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# Oligo- and Polyfructosides from the Bulbs of two *Leucojum* Species

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The structures of the tetrasaccharides of *Leucojum vernum* L. and *Leucojum aestivum* L. correspond to the products which would be formed by transfructosylation to position 1 of a terminal fructofuranosyl group of each of the two trisaccharides found in these plants: β-D-fructofuranosyl-(2-1)-*O*-β-D-fructofuranosyl-(2-1)-α-D-glucopyranoside ($1^F$-kestose) and *O*-β-D-fructofuranosyl-(2-6)-*O*-α-D-glucopyranosyl (1-2)-β-D-fructofuranoside ($6^G$-kestose). The tetrasaccharide containing the $1^F$-kestose structure and one or both of the tetrasaccharides comprising the $6^G$-kestose structure are present.

The polyfructans of the same plants are also of the 2–1 linked type. The glucose found as a component of the fructans (1.33 % *L. vernum*, 1.76 % *L. aestivum*) occurs as terminal glucopyranosyl groups giving tetramethyl-D-glucopyranose after methylation and subsequent hydrolysis. The ratio between tetramethyl-D-fructofuranose and tetramethyl-D-glucopyranose was determined by gas-liquid chromatography and found to be 9:1, a result which probably indicates a certain degree of branching of the fructans. Periodate oxydation leaving no fructofuranose residue unattacked, indicates that branching point linkages are engaging positions 1 and 6 of the fructose unit.

It has been known for some time that subterranean parts of plants belonging to Amaryllidaceae and Liliaceae have a tendency to replace starch as reserve carbohydrate by fructans, and various botanical aspects of this fructan accumulation have received attention from several authors (reviewed by Hegnauer [1]). With a few exceptions little chemical research has been carried out to elucidate the structure of the fructans of these plant families. So few investigations have been made that it is not possible today to make a general statement as to which type(s) of fructan the Amaryllidaceae and the Liliaceae tend to develop; the 2–1 linked (inulin) type, the 2–6 linked (phlein) type, or the branched types comprising both kind of linkages.

This is in contrast to our knowledge of another monocotyledon family, the Gramineae, where the fructans and the simultaneously occurring oligofructosides have been exhaustively examined in several species (reviewed by Schlubach [2–4]).

It is hoped that the fructans of Amaryllidaceae and Liliaceae will now receive more attention, in particular because it has been shown by Bacon [2] and Hammer [6,7] that the oligofructosides of some of these plants are mixtures of saccharides of different structures. The knowledge of the structural relationship between oligo- and polyfructosides of the same plant species is a necessary condition for approaching the problems of the biosynthesis and the biological break-down of both oligo- and polyfructosides. Although the two plant families in question could not compete with the grasses with respect to economical and agricultural importance, it seems likely that progress in the knowledge of the fructosides of Amaryllidaceae and Liliaceae might be useful to agricultural science, *e.g.* to work concerning storage, germination, and frost resistance of the bulbs.

The present paper reports an investigation of the tetrasaccharide fractions and the fructans of *Leucojum vernum* L. and *Leucojum aestivum* L. The trisaccharide fractions of these two plants belonging to the Amaryllidaceae have been examined previously.[6] The preparation of the fructans from the bulbs was complicated by the fact that they also contained polysaccharides of a glucomannan type (and a little starch).

The occurrence of a diversity of water-soluble polysaccharides in the bulbs of these groups of plants has been reported several times in the literature, recently [8] by Mizuno and Hayashi who found in *Lycoris radiata* (bulbs) a fructan, a glucomannan, and some starch.

## THE TETRASACCHARIDES

The oligosaccharides were extracted from the bulbs by methanol and the tetrasaccharide fraction was isolated by chromatography on thick paper.[6]

Transfructosylation to one of the primary alcohol functions of sucrose gives three isomeric fructosyl sucroses known as $1^F$-kestose, $6^F$-kestose, and $6^F$-kestose. The trisaccharide fractions of the two *Leucojum* species consisted of two of these trisaccharides,[6] $1^F$-kestose, and $6^G$-kestose.

If the transfructosylation to primary alcohol groups is supposed to continue with the three kestoses as substrates, a series of nine isomeric tetrasaccharides should be formed hypothetically. A naturally occurring mixture of isomeric tetrasaccharides is likely to be still more difficult to separate chromatographically than the kestoses have proved to be. Only three of the hypothetical tetrasaccharides of the series appear to have been characterized in literature known to the author. A glucose-terminated tetrasaccharide with all fructofuranose residues linked 2 – 1 (formula (I) Fig. 1) has been described by several authors (review [9]) and has been named nystose.[10] The tetrasaccharide having all fructofuranose residues linked 2 – 6 has also been indicated.[9,2] A branched tetrasaccharide of the series is also known, corresponding to the structure which would result from a transfructosylation to position 6 of the central fructofuranose residue in $1^F$-kestose. It has been named bifurcose by Schlubach who detected this sugar in rye [11] and oats.[12]

Schlubach has also found a branched tetrasaccharide of a somewhat different series; it consists of a central glucopyranose residue to which all three fructofuranosyl groups are linked.[12]

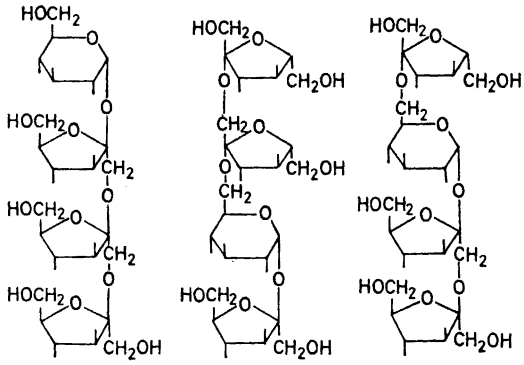


*Fig. 1.* Tetrasaccharides of the kestose series. (I), Nystose, a derivative of $1^F$-kestose; (II), a derivative of $6^G$-kestose; (III) can be derived from both the $1^F$- and $6^G$-kestoses.

Methylation analysis of the tetrasaccharide fractions of the two *Leucojum* species examined gave identical results. In both plants the tetrasaccharide (I) of Fig. 1 must be present, giving rise to tetramethylfructofuranose, tetramethylglucopyranose, and 3,4,6-tri-methylfructose. The characterization of a fourth methylated hexose as 2,3,4-trimethylglucose indicates that the tetrasaccharide fraction also contains some non-terminal glucose residues. Only tetrasaccharides corresponding to formula (II) or (III) of Fig. 1 are able to yield the same methylated fructose derivatives as formula (I), and in addition 2,3,4-trimethylglucose.

The tetrasaccharide fractions examined therefore consist of the sugar of formula (I) (nystose) in admixture with one or both of the sugars (II) and (III). It is seen from Fig. 1 that these tetrasaccharides represent homologues of the two kestoses which we have found in the same species,[7] (I) being a fructofuranosyl-$1^F$-kestose, and (II) as well as (III) being a fructofuranosyl-$6^G$-kestose. The tetrasaccharide (I) is well known from previous works.[10,13] The two isomers (II) and (III) have apparently not been found previously.

Partial enzymatic and acid hydrolysis of the tetrasaccharide fractions gave results which tend to support the above conclusion as to the composition of the tetrasaccharide fractions of the *Leucojum* species examined. The action of invertase, under conditions which minimize the possibility of synthesis of kestoses by transfructosylation to sucrose, liberated fructose from the tetrasaccharides and yielded trisaccharides which were chromatographically indistinguishable from $1^F$-kestose and $6^G$-kestose. $6^F$-kestose could not be detected. Mild acid hydrolysis during a short period gave similar results.

If one presumes that terminal, unsubstituted fructofuranosyl groups of the tetrasaccharides are those most easily split off during enzymatic or acid hydrolysis, the results of the partial hydrolysis are consistent with the conclusions of the methylation analysis as to the composition of the tetrasaccharide fractions. The tetrasaccharide of formula (I) loses a fructofuranosyl group to

yield $1^F$-kestose. The tetrasaccharide (III) is by removal of one of the terminal fructofuranosyl groups transformed to either $1^F$-kestose or $6^G$-kestose. Compound (II) is by loss of the terminal (upper) group in Fig. 1, degraded to $6^G$-kestose, or by attack from the lower side transformed to a reducing, glucose-terminated trisaccharide which, however, was not observed during replicate analysis.

## THE FRUCTANS

The polysaccharide fractions of both *Leucojum* species were submitted to the same analytical methods and their properties thus revealed are almost identical. Their descriptions, therefore, are simplified by the inclusion in one, mainly a singular form description.

The polysaccharides of the bulbs were extracted with water from the dried methanol-extracted material. The material contained some starch which was, however, not extracted by the treatment with cold water. Extraction with water at room temperature gave a viscous solution from which the crude polyfructan could be precipitated by ethanol. The crude fructan contained a comparatively large proportion of other polysaccharide material, presumably a glucomannan. This was removed as its copper complex, and then a purified fructan was obtained by precipitation with ethanol.

The fructan gave by hydrolysis 1.76 % (*L. aestivum*) and 1.33 % (*L. vernum*) of glucose together with 90 % of fructose. The polysaccharide is non reducing. Provided that the polysaccharide has been formed by transfructosylation to sucrose, and does not contain any glucose-free fructan molecules, the analysis of the component monosaccharides would correspond to an average degree of polymerisation of 51 (*L. aestivum*) or 68 (*L. vernum*).

By methylation analysis of the polysaccharide all the glucose is found as tetramethyl-glucopyranose, no trimethyl-glucose being detected. This result indicates that the glucosyl groups are all in terminal position. The predominating product of the methylation procedure was 3,4,6-trimethyl-D-fructose, which by the thin layer chromatography technique used is clearly separated from the 1,3,4-isomer. A certain amount of dimethyl-fructose was observed; it was not further characterised. Tetramethyl-D-fructofuranose was undoubtedly present.

The tetra-methylated fraction, including tetramethyl-glucose, was submitted to a quantitative gas chromatography analysis. The sugars were converted to the corresponding sugar alcohols, which were trisilylated and then submitted to gas chromatography. The ratio between tetramethylfructose and tetramethylglucose was determined as 9:1 for both plants.

If the proportion of tetramethyl-sugars had been 1:1, this would have been a strong indication of unbranched sucrose-terminated polyfructan chains. The proportion found does not, however, unambiguously prove that the polysaccharide has a branched structure. The higher proportion of tetramethylfructose might arise from possible contamination with glucose-free fructan chains like those described by Schlubach,[2-4] or it might be caused by degradation during methylation creating a certain number of shorter fructan chains.

The fully methylated polysaccharide moved as a single spot during thin-layer chromatography. Although this is no conclusive proof of homogeneity,

the author is inclined to accept that the high yield of tetramethylfructose does reflect at least some degree of branching in the natural fructan.

By oxidation of the fructans with periodate they reduced 1.0 mole (*L. aestivum*) and 1.1 mole (*L. vernum*) per fructofuranose unit. The resulting polyaldehyde solutions were treated with borohydride and then hydrolyzed. No intact fructose appeared to be present in the hydrolysates. The periodate oxidations of the fructans indicate that any branching of the fructofuranosyl chains does not engage position 3 or 4 of the sugar residues. If side chains are linked to position 6, periodate oxidation should give results corresponding to our observations.

## COMPARISON OF THE OLIGOMERS AND POLYMERS OF FRUCTOSE

The main feature of the polyfructan from *L. aestivum* and *L. vernum* is that the majority of the fructose units are linked 2 – 1 and that all the glucose present is found in terminal position. Through these features the polysaccharide is structurally related to one of the trisaccharides, $1^F$-kestose, and to one of the tetrasaccharides, nystose, (I) of Fig. 1, occurring in the bulbs of the same plants. The formation of the polysaccharides may be interpreted as a transfructosylation starting with the lower homologous fructosylsucroses, $1^F$-kestose and nystose. The other tri- and tetrasaccharides found in the *Leucojum* bulbs do not possess a terminal glucose unit, and they are obviously not suitable starting points for the biosynthesis by transfructosylation of the fructan structures found in the same plants. The structural difference between the polyfructan and the oligo-saccharides of the $6^G$-kestose type (like (II) and (III) of Fig. 1), probably also synthesised by transfructosylation, indicates specific variations within the group of enzymes responsible for transfructosylation reactions in the bulbs of the two *Leucojum* species examined.

## EXPERIMENTAL

All evaporations were performed under reduced pressure at temperatures below 40°. Paper chromatography separations were performed on Whatman papers No. 1 and No. 3 MM. For thin-layer chromatography (TLC) plates covered with Kieselgel G (0.25 mm) were used. The solvent systems (v/v) were: (A) ethyl acetate-acetic acid-water 3:1:3. (B) benzene-ethanol 20:3. (C) 2-butanone-10 % $NH_3$ 25:1. (D) propanol-nitromethane-water 5:4:1. Spots were revealed by aniline oxalate or urea oxalate.

1. *Extraction* of the bulbs with methanol and separation of the oligofructosides present in the extract are described in a previous paper.[6] The extracted material was dried in an air stream and finally dried at 40°. From this material the polysaccharides were obtained by treatment with water (*cf.* 4).

*The tetrasaccharide fractions* gave, by complete hydrolysis, fructose and glucose in the molecular proportion 2.4:1.0 (calc. 3.0:1.0) as determined by quantitative paper chromatography.[14] The tetrasaccharide fraction of *Leucojum vernum* had $[\alpha]_D = +10.4°$ (*c* 3.8, water) and the corresponding fraction from *L. aestivum* $[\alpha]_D = +4.4°$ (*c* 10, water).

2. *Methylation analysis of the tetrasaccharide fractions.* The tetrasaccharides (5 – 10 mg) were dissolved in dimethyl sulphoxide (0.5 – 1.0 ml) and methylated according to the method described by Anderson and Cree.[15] The process was monitored by TLC (solvent B). The permethylated product was hydrolysed in 1 % oxalic acid (1 ml, 90 – 100°, reflux, 45 min). After neutralisation with calcium carbonate, the mixture was centrifuged and evaporated to dryness. TLC of the residue (solvent B or C) gave spots

indistinguishable from authentic specimens of 1,3,4,6-tetra-*O*-methylfructose, 3,4,6-tri-*O*-methylfructose (estimated ratio tetra/tri 1:1), 2,3,4,6-tetra-*O*-methylglucose and 2,3,4-tri-*O*-methylglucose (estimated ratio tetra/tri 1:1). Very small amounts of dimethyl derivatives were considered insignificant.

3. *Partial hydrolysis of the tetrasaccharides.* a) *Enzymatic.* The saccharides (5—10 mg) were dissolved in 0.1 M acetate buffer (1 ml, pH 5) and mixed with the enzyme solution (1 ml, concentration 5 drops of invertase concentrate in 3 ml buffer). The mixture was left at room temperature for 5—6 min. The enzyme action was then stopped by the addition of barium- and zinc-solutions.[16] After centrifugation the solution was filtered through a carbon-celite column (3 × 0.5 cm). The column was washed with water (50 ml) and then with 20 % ethanol (50 ml). The ethanolic eluate was evaporated to dryness. TLC (solvent D) of the residue dissolved in methanol revealed a predominating spot in the position of $6^G$-kestose and a spot in $1^F$-kestose position. No compound corresponding to $6^F$-kestose was detected. b) *Acid.* A few milligrams of the tetrasaccharides were heated with 0.1 % oxalic acid (0.5 ml, 90—100°, 5—10 min). TLC (solvent D) of the hydrolysate confirmed the above results.

4. *Extraction of the polysaccharides.* If the finely chopped, dried material (from 1) is treated with hot water (about 80°) the aqueous solution will contain some starch. The filtrate gave an intense blue colour with iodine, and the solution lost its ability to produce this colour after contact with α-amylase. Treatment with water at room temperature extracted only negligible amounts of starch.

The finely chopped material (50 g) was treated with water (500 ml) and a little toluene at room temperature (1 h). The solution was filtered to remove tissue fragments and the filtrate was treated with Fehling's solution (23 ml to 100 ml of the filtrate). The precipitate was allowed to settle over night and was then removed by centrifugation. The precipitate proved to be the copper complex of polysaccharides yielding mannose, galactose, glucose, and arabinose by complete hydrolysis. The polysaccharide was obtained after removal of copper, as a crisp powder insoluble in water. It was not further examined.

The supernatant was cooled with crushed ice and carefully adjusted to pH about 6 by N HCl. Most of the copper ions were removed by $H_2S$, and the filtrate was dialysed against running tap water for 3 days. The solution was then concentrated under reduced pressure to a volume less than 50 ml, and 4 times its volume of ethanol was added. The precipitate was removed by centrifugation and then washed repeatedly in 80 % ethanol. The product (250 mg from 50 g of dried bulbs) after drying *in vacuo* appeared as a yellow-white crisp solid, not completely soluble in water. Mild acid hydrolysis (1 h at 80°, 1 % oxalic acid) liberated fructose and a small amount of glucose. A more vigorous hydrolysis did not yield mannose. The amount of fructose in the hydrolysate was determined by the colour reaction with thiobarbituric acid [17] and found to be 90.2 %. Glucose was determined by Glucotest, Boehringer, to 1.76 % in the product from *L. aestivum* and to 1.33 % in the product from *L. vernum*. The hydrolysates exhibited an optical rotation of −68° and −64°, respectively, calculated as specific rotation on the basis of the weight of polysaccharides submitted to hydrolysis.

5. *Methylation analysis of the polysaccharides.* The polysaccharide methylation followed the procedure referred to above. The permethylated products moved as slightly elongated spots when subjected to TLC (solvent B, $R_F$ about 0.40). After boiling in 1 % oxalic acid in methanol (1 h) and subsequent evaporation to dryness, hydrolysis of the resulting methyl products was effected by heating with 1 % oxalic acid (90—100°, reflux, 10 h). The hydrolysates were neutralised (calcium carbonate) centrifuged and evaporated to dryness. TLC of the chloroform solutions gave spots having the mobilities of 1,3,4,6-tetra-*O*-methylfructose, 2,3,4,6-tetra-*O*-methylglucose, 3,4,6-tri-*O*-methylfructose and an unidentified spot in the dimethyl region. The spot intensities indicated large amounts of trimethylfructose, much less tetramethylfructose, corresponding to the amount of dimethyl sugar, and tetramethylglucose as the minor substance.

The methyl derivatives were reduced with sodium borohydride.[18] After co-distillation with methanol the dry residue was treated with a trimethylsilylating agent, and the resulting mixture was subjected to GLC (5 % S.E. 30, nitrogen).

Permethylated sucrose purified by TLC (solvent B) was hydrolysed (1 % oxalic acid, boiling waterbath, 15 min) neutralised (calcium carbonate) and centrifuged. From a portion of the hydrolysate, 1,3,4,6-tetra-*O*-methylfructose and 2,3,4,6-tetra-*O*-methylglucose, were isolated by TLC (solvent B). The remainder of the hydrolysate and the

isolated sugars were prepared for GLC by the method given for the polysaccharides and then used as reference substances. The glucose and fructose derivatives each gave rise to one single peak in the gas chromatograms from which the estimated ratio tetramethylfructose/tetramethylglucose was 9:1.

6. *Periodate oxidation.* The polyfructans of *L. aestivum* and *L. vernum* (5.5 mg and 14.1 mg, respectively) were mixed with 0.01 M sodium periodate (10 ml) and left at 37°. After 2 and 8 d, respectively, UV-measurements [19] of the solutions indicated periodate consumptions of 1.0 (*L. aestivum*) and 1.1 moles (*L. vernum*) per mole anhydrohexose.

The polyaldehyde solutions were mixed with lead acetate and the precipitates of iodate and periodate were separated. Sodium borohydride (15 mg) was added to each solution which was then left at room temperature (1 h). Excess borohydride was destroyed by shaking with ion exchanged (Amberlite IR-120, H). Evaporation and co-distillation with two portions of methanol followed. The residues were hydrolysed in 0.2 % oxalic acid (2 ml, room temperature 18 h, then 90—100°, reflux, 5 h), neutralised (barium carbonate), centrifuged and evaporated to dryness. Methanolic solutions of the residues were subjected to TLC (solvent D). Intact fructose was not detected.

## REFERENCES

1. Hegnauer, R. *Chemotaxonomie der Pflanzen*, Band 2, Basel 1963.
2. Schlubach, H. H. *Angew. Chem.* **69** (1957) 433.
3. Schlubach, H. H. *Fortschr. Chem. Org. Naturstoffe* **19** (1961) 291.
4. Schlubach, H. H. *Fortschr. Chem. Org. Naturstoffe* **23** (1965) 46.
5. Bacon, J. S. D. *Biochem. J.* **73** (1959) 507.
6. Hammer, H. *Acta Chem. Scand.* **22** (1968) 197.
7. Hammer, H. *Acta Chem. Scand.* **23** (1969) 3268.
8. Mizuno, T. and Hayashi, K. *Nippon Nogei-Kagaku Kaishi* **29** (1955) 533, 138; *Chem. Abstr.* **52** (1958) 18682.
9. Schlubach, H. H., Lübbers, H. and Borowski, H. *Ann.* **595** (1955) 232.
10. Binkley, W. W. and Altenburg, F. W. *Intern. Sugar J.* **67** (1965) 110.
11. Schlubach, H. H. and Koehn, H. O. A. *Ann.* **614** (1958) 126.
12. Schlubach, H. H. and Koehn, H. O. A. *Ann.* **647** (1961) 41.
13. Bailey, R. W. *The Oligosaccharides*, London 1965.
14. Sunderwirth, S. G., Olson, G. G. and Johnson, G. *J. Chromatog.* **16** (1964) 176.
15. Anderson, D. M. W. and Cree, G. M. *Carbohyd. Res.* **2** (1966) 162.
16. Colowick, S. P. and Kaplan, N. O. *Methods in Enzymology*, Vol. 1, Academic, New York 1955.
17. Percheron, F. *Compt. Rend.* **255** (1962) 2521.
18. Björndal, H., Lindberg, B. and Svenson, S. *Acta Chem. Scand.* **21** (1967) 1801.
19. Aspinall, G. O. and Ferrier, R. J. *Chem. Ind. (London)* 1957 1216.

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