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A New Tetrasaccharide of the Stachyose Type Extracted from Seeds of *Festuca rubra* L.

SVEIN MORGENLIE

Department of Chemistry, Agricultural College, N-1432 Vollebekk, Norway

A tetrasaccharide containing fructose, glucose, and galactose in the molar ratios 1:1:2 has been isolated from the seeds of *Festuca rubra* L. The results of partial hydrolysis, periodate oxidation, and methylation studies of the tetrasaccharide are all in agreement with the formula *O*-α-D-galactopyranosyl-(1→4)-*O*-α-D-galactopyranosyl-(1→3)-*O*-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside. The presence of raffinose has been confirmed, and stachyose has also been found in fresh seeds.

The isolation of a new trisaccharide of the raffinose family from seeds of *Lolium* and *Festuca* species (Gramineae) was reported by MacLeod and McCorquodale [1] in 1958. In this galactosyl-sucrose the galactopyranosyl group was linked to the glucose moiety of sucrose through an α-(1→3) linkage in contrast to the (1→6) linkage in raffinose. The electrophoretic mobility of the reducing disaccharide obtained by removal of the fructofuranosyl group by partial hydrolysis of the trisaccharide was an important argument for the proposed structure, and the formula of the new trisaccharide was later confirmed by Sömme and Wickström.[2] They found that galactose was liberated from the trisaccharide when it was treated with α-galactosidase, and that periodate oxidation left the glucose unit intact while fructose and galactose were destroyed. In the following the trisaccharide is designated galactosyl-3G-sucrose.

MacLeod and McCorquodale detected chromatographically in the seeds of *Festuca rubra* L. and *Festuca ovina* L. an oligosaccharide which had a lower mobility by paper chromatography than galactosyl-3G-sucrose and raffinose, but which was different from stachyose. They presumed the oligosaccharide was a tetrasaccharide, possibly a homologue of the trisaccharide in analogy with stachyose being a homologue of raffinose.

In the sucrose-raffinose-stachyose series, generally (gal)$_n$-glc-fru, the values of $\log[R_F/(1-R_F)]$ (paper chromatography) fall on a straight line when they are plotted against n,[3] as shown in Fig. 1. We have found that no such relationship exists when going from sucrose through galactosyl-3G-sucrose

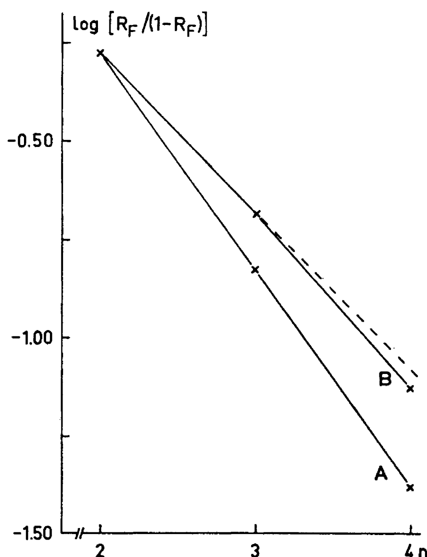
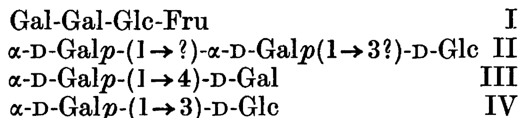


Fig. 1. Relation between $\log[R_F/(1-R_F)]$ and the number of monosaccharide units n. Curve A: Sucrose, raffinose, stachyose. Curve B: Sucrose, galactosyl-3G-sucrose, tetrasaccharide.

to the oligosaccharide of *Festuca rubra* (Fig. 1), indicating that the oligosaccharide is not a regularly (1 – 3) built galactosyl homologue of the trisaccharide. In our opinion it was of interest to isolate the oligosaccharide from *Festuca rubra* for investigation of its composition. The low occurrence and the elaborate isolation process of the oligosaccharide made a characterisation by means of crystalline cleavage products and derivatives impossible, and the investigations of its constitution had to be based on electrophoresis and chromatographic methods.

The oligosaccharide, which was isolated by chromatography on ion-exchange resin and thick filter paper, was non-reducing. Total hydrolysis yielded fructose, glucose and galactose in the ratios 1.2:1:1.9, indicating that the oligosaccharide is a tetrasaccharide, and this is supported by its paper-chromatographic mobility. α-Galactosidase treatment yielded galactose and two oligosaccharides indistinguishable by paper chromatography from galactosyl-3G-sucrose and sucrose, while β-galactosidase left the tetrasaccharide unattacked. When exposed to invertase, the tetrasaccharide split off fructose, giving a reducing oligosaccharide with slightly greater paper chromatographic mobility than the tetrasaccharide. Fructose and this new oligosaccharide, which obviously is a trisaccharide, were also formed by mild hydrolysis. These results are in agreement with the monosaccharide sequence I of the tetrasaccharide. The galactose units must be present in pyranose form since the reducing trisaccharide is resistant to mild hydrolysis.


Gal-Gal-Glc-Fru I
α-D-Gal*p*-(1→?)-α-D-Gal*p*(1→3?)-D-Glc II
α-D-Gal*p*-(1→4)-D-Gal III
α-D-Gal*p*-(1→3)-D-Glc IV

To throw further light upon the structure of the tetrasaccharide, partial acid hydrolysis was applied. On paper chromatographic separation of the products, three oligosaccharide fractions were obtained. Fraction 1, which had the lowest mobility, contained an oligosaccharide chromatographically identical with the reducing trisaccharide mentioned above. Total hydrolysis gave galactose and glucose, visually most of the former. The electrophoretic mobility (M_G 0.70 in borate) indicates a (1→3) or a (1→6) linkage to the reducing end-unit.[4] Borohydride reduction followed by total hydrolysis afforded glucitol and galactose. After partial hydrolysis of borohydride reduced trisaccharide, galactose and an oligosaccharide were the only reducing sugars detected. These results are in accordance with the incomplete formula II of the trisaccharide.

Fraction 2 contained an oligosaccharide inseparable by chromatography and electrophoresis from the oligosaccharide obtained by reduction and partial hydrolysis of the trisaccharide, mentioned above. To this oligosaccharide the formula III was attributed on the basis of the following results: On total hydrolysis only galactose could be detected, and the compound must be a disaccharide; the electrophoretic mobility in borate buffer (M_G 0.48) indicates a (1→2) or a (1→4) linkage.[4] The fact that the triphenyl-tetrazolium chloride reagent gives a red colour with III during conditions which do not give colour with a (1→2) linked disaccharide,[5] and that phenyl hydrazine reacts with III to give a compound, detected by thin layer chromatography, which appears to be a disaccharide osazone, while no monosaccharide osazone could be recognized, makes the possibility of a (1→2) linkage unlikely.[6] Further, the diphenyl amine-aniline-phosphoric acid reagent gives a blue colour with the sugar, and this is reported to be specific for (1→4) linkages.[7] The paper chromatographic mobility is in addition close to that reported for the α-(1→4) linked D-galactopyranosyl-D-galactose in a given solvent.[8] This mobility excludes the possibility of (1→6) linkage (by comparison with the authentic α-(1→6) linked disaccharide), and it makes a (1→3) linkage unlikely, since the α-(1→3) linked disaccharide is reported to have a mobility in the same solvent clearly higher than that observed for III.[8] When aldobionic acids are oxidised with a very dilute periodate solution, glyoxylic acid is reported to result when the linkage is (1→4), (1→5), or (1→6), while (1→2) and (1→3) linked aldobionic acids fail to give a positive glyoxylic acid test.[9] When bromine-oxidised trisaccharide II and disaccharide III were subjected to dilute periodate treatment, the colour test for glyoxylic acid [9,10] was definitely positive for III, but also II gave, though not immediately, a faint colour. The positive result of the colour test is not considered a major argument in favor of a (1→4) linkage in III, since the test is not specific for glyoxylic acid; formaldehyde gives a similar colour, although possibly only when present in somewhat higher concentrations.

Fraction 3, the fastest moving, contained an oligosaccharide (IV) which on hydrolysis yielded galactose and glucose. The compound was paper chromatographically and electrophoretically indistinguishable from authentic 3-*O*-α-D-galactopyranosyl-D-glucose (M_G 0.80 in borate). This great electrophoretic mobility is characteristic for (1→3) and (1→6) linkages,[4] and since IV by chromatographic comparison was shown to be different from authentic

melibiose, it seems reasonable that the compound is 3-*O*-α-D-galactopyranosyl-D-glucose.

The results emerging from the examination of fractions 1, 2, and 3, and of the cleavage products obtained by enzymic and mild hydrolysis, indicate that the tetrasaccharide has the formula V (Fig. 2).

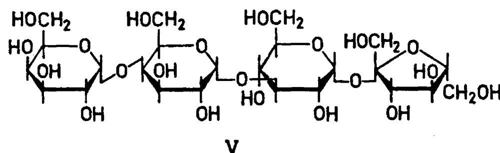


Fig. 2. The proposed structure of the tetrasaccharide.

The supposition that the glucose moiety of the tetrasaccharide is 3-*O*-substituted was strongly supported by the fact that glucose was the only monosaccharide detected after periodate oxidation of the tetrasaccharide followed by hydrolysis. The monosaccharide *O*-methyl ethers obtained by permethylation followed by hydrolysis of the tetrasaccharide are indistinguishable from authentic specimens of the expected *O*-methyl ethers, based on structure V, by thin layer chromatography. None of the methyl ethers migrated during electrophoresis in borate buffer, a fact which excludes the possibility of any 2-*O*-substitution in the galactose and glucose units of the tetrasaccharide, since *O*-methyl ethers of galactose and glucose without a 2-substitution should have migrated by electrophoresis in this electrolyte.

Seeds of *Festuca rubra* which had not been stored too long, contained besides galactosyl-3G-sucrose and the new tetrasaccharide also raffinose and stachyose. MacLeod and McCorquodale [1] reported the presence of raffinose, based only on paper chromatography, but they did not detect stachyose. We found that after storage of one sample of seeds for about one year at room temperature, stachyose had disappeared, and the amount of raffinose had been considerably reduced.

Stachyose and raffinose were isolated on thick filter paper and characterised by means of partial and complete hydrolysis by acid and enzymes. The resulting sugars were identified by chromatography and electrophoresis, and all the compounds were indistinguishable from authentic samples of the expected saccharides.

All the experimental results in the present work are consistent with the formula V of the tetrasaccharide from *Festuca rubra*. We therefore propose the structure of the tetrasaccharide to be *O*-α-D-galactopyranosyl-(1→4)-*O*-α-D-galactopyranosyl-(1→3)-*O*-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside.The galactosyl-sucroses of the raffinose family, among which raffinose and stachyose are present in seeds of *Festuca rubra*, are supposed to be formed from sucrose by successive additions of galactopyranosyl groups from an appropriate donor;[11] the galactosyl groups are then always entering a 6-position. The new tetrasaccharide exhibits unexpected structural features in view of the coexistence in the seeds of raffinose, stachyose, and galactosyl-3G-sucrose.

Provided its biosynthesis implies prolongation of the galactosyl-3G-sucrose molecule by a second galactopyranosyl group, this group does not repeat the (1→3) linkage present in the trisaccharide, nor does it enter the 6-position characteristic of the raffinose family of homologous galactosyl-sucroses, which might have explained the lack of linear relationship of the upper curve of Fig. 1. This work has shown that the explanation is to be found in the formation of a (1→4) linkage between the two galactosyl residues of the tetrasaccharide.

EXPERIMENTAL

Paper chromatograms were run on Whatman No. 1 and 3 MM papers by the descending method in the solvent system (v/v): (A) butanol-pyridine-water, 6:4:3. R_{Gal} values refer to rates of movement relative to galactose in this solvent. Thin layer chromatography was carried out on 0.25 mm layers of Silica gel G with the solvent systems (v/v): (B) propanol-nitromethane-water, 5:3:2, (C) chloroform-acetone-ethanol-water, 46:31:20:3, (D) benzene-ethanol, 20:3, (E) butanone saturated with water containing ammonia (10 %), (F) acetone-benzene, 1:1. Electrophoresis was performed on Munktell's paper for chromatography, No. 302, in 0.1 M borate buffer, pH 10. M_G values refer to rates of mobility relative to glucose. Localisation of the spots was attained with aniline oxalate, diphenyl amine-aniline-phosphoric acid, *p*-anisidine hydrochloride, and aniline hydrogen phthalate reagents for the sugars and with periodate-starch reagent for the sugar alcohols.

1. Extraction of oligosaccharides and isolation of the new tetrasaccharide

Milled seeds (250 g) of *Festuca rubra* (commercial Danish material purchased from Norsk Frö A/S, Oslo) in 1000 ml ethanol (75 %) were heated on a water bath for 30 min. After cooling and filtration the solution was concentrated to 200 ml and 220 ml of a saturated solution of basic lead acetate were added. The solution was set aside for 20 h, then centrifuged, the centrifugate was saturated with hydrogen sulphide for several hours. The filtered solution was evaporated under reduced pressure and the residue dried in a vacuum desiccator, yielding about 5 g of carbohydrate mixture.

The sugar mixture was added in 1 g portions to a column of Dowex 50 W X 4 200—400 mesh (K^+ form) ion exchange resin (120 × 4 cm) and eluted with water. The carbohydrate-containing fractions were localised with phenol-sulphuric acid and the sugars in the different fractions identified by thin layer chromatography in solvent B. The tetrasaccharide fraction was subjected to repeated chromatography on thick filter paper. Each 1 g portion yielded by this procedure about 3 mg of chromatographically pure tetrasaccharide (R_{Gal} 0.20). In some investigations tetrasaccharide obtained directly from column chromatography was used.

2. Characterisation of the tetrasaccharide by hydrolysis

Total hydrolysis. A part of the tetraaccharide was hydrolysed in 1 N sulphuric acid at 100° for 3 h. The resulting monosaccharides were separated on thick filter paper and identified by their chromatographic mobilities and electrophoresis. To determine the molecular ratios between the monosaccharides, the tetrasaccharide was hydrolysed as above, and a modification of the paper chromatographic-colorimetric method described by Wilson [12] was applied. Stachyose was used as a standard. and was hydrolysed under exactly the same conditions as the tetrasaccharide to correct for the destruction of fructose during hydrolysis.

Enzyme hydrolysis. Small amounts of the tetrasaccharide were incubated for 2 h at 37° with α-galactosidase from coffee seeds in 0.1 M acetate buffer of pH 4.8. The enzyme

was destroyed by heating at 100° for a few minutes and removed by centrifugation. β-Galactosidase treatment of the tetrasaccharide was performed under the same conditions. Invertase concentrate BDH (2 drops) was added to 0.2 ml of a tetrasaccharide solution (0.2 %). After 15 min at 35°, the mixture was heated at 100° for 2 min. Paper chromatography was used to detect the sugars formed in the three enzyme experiments.

Mild acid hydrolysis. An oxalic acid solution (1 %) containing small amounts of tetrasaccharide was heated at 100° for 1 h. After removal of oxalic acid with calcium carbonate, the solution was concentrated and the sugars were detected by paper chromatography.

Partial acid hydrolysis. The tetrasaccharide (5 mg) was hydrolysed in 0.3 N sulphuric acid (10 ml) at 100° for 50 min. After neutralisation with barium carbonate and deionisation with Amberlite resin IR-120(H) the solution was concentrated and the sugars separated on thick filter paper.

3. Examination of the products of partial hydrolysis

Fraction 1. The sugar (II), which had R_{Gal} 0.32 and M_G 0.70, was subjected to total hydrolysis as described for the tetrasaccharide; the monosaccharides were detected by paper chromatography. The products obtained by reduction with sodium borohydride followed by partial and total hydrolysis were identified by paper chromatography and electrophoresis; glucitol had M_G 0.83 and was indistinguishable from an authentic sample.

Fraction 2. The sugar (R_{Gal} 0.49, M_G 0.48) was chromatographically indistinguishable from the oligosaccharide (III) obtained by partial hydrolysis of reduced II. Total hydrolysis gave only galactose. A blue colour was obtained when the sugar was sprayed with diphenyl amine-aniline-phosphoric acid reagent.[7] This reagent gave a yellow colour with a (1→2) linked disaccharide (2-*O*-α-D-galactopyranosyl-D-glucose) and a gray-green colour with the (1→3) linked and the (1→6) linked α-D-galactopyranosyl-D-glucoses. After spraying with the triphenyl tetrazolium chloride reagent [5] and heating in a moist chamber to 45°, the sugar developed a red colour. 2-*O*-α-D-galactopyranosyl-D-glucose gave no colour with this reagent under the same conditions.

Phenyl hydrazine (17 mg) was added to a solution of the sugar in water (0.1 ml) and acetic acid (0.13 ml). The mixture was heated at 90° for 2 h, then it was cooled to 2–3° for several hours and centrifuged. The precipitate was dissolved in methanol, and thin layer chromatography (solvent C) showed a compound with an R_F value near that of lactose osazone; no galactose osazone could be seen.

Bromine-oxidation followed by periodate oxidation of fractions 1 and 2. Approximately equal amounts of the sugar of fractions 1 and 2 were each dissolved in 5 ml water, and to both solutions were added saturated bromine water (0.2 ml) and barium carbonate. The mixtures were shaken in the dark for 5 h, filtered and treated with Amberlite resin IR-120(H) and IR-4B(OH); the solutions were then concentrated to about one third of the volume. Periodate oxidation of the bromine oxidation products was subsequently carried out in a 0.4 mM periodate solution (25 ml). After 2 h, barium acetate (0.1 ml of a 0.5 M solution) was added, the solutions were concentrated to about 3 ml, centrifuged, de-ionised with the Amberlite resins and concentrated further to 1 ml. The glyoxylic acid test [10] was applied to two drops of each of the two solutions.

Fraction 3. The sugar (IV) which had R_{Gal} 0.72 and M_G 0.80, was identified as 3-*O*-α-D-galactopyranosyl-D-glucose on the evidence reported above.

4. Oxidation of the tetrasaccharide by periodate

The oxidation was carried out as described by Sömme and Wickström,[3] and the product detected paper chromatographically.

5. Methylation studies

The tetrasaccharide (3–4 mg) was dissolved in dimethyl sulfoxide (2 ml) and sodium hydride (20 mg) was added. After 5 min methyl iodide (1 ml) was added under cooling, and the reaction mixture was shaken for about 2 h at room temperature. The addition

of sodium hydride and methyl iodide was repeated, and after another two hours' shaking, the reaction mixture was poured into 4 ml of water and extracted successively with chloroform (5 ml portions). The chloroform extracts were washed several times with small volumes of water, and the solvent was evaporated at diminished pressure. By preparative thin layer chromatography (solvent D) the permethylated product was separated from small amounts of undermethylated tetrasaccharide and then hydrolysed with 1 N sulphuric acid for 4 h at 100°. The resulting *O*-methyl ethers were identical with 1,3,4,6-tetra-*O*-methyl fructose, 2,3,4,6-tetra-*O*-methyl-galactose, 2,4,6 tri-*O*-methyl-glucose and 2,3,6-tri-*O*-methyl-galactose by thin layer chromatography in the solvents D, E, and F. The two last methyl ethers were only completely separated in solvent D after two developments. The colours obtained with the spray reagents were also identical with those given by authentic samples of the methyl ethers. All the methyl ethers were non-migrating when subjected to paper electrophoresis.

6. Isolation and characterisation of raffinose and stachyose

Raffinose and stachyose were isolated by chromatography on thick filter paper from extracts of new seeds of *Festuca rubra*. The two sugars were obtained chromatographically pure, indistinguishable from authentic raffinose and stachyose.

a) *Stachyose*. Total hydrolysis (1 N sulphuric acid, 4 h, 100°) gave galactose, glucose, and fructose. Hydrolysis with oxalic acid (1 %) at 90° for 1 h yielded fructose and manninotriose, while partial hydrolysis (0.3 N sulphuric acid, 50 min, 100°) gave in addition to fructose, glucose, and galactose, three oligosaccharides, manninotriose, swietenose (6-*O*-α-D-galactopyranosyl-D-galactose) and melibiose. The sugars formed after total and partial hydrolysis were separated by chromatography on thick filter paper and identified by paper chromatography and electrophoresis. Treatment with α-galactosidase and invertase (conditions described above) gave galactose, raffinose, and sucrose with the former, and fructose and manninotriose with the latter enzyme; the products were chromatographically identified.

b) *Raffinose*. Exactly the same treatments as described for stachyose were applied; the compounds formed were indistinguishable by chromatography and electrophoresis from authentic specimens of the expected sugars.

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