TRITERPENE GLYCOSIDES OF Ladyginia bucharica

II. THE STRUCTURE OF LADYGINOSIDES A AND B

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The isolation from the roots of Ladyginia bucharica Lipsky (family Umbelliferae) of five triterpene glycosides which we called ladyginosides A, B, C, D, and E has been reported previously [1].

The present paper gives a proof of the structures of ladyginosides A and B, the least polar glycosides of the Ladyginia and those closest in chromatographic mobility. On repeated chromatography of the combined glycosides on columns of silica gel, we obtained fractions enriched in ladyginosides A and B. These compounds were isolated in the individual state by the preparative separation of these fractions on plates with a fixed layer of silica gel. The amount of each glycoside was approximately 0.1% of the air-dry weight of the roots.

To determine the compositions of the glycosides, each of them separately was hydrolyzed with 18% hydrochloric acid. In the precipitate that deposited from the water-diluted hydrolyzate, oleanolic acid was identified as the aglycone for ladyginoside A and hederagenin for ladyginoside B.

Chromatography in a thin layer of silica gel (TLC) and on paper (PC) showed that the carbohydrate moieties of ladyginoside A and that of ladyginoside B consist of the same sugars, D-glucose and D-glucuronic acid.

The GLC of the silylated methyl glycosides of the sugars showed that in both ladyginosides A and B the D-glucose and D-glucuronic acid are present in a ratio of 1:1 [2]. Consequently, ladyginoside A is a bioside of oleanolic acid and ladyginoside B a bioside of hederagenin.

The partial cleavage of the glycosides A and B with dilute sulfuric acid gave glucuronides of oleanolic acid and of hederagenin, respectively. In this way it was established that the glucuronic acid is attached directly to the aglycone in each case.

The methylation of ladyginosides A and B with diazomethane followed by hydrolysis led to the formation of the methyl esters of oleanolic acid and of hederagenin. On treatment with alkali, ladyginosides A and B underwent no change. This shows that the glycosides studied are not O-acyl glycosides and that the sugar moiety is attached to the hydroxy group in position 3 in each of the aglycones.

In order to determine the position of attachment of the D-glucose to the D-glucuronic acid, ladyginosides A and B were oxidized with sodium periodate. The hydrolysis of the oxidized products with sulfuric acid and a chromatographic study of the substances formed showed that the carbohydrate chains of ladyginosides A and B were completely destroyed. It follows from this that there is no $1 \rightarrow 3$ bond between the monosaccharides in the glycosides studied.

Further information on the structure of the carbohydrate moiety of ladyginosides A and B was obtained by their exhaustive methylation. The hydrolyzates of the permethylates of ladyginosides A and B were found, by comparison with reference materials, to contain 2,3,4,6-tetra-O-methyl-D-glucose and 2,3-di-O-methyl-D-glucuronic acid. From this it may be concluded that the D-glucose occupies the extreme position and is linked to the D-glucuronic acid through the hydroxy at C_4. The $1 \rightarrow 4$ bond is also confirmed by the fact that after chromatography on paper and in a thin layer of silica gel the methylated

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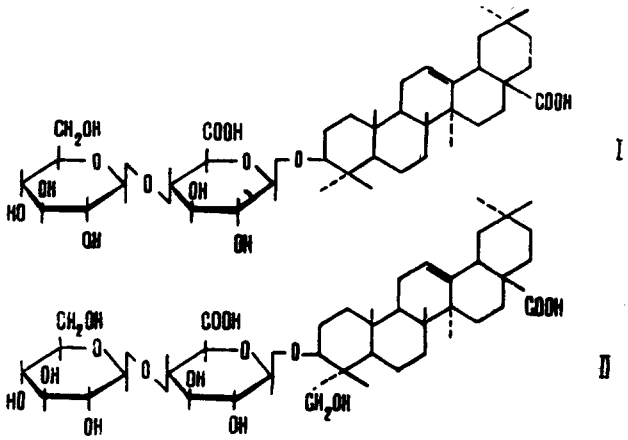
monosaccharides give a negative reaction for an α-glycolic group with the Bonner reagent.

The complete methylated ladyginosides A and B were reduced with lithium tetrahydroaluminate. 2,3-Di-O-methyl-D-glucose and 2,3,4,6-tetra-O-methyl-D-glucose were identified in the products of the hydrolysis of the reduced glycosides.

The calculation of molecular rotation differences for the bioside–monoside–aglycone in the case of ladyginosides A and B showed that the D-glucose is attached to the D-glucuronic acid and the latter to the genin by β-glycosidic bonds (Table 1). In both cases, the increment in the molecular rotation has a negative value. Thus, the structure of ladyginoside A is shown by (I) and that of ladyginoside B by (II):



Ladyginosides A and B proved to be structural isomers of the glycosides isolated by Tschesche et al. from Spinacia oleracea L. [3]. While in the spinasaponins A and B the D-glucose is attached to the hydroxyl at C_3 of the D-glucuronic acid, in ladyginosides A and B the analogous linkage is made through the hydroxyl at C_4.

EXPERIMENTAL

Chromatography was performed with type KSK silica gel and type M (slow) paper of the Leningrad No. 2 paper mill. The following solvent systems were used; for TLC: 1) butan-1-ol–ethanol – 25% ammonia (7:2:5); 2) chloroform–methanol–water (65:35:8); 3) chloroform–methanol (25:1); 4) butan-1-ol–acetone–water (4:5:1); 5) benzene–acetone (3:1); and 6) toluene–ethanol (10:1); and for PC: 7) butan-1-ol–acetic acid–water (4:1:5); and 8) water-saturated methyl ethyl ketone. The glycosides, the genins, and the methylated glycosides were revealed with an alcoholic solution of phosphotungstic acid, and the sugars with o-toluidine salicylate.

TABLE 1

Substance	M	$[\alpha]_D$, deg	$[M]_D = \frac{[\alpha]_D \cdot M}{100}$, deg
Ladyginoside A	795,0	+17,5	+140,7
Oleanolic acid glucuronoside	632,8	+30,8	+194,9
Fraction of the $[M]_D$ of D-glucose	—	—	−54,2
Oleanolic acid [7]	456,7	+80	+375,4
Fraction of the $[M]_D$ of glucuronic acid	—	—	−180,5
Ladyginoside B	811,0	+22,5	+182,5
Hederagenin glucuronoside	648,8	+30	+194,6
Fraction of the $[M]_D$ of D-glucose	—	—	−12,1
Hederagenin [7]	472,7	+82	+387,6
Fraction of the $[M]_D$ of D-glucuronic acid	—	—	−193
Methyl α-D-glucopyranoside [4]	194,2	+158	+307
Methyl β-D-glucopyranoside [4]	194,2	−32	−62
Methyl α-D-glucuronopyranoside [5]	208,2	+129	+268,6
Methyl β-D-glucuronopyranoside [5]	208,2	−55,3	−115,1

Isolation of Ladyginosides A and B. The combined glycosides obtained by extracting an aqueous solution of the extractive substances with n-butanol (10 g) were dissolved in system 1 and deposited on a column containing 1.2 kg of silica gel. Elution was performed with the same solvent system, 30-ml fractions being collected. The process of chromatography was monitored in a thin layer of silica gel in systems 1 and 2. Eluates which consisted of ladyginosides A and B; A, B, and C; C and D; D and E; and D, E, and F, were obtained from five columns. The combined fractions containing the glycosides A and B (500 mg) were chromatographed preparatively on plates (40 × 30 cm) with a fixed layer of silica gel in system 2. The zones with the individual ladyginosides A and B were removed from the plate and eluted with methanol, and the methanolic solutions were boiled

with activated carbon and evaporated. The residues were recrystallized from ethanol. This gave 150 mg of crystalline ladyginoside A, $C_{42}H_{66}O_{14}$, mp 240-242°C (from ethanol), $[\alpha]_D^{20}+17.5\pm2°$ (c 0.8; methanol) and 180 mg of ladyginoside B, $C_{42}H_{66}O_{15}$ in the form of white needles with mp 220-222°C (from ethanol), $[\alpha]_D^{20}+22.5\pm2°$ (c 1.6; ethanol).

Acid Hydrolysis of the Glycosides. 1. Complete Hydrolysis of Ladyginosides A and B. A mixture of 20 mg of ladyginoside A and 5 ml of 15% HCl was heated at 100°C for 6 h. The reaction mixture was diluted with water, and the precipitate that deposited was filtered off and dried. The hydrolyzate was neutralized with AV-17 anion-exchange resin (OH^- form). The neutral solution was evaporated and by TLC silica gel impregnated with a 0.2 M solution of sodium dihydrogen phosphate [6] in system 4 and PC in system 7 showed the presence of D-glucose and D-glucuronic acid and its lactone. The precipitate (10 mg), after recrystallization from absolute ethanol, had mp 306-308°C, $[\alpha]_D^{20}+79\pm2°$ (c 1.8; pyridine). The crystals isolated were identified by a mixed melting point with an authentic sample of oleanolic acid and by TLC in silica gel in system 3.

Methyl oleanolate was obtained by methylating the aglycone with an ethereal solution of diazomethane at room temperature for 24 h and had mp 196-198°C, $[\alpha]_D^{20}+72\pm2°$ (c 1.6; pyridine).

The genin was acetylated with acetic anhydride in dry pyridine at room temperature for 48 h, giving oleanolic acid acetate with mp 270-272°C, $[\alpha]_D^{20}+78\pm2°$ (c 1.5; methanol).

Methyl oleanolate acetate was obtained by methylating the acetate of the genin with diazomethane at room temperature. The excess of diazomethane and ether were eliminated and the methylation product was dissolved in methanol, boiled with activated carbon, and recrystallized from absolute ethanol. Crystals deposited in the form of needles with mp 218-220°C.

The analogous operations were performed with ladyginoside B. D-Glucose and D-glucuronic acid were found in the hydrolyzate of ladyginoside B. The aglycone was shown to be identical with hederagenin by its mp of 326-328°C (ethanol) and $[\alpha]_D^{20}+78\pm2°$ (c 1.8; pyridine). The methyl ester of hederagenin had mp 230-232°C (from ethanol) $[\alpha]_D^{20}+72\pm2°$ (c 0.8; chloroform). Hederagenin acetate was obtained, with mp 166-168°C (from methanol) $[\alpha]_D^{20}+64\pm2°$ (c 2.4; chloroform), and the diacetate of hederagenin methyl ester, with mp 188-190°C, $[\alpha]_D^{20}+62\pm2°$ (c 1.2; absolute ethanol).

The constants found for the oleanolic acid, the hederagenin, and their derivatives corresponded to those given in the literature [7].

2. Partial Hydrolysis of Ladyginosides A and B. The glycoside A (20 mg) was hydrolyzed with 0.25% H_2SO_4 at 70-75°C for 6 h. The reaction mixture was diluted with water and exhaustively extracted with n-butanol. The extract was concentrated and chromatographed in system 2. Oleanolic acid, the initial glycoside, and oleanolic acid glucuronide were found. The last-mentioned substance (10 mg), after purification and recrystallization from ethanol, had mp 212°C, $[\alpha]_D^{20}+30.8\pm2°$ (c 1.0; ethanol). According to the literature, the melting point of oleanolic acid glucuronide is 218°C, $[\alpha]_D^{20}+31.6°$ [8, 9].

Under the same conditions, ladyginoside B yielded hederagenin, the initial substance, and hederagenin glucuronide. Hederagenin glucuronide has not been described in the literature; it consisted of white acicular crystals with mp 198-200°C (from ethanol), $[\alpha]_D^{20}+29\pm3°$ (c 1.1; ethanol), readily soluble in methanol and ethanol and almost insoluble in water. Its elementary analysis corresponded to the formula $C_{36}H_{56}O_{10}$.

When the glucuronides were subjected to acid hydrolysis (6% H_2SO_4) D-glucuronic acid and its lactone were identified in the hydrolyzate by the PC method in system 7.

Methylation of Ladyginosides with Diazomethane. A solution of 42 mg of ladyginoside A and a solution of 40 mg of ladyginoside B, each in 5 ml of dry methanol, were methylated with an ethereal solution of diazomethane at room temperature for 48 h. Each of the products obtained was hydrolyzed with 8-10 ml of 6% H_2SO_4 at the boil for 6 h. The reaction mixture were extracted with chloroform, and the chloroform extracts were subjected to TLC in system 3. The chromatographs showed the presence of methyl oleanolate in the case of ladyginoside A and of the methyl ester of hederagenin in the case of ladyginoside B.

Alkaline Hydrolysis of Ladyginosides A and B. In each case, a solution of 20 mg of the glycoside in 10 ml of methanol was treated with 6 ml of a 10% aqueous ethanolic (1 : 1) solution of caustic soda, and the mixture was heated at 100°C for 6 h. After cooling, the reaction mixture was neutralized with acetic acid. Chromatography of the neutral solutions in a thin layer of silica gel in system 2 showed that neither glycoside had undergone any change.

Periodate Oxidation of Ladyginosides A and B. In each case, 50 mg of the glycoside was oxidized with a 1% solution of sodium periodate at 6°C for 48 h. After ethylene glycol had been added to decompose the excess of periodate, the reaction mixture was evaporated with butanol in vacuum, the salts that had precipitated were separated off, and the reaction product was hydrolyzed with 6% H_2SO_4. No free monosaccharides were detected in the neutralized and purified hydrolyzate by the TLC method in system 4 and by the PC method in system 7.

Hakomori Methylation of Ladyginosides A and B [10]. With stirring, 50 mg of sodium hydride was added in portions to a solution of 50 mg of ladyginoside A in 3 ml of dimethyl sulfoxide over 30 min, and then 4 ml of methyl iodide was added. Stirring was continued for 2 h. Then the reaction mixture was poured into 30 ml of a saturated aqueous sodium thiosulfate and was extracted with chloroform (5×10 ml). The chloroform extract was washed with water and concentrated. The residue was shown by TLC in system 6 to contain a mixture of completely and partially methylated products. To free the mixture from the latter, it was deposited on a column of alumina and was eluted with benzene, 10-ml fractions being collected. The separation was monitored by TLC in system 5. Fractions 6-10 contained the completely methylated glycoside. The methylated product was treated with 3 ml of 6% H_2SO_4 in methanol and heated at 100°C for 3 h. Then the solution was diluted with water, and the aglycone was filtered off. The filtrate was neutralized with AV-17 anion-exchange resin (OH^- form). Using markers, 2,3,4,6-tetra-O-methyl-D-glucose and 2,3-di-O-methyl-D-glucuronic acid were identified in the hydrolyzate by the PC method in system 8 and by TLC in system 5. Methyl oleanolate was found as the genin of the permethylate of ladyginoside A.

Ladyginoside B (42 mg) was methylated by the same method. After the hydrolysis of the fully methylated glycoside, 2,3,4,6-tetra-O-methyl-D-glucose, 2,3-di-O-methyl-D-glucuronic acid, and the methyl ester of 23-O-methylhederagenin were found.

Reduction of the Permethylates of Ladyginosides A and B. Completely methylated ladyginoside A was reduced with an ethereal solution of lithium tetrahydroaluminate. The mixture was heated at the boil for 6 h. The excess of $LiAlH_4$ was destroyed with ethyl acetate and 2% H_2SO_4. The ethereal layer was washed with water and concentrated. The permethylate of ladyginoside B was subjected to precisely the same treatment. Each of the residues was hydrolyzed with 6% H_2SO_4, and the precipitate that deposited was filtered off. 2,3,4,6-Tetra-O-methyl-D-glucose and 2,3-di-O-methyl-D-glucose were identified in the hydrolyzate by PC in system 8. The precipitates were washed with water and dried. It was established by the TLC method in system 3 in comparison with reference samples that the aglycone of the reduced permethylate of ladyginoside A was erythrodiol and that of ladyginoside B was 28-hydroxy-23-methoxy-β-amyrin.

SUMMARY

It has been established that ladyginoside A is the O-β-D-glucopyranosyl-(1 → 4)-β-D-glucuronopyranoside of oleanolic acid and ladyginoside B is the O-β-D-glucopyranosyl-(1 → 4)-β-D-glucuronopyranoside of hederagenin.

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