α-D-xylopyranosyl β-D-fructofuranoside[7]. None of these were substrates for the enzyme, leading workers to assume that dextransucrases belong to a category of sucrases whose specificity is determined by both the glucosyl and fructosyl moieties of the substrate[6]. After the discovery that *O*-β-D-galactopyranosyl-(1→4)-β-D-fructofuranosyl α-D-glucopyranoside[8] and α-D-glucopyranosyl fluoride[9,10] are substrates (glucosyl donors) for these glucosyltransferases, it became apparent that the major requirement for glycosyl donors is the α-D-glucopyranosyl moiety of sucrose. With the finding that α-D-glucopyranosyl

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fluoride is a substrate for these enzymes, it became relatively easy to make substrate analogs by synthesizing D-glycosyl fluorides[10,11]. Although some of the glycosyl fluorides were shown to be inhibitors, none were found to act as substrates for either acceptor reactions or polymerization reactions.

To test this apparent high specificity for the α-D-glucopyranosyl residue by the glucosyltransferases, we developed selective methods for modification at C-6 and C-3 of sucrose[2,3]. Because the D-glucans synthesized by the glucosyltransferases have α-(1→6) and α-(1→3) linkages, we postulated that modifications at C-3 and -6 should have the greatest effect as inhibitors. We have previously tested the C-6 modified analogs as inhibitors and substrates for the soluble-polysaccharide producing enzyme (GTF-S) and the insoluble-polysaccharide producing enzyme (GTF-I), the two enzymes produced by *Streptococcus mutans* 6715, and found that 6-deoxysucrose and 6-deoxy-6-thiosucrose were competitive inhibitors for both GTF-S and GTF-I and that 6-deoxy-6-thiosucrose was a substrate for GTF-I to give (1→3)-6-thio-α-D-glucan[4]. We report here on the results obtained using 3-deoxy-sucrose, 3-deoxy-3-fluorosucrose, and α-D-allopyranosyl β-D-fructofuranoside (allosucrose) as inhibitors and D-glycosyl donors in acceptor reactions for these two glucosyltransferases.

EXPERIMENTAL

Enzymes. — GTF-I and GTF-S were prepared as previously described[4]. Glucansucrase activity was determined as incorporation of label from [U-^{14}C]-sucrose into methanol-insoluble polymer, as previously described[4,12] and is given in International Units (IU), namely, μmol of D-glucose incorporated into glucan per min at pH 5.2 and 37°.

Carbohydrates. — [U-^{14}C]Sucrose was obtained from Schwarz–Mann (Cambridge, MA). 3-Deoxysucrose, 3-deoxy-3-fluorosucrose, and allosucrose were prepared by the methods of Binder and Robyt[3]. Dextran T-10 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden).

Chromatography. — Thin-layer chromatography (t.l.c.) was performed on Whatman K5F 0.25-mm silica gel plates (Whatman Chemical Separations, Clifton, NJ). Chromatograms were developed by three ascents in 9:1 (v/v) acetonitrile–water followed by two ascents in 17:3 (v/v) acetonitrile–water; carbohydrates were detected by spraying with 20% sulfuric acid in methanol and charring for 10 min at 110°.

High-performance liquid chromatography (l.c.) was performed with a Waters Associates ALC/GPC-201 liquid chromatograph and a Whatman Partisil M9/50 polar amino cyano column (9 mm × 50 cm), at 4 MPa pump pressure. The eluant was 70% acetonitrile in water, with a flow rate of 2.8 mL/min; detection was by refractive index.

Enzyme digest conditions. — All digests were conducted at 37° in 50mM sodium acetate buffer, pH 5.2, containing 0.02% sodium azide.

Preparation and characterization of reaction products. — To 30 μL of 300mM 3-deoxysucrose was added either 30 μL of buffer or 30 μL of 300mM maltose. Reaction was initiated by the addition of 30 μL of GTF-S (0.10 IU) or GTF-I (0.05 IU). After 24 h, aliquots from each of the digests were spotted on t.l.c. plates. Similar experiments were carried out for 3-deoxy-3-fluorosucrose and allosucrose, except that they were allowed to incubate for 48 h before aliquots were spotted.

3-Deoxysucrose (200 mg) was added to 5 mL of buffer containing 50 mg of maltose followed by the addition of 1 mL of GTF-S (3.2 IU). This mixture was allowed to incubate for 7 days at 37°, at which time t.l.c. showed that most of the 3-deoxysucrose had been converted into D-fructose and acceptor products. The sample was concentrated to a syrup, dissolved in 1 mL of water, filtered, and the acceptor products isolated by l.c. The fractions containing the tetrasaccharide were pooled, concentrated to dryness under diminished pressure, and dissolved in 1 mL of D_2O. The ^{13}C nuclear magnetic resonance (n.m.r.) spectrum was recorded with a Nicolet NT-300 spectrometer in the Fourier-transform, proton-decoupled mode.

Inhibition kinetics. — A series of 0.18-mL digests were prepared containing 5.0mM [U-^{14}C]sucrose, 7.0 mIU of GTF-S or 2.0 mIU of GTF-I, and one of the following: no inhibitor, 109mM 3-deoxysucrose, 101mM allosucrose, or 97.0mM 3-deoxy-3-fluorosucrose. At various times, 25-μL aliquots were removed and spotted on 1.5 × 1.5-cm pieces of Whatman 3MM filter paper. These papers were washed with five changes of methanol to remove methanol-soluble label, with subsequent liquid-scintillation counting to determine formation of methanol-insoluble product[12]. Initial velocities of polysaccharide formation were calculated and used to determine the percent inhibition caused by each inhibitor.

To determine the kinetic constants for the sucrose analogs, a series of 0.12-mL enzyme digests were prepared, all containing [U-^{14}C]sucrose (3.33–16.7 mM) and dextran T-10 (2 mg/mL). Each GTF-I digest contained 2 mIU of enzyme and one of three inhibitors at the specified concentration: 3-deoxysucrose, 109 and 55mM; 3-deoxy-3-fluorosucrose, 92 and 46mM; or allosucrose, 101 and 51mM. For GTF-S, digests contained 7 mIU of enzyme, 109 and 55mM 3-deoxy-3-fluorosucrose (the only inhibitor whose kinetic constants were determined), and [U-^{14}C]sucrose concentrations as already given. Incorporation of ^{14}C into methanol-insoluble polysaccharide was determined by liquid-scintillation counting, as already described.

RESULTS

To test if any of the sucrose analogs were substrates for the enzymes, a series of digests were prepared containing either GTF-I or GTF-S, and one of the sucrose analogs, in the presence or absence of maltose. Of the three sucrose analogs tested, only allosucrose failed to act as a D-glycosyl donor to maltose. T.l.c. of the results obtained with 3-deoxysucrose and 3-deoxy-3-fluorosucrose is shown in Fig. 1. As may be seen in Fig. 1 (columns A, B, F and G), neither 3-deoxysucrose nor 3-deoxy-3-fluorosucrose reacted to form polysaccharide, as judged by the absence of any

compound at the origin, although a slight amount of hydrolysis of 3-deoxysucrose is indicated by the presence of fructose in those digests (columns F and G). In the presence of maltose, 3-deoxysucrose and 3-deoxy-3-fluorosucrose react to give acceptor products with both GTF-I and GTF-S (C, D, H, and I of Fig. 1). Qualitatively, 3-deoxysucrose is a better glycosyl donor than 3-deoxy-3-fluorosucrose, as more D-fructose was released in 24 h in its digest than was released in 48 h in the 3-deoxy-3-fluorosucrose digest (compare columns H and I with columns C and D of Fig. 1). The structures of the acceptor products were tentatively identified by t.l.c. mobility and the products obtained when sucrose is used as the glycosyl donor[13,14]. Based on this, GTF-S reacts with 3-deoxysucrose in the presence of maltose to give a series of two α-(1→6)-linked acceptor-products, a trisaccharide, 6^2-(3-deoxy-α-D-*ribo*-hexopyranosyl)maltose (**A**, Fig. 2) and a tetrasaccharide, 6^2-(α-D-3,3^2-dideoxyisomaltosyl)maltose (**b**, Fig. 2). GTF-I gives these same products, together with a product of higher mobility, probably an α-(1→3)-linked acceptor product, 3^2-(3-deoxy-α-D-*ribo*-hexopyranosyl)maltose (**C**, Fig. 2).

3-Deoxy-3-fluorosucrose reacted with maltose and GTF-S to give one acceptor product ([d.p. 3]-2, column C, Fig. 1), which is probably 6^2-(3-deoxy-3-fluoro-α-D-glucopyranosyl)maltose (**D**, Fig. 2); GTF-I gave this same product as well as one of higher mobility ([d.p. 3]-1, column D, Fig. 1), which is the corre-

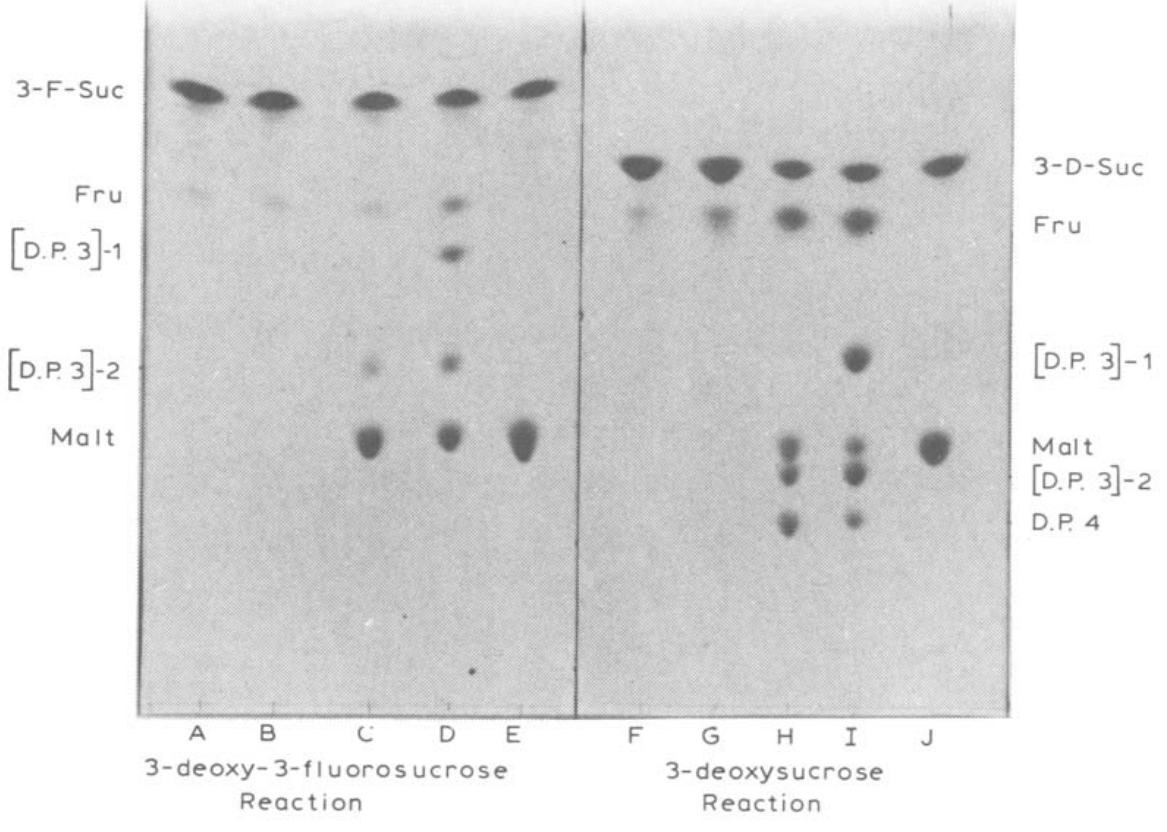
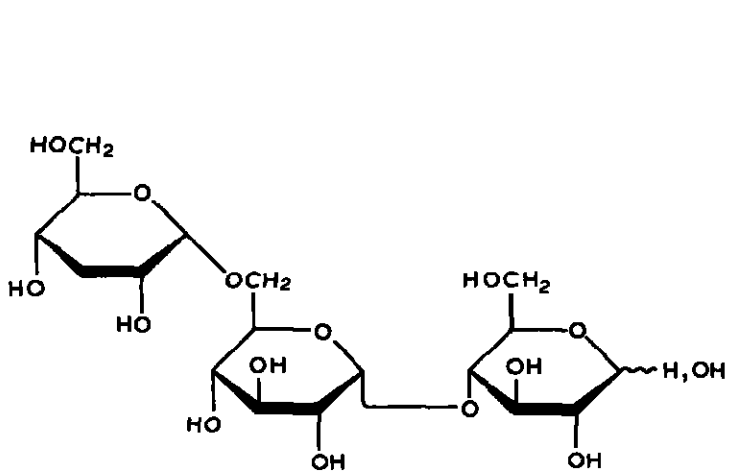
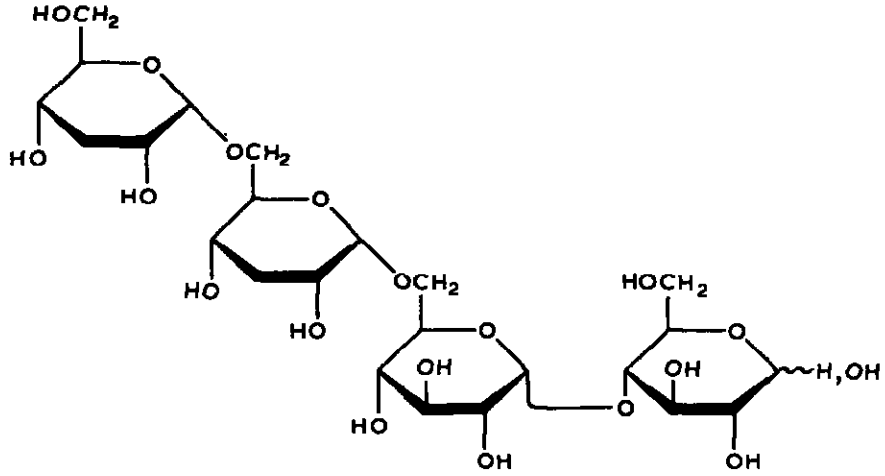
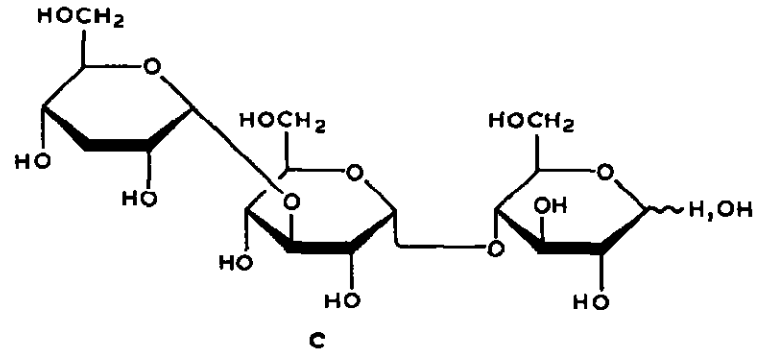
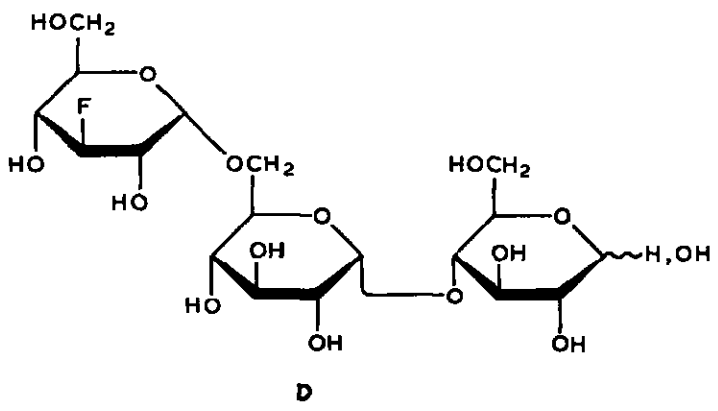


Fig. 1. T.l.c. of the action of *Streptococcus mutans* 6715 glucosyltransferases on 3-deoxy-3-fluoro- and 3-deoxy-sucrose: (A) GTF-S and 3-deoxy-3-fluorosucrose; (B) GTF-I and 3-deoxy-3-fluorosucrose; (C) GTF-S and 3-deoxy-3-fluorosucrose in the presence of maltose; (D) GTF-I and 3-deoxy-3-fluorosucrose in the presence of maltose; (E) 3-deoxy-3-fluorosucrose and maltose standards; (F–I) same series of reactions as (A–D) except that 3-deoxysucrose was used instead of 3-deoxy-3-fluorosucrose; (J) 3-deoxysucrose and maltose standards. [D.P. 3]-1 is a trisaccharide (degree of polymerization of 3) having a terminal α-(1→3) linkage and a mobility greater than [D.P. 3]-2 which is also a trisaccharide having a terminal α-(1→6) linkage and a lower mobility. D.P. 4 is a tetrasaccharide.









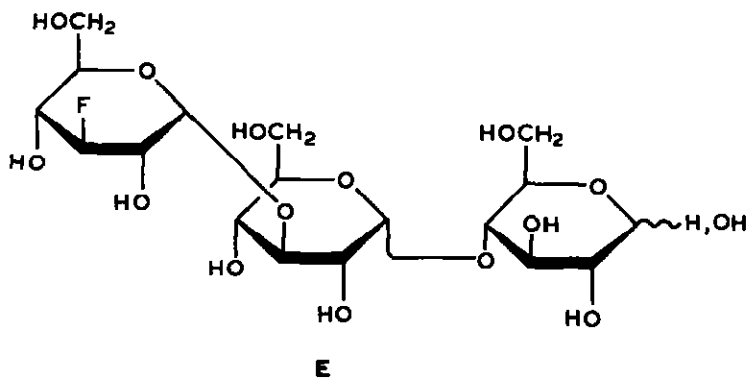


Fig. 2. Proposed structures for the maltose acceptor-products of 3-deoxysucrose and 3-deoxy-3-fluorosucrose reacting with GTF-I and GTF-S. (**A**) [D.P. 3]-2 of columns H and I of Fig. 1 is 6^2-(3-deoxy-α-D-*ribo*-hexopyranosyl)maltose; (**B**) [D.P. 4] of columns H and I of Fig. 1 is 6^2-(3^1,3^2-dideoxyisomaltosyl)maltose; (**C**) [D.P. 3]-1 of column I of Fig. 1 is 3^3-(3-deoxy-α-D-*ribo*-hexopyranosyl)maltose; (**D**) [D.P. 3]-2 of columns C and D of Fig. 1 is 6^2-(3-deoxy-3-fluoro-α-D-glucopyranosyl)maltose; (**E**) [D.P. 3]-1 of column D of Fig. 1 is 3^2-(3-deoxy-3-fluoro-α-D-*ribo*-hexopyranosyl)maltose.

sponding α-(1→3)-linked acceptor-product, 3^2-(3-deoxy-3-fluoro-α-D-gluco-pyranosyl)maltose (**E**, Fig. 2).

To confirm the proposed structures of the acceptor products, we isolated the tetrasaccharide (D.P. 4, columns H and I of Fig. 1) by l.c. and recorded its ^{13}C-n.m.r. spectrum (Fig. 3); the chemical shifts are given in Table I. There are five anomeric carbon resonances (100.8, 98.0, 97.6, 96.8, and 93.0 p.p.m.). Only one of them is above 100 p.p.m., and corresponds to the α-(1→4) linkage in maltose. The resonances at 98.0 and 97.6 p.p.m. correspond to α-(1→6) linkages and the two less-intense resonances at 96.8 and 93.0 p.p.m. are from the reducing-end C-1, which is present in both the α and β configurations[15]. At the other end of the

TABLE I

^{13}C-N.M.R. CHEMICAL SHIFTS (75.5 MHz) FOR TETRASACCHARIDE ACCEPTOR-PRODUCT

Signal	*Chemical shifts p.p.m.*[a]	*Intensity*	*Identification*[b]
1	100.8	181	2, C-1
2	98.0	288	3, C-1
3	97.6	246	4, C-1
4	96.8	196	1, C-1β
5	93.0	116	1, C-1α
6	78.6	98	1, C-4α
7	78.4	154	1, C-4β
8	77.2	171	1, C-3β
9	75.7	178	1, C-5β
10	75.1	166	
11	74.2	288	
12	73.6	292	
13	72.8	106	
14	72.7	167	
15	72.4	253	Resonances not assigned
16	72.4	135	
17	72.2	273	
18	71.2	111	
19	70.6	206	
20	67.6	312	
21	67.6	271	
22	66.8	200	
23	66.5	211	
24	65.2	466	
25	61.9	144	1, C-6α
26	61.8	114	1, C-6β
27	61.6	261	4, C-6
28	50.0	94	(Methanol standard)
29	35.9	113	3 or 4, C-3
30	35.6	138	4 or 3, C-3

[a]Chemical shifts measured in D_2O, with methanol as internal reference. [b]The designation 1 refers to the reducing-end glycosyl residue, with the other residues assigned 2, 3, and 4, in numerical order from the reducing end.

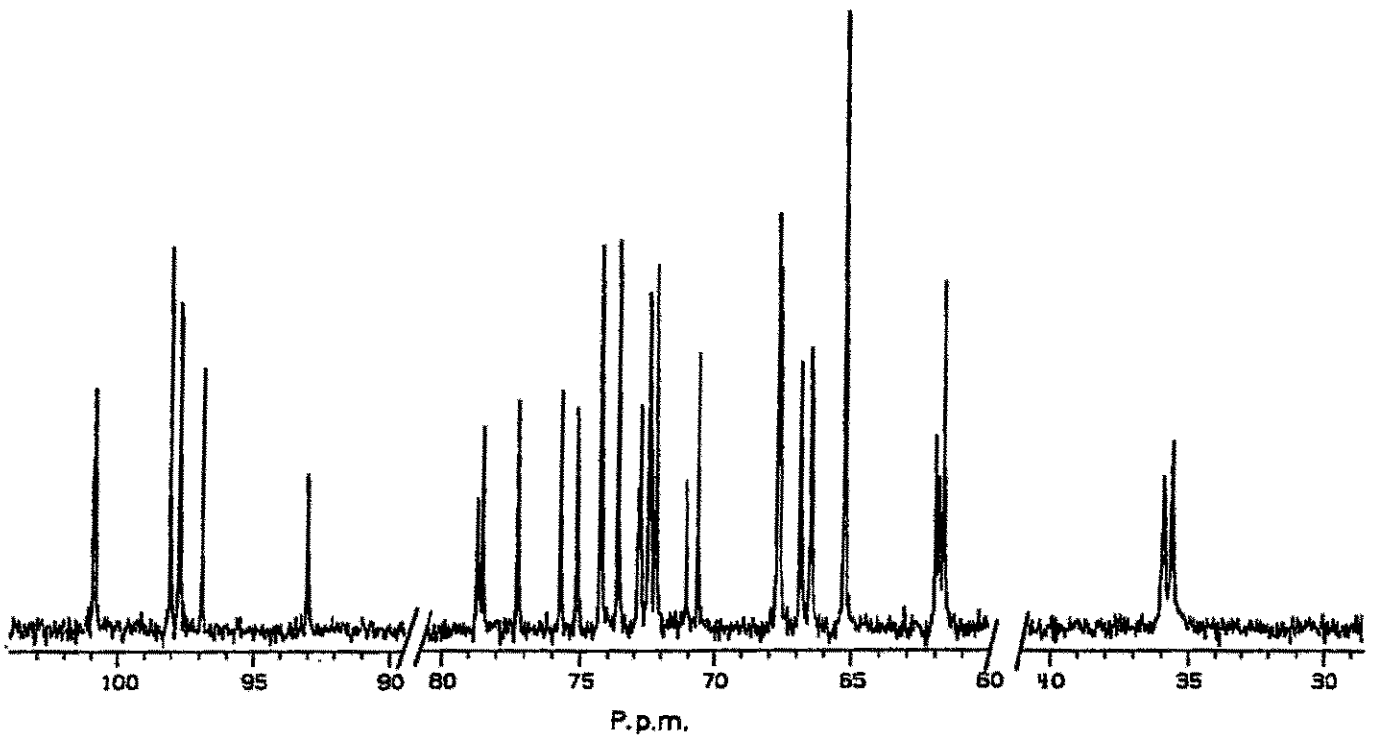


Fig. 3. ^{13}C-N.m.r. spectrum of the tetrasaccharide acceptor-product produced by the action of GTF-S on 6-deoxysucrose in the presence of maltose. Sample concentration 20 mg/mL with methanol as the internal standard.

TABLE II

KINETIC CONSTANTS FOR SUCROSE ANALOGS[a]

Inhibitor, concentration	*Glycosyl donor*	*Inhibition[b] (%)*		*Inhibition constants* (mM) *and type of inhibition*	
		GTF-S	*GTF-I*	*GTF-S*	*GTF-I*
3-Deoxysucrose, 109mM	+	13	51		36.4, 305 mixed[c]
3-Deoxy-3-fluorosucrose, 92mM	+	40	45	60.7 comp.	40.5, 153 mixed[c]
Allosucrose, 101mM	−	8	36		139, 222 mixed[c]
6-Deoxysucrose, 1.5mM	−	67	84	0.18 comp.	0.56 comp.
6-Thiosucrose, 15mM	+	50	62	7.3 comp.	3.4 comp.
6,6′-Dideoxy-6,6′-difluorosucrose (21.9mM)	−	0	55		10 uncomp.

[a]Sucrose K_m values[4]: GTF-S, 5.0mM; GTF-I, 3.7mM. [b]Inhibition at stated concentration, with 5mM sucrose. [c]Mixed-type inhibition is a special type of noncompetitive inhibition in which the lines in a Lineweaver–Burk plot intersect in the second quadrant. This occurs when S can bind with the EI-complex and I can bind with the ES-complex, both giving the nonproductive ESI-complex; EI has a lower; affinity for S than E has for S and the inhibition affects both V_{max} and K_m giving two inhibition constants (ref. 17).

spectrum, only three resonances (61.9, 61.8, and 61.6 p.p.m.) are present, and correspond to free hydroxymethyl carbon atoms. The resonance at 61.6 p.p.m. is from the C-6 at the nonreducing end and the two less-intense resonances (at 61.8, and 61.9 p.p.m.) are from C-6 of the α and β anomers at the reducing end[15]. These results indicate that there are two α-(1→6) linkages and one α-(1→4) linkage present, confirming the structure of **B** proposed in Fig. 2.

Table II gives the kinetic constants obtained for the three C-3-modified sucrose analogs as well as the results from our earlier study of the C-6-modified sucrose analogs[4] for comparison. Only 3-deoxy-3-fluorosucrose inhibited GTF-S and, therefore, only its K_I was determined. All three of the C-3-modified sucrose analogs inhibited GTF-I to some extent and gave mixed-type inhibition. Mixed-type inhibition is a special type of noncompetitive inhibition in which the lines in a Lineweaver–Burk plot intersect in the second quadrant. This occurs when S can bind with the EI-complex and I can bind with the ES-complex, both giving the nonproductive ESI-complex; EI has a lower affinity for S than E has for S and the inhibition affects both V_{max} and K_m, giving two inhibition constants[17].

DISCUSSION

T.l.c. (Fig. 1) of the reactions of the C-3-modified sucroses show that modification at C-3 does not prohibit glycosyltransferase activity, since acceptor products are formed from 3-deoxysucrose and 3-deoxy-3-fluorosucrose in the presence of maltose. 3-Deoxysucrose appears to be a better substrate for acceptor reactions than 3-deoxy-3-fluorosucrose, as more products were formed from it in 24 h than were formed with the fluoro analog under the same conditions in 48 h. This was unexpected, because they both have similar K_I values for GTF-I (Table II), and only the fluoro derivative appears to inhibit GTF-S. A possible explanation for this may be that there is some stabilization of the acetal–acetal bond of sucrose by the presence of the fluoro group at C-3, since in the synthesis of these three analogs[3], we found that the fluoro analog appeared to be the most stable to mild acid hydrolysis (data not shown).

The maltose acceptor-products apparently have the same structure as those produced when sucrose is used as the glycosyl donor[13,14]. ^{13}C-N.m.r. analysis of the tetrasaccharide acceptor-product, confirmed its structure as **B**. In light of the structure of **B**, the known patterns of glucosyltransfer to maltose by *S. mutans* glucosyltransferases[13,14], and the relative chromatographic mobilities of α-D-(1→3)- and α-D-(1→6)-linked glucosyl acceptor-products, we have deduced the structures of the four trisaccharide acceptor-products to be **A, C, D**, and **E** shown in Fig. 2.

The tetrasaccharide (B of Fig. 2) appears to be a terminal acceptor-product for both glucosyltransferases, as no higher saccharides were formed. This is unusual because when sucrose is used as the glycosyl donor with maltose as the acceptor, a series of acceptor products is produced down to at least an octasaccharide[14]. The fact that **B** is a terminal acceptor-product indicates that it is no longer productively

bound by either GTF-I or GTF-S for acceptor reactions because of its 3-deoxy-modified terminal D-glucosyl residue.

Because both 3-deoxysucrose and 3-deoxy-3-fluorosucrose were able to participate in acceptor reactions, we expected them to be fairly good competitive inhibitors of the glucosyltransferases. The data of Table II show this not to be the result. GTF-S was inhibited only by 3-deoxy-3-fluorosucrose and its K_I was 60.7mM, tenfold greater than the K_m value for sucrose. This suggests that the 3-hydroxyl group is important for binding at the active site. The fact that 3-deoxysucrose inhibited GTF-S even less than 3-deoxy-3-fluorosucrose may also indicate the fluoro group is acting as a hydrogen-bond acceptor at the active site.

GTF-I was slightly inhibited by all three C-3-modified sucrose analogs and the inhibition was of a mixed type. The K_I values ranged from 36.4 to 139mM, whereas the K_{II} values were all >150mM. These inhibition constants are much higher than the K_m value for sucrose, with 3-deoxysucrose and 3-deoxy-3-fluoro-sucrose having similar K_I values. The mixed inhibition observed may be caused by the sucrose analogs binding in more than one way at the active site at high concentrations. A further suggestion of this comes from reports that *Leuconostoc*[18,19] and *Streptococcus*[20] glucosyltransferases are inhibited by sucrose at concentrations ranging from 50–200mM and that the inhibition is relieved by adding acceptors[21].

Modification at C-3 decreased binding of the sucrose analogs to GTF-S and GTF-I, whereas in our earlier study[4], deoxy- or thio-modifications at C-6 gave inhibitors that were either bound better or equaled the binding of sucrose. The comparison of the binding of C-3-modified analogs with the C-6-modified analogs would further suggest that the 3-hydroxyl group plays an important role in the binding of sucrose at the active site. The fact that all of the C-3-modified sucrose analogs inhibited GTF-I to a greater extent than GTF-S is probably related to the type of polysaccharide each of these synthesizes. GTF-I synthesizes an α-(1→3)-linked glucan and can utilize the 3-hydroxyl group as a hydrogen-bond donor, as O-3 must act as a nucleophile in the polymerization mechanism[16,22,23]. In contrast, GTF-S, which synthesizes a (1→6)-α-D-glucan, can utilize the 3-hydroxyl group at C-3 for binding as either a hydrogen-bond donor or acceptor. The fact that both 3-deoxysucrose and 3-deoxy-3-fluorosucrose have similar K_I values for GTF-I would also support this conclusion, since neither analog has a hydroxyl hydrogen atom to donate for hydrogen bonding, while with GTF-S only the fluoro derivative can act as a hydrogen-bond acceptor; this may explain why it is the best C-3-modified sucrose-analog inhibitor for this enzyme.

In the earlier study by Grier and Mayer[11] with D-glycopyranosyl fluorides, the only sucrose analogs they studied that correspond to our C-3- and C-6-modified sucrose analogs were 6-deoxy-α-D-glucopyranosyl and α-D-allopyranosyl fluoride, and these were not found to be glycosyl donors for glucosyltransferases. We have found that 6-deoxysucrose[4] and allosucrose were not able to donate glycosyl residues, and this agrees with their results on the specificity of the glucosyltransferases, but we have shown that 6-deoxy-6-thiosucrose[4], 3-deoxysucrose, and

3-deoxy-3-fluorosucrose are able to donate glycosyl residues in acceptor reactions with maltose. A comparable glycosyl fluoride study with C-3- and C-6-modified α-D-glucopyranosyl fluoride has not been done, and so it is not known if any glycosyl fluoride other than α-D-glucopyranosyl fluoride will act as a glycosyl donor for GTF-I and GTF-S.

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