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INHIBITION OF HIGHLY PURIFIED PORCINE KIDNEY Na,K-ATPase BY STEROID GLYCOSIDES OF THE SPIROSTAN AND FUROSTAN SERIES AND A STUDY OF STRUCTURE–ACTIVITY RELATIONSHIPS

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A comparative investigation has been made of the action of 14 steroid glycosides of the spirostan and furostan series on highly purified Na,K-ATPase from the medullary layer of porcine kidneys (~90% purity in terms of protein). It has been shown that alliospirosides A, B, and D, isolated from the collective fruit of Allium sepa *L., are capable of inhibiting the activity of the Na,K-ATPase The inhibition of the activity of the transport enzyme by alliospirosides A and B is of the uncompetitive type and by alliospiroside D of the competitive type. It is desirable to test alliospirosides on the intact organism.*

The search for promising medicinal compounds is being conducted by determining their action on the animal organism as a whole, and also by using the enzyme method, which is based on an analysis of the inhibiting effect on Na,K-ATPase. The latter method arouses definite interest of research workers because of its economy and informativeness [1-6].

The enzyme method possesses a number of advantages [5, 6], since a specific activity undistorted by secondary factors is determined. Minute amounts of the compounds are sufficient for testing; at the same time, it is the direct mechanism of the interaction of the substances under test with the chosen enzyme that is investigated.

In the present work, as the target enzyme we selected highly purified Na,K-ATPase which has a direct effect on the active transport of Na^+ and K^+ ions through plasmatic membranes.

Modern biology and medicine connect the successful development of many theoretical and applied directions with the elucidation of the action of Na,K-ATPase. Drugs inhibiting the activity of Na,K-ATPase may find use in the treatment of diseases connected with a disturbance of the active tranport of ions the action of which is determined by the work of a transport enzyme. Such are diseases of the cardiovascular system, the kidneys, the immune system, and others [1-4, 7].

We have conducted a search for physiologically active compounds from the group of steroid glycosides of the spirostan and furostan series. Testing was carried out on highly purified preparations of Na,K-ATPase in the range of concentrations from $1 \cdot 10^{-4}$ M to $1 \cdot 10^{-7}$ M.

We investigated compounds isolated from the collective fruit of the Turkestan onion *Allium karataviense* Rgl. and the garden onion *Allium cepa* L., and products of the modification of karatavioside A. Of the 14 substances studied, 10 glycosides were steroids of the spirostan series (compounds **(1)**-**(10)**) and 4 were furostan glycosides (compounds **(11)**-**(14)**; Table 1). As was found, all the compounds studied affected the activity of highly purified Na,K-ATPase, being capable of inhibiting or, in some cases, activating it.

The following relationships were observed in the spirostan series: presence of a hydroxy group in ring F at C-24 led to a fall in the percentage inhibition of Na,K-ATPase. For example: karatavioside A (**1**) (19.8% inhibition)–karatavioside E (**3**) (1.7%); alliospiroside A (**5**) (99.7%)–alliospiroside C (activation; +13.4%); alliospiroside B (**6**) (76.3%)–allio-spiroside D (**8**) (67.1%).

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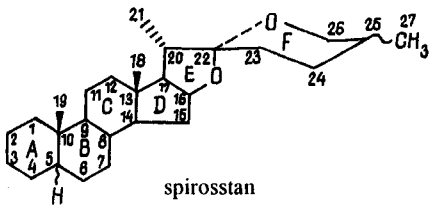
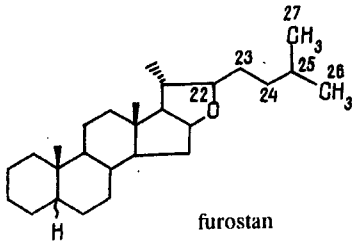
TABLE 1. Influence of Steroid Glycosides of the Spirostan and Furostan Series on the Activity of Highly Purified Na,K-ATPase (inhibition, %; +, activation, %)



spirosstan



furostan

	Trivial name, composition	Structure of the carbohydrate component	Concentration of the glycoside, M				Literature
			$1 \cdot 10^{-4}$	$1 \cdot 10^{-5}$	$1 \cdot 10^{-6}$	$1 \cdot 10^{-7}$	
1	2	3	4	5	6	7	8
		GLYCOSIDES OF THE SPIROSTAN SERIES					
		glycosides of yuccagenin — (25R)-spirost-5-ene-2α,3β-diol					
1.	Karatavioside A $C_{50}H_{80}O_{23}$	β-*D*-Glc$^{2}\rightarrow$β-*D*-Glc$^{4}\rightarrow$β-*D*-Gal$^{3}\rightarrow$(Ag)* $^{1}3$ β-*D*-Xyl lycotetraose (Lyc)	19,8	10,2	4,3	1,2	[9]

TABLE 1. (Continued)

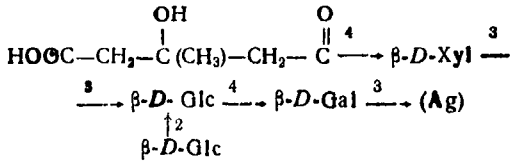
1	2		3	4	5	6	7	8
2.	Karatavioside $C_{56}H_{88}O_{27}$	B		32,4	19,4	7,3	4,3	[10]
			Glycosides of karatavigenin C — (24S,25S)-spirost-5-ene-2α,3β,24-triol					
3.	Karatavioside $C_{50}H_{80}O_{24}$	E	Lyc $\xrightarrow{3}$ (Ag)	1,7	+1,6	+1,3	+0,1	[12]
4.	Karatavioside $C_{56}H_{90}O_{29}$	F	Lyc $\xrightarrow{3}$ (Ag) $\xleftarrow{24}$ β-*D*-Glc	7,5	0,9	0,5	0,2	[12]
			Glycosides of (25S)-ruscogenin — (25S)-spirost-5-ene-1β,3β-diol					
5.	Alliospiroside $C_{38}H_{60}O_{12}$	A	α-*L*-Rha-$\xrightarrow{2}$ α-*L*- Ara $\xrightarrow{1}$ (Ag)	99,7	33,0	0,2	+0,9	[13]
6.	Alliospiroside $C_{39}H_{62}O_{13}$	B	α-*L*-Rha $\xrightarrow{2}$ β-*D*-Gal $\xrightarrow{1}$ (Ag)	76,3	10,2	0,6	+0,4	[14]
			Glycosides of cepagenin — (24S,25R)-spirost-5-ene-1β,3β,24-triol					
7.	Alliospiroside $C_{38}H_{60}O_{13}$	C	α-*L*-Rha $\xrightarrow{2}$ α-*L*- Ara $\xrightarrow{1}$ (Ag)	+13,4	+5,4	+3,8	+1,2	[15]
8.	Alliospiroside $C_{39}H_{62}O_{14}$	D	α-*L*- Rha $\xrightarrow{2}$ β-*D*-Gal $\xrightarrow{1}$ (Ag)	67,1	1,2	+0,8	+1,2	[15]
			Glycoside of alliogenone — 2α,3β,5-trihydroxy-(25R)-5α-spirostan-6-one					
9.	Alliogenone lycotetraoside $C_{50}H_{82}O_{25}$		Lyc $\xrightarrow{3}$ (Ag)	11,0	8,9	4,8	1,5	[16]
			Glycoside of alliogenin — (25R)-5α-spirostan-2α,3β,5,6β-tetraol					
10.	Lycotetraose Lycotetraose $C_{50}H_{82}O_{26}$		Lyc $\xrightarrow{3}$ (Ag)	7,8	0,6	+1,2	+0,7	[16]

TABLE 1. (Continued)

1	2	3	4	5	6	7	8
		GLYCOSIDES OF THE FUROSTAN SERIES					
		Glycoside of (22R,25R)-furost-5-ene-2α,3β, 26-triol					
11.	Dihydroyuccagenin lycotetraoside $C_{50}H_{82}O_{23}$	Lyc $\xrightarrow{3}$ (Ag)	10,0	+0,3	+0,2	0	[16]
		Glycoside of (25R)-furost-5-ene-2α,3β,22R,26-tetraol					
12.	Karatavioside C $C_{56}H_{92}O_{29}$	Lyc $\xrightarrow{3}$(Ag)$\xleftarrow{26}$ β-*D*-Glc	4,9	1,2	+3,4	+4,0	[11]
		Glycoside of (25S)-furost-5-ene-1β,3β,22R,26-tetraol					
13.	Alliospiroside $C_{44}H_{72}O_{18}$	α-*L*-Rha $\xrightarrow{2}$ α-*L*- Ara $\xrightarrow{1}$(Ag) $\xleftarrow{26}$ β-*D*-Glc	3,8	+1,9	+0,7	+0,2	[13]
		Glycoside of (22R,25R)-furostan-2α,3β,5α,6β,26-pentaol					
14.	Dihydroalliogenein lycotetraoside $C_{50}H_{84}O_{25}$	Lyc $\xrightarrow{3}$ (Ag)	2,5	1,8	0,9	0,3	[16]

*(Ag) — aglycon.

TABLE 2. Dependence of the Inhibition of the Activity of Highly Puridied Na,K-ATPase from Porcine Kidneys by Alliospirosides A, B, and D on the Concentration of ATP (inhibition, %; +, activation, %)

Compound	Concentration of the compound, M	Concentration of ATP, mM				
		1,00	0,50	0,33	0,25	0,20
5	$1 \cdot 10^{-5}$	33,0	12,3	—	+7,3	+8,4
	$2 \cdot 10^{-6}$	51,0	39,0	28,0	19,0	12,0
6	$1 \cdot 10^{-5}$	10,2	9,9	2,2	+10,4	+8,2
	$1,5 \cdot 10^{-6}$	21,8	11,9	2,0	+4,3	+5,5
	$3 \cdot 10^{-5}$	44,0	34,3	21,9	16,4	5,3
	$6 \cdot 10^{-5}$	65,9	54,3	47,3	46,9	33,3
8	$1 \cdot 10^{-5}$	1,2	2,5	4,9	19,8	32,4
	$2 \cdot 10^{-5}$	8,1	11,0	16,7	28,3	38,6
	$5 \cdot 10^{-5}$	28,1	30,0	37,1	40,4	45,6

A keto group at C-6 of the steroid part of the molecule slightly increased the inhibition of Na,K-ATPase: alliogenin lycotetraoside (**10**) (7.8%)—alliogenone lycotetraoside (**9**) (11.0%).

On passing from spirostan glycosides, which have a closed ring F, to compounds of the furostan series with an opened ring F and containing a hydroxy group at C-25 to which a sugar residue is attached, a decrease was observed in inhibition of Na,K-ATPase or, at certain concentrations, even its activation (Table 1): alliospiroside A (**5**) (99.7%)—alliofuroside (**13**) (3.8%)—karatavioside A (**1**) (19.8%)—karatavioside C (**12**) (4.9%).

It can be seen from Table 2 that the maximum inhibition was observed in the presence of alliospiroside a (**5**), with a somewhat weaker effect in thew cases of alliospirosides B (**6**) and D (**8**). For these compounds we determined the inhibition constants, J_{50}, which were $1 \cdot 10^{-5}$ M for alliospiroside A, $3.4 \cdot 10^{-5}$ M for alliospiroside B, and $8.8 \cdot 10^{-5}$ M for alliospiroside D.

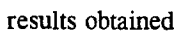
Except for karatavioside B (**2**), the other substances — (**1**), (**3**), (**4**), (**7**), and (**9**)-(**14**) (Table 1) inhibited Na,K-ATPase only slightly or, in some cases, conversely, caused its activation.

On the basis of what has been said above, for further work we selected alliospirosides A, B, and D.

To investigate the mechanism of the action of the group of steroid glucosides of the spirostan and furostan series the determination of the position of attachment to the Na,K-ATPase of the highly active compounds possessing an inhibiting effect is of great importance. We have made an attempt to elucidate the possibility of the attachment of these compounds to the active center of the enzyme. The competition of alliospirosides A, B, and D with ATP for the corresponding centers was studied (Table 2). For these purposes we determined the dependence of the effect of inhibiting the activity of the enzyme by these compounds on the concentration of ATP. For alliospiroside A (**5**) (at a concentration of the glycoside of $2 \cdot 10^{-5}$ M) an increase in the percentage inhibition from 12 to 51 was observed with a change in the concentration of ATP from 0.2 to 1.0 mM, respectively. At concentrations of glycoside (**5**) of $1 \cdot 10^{-5}$, however, the effect passed from activating (+8.4%) to inhibition (25.9%). For alliospiroside B (**6**), the dependence of the inhibitory effect on the concentration of ATP was somewhat weaker than for substance (**5**). At a concentration of compound (**6**) of $6 \cdot 10^{-5}$ M, a rise in the percentage inhibition from 33.3 to 65.9 was observed with a change in the concentration of ATP from 0.2 to 1.0 mM, respectively. At $3 \cdot 10^{-5}$ M, the change was from 5.3 to 44.0%; at $1.5 \cdot 10^{-5}$, from an activating effect (+5.5%) to 21.8% inhibition; and at $1 \cdot 10^{-5}$ M, from an activating effect (+8.2%) to 10.2% inhibition.

Thus, differences have been revealed in the interaction of alliospirosides A and B with the enzyme in high and low concentrations. In the presence of high concentrations of alliospirosides A and B a decrease in enzymatic activity was found for all levels of ATP, while at low concentrations of the compounds under study a decrease in the activity of Na,K-ATPase was characteristic only for high levels of ATP. Some stimulation of activity was observed in the region of low ATP levels.

For alliospiroside D (in a concentration of $5 \cdot 10^{-5}$ M) the opposite process was observed; i.e. there was a fall in the percentage inhibition from 45.6 to 28.1 with a change in the concentration of ATP from 0.2 to 1.0 mM, respectively.

The kinetics of the inhibition by steroid glycosides of the spirostan series of the activity of highly purified Na,K-ATPase were calculated (Fig. 1). The results obtained show that the magnitude  is nonmonotypical and depends on which compound is under study [8]. In the case of alliospirosides A and B, a decrease in the value of is observed with a rise in the concentration of ATP from 0.2 to 1.0 mM (Fig. 1) — for alliospiroside A from 14.66 to 1.92, and for alliospiroside B from 12.0 to 3.12. This shows a rise in the capacity of ATP for interacting with the preparations as the

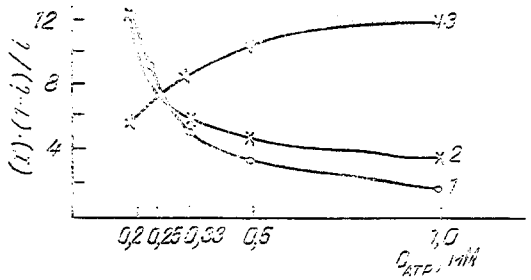


Fig. 1. Competition of alliospirosides A, B, and D with ATP for the corresponding centers of Na,K-ATPase (~90% pure in terms of protein): 1) alliospiroside A in a concentration of $2 \cdot 10^{-5}$ M; 2) alliospiroside B in a concentration of $6 \cdot 10^{-5}$ M; 3) alliospiroside D in a concentration of $5 \cdot 10^{-5}$ M.

ionic strength of the incubation medium is increased and the surface charge or the activation constant of the Na,K-ATPase changes. Consequently, alliospirosides A and B do not compete with ATP for addition to the active center of the enzyme. The dependence of the activity of Na,K-ATPase on the concetration of ATP in the presence of alliospirosides A and B is shown in Fig. 1.

So far as concerns alliospiroside D, it is characterized by an increase in the magnitude $\frac{(I) \cdot (1 - i)}{i}$, from 5.96 to 12.74 with an increase in the concentration of ATP from 0.2 to 1.0 mM, respectively (Fig. 1). This is characteristic for the competitive type of inhibition of an enzyme.

The results obtained on the kinetics of the inhibition by steroid glycosides of the spirostan series of the activity of highly purified porcine kidney Na,K-ATPase show that the nature of their inhibition is nonmonotypical. The greatest interest is aroused by alliospiroside D; for this the competitive type of inhibition in relation to ATP is observed which is an indication of the attachment of this compound to the active enter of the enzyme, located on the intracellular side.

Competition with ATP for the active enter of the enzyme is not characteristic for alliospirosides A and B, which do not become attached to it. From the point of view of a difference in chemical structure, attention turns to the circumstance that the comparatively small structural difference between the aglycon of alliospiroside D — cepagenin (which has an additional hydroxy group at C-24) — and (25S)-ruscogenin — the aglycon of alliospirosides A and B — has led to a striking change in their interaction with the enzyme. At the same time, it must be added that the dependence of the inhibitory effect on the concentration of ATP shows the possibility of an interaction of alliospirosides A and B with Na,K-ATPase on the intracellular side.

Against the background of the well-followed structure–action relationship, the behavior of alliospiroside C (**7**) appears somewhat anomalous. This glycoside has the same aglycon — cepagenin — as alliospiroside D (**8**) but, in contrast to the latter, does not inhibit Na,K-ATPase.

In the final account, we have come to the conclusion that alliospirosides A, B, and D are, unconditionally, physiologically active compounds capable of inhibiting the activity of Na,K-ATPase, while the inhibition of the activity of the transport enzyme by alliospirosides A and B takes place in the uncompetitive manner, and that by alliospiroside D in the competitive manner. Thus, alliospirosides A, B, and D may be recommended for a more profound pharmacological study on the intact organism.

EXPERIMENTAL

The glycosides of the spirostan and furostan series were isolated from plants of the genus *Allium* (family *Liliaceae*) in the free state or were obtained by the partial modification of native compounds. The method of isolation and the determination of the structure of each compound are given in the original source.

Karataviosides A, B, C, E, and F were isolated from the collective fruit of the Turkestan onion *Allium karataviense* Rgl. [9-12]. Alliospirosides A, B, C, and D, and alliofuroside were isolated from the collective fruit of the garden onion *Allium cepa* L. [13-15]. The lycotetraosides of alliogenin, alliogenone, and dihydroyuccagenein were obtained by the modification of karatavioside A [16].

The purity of the compounds was checked by thin-layer chromatography on Silufol plates (Czechoslovakia) and in a fixed layer of KSK and silica gel (particle size < 63 μm). The revealing agent was vanillin–phosphoric acid.

Microsomes were isolated from the medullary layer of porcine kidneys by Skou's method [17]. After preliminary comminution, 50 g of porcine kidney medullary layer from freshly slaughtered animals was homogenized in the isolation medium (130 mM Tris-HCl, pH 7.5; 250 mM sucrose; 1 mM EDTA). The homogenate was diluted with the isolation medium in a proportion of 10 parts of buffer to 1 part of tissue, and was centrifuged at 6000 g for 20 min. The supernatant was centrifuged at 12,000g for 20 min to eliminate the nuclei of the mitochondrial cell fragments. The microsomes were obtained by centrifugation at 40,000 g for 60 min.

Highly purified Na,K-ATPase from porcine kidney medullary layer (~90% purity in terms of protein) was isolated by Jorgensen's method [18] in the modification of Chetverin et al. [19]. With constant stirring by a magnetic stirrer, 14.5 ml of a freshly prepared solution of sodium dodecyl sulfate (Na-DDS) was added dropwise to 35.5 ml of the material (70 mg of protein). The ratio of the concentrations of protein and Na-DDS was always kept constant (2.41:1). The operations were conducted in a medium containing 50 mM Tris-HCl, pH 7.5; 3 mM ATP; 3 mM EDTA at room temperature for 30 min. Further purification was achieved in a sucrose density gradient of from 15 to 45% prepared in a buffer with 25 mM Tris-HCl, pH 7.5; 1 mM EDTA.

Centrifugation was carried out in a Beckman L5-65 centrifuge using a Ti-14 rotor with a volume of 650 ml at 170,000g for 2.5 h. The samples were displaced from the rotor with 45% sucrose, and 12-ml fractions were collected at the rate of 20 ml/min. The optical densities of the fraction at 280 nm were measured, the Na,K-ATPase activities were determined, and the form of the sucrose gradient was found in a refractometer.

The fractions used in the investigation had a sucrose density of 1.12-1.13 (~30% sucrose) and a high specific activity. The fractions that had been collected were diluted with a buffer containing 25 mM Tris-HCl, pH 7; 3 mM ATP; 1 mM EDTA; and about 15% of sucrose and were centrifuged at 190,000g in a 50.2 Ti rotor. The deposit was stored at 4°C in a medium consisting of 25 mM Tris-HCl, pH 7.5; 1 mM EDTA.

The activity of the enzyme was determined in a medium containing 30 mM Tris-HCl, pH 7.5; 130 mM NaCl; 5 mM KCl; 4 mM $MgCl_2$; 2 mM ATP.

Inorganic phosphorus was determined by Dalsan's method in the modification of Danusz et al. [20], and protein by a modified Lowry method [21].

The compounds to be tested were initially dissolved in dimethyl sulfoxide (DMSO) and were transferred into a tube in the necessary concentration. DMSO in the corresponding concentration was added to control tubes.

The concentrations corresponding to 50% inhibition of the activity of Na,K-ATPase (J_{50}) were calculated by a specially compiled programs using an Élektronik MK-54 microcomputer.

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TRITERPENE SAPONINS FROM *Thalictrum minus*. VII. STRUCTURE OF THALICOSIDE E

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A new minor cycloartane glycoside — thalicoside E, 9,19-cyclo-20(S)-lanost-23-ene-3β,16β,22ξ,25,29-pentaol 3-O-β-D-galactoside 29-O-β-D-glucopyranoside — has been isolated from the epigeal part of Thalictrum minus *L. (Ranunculaceae).*

Continuing a study of the triterpene glycosides of *Thalictrum minus* [1-4], we have isolated a new glycoside. The present paper is devoted to the establishment of the structure of a triterpene glycoside containing a cycloartane genin that has not previously been described in the literature. The compound, which we have called thalicoside E (I) is a minor triterpene glycoside of low meadow rue.

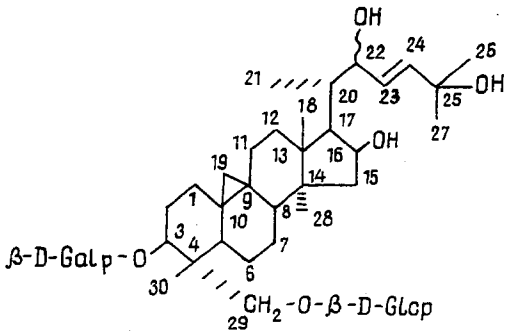
It followed from the ^1H and ^{13}C NMR spectra of thalicoside E that the genin contains fragments showing the triterpene nature of the compound isolated: six methyl groups, one of which is secondary, a cyclopropane ring, a trisubstituted double bond, and five hydroxy groups (Table 1).

The glycosidic nature of the substance was shown by the results of mass spectrometry. Thus, the FAB mass spectrum of glycoside (I) contained a quasi-molecular peak with m/z 837 ($M + Na^+$) and cluster ions with m/z 615 ($M + Na^+ - Hex$-60) and 453 ($M + Na^+ - 2Hex$-60), showing the splitting out of one or two molecules of a hexose, respectively. It follows from the facts presented that a triterpene genin with a mass of 490 is glycosylated by two hexose molecules.

The CSs in the ^{13}C NMR spectrum for the carbohydrates (Table 2) corresponded to a terminal β-D-galactopyranoside and a terminal β-D-glucopyranoside rsidue [5]. A comparison of the ^{13}C NMR spectra of thalicoside E and of cycloartanol [6] showed that the new compound was a cycloartane derivative. Differences between them appeared in the structure of the side-chain and in substitution at the C-4 (ring A) and C-16 (ring D) atoms.

By using two-dimensional TOCSY and COSY experiments [7, 8], we identified the spin systems of rings A and D, The spin system of ring A includes the following protons: 1α (1.10) 1β (1.28)-2α (1.89) 2β (2.31)-3α (4.28 ppm). The signal of a hydroxymethylene proton (4.28 ppm) is in $^{13}C—^1H$ correlation with the signal of the C-3 carbon atom (81.91 ppm)

Formula



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