

GLYCOSIDES OF *VACCARIA SEGETALIS*

## IV. Isosaponarin

V. I. Litvinenko, K. Amanmuradov, and N. K. Abubakirov

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Among the total extractable substances from the seeds of *Vaccaria segetalis* (Neck.) Carcke (family Caryophyllaceae), a flavonoid compound previously given the name of substance C has been found in addition to triterpene glycosides [1] by paper chromatography. Subsequently, a method was developed which enabled this compound to be obtained in the crystalline state without the use of chromatographic separation.

The compound that we have obtained gives a positive cyanidin reaction. The orange pigment formed in this reaction does not pass into the octanol layer [2]. The products of the acid hydrolysis of substance C reduce Fehling's solution. The water solubility and the nature of the coloration of the pigment after the cyanidin reaction and also the positive reaction with Fehling's solution showed that the substance under investigation was a flavone glycoside.

The red orange coloration with ferric chloride showed the presence of a complex-forming phenolic hydroxy group. The yellow coloration with zirconyl nitrate, disappearing in the presence of citric acid, showed that this hydroxy group is in the C-5 position [3]. The glycoside did not form precipitates with a solution of neutral lead acetate, a property of flavonoids with a 3', 4'-dihydroxy grouping [4].

Table 1  
Physicochemical Properties of the Flavonoid Glycoside from *V. segetalis* and its Derivatives

Substance	mp, °C	$[\alpha]_D^{18}$ , deg	M	Composition	$\lambda_{\max}$
Glycoside from <i>V. segetalis</i>	236-237	-92.0	578	$C_{27}H_{30}O_{14}$	$\left\{ \begin{array}{l} 335(\log \epsilon 3.97) \\ 272(\log \epsilon 3.93) \end{array} \right.$
Isosaponarin [5]	—	—	578	$C_{27}H_{30}O_{14}$	$\left\{ \begin{array}{l} 336 \\ 272 \end{array} \right.$
Aphroside [6]	242-243	—	578	$C_{27}H_{30}O_{14}$	$\left\{ \begin{array}{l} 336 \\ 272 \end{array} \right.$
Hydrolysis product (I)	260-262	+50.0	432	$C_{21}H_{20}O_{10}$	$\left\{ \begin{array}{l} 336(\log \epsilon 4.32) \\ 280(\log \epsilon 4.30) \end{array} \right.$
Saponaretin [7]	260-262	+55.0	432	$C_{21}H_{20}O_{10}$	$\left\{ \begin{array}{l} 336(\log \epsilon 4.25) \\ 280(\log \epsilon 4.28) \end{array} \right.$
Hydrolysis product (II)	263-265	-16.0	432	$C_{21}H_{20}O_{10}$	$\left\{ \begin{array}{l} 334(\log \epsilon 4.31) \\ 265(\log \epsilon 4.28) \end{array} \right.$
Vitexin [7]	264-265	-15.0	432	$C_{21}H_{20}O_{10}$	$\left\{ \begin{array}{l} 334(\log \epsilon 4.30) \\ 265(\log \epsilon 4.28) \end{array} \right.$
Aglycone of the glycoside from <i>V. segetalis</i>	343-345	—	270	$C_{15}H_{10}O_5$	$\left\{ \begin{array}{l} 336(\log \epsilon 4.40) \\ 270(\log \epsilon 4.38) \end{array} \right.$
Apigenin [7]	345-347	—	270	$C_{15}H_{10}O_5$	$\left\{ \begin{array}{l} 336 \\ 270 \end{array} \right.$

On acid hydrolysis of the flavone glycoside from *V. segetalis* two substances of a flavonoid nature differing from the initial compound were obtained together with a carbohydrate component which was identified chromatographically as D-glucose. The sum of the new flavonoid compounds formed on hydrolysis amounted to 73.0% of the weight of the initial glycoside. It was calculated that if one molecule of glucose was split off the weight of the residue should be 74.7%. Consequently, on acid hydrolysis only one molecule of D-glucose is split off from the flavone glycoside from *V. segetalis*. The flavonoid products of acid hydrolysis were isolated on a layer of polyamide and separated on a column of cellulose powder (Table 1).

Their qualitative reactions and chromatographic behavior showed that the hydrolysis products (I) and (II) also possessed the properties of glycosides. However, on ordinary acid hydrolysis the hydrolysis products were not split to form aglycones but were mutually isomerized, forming an equilibrium mixture of both substances. On enzymatic hydrolysis, both compounds remained unchanged. As is well known, such properties are those of glucoflavonoids or the so-called C-glycosides of flavonoids [8].

To determine the positions of the free phenolic hydroxy groups, we carried out a spectroscopic investigation of

glycoside C and the products of its hydrolysis in the UV region, with ionizing and complex-forming reagents [9, 10] (Table 2). On analyzing the spectra of the compounds considered, it can be seen that the maxima of the short- and long-wave bands are of almost equal intensity (see Table 1), which is characteristic for flavone compounds [11]. In the presence of sodium acetate a considerable bathochromic shift of the maximum of the long-wave band (by 44–60 m $\mu$ ) is found in the UV spectra of the glycosides and the aglycone, which is due to a free 7-hydroxy group. In all the substances, the second phenolic hydroxy group, judging from the bathochromic shift of the maximum of the long-wave band by 42–50 m $\mu$  on complex formation with zirconyl nitrate, is located at C-5 (Table 2). A bathochromic shift of the long-wave maximum under the influence of sodium acetate is found for all the compounds, but this shift of 64–69 m $\mu$  is accompanied by a considerable increase in intensity only in product (I) and in the aglycone, indicating, therefore, that the carbohydrate substituent in the glycoside C investigated is attached to a hydroxyl at C'-4.

Table 2  
Spectral Characteristics in the UV Region of Glycoside C from *V. segetalis* and its Derivatives (Maxima and Shifts, m $\mu$ )

Solutions and reagents	Glycoside C		Isosaponarin		Product (I)		Aglycone of glycoside C	
	$\lambda$	$\Delta\lambda$	$\lambda$	$\Delta\lambda$	$\lambda$	$\lambda\Delta$	$\lambda$	$\lambda\Delta$
2 · 10 <sup>-5</sup> M solution in anhydrous ethanol	335	—	336	—	336	—	336	—
	272	—	272	—	280	—	270	—
The same + sodium acetate	395	60	390	54	385	49	380	44
	270	—2	272	0	280	0	276	6
The same + sodium ethoxide	400	65	394	58	405	69	400	64
	270	—2	277	5	280	0	275	5
The same + zirconyl nitrate	380	45	378*	42	380	44	386	50
	275	3	278	6	282	2	276	6

\* The spectrum was taken in the presence of aluminum chloride [5].

The acid hydrolysis of products (I) and (II) by Kiliani's method [12] gave an aglycone with free hydroxy groups in the 5, 7, and 4' positions (cf. Table 2) and D-glucose with a small amount of D-arabinose. On alkaline degradation, the aglycone gave phloroglucinol and p-hydroxybenzoic acid and is therefore apigenin.

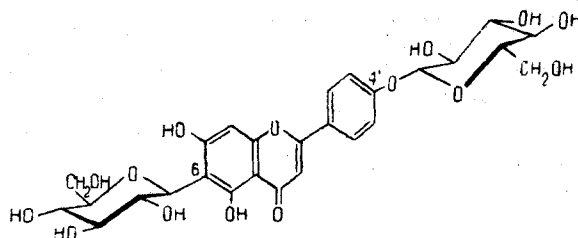
It follows from this that substances (I) and (II) are C-glycosides of apigenin and, consequently, they can be identified as saponaretin and vitexin [7], respectively (Table 1). Then glycoside C must be a 4'-glycoside of saponaretin or vitexin, since on acid hydrolysis of the glycoside a mixture of these substances was obtained. As already mentioned, on acid hydrolysis both hydrolysis products isomerize, being converted into one another. To answer the question of which of these compounds is the basis of the glycoside that we investigated, we compared their properties with those of known O-glycosides of saponaretin and vitexin—isosaponarin [5] and aphroside [6]. These glycosides were selected since both of them, like the glycoside described, are 4'-glucosides of glucoflavonoids. We ought to point out that aphroside was characterized by Shaleby et al., as vitexin 7-O-glucoside. However, on critically evaluating the spectral data given in the paper mentioned, we tend to consider that this glycoside is vitexin 4'-O-glucoside. Because of the inadequate information on isosaponarin and aphroside, the results of our comparisons did not lead to an unambiguous solution of this problem. We then carried out the enzymatic degradation of glycoside C. This gave us only one substance, which was identified as saponaretin. Consequently, the flavonoid glycoside from *V. segetalis* is saponaretin 4'-glucoside, i.e., isosaponarin.

This name was first given to a glucoside isolated from *Spirodela oligorrhiza* (family Lemnaceae) [5]. This flavonoid was found almost simultaneously in *Crataegus oxyacantha* (family Rosaceae) [13]. No physicochemical constants of the glycoside whatever were characterized. Its structure was established on the basis of a comparison of the absorption spectra in 95% ethanol with various additives. Geissman, et al., [5] considered that the aglycone of isosaponarin is vitexin. However, in Harborne's monograph [8] the aglycone of isosaponarin is given as saponaretin, but it is impossible to agree with this.

The structural features of the carbohydrate substituents were studied by means of differential analysis in the infrared region of the spectrum [14]. For this purpose, the spectrum of saponaretin was eliminated from the spectrum of isosaponarin and the spectrum of epigenin from that of saponaretin. It was found that the O- and C-glucosidic substituents in isosaponarin are present in the pyranose form (1027, 1050, and 1090 cm<sup>-1</sup>) and are attached to the nucleus

by a  $\beta$ -glucosidic link ( $893-895\text{ cm}^{-1}$ ).

Thus, isosaponarin is 5, 7, 4'-trihydroxyflavone (6)-C- $\beta$ -D-glucopyranosido-(4')-O- $\beta$ -D-glucopyranoside and its structural formula can be shown in the following form:



### Experimental

The following systems were used in paper chromatography: 1) 15% acetic acid; 2) 1-butanol-acetic acid-water (4:1:5); 3) benzene-ethyl acetate-acetic acid-formamide (24.5:73.5:2:1).

**Isolation of isosaponarin.** A solution of 10 g of the total extractive substances obtained by methanolic extraction of the defatted seeds of *V. segetalis* (mixture of saponins [1]) in 25 ml of methanol was treated with 50 ml of 5% hydrochloric acid and the solution was heated at  $100^{\circ}\text{C}$  for 15 min. The precipitate which formed (crude vaccaroside) was separated off. The dark mother liquor, on standing for several days at room temperature, deposited a yellow crystalline precipitate. After several recrystallizations from methanol and aqueous ethanol, the isosaponarin was obtained in the form of lustrous yellow needle-like crystals with mp  $236^{\circ}-237^{\circ}\text{C}$ ,  $[\alpha]_{\text{D}}^{18} -92^{\circ}$  (c 0.05; methanol).

Found, %: C 56.08, 56.18; H 5.22, 5.14. Calculated for  $\text{C}_{27}\text{H}_{30}\text{O}_{14}$ , %: C 56.05; H 5.19.

The decaacetate of isosaponarin was obtained by heating the glycoside with acetic anhydride in pyridine for 6 hr. The melting point of the needle-like crystals from ethanol was  $190^{\circ}-192^{\circ}\text{C}$ ,  $[\alpha]_{\text{D}}^{20} -51^{\circ}$  (c 0.3; chloroform). The substance gave no coloration with ferric chloride.

Found, %: C 56.60, 56.45; H 5.15, 5.08;  $\text{COCH}_3$  43.00, 43.12. Calculated for  $\text{C}_{47}\text{H}_{50}\text{H}_{24}$ , %: C 56.51; H 5.11;  $\text{COCH}_3$  43.08.

**Acid hydrolysis of isosaponarin.** 0.2 g of the glycoside from *V. segetalis* was dissolved in 20 ml of 50% ethanol containing 10% sulfuric acid. Hydrolysis was carried out in the water bath for 10 hr, the process being checked hourly by paper chromatography in system 1. After the solution had been diluted with water to 100 ml, the hydrolysis products were transferred to a column of polyamide sorbent. The column was washed with water to neutrality, and the flavonoids were eluted with ethanol. The combined ethanolic eluates were evaporated to dryness. Yield 0.146 g. The product was dissolved in 0.5 ml of glacial acetic acid and the solution was diluted with water to 3 ml and transferred to a column of cellulose powder ( $20 \times 400\text{ mm}$ ). The column was eluted with 15% acetic acid until the substances had been separated into two zones. Then the column was cut into two parts. The sorbent was eluted with water to neutrality and the flavones were eluted with 70% alcohol. On chromatography in systems 1 and 2, the substance eluted from the lower zone was identified with an authentic sample of saponaretin, and after recrystallization from aqueous ethanol it had mp  $260^{\circ}-262^{\circ}\text{C}$ ,  $[\alpha]_{\text{D}}^{18} +50.0^{\circ}$  (c 0.01; methanol).

Found, %: C 58.47, 58.28; H 4.44, 4.80. Calculated for  $\text{C}_{21}\text{H}_{20}\text{O}_{10}$ , %: C 58.33; H 4.63.

The upper zone yielded a substance chromatographically identical in systems 1 and 2 with vitexin, mp  $263^{\circ}-265^{\circ}\text{C}$ ,  $[\alpha]_{\text{D}}^{18} -16.0^{\circ}$  (c 0.01; methanol).

Found, %: C 58.35, 58.68; H 4.50, 4.72. Calculated for  $\text{C}_{21}\text{H}_{20}\text{O}_{10}$ , %: C 58.33; H 4.63.

The aqueous part of the hydrolyzate was neutralized with Amberlite IR-45 ( $\text{OH}^-$ ) ion exchange resin and was evaporated to 0.5 ml. The resulting solution was used to analyze the sugars in several systems of solvents. When the chromatograms were treated with aniline phthalate, only one spot was found, at the same level as D-glucose.

**Acid hydrolysis of saponaretin and vitexin.** Separately, 0.02 g of each of the C-glycosides obtained in the preceding experiment was suspended in 4 ml of a mixture of ten parts of concentrated hydrochloric acid, 3.5 parts of glacial acetic acid, and 5.5 parts of water. Hydrolysis was carried out on a water bath for 20 hr. The mixture was diluted with water to 10 ml and the aglycone was separated on a layer of polyamide sorbent. The two compounds gave one and the same aglycone, which, after recrystallization from aqueous ethanol, had mp  $343^{\circ}-345^{\circ}\text{C}$ . A mixture

of the aglycone and apigenin gave no depression of the melting point. On chromatography in systems 1 and 2, the substance that we had obtained was again identical with apigenin. The aqueous part of the hydrolyzate was neutralized with Amberlite IR-45(OH<sup>-</sup>), the solution was evaporated to dryness, and the residue was analyzed for sugars by paper chromatography. This showed the presence of D-glucose and a small spot of D-arabinose.

Fermentation of isosaponarin with emulsin. A solution of 0.1 g of the glycoside from *V. segetalis* in 10 ml of 50% ethanol was diluted with water to slight turbidity, 0.05 g of emulsin in 2 ml of water was added, and the mixture was left overnight. Then it was evaporated by boiling to 10 ml and ethanol was added until the separation of a precipitate ceased. The filtered solution was diluted with water to turbidity and passed through a column of Kapron. The adsorbed flavonoid was washed with water and eluted with ethanol. The ethanolic eluates were concentrated and the residue was crystallized from aqueous ethanol. This gave light yellow crystals with mp 261°–263° C,  $[\alpha]_D^{18} +52.0^\circ$  (c 0.01; methanol), identical with saponaretin on chromatography in systems 1 and 2. Analysis of the hydrolyzate for sugars showed the presence of D-glucose.

Alkaline degradation of apigenin. A solution of 0.01 g of the aglycone obtained by the acid hydrolysis of saponaretin and vitexin by Kiliani's method in 10 ml of 20% aqueous caustic potash was boiled for 3 hr. The reaction mixture was neutralized with 20% hydrochloric acid to pH 4–5. The degradation products were extracted with ether. The ethereal extract was washed with water, dried with anhydrous sodium sulfate, and evaporated to dryness. The dry residue was dissolved in a few drops of ethanol and analyzed by paper chromatography in system 3. When treated with diazotized sulfanilic acid and alkali, the chromatogram revealed two spots. The first was brown and at the same level as phloroglucinol and the second yellow at the same level as p-hydroxybenzoic acid.

### Summary

The glucoflavonoid glycoside isosaponarin has been isolated from the seeds of *Vaccaria segetalis*. It has been shown that isosaponarin has the structure 5, 7, 4'-trihydroxyflavone (6)-C-β-D-glucopyranosido-(4)-O-β-D-glucopyranoside.

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Khar'kov Chemical and Pharmaceutical Scientific  
Research Institute,  
Institute of the Chemistry of Plant Substances, AS UkrSSR