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Note

The detection of isokestose and neokestose in plant extracts by ^{13}C-n.m.r. spectroscopy*

KATHLENE L. FORSYTHE AND MILTON S. FEATHER
Department of Biochemistry, University of Missouri, Columbia, Missouri 65211 (U.S.A.)

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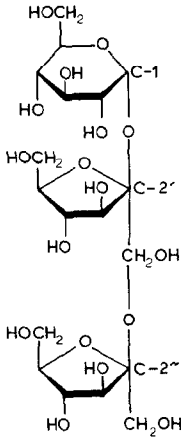
Kestoses (D-fructofuranosyl derivatives of sucrose) are produced by a variety of plants, including tulip bulbs[1], Jerusalem artichoke[2], asparagus[3], and banana fruit[4]. Kestoses have also been reported to be produced by the action of invertase (and related hydrolase enzymes) on incubation with sucrose[5–9]. In higher plants, kestoses are thought to be produced *via* the action of a specific enzyme, namely, sucrose-sucrose 1-fructosyltransferase (SST)[10]. In this case kestose formation appears to be the first step in the synthesis of D-fructans, which are thought[11] to be produced by sequential addition of D-fructofuranosyl groups to the kestose by an enzyme system different from SST. Because the type of kestose (or kestoses) produced by a plant species may determine (or be an indicator of) the type of D-fructan that is ultimately synthesized, a reliable method for the detection of these materials



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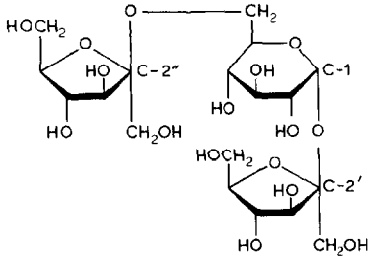

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in plants would be highly desirable. Two of the more common kestoses, isokestose (**1**) and neokestose (**2**) (shown with each anomeric carbon atom coded), constitute the subject of this study.

Published proton-decoupled ^{13}C-n.m.r. data on isokestose, sucrose, and related D-fructofuranosyl-containing compounds[12–14] suggest that this technique might provide a useful approach to the identification of kestoses present in various plant extracts. The C-1 and C-2 signals of the respective D-glucopyranosyl group and D-fructofuranosyl groups or residues are well separated from the other signals in the spectrum and, therefore, could be used to determine the number (and possibly the identity) of kestoses present in a preparation.

Asparagus is the most intensively studied D-fructan-producing plant. Shiomi and co-workers[3] have separated oligosaccharides from asparagus extracts by charcoal chromatography and, based on methylation analysis of some of the fractions obtained, have shown that both **1** and **2** are produced by this plant. They have also isolated the SST enzyme and have shown that, on incubation with sucrose[11], it catalyzes the formation of a kestose. Using this plant as a model system, we have investigated the utility of ^{13}C-n.m.r. spectroscopy as a method for detecting and identifying kestoses in extracts thereof.

EXPERIMENTAL

Asparagus-root cultures. — Asparagus seeds (20) were sterilized with 10% hypochlorite solution for 10 min, washed with distilled water, and allowed to germinate on a standard agar-containing petri dish at 37°. Shoot tips (1–2 mm in length) were then excised from the germinating seeds, placed on a full strength LS-modified medium[15], and kept in the dark for 4 weeks at room temperature to allow for callus development. Callus was subcultured three times on the same medium. For preferential inducement of roots, the callus was transferred to liquid-modified LS medium[16], 1 μM in IBA, in 250-mL Erlenmeyer flasks, and shaken for one month in the dark. Kestose (and D-fructan) synthesis was stimulated by the addition of exogenous sucrose to a concentration of 10%, and the culture was shaken for three days.

Extraction of kestoses. — Asparagus roots (~100 g) were washed with distilled water, placed in a mortar with 200 mL of 80% aqueous ethanol, and the sugars extracted by grinding. The extract was filtered through Whatman No. 1 filter paper, and the filtrate concentrated *in vacuo* at 35° to 50 mL, and dialyzed overnight against distilled water, using a 3500 molecular weight cut-off membrane. The resulting dialyzate was evaporated to dryness *in vacuo*.

Separation of the sugars as the acetates. — The sugars extracted were acetylated with acetic anhydride–pyridine, the solution poured into ice-water and the acetates isolated by extraction with chloroform, and evaporation of the extract; the acetates were obtained as a pale-yellow syrup. Separation was accomplished by

loading 800 mg of the syrup onto a column (26 mm by 130 mm) of silica gel and eluting with 1:1 (v/v) ethyl acetate–toluene.

This procedure removes most of the acetylated D-glucose, D-fructose, and sucrose from the higher-molecular-weight components. Subsequent chromatography using 2:3 (v/v) ethyl acetate–toluene separated the higher-molecular-weight materials and allowed the isolation of kestose-containing fractions. In all cases, 10-mL fractions were collected, and the fractions were examined by t.l.c. on plates of silica gel using 3:1 (v/v) ethyl acetate–toluene as the irrigant and sulfuric acid spray for detection.

RESULTS AND DISCUSSION

Thin-layer chromatography (t.l.c.) of the acetylated extract (prior to separation) revealed the acetates of glucose, fructose, and sucrose as the major components of the extract, and slower-moving components that had R_F values consistent with tri-, tetra-, and higher-d.p. oligomers. Acetylation of a mixture of D-glucose, D-fructose, and sucrose, followed by isolation using the aforementioned procedures showed that, in the reaction, no artifacts are produced that affect the n.m.r. data or interfere with the t.l.c. procedures. The acetylated trisaccharide fraction was isolated in pure form with no lower-molecular-weight contaminants visible, and moved as a single spot (R_F 0.42) in t.l.c. The n.m.r. spectrum, however, clearly showed that the material was heterogeneous. Two glucopyranosyl C-1 signals were evident, at δ 89.3 and 90.2. In addition, four fructofuranosyl C-2 signals could be detected, at δ 103.4, 104.2, and 102.9 (two signals, determined by quantitative integration). At these conditions, the spectrum of sucrose octaacetate shows the resonance for the glycopyranosyl C-1 atom to be at 89.8 p.p.m. and that of the fructofuranosyl C-2 atom to be at 103.9 p.p.m.

In order to establish more firmly that the materials in the preparation were kestose molecules, the fraction was *O*-deacetylated using sodium methoxide in methanol, and the sugars were isolated in the usual way. G.l.c. of the trimethylsilyl ethers (using an OV-17 capillary column) indicated that some hydrolysis had occurred during *O*-deacetylation. Sucrose and fructose were detected, along with two slower-migrating components (the major components) whose retention time was consistent with their being trisaccharides. Complete acid hydrolysis, using a cation-exchange resin as the catalyst, gave a solution that contained fructose and glucose in the ratio of 2:1 (determined by g.l.c.). The foregoing analytical data, and the previous reports on the composition of asparagus extracts[3], suggested that the fraction isolated was, in fact, composed of kestoses.

Jarrell and co-workers[13] collected ^{13}C-n.m.r. data on isokestose (**1**), and assigned the signal observed at 93.7 p.p.m. to glucopyranosyl C-1, that at 104.9 p.p.m. to fructofuranosyl C-2′ (identical to the fructofuranosyl C-2′ signal of sucrose), and that at 104.5 p.p.m. to fructofuranosyl C-2″. These data are for samples in deuterium oxide solutions. The n.m.r. spectrum of the *O*-deacetylated

preparation obtained in this study (also in deuterium oxide solution) showed signals at 93.5, 104.7, (more than two carbon atoms by quantitative integration), 104.3, 93.0, and 105.0 p.p.m. We experimentally determined the fructofuranosyl C-2′ signal of sucrose to be at 104.7 p.p.m., and that of glucopyranosyl C-1 to be at 93.3 p.p.m. The signals at 93.5, 104.7, and 104.3 agree well with the assignments made by Jarrell and co-workers[13], and can be assigned to isokestose. Because prior methylation studies had shown neokestose to be a component of asparagus as well, the remaining signals, at 93.0, 104.7, and 105.0 p.p.m., can be assigned to this structure.

With respect to the spectra of the initially isolated acetates, some tentative assignments can be made, taking into account the shielding effect of acetyl groups on the chemical shift of nearby carbon atoms, as well as the assignments made for the unacetylated compounds and for sucrose and the octaacetate derivative thereof. The signals at 90.2 and 89.3 p.p.m. (because of their chemical shift) can be assigned to the glucopyranosyl C-1 atoms of, respectively, isokestose and neokestose by analogy with the spectra of the unacetylated materials. The fructofuranosyl C-2′ atom of **1** is the least shielded anomeric carbon atom of the four acetylated fructofuranosyl residues and its signal would be expected to be the most downfield of the four observed (104.2 p.p.m.). The two fructofuranosyl C-2 atoms of **2**, because the residues are found in reasonably different chemical environments, would not be expected to have identical chemical shifts. Therefore, one of the signals at 102.9 p.p.m. can be assigned to the remaining, unassigned fructofuranosyl C-2″ atom of **1**, and the other to one of the fructofuranosyl C-2 atoms of **2**. By default, the remaining resonance, at 103.4 p.p.m., can be assigned to **2**. In summary, the signals at 90.2, 104.2, and 102.9 p.p.m. can be assigned to **1**, and the signals at 89.3, 102.9, and 103.4 p.p.m., to **2**. Lacking additional, more sophisticated approaches to the problem, specific assignments cannot be reliably made.

The collected data show that, given an appropriately purified sample of a plant extract, ^{13}C-n.m.r. spectroscopy would be a useful tool with which to detect and identify isokestose, neokestose, or a mixture thereof in a preparation. Separation of the acetates proved to be a much more facile technique than using charcoal or other chromatographic methods that require aqueous systems. An acetylated extract can be easily fractionated, and the fractions isolated by flash evaporation in a relatively short time. For n.m.r. spectra, sample sizes were on the order of 50 mg of acetylated material per mL of chloroform-*d*, which allowed spectral data to be collected using ~1 h of instrument time. Significantly smaller samples could be used, but a corresponding increase in instrument time would be necessary. The data reported herein were collected by using a Nicolet 5-mm single-frequency (75.5 MHz) probe and a Doty Scientific single-frequency ^{13}C preamplifier.

In preliminary experiments, we have examined the trisaccharide fraction of ripe banana fruit using the procedures developed in this study. Banana (a non-D-fructan-producing plant) has been reported[4] to produce neokestose (**2**), presumably as a result of the action of endogenous invertase acting on sucrose. Ripe banana

fruit was extracted as described by Henderson and co-workers[4], and the extract acetylated and fractionated as already described. T.l.c. indicated a complex mixture of trisaccharides that contained at least four components (R_F 0.50, 0.45, 0.40, and 0.37) which were not resolvable into pure fractions. N.m.r. spectroscopy, however, revealed major signals at 89.3, 102.9, and 103.4 p.p.m., consistent with the presence of neokestose in this preparation, thus supporting the original conclusions made by these workers[4].

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