THE GLYCOSIDES OF APOCYNUM ANDROSAEMIFOLIUM L.
II. THE STRUCTURE OF APOBIOSIDE

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Khimiya prirodnykh soedinenii, Vol. 1, No. 1, pp. 15-22, 1965

The roots of the plant Apocynum androsaemifolium L. contain a new steroid diglycoside, apobioside, which has the structure cannogenin-(3)-β-D-cymaropyranoside-(4)-β-D-glucopyranoside.

It has previously been reported [1] that the roots of Apocynum androsaemifolium L. (androsaemifolium dogbane) of the Apocynaceae family contain the cardiac glycosides k-strophanthin-β and cymarin. Continuing the previous investigations, the present authors found that an alcohol extract of the root of the plant contains yet another glycoside, not described in the literature, which has been named apobioside. It has the formula $C_{36}H_{54}O_{13}$, mp 216-218°, and $[\alpha]_D^{20}$ 15.7 ± 1.5° (in methanol).

The IR spectrum of the glycoside contains bands corresponding to a hydroxyl group (3440 cm^{-1}), a butenolide ring (1780, 1750, and 1630 cm^{-1}), and an aldehyde group (2700 and 1725 cm^{-1}). The UV spectrum exhibits a distinct absorption maximum (λ_{max} 218 mμ, log ε 4.16) characteristic of a lactone ring, and the absorption band of an aldehyde group at about 300 mμ, diffuse and sloping in shape. The spectrum in concentrated sulfuric acid contains two clearly defined maxima at 240 mμ (log ε 4.30) and 430-440 mμ (log ε 4.15) reminiscent of the principal absorption maxima of two compounds closely related to strophanthidin, pachygenin and cannogenin. These maxima can hardly be ascribed to strophanthidin, since its most intense absorption maximum is at 410-415 mμ [2] and not 430-440 mμ. Besides the above two bands, the spectrum of apobioside in sulfuric acid exhibits an intense absorption band at 330 mμ (log ε 4.06), which must be ascribed to a 2-hydroxy sugar [3] in the glycoside molecule.

With acetic anhydride the glycoside gives a crystalline acetyl derivative, and measurement of the total integral carbonyl absorption in the IR spectrum indicates the presence of four acetyl groups.

The structure of apobioside was determined by the following reactions. The pancreatic juice of the snail Helix plectotropis hydrolyzes apobioside to give a monoglycoside and a sugar. The latter, isolated by paper chromatography (in three different systems), has been identified as D-glucose, and it is this which gives the 330 mμ maximum in the spectrum of apobioside in sulfuric acid [Fig. 1].

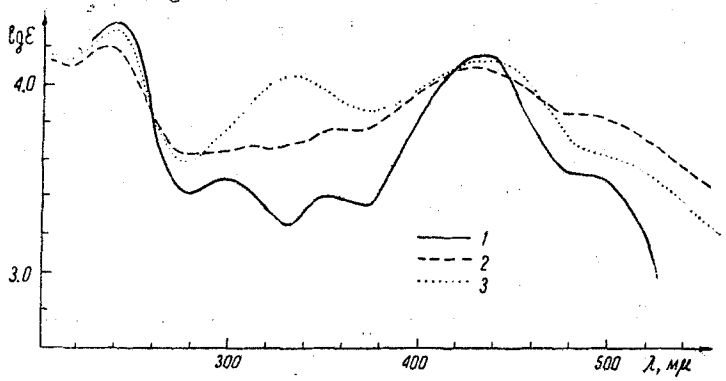


Fig. 1. Absorption spectra in concentrated sulfuric acid: 1) Cannogenin; 2) Apocannoside; 3) Apobioside.

The formula of the monoglycoside is $C_{30}H_{44}O_8$, mp 128-132°, $[\alpha]_D^{20}$ − 10.5 ± 2° (in methanol) with UV absorption spectrum maxima in alcohol at 216 mμ (log ε 4.20) and 296 mμ (log ε 1.54). These values are rather close to the physico-chemical constants for apocannoside isolated from the roots of Apocynum cannabinum L., and first described by Trabert [4, 5]. Direct comparison by paper chromatography of two specimens of apocannoside* from the two related plants confirmed their identity. Hydrolysis of apocannoside in 0.1 N sulfuric acid produces cannogenin and D-cymarose. Direct hydrolysis of apobioside with dilute sulfuric acid gives cannogenin and a disaccharide. From the paper chromatography R_f value of the latter, its absorption spectrum in sulfuric acid [3], and comparison of the physico-chemical properties of penta-acetates, it was identified as the sugar moiety of k-strophanthin-β, viz. strophanthobiose. [6]. This latter is identical with the disaccharide of periplogenin, periplobiose [7], which has the structure 4-O-(β-D-glucopyranosid-D-3-O-methyldigitoxypyranose [8], or, to put it in another form, 4-O-(β-D-glucopyranosid)-

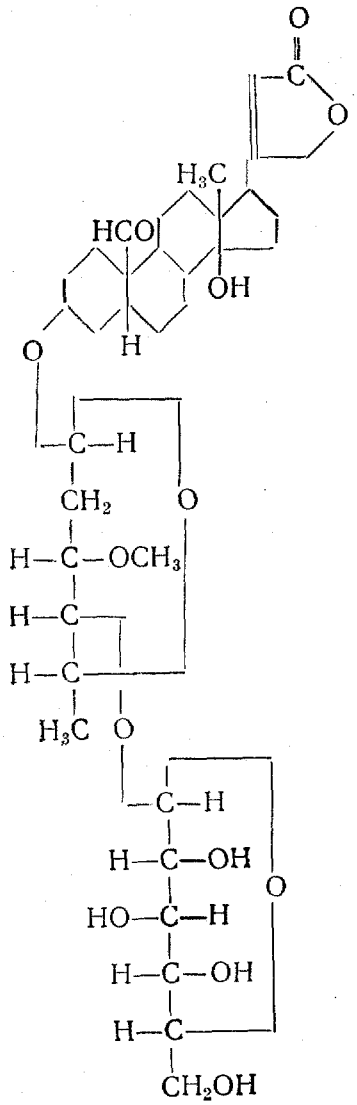
* Dr. S. G. Trabert (Karlsruhe, German Federal Republic) and Prof. T. Reichstein (Basel, Switzerland), kindly supplied us with samples of apocannoside, cyanocannoside, and cannogenin from Apocynum cannabinum L.

D-cymaropyranose.

Substance	M Mol. wt.	$[\alpha]_D$ in methanol	$(M)_D = \frac{M \cdot [\alpha]_D}{100}$
Apobioside	694.83	− 15.7±2°	−109±14°
Apocannoside	532.68	− 10.5±2°	− 56±11°
Cannogenin	388.51	− 10.2±2°	− 40±8°
Optically active D-glucose moiety	—	—	− 53±25°
Optically active D-cymarose moiety	—	—	+ 16±19°
α-methyl-D-glucoside [9]	194.19	+158.9±?	+309±?
β-methyl-D-glucoside [9]	194.19	− 34.2±?	− 66±?
α-methyl-D-cymaroside [10]	176.22	+212.0±2°	373±3°
β-methyl-D-cymaroside [10]	176.22	?	+ 20° [11]

The existence of a β-glucoside linkage between D-glucose and D-cymarose is evident from the difference between the molecular rotations of apobioside and apocannoside, and from the known structure of strophanthobiose [8] (table). The existence of a β-glucoside linkage between D-cymarose and cannogenin is less certain. According to the results of Golab and coworkers [5], the absolute value of the specific rotation of cannogenin in chloroform is higher than that of apocannoside, which is rather extraordinary. According to the results of the present authors the specific rotations of apocannoside and cannogenin are approximately the same, and this is more definite evidence for the existence of a β-glucoside linkage for D-cymarose in apocannoside. The specific rotation of β-methyl-D-cyamaroside is not known, but its molecular rotation is known from approximate data [11].

Thus, apobioside is cannogenin-(3)-β-D-cymaropyranosyl-(4)-β-D-glucopyranoside, the structural formula being shown below.



EXPERIMENTAL

The IR spectra were measured using a UR-10 double beam spectrophotometer. The solid substance was mixed with KBr in the ratio 1 : 10, and the mixture compressed. UV and visible spectra were obtained on a SF-4 spectrophotometer. Measurements in sulfuric acid were begun 30 min after introducing the substance into the acid, and were made every 5 mμ [3]*.

Leningrad No. 2 Paper Mill Grade B paper was used for chromatographing, in conjunction with the following systems: -

1) Toluene-butanol (4:1)-water
2) Chloroform-ethanol (9:1)-formamide
3) Benzene-chloroform-formamide (7:5)
4) Butanol-pyridine-water (6:4:3)
5) Butanol-benzene-pyridine-water (5:1:3:3)
6) Butanol saturated with water.

Systems 4 and 5 are ascending ones, the rest descending. To save space only the numbers of the system will be given below. The visualizers used were Raimond's reagent for cardenolides, a mixture of equal volumes of 3% perchloric acid and a 1% solution of vanillin in ethanol, and a solution containing 0.4 g salicylic acid plus 0.5 ml O-toluidine in 10 ml ethanol for D-glucose. Melting points were determined in capillaries without emergent stem correction.

Isolation of apobioside. 7 kg of ground roots of A. androsaemifolium L. was extracted 10 times with methanol at room temperature. The total methanol extract was concentrated in a vacuum. The copious granular precipitate of triterpene acids which separated was removed and washed with methanol. The washings were added to the main extract, which was then evaporated to 1.5 liters and diluted with an equal volume of water. Inert materials were precipitated by washing with an aqueous solution of lead acetate (130 g), excess lead ions being precipitated by an ammonium sulfate solution. The water-methanol abstract was then evaporated to 2 *l*, and successively extracted with equal volumes of ether (twice), chloroform (four times), and exhaustively with chloroform-methanol (2:1 by volume):

17.5 g of the dry extract obtained by evaporating the chloroform fraction was mixed with 50 g alumina, and poured on to a column with 200 g of alumina for chromatography. The column was washed with a mixture of equal volumes of

* The IR spectra were determined by Ya. V. Rashkes, and the UV and visible region spectra by S. D. Nikonovich.

benzene and butanol saturated with water, and the glycosides divided into 16 fractions.

The qualitative composition of the glycosides in each fraction was determined by paper chromatography, using a toluene-butanol (4:1)-water (Fig. 2). The toluene-butanol (1:1)-water previously used for this purpose [12] was useless because the apobioside moved together with the cymarin. Fractions enriched in apobioside, but which still contained cymarin and traces of k-strophanthin-β, were combined (total weight 7.5 g) and resolved on a chromatography column consisting of 140 g alumina (activity grade II). The column was washed with benzene, chloroform, and then with chloroform containing increasing amounts of ethanol (1, 2, 3%, ...). The eluates were chromatographed on paper using solvent system 1. Subsequent washing of the column with chloroform containing 4-7% ethanol afforded fractions containing apobioside only. The yield of crude glycoside was 1.18 g.

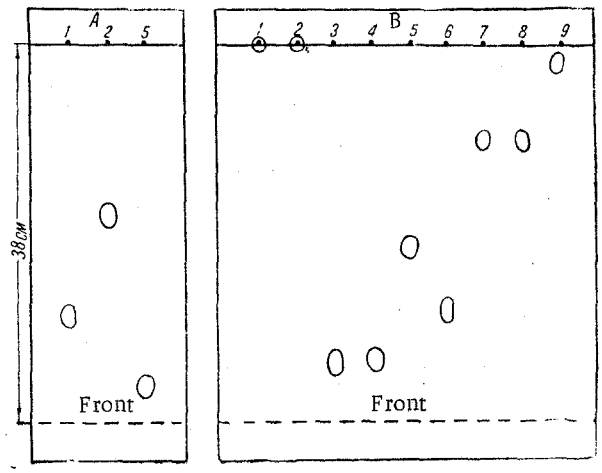


Fig. 2. Check paper chromatograms of cardenolides. Chromatogram A, toluene-butanol (4:1)-water, 6 hr, 20°. Chromatogram B, benzene-chloroform (7:5)-formamide, 3.5 hr., 20°. 1) Apobioside; 2) k-strophanthidin-β; 3) Apocannoside from A. androsaemifolium; 4) Apocannoside from A. cannabinum; 5) Cymarin; 6) Cynocannoside; 7) Cannogenin from A. androsaemifolium; 8) Cannogenin from A. cannabinum; 9) Strophanthidin.

Apobioside. Glycoside which was chromatographically homogeneous but still amorphous was dissolved in a small amount of ethanol and reprecipitated by adding ether to the alcohol solution until turbidity appeared. The bulk of the material came down as viscous oily drops, but a small quantity of stellate crystals appeared on the walls of the vessel, and in subsequent reprecipitations these were used for seeding. The glycoside was reprecipitated by ether from an ethanol solution until quite homogeneous needle-shaped crystals were obtained, and after drying in a vacuum over boiling toluene the apobioside had mp 216-218°, $[\alpha]_D^{20}$ − 15.7 ± 1.5° (c 2.09, methanol). Apobioside was readily soluble in methanol and ethanol, slightly soluble in water, and insoluble in benzene and ether. It gave a negative Keller-Kiliani reaction, but a positive Webb-Levy reaction for a 2-desoxy sugar. The color reaction with 84% sulfuric acid was as follows: 0 min, green; 1 min, yellow-green; 2 min, brown-green; 30 min, brown; 1 hr, light brown.

The average lethal dose for cats was 0.154 mg/kg (6480 cat activity units); the activity for frogs was 30 550 frog activity units.

Found %: C 62.11, 62.04; H 8.22, 7.78. $C_{36}H_{54}O_{13}$. Calculated %: C 62.25; H 7.83.

Tetra-O-acetylapobioside. 200 mg apobioside were acetylated with 1.2 ml acetic anhydride in 2 ml pyridine by heating at 37° for 24 hr. The product was treated in the usual way and recrystallized to give needle-shaped crystals with mp 168-171°, $[\alpha]_D^{20}$ − 16.7 ± 1° (c 1.83, methanol).

Found %: C 61.50; H 7.45. $C_{44}H_{62}O_{17}$. Calculated %: C 61.20; H 7.24.

Enzymatic hydrolysis of apobioside to apocannoside and D-glucose. A solution of 200 mg apobioside in 1.5 ml ethanol was poured into 100 ml water containing 0.3 ml of the pancreatic juice of Helix plectrotropis, and the reaction mixture held at 37° for 3.5 days. Following this, the solution was vacuum dried, the residue dissolved in 4 ml ethanol, the enzymes precipitated in the residue filtered off, the filtrate diluted with 5 ml water and then extracted a few times with chloroform. The residue, after distilling off the chloroform, was dissolved in 1 ml methanol, the solution filtered and diluted with a few milliliters of ether until turbidity appeared. On standing, needle-shaped crystals of apocannoside came down, yield 108 mg.

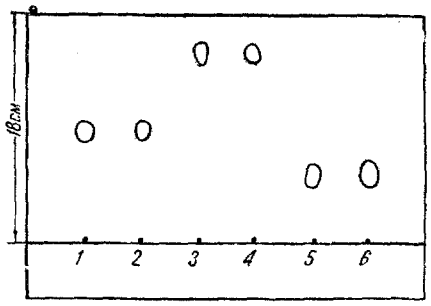


Fig. 3. Check paper chromatograms of sugars. Butanol-benzene-pyridine-water, 16 hr, 20°, 1. Strophanthobiose from apobioside; 2. Strophanthobiose from k-strophanthin-β; 3. D-cymarose from apocannoside; 4. D-cymarose from cymarin; 5. D-glucose from apobioside; 6. D-glucose control.

The water-ethanol solution remaining after extracting the apocannoside with chloroform was concentrated and paper chromatographed. A single sugar was found in the hydrolyzate, using solvent systems 4, 5, and 6, and this sugar moved forward at the same rate as D-glucose (Fig. 3).

Apocannoside. Glycoside twice recrystallized from methanol-ether formed long needles mp 128-132°, $[\alpha]_D^{20}$ − 10.5 ± 2° (c 2.09; methanol. The substance was readily soluble in methanol, ethanol, chloroform, slightly soluble in water, and insoluble in ether. It gave a positive Keller-Kiliani reaction. The color reaction with 84% H_2SO_4 was: 0 min, brown-yellow; 5 min, brown-yellow, edges yellow-green; 10 min, brown, edges yellow; 30 min, dark brown; 1 hr, black. Principal IR spectrum frequencies: 3460 (OH group); 2730, 1720 (aldehyde group); 1782, 1748, and 1630 (butenolide ring) cm^{-1}. Absorption maxima in concentrated H_2SO_4 (log ε in parentheses): 235 (4.22); 355 (3.78); 425-485 (3.90) mμ (Fig. 1).

When apocannoside obtained by the hydrolysis of apobioside was paper chromatographed using solvent systems 2 and 3, it moved at the same rate as apocannoside isolated from A. cannabinum [4, 5] (Fig. 2).

Found %: C 67.35, 67.59; H 8.70, 8.74; OCH_3 6.20. $C_{30}H_{44}O_8$. Calculated %: C 67.65; H 8.34; OCH_3 5.80.

Hydrolysis of apocannoside with 0.1 N H_2SO_4 gave cannogenin, identified by paper chromatography using solvent systems 2 and 3, together with D-cymarose, identified using solvent systems 4 and 5 (Fig. 3).

Hydrolysis of apobioside to cannogenin and strophanthobiose. 200 mg apobioside, 4 ml methanol, and 6 ml 0.1 N H_2SO_4 were heated for 30 min on a steam bath. The solution was cooled and repeatedly extracted with chloroform. The chloroform solution was washed with water until neutral and evaporated to dryness, and this produced 96 mg of an amorphous powder, cannogenin.

The acid solution remaining after chloroform extraction of cannogenin was neutralized with freshly precipitated $BaCO_3$. The precipitate of $BaSO_4$ was filtered off, and the filtrate vacuum dried. The residue was dissolved in 2 ml water, filtered to remove the sediment, and the filtrate again evaporated to dryness. The resultant white powder was dissolved in hot acetone, and on cooling needle-shaped crystals of strophanthobiose precipitated.

Cannogenin was recrystallized by covering an acetone solution with ether which had been carefully freed from peroxides, when small flaky crystals were obtained, mp 141-145°, $[\alpha]_D^{20}$ − 10.9 ± 2° (c 2.39, chloroform), $[\alpha]_D^{20}$ − 10.2 ± ± 2.5° (c 1.71, methanol). The genin rapidly autoxidized in solution. Color reaction with 84% H_2SO_4: 0 min, lemon-yellow; 1 min, yellow, light edges; 5-10 min, light orange; 30 min, almost colorless; 1 hr, colorless. Absorption maxima in concentrated H_2SO_4 (log ε in parentheses): 240 (4.35); 355 (3.46); 435-440 (4.21)mμ (Fig. 1). Paper chromatography using solvent systems 2 and 3 showed that the cannogenin obtained by hydrolysis of apobioside was the same as the genin of apocannoside [4, 5] (Fig. 2).

Strophanthobiose obtained by hydrolyzing apobioside did not melt sharply even after repeated crystallization. It sintered at 120°, gave a turbid melt at 138-140°, and ran to a clear liquid at 170-175°, $[\alpha]_D^{20}$ +32.4 ± 1.5° (c 1.48, water). It proved impossible to obtain the high-melting forms of strophanthobiose [6, 8]. The Keller-Kiliani reaction was negative, but the Webb-Levy reaction positive. With solvent systems 4 and 5, strophanthobiose obtained by hydrolyzing apobioside and the strophanthobiose prepared from k-strophanthin-β moved at the same rate (Fig. 3). The absorption spectra of the strophanthobioses from the two sources in concentrated sulfuric acid coincided, and showed maxima at 260 (3.32); 335 (3.96); 400 (3.28) and 450-460 (2.90) mμ (log ε in parentheses).

Tetra-O-acetylstrophanthobiose. 80 mg strophanthobiose obtained by hydrolyzing apobioside was acetylated by treating for 24 hr at 37° with 3 ml acetic anhydride in 3.2 ml pyridine. The pyridine and excess acetic anhydride were distilled off in a vacuum, leaving the bottom of the vessel covered with small needle-shaped crystals, which after recrystallization from anhydrous alcohol had mp 160-162°, $[\alpha]_D^{24}$ + 12.9 ± 1.5° (c 2.17, chloroform). There was no depression of the melting point on mixing with an authentic specimen of tetra-O-acetylstrophanthobiose (mp 165°), and the absorption spectra in concentrated sulfuric acid were identical.

Found %: C 51.40, 51.60; H 6.36, 6.30. $C_{23}H_{34}O_{14}$. Calculated %: C 51.68; H 6.41.

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13 July 1964

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