THE FLAVONOIDS OF THE MILK VETCHES

Astragaloside

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Continuing an investigation of the flavonoid composition of Astragalus pubiflorun D.C. [1], we have isolated the total flavonoid compounds, which consist of two substances.

A new flavonoid glycoside, which we have called astragaloside (I) was obtained by chromatography on a column of polyamide sorbent [2]. A chemical study of astragaloside has shown that it is a derivative of isorhamnetin; its sugar component is a biose (V) consisting of 2 molecules of glucose (IV). The results of Table 1 permit the assumption that in astragaloside, its monoside, and the aglycone, free hydroxy groups are present in the 5, 7, and 4' positions, while in the aglycone there is an additional one in position 3.

Table 1

Physicochemical Properties of Astragaloside and its Derivatives

Properties	Astragaloside	Isorhamnetin glucoside	Aglycone	Isorhamnetin
Mp, °C	200–204	168–172	304–307	305–307
Formula	$C_{28}H_{32}O_{17} \cdot 2H_2O$	$C_{22}H_{22}O_{12}$	$C_{16}H_{12}O_7$	$C_{12}H_{12}O_7$
$[\alpha]_D^{20}$, deg	−56.6 (c 0.129; dimethylformamide	—	—	—
Mol. wt.	676	478	316	316
Qualitative reactions (coloration)				
Bryant's cyanidin reaction [3]	Red (in water)		Red (in octanol)	
Taubӧcks-Wilson reaction [4, 5]	Yellow			
with ferric chloride	Dark green			
with zirconyl nitrate and citric acid [6]	Negative		Positive	
Rf in systems*				
1	0.76	0.65	0.07	0.07
2	0.24	0.56	0.72	0.72
3	0.70	0.58	0.81	0.81

*1) 25% acetic acid; 2) butan-1-ol–acetic acid–water (4:1:5); 3) ethyl acetate–formic acid–water (10:2:3).

The negative reaction of astragaloside and the monoside with zirconyl nitrate in the presence of citric acid and the positive reaction of the aglycone show that the hydroxy group in position 3 has been replaced by the carbohydrate component.

To determine the free hydroxy groups and the position of the carbohydrate substituent in the glycoside more accurately, we carried out a spectroscopic investigation in the UV region using ionizing and complex-forming reagents [8, 9]. As can be seen from Table 2, the glycoside and aglycone reveal free hydroxy groups in position 7 (from the bathochromic shift of the long-wave band by 10 mμ under the influence of sodium acetate), in position 5 (from the bathochromic shift of 50 mμ with zirconyl nitrate and the absence of a shift with zirconyl nitrate and citric acid), and in position 4' (from the 70-mμ bathochromic shift of the long-wave band under the influence of sodium methoxide). Thus, the data presented confirm the assumption that the glycoside has free hydroxy groups in positions 5, 7, and 4' and a carbohydrate substituent in position 3, and the aglycone has, in addition, a hydroxy group in position 3 (from the 80-mμ bathochromic shift of the long-wave band under the influence of zirconyl nitrate and the 50-mμ shift with zirconyl nitrate and citric acid).

The IR spectrum of astragaloside (Fig. 1), taken in a UR-10 spectrograph, has absorption bands characteristic for hydroxy groups (3300, 3380, 3560 cm^{-1}), for a carbonyl group (1660 cm^{-1}), and for a methoxy group (2945 cm^{-1}) [10].

Stepwise acid hydrolysis with 10% hydrochloric acid and 10% acetic acid and also enzymatic hydrolysis with a preparation from the fungus Aspergillus oryzae and rhamnodiastase gave a flavonoid glycoside which proved to be isorhamnetin 3-glucoside (II). From its physicochemical properties, the products of alkaline degradation [phloroglucinol (VI) and vanillic acid (VII)], and its chromatographic behaviour, the aglycone obtained on acid hydrolysis was identified as isorhamnetin (III) (cf. Table 1).

Table 2

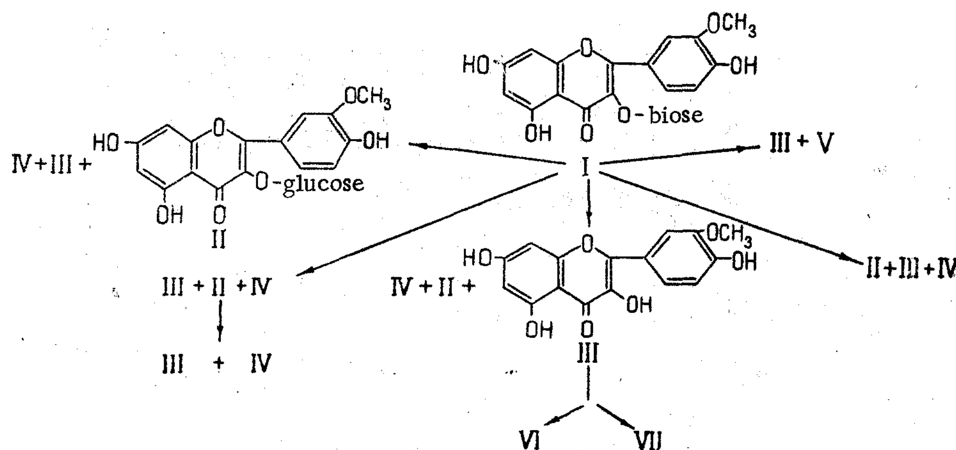
Spectroscopic Characteristics of Astragaloside and its Aglycone

Medium	Bands	Astragaloside		Aglycone	
		mμ			
		λ_{max}	$\Delta\lambda$	λ_{max}	$\Delta\lambda$
2×10^{-5} M in abs. ethanol	I	355	—	375	—
	II	255,266	—	254,266	—
The same + sodium acetate	I	365	10	385	10
	II	255,266	0	256,266	2
The same + zirconyl nitrate	I	405	50	455	80
	II	264	2	270	4
The same + zirconyl nitrate + citric acid	I	355	0	425	50
	II	255	0	256	2
The same + sodium ethoxide	I	425	70	425	70
	II	255	0	256,266	2

To determine the position of attachment of the hydrocarbon moiety to the aglycone and to establish the arrangement of the bond between the sugars, the glycoside was oxidized with hydrogen peroxide in an ammoniacal medium and the resulting products were identified by their coloration with specific reagents.

The biose produced by hydrogen peroxide oxidation was compared with respect to its chromatographic behaviour with authentic samples of the bioses and monoses most frequently found in flavone glycosides. The coloration of the biose of astragaloside with diphenylamine reagents apparently shows a 1–6 bond between the sugars [11], which is also confirmed by the hydrolysis of astragaloside with rhamnodiastase [12].

It has been established that astragaloside and its monoside are hydrolyzed by an enzyme from the fungus Aspergillus oryzae and rhamnodiastase, which is specific for a β-glycosidic bond. This enables us to assume that astragaloside is glycosidated in position 3 with O-6-β-D-glucosyl-D-glucoside), and its reactions can be shown by the following scheme:



Experimental

Isolation of astragaloside. 170 g of the air-dried flowers of Astragalus publiflorus D.C. gathered in the Zaporozh'e Oblast (May-June 1965) was exhaustively extracted with 2-*l* portions of ethanol. The combined ethanolic extracts were evaporated under vacuum. The residue was treated with hot distilled water and left for 12 hr. The precipitates that had deposited were filtered off. To purify the aqueous extract, it was treated with petroleum ether and then with chloroform.

The total flavonoids separated out at the interphase between the layers in the form of a yellow powder. Chromatographic analysis on paper (Leningrad type "M") in 15% acetic acid and butan-1-ol—acetic acid—water (4:1:5) showed that the total product contained two glycosides of flavonoid nature with R_f 0.68 and 0.37, and 0.24 and 0.64, respectively. Recrystallization of the total flavonoids from ethanol gave a crystalline precipitate in which one flavonoid with R_f 0.68 and 0.24 was detected chromatographically.

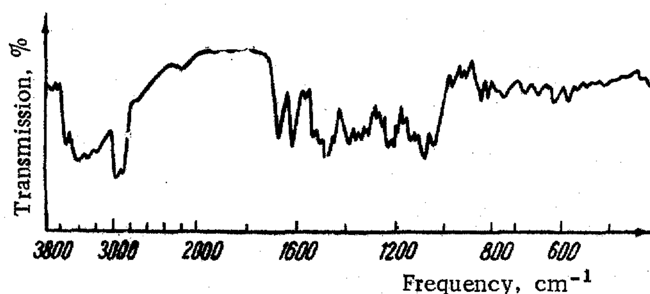


Fig. 1. IR spectrum of astragaloside (mull of the substance in paraffin oil).

For further purification, the precipitate was dissolved in the minimum amount of ethanol and mixed with kapron powder. The mixture was left until the solvent had evaporated off and was then chromatographed on a column of polyamide. Elution with 50% ethanol gave eluate fractions with a dark brown fluorescence in UV light. The eluates were combined and evaporated in vacuum to small bulk. Crystals of the flavonoid deposited, and these were separated off and recrystallized from methanol. The flavonoid consisted of fine yellow acicular crystals readily soluble in dimethylformamide, pyridine, methanol, dioxane, and ethanol, and sparingly soluble in water, chloroform, and petroleum ether. Mp 200°–204° C (Kofler block).

Found, %: C 49.83; 50.07; H 5.59; 5.64; OCH_3 6.51. Calculated for $C_{28}H_{32}O_{17} \cdot 2H_2O$, %: C 49.70; H 5.32; OCH_3 4.58.

Stepwise acid hydrolysis of astragaloside. A solution of 0.05 g of astragaloside in 20 ml of 50% methanol containing 10% of hydrochloric acid was heated in a boiling water bath for 6 hr, the course of the hydrolysis being followed chromatographically on paper in the isopropanol—formic acid—water (2:5:5) system every 5 min for 30 min, every 10 min for 1 hr, and every 30 min subsequently. When the products of the acid hydrolysis were analyzed chromatographically, an intermediate substance with R_f 0.34 was found, appearing 5 min after the beginning of hydrolysis. This is probably a monoside of isorhamnetin. To determine the properties of this substance, we isolated it from the products of acid hydrolysis by preparative paper chromatography. It formed yellow crystals with mp 168°–172° C from aqueous ethanol.

Found, %: C 55.14; 55.13; H 4.50; 4.53, OCH_3 6.53. Calculated for $C_{22}H_{22}O_{12}$, %: C 55.23; H 4.60; OCH_3 6.48.

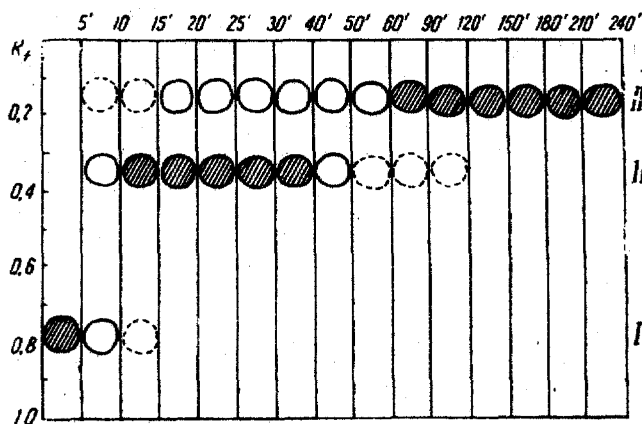


Fig. 2. Scheme of the chromatograms of the stepwise acid hydrolysis of astragaloside in the isopropanol—formic acid—water (2:5:5) system.

The enzymatic hydrolysis of the substance obtained with a preparation from the fungus Aspergillus oryzae and with rhamnodiastase led to the formation of isorhamnetin and D-glucose. In the acid hydrolysis of astragaloside, in addition

to the intermediate substance, the aglycone with R_f 0.16 (Fig. 2) appeared after 10 min.

The isolated aglycone was filtered off and recrystallized from 96% ethanol. It formed small yellow acicular crystals, mp 304°–307° C.

Found, %: C 60.77; 61.01; H 3.83; 4.03; OCH_3 9.78. Calculated for $C_{16}H_{12}O_7$, %: C 60.75; H 3.79, OCH_3 9.81.

The substance obtained was chromatographically homogeneous; it was identified by qualitative reactions, spectroscopic characteristics, and a mixed melting point test as isorhamnetin (Table 1). The acid hydrolysate after neutralization with KU-2 ion exchanger in the OH form was analyzed for its content of sugars by paper chromatography in the butan-1-ol–acetic acid–water (BAW) (4:1:5) system. The analysis showed the presence of an isorhamnetin glucoside, isorhamnetic, and glucose. The quantitative hydrolysis of 0.4230 g of astragaloside with 10% hydrochloric acid solution gave 0.1977 g of the aglycone, which corresponds to 46.73% (calculated 46.74%).

Enzymatic hydrolysis of astragaloside. With heating, 0.03 g of astragaloside was dissolved in 5 ml of methanol, and the solution was diluted with water, treated with 0.03 g of an enzyme preparation from the fungus *Aspergillus oryzae*, and left for 24 hr at 33° C. After the hydrolysis, the enzyme was precipitated by boiling, the precipitate was filtered off, and the filtrate was analyzed in the BAW (4:1:5) system. An isorhamnetin glucoside, isorhamnetin, and D-glucose were found.

Enzymatic hydrolysis of astragaloside with the enzyme of rhamnodiastase. A solution of 0.01 g of astragaloside in the minimum amount of methanol was diluted with water, a solution of rhamnodiastase in water (0.01 g) was added, and fermentation was carried out at 38° C for 24 hr. The hydrolysis products were analyzed by chromatography in the BAW (4:1:5) system. An isorhamnetin glucoside, isorhamnetin, and D-glucose were found.

Oxidation of astragaloside. A solution of 0.01 g of astragaloside in 2 ml of methanol was treated with 1 ml of a 0.1 N solution of ammonia and then with 0.4 ml of a 30% solution of hydrogen peroxide; after 4 hr freshly prepared lead sulfide was added to decompose the excess of hydrogen peroxide. The precipitate was filtered off, washed with water, and heated for 5 min with 0.5 ml of ammonia (d = 0.880) [12]. The solution was evaporated and analyzed for its content of sugars in the BAW system with authentic samples of cellobiose, lactose, and glucose. The chromatograms were visualized with aniline phthalate, diphenylamine + urea, and diphenylamine + p-anisidine (Table 3). A biose was found.

Alkaline degradation of the aglycone. A solution of 0.03 g of the aglycone in 30 ml of 20% caustic potash was heated in the boiling water bath for 30 min and was then cooled and neutralized with 10% sulfuric acid to pH 4–5, after which it was extracted with five or six 10-ml portions of ether. The ethereal extracts were evaporated and chromatographed in the benzene–ethyl acetate–acetic acid–formamide (23.5:74.5:2:1) system. The products of alkaline degradation were identified as phloroglucinol and vanillic acid, which correspond to the structure of isorhamnetin.

Table 3

Sugar	R_f with respect to glucose*	Coloration in the following visualizing agents		
		Aniline phthalate	Diphenylamine + p-anisidine	Diphenylamine + urea
Glucose	1.00	Brown	Green	Pink
Lactose	0.32	Brown	Blue green	Orange-brown
Cellobiose	0.38	Brown	Blue green	Orange-brown
Biose of astragaloside	0.44	Pink	Blue green	Bright pink

*In the BAW (4:1:4) system.

Summary

1. A new flavonoid glycoside that we have called astragaloside has been isolated from the flowers of *Astragalus publiflorus* D.C.; it has the structure of isorhamnetin 3-(β-D-glucosyl-6-β-D-glucoside).

2. Stepwise acid hydrolysis and enzymatic hydrolysis with *Aspergillus oryzae* has given a flavonol glycoside, isorhamnetin 3-β-D-glucoside.

3. The carbohydrate component is O-6-β-D-glucosyl-D-glucose.

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