Pergamon

Tetrahedron 57 (2001) 9563–9568

TETRAHEDRON

Patagonicoside A: a novel antifungal disulfated triterpene glycoside from the sea cucumber *Psolus patagonicus*

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Received 21 August 2001; accepted 3 October 2001

Abstract—A new triterpene glycoside, patagonicoside A (**1**), has been isolated from the sea cucumber *Psolus patagonicus* and its structure has been elucidated by 1D and 2D NMR (^{1}H-, ^{13}C-, ^{1}H–^{1}H COSY, HETCOR, COLOC and NOESY spectra), FAB-MS and chemical evidence. Compound **1** is a disulfated tetrasaccharide with a new aglycon moiety. Patagonicoside A (**1**) exhibits considerable antifungal activity against the pathogenic fungus *Cladosporium cucumerinum*.

1. Introduction

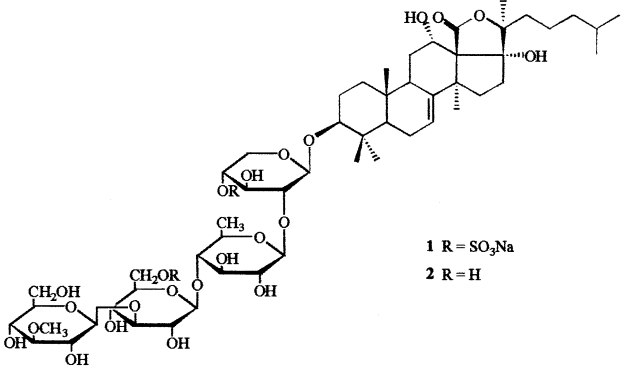
Sea cucumber saponins, triterpene glycosides commonly known as holothurins, have drawn attention because of their wide spectrum of biological effects: antifungal, cytotoxic, hemolytic, cytostatic and immunomodulatory activities.[1] Recently, we have demonstrated the virucidal activity of two new trisulfated triterpene glycosides isolated from the antarctic holothuroid *Staurocucumis liouvillei*.[2] Although several species of various families of this class of echinoderms have been studied, only three species of the genus *Psolus* have been investigated.[3–5]

As a continuation of our search for bioactive metabolites from cold water echinoderms of the South Atlantic[6,7] we have focused our attention on the polar extract of *Psolus patagonicus* (Ekman, 1925) (Psolidae, Dendrochirotida), an holothurian collected off Bahía Ensenada, Ushuaia. We have isolated the triterpene glycosides mixture from an alcoholic extract of *P. patagonicus* and have established the structure of the main component, patagonicoside A (**1**), a disulfated tetrasaccharide with a new aglycon moiety. Antifungal activity of patagonicoside A (**1**) and its desulfated derivative, ds-patagonicoside A (**2**) against the pathogenic fungus *Cladosporium cucumerinum* was studied.

2. Results and discussion

The ethanolic extract of *P. patagonicus* was sequentially submitted to vacuum-dry column chromatography on C_{18} reversed-phase Si gel and preparative reversed-phase HPLC affording pure patagonicoside A (**1**), the main component of the glycosidic fraction.

The ^{1}H and ^{13}C NMR spectra of compound **1** recorded in CD_3OD suggested the presence of a triterpenoid aglycon bonded to a tetrasaccharide chain (Tables 1 and 2). The structure of the aglycon of **1** was determined on the basis of spectroscopic data and by their comparison with those of known triterpenes.[8,9] The ^{13}C and ^{1}H NMR data (Table 1) indicated the presence of a trisubstituted double bond [δ_H 5.62 ppm (1H, br s, H-7) and δ_C 121.4 and 148.4 ppm, characteristic of a Δ^7 double bond], a carbonyl lactone group (δ_C 178.5 ppm) and seven methyl groups [δ_H 1.01 ppm (3H, s, H-19)/δ_C 24.4 ppm (C-19), δ_H 1.55 ppm



Keywords: *Psolus patagonicus*; Holothuroidea; glycosides; triterpenes; antifungal activity.

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0040–4020/01/$ - see front matter
PII: S0040-4020(01)00970-X

Table 1. ^{1}H and ^{13}C NMR data for the aglycon moiety of patagonicoside A (**1**) and its desulfated analog (**2**)

Carbon	**1**		**2**	
	δ_C mult[a]	δ_H mult[b] (J in Hz)	δ_C mult[a]	δ_H mult[b] (J in Hz)
1	37.3 t	1.44 m; 1.70 m	37.3 t	1.45 m; 1.71 m
2	27.8 t	1.87 m	27.8 t	1.87 m
3	90.8 d	3.11 m	90.9 d	3.09 m
4	40.4 s	–	40.4 s	–
5	50.2 d	0.95 m	50.2 d	0.93 m
6	24.0 t	1.97 m	24.0 t	1.97 m
7	121.4 d	5.62 br s	121.4 d	5.63 br s
8	148.4 s	–	148.4 s	–
9	46.1 d	3.02 br d (15.2)	46.1 d	3.03 br d (14.0)
10	36.4 s	–	36.4 s	–
11	35.9 t	1.70 m; 2.31 m	35.9 t	1.71 m; 2.32 m
12	73.6 d	4.44 m	73.6 d	4.45 t (7.95)
13	60.2 s	–	60.2 s	–
14	52.0 s	–	52.0 s	–
15	35.7 t	1.31 m; 1.88 m	35.7 t	1.32 m; 1.90 m
16	36.5 t	2.06 m; 2.45 dd (9.8, 14.6)	36.5 t	2.08 m; 2.47 dd (9.5,14.7)
17	90.7 s	–	90.7 s	–
18	178.5 s	–	178.5 s	–
19	24.4 q	1.01 s	24.4 q	1.02 s
20	88.0 s	–	88.0 s	–
21	23.0 q	1.55 s	23.0 q	1.56 s
22	39.2 t	1.72 m; 1.68 m	39.2 t	1.71 m
23	23.0 t	1.48 m; 1.38 m	23.0 t	1.48 m; 1.38 m
24	40.7 t	1.21 m	40.7 t	1.22 m
25	29.0 d	1.56 m	29.0 d	1.57 m
26	23.0 q	0.90 d (6.6)	23.0 q	0.91 d (6.6)
27	22.9 q	0.90 d (6.6)	22.9 q	0.91 d (6.6)
30	29.3 q	1.09 s	29.3 q	1.10 s
31	17.7 q	0.94 s	17.7 q	0.95 s
32	31.2 q	1.50 s	31.2 q	1.51 s

[a] Recorded at 125 MHz in methanol-d_4; multiplicity by DEPT.
[b] Recorded at 500 MHz in methanol-d_4.

Table 2. ^{1}H and ^{13}C NMR data for the sugar moieties of patagonicoside A (**1**) and its desulfated analog (**2**)

Carbon	**1**		**2**	
	δ_C[a,b]	δ_H[c] (J in Hz)	δ_C[a,b]	δ_H[c] (J in Hz)
1′	105.6 d	4.46 d (7.9)	106.0 d	4.40 d (6.9)
2′	*82.7* d	3.55 m	*83.2* d	3.45 m
3′	75.1 d	3.78 m	77.7 d	3.38 m
4′	**77.1** d	4.23 m	71.1 d	3.52 m
5′	63.8 t	3.37 m; 4.17 m	66.4 t	3.18 m; 3.85 m
1″	104.8 d	4.61 d (7.6)	105.3 d	4.57 d (7.7)
2″	76.3 d	3.36 m	76.5 d	3.30 m
3″	75.6 d	3.55 m	76.1 d	3.47 m
4″	*87.3* d	3.23 m	*86.8* d	3.17 m
5″	72.5 d	3.49 m	72.5 d	3.46 m
6″	18.0 q	1.35 d (6.1)	18.1 q	1.37 d (5.9)
1‴	104.8 d	4.45 (6.9)	104.7 d	4.42 d (7.9)
2‴	74.3 d	3.41 m	74.5 d	3.42 m
3‴	*87.1* d	3.60 m	*87.7* d	3.56 m
4‴	70.2 d	3.42 m	69.9 d	3.42 m
5‴	75.2 d	3.69 m	77.9 d	3.50 m
6‴	**68.5** t	4.12 m; 4.38 m	62.4 t	3.68 m; 3.47 m
1⁗	105.2 d	4.57 d (7.9)	105.3 d	4.57 d (7.7)
2⁗	75.4 d	3.31 m	75.4 d	3.33 m
3⁗	87.6 d	3.10 m	87.6 d	3.11 m
4⁗	71.1 d	3.34 m	71.1 d	3.36 m
5⁗	78.0 d	3.32 m	78.1 d	3.34 m
6⁗	62.5 t	3.65 m; 3.85 br d (10.5)	62.5 t	3.63 m; 3.85 m
OCH_3	61.1 q	3.62 s	61.1 q	3.63 s

[a] Recorded at 125 MHz in methanol-d_4; multiplicity by DEPT.
[b] Italics=interglycosidic positions, bold=sulfate positions.
[c] Recorded at 500 MHz in methanol-d_4.

(3H, s, H-21)/δ_C 23.0 ppm (C-21), δ_H 1.09 ppm (3H, s, H-30)/δ_C 29.3 ppm (C-30), δ_H 0.94 ppm (3H, s, H-31)/δ_C 17.7 ppm (C-31), δ_H 1.50 ppm (3H, s, H-32)/δ_C 31.2 ppm (C-32) and δ_H 0.90 ppm (6H, d, J=6.6 Hz, H-26, H-27)/δ_C 23.0 and 22.9 ppm (C-26, C-27)]. The presence of two hydroxy groups attached to C-12 and C-17 was evidenced by two signals in the downfield region of the ^{13}C NMR spectrum, δ_C 73.6 and δ_C 90.7 ppm, respectively, together with a signal at δ_H 4.44 ppm (1H, m) in the ^{1}H NMR spectrum corresponding to H-12. ^{1}H–^{1}H COSY and HETCOR spectra allowed the assignment of all the proton and carbon resonances. The position of the methyl groups in the aglycon was confirmed by the correlations for H-30 to C-4 and C-5, H-31 to C-4, H-19 to C-9, H-21 to C-20 and for H-32 to C-13, C-14 and C-15 in a COLOC experiment. The relative stereochemistry of all chiral centers of the aglycon was established with the aid of a NOESY experiment. Thus, as is depicted in Fig. 1, H-3 showed correlations with H-1′, H-1α (δ_H 1.44 ppm), H-5α and H-31 confirming the β configuration at C-3. Correlations between H-12/H-9 and H-9/H-19 revealed the α configuration of the hydroxy group at C-12. A correlation between H-12 and H-21 evidenced the α configuration of the hydroxy group at C-17 and consequently the *S* configuration of C-20. In this way we were able to confirm the stereochemistry assigned previously to these carbons by Kitagawa et al.[8,10] only on the basis of solvent-induced shifts in the ^{1}H NMR spectra of the corresponding sapogenols. Correlations between H-7/H-32 and H-7/H-15α (δ_H 1.88 ppm) were also observed.

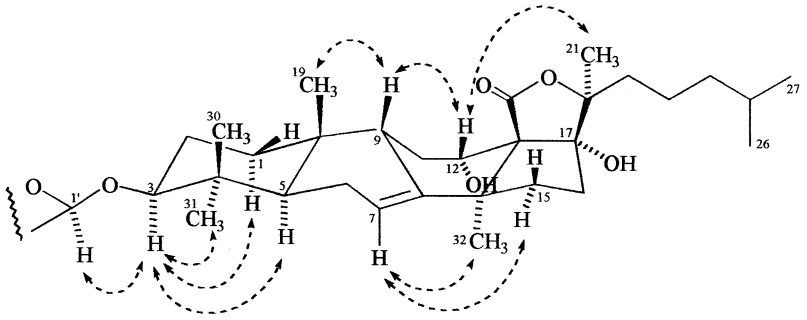

Figure 1. NOESY correlations of the aglycon moiety of patagonicoside A (**1**).

The proton and carbon resonances corresponding to the sugar part of the molecule (Table 2) showed signals for anomeric carbons at δ_C 105.6, 104.8, and 105.2 ppm and four anomeric protons at δ_H 4.46, 4.61, 4.45, and 4.57 ppm that resonate as doublets with coupling constants (J=6.9–7.9 Hz) indicating a β stereochemistry at the anomeric carbons.

The presence of 3-*O*-methylglucose, glucose, quinovose and xylose in a ratio 1:1:1:1 was established by acid hydrolysis with aqueous 2N trifluoroacetic acid followed by GC analysis of the corresponding peracetylated alditols. A doublet at δ_H 1.35 ppm (3H, J=6.1 Hz, H-6″) and a singlet at δ_H 3.62 ppm (3H, $-OCH_3$) in the ^{1}H NMR spectrum of **1** corroborated the presence of quinovose and 3-*O*-methylglucose.

The molecular formula of patagonicoside A (**1**) was determined as $C_{54}H_{86}O_{29}S_2Na_2$ by the pseudomolecular ions at m/z 1307 $[M_{NaNa}-H]^-$ and 1285 $[M_{NaNa}-Na]^-$ in its FABMS (negative ion mode). Hydrolysis of glycoside **1** gave the desulfated derivative, ds-patagonicoside A (**2**). Comparison of ^{13}C NMR spectroscopic data of patagonicoside A (**1**) with those of its desulfated derivative **2** confirmed the presence of two sulfate groups. Thus, the downfield shift (by 6.0 ppm) of C-4′ signal and the upfield shift of C-5′ (by 2.6 ppm) and C-3′ (by 2.6 ppm) signals of the xylose residue in the spectrum of **1** in comparison with those of **2**, indicated the attachment of one sulfate group to C-4′ of xylose. Similarly, the downfield shift (by 6.1 ppm) of C-6‴ signal and the upfield shift (by 2.7 ppm) of C-5‴ signal of the glucose residue in the spectrum of **1** in comparison with those of **2**, indicated the attachment of the second sulfate group to C-6‴ of glucose. This was also confirmed by the upfield shifts of both H-6‴ protons at δ 3.68 and 3.47 ppm in the desulfated analog **2**.

The sequence of the monosaccharide units in the carbohydrate chain of glycoside **1** was determined by fragment ion peaks at m/z 1109, 845 and 699 in the FABMS (negative ion mode) spectrum corresponding to the sequential losses of 3-*O*-methylglucosyl, sulfated glucosyl, and quinovosyl units, respectively, and m/z 820 [3-*O*-Me-Glc-*O*-Glc-OSO_3Na-*O*-Qui-*O*-Xyl-OSO_3Na$-H]^-$.

All proton and carbon chemical shifts of the oligosaccharide chain of **1** (Table 2) could be assigned using ^{1}H–^{1}H COSY and HETCOR experiments. Location of the interglycosidic linkages in the oligosaccharide chain was deduced from the chemical shifts of C-2′ (δ_C 82.7 ppm), C-4″ (δ_C 87.3 ppm), C-3‴ (δ_C 87.1 ppm) and C-3⁗ (δ_C 87.6 ppm), assigned on the basis of crosspeaks δ 3.55/82.7 (H-2′/C-2′), δ 3.23/87.3 (H-4″/C-4″), δ 3.60/87.1 (H-3‴/C-3‴) and δ 3.10/87.6 (H-3⁗/C-3⁗) in the HETCOR spectrum. The carbons involved in the interglycosidic linkages gave values shifted downfield from those expected for the corresponding methylglycopyranosides.[11] These results were confirmed by methylation of glycoside **1** followed by hydrolysis and GC-MS analysis of the partially methylated alditol acetates derived from 2-linked xylopyranose, 4-linked quinovopyranose, 3-linked glucopyranose and terminal 3-*O*-methylglucopyranose.[12]

The NOESY spectrum of **1** showed the correlations H-1′/H-3, H-3′, H-5′$_{ax}$ (δ_H 3.37 ppm); H-1″/H-2′, H-3″, H-5″; H-1‴/H-4″, H-3‴, H-5‴ and H-1⁗/H-3‴, H-3⁗, H-5⁗ (Fig. 2) confirming the interglycosidic linkages in the oligosaccharide chain. Considering our previous experience in

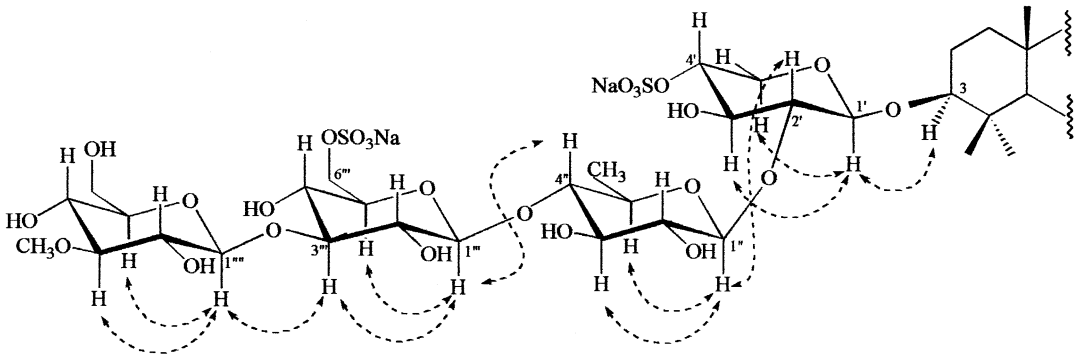

Figure 2. NOESY correlations of the oligosaccharide chain of patagonicoside A (**1**).

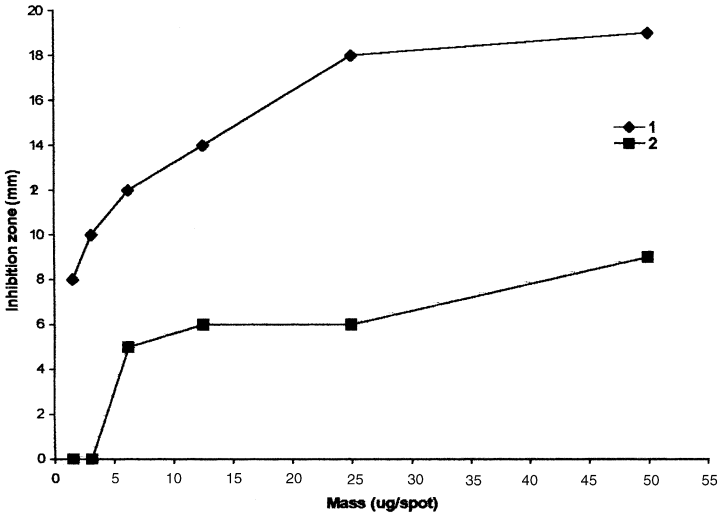

Figure 3. Dose-response curves for antifungal activity of patagonicoside A (**1**) and ds-patagonicoside A (**2**) against *C. cucumerinum*.

the determination of the absolute configuration of the carbohydrate units of analog sugar chains, afforded by the GC analysis of the 1-[(*S*)-*N*-acetyl-(2-hydroxypropylamino)]-1-deoxyalditol acetate derivatives, we assume a D-configuration for all the monosaccharide units of patagonicoside A (**1**).[2] Additional confirmation of the monosacharide sequence in the sugar chain was achieved by the correlations observed in the COLOC spectrum.

On the basis of all the above data, the structure of patagonicoside A (**1**) was established as 3-*O*-[3-*O*-methyl-β-D-glucopyranosyl-(1→3)-6-*O*-sodium sulfate-β-D-glucopyranosyl-(1→4)-β-D-quinovopyranosyl-(1→2)-4-*O*-sodium sulfate-β-D-xylopyranosyl]-holost-7-en-3β,12α,17α-triol.

The sugar part of **1** is a linear disulfated tetrasaccharide belonging to the group of most evolved carbohydrate chains of glycosides from sea cucumbers.[13] In this case it is the same sugar chain found in cucumechinosides A and C isolated from the holothurian *Cucumaria echinata*.[11] On the other hand, the aglycon moiety of **1** has not been previously reported. There are some examples of holothurins having one,[9,10,14] two[8,14,15] or three[16] hydroxy groups in the aglycon but all of them have a Δ^9 double bond. Patagonicoside A (**1**) is the first example of an holothurin with a Δ^7, 3β, 12α, 17α trihydroxy holostane type aglycon.

It is to note that the structures of these related holothurins having hydroxy groups were assigned, in most of the cases,[8–10,15] on the basis of ^{13}C NMR data and laborious chemicals transformations of the aglycon part leading to artifact sapogenols. In this case, the structure and the complete stereochemistry of patagonicoside A (**1**) have been established by a thorough analysis of the spectral data including mass spectrometry (FAB), ^1H and ^{13}C NMR, ^1H–^1H COSY, HETCOR, COLOC and NOESY experiments. All the proton and carbon signals could be unambiguously assigned. The analysis of the spectral data obtained for ds-patagonicoside A (**2**) corroborated the previous assignment done for glycoside **1**.

Since some holothurins with at least one oxygen function in positions 12, 16 or 17 of the aglycon moiety (e.g. holotoxins A and B,[17] holothurins A[15] and B,[18] echinosides A and B,[8] pervicosides A–C,[10] bivittosides A–D[9]) are known to exhibit antifungal activities, patagonicoside A (**1**) and ds-patagonicoside A (**2**) were examined against the pathogenic fungus *C. cucumerinum* by a bioautography technique.[19] Saponins **1** and **2** showed a marked difference in their antifungal properties. Patagonicoside A (**1**) resulted to be considerably active, in a concentration dependent manner (Fig. 3), showing inhibitions zones of 8–19 mm at the tested concentrations (1.5–50 μg/spot). On the other hand, ds-patagonicoside A (**2**) 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). As compounds **1** and **2** differ only in the substitutions at C-4′ and C-6‴, these results suggest that the presence of sulfate groups in the oligosaccharide chain plays an important role in the antifungal activity of these triterpene glycosides.

3. Experimental

3.1. General methods

^1H and ^{13}C NMR spectra were recorded in CD_3OD on a Bruker AM 500 spectrometer.The FABMS (negative ion mode) were obtained on a VG-ZAB mass spectrometer, on a glycerol matrix. Optical rotations were measured on a Perkin–Elmer 343 polarimeter. Preparative HPLC was carried out on a SP liquid chromatograph equipped with a Spectra Series P100 solvent delivery system, a Rheodyne manual injector and a refractive index detector using a Bondclone 10 μ column (30 cm×7.8 mm i.d.). TLC was performed on precoated Si gel F254 (*n*-BuOH–HOAc–H_2O (12:3:5)) and C_{18} reversed-phase plates (60% MeOH–H_2O) and detected by spraying with *p*-anisaldehyde (5% EtOH). GC was performed on a Hewlett–Packard 5890A chromatograph equipped with a flame ionization detector and a SP-2330 column (25 m×0.2 mm i.d.). GC-MS was performed on a TRIO-2 VG mass spectrometer

coupled to a Hewlett–Packard 5890A chromatograph with an SP-2330 column (30 m×0.25 mm i.d.).

3.2. Animal material

Specimens of *P. patagonicus* were collected off Bahía Ensenada, Ushuaia, Tierra del Fuego province, Argentina. The organisms were identified by Dr C. Muniaín. A voucher specimen is preserved at the Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia', Buenos Aires, Argentina (MACN no. 34776).

3.3. Extraction and isolation

The sea cucumbers (83 g), frozen prior to storage, were homogenized in EtOH (2.5 L) and centrifuged. The EtOH was evaporated and the residue was partitioned between MeOH–H_2O (90:10) and cyclohexane. The glassy material obtained after evaporation of the methanolic extract was subjected to vacuum dry column chromatography[20] on Davisil C_{18} reversed-phase (35–70 μ) using H_2O, H_2O–MeOH mixtures with increasing amounts of MeOH and finally MeOH as eluents. Fractions (250 mL) were analyzed by TLC and the triterpene glycosides were eluted with 50 and 40% MeOH. These fractions were combined and submitted to reversed-phase HPLC to give the pure glycoside **1** (45 mg) as the main component.

3.3.1. Patagonicoside A (1). White amorphous powder, mp 204–206°C, $[\alpha]^{20}_D=-30°$ (*c* 0.5, MeOH). ^1H and ^{13}C NMR, see Tables 1 and 2. FABMS (negative ion mode), *m/z* 1307 $[M-H]^-$, 1285 $[M-Na]^-$, 1183 $[M-SO_3Na+H-Na]^-$, 1109 [M−3-*O*-Me-Glc+H−Na]$^-$, 1091 [M−3-*O*-Me-Glc-*O*−H−Na]$^-$, 845 [M−3-*O*-Me-Glc-*O*-Glc-OSO_3Na+H−Na]$^-$, 827 [M−3-*O*-Me-Glc-*O*-Glc-OSO_3Na-*O*−H−Na]$^-$, 699 [M−3-*O*-Me-Glc-*O*-Glc-OSO_3Na-*O*-Qui+H−Na]$^-$, 681 [M−3-*O*-Me-Glc-*O*-Glc-OSO_3Na-*O*-Qui-*O*−H−Na]$^-$, 820 [3-*O*-Me-Glc-*O*-Glc-OSO_3Na-*O*-Qui-*O*-Xyl-OSO_3Na−H]$^-$.

3.3.2. Desulfation of patagonicoside A (1). A solution of **1** (13 mg) in pyridine (0.3 mL) and dioxane (0.3 mL) was heated at 120°C for 2.5 h in a stoppered reaction vial. The reaction mixture was cooled, poured into water and extracted with *n*-BuOH. The butanolic extract was evaporated to dryness at reduced pressure and the residue was subjected to reversed-phase HPLC to give the pure desulfated glycoside, ds-patagonicoside A (**2**) (7.4 mg).

3.3.3. Ds-patagonicoside A (2). White amorphous powder, mp 246–248°C, $[\alpha]^{20}_D=-33°$ (*c* 0.3, MeOH). ^1H and ^{13}C NMR, see Tables 1 and 2. FABMS (negative ion mode), *m/z* 1103 $[M-H]^-$, 909 [M−3-*O*-Me-Glc-*O*−2H]$^-$, 747 [M−3-*O*-Me-Glc-*O*-Glc-*O*−2H]$^-$, 601 [M−3-*O*-Me-Glc-*O*-Glc-*O*-Qui-*O*−2H]$^-$, 634 [3-*O*-Me-Glc-*O*-Glc-*O*-Qui-*O*-Xyl-*O*+H]$^-$.

3.3.4. Acid hydrolysis of patagonicoside A (1). Glycoside **1** (5 mg) was heated in a screwcap vial with 2N trifluoroacetic acid (1 mL) at 120°C for 2 h. The aglycon was extracted with CH_2Cl_2 and the aqueous residue was evaporated under reduced pressure. The sugar mixture was treated with 0.5 M NH_3 (0.5 mL) and $NaBH_4$ (5 mg) at room temperature for 18 h. After acidification with 1 M AcOH, the reaction mixture was treated with MeOH (0.5 mL) and evaporated under reduced pressure. The alditol mixture was peracetylated with Ac_2O (0.5 mL) and pyridine (0.5 mL) at 100°C for 45 min. The reaction mixture was cooled and poured into $CHCl_3$–H_2O (1:1) and the aqueous phase was extracted with $CHCl_3$. The combined chloroform extracts were washed with H_2O (0.5 mL), saturated $NaHCO_3$ solution (0.5 mL) and H_2O (0.5 mL) and evaporated to dryness under nitrogen. The mixture of peracetylated alditols was analyzed by GC using standard peracetylated alditols as reference samples.

3.3.5. Methylation of 1 followed by hydrolysis. A solution of **1** (5 mg) in anhydrous DMSO (1.3 mL) was treated with NaOH (63 mg) and stirred at room temperature for 20 min. The reaction mixture was treated with CH_3I (0.4 mL) and stirred for further 30 min. After addition of water (4 mL) the mixture was extracted with CH_2Cl_2 and evaporated to dryness under nitrogen. The permethylated glycoside was submitted to acid hydrolysis following the same procedure described above for the hydrolysis of **1**. The mixture of methylated alditol acetates was analyzed by GC-MS.[12] The following carbohydrates could be detected: 2-linked xylose (1,2,4,5-tetra-*O*-acetyl-3-*O*-methylxylitol; t_R= 33.5 min; *m/z* (%)=231 (1.4), 189 (52.3), 129 (100.0), 87 (64.4); 4-linked quinovose (1,4,5-tri-*O*-acetyl-2,3-di-*O*-methylquinovitol; t_R=29.4 min; *m/z* (%)=203 (18.2), 143 (39.6), 117 (100.0), 101(69.8); 3-linked glucose (1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylglucitol; t_R=35.5 min; *m/z* (%)=305 (1.4), 233 (11.2), 189 (18.6), 129 (50.5), 117 (100.0), 87 (27.1); terminal 3-*O*-methylglucose (1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol; t_R=28.5 min; *m/z* (%)=205 (12.8), 161 (32.5), 145 (26.3), 129 (38.4), 117 (53.7), 101 (100.0).

3.4. Antifungal assay

Geometric dilutions were obtained from freshly prepared stock solutions of patagonicoside A (**1**), ds-patagonicoside A (**2**) and reference compound (benomyl) at concentrations of 1–10 mg mL^{-1} in an appropriate solvent. Of these solutions, 10 μL were applied on the TLC plates using graduated capillaries. After that, the plates were sprayed with a suspension of *C. cucumerinum* in a nutritive medium and incubated 2–3 days in a glass box with a moist atmosphere.[19] Clear inhibitions zones appeared against dark grey background. All samples were measured in duplicate. Data given in Fig. 3 are averages of these measurements.

Acknowledgements

This work was supported by the International Foundation for Science, Stockholm, Sweden, and the Organization for the Prohibition of Chemical Weapons, The Hague, The Netherlands, through a grant to M. S. Maier. We also wish to thank CONICET, ANPCyT and the Universidad de Buenos Aires for financial support of this work. We are indebted to UMYMFOR (CONICET-FCEN, UBA) for NMR spectra and LANAIS-EMAR (CONICET-FCEN, UBA) for mass spectra. M. S. M. and A. M. S. are Research

Members of the National Research Council of Argentina (CONICET).

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