Atta-ur-Rahman (Ed.) *Studies in Natural Products Chemistry, Vol. 28*

BIOLOGICALLY ACTIVE TRITERPENE GLYCOSIDES FROM SEA CUCUMBERS (HOLOTHUROIDEA, ECHINODERMATA)

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ABSTRACT: Sea cucumbers are characterized by their content in holothurins, triterpenoid glycosides that are responsible for the toxicity of these echinoderms. Nearly 100 holothurins isolated in the last twenty years are grouped into three main aglycone structural types: 3β-hydroxyholost-9(11)-ene, 3β-hydroxyholost-7-ene and non-holostane based aglycones. This communication offers a general view of the structural characteristics of these saponins and the spectral features in their ^{1}H- and ^{13}C-NMR and FAB-MS spectra. Recent advances in the unambiguous spectroscopic characterization of the triterpenoid skeleton, the substitution patterns and the complete structure of the oligosaccharide chain are discussed.

INTRODUCTION

The phylum Echinodermata (Greek *echinos*, spiny; *derma*, skin) comprises some of the most familiar seashore animals. There are about 7,000 living species widely distributed in all oceans at all depths. The phylum is divided into five classes: Holothuroidea (sea cucumbers or holothurians), Asteroidea (starfishes or sea stars), Ophiuroidea (brittle stars), Crinoidea (sea lilies and feather stars) and Echinoidea (sea urchins). Triterpenoid and steroid oligoglycosides are predominant and characteristic secondary metabolites of sea cucumbers and starfishes and are responsible for their general toxicity [1-6]. Both classes of echinoderms contain also glycosphingolipids, such as monohexosylceramides (cerebrosides) and gangliosides [7]. Brittle stars contain sulfated polyhydroxysteroids [4,8,9] and only two sulfated steroidal monoglycosides have been reported in the brittle star

Ophioderma longicaudum [10]. On the contrary, there is no report of steroid or triterpenoid glycosides in the classes Echinoidea and Crinoidea.

Several reviews concerning the structures, taxonomic distribution, evolution and biological activities of sea cucumber triterpenoid oligoglycosides have been published [11-14]. The purpose of the present communication is to offer a general view of the methods applied in the structural elucidation of these complex molecules, focusing on recent examples of cytotoxic, antifungal and virucidal triterpenoid oligoglycosides from our laboratory.

TRITERPENOID GLYCOSIDES

Triterpenoid saponins are typical metabolites of plant origin, but extensive investigation on marine organisms as sources of new bioactive metabolites has shown that triterpenoid glycosides are widely distributed in sea cucumbers. It has been suggested that these saponins have a defensive role due to their membranotropic action [11]. Penta- and tetraglycosides containing a norlanostane triterpenoid have been encountered rarely also in sponges [15].

Most of the triterpenoid glycosides isolated so far from holothurians present a sugar chain of two to six monosaccharide units linked to the C-3 of the aglycone, which is usually based on a "holostanol" skeleton [3β,20*S*-dihydroxy-5α-lanostano-18,20-lactone] (**1**), Fig.(**1**) [1].

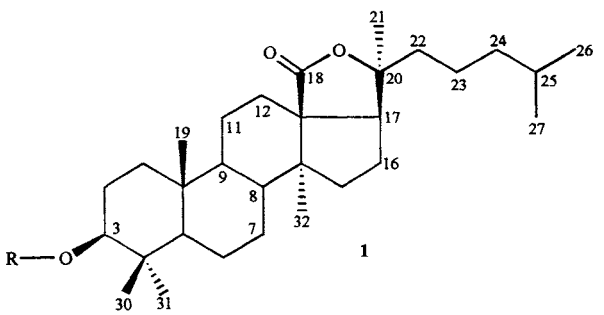


Fig. (1). Structure of hypothetical holostanol

Only quinovose, glucose, 3-*O*-methylglucose, xylose and 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. In comparison to steroidal

oligoglycosides from starfishes which always contain a sulfate group attached to C-3 of the aglycone, sixty percent of the triterpenoid glycosides isolated so far from sea cucumbers have sulfate groups linked to the monosaccharide units of the oligosaccharide chain. Although most of them are monosulfated oligoglycosides, several di- and trisulfated glycosides have been isolated, mainly from the order Dendrochirotida.

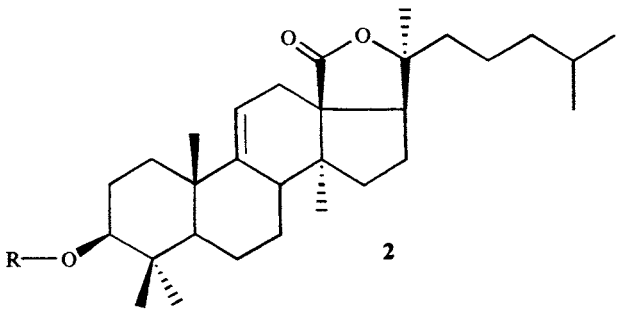
Triterpene glycosides are specific for different taxonomic groups of sea cucumbers and represent good models for studies on biochemical evolution [16]. They have a wide spectrum of biological effects: antifungal, cytotoxic, hemolytic, cytostatic and immunomodulatory activities [12]. These biological activities are a consequence of their membranotropic action against any cellular membrane containing Δ^5-sterols. Triterpene glycosides form complexes with these sterols that lead to the development of single ion channels and larger pores, which cause significant changes in the physico-chemical properties of membranes [13]. Sea cucumber cell membranes are resistant to their own toxins due to the presence of Δ^7- and $\Delta^{9,11}$-sterols, sulfated Δ^5-sterols and β-xylosides of sterols instead of the free Δ^5-sterols [17].

CHEMICAL STRUCTURES

Nearly 100 different chemical structures of these toxins have been published in the last 20 years. 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. Usually aglycones that have a $\Delta^{9,11}$ double bond are characteristic of sea cucumbers belonging to the order Aspidochirota, while those with a Δ^7 unsaturation were generally isolated from animals of the order Dendrochirotida.

3β-Hydroxyholost-9(11)-ene aglycones

Bivittoside C, Fig. (**2**), a hexaglycoside isolated from the sea cucumber *Bohadschia bivittata* [18] is the simplest triterpene glycoside with a $\Delta^{9,11}$ double bond:

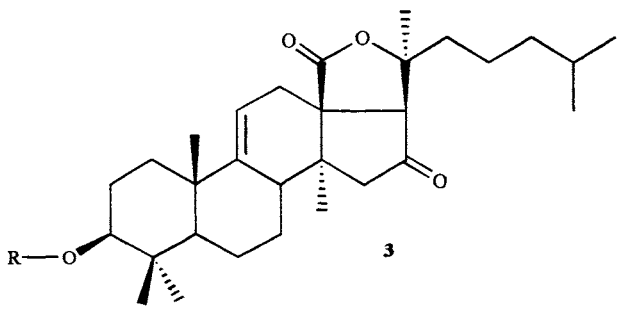


2

2 Bivittoside C [18] R = [3-*O*-Me-Glc-(1→3)-Glc-(1→4)]-[3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)]-Xyl

Fig. (2). Structure of Bivittoside C

A number of glycosides containing aglycones of this series show a carbonyl group at C-16 (Structure **3**, Fig. (**3**)). With exception of glycoside **3a**, all have an additional Δ^{25} double bond in the side chain.

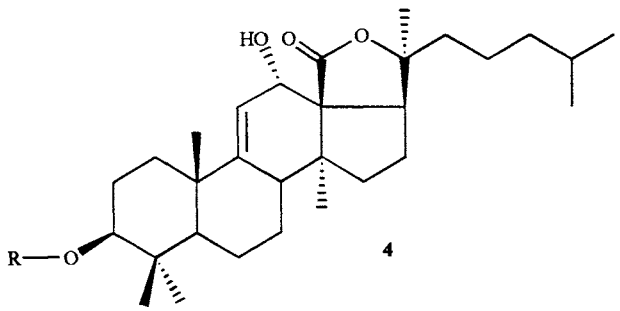


3

3a Ds-Penaustroside D [19] R = [3-*O*-Me-Xyl-(1→3)-Glc-(1→4)]-[Qui-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl
3b Holotoxin A_1 [20] R = [3-*O*-Me-Glc-(1→3)-Glc-(1→4)]-[3-*O*-Me-Glc-(1→3)-Xyl-(1→4)-Qui-(1→2)]-Xyl; Δ^{25}
3c Holotoxin A [21] R = [3-*O*-Me-Glc-(1→3)-Glc-(1→4)]-[3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)]-Xyl; Δ^{25}
3d Holotoxin B_1 [20] R = [Glc-(1→3)-Glc-(1→4)]-[3-*O*-Me-Glc-(1→3)-Xyl-(1→4)-Qui-(1→2)]-Xyl; Δ^{25}
3e Holotoxin B [21] R = [Glc-(1→3)-Glc-(1→4)]-[3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)]-Xyl; Δ^{25}
3f Neothynidioside [22] R = 3-*O*-Me-Glc-(1→3)-Xyl-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; Δ^{25}
3g Psolusoside A [23] R = 6-OSO_3Na-3-*O*-Me-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)-Qui-(1→2)-Xyl; Δ^{25}
3h Cladoloside A [24] R = 3-*O*-Me-Glc-(1→3)-Xyl-(1→4)-Qui-(1→2)-Xyl; Δ^{25}
3i Cladoloside B [24] R = [Glc-(1→4)]-[3-*O*-Me-Glc-(1→3)-Xyl-(1→4)-Qui-(1→2)]-Xyl; Δ^{25}
3j Ds-Penaustroside C [19] R = [3-*O*-Me-Xyl-(1→3)-Glc-(1→4)]-[Qui-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl; Δ^{25}
3k Hemoiedemoside A [25] R = 3-*O*-Me-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; Δ^{25}
3l Hemoiedemoside B [25] R = 6-OSO_3Na-3-*O*-Me-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; Δ^{25}
3m Caudinoside A [26] R = 3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)-Xyl; Δ^{25}

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

Another structural feature is the presence of a 12α-hydroxyl group in the aglycone, Fig. (**4**):

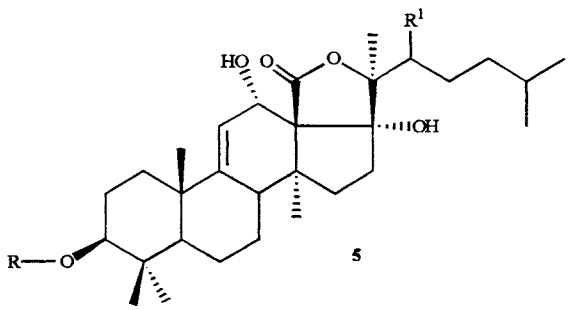


4

4a Bivittoside A [18] R = Qui-(1→2)-Xyl
4b Bivittoside B [18] R = [3-*O*-Me-Glc-(1→3)-Glc-(1→4)]-[Qui-(1→2)]-Xyl
4c Bivittoside D [18] R = [3-*O*-Me-Glc-(1→3)-Glc-(1→4)]-[3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)]-Xyl
4d Pervicoside C [27] R = 3-*O*-Me-Glc-(1→3)-Glcl-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl
4e Pervicoside B [27] R = 3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; Δ^{24}

Fig. (4). Structure of 3β,12α-dihydroxyholost-9(11)-ene aglycone based glycosides

Some glycosides contain two hydroxyl groups at positions 12α and 17α of the holostanol skeketon, Fig. (**5**):

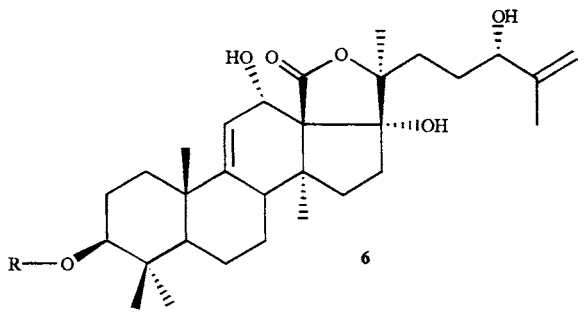


5

5a Echinoside B [28] R = Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = H
5b Echinoside A [28] R = 3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = H
5c 22-Acetoxy-echinoside A [29] R = 3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = OAc
5d Holothurin A_1 [30] R = 3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = OH
5e 24-Dehydroechinoside B [31] R = Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = H; Δ^{24}
5f 24-Dehydroechinoside A [31] R = 3-*O*-MeGlc-(1→3)-Gcl-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = H; Δ^{24}
5g 22-Hydroxy-24-dehydroechinoside A [29] R = 3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = OH; Δ^{24}

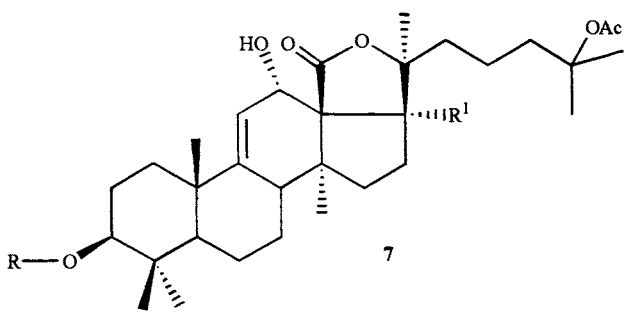
Fig. (5). Structure of 3β,12α,17α–trihydroxyholost-9(11)-ene aglycone based glycosides

Glycosides **5c**, **5d** and **5g** together with glycosides **6**, Fig. (**6**) and **7**, Fig. (**7**) are characterized by additional acetoxy or hydroxy groups in the side chain.



6 24(*S*)-hydroxy-25-dehydroechinoside A [29] R = 3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl

Fig. (6). Structure of a sulfated tetraglycoside isolated from the sea cucumber *Actinopyga flammea*

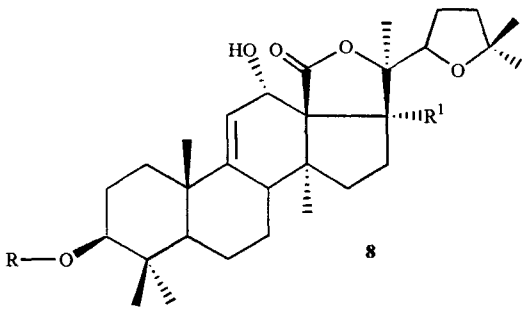


7a Holothurinoside B [32] R = [3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)]-[Glc-(1→4)]-Xyl; R^1 = OH; Δ^{22}
7b Pervicoside A (Neothyoside A) [27] R = 3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = H
7c Neothyoside B [33] R = Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = H

Fig. (7). Structure of 25-acetoxi-3β,12α–dihydroxyholost-9(11)-ene aglycone based glycosides

Holothurins A (**8a**) and B (**8b**) isolated from the sea cucumber *Holothuria leucospilota* [34] as well as Desholothurin A (**8d**), and Holothurinosides A (**8c**), C (**8e**) and D (**8f**), Fig. (**8**) from *Holothuria forskali* [32] are the only examples of glycosides containing the side chain in a furan form.

Compounds **3a**, **3g-3l** and **7c** are the only $\Delta^{9,11}$-glycosides isolated from sea cucumbers belonging to the order Dendrochirotida. In general, 3β-hydroxyholost-9(11)-ene based aglycones were characterized in holothurins isolated from animals of the order Aspidochirota.



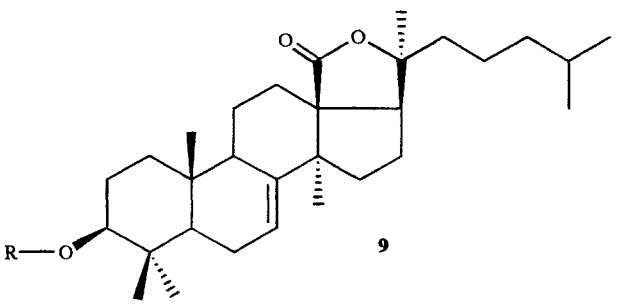
8

8a Holothurin B [34] R = Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = OH
8b Holothurin A [34] R = 3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = OH
8c Holothurinoside A [32] R = [Glc-(1→4)]-[3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)]-Xyl; R^1 = OH
8d Desholothurin A [32] R = 3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)-Xyl; R^1 = OH
8e Holothurinoside C [32] R = 3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)-Xyl; R^1 = H
8f Holothurinoside D [32] R = Qui-(1→2)-Xyl; R^1 = H

Fig. (8). Structures of glycosides isolated from the sea cucumbers *Holothuria leucospilota* and *Holothuria forskalii*

3β-Hydroxyholost-7-ene aglycones

Frondoside B (**9a**), Cucumariosides A_2-4 (**9b**) and A_7-3 (**9c**), Fig. (**9**) as well as several triterpene glycosides isolated from the sea cucumbers *Stichopus chloronotus* (**10a-10h**) and *Thelenota ananas* (**10i, 10j**), Fig. (**10**) contain the simple 3β-hydroxyholost-7-ene as the aglycone. An additional acetoxyl group in the side chain is present in compounds **10a-10j**.

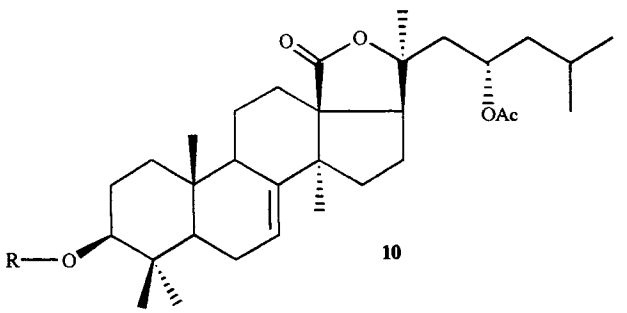


9

9a Frondoside B [35] R = [3-*O*-Me-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)]-[Xyl-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl; Δ^7; Δ^{24}
9b Cucumarioside A_2-4 [36] R = [3-*O*-Me-Glc-(1→3)-Glc-(1→4)]-[Xyl-(1→2)]-Qui-(1→2)-Xyl; Δ^7; Δ^{25}
9c Cucumarioside A_7-3 [36] R = [6-OSO_3Na-3-*O*-Me-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)]-[Xyl-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl; Δ^7; Δ^{25}

Fig. (9). Structures of glycosides isolated from the sea cucumbers *Cucumaria frondosa* and *Cucumaria japonica*

Glycosides **10a-10j** were isolated from *Stichopus chloronotus* and *Thelenota ananas*, two sea cucumbers belonging to the order Aspidochirota [37].

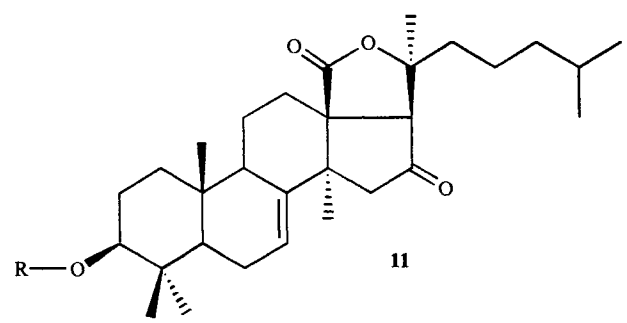


10

10a Stichloroside C_1 (Stichoposide C) [37] R = [3-*O*-Me-Glc-(1→3)-Glc-(1→4)]-[3-*O*-Me-Glc-(1→3)-Xyl-(1→4)-Quin-(1→2)]-Xyl
10b Stichloroside B_1 (Stichoposide D) [37] R = [3-*O*-Me-Glc-(1→3)-Glc-(1→4)]-[3-*O*-Me-Glc-(1→3)-Xyl-(1→4)-Glc-(1→2)]-Xyl
10c Stichloroside A_1 [37] R = [3-*O*-Me-Glc-(1→3)-Glc-(1→4)]-[3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Xyl-(1→2)]-Xyl
10d Stichoposide A [37] R = Qui-(1→2)-4-OSO_3Na-Xyl
10e Stichoposide B [37] R = Glc-(1→2)-Xyl
10f Stichloroside C_2 [37] R = [3-*O*-Me-Glc-(1→3)-Glc-(1→4)]-[3-*O*-Me-Glc-(1→3)-Xyl-(1→4)-Qui-(1→2)]-Xyl; Δ^{25}
10g Stichloroside B_2 [37] R = [3-*O*-Me-Glc-(1→3)-Glc-(1→4)]-[3-*O*-Me-Glc-(1→3)-Xyl-(1→4)-Glc-(1→2)]-Xyl; Δ^{25}
10h Stichloroside A_2 [37] R = [3-*O*-Me-Glc-(1→3)-Glc-(1→4)]-[3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Xyl-(1→2)]-Xyl; Δ^{25}
10i Thelenotoside A [37] R = 3-*O*-Me-Glc-(1→3)-Xyl-(1→4)-Qui-(1→2)-Xyl
10j Thelenotoside B [37] R = 3-*O*-Me-Glc-(1→3)-Xyl-(1→4)-Glc-(1→2)-Xyl

Fig. (10). Structures of glycosides isolated from the sea cucumbers *Stichopus chloronotus* and *Thelenota ananas*

3β-Hydroxyholost-7-ene aglycones with a carbonyl group at C-16 have been isolated exclusively from the sea cucumber *Cucumaria japonica*, Fig. (**11**).



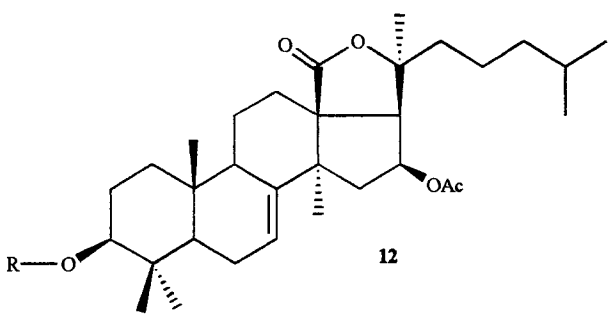
11

11a Cucumarioside A_2-3 [36] R = [3-*O*-Me-Glc-(1→3)-Glc-(1→4)]-[Xyl-(1→2)]-Qui-(1→2)-Xyl

11b Cucumarioside A_7-2 [36] R = [6-OSO_3Na-3-*O*-Me-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)]-[Xyl-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl
11c Cucumarioside A_0-3 [38] R = [3-*O*-Me-Glc-(1→3)-Xyl-(1→4)]-[Xyl-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl; Δ^{25}
11d Cucumarioside A_1-2 [38] R = [6-OAc-Glc-(1→3)-Glc-(1→4)]-[Xyl-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl; Δ^{25}
11e Cucumarioside A_2-2 [36] R = [3-*O*-Me-Glc-(1→3)-Glc-(1→4)]-[Xyl-(1→2)]-Qui-(1→2)-Xyl; Δ^{25}
11f Cucumarioside A_7-1 [36] R = [6-OSO_3Na-3-*O*-Me-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)]-[Xyl-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl; Δ^{25}
11g Cucumarioside A_3 [39] R = [3-*O*-Me-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)]-[Xyl-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl; Δ^{25}
11h Cucumarioside A_6-2 [39] R = [6-OSO_3Na-3-*O*-Me-Glc-(1→3)-Glc-(1→4)]-[Xyl-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl; Δ^{25}
11i Cucumarioside A_4-2 [36] R = [Glc-(1→3)-Glc-(1→4)]-[Xyl-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl; Δ^{25}

Fig. (11). Structures of glycosides isolated from the sea cucumber *Cucumaria japonica*

Another structural feature that has been found only in this series of aglycones is the presence of an acetoxyl group at C-16. Glycosides with a β-configuration for this group are shown in Fig. (**12**).

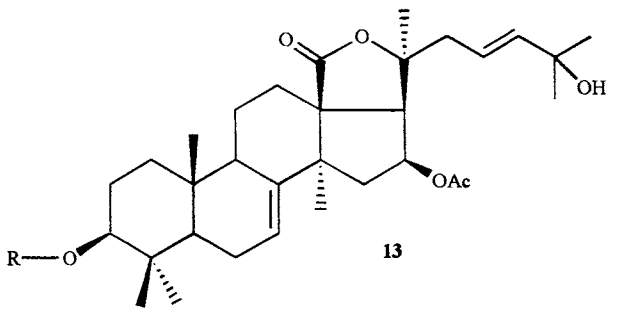


12

12a Frondoside A [40] R = [3-*O*-Me-Glc-(1→3)-Xyl-(1→4)]-[Xyl-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl
12b Frondoside A_1 [41] R = 3-*O*-Me-Glc-(1→3)-Xyl-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl
12c Liouvilloside B [42] R = 6-OSO_3Na-3-*O*-Me-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl
12d Cucumarioside A_0-2 [38] R = [3-*O*-Me-Glc-(1→3)-Xyl-(1→4)]-[Xyl-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl; Δ^{25}
12e Neothyonidioside C [43] R = 6-OSO_3Na-3-*O*-Me-Glc-(1→3)-Xyl-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; Δ^{25}
12f Cucumarioside G_1 [44] R = 3-*O*-Me-Xyl-(1→3)-Glc-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; Δ^{24}
12g Liouvilloside A [42] R = 6-OSO_3Na-3-*O*-Me-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; Δ^{24}
12h Cucumarioside C_2 [45] R = [3-*O*-Me-Xyl-(1→3)-Glc-(1→4)]-[Xyl-(1→2)]-Qui-(1→2)-Xyl; 22*E*; Δ^{24}
12i Cucumarioside H [46] R = 3-*O*-Me-Xyl-(1→3)-Glc-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; 22*E*; Δ^{24}
12k Cucumarioside C_1 [45] R = [3-*O*-Me-Xyl-(1→3)-Glc-(1→4)]-[Xyl-(1→2)]-Qui-(1→2)-Xyl; 22*Z*; Δ^{24}
12l Cucumarioside G_3 [47] R = 3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; 22*Z*; Δ^{24}

Fig. (12). Structure of 16β-acetoxy-3β-hydroxyholost-7-ene aglycone based glycosides

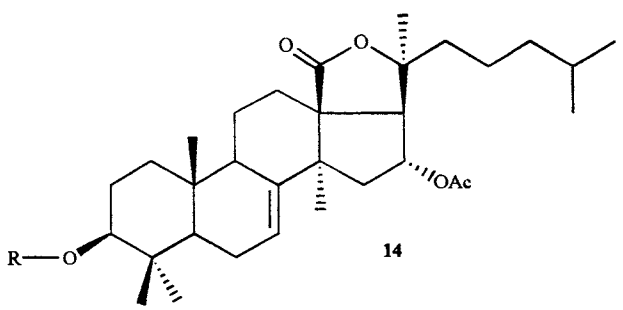
Some of the glycosides containing a 16β-acetoxy group also present an allylic hydroxyl group at C-25, Fig. (**13**).



13a Cucumarioside G_4 [47] R = 3-*O*-Me-Xyl-(1→3)-Glc-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl
13b Eximisoside A [48] R = 3-*O*-Me-Glc-(1→3)-Xyl-(1→4)-Glc-(1→2)-Xyl
13c Calcigeroside E [49] R = [6-OSO_3Na-3-*O*-Me-Glc-(1→3)-Glc-(1→4)]-[Glc-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl

Fig. (13). Structure of 16β-acetoxy-3β,25-dihydroxyholosta-7,22-diene aglycone based glycosides

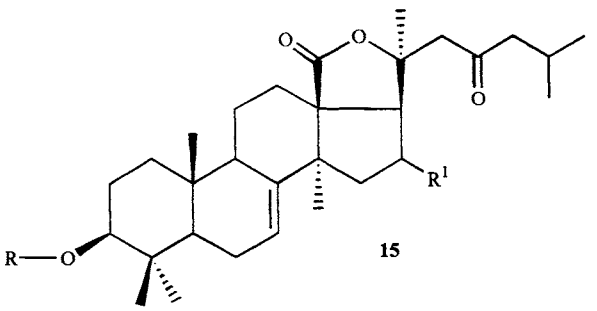
Four glycosides isolated from the sea cucumber *Cucumaria lefevrei* [50] are the only examples of holothurins with a 16α-acetoxy group in their aglycones, Fig. (**14**). Lefevreiosides A_2 (**14b**), B (**14c**) and C (**14d**) show the same monosulfated tetrasaccharide chain and differ in the degree of unsaturation or the position of the double bond in their side chains. Lefevreioside A_1 (**14a**) is the desulfated analog of glycoside **14b**.



14a Lefevreioside A_1 [50] R = 3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)-Xyl
14b Lefevreioside A_2 [50] R = 3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl
14c Lefevreioside B [50] R = 3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; Δ^{24}
14d Lefevreioside C [50] R = 3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; Δ^{25}

Fig. (14). Structures of glycosides isolated from the sea cucumber *Cucumaria lefevrei*

Several triterpene glycosides isolated from the sea cucumbers *Cucumaria echinata* and *Pentamera calcigera* contain a carbonyl group at C-23 in the side chain, Fig. (**15**). This structural feature is absent in 3β-hydroxyholost-9(11)-ene aglycones.

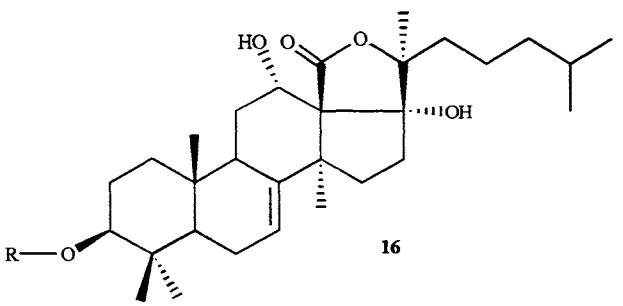


15

15a Cucumechinoside C [51] R = 3-*O*-Me-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = H
15b Cucumechinoside F [51] R = 6-OSO_3Na-3-*O*-Me-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = H
15c Calcigeroside C_2 [52] R = [3-*O*-Me-Xyl-(1→3)-Glc-(1→4)]-[Glc-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = H
15d Calcigeroside D_2 [49] R = [3-*O*-Me-Xyl-(1→3)-6-OSO_3Na-Glc-(1→4)]-[Glc-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = H
15e Cucumechinoside A [51] R = 3-*O*-Me-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = O
15f Cucumechinoside B [51] R = 3-*O*-Me-Glc-(1→3)-2-OSO_3Na-Xyl-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = O
15g Cucumechinoside D [51] R = 6-OSO_3Na-3-*O*-Me-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = O
15h Cucumechinoside E [51] R = 6-OSO_3Na-3-*O*-Me-Glc-(1→3)-2-OSO_3Na-Xyl-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = O
15i Cucumarioside A_0-1 [38] R = [3-*O*-Me-Glc-(1→3)-Xyl-(1→4)]-[Xyl-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = β-OAc

Fig. (15). Structures of glycosides isolated from the sea cucumbers *Cucumaria echinata* and *Pentamera calcigera*

Recently, we have isolated an antifungal holothurin from the sea cucumber *Psolus patagonicus* [53]. Patagonicoside A (**16**), Fig. (**16**) is the first example of a 3β-hydroxyholost-7-ene aglycone substituted with 12α- and 17α-hydroxy groups.



16

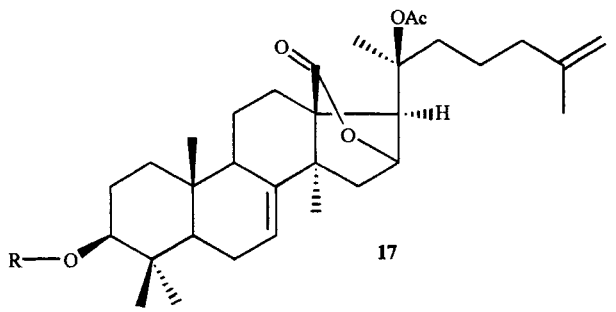
16 Patagonicoside A [53] R = 3-*O*-Me-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl

Fig. (16). Structure of patagonicoside A, an antifungal oligoglycoside isolated from the sea cucumber *Psolus patagonicus*

Non-holostane aglycones

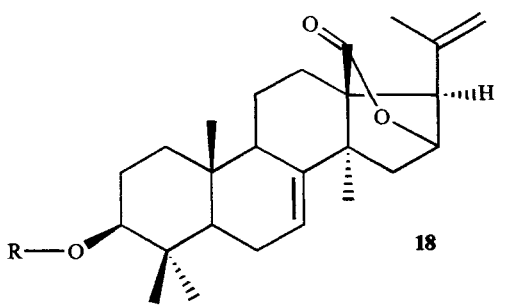
Recently, some examples of holothurins having uncommon non-holostane aglycones have appeared in the literature. These glycosides have been isolated from seven species of sea cucumbers belonging to the order Dendrochirota. All are sulfated compounds, the majority monosulfated at the glucose or xylose units.

Five glycosides contain aglycones with an 18(16)-lactone and a Δ^7-unsaturation, Fig. (**17**) and (**18**).



17 Psolusoside B [54] R = [6-OSO_3Na-Glc-(1→4)]-[Glc-(1→4)-Glc-(1→2)]-Xyl

Fig. (17). Structure of Psolusoside B, isolated from the sea cucumber *Psolus fabricii*

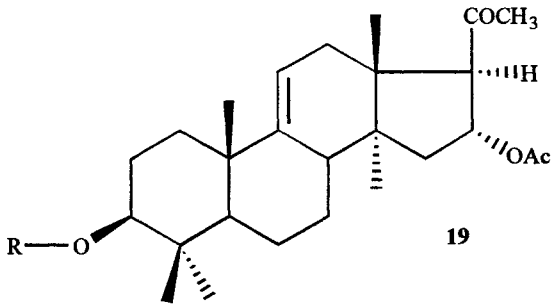


18a Cucumarioside G_2 [55] R = 3-*O*-Me-Xyl-(1→3)-Glc(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl
18b Calcigeroside B [52] R = [3-*O*-Me-Xyl-(1→3)-Glc(1→4)]-[Qui-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl
18c Calcigeroside C_1 [52] R = [3-*O*-Me-Xyl-(1→3)-Glc(1→4)]-[Glc-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl
18d Calcigeroside D_1 [49] R = [3-*O*-Me-Xyl-(1→3)-6-OSO_3Na-Glc(1→4)]-[Glc-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl

Fig. (18). Structures of non-holostane glycosides isolated from the sea cucumbers *Eupentacta fraudatrix* and *Pentamera calcigera*

Avilov et al. [56,57] reported three holothurins that are devoid of a lactone function and have a shortened side chain. Kurilosides A (**19a**) and

C (**19b**) contain a 9(11)-double bond aglycone moiety and 16α-acetoxy group, Fig. (**19**).

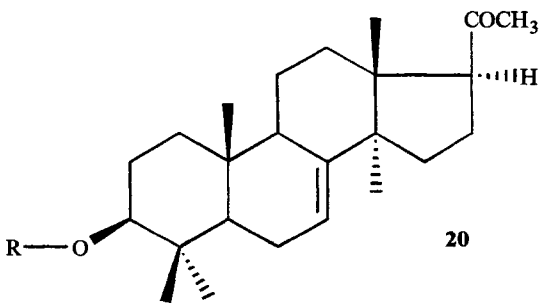


19

19a Kuriloside A [56] R = [3-*O*-Me-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)]-[Glc-(1→4)-Qui-(1→2)]-Xyl
19b Kuriloside C [56] R = [3-*O*-Me-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)]-[Qui-(1→2)]-Xyl

Fig. (19). Structures of glycosides isolated from the sea cucumber *Duasmodactyla kurilensis*

Koreoside A (**20**) isolated from *Cucumaria koraiensis* is one of the two examples of non-holostane glycosides with three sulfate groups in the oligosaccharide chain, Fig. (**20**).

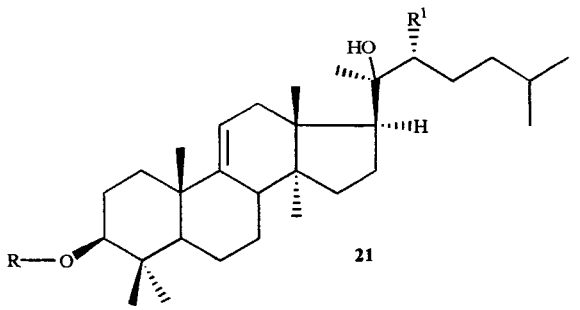


20

20 Koreoside A [57] R = [6-OSO_3Na-3-*O*-Me-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)]-[Xyl-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl

Fig. (20). Glycoside isolated from the sea cucumber *Cucumaria koraiensis*

Ds-Penaustrosides A (**21a**) and B (**21b**), as well as Frondoside C (**21c**), also lack the lactone function and have an additional hydroxyl group at C-20, Fig. (**21**).



21

21a Ds-Penaustroside A [19] R = [3-*O*-Me-Xyl-(1→3)-Glc-(1→4)]-[Qui-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = H
21b Ds-Penaustroside B [19] R = [3-*O*-Me-Xyl-(1→3)-Glc-(1→4)]-[Qui-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = H; Δ^{25}
21c Frondoside C [58] R = [3-*O*-Me-Xyl-(1→3)-Glc-(1→4)]-[Qui-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = OAc; Δ^{24}

Fig. (21). Structures of non-holostane glycosides isolated from the sea cucumbers *Pentacta australis* and *Cucumaria frondosa*

Most of sea cucumber triterpene glycosides are tetra- or pentaglycosides. The few disaccharides that have been isolated show a Qui-(1→2)-4-OSO_3Na-Xyl chain attached to C-3 of the triterpenoid aglycone [28, 31, 33, 34, 37]. Bivittoside A (**4a**) and Holothurinoside D (**8f**) show no sulfate group while Stichoposide B (**10e**) is the only example of a disaccharide with a glucose unit attached to C-2 of the xylose unit. Some hexasaccharides have been isolated from sea cucumbers of the order Aspidochirota: *Stichopus japonica* [21], *Stichopus chloronotus* [37], *Parastichopus californius* [20] and *Bohadschia bivittata* [18]. 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 example with a glucose unit instead of the terminal 3-*O*-Me-glucose is Holotoxin B_1 (**3d**).

Most tetrasaccharides show a linear chain with the most common 3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)-Xyl structure. In some tetrasaccharides the glucose unit is replaced by a xylose [22, 24, 37, 38, 40, 43, 51] while Cucumariosides G_1 (**12f**) and G_4 (**13a**) show a terminal 3-*O*-Me-xylose unit. Thelenotoside B (**10j**) and Eximioside A (**13b**) show a different tetrasaccharide chain: 3-*O*-Me-Glc-(1→3)-Xyl-(1→4)-Glc-(1→2)-Xyl with no quinovose unit. Non-holostane triterpenoids, such as Psolusoside B (**17**), Kuriloside C (**19b**) and Bivittoside B (**4b**) are the only examples of tetrasaccharides with a non-linear chain. Most tetrasaccharides are sulfated at C-4 of the xylose unit. Additional sulfate

groups at C-6 of the 3-*O*-Me-glucose unit and at C-6 of the glucose unit have been found in trisulfated tetraglycosides.

Pentaglycosides isolated from sea cucumbers show a variety of carbohydrate chains, Fig. (**22**). Most glycosides contain chains **I-IV**. Chain **IV** is typical for glycosides isolated from the sea cucumber *Pentamera calcigera*: Calcigerosides C_1 (**18c**), C_2 (**15c**), D_1 (**18d**), D_2 (**15d**) and E (**13c**). Cucumarioside A_1-2 (**11d**) is the only example of a triterpene glycoside containing an acetate group at C-6 of the terminal glucose unit (chain **XII**). Pentasaccharide chains with glucose as the terminal sugar are uncommon and were found in a few glycosides, such as Cucumarioside A_4-2 (**11i**) (chain **VII**), Cladoloside B (**3i**) (chain **X**) and Holothurinoside A (**8c**) (chain **XI**).

[3-*O*-Me-Glc-(1→3)-Glc-(1→4)]-[Xyl-(1→2)]-Qui-(1→2)]-Xyl-aglycone (I)
[3-*O*-Me-Glc-(1→3)-Xyl-(1→4)]-[Xyl-(1→2)]-Qui-(1→2)]-Xyl-aglycone (II)
[3-*O*-Me-Xyl-(1→3)-Glc-(1→4)]-[Glc-(1→2)]-Qui-(1→2)]-Xyl-aglycone (III)
[3-*O*-Me-Xyl-(1→3)-Glc-(1→4)]-[Qui-(1→2)]-Qui-(1→2)]-Xyl-aglycone (IV)
[3-*O*-Me-Xyl-(1→3)-Glc-(1→4)]-[Xyl-(1→2)]-Qui-(1→2)]-Xyl-aglycone (V)
[3-*O*-Me-Glc-(1→3)-Glc-(1→4)]-[Glc-(1→4)]-Qui-(1→2)]-Xyl-aglycone (VI)
[Glc-(1→3)-Glc-(1→4)]-[Xyl-(1→2)]-Qui-(1→2)]-Xyl-aglycone (VII)
[3-*O*-Me-Xyl-(1→3)-Glc-(1→4)]-[Qui-(1→2)]-Qui-(1→2)]-Xyl-aglycone (VIII)
[3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)]-[Glc-(1→4)]- Xyl-aglycone (IX)
[Glc-(1→4)]-[3-*O*-Me-Glc-(1→3)-Xyl-(1→4)-Qui-(1→2)]- Xyl-aglycone (X)
[Glc-(1→4)]-[3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)]- Xyl-aglycone (XI)
[6-*O*-Ac-Glc-(1→3)-Glc-(1→4)]-[Xyl-(1→2)]-Qui-(1→2)]-Xyl-aglycone (XII)

Fig. (**22**). Pentaglycoside chains in holothurins

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-*O*-Me-glucose and glucose units have been isolated [35, 36, 39, 42, 49, 57].

STRUCTURAL ELUCIDATION

Sea cucumber triterpene glycosides are quite fragile molecules. Acidic hydrolysis of intact holothurins results in the production of artifacts of the original aglycones due to migration of double bonds and dehydration reactions [59, 28]. Aqueous acid hydrolysis of glycosides containing a 25(26)-double bond in the aglycone side chain has led to the formation of artificial 25-hydroxy-genines [21, 60]. To overcome these difficulties ^{1}H- and ^{13}C-NMR spectroscopy have been extensively used to determine the

structure of the native aglycones as well as the glycosidic linkages in the oligosaccharide chain without degradation of the glycosides. Besides, the development of soft ionization methods, such as fast atom bombardment (FAB) [61] allowed the mass spectrometric analysis of polar thermally labile molecules of masses of up to a few thousand Daltons, in particular for samples which exist as preformed ions in solution. FAB-MS in positive- and negative-ion modes has been applied to obtain information on the molecular weight of underivatized glycosides of starfish and sea cucumbers on the basis of quasi-molecular ions $[M+H]^+$, $[M+Na]^+$and $[M-H]^-$ and $[M-Na]^-$, respectively, together with useful information on the saccharide sequence [2].

Nuclear magnetic resonance (NMR) has proved to be a very useful tool for structural elucidation of natural products. Recent progress in the development of two-dimensional ^{1}H- and ^{13}C-NMR techniques has contributed to the unambiguously assignment of proton and carbon chemical shifts, in particular in complex molecules. The more used techniques include direct correlations through homonuclear (COSY, TOCSY, ROESY, NOESY) [62-65] and heteronuclear (HMQC, HMBC) [66, 67] couplings.

^{1}H-NMR spectra of triterpene glycosides are complicated due to extensive interproton coupling. The first complete holothurin structures published in the literature [59, 68] reported only some characteristic proton signals, such as those due to methyl groups of the triterpenoid skeleton (19-CH_3, 21-CH_3, 26-CH_3, 27-CH_3, 30-CH_3, 31-CH_3, 32-CH_3), olefinic protons at C-7, C-11 or those present in the side chain, and doublets ascribable to the anomeric protons of the oligosaccharide moiety. Originally, structural elucidation of holothurins was based mainly on ^{13}C-NMR data, acid hydrolysis and enzymatic and degradation reactions. In the last ten years bidimensional NMR experiments have allowed the assignment of all proton and carbon resonances of the aglycone and the oligosaccharide chain [32, 35, 39, 40, 48, 52, 57]. NOESY experiments [69] on the intact glycosides have been useful in determining the relative stereochemistry of all chiral centers of the aglycone. Recently, we have successfully assigned all proton and carbon resonances of a new aglycone in the disulfated tetraglycoside patagonicoside A (**16**) by a combination of ^{1}H-^{1}H COSY, COLOC, HETCOR and NOESY experiments [53]. Fig. (**22**) shows the NOESY correlations of the aglycone moiety of Patagonicoside A. Correlations of

H-3 with H-1′, H-1α, H-5α and H-31 confirmed the β-configuration at C-3. Of particular interest is the β-configuration of H-9 in 3β-hydroxyholost-7-ene based aglycones instead of the characteristic 9α-configuration in natural steroids and triterpenoids. This proton showed a characteristic broad doublet at δ 3.02 ppm (in CD_3OD) and a strong NOE correlation with H-19 and H-12β. This last correlation revealed the α-configuration of the hydroxyl group at C-12. Correlations between H-12 and H-21 evidenced the α-configuration of the hydroxyl group at C-17 and consequently the *S* configuration at C-20. In this way we were able to confirm the stereochemistry assigned previously to these carbons by Kitagawa et al. [27, 28] only on the basis of solvent-induced shifts in the ^{1}H-NMR spectra of the corresponding sapogenols obtained by hydrolysis of the native saponin.

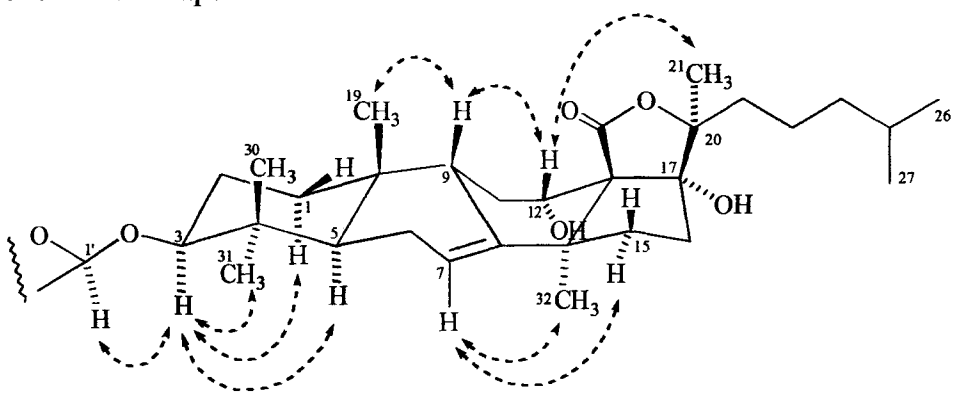


Fig. (22). NOESY correlations of the aglycone moiety of Patagonicoide A

Triterpene glycosides of sea cucumbers show characteristic signals due to the aglycone and the sugar moieties in their ^{13}C-NMR spectra. The chemical shifts of aglycone carbons of representative holothurins containing holostane aglycones are shown in Table 1. Characteristic resonances for C-3 (δ *ca.* 88-91 ppm) and C-20 (δ *ca.* 83-88 ppm) are observed. Both signals are easily distinguished by DEPT analysis [70]. The presence of a signal at δ *ca.* 175-180 ppm is typical for a lactone carbonyl group ascribable to C-18. The two series of aglycones differing in the position of the double bond in the triterpenoid skeleton can be readily distinguished by the chemical shifts of their olefinic carbons. Holothurins containing a 3β-hydroxyholost-9(11)-ene based aglycone such as glycoside **3k** show characteristic resonances for a trisubstituted

9(11)-double bond at δ 151.0 ppm (s, C-9) and 111.0 ppm (d, C-11). The presence of an allylic hydroxyl group at C-12α shifts the resonance of C-11 to δ *ca.* 116 ppm (glycosides **4e**, **5b**, **6** and **8e**). Besides, aglycones with a trisubstituted 7(8)-double bond, as in glycosides **9b**, **11f**, **12a**, **13c**, **15c** and **16**, show typical resonances at δ *ca.* 120-121 ppm (d, C-7) and 143-148 ppm (s, C-8).

Table 1. ^{13}**C NMR chemical shifts of holostane aglycones**

C	3k [a]	4e [b]	5b [b]	6 [b]	8e [b]	9b [a]	11f [a]	12a [a]	13c [a]	15c [a]	16 [c]
1	36.1	36.8	36.4	36.1	36.4	36.0	36.8	36.0	35.8	36.0	37.3
2	26.8	27.3	27.0	27.1	27.3	26.9	27.7	26.8	26.6	26.8	27.8
3	88.4	89.1	88.5	88.8	88.8	88.9	90.0	89.2	88.9	88.7	90.8
4	39.5	40.2	39.9	40.1	40.1	39.5	40.4	39.5	39.2	39.4	40.4
5	52.7	53.2	52.7	52.8	52.8	47.9	49.5	48.0	47.7	47.8	50.2
6	20.9	21.4	21.2	21.3	21.2	23.2	24.2	23.3	23.0	23.2	24.0
7	28.3	29.0	28.2	28.4	28.7	119.8	122.7	120.4	120.2	119.8	121.4
8	38.6	40.4	40.8	41.0	40.8	146.6	144.8	145.8	143.2	146.5	148.4
9	151.0	153.6	154.0	154.1	153.5	47.2	48.2	47.2	47.3	47.2	46.1
10	39.7	39.8	39.6	39.8	40.1	35.4	36.7	35.5	35.2	35.4	36.4
11	111.0	116.4	115.6	115.7	116.1	22.8	23.3	22.6	22.3	22.7	35.9
12	31.9	68.5	71.3	71.5	71.5	30.2	30.7	31.4	31.0	30.0	73.6
13	55.7	64.4	58.5	57.8	63.7	58.6	57.9	59.5	59.1	57.5	60.2
14	41.9	46.8	46.3	46.5	46.2	51.2	46.7	47.5	46.9	51.2	52.0
15	51.9	24.3	27.0	36.5	23.6	24.4	53.0	43.6	43.4	34.0	35.7
16	213.4	37.3	35.8	36.8	38.4	34.2	215.3	75.4	74.9	25.5	36.5
17	61.2	47.2	89.1	89.5	47.6	53.0	64.6	54.5	54.2	53.4	90.7
18	176.1	177.5	174.7	174.8	177.5	180.2	180.3	180.6	180.1	179.8	178.5
19	21.9	18.3	20.0	20.1	22.0	23.9	25.0	24.0	23.7	23.9	24.4
20	83.2	84.7	86.9	87.4	83.6	84.1	84.9	85.9	84.9	82.5	88.0
21	26.7	26.4	23.0	23.3	18.7	25.9	27.2	28.4	28.7	27.1	23.0
22	38.3	39.6	36.5	35.2	80.2	39.2	39.2	39.1	41.7	51.8	39.2
23	22.2	23.4	22.2	30.7	28.7	22.9	23.2	22.8	143.2	207.5	23.0
24	37.9	124.5	38.8	75.4	36.7	123.3	38.8	39.6	120.5	52.0	40.7
25	145.4	132.1	28.2	150.0	81.2	131.8	146.5	28.1	70.0	24.3	29.0
26	110.4	25.7	22.6	110.6	28.7	25.5	111.4	22.4	29.6	22.3	23.0
27	22.1	17.7	22.6	18.2	28.0	17.8	23.2	22.9	29.8	22.3	22.9
30	16.4	16.8	16.7	16.8	16.7	17.3	18.4	17.5	17.2	17.3	29.3
31	27.8	28.3	28.0	28.2	27.2	28.6	29.8	28.8	28.1	28.6	17.7
32	20.5	22.1	22.7	22.7	20.1	30.8	32.8	32.4	32.0	30.6	31.2
AcO								171.0	171.0		
								21.5	21.1		

[a] In Py-d_5-D_2O, [b] In Py-d_5, [c] In CD_3OD

The position of additional double bonds in the side chains can be determined from the carbon resonances of the olefinic carbons. A terminal isopropenyl group in the side chain shows characteristic signals for the olefinic carbons at δ *ca.* 123-124 ppm (C-24) and 132 ppm (C-25) as well as for the methyl groups attached to C-25 at δ *ca.* 25 ppm (C-26) and 18 ppm (C-27) (glycosides **4e** and **9b**). The presence of these two methyl vinyl groups is easily confirmed by the downfield shift of the methyl singlets in the ^{1}H-NMR spectrum with respect to the corresponding doublets in a saturated chain. Liouvilloside A (**12g**), a virucidal trisulfated triterpene glycoside isolated from the Antarctic sea cucumber *Staurocucumis liouvillei* shows two singlets at δ 1.54 ppm (H-26) and 1.64 ppm (H-27), while Liouvilloside B (**12c**), the saturated analog, shows two nearly overlapped doublets (J = 6.6 Hz) at δ 0.83 and 0.84 ppm [42]. Aglycones with a Δ^{25}-double bond (**3k**, **6** and **11f**) are characterized by olefinic carbon resonances at δ ca. 145-150 ppm (C-25, s) and 110-111 ppm (C-26, t). This disubstituted terminal double bond shows a diagnostic multiplet for H-26 at δ 4.75 ppm (2H) and a vinyl methyl signal at δ 1.68 ppm (s, H-27) in the ^{1}H-NMR spectrum [25].

Holothurins that contain a carbonyl group at C-16 (**3k** and **11f**) show a ketone carbonyl signal at δ *ca.* 213-215 ppm. Aglycones substituted with hydroxyl groups at C-12 and C-17 with α-configurations (**5b**, **6**, **16**) show a signal at δ 89-90 ppm due to the quaternary C-7. This carbon signal can be readily distinguished from the C-3 signal by a DEPT experiment. Holost-7-ene aglycones containing an acetate group at C-16 (**12a**, **13c**) are characterized by the presence of a singlet at δ 2.0 ppm ($C\underline{H}_3CO_2$) in their ^{1}H-NMR spectra as well as signals at δ *ca.* 171 and 21 ppm for the carbonyl and methyl groups of the acetate group. Recently, we have deduced the position of the acetoxyl group at C-16 in Liouvilloside A (**12g**) 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 ($J_{15\alpha,16\alpha}$), 7.4 ($J_{15\beta,16\alpha}$) and 8.9 Hz ($J_{16\alpha,17\alpha}$) for the most stable conformation of 16β-acetoxyholosta-7,24-dien-3β-ol obtained by molecular mechanics were coincident with experimental and reported values [40] and differed

considerably from those calculated for the 16α-isomer (4.1, 6.9 and 1.2 Hz, respectively) [42].

^{13}C-NMR data for non-holostane triterpenoid aglycones in intact glycosides are shown in Table 2.

Table 2. ^{13}C-NMR chemical shifts of non-holostane aglycones

Carbon	17 [a]	18b [b]	Ds-19a [a,c]	20 [b]	21a [b]
1	35.0	35.6	36.5	35.6	36.6
2	26.1	26.6	27.2	27.0	27.3
3	87.8	88.7	88.6	89.1	88.9
4	38.4	39.2	39.5	39.4	39.4
5	46.9	47.2	53.0	48.5	53.2
6	22.4	23.1	21.4	23.4	21.6
7	146.5	122.4	28.6	122.5	28.5
8	121.7	147.3	41.5	147.7	41.7
9	45.2	46.3	149.0	49.3	149.0
10	34.8	35.3	40.0	35.7	40.0
11	20.9	21.6	114.0	22.4	115.3
12	19.3	19.9	35.8	33.6	38.1
13	53.9	56.7	46.4	45.1	45.2
14	45.0	46.0	46.8	53.2	47.6
15	43.7	43.6	42.7	33.6	33.9
16	78.3	81.0	75.9	22.5	22.7
17	59.4	59.1	66.0	62.1	53.3
18	180.2	181.9	16.7	24.9	16.6
19	23.5	23.7	22.4	24.7	23.0
20	82.9	139.9	206.5	211.5	74.2
21	23.0	23.0	30.9	30.7	26.3
22	38.7	113.8			45.7
23	20.7				22.6
24	37.1				40.1
25	144.5				28.3
26	110.1				22.5
27	21.7				22.6
30	16.6	17.1	28.2	17.5	17.0
31	28.1	28.5	17.4	29.0	28.2
32	33.0	33.9	19.6	30.5	19.1
CH_3CO	169.0		170.2		
CH_3CO	21.5		21.0		

[a] In DMSO-d_6 [b] In Py-d_5-D_2O (4:1) [c] Desulfated analog of **19a**

Aglycones with a 7(8)-double bond, as structures **17**, **18** and **20**, show typical olefinic carbon resonances at δ 146-147 ppm (C-8) and *ca.* 122 ppm (C-7), while those containing a $\Delta^{9(11)}$-trisubstituted double bond (**19a**, **19b**, **21a**, **21b** and **21c**) are characterized by signals at δ 149.0 ppm (C-9) and 114-115 ppm (C-11). Psolusoside B (**17**), the first reported structure of a non-holostane glycoside with an 18(16)-lactone as well as holothurins **18a-18d** show signals at δ *ca.* 180-182 ppm (C-18, s) and 78-81 ppm (C-16, d). These signals are easily distinguished from the chemical shifts of C-18 (δ *ca.* 178-180 ppm) and C-20 (δ *ca.* 83-88 ppm) in a Δ^7-holostane aglycone with an 18(20)-lactone.

Characterization of the oligosaccharide chain of holothurins requires: a) the identification of each monosaccharide and its anomeric configuration in the oligosaccharide chain, b) the interglycosidic linkages and the sequence, c) the position of sulfate groups, and finally the site of attachment of the oligosaccharide chain to the triterpene aglycone. The identification of the monosaccharide composition is easily accomplished by acid hydrolysis of the intact holothurin, derivatization of each monosaccharide and further analysis by GC and comparison with standards [42].

FAB-MS is a very useful technique for the determination of the sequence of monosaccharides in the carbohydrate chain of a glycoside. As shown in Fig. (**24**) for Liouvilloside A (**12g**), cleavages can occur on both sides of the glycosidic linkages with proton transfer. These cleavages give characteristic fragments that correspond to the sequential losses of each monosaccharide. In sulfated holothurins, fragment ion peaks due to the loss of SO_3Na are diagnostic for the presence of sulfate groups in the glycosides. For example, Liouvilloside A (**12g**) showed fragment ion peaks at *m/z* 1355 $[M - SO_3Na + H + Na]^+$, 1253 $[M - 2SO_3Na + H + Na]^+$and 1151 $[M - 3SO_3Na + 3H + Na]^+$, corresponding to the sequential losses of three sulfate groups.

Although FAB-MS gives information on the sequence of monosaccharides in the oligosaccharide moiety, it is not possible to determine the location of the interglycosidic linkages by this method. NMR has been the method of choice for complete characterization of the oligosaccharide chain.

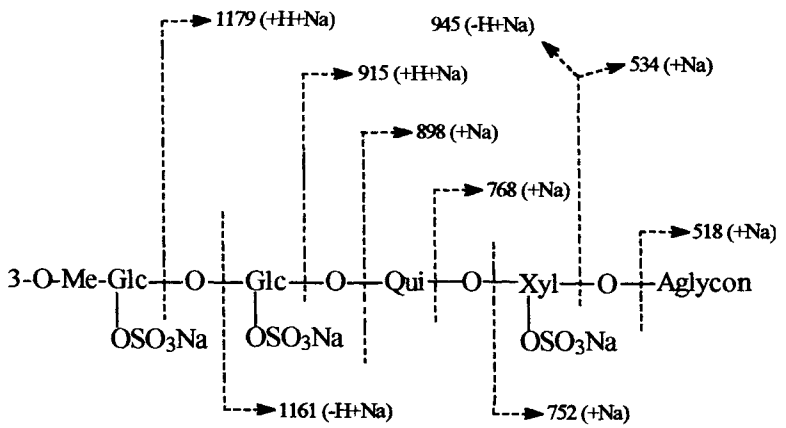


Fig. (24). Positive FAB-MS fragmentation of Liouvilloside A

The ^{1}H-NMR spectra of holothurins show complex and overlapping signals for hydroxymethine and hydroxymethylene protons of sugar residues in the downfield region at δ 3.0-5.0 ppm. · Nevertheless, the anomeric signals usually appear as almost separated doublets at δ 4.7-5.3 ppm in C_5D_5N:D_2O (5:1) with $^{3}J_{1,2}$ of *ca.*7.7 Hz and are indicative of the β-anomers of the pyranose sugars with *gluco* and *galacto* configurations [72]. Another characteristic signal in the ^{1}H-NMR spectra of holothurins is the methyl doublet resonance of the 6-deoxyhexose quinovose at δ *ca.* 1.6 ppm in C_5D_5N:D_2O (5:1). The resonance of the methyl carbon of quinovose is observed at δ *ca.* 18 ppm. Glycosides containing a methoxyl group attached to C-3 of a glucose or a xylose unit show a typical singlet at δ 3.6 ppm in their ^{1}H-NMR spectra as well as the corresponding carbon resonance at δ *ca.* 60 ppm.

Comparison of ^{13}C-NMR data of the oligosaccharide chain of holothurins with those of reference methyl glycosides has been the method of choice to determine the interglycosidic linkages [73]. Terminal sugar residues exhibit remarkable resemblance of their ^{13}C-NMR data with those of their respective methyl glycosides. Internal sugar moieties show deviations in their carbon resonances with respect to the corresponding methyl glycosides due to glycosidation. Measurement of the relaxation times (T_1) of the sugar units aided in some cases in the assignment of carbon resonances of the oligosaccharide chain [28, 60]. Permethylation of the intact non sulfated glycosides or the desulfated derivatives of sulfated holothurins followed by GC-MS analysis of the

partially methylated alditol acetates [21, 28, 31] has also been used in order to determine the interglycosidic linkages.

Table 3 shows the ^{13}C-NMR data for the sugar units of sea cucumber glycosides containing different pentasaccharide chains.

Table 3. ^{13}C-NMR data for the sugar moieties of holothurins with pentaglycosidic chains

Carbon	12a[a]	8c[b]	9a[c]	Des-18b [b,e]
1'	104.5	103.5	105.7	104.6
2'	81.6	83.3	81.7	83.0
3'	75.3	75.7	75.3	76.9
4'	76.3	77.9	75.8	69.5
5'	64.2	64.1	64.2	65.9
1''	102.2	105.5	102.2	103.0
2''	82.6	75.8	83.9	83.4
3''	75.2	76.3	74.7	75.1
4''	85.3	87.2	87.1	85.5
5''	71.2	71.7	70.6	71.0
6''	18.0	18.0	17.5	17.9
1'''	104.7	104.9	104.3	103.9
2'''	73.6	73.7	73.4	73.5
3'''	86.2	87.8	86.4	86.5
4'''	68.9	69.8	69.7	69.0
5'''	66.0	77.3	74.9	77.1
6'''		62.1	67.6	61.5
1''''	104.5	105.6	104.9	105.1
2''''	74.7	74.9	74.7	74.1
3''''	87.0	87.7	87.5	86.6
4''''	70.6	70.5	78.0	69.6
5''''	77.6	78.3	70.4	66.3
6''''	61.9	62.5	61.8	
OMe	60.9	60.7	60.7	60.6
1'''''	105.4	105.3	105.9	105.2
2'''''	74.9	74.3	75.2	75.8
3'''''	76.6	78.7	76.6	76.2
4'''''	70.2	71.7	69.7	75.6
5'''''	66.6	78.1	64.2	73.4
6'''''		62.2		17.9

[a] In Py-d_5-D_2O (5:1) [b] In Py-d_5 [c] Py-d_5-D_2O (8:2) [d]Desulfated analog of **18b** [e] In Py-d_5-D_2O (4:1)

One common structural feature is the presence of a xylose unit attached to C-3 of the aglycone and substituted at C-2' with a quinovose unit. As shown in Table 2, carbons involved in the interglycosidic linkages show chemical shifts at δ *ca.* 82-88 ppm, shifted downfield from those expected for the corresponding methyl glycopyranosides.

Glycosides containing a terminal glucose (**12a**, **8c**, **9a**) or xylose (**Des-18b**) substituted with a methoxyl group at C-3 show an additional signal at δ *ca.* 86-88 ppm due to the substitution at this carbon. Frondosides A (**12a**) and B (**9a**) and the desulfated derivative of Calcigeroside B (**Des-18b**) present a branched 2,4-disubstituted quinovose residue with a xylose unit attached to C-2'' in **9a** and **12a** and a quinovose unit in **Des-18b**. This substitution pattern is deduced from the downfield shifts of C-2'' and C-4'' of the 2,4-disubstituted quinovose in comparison with those carbon resonances in a terminal quinovose unit (**Des-18b**). On the other hand, Holothurinoside A (**8c**) with a 2,4-disubstituted xylose unit attached at C-3 of the aglycone shows signals at δ 83.3 ppm (C-2') and 77.9 ppm (C-4') for the carbon atoms involved in the glycosidic bonds.

As shown in Table 3, due to the proximity of carbon resonances of the different sugar units in the oligosaccharide chain, it is difficult to assign unambiguously each signal on the only basis of comparison with published data, sometimes performed in different solvents or solvent mixtures. Recent application of two-dimensional NMR techniques (^{1}H-^{1}H COSY, relay COSY, HETCOR, COLOC, HMBC and HMQC) to the structural elucidation of holothurins [32, 35, 39, 40, 48, 52, 57] has allowed the unambiguous assignment of all ^{1}H and ^{13}C resonances of the oligosaccharide chain. The NOESY spectrum of Patagonicoside A clearly showed the correlations between the protons (Fig. (**23**)) of the oligosaccharide chain. These correlations confirmed the interglycosidic linkages deduced previously from analysis of ^{1}H-^{1}H COSY and HETCOR spectra as well as the site of attachment of the sulfated xylose unit to the C-3 of the aglycone [53].

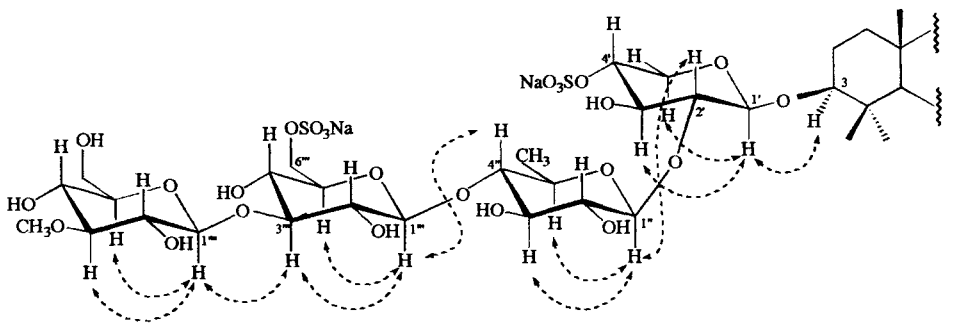


Fig. (23). NOESY correlations of the oligosaccharide moiety of patagonicoide A

Another common structural feature in holothurins is the presence of one, two or three sulfate units attached to the sugar residues of the oligosaccharide chain. The location of these groups has been determined by comparison of ^{13}C-NMR data of the native glycosides and the corresponding desulfated derivatives. Desulfation of the native holothurins is easily achieved by hydrolysis in a mixture of pyridine and dioxane at 120°C and further purification of the desulfated derivatives by HPLC [53]. Those holothurins containing an acetoxyl group at C-16, as Liouvilloside A (**12g**), are desulfated by acid hydrolysis in HCl-MeOH in order to prevent hydrolysis of the acetate group [42].

Table 4 shows the ^{1}H- and ^{13}C-NMR data for two glycosides, Patagonicoside A (**16**) and Hemoiedemoside A (**3k**), containing the same disulfated tetrasaccharide chain and the trisulfated Liouvilloside A (**12g**), that differs from **16** and **3k** in the presence of an additional sulfate group at C-6 of the terminal 3-*O*-Me-glucose unit. The three glycosides differ in their aglycone structures. As observed in Table 4 the esterified carbons with a sulfate group show downfield shifts of *ca.* 4-6 ppm with respect to their desulfated derivatives, while upfield shifts of *ca.* 2-3 ppm are observed for the vicinal carbons. The chemical shifts of these carbons vary with the solvent used for performing the spectra. For example, the xylose unit with a sulfate group at C-4', common to all sulfated holothurins, shows a characteristic signal for C-4' at δ 77.1 ppm in CD_3OD, while the same carbon resonance is shifted downfield to δ 75.8 and 74.4 ppm in C_5D_5N:D_2O (5:1) and DMSO-d_6, respectively. Sulfate groups at C-6 of glucose or a 3-*O*-Me-glucose residue show typical signals for C-6 at δ 65.6-68.5 ppm in these deuterated solvents.

Table 4. ^{1}H- and ^{13}C-NMR data for the Sugar Moieties of Patagonicoside A (16), Hemoiedemoside A (3k) and Liouvilloside A (12g).

	16		3k		12g	
C	δ_C [a,b]	δ_H [c] (*J* in Hz)	δ_C [b,d]	δ_H [e] (*J* in Hz)	δ_C [b,f]	δ_H [g] (*J* in Hz)
1'	105.6	4.46 d (7.9)	104.9	4.69 d (7.1)	104.2	4.32 d (7.3)
2'	*82.7*	3.55 m	*82.4*	3.69 m	*82.0*	3.36 m
3'	75.1 (+2.3)	3.78 m	74.8 (+2.6)	4.27 dd (9, 8.7)	74.7 (+2)	3.54 m
4'	77.1 (-6)	4.23 m	**75.8** (-5.5)	5.11 m	**74.4** (-4.8)	3.97 m
5'	63.8 (+2.6)	3.37 m; 4.17 m	63.9 (+2.3)	3.72 m; 4.75 m	63.2 (+2.3)	3.19 m
1"	104.8	4.61 d (7.6)	104.6	4.92 d /7.7)	103.8	4.49 d (8)
2"	76.3	3.36 m	75.5	3.88 dd	74.9	3.10
3"	75.6	3.55 m	75.6	3.97 m	74.2	3.32 m
4"	*87.3*	3.23 m	*87.8*	3.44 dd (8.7, 8.9)	*86.2*	3.03
5"	72.5	3.49 m	71.3	3.66 m	70.4	3.34 m
6"	18.0	1.35 d (6.1)	17.8	1.63 d (6.1)	17.4	1.25 d (5.3)
1"'	104.8	4.45 (6.9)	104.6	4.76 d (7.7)	103.1	4.40 d (7.8)
2"'	74.3	3.41 m	74.3	3.95	72.6	3.25 m
3"'	*87.1*	3.60 m	*86.5*	4.25	*85.9*	3.49 m
4"'	70.2	3.42 m	69.9	3.79	68.7	3.20 m
5"'	75.2 (+2.7)	3.69 m	74.7 (+2.7)	4.21	74.2 (+2.1)	3.53 m
6"'	**68.5** (-6.1)	4.12 m; 4.38 m	**67.5** (-5.7)	4.68 m, 5.14 dd (2, 10.7)	**65.9** (-4.9)	3.78 m, 4.04 dd (18, 10)
1""	105.2	4.57 d (7.9)	104.9	5.29 d (7.8)	103.8	4.47 d (7.9)
2""	75.4	3.31 m	74.5	3.96 m	73.6	3.15 m
3""	87.6	3.10 m	87.4	3.71 m	85.8	3.00
4""	71.1	3.34 m	70.3	4.02 dd (8.9, 9.3)	69.3	3.21 m
5""	78.1	3.32 m	77.9	3.95 m	75.1 (+1.8)	3.34 m
6""	62.5	3.65 m; 3.85 bd (10.5)	61.8	4.19 m, 4.43 dd (2, 11.9)	**65.6** (-4.6)	3.83 m,4.04 dd (18, 10)
OCH_3	61.1	3.62 s	60.6	3.85 s	60.1	3.49 s

[a] Recorded at 125 MHz in Methanol-d_4; [b] Italics = interglycosidic positions, bold = sulfate positions; (Δ_C = δ_C - $\delta_{C\text{desulfated analog}}$); [c] Recorded at 500 MHz in Methanol-d_4; [d] Recorded at 125 MHz in Py-d_5:D_2O (5:1); [e] Recorded at 500 MHz in Py-d_5:D_2O (5:1); [f] Recorded at 125 MHz in DMSO-d_6; [g] Recorded at 500 MHz in DMSO-d_6

The determination of the position of sulfate groups in holothurins is important in order to establish structure-activity correlations. Recently, we have evaluated the antifungal activity of di- and trisulfated glycosides and their semi-synthetic desulfated analogs against the phytopathogenic fungus *Cladosporium cucumerinum* [25, 53]. We have found that Hemoiedemosides A (**3k**) and B (**3l**) and Patagonicoside A (**16**) were more active than their desulfated analogs. On comparing the antifungal

activities of the disulfated glycosides **3k** and **16**, Hemoiedemoside A resulted more active. Both glycosides present the same oligosaccharide chain and differ in the aglycone structure. On the other hand, Hemoiedemoside B (**3l**) differing from **3k** in the presence of a third sulfate group at C-6 of the terminal 3-*O*-methylglucose residue is less active than **3l**. These results suggest that both the aglycone structure and the presence and number of sulfate groups at the oligosaccharide chain play an important role in the antifungal activity of holothurins.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge grants from the International Foundation for Science, Stockholm, Sweden, the Organization for the Prohibition of Chemical Weapons, The Hague, The Netherlands, CONICET, ANPCyT and the Universidad de Buenos Aires. H. D. Chludil thanks FOMEC-UBA for a fellowship. M. S. M. and A. M. S. are Research Members of the National Research Council of Argentina (CONICET).

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