ISOLATION AND IDENTIFICATION OF NYSTOSE FROM SEEDS OF THE HORSE CHESTNUT (*Aesculus hippocastanum* L.)

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ABSTRACT

From the seeds of the horse chestnut (*Aesculus hippocastanum* L.), a tetrasaccharide containing 1 mol. of glucose and 3 mol. of fructose has been isolated and shown to be nystose [β-D-Fru*f*-(2→1)-β-D-Fru*f*-(2→1)-β-D-Fru*f*-(2↔1)-α-D-Glc*p*·H_2O].

INTRODUCTION

Recently, the isolation and characterisation of several free saccharides present in aqueous, ethanolic extracts of freshly matured seeds of the horse chestnut (*Aesculus hippocastanum* L). have been described[1–4]. In addition to D-glucose, sucrose, and maltose, the higher oligosaccharides, maltotriose, maltotetraose, maltopentaose, maltoheptaose, malto-octaose, stachyose, 1-kestose, and 6-kestose were identified. These saccharides can be divided into three types, namely, the amylose series, the galactosyl-sucrose series, and the fructosyl-sucrose series.

We now describe the characterization of another representative of the fructosyl-sucrose series, namely, nystose [*O*-β-D-fructofuranosyl-(2→1)-*O*-β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl α-D-glucopyranoside].

EXPERIMENTAL AND RESULTS

Paper chromatography was performed on Whatman No. 1 paper, using *A* 1-butanol–pyridine–water (9:5:4) and *B* 1-butanol–pyridine–water (10:8:7). Thin-layer chromatography (t.l.c.) was performed on Silica gel GF_{254} (Merck), using *C* 1-butanol saturated with water–methanol[5] (100:60) or *D* toluene–chloroform–ethanol (95:20:7), on Kieselgel (Merck), using *E* 1-propanol–cyclohexane–ethyl acetate–water (9:2:3:2), and on precoated plates (TLC-Ready Plastic Sheets F1500 Silica gel, Carl Schleicher Schüll), using *F* toluene–ethanol (80:20), *G* benzene–ethanol[5] (20:3), and *H* butanone saturated with 6% aqueous ammonia[5]. The spots were visualized by spraying with D.A.P.[6] (free saccharides), and orcinol and aniline oxalate reagents

(methylated saccharides). Gas–liquid chromatography (g.l.c.) of Me_3Si derivatives was performed on a F and M Gas Chromatograph Model 700, equipped with a dual flame-ionization detector and coiled stainless-steel columns (2.70 m × 3.2 mm), using 3% OV-17 or 3% OV-25 on Chromosorb W (HP), 80–100 mesh (Pierce Chemicals Company). The injection-port temperature was 290° and the detector temperature 310°. The gas flow-rates for hydrogen and air were 45 and 375 ml/min, respectively. The gas flow-rate of nitrogen was 18 ml/min for 3% OV-17, and 5 ml/min for 3% OV-25. Optical rotations were measured with an LEP A1 Polarimeter (Carl Zeiss). P.m.r. spectra were recorded at 100 MHz, for solutions in acetone-d_6, using a Varian HA-100 spectrometer. Mass spectra (70 eV) were recorded with an AEI MS-9 mass spectrometer at an ion-source temperature of ~160°. The X-ray analyses were performed with a Debye–Scherrer apparatus with Cu-$K\alpha$ radiation (1.5418 Å).

Isolation of the saccharide. — The preparation of the sugar extract from the seeds of the horse chestnut and the separation of this extract on a charcoal–Celite column were described previously[1,4]. The saccharide, obtained from fractions IX and X (see ref. 4) by fractional crystallization from methanol–ethanol (1:1), had m.p. ~134°, $[\alpha]_D$ +10° (*c* 0.36, water) (Found: C, 42.08; H, 6.75. $C_{24}H_{42}O_{21}\cdot H_2O$ calc.: C, 42.11, H, 6.43%). The purity of the saccharide was tested by paper chromatography (solvents *A* and *B*), t.l.c. (solvents *C* and *E*), and g.l.c. of the Me_3Si derivative (3% OV-25, 290°). The saccharide gave no reaction in the triphenyltetrazolium test, indicating that it was non-reducing.

Total, acid hydrolysis. — In order to determine the ratio of glucose to fructose, 1 mg of the saccharide was hydrolyzed in 1 ml of 0.1M HCl for 1 h at 100°. Standard mixtures containing glucose and fructose in different ratios were treated in a similar way. The solutions were neutralized and lyophilized, and subsequently the residues were trimethylsilylated[7]. The mixtures were analyzed by g.l.c. (3% OV-17, 160°). From comparisons of peak areas, it was established that the ratio of glucose to fructose was 1:3.

Partial, acid hydrolysis. — A portion (1 mg) of the saccharide was hydrolyzed in 1 ml of mM HCl for 5 min at 100°. After neutralization, lyophilization, and trimethylsilylation, a peak with the same retention time as the Me_3Si derivative of sucrose was detected by g.l.c. (3% OV-17, 228°).

Mass spectrometry. — A portion (1 mg) of the saccharide was trimethylsilylated[7] and subjected to mass spectrometry. The spectrum (Fig. 1) contained a highest mass peak at *m/e* 1659 corresponding to $(M-CH_3)^+$ and indicative of a tetrasaccharide structure[8]. The peak at *m/e* 1571 corresponded to $(M-CH_2OSiMe_3)^+$. The ratio of the intensities of the peaks at *m/e* 217 ($Me_3SiO–CH=CH–\overset{+}{C}H–OSiMe_3$) and 204 ($Me_3SiO–\dot{C}H–\overset{+}{C}H–OSiMe_3$) was greater than unity, indicating the presence of furanose units. The relatively intense peak at *m/e* 437 suggested that the tetrasaccharide contained an ($x\rightarrow2$)-linked fructofuranoside unit at one end of the molecule. The presence of a distinct peak at *m/e* 811 demonstrated that the compound was closely related to 1-kestose, and not to 6-kestose or *neo*-kestose. The peaks at *m/e* 671,

1049, and 1427 indicated that the three fructose units were linked to each other linearly (Kamerling *et al.*[7,8]).

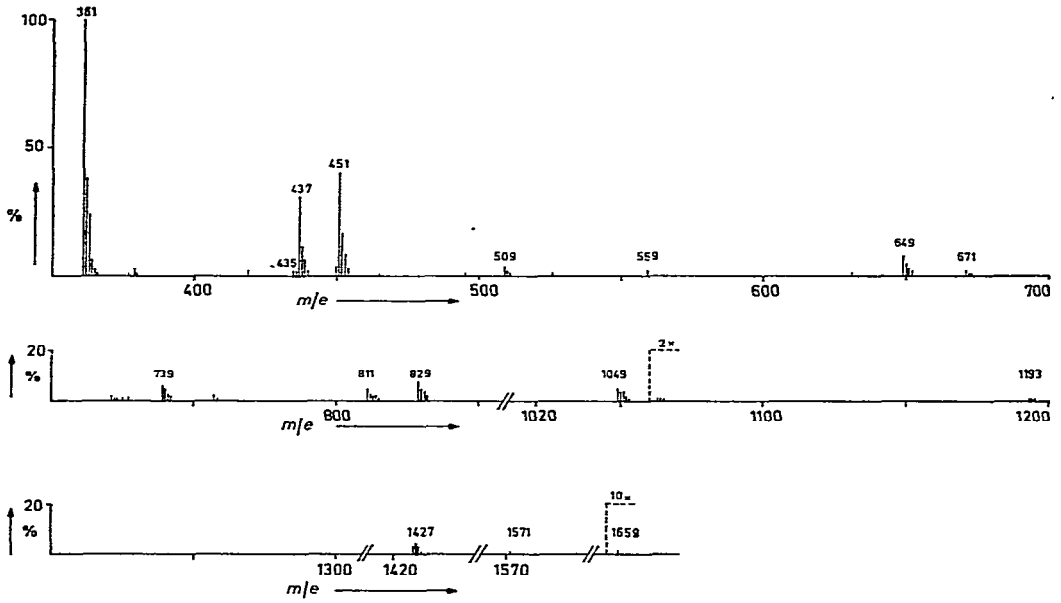

Fig. 1. Mass spectrum (70 eV) of *O*-trimethylsilylated nystose. Only *m/e* values higher than 360 are given.

Permethylation. — The dry, crystalline tetrasaccharide (30 mg) was methylated with methyl iodide and the methylsulphinyl anion in methyl sulphoxide (Hakomori[9]). The course of the methylation was monitored by t.l.c. (solvent *D*). The methylation procedure had to be repeated to obtain a fully methylated product. The permethylated compound (25 mg) was dissolved in a few drops of ethanol and hydrolyzed with 2 ml of M H_2SO_4 for 2 h at 100° in a sealed glass tube. The hydrolysate was neutralized with $BaCO_3$, filtered, and concentrated under reduced pressure at 30–40°. The residue was dissolved in methanol and investigated by t.l.c. (solvents *F*, *G*, and *H*). In some cases, double development was applied to improve the separation. Comparisons with reference compounds demonstrated that 2,3,4,6-tetra-*O*-methyl-D-glucose, 1,3,4,6-tetra-*O*-methyl-D-fructose, and 3,4,6-tri-*O*-methyl-D-fructose were present.

Periodate oxidation. — The tetrasaccharide (3 mg) was dissolved in 5 ml of water and oxidized with 1 ml of 0.25M sodium metaperiodate at room temperature in the dark for 24 h. A consumption of 5.22 $\pm$0.03 mol. of oxidant per mol. of the tetrasaccharide was determined by the Fleury–Lange method[10].

Proton magnetic resonance spectroscopy. — The p.m.r. spectrum of the trimethylsilylated tetrasaccharide (from 10 mg of saccharide) showed a doublet at δ 5.45 ($J_{1,2}$ 3.2 Hz) (Fig. 2). Taking into account the fact that the tetrasaccharide is

non-reducing and that it contains 1 glucose and 3 fructose residues, the configuration of H-1 of the D-glucose residue must be α (Kamerling *et al.*[11,12]).

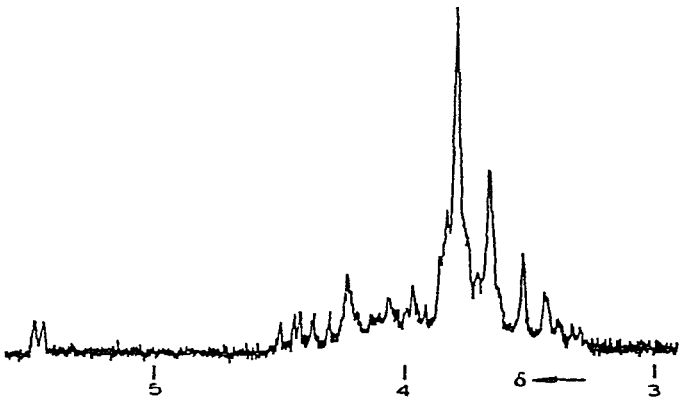

Fig. 2. P.m.r. spectrum at 100 MHz in acetone-d_6 of *O*-trimethylsilylated nystose. The signals of the Me_3Si groups are not given.

Hydrolysis with β-D-fructofuranosidase. — The saccharide (2 mg) dissolved in 0.1 ml of water was incubated with 2 mg of invertase for 24 h at 32°. The solution was then heated for a few minutes at 80° to inactivate the enzyme. After lyophilization and trimethylsilylation, the mixture was analysed by g.l.c. (3% OV-17). Only the Me_3Si derivatives of glucose and fructose could be detected, in the ratio 1:3. Therefore, all fructose residues in the tetrasaccharide must have the β-D configuration.

DISCUSSION

For the oligosaccharide, the following points have been established. *1.* It is non-reducing and contains 1 mol. of glucose and 3 mol. of fructose (total acid hydrolysis; mass spectrometry). *2.* It contains a β-D-fructofuranosyl α-D-glucopyranoside unit (partial, acid hydrolysis). *3.* It has an (*x*→2)-linked fructofuranoside unit at one end of the molecule and resembles β-D-Fru*f*-(2→1)-β-D-Fru*f*-(2↔1)-α-D-Glc*p*. The three fructose residues are linked to each other linearly (mass spectrometry). *4.* The partially methylated monosaccharides are identified as 2,3,4,6-tetra-*O*-methyl-D-glucose, 1,3,4,6-tetra-*O*-methyl-D-fructose, and 3,4,6-tri-*O*-methyl-D-fructose (permethylation). Therefore, *x* in the (*x*→2)-fructofuranoside unit must be 1. *5.* The periodate consumption amounts to 5 mol. *6.* The configuration of the glycosidic bond in the D-glucose residue is α (p.m.r. spectroscopy). *7.* The configurations of the glycosidic bonds in the D-fructose residues are β (invertase).

Therefore, the structure of the oligosaccharide must be β-D-Fru*f*-(2→1)-β-D-Fru*f*-(2→1)-β-D-Fru*f*-(2↔1)-α-D-Glc*p*. The X-ray powder diffraction data are in accordance with those published for nystose by Binkley *et al.*[13,14].

The occurrence of the tetrasaccharide described above was reported earlier by

two other groups of investigators. Binkley *et al.*[13] described the formation of this saccharide by the action of a transfructosylase (Clarase 900, a fungal alpha-amylase) on sucrose, whereas Tsuchida *et al.*[15] found that *Dematium pullulans* cultured on a sucrose medium also produced the tetrasaccharide. We have now shown that nystose is present in higher organisms. The tetrasaccharide crystallised as a monohydrate, whereas the previous preparations were a trihydrate[15] and an anhydrous product[13]. When our tetrasaccharide was dried over silica gel at 90° *in vacuo*, decomposition was observed, yielding 1-kestose, sucrose, and fructose.

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