BIOLOGICAL ACTIVITIES OF SULFATED GLYCOSIDES FROM ECHINODERMS

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ABSTRACT: The classes Asteroidea (starfishes) and Holothuroidea (sea cucumbers) belonging to the phylum Echinodermata are characterized by their content in toxic saponins. Asterosaponins from starfishes are sulfated steroidal glycosides whereas holothurins from sea cucumbers are triterpenoid glycosides with sulfate groups attached to the monosaccharide residues in sixty percent of the saponins isolated so far. Starfishes also contain steroidal mono- and diglycosides which occur as complex mixtures with asterosaponins. Due to their toxicity and membranotropic action, these polar compounds have attracted the attention of chemists and pharmacologists and a wide spectrum of biological activities has been found for these saponins. The purpose of this communication is to review the structural characteristics and biological properties of the saponins isolated from starfishes and sea cucumbers in the last five years, focusing on the structural elucidation and evaluation of antifungal, cytotoxic and antiviral properties of some examples of the author’s laboratory isolated from starfishes and sea cucumbers collected near the Patagonian shore and Antarctica.

INTRODUCTION

Marine organisms are sources of new natural products with unusual structural features, many of which have no counterpart among secondary metabolites of terrestrial origin [1]. The interest in marine chemicals lies in the pharmacological and toxicological properties of many of these secondary metabolites [2] and in their biological role in the natural environment [3]. Echinoderms belonging to the classes Asteroidea (starfishes or sea stars) and Holothuroidea (sea cucumbers) produce complex mixtures of saponins that are responsible for their general toxicity and that may play a defensive role due to their membranotropic action [4, 5]. These secondary metabolites are very common in plants but their presence in the animal kingdom is very rare. While the occurrence of saponins is constant in starfishes and sea cucumbers, they have been
found only rarely in some sponges, gorgonians, alcyonarians, green alga and in fishes of the genus *Pardachirus* [6].

Starfishes and sea cucumbers also contain glycosphingolipids (cerebrosides and gangliosides) that have a wide range of biological activities, presumably related to the amphipathic nature of these molecules [7, 8].

Several structural features of the aglycones and the oligosaccharide chains differentiate starfish saponins (asterosaponins) from those isolated from sea cucumbers (holothurins). Asterosaponins contain a steroidal nucleus while holothurins are characterized by a triterpenoid aglycone. All asterosaponins contain a sulfate group attached at C-3 of the aglycone while approximately sixty percent of the holothurins isolated so far have sulfate groups linked to the monosaccharide units of the oligosaccharide chain. It is suggested that sulfated compounds could have a role as stabilizers for hydroxyl or amino groups or as detoxifiers for non sulfated toxic compounds [9].

In addition to Asteroidea and Holothuroidea, the phylum Echinodermata (Greek *echinos*, spiny; *derma*, skin) comprises the classes Ophiuroidea (brittle stars), Crinoidea (sea lilies and feather stars) and Echinoidea (sea urchins). There is no report of occurrence of steroid or triterpenoid glycosides in sea lilies, feather stars or sea urchins. Brittle stars contain sulfated polyhydroxylated steroids [10-12] and only two sulfated steroidal monoglycosides have been isolated from the brittle star *Ophioderma longicaudum* [13].

Starfish and sea cucumber saponins have shown a wide spectrum of biological effects: antifungal, cytotoxic, hemolytic, antiviral, antibacterial and immunomodulatory activities [14, 15]. These biological activities are a consequence of their membranotropic action against $\Delta^5$ sterols in cellular membranes. Saponins form complexes with these sterols, developing single ion channels and larger pores which cause an alteration of the physico-chemical properties of membranes [16]. Starfish and sea cucumber cell membranes are resistant to their own saponins due to the presence of $\Delta^7$- and $\Delta^{9,11}$ sterols, sulfated $\Delta^5$ sterols and $\beta$-xylosides of sterols instead of the free $\Delta^5$ sterols [17].

Initially, most of the work on biological activities of asterosaponins has been performed on purified extracts of starfish and only few pharmacological studies have been carried out on the pure glycosides. This trend has changed and most of the new structures published
nowadays in the literature include biological studies that may contribute to a better understanding of the role of these secondary metabolites in starfish and to establish structure-activity correlations.

Several monographs concerning the structures and biological activities of asterosaponins and holothurins have been published [5, 14, 15, 18-20]. The aim of the present communication is to discuss the most recent findings in the field, focusing on the structural characteristics and biological activities of starfish steroidal glycosides and sea cucumber triterpene glycosides. Structural elucidation of some examples of the author’s laboratory will be presented.

**STEROIDAL GLYCOSIDES FROM ASTEROIDEA**

**Asterosaponins**

Starfishes are rich in sulfated steroidal oligoglycosides which occur as complex mixtures of highly oxygenated compounds, such as steroidal mono- and diglycosides and free and sulfated polyhydroxysteroids [18]. The term asterosaponin has been applied to designate the oligoglycosides containing a $\Delta^{9,11}$-3$\beta$6$\alpha$-dihydroxy steroidal aglycone with a sulfate group at C-3 and an oligosaccharide chain, usually made up of five or six units, glycosidically linked to C-6. The most common steroidal side chain in asterosaponins contains 20$\alpha$-hydroxy and 23-oxo functionalities, as in thornasterol A sulfate (1), the most common aglycone in asterosaponins, Fig. (1).

![Fig. (1). Structure of Thormasterol A sulfate](image)

Steroid 1 has been isolated from the starfish *Aphelasterias japonica* and showed hemolytic activity to mouse erythrocytes with an ED$_{50}$ value
of $1.1 \times 10^{-4}$ M [21]. Thornasterol A has also been isolated as the 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinolinium (salsinol) salt from the starfish *Lethasterias nanimensis chelifera* [22]. Comparison of the action of the sodium and salsinol salts of thomasterol A sulfate on the development of the sea urchin *Strongylocentrotus nudus* showed that the sodium salt was toxic with an EC$_{50}$ value of 40 μg/ml in contrast with the salsinol salt which was non-toxic up to a concentration of 100 μg/ml.

The most common monosaccharide units in asterosaponins are D-xylose, D-quinovose, D-fucose and D-galactose, always in their pyranose form with $\beta$-anomeric configuration ($\alpha$ for L-arabinose). The large majority of asterosaponins are pentaglycosides and the first monosaccharide unit (usually D-glucosyl or D-quinovosyl) linked to the aglycone is always glycosylated at C-3 by a branching glycosyl group (usually D-xylosyl or D-quinovosyl) with a terminal quinovose unit linked to C-2 [19, 20]. The branching sugar is glycosylated at C-4 by the remaining part of the oligosaccharide chain. An example of these characteristic features is thomasteroside A (2), the asterosaponin isolated from the common Pacific starfish *Achantaster planci* and the most widely distributed oligoglycoside in starfishes, Fig. (2) [19].

![Structure of asterosaponins](image)

2 Thomasteroside A $R = [\text{Fuc-(1→2)-Gal-(1→4)}]-(\text{Qui-(1→2)})-[\text{Xyl-(1→3)-Qui}]

3 Labidiasteroside A $R = [\text{Fuc-(1→2)-Qui-(1→4)}]-(\text{Qui-(1→2)})-[\text{Glc-(1→2)-Glc}]

4 Luidiaquinovoside A $R = [\text{Fuc-(1→2)-Qui-(1→4)}]-(\text{Qui-(1→2)})-[\text{Glc-(1→3)-Qui}]

5 Psilasteroside $R = [\text{Ara-(1→3)}]-[\text{Fuc-(1→2)-Gal-(1→4)}]-(\text{Qui-(1→2)})-[\text{Xyl-(1→3)-Qui}]

Fig. (2). Structure of asterosaponins from the starfishes *Acanthaster planci*, *Labidiaster annulatus*, *Luidia quinaria* and *Psilaster cassiscape*

Several reviews on steroidal glycosides from starfishes with descriptions of their structures, distribution and spectroscopic characteristics have been published [10, 18, 19].
Recently, we have isolated from the polar extracts of the Antarctic starfish *Labidiaster annulatus* the pentaglycoside labidisteroside A (3), the first asterosaponin with a 2,4-disubstituted glucose unit as a branching point in the oligosaccharide chain, Fig. (2) [23]. Asterosaponin 3 and the recently isolated luidiaquinovoside A (4) are the only two examples reported so far of asterosaponins containing a branched glucose in the oligosaccharide chain, Fig. (2) [24].

Luidiaquinovoside (4) and psilasteroside (5), isolated from *Psilaster cassiope*, Fig. (2) showed marginal in vitro cytotoxicity against RBL2H3 (rat basophilic leukemia) cell lines (IC$_{50}$ 31.3 and 5.4 µg/ml, respectively) [25].

A few asterosaponins containing hexasaccharide chains have been described. Versicoside A (6) was the first example of a group of hexaglycosides containing a single branching sugar unit [26] while pectinioside E was the first asterosaponin of a second group of hexasaccharides with two branching points in the sugar chain [27]. Anasterosides A (7) and B (8) are further examples of new hexasaccharides isolated together with versicoside A from the Patagonian starfish *Anasterias minuta*, Fig. (3) [28].

The saponins were isolated by bioactivity-guided fractionation of the ethanolic extract of the starfish using the brine shrimp (*Artemia salina* L.)
larvae mortality assay. The three hexaglycosides differed in the aglycone side chain, but contained the same hexasaccharide chain, composed of galactose, fucose, xylose and quinovose in the ratio 2:1:1:2. Anasteroside A (7) contained 23-oxo-5α-cholest-9(11)-en-3β,6α-diol 3-sulfate, an aglycone present in asterosaponins isolated from the starfishes *Marthasterias glacialis*, *Coscinasterias tenuispina*, *Luidia maculata* and *Neosmilaster georgianus* [19].

Anasteroside B (8) contained 5α-pregn-9(11)-en-3β,6α-diol 3-sulfate (3-O-sulfoasterone), which has been isolated for the first time as a natural product from the starfish *Aphelasterias japonica* [21]. Asterone has been reported as an artifact easily obtained by retroaldol cleavage of glycosides containing steroidal nucleus with 20-hydroxy-23-oxo side chains. The mild extraction and processing conditions employed in the isolation of the asterosaponins of *Anasterias minuta*, suggested that anasteroside B is a naturally occurring hexaglycoside. Two further examples of hemolytic monoglycosides containing 3-O-sulfoasterone were recently isolated from the starfish *Aphelasterias japonica* [21]. Recently, the 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinolinium (salsinol) salt of 3-O-sulfoasterone has been isolated from the starfish *Lethasterias nanimensis chelifera* [22].

The structural elucidation of the hexaglycosides of *A. minuta* required a combination of spectroscopic analysis (NMR and FABMS), chemical transformations and enzymatic degradations. Characteristic signals in the highfield region of the $^1$H-NMR spectra gave information on methyl groups belonging to the aglycones and the 6-desoxy sugars, fucose and quinovose. $^1$H- and $^{13}$C-NMR data were consistent with the presence of a steroidal aglycone with a 9(11)-double bond [$δ_C$ 145.3 (s, C-9) and 116.2 (d, C-11); $δ_H$ 5.19 (1H, m, H-11)], one sulfated oxomethine [$δ_C$ 78.0 (d, C-3); $δ_H$ 4.84 (1H, m, H-3)] and one oxomethine [$δ_C$ 79.5 (d, C-6); $δ_H$ 3.82 (1H, m, H-6)]. The methyl singlet at $δ$ 2.17 ppm in the $^1$H-NMR spectrum of 8 and the signals at $δ$ 210.0 (C-20) and 31.0 ppm (C-21) in the $^{13}$C-NMR spectrum confirmed the presence of asterone as the aglycone. Six doublets at $δ$ 4.82, 4.86, 4.90, 5.06, 5.09 and 5.18 ppm were assigned to the anomic protons. The $β$-stereochemistries at the anomic carbons were deduced from the coupling constant values ($J = 7.3-7.7$ Hz). The position of the interglycosidic attachments was determined using a combination of $^1$H-$^1$H COSY, relayed COSY, $^{13}$C-NMR and HETCOR experiments. These data were further confirmed by methylation of the
saponin 6 followed by acid hydrolysis and GC-MS analysis of the partially methylated alditol acetates derived of each sugar unit.

Some saponins have been reported to show antifungal properties [14]. Therefore, the hexaglycosides 6-8 were evaluated by a bioautographic technique [29] for their antifungal activity against the plant pathogenic fungus Cladosporium cucumerinum. In order to evaluate the influence of the oligosaccharide chain moiety on the antifungal activity, we hydrolyzed versicoside A (6) with Charonia lampas glycosidase mixture to obtain, after separation by HPLC, the pentaglycoside thomasteroside A (6a) and the triglycoside forbeside H (6b). The hexaglycosides versicoside A (6) and anasteroside A (7) as well as the pentaglycoside thomasteroside A (6a) were active in a concentration-dependent manner. Anasteroside A (7), lacking the hydroxyl group at C-20 was the most active saponin, while anasteroside B (8), with a pregnane side chain was inactive in all the concentrations range. On the other hand, the triglycoside forbeside H containing the same aglycone as thomasteroside A and versicoside A showed no activity at all the tested concentrations. Desulfation of versicoside A by solvolysis in dioxane/pyridine (1:1) rendered a totally inactive saponin. These results suggest that the sugar chain, together with the side chain in the steroidal aglycone moiety and the presence of a sulfate group at C-3 play an important role in the antifungal activity of these saponins.

Culcita novaeguineae, an abundant starfish distributed in the South China Sea, is used as a folk medicine for the treatment of rheumatism and as a tonic in China. The n-BuOH extract of this starfish showed significant deforming effect against the plant pathogenic fungus Pyricularia oryzae P-2b. Bioassay-guided fractionation led to the isolation of three new asterosaponins (9-11), together with four known pentaglycosides, regularosides A and B, thomasteroside A and marthasteroside A1, Fig. (4) [30, 31].

Asterosaponins 9, 10 and 11 were tested for in vitro cytotoxicity against human leukemia K-562 cells (IC<sub>50</sub>'s 8.60, 4.90 and 4.95 μg/ml, respectively) and human hepatoma BEL-7402 cells (IC<sub>50</sub>'s 9.2, 4.1 and 3.4 μg/ml, respectively).

Further analysis of the n-BuOH extract of Culcita novaeguineae resulted in the isolation of three new bioactive asterosaponins, Fig. (5) [32]. Compounds 12-14 possessed the same pentasaccharide moiety
linked to C-6 of the steroidal aglycone and differed from each other in the side chains.

Fig. (4). Structures of asterosaponins with a 22,23-epoxide in the side chain isolated from the starfish *Culcita novaeguineae*

Asterosaponins 12 and 13 showed significant cytotoxicity against two cancer cell lines, K-562 (IC$_{50}$'s 3.57 and 3.75 μg/ml, respectively) and BEL-7402 (IC$_{50}$'s 2.55 and 1.89 μg/ml, respectively).

Fig. (5). Structures of asterosaponins from the starfish *Culcita novaeguineae*
On the contrary, asterosaponin 14 possessed no cytotoxicity. Compounds 12 and 13 showed hemolytic activity to rabbit erythrocytes with ED\textsubscript{50} values of 16 and 31 \(\mu\)g/ml, respectively while 14 was inactive. These results are coincident with the lack of antifungal activity of anasteroside B (8), possessing the same 20-cetopregnane chain as asterosaponin 14.

**Glycosides of polyhydroxylated steroidal glycosides**

Besides asterosaponins, glycosides of polyhydroxysteroids are widespread in starfishes. These compounds usually occur as complex mixtures present in minor amounts. They are composed of a polyhydroxylated steroidal aglycone and a carbohydrate portion usually made up from one or two monosaccharide units, often linked to each other. Although the most common glycosylation position is C-24 of the steroidal aglycone, several glycosides with the sugar unit attached at C-3, C-26, C-28 or C-29 have been reported. The most common monosaccharide units are D-xylopyranose, often methylated at positions 2 and/or 4 and L-arabinose in its furanose form. Polyhydroxysteroid glycosides show a much larger structural variability than asterosaponins due to the hydroxylation pattern of the steroidal nucleus, the identity and location of the sugar residue and the presence of sulfate groups [18]. Usually, steroidal aglycones contain hydroxyl groups at positions 3\(\beta\), 6 (\(\alpha\) or \(\beta\)), 8, 15 (\(\alpha\) or \(\beta\)), 16 (\(\alpha\) or \(\beta\)) and 24 of the aglycone with additional hydroxyl groups at 4\(\beta\), 5\(\alpha\) and 7\(\alpha\). Hydroxylation at C-3 (\(\beta\)) and C-8 is a characteristic feature of the large majority of these compounds. Several examples of polyhydroxysteroid glycosides contain sulfate groups attached to the aglycone, as in the diglycoside antarcticoside P [33] or at the sugar unit, as in luridoside A, isolated from the Patagonian starfish *Cosmasterias lurida* [34]. The most common positions are C-3, C-6, C-15 and C-24 of the aglycone and C-5 of the pentose unit. Only three sulfated compounds with additional fosfate residues at C-6 (\(\alpha\)) have been isolated from the deep-water starfish *Tremaster novaecaledoniae* [35].

More than one hundred polyhydroxylated steroidal glycosides have been characterized but only few reports on their biological activity have been published. Recently, several examples of new bioactive mono- and diglycosides of polyhydroxysteroids as well as biological studies on known compounds have been reported.
Two new 24-\textit{O}-xylosides, rathbuniosides R\textsubscript{1} (15) and R\textsubscript{2} (16) have been isolated from the starfish \textit{Asterias rathbuni}, Fig. (6) [36]. The 3\beta,6\alpha,15\beta,24-tetrahydroxy substitution pattern in 16 has been found for the first time in polyhydroxysteroids from starfish.

![Fig. (6). Monoglycosides isolated from the starfish \textit{Asterias rathbuni}](image-url)

Rathbuniosides R\textsubscript{1} (15) and R\textsubscript{2} (16) as well as six related diglycosides (Structures 17-22, Fig. (7) and (8)) isolated from the starfishes \textit{Mediaster murrayi} [37], \textit{Ceramaster patagonicus} [38] and \textit{Culcita novaeguineae} [39] showed inhibition of cell division of the fertilized eggs of the sea urchin \textit{Strongylocentrotus intermedius} as well as hemolytic activity [40].

![Fig. (7). Diglycosides isolated from the starfish \textit{Mediaster murrayi}](image-url)

Differences in the activity of the eight compounds were correlated with the number of hydroxyl groups in the aglycones. Cytotoxic effects of the compounds were the result of cell membrane damage due to their ability to bind to membrane cholesterol.
The starfish *Certonardoa semiregularis* has shown to be a rich source of bioactive sterols and glycosides of polyhydroxylated steroids. Purification of the brine shrimp active fraction of the methanolic extract of *C. semiregularis* led to the isolation of nine new sulfated polyhydroxylated steroidal diglycosides [41].

Fig. (9). Structures of Certonardosides A – E
Certonardosides A-E (23-27), Fig. (9) are sulfated at C-3’ of the xylopyranose unit attached to C-26 of the steroidal side chain, while certonardosides F-I (28a, 28b, 29a, 29b), Fig. (10) contain a sulfate group at C-6 (α) of the steroidal aglycone. The isolated compounds were evaluated for their antiviral activity against HIV, HSV, CoxB, EMCV and VSV viruses. Certonardosides A-J were inactive within the range of non cytotoxic concentrations. Only weak antiviral activity against HSV was observed in compounds 29a and 29b and the desulfated analog of 28b.

Fig. 10. Structures of Certonardosides F – I

Eight new polyhydroxysteroid glycosides (32a-32e, 33a-33c), Fig. (11), glycosylated at C-28 and C-29, and the C-24 glycosides Certonardosides H₃ and H₄ (34 and 35), Fig. (12), together with twenty two new polyhydroxysteroids were further isolated from the brine shrimp active fraction of the starfish *Certonardoa semiregularis* [42, 43].

The isolated compounds were tested for cytotoxicity against a small panel of human solid tumor cell lines (A549, SK-OV-3, SK-MEL-2, XF498, CNS, HCT 15). The polyhydroxysteroids were more potent than
the corresponding glycosides except certonardosides $P_1$ (32a) and $J_3$ (32c).

Fig. (11). Certonardosides $O_1$ – $B_3$ from *Certonardoa semiregularis*

Fig. (12). Certonardosides $H_3$ and $H_4$ from *Certonardoa semiregularis*
Asterosaponins are usually considered to be responsible for the general toxicity of starfish. However, this investigation showed that sterols and another class of minor chemical components, such as glycosides of polyhydroxysteroids also play an important role in the general toxicity.

Recently, a new sulfated steroidal monoglycoside phrygioside B (36) together with its desulfated analog borealoside C (36a) [44] and the cyclopropane-containing steroid phrygiasterol (37), Fig. (13) have been isolated from the Pacific starfish *Hippasteria phrygiana* [45]. Compound 37, the first cyclopropane-containing steroid isolated from echinoderms, inhibited the growth of Ehrlich carcinoma cells with an IC<sub>50</sub> of 50 μg/ml, whereas borealoside C induced apoptosis of the same cells (EC<sub>50</sub> = 70 μg/ml) and inhibited Ca<sup>2+</sup> influx into mouse spleenocytes (EC<sub>50</sub> = 20 μg/ml).

![Diagram of compounds](image)

**Fig. (13).** Structures of phrygiasterol and related compounds from *Hippasteria phrygiana*

Bioactivity-guided fractionation of the alcoholic extract of the Okinawan starfish *Linckia laevigata* led to the isolation of five new neuritogenic polyhydroxysteroid diglycosides, Fig. (14) [46, 47]. These compounds are further examples of a group of rare steroidal glycosides in which the monosaccharide residues are located in two different positions of the steroidal nucleus [48]. Each of the linckosides possesses one 2-O-methylxylopyranose unit attached to C-3 of a 3β,6β,8β,16β-tetrahydroxy substituted steroidal aglycone and an additional monosaccharide unit at the aglycone side chain (C-24, C-28 or C-29).
Linckosides A (38) and B (39) differ in the sugar unit attached to C-29, an α-arabinofuranosyl in 38 and a xylose unit in 39. Linckosides C (40) and D (41) are the first steroids that possess a hydroxyisopropyl substituent at C-24 of the side chain. The neuritogenic activity of diglycosides 38-42 was evaluated using rat pheochromocytoma (PC12) cells in comparison with NGF (nerve growth factor). Linckosides B, C and D with a xylose unit in the side chain showed significant neuritogenic activities of 62%, 56% and 73%, respectively, comparable with that of NGF.

On the other hand, linckosides A and B with an arabinose unit at the side chain exhibited lower activities (33% and 29%, respectively) than the others. This result suggests that the nature of the sugar moiety at the side chain plays an important role for the neuritogenic activity and that other structural factors, such as the presence or absence of a methyl group at C-28 and the double bond at C-22, seemed not to be important. All the linckosides showed significant synergistic effects on the NGF-induced neuronal differentiation of PC12 cells.

Bioactivity-guided fractionation of the ethanolic extract of the Patagonian starfish Anasterias minuta using the brine shrimp (Artemia salina L.) larvae mortality assay led us to the isolation of three sulfated
polyhydroxylated steroidal xylosides, the new minotosides A (43) and B (44), together with the known pycnopodioside B (45), Fig. (15) [49]. The structures were elucidated by extensive 1D and 2D NMR experiments as well as FABMS analysis and chemical methods.

The molecular formula of compound 44 was established as \( \text{C}_{35}\text{H}_{58}\text{O}_{12}\text{NSNa} \) on the basis of the pseudomolecular ion peak at \( m/z \) 762.3481 [M + Na]\(^+\) in the HRFABMS. Examination of the \(^1\text{H}-\) and \(^{13}\text{C}-\)NMR spectra indicated that 44 possessed the same \( 3\beta,6\alpha,8,15\beta \)-tetrahydroxy steroidal nucleus as glycosides 43 and 45 bonded to a monosaccharide unit.

![Structural formulas](image)

Fig. (15). Bioactive monoglycosides from the starfish *Anasterias minuta*

The relative stereochemistry of all chiral centers was established by analysis of selected NOE correlations. The hydroxyl groups at C-3 and C-6 were assigned to \( 3\beta \) and \( 6\alpha \) on the basis of NOEs between H-3 and H-
5α and between H₃-19 and H-6. Correlations between H-14/H-15 and H-15/H-16α revealed the β-configuration of the hydroxyl group at C-15. The ¹H NMR spectrum contained three methyl doublets at δ 0.95, 0.96 and 1.07 ppm which were assigned to CH₃-28, CH₃-21 and CH₃-27, respectively by analysis of the ¹H-¹H COSY, HMQC and HMBC spectra. The ¹H-NMR spectrum of 44 also contained two methylene signals at δ 2.96 and 3.53 ppm coupled to each other in the ¹H-¹H COSY spectrum. Correlation of these protons in the HMQC spectrum to the signals at δ 51.5 (C-1') and 36.6 ppm (C-2'), together with the correlation of the triplet at δ 2.96 (H₂-1') to the Me-27 (δH 1.07) in the NOESY spectrum as well as the chemical shift of the carbonyl group (δC 178.5) suggested the presence of a Δ²₂E, 26-amide ergostane side chain [50]. The strong bands at 1212 and 1050 cm⁻¹ in the FTIR spectrum, characteristic of a sulfonic acid salt, together with the fragment ion at m/z 455 [M - SO₃Na - Xyl + Na]^+ in the positive FABMS and the NMR data of 44 suggested the presence of a taurine residue in the side chain. This side chain has been previously identified in a polyhydroxysteroid isolated from the starfish Myxoderma platyacanthum [50]. Recently, the aglycone of minutoside B (44), triseramide, has been isolated from the starfish Astropecten triseriatus [51].

Analysis of the monosaccharide signals in the ¹³C-NMR spectrum (103.1, 77.9, 75.0, 71.3 and 66.9) and further assignment of all proton and carbon chemical shifts using ¹H-¹H COSY and HMQC experiments indicated the presence of a xylose residue. The presence of xylose was confirmed by acid hydrolysis of 44 with aqueous 2N trifluoroacetic acid followed by GC analysis of the corresponding peracetylated alditol. The D-configuration was determined by GC analysis of the 1-[(S)-N-acetyl-(2-hydroxypropylamino)]-1-deoxyalditol acetate derivative as for minutoside A (43). The chemical shift of the anomic carbon (δC 103.1) and the coupling constant of the anomic proton (δH 4.36, J₁',₂' = 7.7 Hz) suggested that the sugar had a β-configuration. The location of the sugar at C-3 of the aglycon was established on the basis of the correlation between the anomic proton and H-3α in the NOESY spectrum of 44. Minutoside B (44) is the first example of a steroidal xyloside containing an amide function in the aglycone obtained from a natural source. Only a few examples of polyhydroxylated sterols with an amide function in their side chains have been reported previously from the starfishes Myxoderma platyacanthum [50] and Styra...
Since some polyhydroxylated steroid glycosides have shown interesting biological activities, compounds 43-45 and the desulfated analogs 43a and 45a were examined against the pathogenic fungi Cladosporium cucumerinum and Aspergillus flavus by a bioautographic technique [29]. Minutoside A (43) and pycnopodioside B (45) were moderately active (inhibition zones of 7–10 mm) against C. cucumerinum at the tested concentrations (10–60 µg/spot), while minutoside B (44) was inactive at the lowest concentration (10 µg/spot) and weakly active (inhibition zones of 3–4 mm) at the highest concentrations (20–60 µg/spot). The three glycosides (1-3) were moderately active against A. flavus showing inhibition zones of 5–10 mm at the highest tested concentrations (20–60 µg/spot). The compounds were found to be less active than benomyl, a commercially available fungicide, which showed inhibition zones of 15 and 14 mm at a concentration of 5 µg/spot for C. cucumerinum and A. flavus, respectively. The three xylosides were also less active against C. cucumerinum than the asterosaponins versicoside A (6) and anasteroside A (8) isolated from A. minuta. As for the asterosaponins, the desulfated analogs 43a and 45a were inactive against C. cucumerinum and A. flavus at all the tested concentrations. These results suggest that the presence of a sulfate group in the aglycon moiety may play an important role in the antifungal activity of these monoglycosides.

Recently, two related monoglycosides, the known henricioside H₂ (46) and the new leviusculoside J (47) have been isolated from the Far Eastern starfish Henricia leviuscula, Fig. (16) [53].

Fig. (16). Bioactive monoglycosides from the starfish Henricia leviuscula

46 Henricioside H₂, R = A
47 Leviusculoside J R = B
Both 3,6-di-\(O\)-methylxylanosides differ in the aglycone side chain. Compounds 46 and 47 showed 70\% and 100\% hemolysis of mouse erythrocytes, respectively, at a concentration of \(8.0 \times 10^{-5} \text{M}\).

**TRITERPENOID GLYCOSIDES FROM HOLOTHUROIDEA**

Holothurins isolated from sea cucumbers are triterpenoid oligoglycosides that contain an aglycone based on a “holostanol” skeleton \([3\beta,20S\text{-dihydroxy-5α-lanostano-18,20-lactone}] (48), Fig. (17)\) and a sugar chain of two to six monosaccharide units linked to the C-3 of the aglycone \([54]\). Quinovose, glucose, 3-\(O\)-methylglucose, xylose and, rarely 3-\(O\)-methylxylose are present in the carbohydrate moieties of these glycosides. The first monosaccharide unit is always xylose, while 3-\(O\)-methylglucose and 3-\(O\)-methylxylose are always terminal.

![Fig. (17). Structure of hypothetical holostanol](image)

Most of sea cucumber triterpene glycosides are tetra- or pentaglycosides. The majority of tetrascarbohydrates show a linear chain with the most common 3-\(O\)-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)-Xyl structure. The few disaccharides that have been isolated show a Qui-(1→2)-4-\(\text{OSO}_3\)Na-Xyl chain attached to C-3 of the triterpenoid aglycone. Some hexascarbohydrides have been isolated from sea cucumbers of the order Aspidochirota: *Stichopus japonica*, *Stichopus chloronotus*, *Parastichopus californius* and *Bohadschia bivittata* \([55]\). They are non-sulfated glycosides with a linear 3-\(O\)-Me-Glc-(1→3)-Glc-(1→4)-Xyl chain and a branching of a linear trisaccharide at C-2 of the xylose unit.
Sixty percent of the triterpene glycosides isolated so far from sea cucumbers have sulfate groups linked to the monosaccharide units of the oligosaccharide chain. Most of them are monosulfated oligoglycosides, but several di- and trisulfated glycosides have been isolated, mainly from the order Dendrochirotida. Most tetrasaccharides are sulfated at C-4 of the xylose unit. Additional sulfate groups at C-6 of the 3-0-Me-glucose unit and at C-6 of the glucose unit have been found in trisulfated tetruglycosides. Most of the pentasaccharide chains are monosulfated at C-4 of the xylose unit linked to the aglycone. Only a few disulfated or trisulfated pentaglycosides with additional sulfate groups at C-6 of the 3-0-Me-glucose and glucose units have been isolated [55].

More than 100 different holothurins have been isolated from sea cucumbers in the last 25 years. These saponins differ in the composition and number of the sugar units, the number and positions of the sulfate groups and the structural characteristics of the aglycone. Some examples of holothurins having non-holostane aglycones have been found in seven species of sea cucumbers belonging to the order Dendrochirotida. The majority are monosulfated at the glucose or xylose units [55]. Triterpene glycosides are produced in the skin and in the Cuvier’s tubules of sea cucumbers and are ejected when the animals are disturbed. This behavior may be associated to a defensive function due to the ability of holothurins to form complexes with cholesterol and other \( \Delta^5 \)-sterols from cell membranes. This membranotropic action determines the wide spectrum of their biological activities [15], Sea cucumbers are resistant to their own toxins due to the presence of \( \Delta^7 \), 14\( \alpha \)-methyl- and 14\( \alpha \)-dimethyl- \( \Delta^{9,11} \)-sterols as well as their conjugated forms such as steryl sulfates and steryl xylosides [56].

Several holothurins are specific for different taxonomic groups of sea cucumbers and structural characteristics of triterpene glycosides have been used to resolve taxonomic problems in the class Holothuroidea [57, 58]. For example, the triterpenoid glycosides distribution has been successfully applied in the reclassification of \textit{Stichopus mollis} into the genus \textit{Australostichopus} [59] and in the taxonomy of sea cucumbers belonging to the genus \textit{Cucumaria} [60].

Recently, we have reviewed for the first time the chemical structures of sea cucumber glycosides and the principal spectral features of the aglycones and oligosaccharide chains in their \( ^1H \)- and \( ^{13}C \)-NMR spectra [55]. Most of these triterpenoid oligoglycosides contain an aglycone
based on a “holostanol” skeleton and two main series can be distinguished: glycosides based on a 3β-hydroxyholost-9(11)-ene aglycone and those containing a 3β-hydroxyholost-7-ene skeleton. Only a few examples of holothurins containing a non holostane aglycone have been isolated from sea cucumbers of the order Dendrocirotida. Usually aglycones that have a $\Delta^{9,11}$ double bond are characteristic of sea cucumbers belonging to the order Aspidochirota, while those with a $\Delta^7$ unsaturation were generally isolated from animals of the order Dendrochirotida.

### 3β-Hydroxyholost-7-ene aglycones

Several triterpene glycosides isolated from the sea cucumbers *Cucumaria frondosa*, *Cucumaria japonica*, *Stichopus chloronotus* and *Thelenota ananas* contain the simple 3β-hydroxyholost-7-ene as the aglycone (49) [55]. Recently, three new monosulfated pentaglycosides, frondosides A2-2 (49a) A2-3 (49b) and A2-4 (49c), Fig. (18) were isolated from the sea cucumber *Cucumaria frondosa* [61, 62].

![Structure of holostane aglycone](image)

**Fig. (18).** Sulfated glycosides isolated from the sea cucumber *Cucumaria frondosa*

The three holothurins possess the same pentasaccharide moiety and differ from each other in the aglycone side chain. Frondoside A2-3 (49b) contains a $\Delta^{23}$ double bond and a hydroxy group at C-25. This side chain has been previously identified in the aglycones of cucumarioside G4 from
Eupentactafraudatrix [63] and Eximisoside A from Psolus eximius [64]. In frondoside A₂-2 (49a), the simultaneous presence of a 24-ketone conjugated with a Δ²⁵ double bond in the side chain is a unique characteristic for holothurins.

Dendrochirotida sea cucumbers belonging to the family Cucumariidae were extensively studied during the last years. Recently, the cytotoxic activities of the known pentaglycosides cucumariosides A₂-2 (50a) and A₇-1 (50b), Fig. (19) isolated from the sea cucumber Cucumaria japonica [65] were investigated using embryos of the sea urchin Strongylocentrotus nudus [66]. Cucumariosides A₂-2 and A₇-1 were highly toxic and induced morphological abnormalities and delay of development. EC₅₀ values of glycosides 50a and 50b were determined as 0.3 and 1.98 μg/ml, respectively, whereas the aglycone was completely inactive at 100 μg/ml. Both cucumariosides differ only in the number of sulfate groups in the oligosaccharide chain. Cucumarioside A₂-2 is a monosulfated glycoside while A₇-1 is a trisulfated holothurin.

![Glycosides with a 3β-hydroxyholost-7-en-16-one aglycone](image)

Disulfated cucumariosides A₃ (50c) and A₆-2 (50d), Fig. (19) differ from the monosulfated cucumarioside A₂-2 (50a) in the presence of an additional sulfate group at C-6 of the glucose unit in 50c and at C-6 of the
3-O-methyl glucose unit in 50d. Both holothurins showed cytotoxicity in vitro at IC50 = 1μg/ml against a selection of five human and mice tumoral cell lines [67].

Recently, a new monosulfated holothurin has been isolated from the sea cucumber *Cucumaria frondosa* [61]. The structure of frondoside A2-1 (50e) is very similar with that of cucumarioside A2-2 (50a), the only difference being the presence of a Δ24 double bond instead of a Δ25 one. Another new sulfated tetraglycoside, mollisoside B2 (50f) was isolated from the sea cucumber *Australostichopus mollis*, collected at New Zealand and southern Australia [68]. This holothurin has the same aglycone as Cucumariosides A2-2, A7-1, A3 and A6-2 and differs from them in the oligosaccharide chain. Mollisoside B2 (50f) has a linear terasaccharide chain monosulfated at C-4 of the xylose unit.

Several triterpene glycosides isolated from the sea cucumbers *Cucumaria echinata* and *Pentamera clacigera* contain aglycones with a carbonyl group at C-23 in the side chain. This structural feature is absent in 3β-hydroxyholost-9(11)-ene aglycones [55]. Two new glycosides containing this aglycone have been isolated from the sea cucumber *Pentamera calcigera*. Calcigerosides C2 (51a) [69] and D2 (51b) [70], Fig. (20) have a branched pentasaccharide chain with 3-O-methyl xylose as a terminal residue. Glycoside 51a contains a terminal glucose attached to the quinovose residue. This feature has not been found before in sea cucumbers.

![Fig. (20). Bioactive glycosides isolated from the sea cucumber Pentamera calcigera](image-url)
Calcigeroside C₂ and its desulfated derivative were individually tested in vitro against four standard human and mouse tumoral cell lines (P-388, A-549, HT-29 and Mel-98). Only the desulfated derivative showed moderate cytotoxicity (IC₅₀ = 5 μg/ml).

Another example of an holothurin containing aglycon 51 with a ß-acetoxy group at C-16, Cucumarioside A₂-5 (51c), was isolated from the sea cucumber *Cucumaria conicospermium*, collected in the Sea of Japan [71]. The same aglycone has been found earlier only in Cucumarioside A⁰-1, isolated from *Cucumaria japonica* [72].

Five non-sulfated triterpene glycosides, synallactosides A₁ (52a), A₂ (52b), B₁ (52c), B₂ (52d) and C (52e), Fig. (21) were isolated from the deep-water North Pacific sea cucumber *Synallactes nozawai* [73]. Synallactoside A₁ is the 25,26-dehydro derivative of thelenotoside A, isolated from *Thele­nota ananas* as a mixture with thelenotoside A [74]. Compounds 52b-52e are new compounds with interesting features in the carbohydrate chains of synallactosides A₂, B₁, and B₂. Synallactoside A₂ (52b) is the first glycoside isolated from sea cucumbers with two terminal 3-O-methylxylose residues in the oligosaccharide chain. On the other hand, the carbohydrate chains of synallactosides B₁ (52c) and B₂ (52d) have unprecedent structures among holothurins.

![Fig. (21). Glycosides isolated from the sea cucumber *Synallactes nozawai*](image)

Recently, two new sulfated holothurins, pseudostichoposide B (53a) and mollisoside A (53b), Fig. (22) were isolated from two sea cucumbers belonging to the order Aspidochirotida, *Pseudostichopus trachus* [75] and...
Australostichopus mollis [68]. Both glycosides contain an aglycone with a 22-keto group in the side chain, a novel feature for sea cucumber glycosides. Pseudostichoposide B (53a) contains a sulfate group at C-4 of the first xylose residue and an additional sulfate group at C-3 of the quinovose residue, a feature not earlier found in holothurins. The isolation of mollisosides A (53b) and B2 (50f) together with the known neothyonidioside reported earlier from the sea cucumber Neothyonidium magnum [76] as well as morphological features justified the reclassification of Stichopus mollis in the new genus Australostichopus Levin [59].

Pseudostichoposide B $R = \text{3-0-Me-Glc-(1\rightarrow3)-Xyl-(1\rightarrow4)-3-OSO}_3\text{Na-Quin-(1\rightarrow2)-4-OSO}_3\text{Na-(1\rightarrow4)-Xyl}$

Mollisoside A $R = \text{3-0-Me-Glc-(1\rightarrow3)-Glc-(1\rightarrow4)-Quin-(1\rightarrow2)-4-OSO}_3\text{Na-Xyl}$; $\Delta^{35}$

Fig. (22). Glycosides with a 22-keto-holost-7-en-3β-ol aglycone

Calcigeroside E (54), Fig. (23) is a new disulfated pentaglycoside isolated from the sea cucumber Calcigera pentamera [70].

Calcigeroside E $R = \text{[6-OSO}_3\text{Na-3-0-Me-Glc-(1\rightarrow3)-Glc-(1\rightarrow4)-[Glc-(1\rightarrow2)]-Quin-(1\rightarrow2)-4-OSO}_3\text{Na-Xyl}}$

Fig. (23). Structure of Calcigeroside E isolated from Calcigera pentamera
The aglycone of this glycoside has been previously found in only two holothurins, cucumarioside G₄ [63] and eximisoside A [64].

One structural feature that has been found only in the 3ß-hydroxyholost-9(11)-ene aglycones is the presence of an acetoxyl group at C-16 (α or β). Recently, nine new bioactive sulfated tetracylglosides (compounds 55a-55i, (Fig. (24)) containing a 6ß-acetoxyl group at C-16 have been isolated from the sea cucumber Mensamaria intercedens [77, 78]. Intercedensides A (55a), C (55c), D (55d), E (55e), G (55g) and H (55h) contain a conjugated double bond (22,24-diene) in the aglycone side chain. This feature is very rare among sea cucumber glycosides. Analogous side chains were found only in cucumariosides C₁, C₂ [79] H [80] and G₃ [63]. Intercedensides C (55c), D (55d), E (55e), F (55f), H (55h) and I (55i) have a 17α-hydroxy group that is not characteristic for sea cucumbers of the Dendrochirotida order with exception of patagonicoside A, isolated previously from Psolus patagonicus [81].

![Fig. (24). Sulfated glycosides from Mensamaria intercedens, Staurocucumis liouvillei and Thyone aurea](image)

Intercedensides A-H showed significant in vitro cytotoxicity against 10 human tumor cell lines (A549, MCF-7, IA9, CAKI-1, U-87-MG, PC-3,
KB, KB-VIN, SK-MEL-2, HCT-8) with ED$_{50}$ values in the range 0.6-4.0 µg/ml. Intercedenside A exhibited significant in vivo antineoplastic activity against mouse Lewis lung cancer and mouse S180 sarcoma.

Two new trisulfated tetraglycosides, Liouvillosides A (55j) and B (55k) were isolated from the Antarctic sea cucumber *Staurocucumis liouvillei* [83]. Both glycosides differ only in the presence of a Δ$^{24}$ double bond in compound 55j. The terminal isopropenyl group in the side chain of liouvilloside A showed characteristic signals in the $^{13}$C-NMR spectrum for the olefinic carbons at δ 123.9 ppm (C-24) and 131.3 ppm (C-25) as well as for the methyl groups attached to C-25 at δ 25.6 ppm (C-26) and 17.8 ppm (C-27). The presence of these two methyl vinyl groups was easily confirmed by the downfield shift of the methyl singlets in the $^1$H-NMR spectrum at δ 1.54 ppm (H-26) and 1.64 ppm (H-27), while liouvilloside B (55k), the saturated analog, showed two nearly overlapped doublets ($J$ = 6.6 Hz) at δ 0.83 and 0.84 ppm. Glycosides 55j and 55k showed a characteristic singlet at δ 2.0 ppm (CH$_3$CO$_2$) as well as signals at δ 169.4 and 21.1 ppm for the carbonyl and methyl groups of the acetate moiety. The position of the acetoxy group at C-16 was deduced from the chemical shift of the H-16 signal (δ 5.63 ppm) and its correlation with H-17, H-15α and H-15β in the $^1$H-$^1$H COSY spectrum. The 16β-configuration was assigned by a NOESY experiment and by coupling constant analysis for the C-16 proton with the C-17α and C-15 protons. Calculated coupling constant values of 8.9 ($J_{15α,16α}$), 7.4 ($J_{15β,16α}$) and 8.9 Hz ($J_{16α,17α}$) for the most stable conformation of 16β-acetoxyholosta-7,24-dien-3β-ol obtained by molecular mechanics were coincident with experimental and reported values [83] and differed considerably from those calculated for the 16α-isomer (4.1, 6.9 and 1.2 Hz, respectively).

In addition to the aglycone signals, the $^1$H-NMR spectra of 55j and 55k showed four anomic protons at δ 4.32 (xylose), 4.40 (glucose), 4.47 3-O-methylglucose) and 4.49 ppm (quinovose) that correlated with the anomic carbon signals at δ 104.2, 103.1, 103.8 ppm. The β-stereochemistries at the anomic carbons were deduced from the coupling constant values ($J$ = 7.1–7.9 Hz). The position of the interglycosidic attachments was determined by a combination of $^1$H-$^1$H COSY, relayed COSY and HETCOR experiments. The four carbohydrate units were determined to belong to the D-series by GC analysis of the mixture of 1-[(S)-N-acetyl-(2-hydroxypropylamino)]-1-deoxyalditol
acetate derivatives. The position of the sulfate groups at C-6 of both glucose units and at C-4 of the xylose unit in the sugar chain was determined by comparison of $^{13}$C-NMR data of both glycosides with those of the corresponding desulfated derivatives obtained by hydrolysis in anhydrous 0.15% HCl-MeOH at room temperature. This work is the first study of the glycosidic content of an Antarctic sea cucumber belonging to the genus *Staurocucumis* (family Cucumariidae, order Dendrochirotida).

Evaluation of the cytotoxicity of liouvilloides A and B at concentrations ranging from 6.25 to 50 μg/ml showed little or no cytotoxicity within eight hours of cell exposure to the compounds, but both saponins were cytotoxic following prolonged incubation periods. According to these results, the virucidal activity of both glycosides was then evaluated by incubation of a suspension of herpes simplex virus 1 (HSV-1) with the holothurins at concentrations below 10 μg/ml. Both saponins exerted an irreversible virucidal effect on HSV-1, but with different effectiveness: liouvilloide A produced a weak inactivation of HSV-1 since at the maximum concentration tested the residual infectivity was 24% with respect to the control virus sample, whereas after treatment with liouvilloide B in the same experimental conditions the remaining infectivity was 10-fold lower (2.5%). These results are in agreement with previous reports on the activity of triterpenoid compounds against several viruses, including important pathogens such as HSV and human immunodeficiency virus. In particular, naturally occurring saponins with different structures were found to be inhibitory of HSV through either a direct virucidal effect or by interference with an early step of the viral replicative cycle.

Two further examples of new sulfated holothurins containing aglycone 55 with a Δ$^{25}$ double bond were isolated from the sea cucumber *Thyone aurea*, collected in Namibia [84]. Thyonosides A (551) and B (55m) differ from each other in one glycosidic residue, xylose in glycoside 55m instead of glucose-6-OSO$_3$Na in 551 and in the β-(1→4) linkage between 3-O-methylxylose and xylose in Thyonoside B. Both glycosides contain the rare terminal 3-O-methylxylose.

Purification of the triterpenoid glycosides mixture of the Patagonian sea cucumber *Psolus patagonicus* led us to isolate patagonicoside A (56a), Fig. (24), the main holothurin in the mixture [81]. This disulfated tetruglycoside has an uncommon aglycone with two hydroxyl groups at
C-12α and C-17α, a structural feature characteristic for aspidochirotid sea cucumbers. This is the first report of an aglycone with simultaneous presence of 12α- and 17α-hydroxy groups and a Δ⁷ double bond. The presence of two hydroxy groups attached to C-12 and C-17 was evidenced by two signals in the ¹³C-NMR spectrum at δ 73.6 and 90.7 ppm, respectively. The C-7 signal at δ 90.7 ppm could be readily distinguished from the C-3 signal (δ 90.8 ppm) by a DEPT experiment.

\[
\text{56a Patagonicoside A } R = 3-\text{OMe-Glc-(1→3)-6-OSO₃Na-Glc-(1→4)-Quin-(1→2)-4-OSO₃Na-Xyl}
\]
\[
\text{56b Ds-Patagonicoside A } R = 3-\text{OMe-Glc-(1→3)-Glc-(1→4)-Quin-(1→2)-Xyl}
\]

Fig. (24). Structure of Patagonicoside A, an antifungal holothurin isolated from the sea cucumber \textit{Psolus patagonicus} and its desulfated derivative

Since some holothurins with at least one oxygen function in positions 12, 16 or 17 of the aglycone moiety (e.g. holotoxins A and B, holothurins A and B, echinosides A and B, pervicosides A-C and Bivittosides A-D [55]) exhibit antifungal activities, patagonicoside A (56a) and its desulfated derivative, ds-patagonicoside A (56b) were examined against the phytopathogenic fungus \textit{Cladosporium cucumerinum} by a bioautographic technique. Both saponins showed a marked difference in their antifungal properties. Patagonicoside A (56a) resulted to be considerable active, in a concentration dependent manner, showing inhibition zones of 8-19 mm at the tested concentrations (1.5-50 μg/spot). On the other hand, ds-patagonicoside A (56b) was inactive at the lowest concentrations (1.5 and 3 μg/spot) and weakly active (inhibition zones of 5-9 mm) at the highest tested concentrations (6-50 μg/spot). These results suggest that the presence of sulfate groups in the oligosaccharide chain may play an important role in the antifungal activity of these glycosides.
3β-Hydroxyholost-9(11)-ene aglycones

In the last four years, five new sulfated and two new non sulfated triterpene glycosides containing the aglycone 3β-hydroxyholost-9(11)-en-16-one (Structure 57), Fig. (25) have been isolated from sea cucumbers.

![Structure 57](image)

57a Mollisoside B1, R = 3-OMe-Glc-(1→3)-Xyl-(1→4)-Glc-(1→2)-4-OSO3Na-Xyl; Δ25
57b Neothyonidioside C R = 3-OMe-Glc-(1→3)-Xyl-(1→4)-Qui-(1→2)-4-OSO3Na-Xyl; Δ25
57c Frondoside A2-6 R = [3-OMe-Glc-(1→3)-Glc-(1→2)]-Xyl-(1→2)-4-OSO3Na-Xyl; Δ25
57d Hemoiedemoside A R = 3-OMe-Glc-(1→3)-6-OSO3Na-Glc-(1→4)-Qui-(1→2)-4-OSO3Na-Xyl; Δ25
57e Hemoiedemoside B R = 6-OSO3Na-3-OMe-Glc-(1→3)-6-OSO3Na-Glc-(1→4)-Qui-(1→2)-4-OSO3Na-Xyl; Δ25
57f Parvimoside A R = [Glc-(1→3)-Glc-(1→4)]-3-OMe-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)-Xyl
57g Parvimoside B R = [Glc-(1→3)-Glc-(1→4)]-3-OMe-Glc-(1→3)-Xyl-(1→2)-Xyl
57h Parvimoside C R = [Glc-(1→3)-Glc-(1→4)]-3-OMe-Glc-(1→3)-Xyl-(1→2)-Qui-(1→2)-Xyl
57j Holotoxin B R = [Glc-(1→3)-Glc-(1→4)]-3-OMe-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)-Xyl; Δ25

Fig. (25). Structures of 3β-hydroxyholost-9(11)-en-16-one aglycone based glycosides

The monosulfated tetraglicoside mollisoside B1 (57a) was isolated from the sea cucumber *Stichopus mollis* [68] together with the major component of its glycosidic fraction, the known neothyonidioside C (57b) [59]. Glycosides 57a and 57b have holotoxinogenin as aglycone and a carbohydrate chain with a sulfate group at C-4 of the xylose group attached to C-3 of the aglycone, an uncommon feature for glycosides from sea cucumbers belonging to the family Stichopodidae.

The sea cucumber *Cucumaria frondosa* contains a complex mixture of Δ7- and Δ9,11-holothurins. Frondoside A2-6 (57c) [61] as well as the minoritary monosulfated pentaglycosides frondosides A2-1 (50e), A2-2 (49a), A2-3 (49b) and A2-4 (49c) [61, 62] have a glucose residue as the third monosaccharide unit in the carbohydrate chain in contrast to frondoside A, the major component of the glycosidic fraction [85].

Recently, we have isolated two new sulfated tetraglycosides, Hemoiedemoside A (57d) and B (57e), Fig. (25) from the sea cucumber *Hemoiedema spectabilis*, collected in the South Atlantic near the
Patagonian shore [86]. Both glycosides have the same aglycone, holotoxinogenin and a linear tetrasaccharide chain and differ in the number of sulfate groups attached to the monosaccharide residues. Glycoside 57d is a disulfated compound while 57e has three sulfate groups attached to C-6 of the two glucose units and C-4 of the xylose residue.

The assignment of the NMR signals associated with the aglycone moiety of 57d and 57e was derived from $^1$H-$^1$H COSY, HETCOR and NOESY experiments. The $^1$H-NMR signals of 3β-hydroxyholosta-9(11),25-dien-16-one were unambiguously assigned for the first time by application of the standard 2D NMR methods mentioned above. Analysis of NMR data indicated the presence of xylose, quinovose, glucose and 3-O-methylglucose in a ratio 1:1:1:1 in the oligosaccharide chain of both holothurins. This was confirmed by acid hydrolysis with aqueous 2N trifluoroacetic acid followed by GC analysis of the corresponding alditol peracetates. Analysis of the fragment ion peaks of 57d in the FABMS (positive ion mode) at $m/z$ 1209 [M - SO$_3$Na + H + Na]$^+$ and $m/z$ 1083 [M - 2SO$_3$Na + H]$^+$ indicated the presence of two sulfate groups. This was confirmed by solvolytic desulfation of 57d and comparison of its $^{13}$C-NMR spectrum with that of the desulfated derivative. The pseudomolecular ion at $m/z$ 1413 [M + Na]$^+$ of 57e in the FABMS (positive ion mode) and fragment ions at $m/z$ 1311 [M - SO$_3$Na + H + Na]$^+$, 1209 [M - 2SO$_3$Na + 2H + Na]$^+$ and $m/z$ 1107 [M - 3SO$_3$Na + 3H + Na]$^+$ confirmed the presence of three sulfate groups in the saponin.

Hemoiedemosides A (57d) and B (57e) and their semisynthetic desulfated analog were evaluated for antifungal activity against the phytopathogenic fungus Cladosporium cucumerinum. Benomyl, a commercially available fungicide was used as reference compound. The three saponins were active in a concentration-dependent manner and the natural sulfated glycosides were more active than their desulfated analog which was inactive at the lowest concentrations (1.5–5 µg/spot) and weakly active at the higher ones (7.5–50 µg/spot). The disulfated holothurin was more active than benomyl at the higher concentrations (20–50 µg/spot), while trisulfated glycoside 57e was slightly more active than the reference compound at 40 and 50 µg/spot. On comparing the antifungal activities against C. cucumerinum of hemoiedemoside A (57d) and patagonicoside A (56a), containing the same disulfated tetrasaccharide chain, glycoside 57d showed inhibition zones of 8–33 mm
at the tested concentrations (1.5–50 µg/spot), while patagonicoside A was less active (8–19 mm) at the same concentrations. On the other hand, hemoiedemoside B (57e) was less active that 57d. These results suggest that both the structure of the triterpenoidal aglycone and the presence and number of the sulfate groups at the oligosaccharide chain may play a significant role in the antifungal activity of these saponins. Further evaluation of the zootoxicities of the holothurins 57d and 57e and their desulfated analog using the brine shrimp (Artemia salina L.) larvae mortality bioassay showed that hemoiedemoside A (57d) had a noteworthy toxicity (LC50 18.7 ppm). Hemoiedemoside B (57e) was 2 times less active (LC50 47.5 ppm) than 57d and nearly 10 times more active than the desulfated derivative (LC50 424.5 ppm). These results correlated with the data on antifungal activity.

Most of the members of the Stichopodidae family (Stichopus japonica [87], Stichopus chloronotus [88], Parastichopus californius [89] and Bohadschia bivittata [90]) have been shown to produce hexosides as major components of the glycosidic fraction. They are non-sulfated glycosides with a linear 3-O-Me-Glc-(1→3)-Glc-(1→4)-Xyl chain and a branching of a linear trisaccharide at C-2 of the xylose unit. The only examples with a glucose unit instead of the terminal 3-O-Me-glucose are holotoxin B1 (57h) and B (57i) [87, 89]. Recently, two new non-sulfated hexoglycosides, parvimosides A (57f) and B (57g), Fig. (25) were isolated from the sea cucumber Stichopus parvimensis [91]. Holothurin 57f is related to holotoxin B, the difference being the absence of a double bond at position 25 in the aglycone of parvimoside A (57f). On the other hand, parvimoside B (57g) is the 25-dihydroderivative of holotoxin B1 (57h).

Another structural feature in 3β-hydroxyholost-9(11)-ene aglycones is the presence of a 12α-hydroxyl group, such as in the hexaglycoside bivittoside D (Structure 58), Fig. (26) [90]. Bivittoside D, widespread in sea cucumbers belonging to the genus Bohadschia (Holothuriidae) was described by Hedge et al. [92] as a new compound in the sea cucumber identified as Telenata ananas. Kalinin et al. [58] considered that this taxonomic classification relates to Thelenota ananas since the genus Telenata has never been described for sea cucumbers.
T. ananas also contained a new sulfated triglycoside (59a), Fig. (27) with a 12α,17α-dihydroxyl and 22(25)-epoxy group in the aglycone, a very common for sea cucumbers from the family Holothuriidae [93, 94]. Holothurin 59a contains a very uncommon trisaccharide carbohydrate chain.

Bivittoside D (58) and triglycoside 59a exhibited inhibitory activity in a chemokine receptor subtype 5 (CCR5) assay at concentrations of 30 and 5 μM, respectively [92]. Holothurin 59a having only three monosaccharide residues is significantly lesser active than the tetraglycoside bivittoside D (58).

Holothurin B₃ (59b), Fig. (27), a new sulfated diglycoside, was isolated from the sea cucumber Holothuria polii [95]. This saponin contains the
same monosulfated disaccharide chain as holothurin B [94] and differs from it the absence of a 17α-hydroxyl group.

The sea cucumber *H. polii* also contained the monosulfated disaccharides holothurin B₂ (60), Fig. (28) and holothurin B₄ (61), Fig. (29) [95]. Both saponins have the same disaccharide chain as holothurin B₃ (59b).

![Holothurin B₂](image1)

**Fig. (28).** Structure of Holothurin B₃ from the sea cucumber *Holothuria polii*

Saponin 60 has the aglycone of holothurin A₁ [96] whereas holothurin B₃ has the same aglycon side chain as the 3β-hydroxyholost-7-ene aglycones in cucumarioside G₄ [63], eximisoside A [64] and calcigeroside E [69].

![Holothurin B₄](image2)

**Fig. (29).** Structure of Holothurin B₄ from the sea cucumber *Holothuria polii*

**Non-holostane aglycones**
Some examples of sulfated holothurins with uncommon non-holostane aglycones have been isolated from eight species of sea cucumbers belonging to the order Dendrochirotida. Glycosides 62a-62e, Fig. (30) contain aglycones with a 18(16)-lactone [69, 70, 97, 98].

Calcigeroside B (62b) has a quinovose terminal residue attached to another quinovose unit. This structural feature is unique for sea cucumber glycosides [69]. Psolusoside B (62a) showed inhibition of rat brain Na$^+$, K$^+$-ATPase with an $I_{50}$ value of $3 \times 10^{-4}$ M [99]. Calcigerosides B (62b), C$_1$ (62c) and C$_2$ (62e) were individually tested in vitro against four standard human and mouse tumoral cell lines (P-388, A-549, HT-29 and Mel-98). Only their desulfated derivatives showed moderate cytotoxicity ($IC_{50} = 5 \mu g/ml$) [70].
Six holothurins that lack a lactone function and have a shortened side chain have been isolated from sea cucumbers *Cucumaria koraiensis*, *Cucumaria conicospermium* and *Duasmodactyla kurilensis*. Cucumarioside A3-2 (63a) [71] and Koreoside A (63b) [100], Fig. (31) contain a $\Delta^7$ double bond while Cucumarioside A3-3 (64a) [71], Isokoreoside A (64b) [71] and Kurilosides A (64c) and C (64d) [101], Fig. (32) contain a $\Delta^9$ double bond. Kurilosides A (64c) and C (64d) contain a 16α-acetoxy group.

![Diagram of 64a Cucumarioside A3-3 and 64b Isokoreoside A]

Recently, frondoside A2-8 (65), a new saponin with a non-holostane aglycone containing a $\Delta^7$ double bond, a hydroxyl group at C-20 and an acetoxy group at C-22 was isolated from the sea cucumber *Cucumaria frondosa*, Fig. (33) [62].

![Diagram of Frondoside A2-8]

**Fig. (32).** Structures of non-holostane glycosides from the sea cucumbers *Cucumaria conicospermium* and *Duasmodactyla kurilensis.*

**Fig. (33).** Structure of Frondoside A2-8 from the sea cucumber *Cucumaria frondosa*
C. frondosa also contained frondosides C (66b) [58] and A2-7 (66c) [62], two holothurins related to frondoside A2-8 (65), Fig. (34) that differed from this holothurin in the presence of a \( \Delta^9,11 \) double bond and in the oligosaccharide chain. Previously, two saponins, Ds-penaustrosides A (66c) and B (66d), Fig. (34) lacking the acetoxy group at C-22 were isolated from the sea cucumber Pentacta australis [103].

![Image](image.png)

66a Frondoside C \( R = [3-O\text{-Me}-Xyl-(1\rightarrow3)-Glc(1\rightarrow4)]-\text{Quin}(1\rightarrow2)-\text{Quin}(1\rightarrow2)-4-\text{OSO}_3\text{Na}-Xyl; R^1 = \text{OAc}; \Delta^{24} \)
66b Frondoside A2-7 (2) \( R = [3-O\text{-Me}-\text{Glc}(1\rightarrow3)-\text{Glc}(1\rightarrow4)]-\text{Xyl}(1\rightarrow2)-\text{Quin}(1\rightarrow2)-4-\text{OSO}_3\text{Na}-Xyl; R^1 = \text{OAc}; \Delta^{24} \)
66c Ds-Penaustroside A \( R = [3-O\text{-Me}-\text{Xyl}(1\rightarrow3)-\text{Glc}(1\rightarrow4)]-[\text{Qui}(1\rightarrow2)]-[\text{Qui}(1\rightarrow2)-4-\text{OSO}_3\text{Na}-Xyl; R^1 = \text{H} \)
66d Ds-Penaustroside B \( R = [3-O\text{-Me}-\text{Xyl}(1\rightarrow3)-\text{Glc}(1\rightarrow4)]-[\text{Qui}(1\rightarrow2)]-[\text{Qui}(1\rightarrow2)-4-\text{OSO}_3\text{Na}-Xyl; R^1 = \text{H}; \)

Fig. (34). Structures of non-holostane glycosides from the sea cucumbers Cucumaria frondosa and Pentacta australis

The desulfated derivative of frondoside C (66a) showed intense cytotoxic activity (IC$_{50}$ = 1\( \mu \)g/ml) when tested in vitro against several standard mice and human tumoral cell lines (P-388, Schabel, A-549, HT-29 and Mel-28).

**CONCLUSIONS**

In the last five years only a few examples of asterosaponins have been published in the literature. These include new pentaglycosides from the starfishes Labidiaster annulatus, Luidia quinaria, Psilaster cassiope and Culcita novaeguineae, together with three new hexaglycosides from the Patagonian starfish Anasterias minuta. Cytotoxic and antifungal activities were reported for some of these saponins as well as structure-activity
correlations for the asterosaponins of *Anasterias minuta* and *Culcita novaeguineae*.

On the contrary, many new glycosides of polyhydroxylated steroids have been isolated in this period and the biological properties of most of them have been evaluated. Correlations between the number of hydroxyl groups in the aglycones and the hemolytic activity and inhibition of cell division of fertilized sea urchin eggs were established for glycosides isolated from *Asterias rathbuni*, *Mediaster murrayi*, *Ceramaster patagonicus* and *Culcita novaeguineae*. The starfish *Certonardoa semiregularis* has shown to be an extremely rich source of new glycosides of polyhydroxylated steroids: ten diglycosides and eight monoglycosides were isolated, together with twenty two new polyhydroxysteroids. Evaluation of the cytotoxicity of the individual compounds against a panel of human solid tumor cell lines revealed that the polyhydroxysteroids were more potent than the corresponding glycosides. This investigation showed that not only asterosaponins but minoritary components such as polyhydroxysterols and their glycosides may play an important role in the toxicity of starfishes.

Evaluation of the neuritogenic activity of five new monoglycosides isolated from the starfish *Linckia laevisata* demonstrated that the nature of the sugar moiety attached to the side chain seems to be an important factor for the neuritogenic activity.

During these years many new examples of sea cucumber glycosides were isolated, in particular those containing a 3β-hydroxyholost-7-ene aglycone. Some structural characteristics such as a 24-ketone conjugated with a Δ^{25} double bond as in frondoside A2-2 and a 22-keto group in the side chains of mollisoside A and pseudostichoposide B are novel features for holothurins. Another new feature for 3β-hydroxyholost-7-ene aglycones is the presence of an acetoxy group at C-16, present in nine glycosides isolated from *Mensamaria intercedens*. Intercedensides C, D, F, H and I also contain a 17α-hydroxy group, not characteristic for sea cucumbers of the order Dendrochirotida with exception of patagonicoside A that contains an uncommon aglycone with the simultaneous presence of two hydroxyl groups at C-12α and C-17α.
Some of the sulfated holothurins isolated in this period were evaluated for their biological activities. Holothurins isolated from the sea cucumber Cucumaria japonica were highly toxic and induced abnormalities in the development of sea urchin embryos. Mollisoside A, pseudostichoposide B and the intercedensides A-H showed in vitro cytotoxicity against human tumor cell lines. Liouvillosides A and B isolated from Staurocucumis liouvillei exerted virucidal effect against herpes simplex virus 1. Evaluation of the antifungal activity of the sulfated hemoiedemosides A and B and patagonicoside A as well as their desulfated analogues allowed us to conclude that the aglycone structure and the presence and number of the sulfate groups at the oligosaccharide chain are important features for their antifungal properties against the phytopathogenic fungus Cladosporium cucumerinum.

Much work has been done in the last thirty years on the isolation and structural characterization of saponins and polyhydroxylated steroid glycosides from starfishes and sea cucumbers. The wide spectrum of biological activities these compounds show must be related to their role in the organisms that produce them and this task must be addressed in future investigations.

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