# THE STRUCTURE OF NUTANOSIDE

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We have previously reported the isolation from the roots of Silene nutans L. of a triterpene glycoside – nutanoside – containing gypsogenin as the aglycone and having nine monosaccharide residues [1]. In the present paper we give experimental results permitting the complete structure of this glycoside to be established. Nutanoside permethylate, obtained by treating the initial glycoside with methyl iodide and sodium hydride gave on hydrolysis a mixture of various methylated monosaccharides which were separated preparatively by paper chromatography and were identified by gas-liquid chromatography (GLC) by the constants of the compounds, and also by some chemical reactions. The following were obtained: 2 moles of 2,3,4,6-tetra-O-methyl-D-glucose, and 1 mole each of 2,3,4-tri-O-methyl-D-glucose, 2,3,4-tri-O-methyl-L-arabinose, 3,4,6-tri-O-methyl-D-galactose, 2,3-di-O-methyl-D-fucose, 2,3-di-O-methyl-D-galactose, and 2-O-methyl-L-rhamnose. The isolation of four completely methylated sugars shows the highly branched nature of the carbohydrate chains of nutanoside. It is obvious that the branching centers are galactose and rhamnose.

Further information on the structure of each of the carbohydrate chains of nutanoside was obtained by by the methylation of the acid glycoside and pentasaccharide formed on alkaline hydrolysis [1]. In the hydrolysate of the permethylate of the acid glycoside 2,3,4-tri-O-methyl-D-xylose, 2,3,4-tri-O-methyl-L-arabinose and 2,3-di-O-methyl-D-galactose were identified.

It was necessary to determine to which of the hydroxyls of the galactose the arabinose residue was attached and to which the xylose residue. To solve this question, partial hydrolysis of the acid glycoside was performed. A trioside was obtained which contained, in addition to gypsogenin and glucuronic acid, one mole each of galactose and xylose. The hydrolysis of the permethylate of this glycoside showed that the xylose was attached to the galactose by a 1-4 bond, since of the two possible compounds [2,3,6- and 2,3,4-tri-O-methyl-D-galactose ($R_g$ 0.64 and 0.71, respectively)], the latter was found. On partial hydrolysis, a progenin was also isolated, the constants and composition of which agreed with the vaccaroside described previously [2]. The periodate oxidation of nutanoside by Smith's method gave a glycoside containing, in addition to the aglycone, glucuronic acid and rhamnose [1]. Consequently, the glucuronic acid is attached directly to the hydroxyl of the gypsogenin and rhamnose to the carboxyl, and the trisaccharide chain in the acid glycoside is attached to the hydroxyl at the C-3 atom of the glucuronic acid. The enzymatic hydrolysis of nutanoside with diastase also showed only this glycoside, which confirms the presence of a bond of the glucuronic acid and rhamnose with galactose or glucose.

This structure of the carbohydrate chain attached to the carboxyl of gypsogenin was established from the results of a comparison of periodate oxidation, methylation, and enzymatic hydrolysis. When the permethylate of the pentasaccharide was heated with acids, 2-O-methyl-L-rhamnose, 3,4,6-tri-O-methyl-D-galactose, 2,3-di-O-methyl-D-fucose, and 2 moles of 2,3,4,6-tetra-O-methyl-D-glucose were formed. As has been shown above, the rhamnose is attached directly to the carboxyl of the gypsogenin and has branching at the hydroxyls on carbon atoms 3 and 4. The enzymatic hydrolysis with diastase of the pentasaccharide gave a disaccharide containing rhamnose and glucose. Its permethylate was reduced with sodium tetrahydroborate and hydrolysed with hydrochloric acid. The resulting 2,3-dimethylrhamnitol gave a positive reaction for an $\alpha$-diol grouping, which it would not do in the case of 2,4-dimethylrhamnitol. Consequently, the terminal glucose is attached to the hydroxyl at the C-4 atom of the rhamnose residue. The hydroxyl at its C-3 atom bears a straight chain consisting of three monosaccharide residues – galactose,

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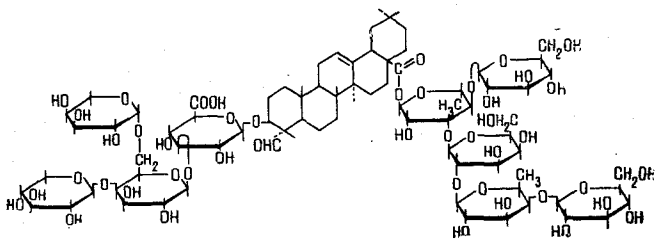
fucose, and glucose. This is shown by the methylation of the pentasaccharide. In addition, this trisaccharide was obtained in the enzymatic hydrolysis of nutanoside. Since in the trisaccharide on reduction with sodium tetrahydroborate the galactose is converted into dulcitol and the other two monosaccharide residues are unchanged, it is clear that the galactose is attached to the C-3 atom of the rhamnose, the intermediate link is the fucose residue, and the chain is terminated by the glucose residue.

The configurations of the glycosidic centers of the linear chains were calculated by Klyne's rule taking into account the molecular rotations of nutanoside, the intermediate glycosides, the aglycone, and the di- and trisaccharides with reduced terminal units. Thus, the facts given above permit the following proposal for the full structure of nutanoside:



## EXPERIMENTAL

Chromatography was carried out with ASK silica gel, Schleicher und Schüll No. 2043 paper, and with the following systems of solvents: 1) butan-1-ol–ethanol–water (4:1:5); 2) benzene–butan-1-ol–pyridine–water (1:5:3:3); 3) butan-1-ol–acetic acid–water (5:1:4); 4) ethyl acetate–methanol–water–acetic acid (10:4:2:1); and 5) chloroform–methanol (20:1.5). Gas-liquid chromatography was performed in a "Tswett-1" chromatograph with a flame ionization detector at 175° C in a column 1 m long and 4 mm in diameter [Celite-545 treated with an alkali, with 15% of poly(butane-1,4-diol succinate)].

Methylation of Nutanoside. With stirring, 0.6 g of NaH and 7 ml of $Ch_3I$ were added over an hour to a solution of 0.7 g of nutanoside in 50 ml of dimethylformamide. Then the mixture was stirred for about 2 h, after which it was poured into water and extracted with chloroform (3 × 75 ml). The extract was washed with thiosulfate solution and with water and was evaporated. The residue (0.33 g) was transferred to a column of alumina (3 g) and eluted with 30 ml of chloroform. This gave 0.3 g of nutanoside permethylate with mp 120-125° C $[\alpha]_D$ + 5° (c 1; pyridine).

A mixture of 0.1 g of this product and 3 ml of absolute methanol containing 5% of HCl was heated at 85° C for 4 h. The mixture of completely-methylated monosaccharides was extracted with chloroform and analyzed by GLC. Peaks were found with retention times in relation to methyl β-2,3,4,6-tetra-O-methyl-D-glucoside as follows: 1, 1.38, 1.05, 0.45, and 0.52. Literature data: 1 and 1.43 – methyl α- and β-2,3,4,6-tetra-O-methyl-D-glucosides; 1.04 – methyl β-2,3,4-tri-O-methyl-L-arabinoside; 0.46 and 0.57 – methyl α- and β-2,3,4-tri-O-methyl-D-xylosides [3].

Nutanoside permethylate (0.2 g) was heated in 5 ml of methanol containing 5% of hydrochloric acid for 3 h. The mixture of methylated monosaccharides was separated preparatively on sheets of paper (29 × 58 cm) in system 1. This gave the following sugar methyl ethers.

2,3,4,6-Tetra-O-methyl-D-glucose (12 mg), $[\alpha]_D$ + 82° (c 1; water) $R_g$ 1.0. Literature data: $R_g$ 1.0 [4], $[\alpha]_D$ + 83.3° (water) [5].

2,3,4-Tri-O-methyl-D-xylose (8 mg), $[\alpha]_D$ + 60° (c 1; water), $R_g$ 0.93. Literature data: $[\alpha]_D$ + 64.5° (water), $R_g$ 0.94 [6].

2,3,4-Tri-O-methyl-L-arabinose (7 mg), $[\alpha]_D$ + 120° (c 0.5; water), $R_g$ 0.88. Literature data: $[\alpha]_D$ + 133.4° (water) [7].

3,4,6-Tri-O-methyl-D-galactose (14 mg) $[\alpha]_D$ + 40° (c 1; water), $R_g$ 0.72. The substance gave positive reactions with triphenyltetrazolium chloride and with the $KIO_4$–benzidine reagent [8]. On demethylation with boron trichloride [9], galactose was identified by paper chromatography in systems 2 and 3.

2,3-Di-O-methyl-D-fucose (13 mg), $[\alpha]_D$ + 60° (c 0.5; water), $R_g$ 10.63. The reaction for an α-diol grouping was negative, but after reduction with $NaBH_4$ it was positive. The triphenyltetrazolium chloride test was negative [8].

2-O-Methyl-L-rhamnose (11 mg), mp 106-108° C $[\alpha]_D$ + 33.3° (c 0.6; water), $R_g$ 0.54. Literature data: mp 113-114° C $[\alpha]_D$ + 31° (water) [10]. The reaction for an α-diol grouping was positive. The triphenyltetrazolium test was negative. A mixture of 2 mg of the substance and 1 ml of absolute methanol containing 4% of HCl was heated at 100° C for 1 h. The methyl glycoside gave a positive reaction for an α-diol grouping.

2,3-Di-O-methyl-D-galactose (12 mg), $[\alpha]_D$ + 62° (c 0.8; water), $R_g$ 0.5. Literature data: $R_g$ 0.55 [11], $[\alpha]_D$ + 57 → + 105° (water) [12]. On demethylation with boron trichloride, galactose was identified. The $KIO_4$–benzidine reaction was negative, but after reduction with sodium tetrahydroborate it was positive. The triphenyltetrazolium chloride test was negative.

Methylation of the Acid Glycoside. The methylation of 0.1 g of the compound was performed as described above. This gave 0.087 g of permethylate with mp 120-125° C $[\alpha]_D$ + 30° (c1; pyridine). The product was heated in 3 ml of 5% aqueous ethanolic hydrochloric acid for 3 h. 2,3,4-Tri-O-methyl-D-xylose, 2,3,4-tri-O-methyl-L-arabinose, and 2,3-di-O-methyl-D-galactose were identified in the hydrolysate by paper chromatography.

Partial Hydrolysis of the Acid Glycoside. A solution of 0.14 g of the glycoside in 40 ml of isopropanol–water–sulfuric acid (2 : 1 : 0.1) was heated at 85° C for 15 min. After neutralization with $Ba(OH)_2$, the hydrolysis products were extracted with butanol (3 × 100 ml). The solvent was evaporated off and the residue, amounting to 0.11 g, was transferred to a column containing 30 g of silica gel. Elution was performed first with 220 ml of chloroform–ethyl acetate (1 : 3) and then with system 4, 1.5-ml fractions being collected. Fractions 6-14 yielded 10 mg of vaccaroside with mp 186-189° C, $[\alpha]_D$ + 26.6° (c 1.5; pyridine). After hydrolysis with Kiliani's mixture, glucuronic acid and its lactone were identified by paper chromatogrography in system 3, and gypsogenin by thin-layer chromatography in system 5 with an authentic sample. Fractions 15-18 contained 25 mg of a trioside with mp 205-207° C, $[\alpha]_D$ + 10° (c 3; pyridine).

Part of the trioside was heated in 1 ml of 5% hydrochloric acid for 3 h. Glucuronic acid, galactose, and xylose were identified in the hydrolysate by paper chromatography in systems 2 and 3.

A solution of 20 mg of the trioside in 1 ml of dimethylformamide was treated with 1 ml of $CH_3I$ and 0.5 g of BaO, and the mixture was placed in a tube and heated at 100° C for 19 h. The product was dissolved in 3 ml of 5% aqueous methanolic hydrochloric acid and heated at 80° C for 3 h. 2,3,4-Tri-O-methyl-D-xylose with $R_g$ 0.91 and 2,3,6-tri-O-methyl-D-galactose with $R_g$ 0.73 were identified in the hydrolysate by paper chromatography in system 1. Literature data: $R_g$ 0.93 and 0.71, respectively [4].

Methylation of the Pentasaccharide. The methylation of 0.1 g of the substance was performed as described above. The permethylate (0.07 g) was heated in 7 ml of 5% hydrochloric acid for 3 h. In the hydrolysate by paper chromatography in system 1 with authentic samples the following sugar derivatives were identified: 2,3,4,6-tetra-O-methyl-D-glucose, 3,4,6-tri-O-methyl-D-galactose, 2,3-di-O-methyl-D-fucose, and 2-O-methyl-L-rhamnose.

Enzymatic Hydrolysis of the Pentasaccharide. A solution of 0.17 g of the pentasaccharide in 20 ml of phosphate buffer was treated with 17 mg of diastase, and the mixture was left at 37° C for 14 h. The substrate was separated preparatively on sheets of paper (29 × 58 cm) in system 2. This gave 17 mg of disaccharide as an amorphous powder; after reduction with sodium tetrahydroborate, $[\alpha]_D$ 0° (c 0.6; water). The disaccharide was methylated as described above. Part of the permethylate was reduced and hydrolyzed. The product was placed on a paper chromatogram; the 2,3-dimethylrhamnitol gave a positive reaction for an α-diol grouping. Another part of the permethylate was heated with 3% hydrochloric acid for 1 h, and the hydrolysate was found to contain 2,3,4,6-tetra-O-methyl-D-glucose with $R_g$ 1.0, and 2,3-di-O-methyl-L-rhamnose with $R_g$ 0.74.

Enzymatic Hydrolysis of Nutanoside. A solution of 0.5 g of nutanoside in 30 ml of phosphate buffer was treated with 13 mg of diastase and the mixture was kept at 37° C for 38 h. Two glucosides were found in the substrate by thin-layer chromatography in system 4. The addition of an excess of ethanol gave 0.1 g of the initial nutanoside, $R_f$ 0.05. The filtrate was evaporated and the residue was dissolved in water and extracted with butanol.

The butanolic extract was evaporated until a precipitate deposited, and this was filtered off giving 0.1 g of a glycoside with mp 205-207° C, $[\alpha]_D$ + 20° (c 1; pyridine), $R_f$ 0.3. After hydrolysis with 5% hydrochloric acid, glucuronic acid and rhamnose were found in the hydrolysate by paper chromatography in systems 2 and 3.

The aqueous extract was evaporated. The 0.2-g residue was separated preparatively on sheets of paper in system 3. This gave 25 mg of a trisaccharide with $[\alpha]_D$ + 60° (c 0.5; water). A mixture of 10 mg of the product and 1 ml of 3% hydrochloric acid was heated at 90° C for 1.5 h. Galactose, glucose, and fucose were found in the hydrolyzate. The trisaccharide (13 mg) was reduced with sodium tetrahydroborate. The product, with $[\alpha]_D$ – 24° (c 0.75; water) was hydrolyzed. On a paper chromatogram run in system 2 with markers, aniline phthalate showed the presence of glucose and fucose, and the $KIO_4$–benzidine reagent showed the presence of glucose, fucose, and dulcitol.

## SUMMARY

The complete structure of nutanoside – a triterpene glycoside isolated from the roots of Silene nutans L. – has been established.

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