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SELF-ASSOCIATION AND COMPLEXATION OF TRITERPENE GLYCOSIDES AND CHOLESTEROL

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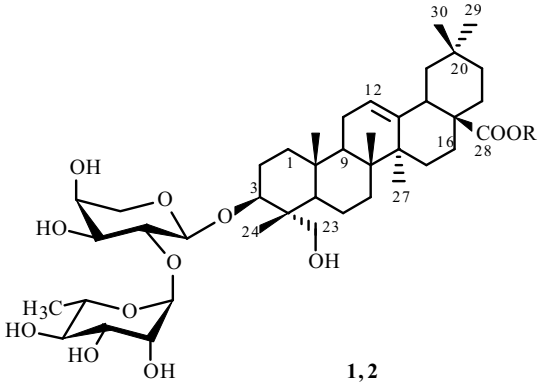
Self-association of hederagenin 3-O-α-*L-rhamnopyranosyl-(1→2)*-O-α-*L-arabinopyranoside (α-hederin) and its 28*-O-α-*L-rhamnopyranosyl-(1→4)*-O-β-*D-glucopyranosyl-(1→6)*-O-β-*D-glucopyranosyl ether (hederacoside C) with cholesterol was studied using mass spectrometry (ESI MS). Possible complexation of the glycosides with cholesterol was examined. The ichtyotoxic activity of the glycosides and their mixtures with cholesterol to* Barbus fefrozona *was studied.*

Keywords: self-association, triterpene glycosides, cholesterol, complexation, mass spectrometry, ichtyotoxic activity, *Barbus fefrozona*.

Complexation of several pharmacophores with various saponins has recently been widely studied. Glycosides contain polar functional groups that give them the capability to associate with and bind to drugs. The resulting complexes exhibit several useful properties that enable the drug dose to be reduced, side effects to be diminished, and the action to be prolonged. Molecular encapsulation of drugs has been examined in most detail mainly only for glycyrrhizic acid [1, 2], the main triterpene glycoside of plants of the genus *Glycyrrhiza*. It was found that glycyrrhizic acid in aqueous solutions forms rod-shaped associates [1]. Acanthophylloside B, which was isolated from *Acanthophyllum gypsophyloides* roots [1], was also examined as a clathrating agent. Complexes of steroidal glycosides with amino acids and nucleosides were also recently prepared [3].

We examined some of the most common triterpene glycosides of plants from the family Araliaceae Juss. as promising complexants. These included the glycosides α-hederin (glycoside **1**) and hederacoside C (glycoside **2**). Glycoside **1** is hederagenin 3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-α-L-arabinopyranoside; **2**, hederagenin 3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-α-L-arabinopyranosyl-28-*O*-α-L-rhamnopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→6)-*O*-β-D-glucopyranoside.



1, 2

1: R = H

2: R = ←*β*Glc*p*-(6←1)-*β*Glc*p*-(4←1)-*α*Rha*p*

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TABLE 1. Characteristic Ions in Mass Spectra of **1** and **2**, Cholesterol, and Their Mixtures

Ion structure	*m/z* ratio (I_{rel}, %)	Ion structure	*m/z* ratio (I_{rel}, %)
Glycoside **1** (MW 750.453 Da)		Glycoside **2** (MW 1220.616 Da)	
$[M–H]^-$	749.463 (100.0)	$[M–H]^-$	1219.642 (100.0)
$[(M–H)+M]^-$	1499.957 (10.7)	$[M–2H]^{2-}$	609.304 (34.9)
$[M–2H]^{2-}$	374.218 (26.7)	$[M+Na]^+$	1243.765 (100.0)
$[(M–H)+(M–H)]^{2-}$ или $[M+(M–2H)]^{2-}$	749.463 (100.0)	$[M+K]^+$	1259.738 (75.7)
$[2(M–H)+M)]^{2-}$ или $[2M+(M–2H)]^{2-}$	1124.710 (6.8)	$[2M+Na+K]^{2+}$	1251.751 (17.1)
$[2M+2(M–H)]^{2-}$ или $[3M+(M–2H)]^{2-}$	1499.957 (10.7)	$[3M+Na+K]^{2+}$	1860.614 (3.5)
$[3M+2(M–H)]^{2-}$	1875.213 (45.8)	Cholesterol (MW 386.353 Da)	
$[4M+2(M–H)]^{2-}$	2250.479 (49.6)	$[M+H]^+$	387.410 (100.0)
$[M+Na]^+$	773.566 (100.0)	Glycoside **1** and cholesterol mixture	
$[M+K]^+$	789.540 (63.3)	$[2M(1)+Na]^+$	1524.035 (100.0)
$[2M+Na]^+$	1524.035 (11.3)	$[2M(1)+H+2M(cholesterol)]^+$	2274.445 (4.9)
$[3M+H]^+$	2274.490 (4.6)	Glycoside **2** and cholesterol mixture	
$[3M+2Na]^{2+}$	1148.804 (10.2)	$[M(2)+Na]^+$	1243.765 (100.0)
$[3M+2K]^{2+}$	1156.796 (8.3)	$[(M(2)–H)+2H_2O]^-$	1255.639 (100.0)
$[5M+2Na]^{2+}$	1899.273 (0.4)	$[(M(2)–2H)+4H_2O]^{2-}$	645.331 (44.3)

Glycosides **1** and **2** were found in plants of various species of *Hedera* and *Kalopanax*, in *Aralia elata* and *Acanthopanax sieboldianus* [4–6]; **1** was also found in *Polyscias dichroostachya* [4]; **2**, *Schefflera octophylla* [7]. Glycosides **1** and **2** are included in the well-known drugs for treating coughs Gedeliks® and Prospan®, which are formulated from *Hedera helix* leaves [8].

A characteristic feature of saponins is their ability to form complexes with sterols [4, 6, 9]. Binding to them alters the permeability of cell membranes, which is responsible for some of the biological activity of saponins. This effect was found previously for **1** [9]. However, it was shown [10] that it does not form complexes with cholesterol. Therefore, we examined the ability of **1** and **2** to form complexes with cholesterol. Self-association of **1**, **2**, and cholesterol in addition to the ability to form complexes was studied using electrospray mass spectrometry (ESI–MS).

Glycoside **1** was a monocarboxylic acid. Therefore, the peak for its dianion $[M(\mathbf{1}) – 2H]^{2-}$ with *m/z* 374.218 in the mass spectrum was almost four times weaker than that of its monoanion $[M(\mathbf{1}) – H]^-$ with *m/z* 749.463 (Table 1). Tribasic glycyrrhizic acid under the same conditions gives preferentially the dianion. The monoanion is half as strong; the trianion, 50 times weaker [11].

Analysis of the spectral data showed that self-association was characteristic of **1**. The highest recorded association coefficient for it was 6 (self-associate with *m/z* 2250.479).

Glycoside **2** was more difficult than **1** to ionize to the anion. Therefore, its strongest peaks were an order of magnitude weaker. The principal peaks corresponded to the dianion $[M(\mathbf{2}) – 2H]^{2-}$ with *m/z* 609.304 and the monoanion $[M(\mathbf{2}) – H]^-$ with *m/z* 1219.642. Reliable evidence that **2** formed self-associates under the selected ionization conditions was not observed.

Cholesterol does not characteristically form in negative-ion mode a pseudomolecular ion $[M – H]^-$ with *m/z* 385.35 or associates $[2M – H]^-$, confirming its high lipophilicity. In positive-ion mode, it formed a pseudomolecular ion $[M + H]^+$ with *m/z* 387.41 (intensity 14% of cluster peaks of CH_3CN). The defragmentation peak proposed for a cycloaliphatic alcohol $[(M – H_2O) + H]^+$ with *m/z* 369 was missing. Self-associates of cholesterol were also not observed. However, the deficit of cholesterol ions did not exclude the possibility that complexes of ions of **1** or **2** with cholesterol formed.

Complexes of **1** with cholesterol in negative-ion mode were sought for mono-, di-, tri-, and tetraanions of composition 1:1, 1:2, 2:1, 3:1, 4:1, 3:2, and 2:3. The corresponding peaks were not observed.

The mass spectrum of a mixture of **1** and cholesterol in positive-ion mode contained a peak for the dimeric associate and a sodium cation $[2M(\mathbf{1}) + Na]^+$ in addition to a peak for a dimeric complex of **1**, cholesterol, and a proton $[2M(\mathbf{1}) + H + 2M(cholesterol)]^+$ with *m/z* 2274.445. Other associates of **1**, cholesterol, and sodium or hydrogen cations were missing, which says something about the required stereochemistry and size of the cation. The peak for the $[2M(\mathbf{1}) + Na]^+$ complex was 20.4 times stronger than that for $[2M(\mathbf{1}) + H + 2M(cholesterol)]^+$. Therefore, the concentration of the complex of **1** and lipophilic cholesterol was an order of magnitude less than that with ion –dipole binding.

All principal peaks in positive-ion mode corresponded to complexes of **2** with sodium and potassium cations. Peaks of dicationic complexes $[2M(\mathbf{2}) + Na + K]^{2+}$ and $[3M(\mathbf{2}) + Na + K]^{2+}$ were detected. A comparison of their intensities showed that $[2M(\mathbf{2}) + Na + K]^{2+}$ was five times more stable than $[3M(\mathbf{2}) + Na + K]^{2+}$.

The formation of a complex of **2** with only a set of Na^+ and K^+ cations and not with two Na^+ or two K^+ cations or with a different number of them proves clearly that the cations bind namely with the cavity formed by two molecules of **2** and not to the external surface of this podand. The size of the M(**2**) + M(**2**) cavity can be roughly estimated taking into account the stoichiometry of $[2M(\mathbf{2}) + Na + K]^+$, the diameters of Na^+ (1.90 Å) and K^+ (2.66 Å), and the buffer zone between them. Such an approach was validated previously for homo- and heteronuclear complexes of crown ethers and coronates [12].

The search for a complex of **2** and cholesterol in positive-ion mode found that the mass spectrum of a mixture of these compounds contained a peak for $[M(\mathbf{2}) + Na]^+$ but not for $[nM(\mathbf{2}) + zH + mM(\text{cholesterol})]^{z+}$, where *n*, *m*, and *z* are natural numbers. Apparently cholesterol is too large to bind to the lipophilic part of the aglycon of **2**, which is bordered on the sides by the carbohydrate chains on C-3 and C-28. The two carbohydrate chains enable **2** to bind to a sodium cation in a 1:1 ratio (i.e., $[M(\mathbf{2}) + Na]^+$) whereas the single disaccharide group of **1** gives a 2:1 ratio ($[2M(\mathbf{1}) + Na]^+$).

In negative-ion mode, complexes also did not form in a mixture of **2** and cholesterol. The mass spectrum showed peaks for the dihydrate of the anion of **2** $[(M(\mathbf{2}) - H) + 2H_2O]^-$ and the tetrahydrate $[(M(\mathbf{2}) - 2H) + 4H_2O]^{2-}$.

In order to confirm the conclusions about the complexation of **1** and **2** with cholesterol, we examined their toxicity to the fish *Barbus fefrozona* (Cyprinidae), which is distributed in aquifers of Asia, Europe, and Africa. About 10 species are indigenous to the CIS. Barbus fish are commonly used as aquarium species [13]. The results from the study of the ichthyotoxicity of **1** and **2** and their mixtures with cholesterol confirmed the conclusions obtained from mass spectrometry:

Compound	*Exposure time* $t_{LD_{100}}$ *until death, min*
1	6.5±0.3
2	25.2±0.4
1– cholesterol	11.5±0.4
2– cholesterol	23.8±0.3.

The toxicity of the mixture of **1** with cholesterol was substantially decreased compared with the pure glycoside. On the other hand, the activity of **2** in the mixture with cholesterol was practically the same, which was related to the lack of stable complexation between them. Obviously, cholesterol binds to monodesmoside triterpene glycosides with a carbohydrate chain on C-3 of the aglycon and a free C-17 COOH group of hederagenin. A COOH group on the other site of the aglycon does not prevent complexation with cholesterol, as was recently shown for glycyrrhizic acid, for which the COOH in the aglycon (glycyrrhetic acid) is located on C-20 [14].

Thus, it was found that glycoside **1** forms self-associates in negative-ion mode whereas **2** and cholesterol do not. Cholesterol forms a 2:2 molecular complex with **1** whereas complexes of **2** were not observed. This was confirmed by a study of the ichthyotoxicity. Complexes of **1** and **2** with Na^+ and K^+ were observed. Glycoside **2** formed a clathrate with cations, the guests Na^+ and K^+, in which the host cavity consisted of a dimer of **2**.

EXPERIMENTAL

Isolation of Glycosides and Preparation of Complexes. Glycosides **1** and **2** were isolated from *Hedera taurica* Carr. and *H. canariensis* Willd. by the reported methods [15]. TLC was performed on Sorbfil PTSKh-P-A-UF-254 silica gel analytical plates (5–7 μm, STKh-1A) (Russian Federation). We used solvent systems $CHCl_3$:CH_3OH:H_2O (100:26:3 and 100:31:5) and $CHCl_3$:CH_3OH:NH_4OH (25%) (100:20:3 and 100:30:5). The R_f values for **1** were 0.46, 0.51, 0.12, and 0.49; for **2**, 0.04, 0.09, origin, and 0.09 (in the solvent systems, respectively). The developer was *p*-hydroxybenzaldehyde (0.2%) in H_2SO_4 (2 N) [16] with heating to 100°C.

Complexes were prepared by mixing solutions containing glycoside (1 mmol) and cholesterol (2 mmol) [solvent: aqueous ethanol (70%) and $CHCl_3$, 3:1, v/v] The resulting mixture was held at 50°C for 1.5 h with constant stirring. Organic solvents were removed by vacuum distillation.

Mass Spectral Studies. Measurements were made using a Bruker Daltonics microTOF-Q mass spectrometer with a direct sample probe, electrospray ionization, negative- and positive-ion detection in the range 50 –3000 Da with an accuracy of at least 1×10^{-2} Da. The spray capillary potential was ±4200 V. The drying gas parameters (Pure N_2, 5 L/min, 200°C) and

quadrupole energy of ions (5.0 eV) were optimized for detection of peaks for pseudomolecular and associate ions. Solutions of the compounds in CH_3CN (Merck, HPLC/MS grade) at concentrations up to 0.2 mg/mL ($10^{-7} \div 10^{-6}$ M) were used for direct introduction. Table 1 presents the results.

Biological Activity. Ichthyotoxicity was measured for *Barbus fefrozona* (Cyprinidae) using solutions of glycosides and their complexes in distilled water. The effect of each substance was studied using 20 fish. Cholesterol was added to glycoside solutions (10^{-3} M) at a 1:1 molar ratio at 50°C. The mixture was stored for 60 min and then cooled to room temperature. Fish were placed into solutions of the glycosides and complexes. The time t during which 100% of the fish died was determined. The confidence range was calculated to reliability $\alpha = 0.95$.

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REFERENCES

1. T. G. Tolstikova, A. G. Tolstikov, and G. A. Tolstikov, *Vestn. Ross. Akad. Nauk*, **77**, No. 10, 867 (2007).
2. V. A. Stonik and G. A. Tolstikov, *Vestn. Ross. Akad. Nauk*, **78**, No. 8, 675 (2008).
3. V. V. Pilipenko and L. F. Sukhodub, *Biopolim. Kletka*, **18**, No. 2, 139 (2002); V. V. Pilipenko, L. F. Sukhodub, S. A. Bobeyko, V. A. Shvets, and P. K. Kintia, in: *Book of Abstracts, International Conference on Saponins "Phytochemistry and Application of Plant Saponins,"* Pulawy, Poland, 2004, 39.
4. K. Hostettmann and A. Marston, *Saponins*, Cambridge University Press, Cambridge, 1995, p. 548.
5. V. I. Grishkovets, V. Ya. Chirva, V. V. Kachala, and A. S. Shashkov, *Tr. Nikit. Bot. Sada*, **127**, 97 (2007); V. I. Grishkovets, V. Ya. Chirva, V. V. Kachala, and A. S. Shashkov, *Tr. Nikit. Bot. Sada*, **128**, 90 (2007).
6. G. E. Dekanosidze, V. Ya. Chirva, and T. V. Sergienko, *Biological Role, Distribution, and Chemical Structure of Triterpene Glycosides* [in Russian], Metsniereba, Tbilisi, 1984, p. 349.
7. C. Maeda, K. Ohtani, R. Kasai, K. Yamasaki, N. M. Duc, N. T. Nham, and G. K. Q. Cu, *Phytochemistry*, **37**, No. 4, 1131 (1994).
8. L. A. Yakovishin and V. I. Grishkovets, *Khim. Prir. Soedin.*, 417 (2003); B. M. Zuzuk, R. V. Kutsik, and L. I. Zuzuk, *Provizor*, No. 12, 13 (2003); L. A. Yakovishin, M. A. Vozhzhova, A. L. Kuznetsova, and V. I. Grishkoevts, *Zh. Org. Farm. Khim.*, **3**, No. 1 (9), 57 (2005).
9. M. M. Anisimov and V. Ya. Chirva, *Usp. Sovrem. Biol.*, **6**, No. 3, 351 (1980).
10. *Chemical Analysis of Medicinal Plants* [in Russian], N. I. Grinkevich and L. N. Safronich (eds.), Vysshaya Shkola, Moscow, 1983, p. 50.
11. S. N. Borisenko, M. I. Rudnev, and N. I. Borisenko, in: *Materials of the Vth International Scientific-Technical Conference "Current Problems of Theoretical and Applied Biophysics, Physics, and Chemistry (BPPC-2009)"* [in Russian], Sevastopol, Ukraine, 2009, p. 208.
12. *Host Guest Complex Chemistry Macrocycles. Synthesis, Structures, Applications*, F. Vogtle and E. Weber (eds.), Springer-Verlag, Heidelberg, 1985, p. 31.
13. I. N. Agekyan, N. V. Belov, and I. L. Kopylov, *Encyclopedia of Aquariums* [in Russian], AST, Kharvest, Moscow, Minsk, 2002, p. 352.
14. L. O. Yakovishin, V. A. Ertakhova, and K. A. Bazyura, *Ukrainica Bioorg. Acta*, **4**, No. 2, 22 (2006).
15. A. S. Shashkov, V. I. Grishkovets, A. A. Loloiko, and V. Ya. Chirva, *Khim. Prir. Soedin.*, 363 (1987); V. I. Grishkovets, D. Yu. Sidorov, L. A. Yakovishin, N. N. Arnautov, A. S. Shashkov, and V. Ya. Chirva, *Khim. Prir. Soedin.*, 377 (1996).
16. L. A. Yakovishin, *Khim. Prir. Soedin.*, 419 (2003).