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BIOLOGICAL ACTIVITIES OF ASTEROSAPONINS WITH SPECIAL REFERENCE TO STRUCTURE-ACTIVITY RELATIONSHIPS

NOBUHIRO FUSETANI, YUKO KATO, KANEHISA HASHIMOTO,

Laboratory of Marine Biochemistry, Faculty of Agriculture, The University of Tokyo, Tokyo 113

TETSUYA KOMORI,

Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka 812

YOICHI ITAKURA,

Research Laboratories, Wakunaga Pharmaceutical Co. Ltd., Hiroshima 729-64

and TOSHIO KAWASAKI

Faculty of Pharmaceutical Sciences, Setsunan University, Hirakata 571-01, Japan

ABSTRACT.—Seventeen steroidal saponins isolated from the Japanese starfishes, *Acanthaster planci*, *Luidia maculata*, and *Asterias amurensis* [cf.] *versicolor* have been examined for their hemolytic activity and effects on the development of fertilized sea urchin *Hemicentrotus pulcherrimus* and starfish *Asterina pectinifera* eggs. These biological activities have been discussed in terms of structure-activity relationships.

Sulfated steroidal glycosides, sometimes referred to as asterosaponins, are commonly found in starfish (1,2). The saponins are characterized by steroidal aglycones possessing a 9,11-ene-3β,6α-diol structural feature, an oligosaccharide moiety consisting of five or six units of D-fucose, D-quinovose, D-xylose, D-galactose, and D-glucose linked at the C-6 position, and a sulfate group linked to the C-3 position. The compounds have shown a variety of biological activities: lethality in mammals, fish, insects, and other types of organisms (1); hemolytic activity toward erythrocytes of various origins (3); cytotoxicity to tumor cells and viruses (4); blockage of neuromuscular transmission in mammals (5); anti-inflammatory, analgesic, and hypotensive activities (6); and induction of avoidance reactions in marine snails, sea anemones, and other organisms (7).

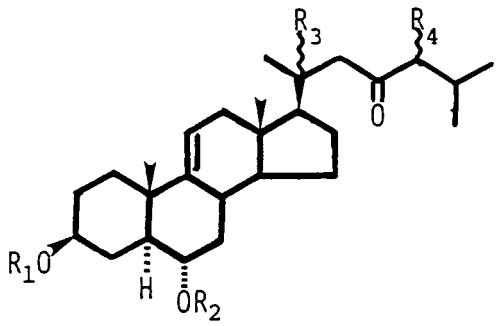
The saponins have also been known to immobilize sea urchin sperm and to inhibit development of fertilized sea urchin eggs (8). It was reported that the assay for fertilized sea-urchin or starfish eggs is activity-selective and may provide substantive information on the site and mode of action of drugs that inhibit cell division (9-12). Recently, we have isolated 17 asterosaponins from Japanese starfish which prompted us to examine their hemolytic activity and effects on cell division in fertilized echinoderm eggs mainly from the viewpoint of structure-activity relationships.

MATERIALS AND METHODS

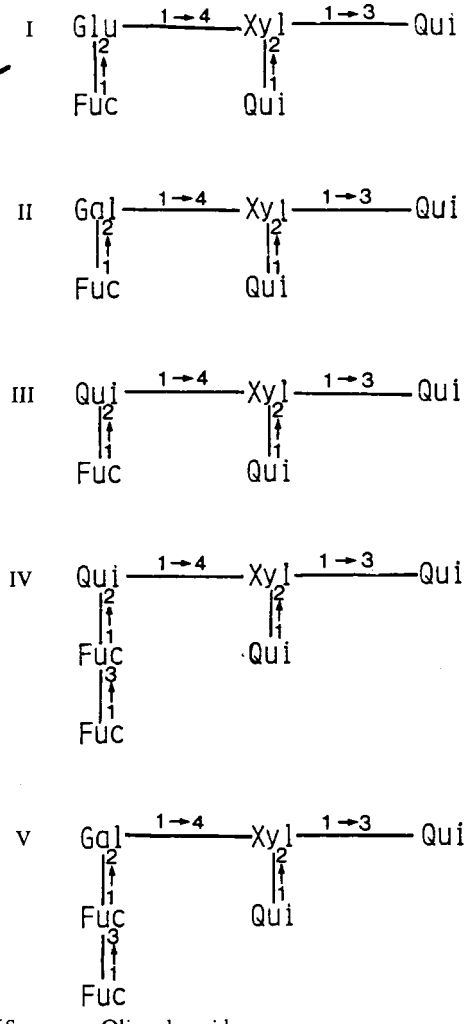
Seventeen oligoglycosides (Scheme 1) were isolated from *Acanthaster planci* L., *Luidia maculata* Müller et Troschel, and *Asterias amurensis* [cf.] *versicolor* Sladen (13-23), were used as test materials. The starfish specimens were identified by Professor T. Kikuchi, Amakusa Marine Biological Laboratory, Kyushu University, Japan. Specimens of the starfish *Asterina pectinifera* collected near Tokyo in April 1983, were kept in an aquarium at 15°, and sea urchin (*Hemicentrotus pulcherrimus*), obtained from Sagami Bay in January 1984, was kept at 10° in an aquarium until used for assay.

STARFISH AND SEA URCHIN EGG ASSAYS.—The methods of Ikegami *et al.* (9) were used. The fertilized eggs were obtained as follows. The starfish ovaries were removed from the animal and washed by decantation with natural seawater. To the egg suspension we added a few drops of 10^{-5} M 1-methyladenine in saline solution. After germinal vesicle breakdown, the eggs were filtered through gauze and suspended again in seawater, to which sperm was added. In the case of the sea urchin, eggs and sperm were collected from the animal by addition of 0.5 M KCl (10), and sperm was added to the washed eggs.

When a fertilized membrane was seen around each fertilized egg, approximately 100 fertilized eggs



Compound	R_1	R_2	R_3	R_4
4	SO_3Na	I	OH	H
5	SO_3Na	II	OH	H
6	H	VI	OH	H
7	SO_3NH_4	III	OH	H
8	SO_3Na	IV	OH	H
9	SO_3Na	V	OH	H
10	SO_3Na	VI	OH	H
11	SO_3Na	VII	OH	H
12	SO_3Na	II	OH	Me
13	SO_3Na	VI	OH	Me
14	SO_3Na	V	OH	Me
16	SO_3Na	VIII	H	H



SCHEME 1. Structures of Seventeen Oligoglycosides

were pipetted into sample wells that contained test solutions. The concentrations after addition of eggs were adjusted to 200, 100, 50, 30, 20, 15, 10, 5, 2, and 1 μg/ml. The dishes were stirred gently and maintained at 20°. The eggs were examined under a microscope at 1.5 and 24 h after fertilization.

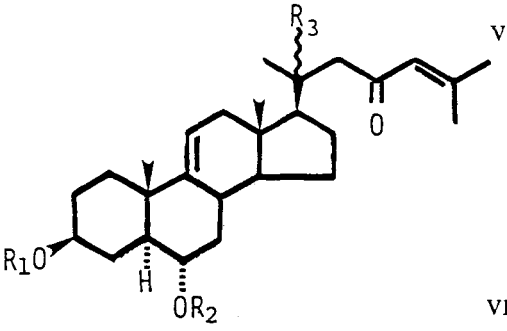
LACTOORCEIN STAINING.—According to the method of Nagano *et al.* (10), a few drops of a lactoorcein solution (10 ml of 2% orcein in glacial HOAc and 8.5 ml of lactic acid) were applied to embryos on a glass slide and squashed with a coverglass. After being allowed to stand at room temperature for 2 h, the embryos were observed under a microscope.

HEMOLYTIC ACTIVITY.—The method of Fujita and Nishimoto (24) was employed to estimate hemolytic activity. A 0.05-ml portion of each test solution (0.3-0.7 ml) containing 0.12-1.2 mg of sample saponins per ml of phosphate buffer (16 g of Na_2HPO_4 and 4.4 g of $NaH_2PO_4 \cdot 2H_2O$ in 1 liter of distilled H_2O, pH 7.4) was placed in a small test tube, to which buffer solution was added to make up to 1 ml. To the test solution we added 1 ml of a 2% suspension of rabbit erythrocytes in phosphate buffer, containing 0.0072% sodium citrate. The mixture was thoroughly mixed and allowed to stand for 6-8 h at room temperature. Hemolytic activity was expressed by a hemolytic index (HI), which was estimated from a minimum concentration inducing complete hemolysis and the following equation:

$$HI = \frac{V}{P/100 \times S}$$

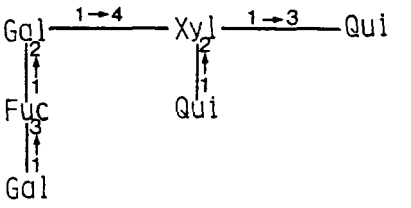
where

V = total volume (ml) of solution in a test tube,
P = % of saponin in test solution, and
S = test solution used (ml).

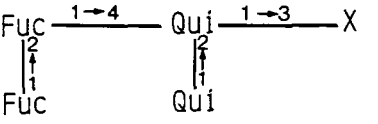


Compound	R_1	R_2	R_3
1	SO_3Na	III	OH
2	H	III	OH
3	SO_3Na	II	OH
15	SO_3Na	VIII	H

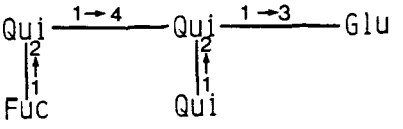
VI



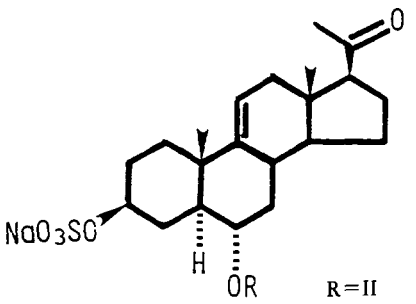
VII



VIII



X=6-deoxy-β-D-xylo-hexos-4-ulose
Qui=D-quinovose
Fuc=D-fucose
Glu=D-glucose
Gal=D-galactose
Xyl=D-xylose



R=II

Compound **17**

SCHEME 1. (Continued)

Standard saponin solution (0.016%, Merck, lot no. 9014073) was also prepared and tested for hemolytic activity. The HI of standard saponin was estimated to be 26,700.

RESULTS AND DISCUSSION

The effects of 17 saponins isolated from Japanese starfish on fertilized sea-urchin and starfish eggs are summarized in Table 1. For the sea urchin egg assay, ED_{50} and LD_{99} values are given which represent doses inducing 50% inhibition of the first cleavage at 1.5 h after fertilization and those inducing 99% death until blastulation, respectively. The doses that induced 50% of abnormal embryos at the blastula or gastrula stages are given for starfish egg assay, because very high doses were required to inhibit or kill starfish embryos. The developmental abnormalities were also observed in sea urchin embryos, as was reported by Ruggieri (25,26). It is evident that starfish eggs are much more resistant to the saponins, which might be partly due to the fact that asterosaponins are retained in the eggs during early embryogeneses (27). Nevertheless, the structure-activity relationships obtained in both tests are comparable. This also holds true for hemolytic activity.

TABLE 1. Effects of Seventeen Saponins on the Development of Sea Urchin and Starfish Embryos

Compound	Sea urchin assay		Starfish assay
	ED_{50} (μg/ml)[a]	LD_{99} (μg/ml)[b]	ED_{50} (μg/ml)[c]
1	50	50	200
2	10	10	100
3	15	5	100
4	15	2	200
5	10	5	100
6	50	10	100
7	15	5	200
8	15	10	200
9	15	5	200
10	30	10	200
11	15	5	200
12	5	2	50
13	10	5	200
14	5	2	100
15	10	5	50
16	5	5	50
17	>50	>50	100

[a]Dose that induced 50% inhibition of the first cleavage (observed at 1.5 h after fertilization).
[b]Dose that induced 99% lethality before the morula stage (24 h).
[c]Dose that induced 50% abnormal embryos at the blastula or gastrula stage (24 h).

Of 17 saponins tested, compound **12** was the most active and **17** the least, which suggests the importance of the structure of the side chain. In the series of saponins possessing similar side-chain features, those which contain a methyl group at C-24 (ergostane-type side-chain) were more active than those with no methyl group (cholestane-type side-chain). It is likely that a hydroxyl group in the side-chain does not influence activity. Also the $\Delta^{24(25)}$ structure appeared to be not important in activity. Further, it is noted that the pentaglycosides are somewhat more active than the hexaglycosides. The terminal D-fucose seems to have a considerable impact on activity. However, the sulfate moiety at C-3 is not important in activity, which is in contrast to the results obtained by Friess (5) in the neuromuscular blockage experiments.

All the saponins except compound **17** inhibited sea urchin embryos from developing further than the morula stage. The affected cells underwent incomplete cytokinesis with shallow cleavage furrows (Figure 1) and contained many nuclei (Figure 2). The same is true for starfish embryos. These phenomena are similar to those produced by cytochalasins (28-30). It is likely that asterosaponins inhibit actin polymerization during embryogenesis.

FIGURE 1. Abnormal starfish embryos induced by compound **12** showing incomplete cytoplasmic cleavage.

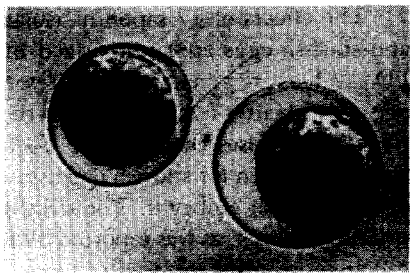


FIGURE 2. Abnormal embryos stained with lactoorcein solution. Small black spots in cell represent nuclei.

The hemolytic activity of nine asterosaponins is tabulated in Table 2. The structure-activity trends are similar to those obtained in the sea urchin and starfish embryo assay. Again compound **12** is the most active, whereas **17** is the least active. However, the sulfate group at C-3 appeared to influence somewhat the hemolytic activity.

TABLE 2. Hemolytic Activity of Starfish Saponins

Compound	Hemolytic Index
1	< 2,420
5	22,000
6	< 2,550
9	11,400
10	5,100
11	17,700
12	31,400
13	10,700
14	21,500
17	< 2,270

ACKNOWLEDGMENTS

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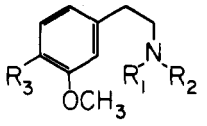
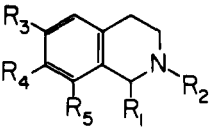
ERRATUM

We inadvertently switched two of the chemical formulae in N.R. Ferrigni, S.A. Sweetana, J.L. McLaughlin, K.E. Singleton, and R.G. Cooks, "Identification of New Cactus Alkaloids in *Backebergia militaris* by Tandem Mass Spectrometry," *J. Nat. Prod.*, **47**, 839.

The corrected placement is:



1 R_1,R_2,R_3=H
2 R_1,R_2=H; R_3=CH_3O
3 R_1=CH_3; R_2=H; R_3=CH_3O
4 R_1,R_2=CH_3; R_3=CH_3O



5 R_1=CH_3; R_2=H; R_3,R_4=CH_3O; R_5=H
6 R_1,R_2=H; R_3,R_4=CH_3O; R_5=H
7 R_1,R_2,R_3=H; R_4,R_5=CH_3O
8 R_1=H; R_2=CH_3; R_3,R_4=CH_3; R_5=H
9 R_1=H; R_2=CH_3; R_3=H; R_4,R_5=CH_3O

We regret any problems this may have caused.—ED.