TABLE II

PRODUCTION OF HEXA-*O*-ACETYL-DIFRUCTOSE ANHYDRIDE FROM ACETYLATED *Ti* POLYFRUCTOSAN BY DEGRADATION WITH FUMING NITRIC ACID FOLLOWED BY DENITRATION AND REACETYLATION

Wt. of *ti* fructosan acetate	$CHCl_3$, ml.	HNO_3, ml.	Time, min.	Temp., °C.	Yield of hexa-*O*-acetyldifructosan Wt., g.	% by wt.
1.0	15	7	120	23	0.04	4.0
5.0	None	25	120	13	.19	3.8

C. The Action of Fuming Nitric Acid on Sucrose Octa-*O*-acetate.—When sucrose octa-*O*-acetate was treated with fuming nitric acid as described above in A there was formed 1-nitro-2,3,4,6-tetra-*O*-acetyl-β-D-glucose, m.p. 150–151° (after crystallization from aqueous ethanol). A small amount of unchanged sucrose octa-*O*-acetate was also isolated, m.p. and mixed m.p. 82°, but no hexa-*O*-acetyl-difructofuranose 2,1′:1,2′-dianhydride was detected.

D. Artichoke (*Helianthus tuberosus*) Inulin Acetate.—(a) To an ice-cold solution of inulin tri-*O*-acetate (20 g.)[4] in chloroform (300 ml., dried over P_2O_5), freshly distilled fuming nitric acid (130 ml.) (prepared by two distillations of fuming nitric acid (d. 1.5) in the presence of concentrated sulfuric acid) was added slowly with vigorous stirring, moisture being excluded from the apparatus. The experiment was conducted at 0° in an atmosphere of dry carbon dioxide. Phosphorus pentoxide (80 g.) was added to the homogeneous solution in 5-g. portions during 1.25 hours. Most of the phosphorus pentoxide remained unchanged and it did not appear to become sirupy in nature. After 1.5 hours the reaction mixture was poured into ice-water and when all the phosphorus pentoxide had dissolved the colorless chloroform layer was separated and washed successively with water and sodium bicarbonate solution. After drying ($MgSO_4$) the chloroform extract removal of solvent gave a pale yellow sirupy product which was dissolved in ethanol from which the hexa-*O*-acetyl-difructofuranose 2,1′:1,2′-dianhydride crystallized. The product was filtered off, washed with ice-cold ethanol and dried (yield 9 g.[4]). Time is an important factor in this experiment and variations from 1.5 hours were found to give reduced yields of the crystalline hexa-*O*-acetyl-difructose dianhydride.

(b) To a solution of inulin acetate (10 g.) in dry chloroform (200 ml.) fuming nitric acid (100 ml.) was added with stirring. The reaction was allowed to proceed for 2 hours at 18–20°. The hexa-*O*-acetyl-difructose 2,1′:1,2′-dianhydride isolated as above amounted to 5 g. (50% of theory). Several experiments carried out for 2 hours at 0° gave lower yields of the crystalline dianhydride.

These yields of the hexa-*O*-acetyl-difructose dianhydride which are higher than those obtained from dahlia inulin acetate are believed to be due to the greater precautions taken to exclude moisture from the reaction mixture and not to any structural differences between the two polysaccharide acetates.

The hexa-*O*-acetyl-difructofuranose 2,1′:1,2′-dianhydride prepared as above and recrystallized from ethanol showed $[\alpha]^{20}D$ −0.5° (approx.) in chloroform (*c* 5), m.p. 128° when rapid heating was applied and m.p. 138° when the heating was slow. From cooling curves the true m.p. was shown to be 128°.

Anal. Calcd. for $C_{24}H_{32}O_{16}$: C, 50.0; H, 5.6; Ac, 44.8. Found: C, 49.7; H, 5.8; Ac, 44.4.

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[CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL BIOCHEMISTRY, UNIVERSITY OF MINNESOTA]

The Constitution of the Glucofructan of the Tuber of the Hawaiian "*TI*" Plant (*Cordyline Terminalis*)[1,2]

BY L. A. BOGGS AND F. SMITH

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The constitution of the glucofructan present in the tuber of the Hawaiian *ti* plant has been shown to be composed of 1 unit of D-glucose and about 13 of D-fructose. The methylated polysaccharide gives upon hydrolysis 1,3,4,6-tetra-*O*-methyl-D-fructose (4 moles), 1,3,4-tri-*O*-methyl-D-fructose (2 moles), 3,4,6-tri-*O*-methyl-D-fructose (5 moles), 3,4-di-*O*-methyl-D-fructose (2 moles) and 2,3,4-tri-*O*-methyl-D-glucose (1 mole). The structural significance of these findings is discussed.

Extraction of the dried tubers of the Hawaiian *ti* plant (*Cordyline terminalis*) with water yields a glucofructan.[3] The latter, obtained in the form of an amorphous white powder, showed $[\alpha]D$ −37° (water) and was non-reducing to Fehling solution.

Upon hydrolysis it gave D-glucose and D-fructose in the approximate ratio of 1:14. The ease with which the polysaccharide underwent hydrolysis indicated that the fructose residues were present in the furanose form.

The *ti* glucofructan, which appeared to be essentially homogeneous from fractionation studies, was transformed into a triacetate with acetic anhydride and pyridine[4] and this was methylated[4,5] with methyl sulfate and sodium hydroxide to give the corresponding trimethyl derivative as a viscous liquid which also appeared to be homogeneous from fractionation studies.

Hydrolysis of the tri-*O*-methyl *ti* glucofructan with oxalic acid under conditions which have been found[5] to hydrolyze 1,2′-linked fructans composed of furanose units failed to effect complete hydrolysis. This observation indicated that *ti* fructan might contain some linkage other than the 1,2′-type.

After completing the hydrolysis by using a higher concentration of oxalic acid and a higher temperature than those required for inulin,[5] the mixture of methylated sugars was resolved by cellulose column chromatography[6] into three fractions,[7] namely, 1,3,4,6-tetra-*O*-methyl-D-fructose, 3,4-di-*O*-methyl-D-fructose and a mixture of trimethylhexoses. This mixture consisted of 1,3,4-tri-*O*-methyl-D-fructose, 3,4,6-tri-*O*-methyl-D-fructose and 2,3,4-tri-*O*-methyl-D-glucose (see Table II).

The 1,3,4,6-tetra-*O*-methyl-D-fructose was iden-

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tified by oxidation with nitric acid to give 3,4,6-tri-*O*-methyl-D-fructofuranuronic acid (3,4,6-tri-*O*-methyl-α-keto-D-gluconic acid). Methylation with silver oxide and methyl iodide afforded the corresponding ester glycoside which when treated with methanolic ammonia yielded the crystalline amide of methyl 3,4,6-tri-*O*-methyl-D-fructofuranoside uronic acid.[8]

The 3,4-di-*O*-methyl-D-fructose was characterized by oxidation with nitric acid followed by methylation and treatment with ammonia whereby there was obtained the crystalline diamide of methyl-3,4-di-*O*-methyl-D-fructofuranoside-1,6-dicarboxylic acid.[9,10]

The tri-*O*-methylhexose fraction consisted of a mixture of three components, 1,3,4-tri-*O*-methyl-D-fructose, 3,4,6-tri-*O*-methyl-D-fructose and what is believed to be 2,3,4-tri-*O*-methyl-D-glucose. The second of these was readily separated as the 1,2-*O*-isopropylidene derivative, by virtue of its greatly enhanced mobility in methyl ethyl ketone:water azeotrope on the cellulose column, and after removal of the isopropylidene group the 3,4,6-tri-*O*-methyl-D-fructose was transformed into the characteristic crystalline osazone.[10,11]

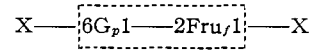
The 1,3,4-tri-*O*-methyl-D-fructose was separated from the tri-*O*-methylhexose fraction by making use of the fact that, unlike the 3,4,6-tri-*O*-methyl derivative, it does not form an isopropylidene derivative and it was freed from the suspected 2,3,4-tri-*O*-methyl-D-glucose by oxidizing the latter with bromine and removing the 2,3,4-tri-*O*-methyl-D-gluconic acid on an anion-exchange resin. The purified 1,3,4-tri-*O*-methyl-D-fructose was then transformed by nitric acid oxidation, followed by methylation and treatment with ammonia into the same diamide of methyl 3,4-di-*O*-methyl-D-fructofuranoside-1,6-dicarboxylic acid as was produced from 3,4-di-*O*-methyl-D-fructose.[9]

The tri-*O*-methylaldohexose component of the tri-*O*-methylhexose fraction was not identified in the form of a crystalline derivative. Since a pure specimen of the tri-*O*-methyl sugar could not be isolated from the hydrolyzate of the methylated *ti* glucofructosan it seemed impractical to prepare either its methyl β-glycoside[12,13] or its anilide.[13]

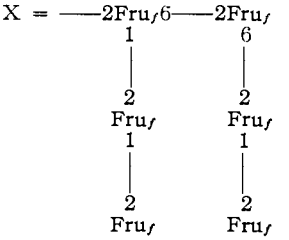
It was therefore oxidized with bromine to the corresponding lactone and it was established that this lactone had the same mobility on filter paper, using three different solvent systems, as an authentic specimen of 2,3,4-tri-*O*-methyl-D-glucono-δ-lactone.

Bearing in mind that D-glucose is known to be present in the parent glucofructan and that this unit is probably the one responsible for the formation of formic acid during periodate oxidation of the polysaccharide, it seems reasonable to suggest that the tri-*O*-methylhexose fragment which gives rise to an acid by bromine oxidation is 2,3,4-tri-*O*-methyl-D-glucose.

The approximate ratios of the cleavage fragments of the methylated *ti* glucofructan from the cellulose chromatographic analysis were as follows: 1,3,4,6-tetra-*O*-methyl-D-fructose (4 moles), 1,3,4-tri-*O*-methyl-D-fructose (2 moles), 3,4,6-tri-*O*-methyl-D-fructose (5 moles), 3,4-di-*O*-methyl-D-fructose (2 moles) and (?) 2,3,4-tri-*O*-methyl-D-glucose (1 mole). A possible structure for *ti* glucofructan, in agreement with these ratios for the components of the hydrolyzate, is shown in formula I.



where



Other possible arrangements of the units are of course not excluded, but this represents the general type of structure in the glucofructan.

Proof that the D-glucose unit is not in a terminal reducing position followed from the fact that bromine oxidation of the *ti* glucofructan and subsequent hydrolysis furnished glucose as well as fructose. Were the glucose located in a terminal reducing position, it would be expected to be transformed into D-gluconic acid.

The presence of glucose in the hydrolyzates of polyfructans has been recognized for a considerable time,[14-21] but it is only relatively recently that its true structural significance has been established.

The disaccharide building unit in I surrounded by dotted lines represents a sucrose unit to which the plant adds fructofuranose units by a process of transfructosidation to give a glucofructan.[2,7] We first encountered[22] this same type of structure, in which glucose forms an integral part of the carbohydrate polymer, in the graminin (levosine) of rye grain[2,7,22] isolated first by Muntz[23] and later by Tanret.[24] This structural feature was recognized by the fact that cleavage of the methylated graminin glucofructan gave not only tetra-, tri- and di-*O*-methyl derivatives of D-fructose indicating a branched structure, but also 2,3,4,6-tetra-*O*-methyl-D-glucose. Since only a tetra-*O*-methyl derivative of glucose was detected, the possibility of the poly-

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fructosan being contaminated with a polyglucan[25,26] was eliminated.

Support for the glucofructan type of structure has been accumulating as a result of both chemical[27-32] and enzymatic[33-37] investigations.

The glucose may be present as a terminal non-reducing group as in the graminin[22] of rye grain, in inulin[38] from dahlia tubers, and in the glucofructan of perennial rye-grass (*Lolium perenne*),[28] and/or it may be located in a non-terminal position as in the *ti* glucofructosan now under discussion. In all these cases where glucose has been detected it is believed to be joined to a furanose unit of fructose as in sucrose.

Experimental

Isolation of Glucofructan from the *Ti* Plant (*Cordyline terminalis*).—A mixture of the dried ground tuber of the *ti* plant (100 g.) and powdered calcium carbonate (8 g.) was divided into four portions and extracted countercurrently with four 100-ml. portions of water at 70°.

The combined aqueous extract (300 ml.) was cooled and poured, with stirring, into a mixture of acetone (1500 ml.) and absolute ethanol (1500 ml.). After 48 hours, the supernatant liquid was decanted and the precipitated polysaccharide was washed successively with acetone, ethanol, diethyl ether, petroleum ether, and dried *in vacuo* to give a white amorphous powder (yield 48 g.) which had $[\alpha]^{28}D$ −37.0° in water (*c* 1.0). The *ti* glucofructan did not reduce Fehling solution.

The combined supernatant liquid and washings were evaporated under reduced pressure to yield a sirupy mixture of sugars (6.9 g.) in which fructose, glucose and sucrose were detected by paper chromatography using phenol saturated with water as the irrigating solvent.

Fractionation of the *Ti* Glucofructan.—The crude glucofructan (45 g.) was dissolved in pyridine (245 ml.) and poured, with stirring, into acetone (735 ml.). The resulting precipitate was filtered off and dried as before (yield 37.8 g.). The partially purified polysaccharide was redissolved in pyridine (250 ml.) and subjected to fractional precipitation, in the usual manner, by the addition of increasing amounts of acetone. The gelatinous precipitates resulting were separated, redissolved in pyridine and precipitated by pouring the solution into three volumes of acetone. The five fractions (1.9, 13.8, 9.7, 4.9 and 3.1 g.) obtained in this way had almost the same rotation $[\alpha]^{21}_{5461}$ −39° in water (*c* 6.5).

Identification of the Component Sugars in *Ti* Glucofructan.—When a solution of the glucofructan (2 g.) in 0.01 *N* sulfuric acid (100 ml.) was heated for 15 minutes on a boiling water-bath, the rotation changed from $[\alpha]^{27}D$ −35° to −75°. Neutralization ($BaCO_3$), filtration and removal of solvent *in vacuo* gave a sirup (yield almost quantitative), $[\alpha]^{18}D$ −71° in water (*c* 1). Paper chromatographic analysis of this sirup using phenol saturated with water as the irrigating solvent and ammoniacal silver nitrate to locate the components indicated the presence of glucose and fructose.

Column chromatography of the hydrolyzate (0.426 g.) in the manner previously reported[6] gave D-glucose (28 mg.), m.p. and mixed m.p. 145°, $[\alpha]^{25}D$ +52.5°, equilibrium value in water (*c* 1), and D-fructose (316 mg.), m.p. and mixed m.p. 98°, $[\alpha]^{25}D$ −91°, equilibrium value in water (*c* 1).

Isolation of 2,3;4,5-Di-*O*-isopropylidene-D-fructose. (a.)—The sirupy hydrolyzate from the *ti* glucofructan above was shaken for 5 days at room temperature with dry acetone (20 vol.) containing 1.6% (v./v.) concentrated sulfuric acid. The mixture was kept in the cold room (−18°) for 2 days, and, while still cold, the acid was neutralized with gaseous ammonia. Filtration and evaporation left a residue which was extracted with chloroform in the presence of water. Evaporation of the dried chloroform extracts gave a crystalline residue which upon recrystallization from petroleum ether (b.p. 30–60°) yielded 2,3;4,5-di-*O*-isopropylidene-β-D-fructose, m.p. and mixed m.p. 95.5°, $[\alpha]^{28}D$ −37.6° in acetone (*c* 3.8).

(b).—Simultaneous cleavage of *ti* glucofructan and formation of 1,2;4,5-di-*O*-isopropylidene-D-fructose, *i.e.*, "acetonolysis," was accomplished by dissolving the polysaccharide (1 g.) in dimethylformamide (10 ml.) by warming to 50° after which the solution was cooled and mixed with a cold solution of concentrated sulfuric acid (1.25 ml.) in acetone (20 ml.). The reaction mixture was stirred for 2 days at 5° and after cooling to −18° it was neutralized with concentrated ammonium hydroxide. Filtration and removal of solvent *in vacuo*, yielded 1,2;4,5-di-*O*-isopropylidene-α-D-fructose m.p. and mixed m.p. 120°, $[\alpha]^{27}D$ −139.5° in chloroform (*c* 1.9); literature m.p. 119–120°.[39]

Isolation of 1,2;4,5-Di-*O*-cyclohexylidene-D-fructose.—The polysaccharide (1 g.) was treated with cyclohexanone (19 ml.) containing sulfuric acid (0.33 ml.) at room temperature overnight, with occasional shaking. After 4 days at −18° followed by neutralization with concentrated ammonium hydroxide (3 ml.) filtration and removal of solvent *in vacuo* yielded di-*O*-cyclohexylidene-D-fructose (0.8 g.), m.p. and mixed m.p. 145°, $[\alpha]^{20}D$ −123° in acetone (*c* 1.9), after recrystallization from acetone.

Anal. Calcd. for $C_{18}H_{28}O_6$: C, 63.5; H, 8.2. Found: C, 63.5; H, 8.5.

Determination of Glucose/Fructose Ratio in *Ti* Glucofructan. (a) **By Periodate Oxidation.**—A solution of *ti* glucofructosan (0.9745 g.) in sodium periodate solution (200 ml. 0.114 *N*) which showed $[\alpha]^{25}D$ −32° was transferred together with a blank to the cold room at 5°. Aliquots were periodically withdrawn for the determination of the periodate consumption and the liberation of formic acid in the usual manner.[40] The results are shown in Table I.

TABLE I

PERIODATE OXIDATION OF *Ti* GLUCOFRUCTAN

Reaction time (days)	Moles of anhydrohexose/mole of formic acid produced	Moles of periodate consumed/mole of anhydrohexose	Reaction time (days)	Moles of anhydrohexose/mole of formic acid produced	Moles of periodate consumed/mole of anhydrohexose
3	19.0	..	10	13.5	..
4	16.6	..	12	13.3	1.01
5	15.5	1.00	13	13.5	..
7	14.6	0.98	17	12.7	1.01
9	13.7	1.00			

From these data it appears that the polysaccharide contains one set of three vicinal hydroxyl groups (anhydroglucopyranose unit) in each chain of from thirteen to fourteen anhydrohexose units. After 13 days the periodate oxidation reaction mixture showed $[\alpha]^{26}D$ −43°.

(b) **By Chromatographic Analysis.**—A solution of the polysaccharide (59 mg.) in 0.1 *N* sulfuric acid (2 ml.) was digested 5.5 hours in a boiling water-bath. The solution was cooled, neutralized by passing through "Duolite A4"[41] anion-exchange resin, and concentrated under reduced pressure to dryness. The residue was dissolved in 50% aqueous ethanol (2 ml.). A qualitative chromatogram, using 1-butanol:ethanol:water (4:1:5) as the irrigating solvent, showed that glucose and fructose were both present.

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(41) A product of the Chemical Process Co., Redwood City, Calif.

A portion (0.104 ml.) of the 50% aqueous ethanol extract was placed on a chromatogram and the two components were separated by irrigation on a strip of Whatman No. 1 filter paper with 1-butanol:ethanol:water (4:1:5) for a period of 70 hours. The positions of the glucose and fructose components were located on marginal strips and each component was extracted from the chromatogram in the usual way[42] using 20 ml. of water for the fructose and 10 ml. for the glucose. Since each component was located on a strip 12.6 × 6.1 cm., a piece of paper of the same size cut from a blank chromatogram similarly treated was extracted with 20 ml. of water for use as a blank correction. For the colorimetric determinations, the glucose extract was diluted to three volumes, the fructose extract was diluted to nine volumes, and the blank extract was used without dilution.

For the colorimetric determination a 2-ml. aliquot of each extract was mixed with 80% phenol (0.1 ml.) and treated with concentrated sulfuric acid (5 ml.). After the mixture had cooled (approximately 1 hour) the intensity of the color at 490 mμ was read with a Coleman Junior instrument and the amount of sugar determined by reference to a standard curve. The results of the experiment, carried out in duplicate with triplicate readings for each colorimetric measurement,[42] showed that the ratio of D-fructose to D-glucose was 13:1.

Bromine Oxidation of *Ti* Glucofructan and of its Hydrolyzate.—A solution of *ti* glucofructan (5.0 g.) in water (15 ml.) was treated for 2 days at room temperature in the dark with bromine (0.2 ml.) in the presence of calcium carbonate (0.8 g.). Residual bromine was removed by aeration under reduced pressure, the mixture was treated with silver carbonate, and the filtrate was passed through a column of the acid form of a cation-exchange resin (IR 120). The volume of the effluent was adjusted with water to 350 ml. and the solution, after adding crystalline oxalic acid (7.8 g.), was heated for 1 hour in a boiling water-bath. The acid was neutralized by digestion with calcium carbonate (12 g.) and the filtrate was deionized by successive passage through columns of ion-exchange resins (Amberlite[43] IR 120 and Duolite A-4). Removal of solvent from the effluent *in vacuo* left a sirupy residue (5.99 g.) which was found by paper chromatography using phenol–water as the irrigating solvent to contain both fructose and glucose.

In another experiment, the polysaccharide (5 g.) was dissolved in 2.25% aqueous oxalic acid and hydrolyzed for 1 hour in a boiling water-bath, to constant rotation $[\alpha]^{24}D$ −72.6°. After heating with excess calcium carbonate (2.7 g.) to neutralize the acid, the mixture was oxidized with bromine (0.3 ml.) as before. Removal of residual bromine, neutralization (Ag_2CO_3) and deionization with exchange resins (Amberlite IR120 and Duolite A-4) and evaporation, afforded a sirupy residue (4.935 g.) which contained fructose but no glucose.

These results indicate that the glucose unit in *ti* glucofructan does not exist as a terminal reducing group.

Methylation Studies on *Ti* Glucofructan

Acetylation of *Ti* Glucofructan.—Purified *ti* polysaccharide (15 g.) was acetylated by dissolving it in pyridine (150 ml.), adding acetic anhydride (150 ml.) and allowing the mixture to stand for 48 hours at room temperature. The product was isolated by pouring the reaction mixture, with stirring, into 10 volumes of water and washing the resulting white amorphous precipitate with water until it was free of pyridine and acetic acid. The dried, powdery, acetylated *ti* glucofructan (21.7 g. or 81% of theory) had $[\alpha]^{22}D$ −30° and $[\alpha]^{21}_{5461}$ −34° in chloroform (*c* 6.4).

Methyl *Ti* Glucofructan.—The acetylated polysaccharide (17.4 g.) was dissolved in acetone (370 ml.) and methylated at 55° in a nitrogen atmosphere with sodium hydroxide (460 ml. of 30%) and methyl sulfate (180 ml.). After the final addition of the reagents, the reaction was completed by heating on a boiling water-bath with gentle stirring. As the solvent distilled, the partially methylated polysaccharide separated on the surface of the solution as a semi-solid. It was separated from the aqueous layer while still hot and remethylated.

After four methylations in the same manner, the product was washed with hot water to remove most of the sodium sulfate and extracted with chloroform in the presence of water. The chloroform extract was dried ($MgSO_4$), filtered, and evaporated *in vacuo* to a viscous sirup. The methylated *ti* glucofructan (10.8 g. or 74% of theory) showed $[\alpha]^{23}D$ −53.3° in chloroform (*c* 6.4). Fractional precipitation of the product from chloroform with light petroleum ether showed that it was essentially homogeneous.

Anal. Calcd. for $C_9H_{16}O_5$: OCH_3, 45.6. Found: OCH_3, 45.3.

Hydrolysis of Methylated *Ti* Glucofructan.—The methylated polysaccharide (5.6 g., OCH_3, 45.3) was hydrolyzed by heating a solution of it in a mixture of methanol (168 ml.) and water (56 ml.) containing oxalic acid dihydrate (2.24 g.) when the rotation changed in 20 hours from $[\alpha]^{23}D$ −58° (initial value) to +26.8° (constant value).

In a parallel experiment, chromatography on a cellulose column as described later, of the methyl sugars so formed and fractional distillation of the corresponding methyl glycosides, showed that the methylated polysaccharide had not been completely hydrolyzed by the above procedure.

The methanol was removed and the incompletely hydrolyzed material was dissolved in water (200 ml.) containing oxalic acid dihydrate (2.26 g., making a total of 4.5 g.) and the solution heated on a water-bath at 85° for 1 hour when the rotation, $[\alpha]^{26}D$ +5.5°, became constant. In a repeat experiment the final rotation was $[\alpha]^{26}D$ +5.8°. Neutralization ($CaCO_3$), filtration and removal of solvent *in vacuo* gave a sirup having $[\alpha]^{26}D$ +4.7° in water (*c* 6) and $[\alpha]^{26}D$ +4° in methanol (*c* 4).

Column Chromatographic Analysis of the Hydrolyzate of Methylated *Ti* Glucofructan.—A portion (0.610 g.) of the sirupy hydrolyzate obtained above was subjected to fractionation on a cellulose column as described elsewhere[7] using methyl ethyl ketone:water azeotrope as the solvent. The data obtained are given in Table II.

Fraction A was identified as 1,3,4,6-tetra-*O*-methyl-D-fructose, fraction D was 3,4-di-*O*-methyl-D-fructose, while the intermediate components, fractions B and C, were shown to consist of a mixture of 1,3,4-tri-*O*-methyl-D-fructose, 3,4,6-tri-*O*-methyl-D-fructose, together with a third component believed to be 2,3,4-tri-*O*-methyl-D-glucose (see below).

The data indicate an approximate ratio of 4 moles of 1,3,-4,6-tetra-*O*-methyl-D-fructose; 8 moles of tri-*O*-methylhexoses; 2 moles of 3,4-di-*O*-methyl-D-fructose. It is significant that the number of end groups is twice the number of branched points.

Alternative Procedure for the Separation of the Methyl Sugars in the Hydrolyzate of Methylated *Ti* Glucofructan.—Another sample (5.6 g.) of the methylated glucofructan was hydrolyzed as described above. The sirupy mixture of sugars was dissolved in water (20 ml.) and treated with bromine (0.15 ml.) in the presence of barium carbonate (0.74 g.) for 3 days in the dark at room temperature. Excess bromine was removed by aeration under reduced pressure. The solution was acidified with hydrochloric acid, neutralized with silver carbonate and passed through a column of cation-exchange resin (Amberlite IR 120) to remove silver ions. The organic acid present in the eluate was removed by passing the solution through an anion-exchange resin (Duolite A4).

The methylated sugar acid was recovered from the anion resin by displacement with dilute sodium hydroxide and the free acid regenerated by passage of the solution through the cation resin (IR 120). Removal of solvent *in vacuo* yielded a sirup (305 mg.) which appeared to be 2,3,4-tri-*O*-methyl-D-glucono-δ-lactone (see below).

A portion (1.445 g.) of the neutral sugars from the bromine oxidized methylated glucofructosan hydrolyzate, not retained by the Duolite A4 anion-exchange resin, was subjected to cellulose column chromatography using methyl ethyl ketone:water azeotrope as the irrigating solvent. The tri-*O*-methyl ketohexose fraction (788 mg.), $[\alpha]^{25}D$ +7° (methanol), R_f 0.68 (methyl ethyl ketone:water azeotrope) corresponding to fraction B, Table II, was dissolved in acetone (10 ml.) containing concentrated sulfuric acid (0.1 ml.) and the mixture was kept for 48 hours at −18°. Concentrated ammonium hydroxide (0.8 ml.) was added to the cold mixture and the ammonium sulfate formed was filtered off and washed with acetone at room temperature. Removal of solvent gave a sirupy product which was subjected to cellulose column chromatography using methyl ethyl ke-

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(43) A product of the Rohm and Haas Chemical Co., Philadelphia, Pa.

TABLE II

CHROMATOGRAPHIC FRACTIONATION OF METHYLATED *Ti* GLUCOFRUCTAN HYDROLYZATE ON A CELLULOSE COLUMN WITH METHYL ETHYL KETONE:WATER AZEOTROPE[7]

Fraction	Component	Tube no.	R_f[a]	$[\alpha]^{23}D$	OCH_3 (%)	Wt., mg.	Mole ratio (approx.)
A	1,3,4,6-Tetra-*O*-methyl-D-fructose	1–19	0.86	+21.6° (MeOH) +19.3° ($CHCl_3$)	52.2	184	4
B	1,3,4-Tri-*O*-methyl-D-fructose, 3,4,6-tri-*O*-methyl-D-fructose and 2,3,4-tri-*O*-methyl-D-glucose			+ 8.3° (MeOH) +12.8° ($CHCl_3$) +13.2° (H_2O)			
		20–40	0.71		39.4	285	8
C	Same as B	41–73		.....		41	
D	3,4-Di-*O*-methyl-D-fructose	74–120	0.39	−22.4° (MeOH) − 6.0° ($CHCl_3$) −53.8° (H_2O)	27.5	86	2
					Total	596	
					Recovery	98%	

[a] Determined on paper using methyl ethyl ketone:water azeotrope.

tone:water azeotrope as the solvent. This yielded a fast moving component (630 mg.), 1,2-*O*-isopropylidene-3,4,6-tri-*O*-methyl-D-fructose, R_f 0.96 (on paper, using methyl ethyl ketone:water azeotrope as the solvent), $[\alpha]^{25}D$ +62° in methanol (*c* 2), $[\alpha]^{25}D$ +63° in acetone (*c* 2), and a slower moving component, 1,3,4-tri-*O*-methyl-D-fructose, R_f 0.59 (on paper, using methyl ethyl ketone:water azeotrope as solvent), $[\alpha]^{25}D$ −13°, equilibrium value in methanol, which crystallized upon nucleation with authentic 1,3,4-tri-*O*-methyl-D-fructose.

The fast moving component of the acetonated reaction mixture, 1,2-*O*-isopropylidene-3,4,6-tri-*O*-methyl-D-fructose (172 mg.), was dissolved in 2.25% oxalic acid (3 ml.) and heated for 2 hours on a water-bath at 80–85°. Neutralization ($CaCO_3$), filtration and concentration *in vacuo* yielded 3,4,6-tri-*O*-methyl-D-fructose, R_f 0.7, using methyl ethyl ketone:water azeotrope, $[\alpha]^{25}D$ +10.8° in methanol (for identification, see below).

Table III summarizes the data obtained in the fractionation of the tri-*O*-methyl sugars from the methylated *ti* glucofructan, on the basis of 8 moles of methylated hexose in this portion of the hydrolyzate.

Since 1.445 g. out of a total of 6.448 g. of the hydrolyzate of the methylated glucofructan was used for the isolation of the two tri-*O*-methylfructoses, the figures given have been multiplied by the factor 4.9 to convert them to the basis of 5.6 g. of methylated glucofructosan subjected to hydrolysis.

TABLE III

MOLAR RATIOS OF 1,3,4-TRI-*O*-METHYL-D-FRUCTOSE, 3,4,6-TRI-*O*-METHYL-D-FRUCTOSE, AND 2,3,4-TRI-*O*-METHYL-D-GLUCOSE IN THE HYDROLYZATE OF METHYLATED *Ti* GLUCOFRUCTAN

Fraction	Wt., mg.	Mole ratio
1,2-*O*-Isopropylidene-3,4,6-tri-*O*-methyl-D-fructose	2829	5.36
1,3,4-Tri-*O*-methyl-D-fructose	831	1.93
(?)2,3,4-Tri-*O*-methyl-D-glucono-δ-lactone	305	0.71

These data of Table III coupled with those of Table II indicate an approximate composition for methylated *ti* glucofructan hydrolyzate of 1,3,4,6-tetra-*O*-methyl-D-fructose (4 moles), 3,4,6-tri-*O*-methyl-D-fructose (5 moles), 1,3,4-tri-*O*-methyl-D-fructose (2 moles), 2,3,4-tri-*O*-methyl-D-glucose (1 mole) and 3,4-di-*O*-methyl-D-fructose (2 moles).

Identification of the Products of Hydrolysis of Methylated *Ti* Glucofructan. (a) **1,3,4,6-Tetra-*O*-methyl-D-fructose.**—A solution of the sirupy product (217 mg., fraction A, Table II) in nitric acid (3 ml., d. 1.42) was heated for 1 hour at 50–55°, followed by 0.5 hour on a boiling water-bath. The mixture was allowed to cool, diluted with water (10 ml.) and distilled *in vacuo* with a series of additions of methanol to remove nitric acid. The resulting sirup was subjected to two methylations with silver oxide (0.5 g.) and methyl iodide (10 ml.) in the presence of Drierite (0.5 g.) the product being isolated each time, after removal of excess methyl iodide, by extraction with acetone. Distillation gave methyl (methyl 3,4,6-tri-*O*-methyl-D-fructofuranoside)-uronate, as a colorless liquid (108 mg.), b.p. (bath temp.) 135°, 0.005 mm. Treatment with methanolic ammonia in the usual manner followed by removal of the solvent *in vacuo* left a crystalline residue which upon recrystallization from acetone–light petroleum ether yielded methyl 3,4,6-tri-*O*-methyl-D-fructofuranoside uronamide,[8] m.p. and mixed m.p. 95–96°, $[\alpha]^{24}D$ −62.5° in water (*c* 0.4); literature values[8] m.p. 100–101° and $[\alpha]D$ −76° (water).

Anal. Calcd. for $C_{10}H_{19}O_6N$: C, 48.2; H, 7.6; N, 5.6; OCH_3, 49.8. Found: C, 48.0; H, 7.7; N, 5.5; OCH_3, 46.4.

(b) **3,4,6-Tri-*O*-methyl-D-fructose.**—The tri-*O*-methyl-D-fructose component of fraction B, Table II, not oxidized by bromine was purified *via* its mono-*O*-isopropylidine derivative (see above) giving a sirup, $[\alpha]^{20}D$ +10.8° in methanol (*c* 3.9), +15.7° in chloroform (*c* 7.8). Treatment of this material (100 mg.) in 20% aqueous acetic acid (2 ml.) with phenylhydrazine (0.1 ml.) for 30 minutes at 65°, and for 1 hour on a boiling water-bath gave an orange-red oil which was washed by decantation with water whereupon it crystallized. After two recrystallizations from ethanol–water, the 3,4,6-tri-*O*-methyl-D-glucosephenylosazone[10,11] (93 mg.) had m.p. 80–82°, $[\alpha]^{28}D$ −62.5° in ethanol (*c* 0.4) after 45 minutes, changing in 23 hours to +5° (constant value); lit. m.p. 80–82°.[11]

Anal. Calcd. for $C_{21}H_{28}O_4N_4$ + 0.5 H_2O: C, 61.6; H, 7.1; N, 13.7; OCH_3, 22.7. Found: C, 61.5; H, 6.9; N, 13.3; OCH_3, 22.3.

When the 3,4,6-tri-*O*-methyl-D-fructose was treated with cyclohexanone in the presence of sulfuric acid in the usual way, a crystalline compound, believed to be the 1,2-*O*-cyclohexylidene derivative, was obtained; m.p. 101° (after recrystallization from petroleum ether (b.p. 30–60°)).

(c) **1,3,4-Tri-*O*-methyl-D-fructose.**—The component of fraction B, Table II, which was not affected by bromine and did not form an isopropylidene derivative (see above), showed $[\alpha]^{23}D$ −13° equilibrium value in methanol (*c* 3.7) and crystallized upon nucleation with 1,3,4-tri-*O*-methyl-D-fructose. A portion (47 mg.) of this fraction was dissolved in nitric acid (3 ml., d. 1.42) and heated in a water-bath for 1 hour at 65° and then for 30 minutes in a boiling water-bath. The reaction mixture was cooled, diluted with water, and freed from nitric acid as described above. The sirupy dicarboxylic acid thus formed was transformed into the corresponding methyl 3,4-di-*O*-methyl-D-fructofuranoside dicarboxylic acid dimethyl ester by two methylations with silver oxide and methyl iodide and this in turn was treated with methanolic ammonia. This provided the corresponding diamide, m.p.[9] and mixed m.p. with a synthetic specimen (see below) 191°, $[\alpha]^{24}D$ −75° in water (*c* 0.4), after recrystallization from ethanol.

Anal. Calcd. for $C_9H_{16}O_6N_2$: C, 43.6; H, 6.5; OCH_3, 42.7. Found: C, 43.7; H, 6.4; OCH_3, 39.6.

(d) **2,3,4-Tri-*O*-methyl-D-glucose.**—A portion (87 mg.) of the methylated sugar lactone formed by bromine oxidation of the hydrolyzate of methylated *ti* glucofructosan (see above) was distilled in high vacuum to give a liquid (64 mg.), $[\alpha]^{26}D$ +20° in methanol (*c* 0.4). Paper chromatographic analysis followed by the hydroxamic acid test spray[44] showed that with three separate solvent systems, phenol saturated with water (R_f = 0.9), 1-butanol:ethanol:water (4:1:5) (R_f = 0.86) and methyl ethyl ketone:water azeotrope (R_f = 0.84) the lactone corresponded to 2,3,4-tri-*O*-methyl-D-glucono-δ-lactone.

Anal. Calcd. for $C_9H_{16}O_6$: equiv. wt., 220. Found: equiv. wt., 232.

An attempt to characterize the unknown lactone as crystalline 2,3,4-tri-*O*-methyl-D-glucosaccharo-1,5-lactone-6-methyl ester by oxidation with nitric acid followed by methanolysis was unsuccessful and treatment of the lactone ester with methanolic methylamine failed to yield the characteristic crystalline bis-methylamide of 2,3,4-tri-*O*-methyl-D-glucosaccharic acid.

Preparation of 3,4-Di-*O*-methyl-D-fructose and its Conversion to the Diamide of Methyl 3,4-Di-*O*-methylfructofuranosido-1,6-dicarboxylic Acid.—Fructose (1.0 g.) was dissolved in dry pyridine (40 ml.) and trityl chloride (6.2 g.) was added. After 2 days at room temperature, acetic anhydride (25 ml.) was added. The next day the mixture was poured with stirring into ice-water (300 ml.) and the resulting flocculent precipitate was separated and triturated with water until the washings were no longer acid to congo red paper. The product, after air-drying, was a powdery solid (10.3 g.) from which attempts to obtain crystalline 1,6-di-*O*-trityl-2,3,4-tri-*O*-acetyl-D-fructose were unsuccessful.

The crude material (9.27 g.) was dissolved in acetone (150 ml.) and methylated, at room temperature, with methyl sulfate and sodium hydroxide. After three successive methylations, the product being extracted after each methylation with chloroform, the crude methylated product was isolated and treated, at room temperature, with methanol (52 ml.) containing 0.5% hydrogen chloride. The gummy material went into solution when the mixture was shaken and soon thereafter trityl methyl ether separated as a copious, crystalline precipitate, m.p. 76°. The solvent was removed from the filtrate under reduced pressure after neutralization with silver carbonate, and impurities were removed by extraction with ether from aqueous solution. Water was removed under reduced pressure and the sirupy residue was extracted with acetone. Removal of the acetone yielded sirupy methyl 3,4-di-*O*-methyl-D-fructoside (0.8 g.).

Anal. Calcd. for $C_9H_{18}O_6$: OCH_3, 41.9. Found: OCH_3, 39.0.

The methyl glycoside (106 mg.) was converted to the free sugar by treatment with 2.25% aqueous oxalic acid for 2 hours in a water-bath at 85°. Neutralization ($CaCO_3$), followed by filtration and removal of the solvent under reduced pressure, yielded a sirupy product (97 mg.) having $[\alpha]^{26}D$ −23° in methanol (*c* 3.2). The 3,4-di-*O*-methyl-D-fructose was converted, by the method described above for the preparation of 3,4,6-tri-*O*-methyl-1,2-*O*-isopropylidene-D-fructose, to 3,4-di-*O*-methyl-1,2-*O*-isopropylidene-D-fructose (117 mg.) which was a sirup having $[\alpha]^{26}D$ −10° in methanol (*c* 4).[9,10] The fact that the rotation in this solvent is negative indicates that the substance is probably in the pyranose form, and hence is 1,2-*O*-isopropylidene-3,4-di-*O*-methyl-D-fructopyranose. Paper chromatograms of this derivative showed that it would afford a means of separating 3,4-dimethylfructose, with methyl ethyl ketone as the irrigating solvent, from dimethylhexoses not forming isopropylidene derivatives. The R_f value found for the isopropylidene derivative was 0.87 as compared with the value 0.29 for the free di-*O*-methyl sugar.

The slow moving component disappeared when the mixture of methylated sugars from methylated *ti* fructosan hydrolyzate was treated with acetone and sulfuric acid, indicating that 3,4-di-*O*-methyl-D-fructose was the sole dimethyl hexose component.

Conversion of the sirupy synthetic methyl 3,4-di-*O*-methyl-D-fructofuranoside (159 mg.) to crystalline methyl 3,4-di-*O*-methyl-D-fructofuranoside-1,6-dicarboxylic acid diamide by oxidation with nitric acid, methylation with methyl iodide and silver oxide, and treatment of the resulting sirupy dimethyl ester in methanolic solution with gaseous ammonia, as previously described, yielded a crude product (55 mg.) which, upon recrystallization from ethanol, had m.p. 192–193° and $[\alpha]^{22}D$ −68° in water (*c* 0.6). There was no depression of the m.p. when mixed with the compound prepared in the same manner from the di-*O*-methyl-D-fructose component from the methylated *ti* glucofructan.

Anal. Calcd. for $C_9H_{16}O_6N_2$: C, 43.55; H, 6.45; N, 11.3. Found: C, 43.3; H, 6.4; N, 11.5.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY AND NUTRITION, COLLEGE OF AGRICULTURE, UNIVERSITY OF NEBRASKA]

The Preparation of 3-O-α-D-Glucopyranosyl-D-glucose[1]

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3-O-α-D-glucopyranosyl-D-glucose has been isolated from a reversion mixture obtained by heating glucose and maltose in acid solution. The specific rotation of the oligosaccharide is +87°. The crystalline phenylosazone of the compound yields an X-ray diffraction pattern identical with that of the phenylosazone of turanose, establishing the presence of an α-1,3-glucosidic bond in the new oligosaccharide.

Studies on the substrate specificity and on the mode of action of the carbohydrases have been greatly facilitated with the increased availability of pure oligosaccharides. These oligosaccharides have, for the most part, been obtained from either enzymatic digests of appropriate substrates with transglycosidases[2] or from partial acid hydrolysates of polysaccharides.[3] A number of glucosyl oligosaccharides[4,5] also have been isolated from the reversion mixture obtained by heating glucose in acid solution.[6] In the present paper, we describe methods for the isolation and characterization of a new oligosaccharide from a reversion mixture of

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