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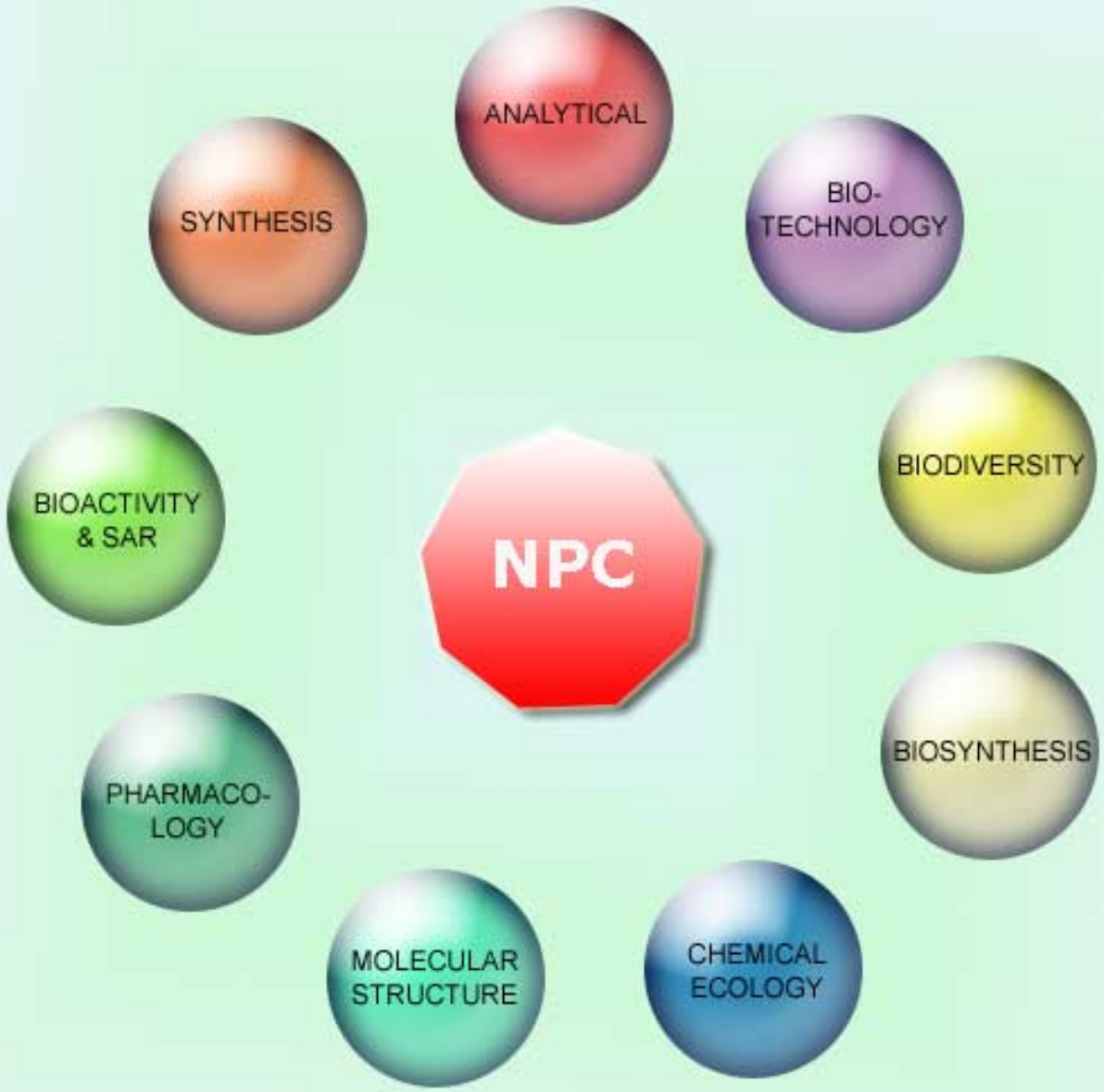

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Cytotoxic Action of Triterpene Glycosides from Sea Cucumbers from the genus *Cucumaria* on Mouse Spleen Lymphocytes. Inhibition of Nonspecific Esterase

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Four triterpene glycosides from sea cucumbers belonging to the genus *Cucumaria*, okhotoside A_1-1 (**1**), cucumarioside A_0-1 (**2**), frondoside A (**3**) and cucumarioside A_2-2 (**4**) inhibit the activity of nonspecific esterase of mouse spleen lymphocytes. The dependence of the inhibitory activity of the glycosides on their structure is similar to that for hemolytic activity. The absence of inhibitory activity for the preparation Cumaside, which is a complex of cucumarioside A_2-2 and related compounds with cholesterol, shows a cholesterol-dependent character of the inhibitory action of the glycosides. The effective inhibitory concentrations of frondoside A and cucumarioside A_2-2 are significantly higher than the immunomodulatory doses of these glycosides.

Keywords: Lymphocytes, cytotoxicity, triterpene glycosides, sea cucumber, *Cucumaria*, nonspecific esterase.

Triterpene glycosides from sea cucumbers (Holothurioidea, Echinodermata) possess a wide spectrum of biological activities caused by their ability to form complexes with cholesterol and other 5,6-unsaturated sterols followed by formation of single ion channels and large pores in cell membranes, depending on concentration. Most of these substances are strong membranolytic agents and have, for example, antifungal and hemolytic activities, are ichthyotoxic, and are cytotoxic to tumor cells [1]. The different kinds of biological activities caused by membranolytic action similarly depend on glycoside structure [1–5]. This dependence may be relatively easily predicted [6].

Recently, biological activities of sea cucumber triterpene glycosides in sub-toxic doses that may not be caused by their interaction with cholesterol have attracted considerable attention. The specific interest is in the action of the glycosides as modulators of cell-immunity. Indeed sub-toxic dosages of cucumarioside A_2-2 and other

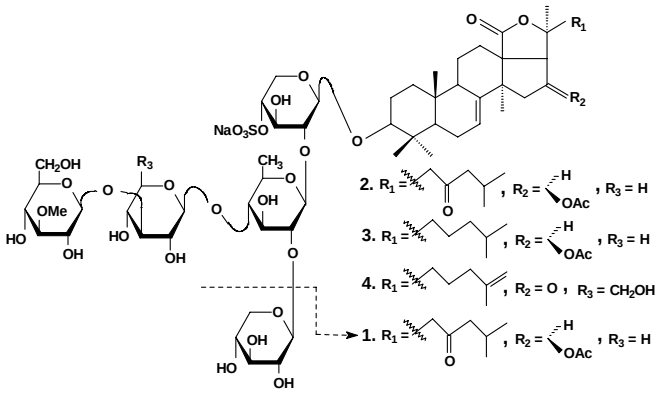

Figure 1: Chemical structures of : **1** – okhotoside A_1-1 from *Cucumaria okhotensis* [14]; **2** – cucumarioside A_0-1 from *C. okhotensis* [14]; **3** – frondoside A from *C. okhotensis* [16]; 4 – cucumarioside A_2-2 from *C. japonica* [17].

monosulfated glycosides from the Far Eastern sea cucumber *Cucumaria japonica* increased lysosomal activity of mouse peritoneal macrophages both *in vivo* and *in vitro* [7,8]. Frondoside A from *C. frondosa* also increased lysosomal activity of

macrophages, and stimulated macrophage phagocytosis and ROS formation in the macrophages [9]. Moreover, the compound increased lymphocyte formation in mouse spleen [9]. The preparation Cumaside, which is a complex of cucumarioside A_2-2 and other monosulfated glycosides with cholesterol, increases lysosomal activity of macrophages and stimulates phagocytosis and ROS formation in macrophages [10]. Cumaside in doses of 0.1–1 μg/mL may restore the level of CD3, CD4 and CD8-antigenes of human blood lymphocytes decreased by pre-incubation of these lymphocytes with the immunodepressant, hydrocortisone [10].

Hence it is of interest to obtain information about the character of the cytotoxic action of glycosides from the sea cucumbers of the genus *Cucumaria* against lymphocytes. To study cytotoxic activity we have chosen hydrolysis of fluorescine diacetate (FDA) with nonspecific esterase, which produces the easily measured fluorescine. This procedure is a simple and convenient method to estimate viability i.e. general level of metabolic activity for different kinds of cells [11], including lymphocytes [12].

The series of monosulfated glycosides isolated from sea cucumbers of the genus *Cucumaria*, having similar structures distinguished only by small differences in the carbohydrate chains and aglycones, was chosen for studying the character of the cytotoxic activity (Figure 1).

Using the procedure of lysosome staining with a molecular probe followed by quantitative analysis of cell fluorescence it was shown that glycosides **1**–**4** inhibit the activity of nonspecific esterase in mouse spleen lymphocytes. However, Cumaside, which has immunomodulatory action comparable to that of glycoside **4**, showed no nonspecific esterase inhibition in doses of 50 μg/mL (Table 1).

The effective immunomodulatory doses *in vitro* for frondoside A (**3**), cucumarioside A_2-2 (**4**), and preparation Cumaside do not exceed 0.1–1 μg /mL [7–10]. The obtained results showed that the cytotoxic activity of the glycosides having evident immunomodulatory activity against immune cells occurred in concentrations significantly higher than the immunomodulatory doses. An immunomodulatory preparation, Cumaside showed no cytotoxic activity at a concentration of 50 μg/mL. This indicates a cholesterol-dependent cytotoxic action of these glycosides against immune cells.

Table 1: Influence of triterpene glycosides on activity of nonspecific esterase of mouse spleen lymphocytes.

Substance	**1**	**2**	**3**	**4**	Cumaside
EC_{50} (μg/mL)	2.4	4.1	5.0	8.0	No activity

The monosulfated tetraoside, okhotoside A_1-1 (**1**) differs from the monosulfated pentaoside, cucumarioside A_0-1 (**2**) only by the absence of a terminal xylose residue attached to a quinovose residue. Studies of hemolytic activity have shown that ED_{50} values for linear tetraosides are three times less than those for glycosides having an additional xylose residue attached to the second monosaccharide residue, quinovose [4]. Hence the stronger cytotoxic action of glycoside **1** against lymphocytes in comparison with glycoside **2** correlates very well with the data on hemolytic activity.

Cucumarioside A_0-1 (**2**) is very similar to frondoside A (**3**) in structure, but differs by the presence of a keto-group at C-23 of the aglycone. Nevertheless, even such a small difference causes higher cytotoxic activity of glycoside **2** against lymphocytes than that of glycoside **3**. This indicates some contribution of the 23-keto-group in cytotoxic activity.

Frondoside A (**3**) differs from cucumarioside A_2-2 (**4**) by the presence of an acetate group at C-16 instead of a keto-group. Moreover, the aglycone of cucumarioside A_2-2 has a 25(26)-terminal double bond in the side chain and glucose instead of xylose as the third monosaccharide residue. Frondoside A has about three times higher hemolytic activity (three times lesser ED_{50}) than cucumarioside A_2-2. [4,5]. Hence the stronger cytotoxic action of frondoside A against lymphocytes compared with that of cucumarioside A_2-2 correlates very well with the data on hemolytic activity.

The hemolytic activity (ED_{50}) of cucumarioside A_2-2 (**4**) is about 2.5 μg/mL, but the corresponding activity for Cumaside is about 50 μg/mL [10]. There also is some correlation between cytotoxic activity against lymphocytes and hemolytic activity.

Hence the cytotoxic activity of these substances against lymphocytes occurs at doses significantly higher than their immunomodulatory doses (about 50–100 times). The dependence of inhibition of nonspecific esterase on glycoside structure is similar to that for the hemolytic activity of these substances

and their structure. This observation and the cholesterol-dependent nature of inhibition of nonspecific esterase of lymphocytes by glycosides in cytotoxic concentrations indicate that the action of sea cucumber glycosides against lymphocytes in cytotoxic doses has the common membranolytic mode for this class of substances. The results confirm that data on dependence of activity of sea cucumber glycosides on their structure obtained for one kind of cells (for example, erythrocytes, fungal, tumor, sea urchin eggs) may be used to predict their cytotoxic action against other kinds of cells if sterol-dependent membranolytic activity is involved [6].

Experimental

Triterpene glycosides: Okhotosides A_1-1 (**1**) and cucumarioside A_0-1 (**2**), previously reported for *C. japonica* [13], were isolated from *C. okhotensis* [14]. Frondoside A, reported for *C. frondosa* [15], was isolated from *C. okhotensis* [16]. Cucumarioside A_2-2 (**4**) was isolated from *C. japonica* [17]. All the glycosides were individual compounds, as shown by HPLC data and ^{13}C NMR spectra. The glycoside structures are presented in Figure 1. Cumaside, a complex of monosulfated glycosides from *C. japonica* and cholesterol (1:2 molar ratio) was produced by a published method [10]. The glycoside / cholesterol molar ratio was checked by the ratio of the signals of methyl groups of cholesterol and aglycones in the ^1H NMR spectrum of Cumaside in C_5D_5N.

Lymphocytes: The total fraction of mouse lymphocytes (spleenocytes) was isolated from the spleen of BALB/C line mice. The spleen was homogenized in PSB solution (pH 7.4) and the cell suspension filtered through nylon voile (280 mesh). The suspension of spleenocytes was washed three times with PSB (pH 7.4) and centrifuged (1500 rpm for 5 min) to remove debris and re-suspended in PSB to make the final cell concentration $2–5\times10^6$ cells/mL.

Determination of activity of nonspecific esterase: The test substance solution (10 μL) and 100 μL of cell suspension was placed in each of a 96-hole plate, which was incubated in a thermostat at 37°C for 1 h. A stock solution of probe fluorescein diacetate **(**FDA, Sigma) in DMSO (1 mg/mL) was prepared. After incubation of the cells with the test compound, 10 μL FDA solution (50 μg/mL) was added into each hole and the plate was incubated in a thermostat at 37°C for 15 min. Fluorescence was measured with a plate reader (Fluoroskan Ascent) at λ_{ex} = 485 nm and λ_{em}= 518 nm. All experiments were repeated in triplicate. The means and standard errors for each treatment were calculated and EC_{50} values were estimated from dose-response plots using SigmaPlot 3.02 software (Jandel Scientific, San Rafael, CA).

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