Antiviral Sulfated Steroids from the Ophiuroid *Ophioplocus januarii*

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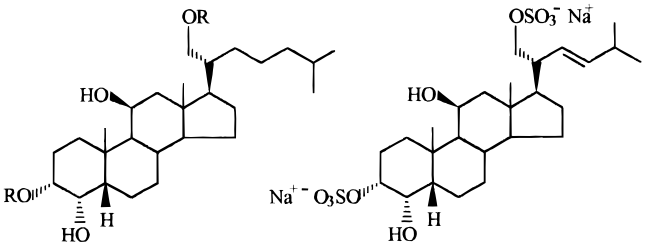
One new and three known sulfated steroidal polyols have been isolated from the ophiuroid *Ophioplocus januarii*, collected at San Antonio Oeste, Río Negro, Argentina. The four compounds possess 4α,11β-dihydroxy-3α,21-disulfoxy substituents and the A/B *cis* ring junction but differ in the side chain. The new compound has been characterized as (22*E*)-5β-24-norcholest-22-ene-3α,4α,11β,21-tetrol 3,21-disulfate (**4**). The structures of the four compounds were determined from spectral data and comparison with those of related steroidal polyols. The four compounds were tested for their inhibitory effect on the replication of one DNA and three RNA viruses. Compounds **2** and **4** were active against respiratory syncytial and polio viruses, and compound **3** inhibited Junin virus, responsible for Argentine hemorrhagic fever.

Sulfated sterols have been described from a wide variety of marine organisms, particularly sponges and echinoderms.[1] Among the echinoderms, starfish contain asterosaponins, steroidal glycosides sulfated at C-3, as well as polyhydroxysteroids and their glycosides sulfated at the steroidal skeleton or the carbohydrate unit. On the other hand, ophiuroids are characterized by their content of polar sulfated steroids and the lack of saponins.[2] Sulfated steroidal polyols have shown a broad spectrum of biological activities, such as antiviral properties,[3] cytotoxic action, and inhibition of protein tyrosine kinases.[4] More recently, McKee *et al.* demonstrated the antiviral activity against HIV-1 and HIV-2 of sulfated sterols isolated from sponges, ophiuroids, and starfish.[5]

In continuation of our studies on echinoderms of cold waters of the South Atlantic[6,7] we have investigated the ophiuroid *Ophioplocus januarii* Luetken (Ophiuroidea), a typical organism from the Patagonian coast of Argentina.[8] We have isolated one new sterol sulfate (**4**) and three known compounds (**1**−**3**) previously isolated from the Pacific ophiuroids *Ophiocoma dentata*, *Ophiarthrum elegans*, and *Ophiarachna incrassata*.[9] The antiviral activity of the four compounds was tested against different pathogenic viruses of humans: Herpes simplex virus type 1 (HSV-1), Junin virus (JV), respiratory syncytial virus (RSV), and polio virus (PV). HSV-1 is the agent responsible for primary and recurrent infections of mucous membranes (gingivostomatitis, herpes labialis infections), skin lesions, keratoconjunctivitis, neonatal and visceral infections, and encephalitis.[10] JV causes a severe disease in humans known as Argentine hemorrhagic fever.[11] RSV is commonly associated with bronchitis, pneumonia, and bronchiolitis in infants,[12] and PV with flaccid paralysis due to lower motor neuron damage.[13]

The ophiuroid *O. januarii* was homogenized and extracted with MeOH followed by centrifugation and concentration. The extract was partitioned between *n*-hexane and H_2O. The aqueous phase was then extracted with *n*-BuOH. Separation and isolation of the individual compounds from the *n*-BuOH extract was achieved by chromatography on Sephadex LH 20, followed by reversed-phase HPLC.



1 R = SO_3^- Na^+
2 R = SO_3^- Na^+, Δ^{22}
3 R = SO_3^- Na^+, 24-Me, $\Delta^{24(28)}$

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The polar steroids isolated from *O. januarii* are characterized by the presence of three−OCHR− units and one $-OCH_2-$ unit as confirmed by ^{13}C-NMR and DEPT measurements. The ^{1}H-NMR spectra of compounds **1**−**4** showed signals at δ 3.95 (1H, dd, J = 9.2, 6.2 Hz) and 4.19 (4H, bb), typical for those observed in the spectra of steroids characterized by the presence of C-21 and C-3α sulfate groups, *cis* A/B ring fusion, and hydroxy groups located at C-4α and C-11β.[9] The *cis* A/B ring junction follows from the lowfield chemical shift of the 19-methyl protons (δ_H 1.15 ppm) and the 19-methyl carbon (δ_C 27.4 ppm) as calculated on the basis of the substituent effects published for polyhydroxy steroids.[14] Otherwise, the 19-methyl carbon for the alternate 5α-cholestane-3β,4β,11β,21-tetrol should be 17.9 ppm,[9] which is far from the value obtained for C-19 (δ_C 27.4 ppm) in **1**−**4**. The known compounds (**1**−**3**) were identified by comparison of their ^{1}H- and ^{13}C-NMR, FABMS, and optical rotation data with published data.[9] Spectral data indicated that steroids **1**−**4** possessed identical nuclei and one 21-sulfoxy substituent, but differed in the side chain. In order to confirm the nuclear substitution pattern of the steroidal skeleton, we solvolyzed compound **1** (the major polar steroidal component). In the ^{1}H-NMR spectrum of **1a**, derived from **1** upon solvolysis, the resonances of the protons of the hydroxy-bearing carbons gave rise to five isolated signals (see Experimental Section). ^{1}H-NMR assignments were derived from ^{1}H−^{1}H COSY data. Doublet-of-doublet signals at δ 3.69 and 3.74 correlated with

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each other and were assigned to the 21-hydroxymethylene protons. The resonance frequencies of the hydroxymethine protons were observed at δ 3.60, 3.90, and 4.22 ppm. From the $^1H-^1H$ COSY spectrum it was determined that the apparent quartet at δ 4.22 was coupled to two protons at δ 2.05 and 1.50 (H-12 and H-9, respectively) and assigned to H-11α. The axial orientation of the hydroxy group at C-11 was supported from the chemical shifts of H-18 and H-19 (0.91 and 1.13, respectively) and the significant shifts for both angular methyl resonances when the ^1H-NMR spectrum of **1** was measured in pyridine-d_5 (Δ +0.36), which indicated a 1,3-diaxial interaction between both angular methyl groups and the hydroxyl group at C-11.[15] The doublet of triplets signal at δ 3.60 and the triplet at δ 3.90 were correlated with each other in the $^1H-^1H$ COSY spectrum of **1a** and assigned to H-3β and H-4β, respectively. The stereochemistry at C-3 and C-4 was defined by coupling constants for H-3 (dt, J = 11.3, 4.4 Hz) and H-4 (t, J = 3.1 Hz). The sulfate groups were assigned to C-3 and C-21 on comparison of ^1H- and ^{13}C-NMR spectra of **1** and **1a**.

The new metabolite (**4**) was characterized as (22*E*)-5β-24-norcholest-22-ene-3α,4α,11β,21-tetrol 3,21-disulfate. The ^{13}C-NMR spectrum of **4** showed 26 carbon atoms (see Experimental Section), and DEPT measurements revealed the presence of four methyl groups, seven methylene groups, seven methine groups, two quaternary carbons, three $-$OCHR$-$ units, one $-OCH_2-$ unit, and a disubstituted double bond. Negative-ion FABMS showed a molecular ion species at m/z 601 [M(SO_3Na)(SO_3^-)] and intense fragments with m/z 497 and 479, which correspond to the loss of $NaSO_3$ (+H) and $NaHSO_4$. The ^1H-NMR spectrum of **4** contained two methyl singlet peaks at δ 0.93 and 1.15 ppm, assigned to Me-18 and Me-19, respectively; two overlapping methyl doublet signals at δ 0.96 (6H, d, J = 6.6 Hz), assigned to the isopropyl methyl groups (C-26 and C-27), and the signals at δ 3.90 (1H, dd, J = 9.2, 6.2 Hz) and 4.17 (4H, m), corresponding to the protons of the oxygen-bearing carbons. These spectral data established the presence of the same tetracyclic nucleus found in compounds **1**$-$**3** and a side chain that was shorter. The ^1H-NMR spectrum showed two well separated olefinic protons at δ 5.25 (1H, dd, J = 15.1, 7.8 Hz) and 5.43 (1H, dd, J = 15.1, 6.2 Hz), which indicated a *trans* double bond. In the $^1H-^1H$ COSY spectrum of **4**, both olefinic protons were correlated with each other, and the one at δ 5.43 was coupled to H-25 at δ 2.21, which correlated with the isopropyl methyl groups at δ 0.96 (d, J = 6.6 Hz). The downfield shifts of C-20 and C-21 to 46.6 and 71.5 ppm, respectively, also observed in compound **2**,[9] confirmed the presence of the Δ^{22} double bond. The spectral data for compound **4** discussed above were in good agreement with those reported for related compounds containing the same side chain as **4**.[16,17]

Compounds **1**$-$**4** were tested for their inhibitory effect on the replication of RNA (PV, JV, and RSV) and DNA (HSV-1) viruses. As shown in Table 1 compounds **2** and **4** elicited a marked RSV inhibitory activity and also proved to be active against PV at a concentration of 40 μg/mL. Compound **3** was less active against RSV and weakly active toward PV. Most of the compounds showed similar activity against HSV-1 and JV, except **3** which inhibited 54% of JV replication. In general, HSV-1 and JV were less sensitive to the sulfated sterols. Compound **1** was weakly active to all the viruses employed. No cytotoxicity was observed at the concentrations assayed.

Table 1. Antiviral and Cytotoxic Activity of the Compounds Isolated from *O. januarii*

compound	concentration μg/mL	antiviral activity[a] HSV-1	PV	JV	RSV
1	40	35.0 ± 0.7	30.0 ± 2.1	33.0 ± 3.5	25
	20	8.0 ± 1.8	24.0 ± 4.4	28.0 ± 1.0	25
	10	7.0 ± 0.6	0	26.0 ± 4.5	0
	5	2.0 ± 0.3	0	24.0 ± 0.7	0
2	40	39.0 ± 0.7	68.0 ± 1.4	47.0 ± 4.2	75
	20	14.0 ± 2.8	35.0 ± 1.4	36.0 ± 6.3	50
	10	0	0	31.0 ± 4.9	25
	5	0	0	15.0 ± 2.8	0
3	40	36.0 ± 0.7	31.0 ± 5.6	54.0 ± 2.1	50
	20	20.0 ± 6.6	16.0 ± 1.7	38.0 ± 3.5	25
	10	11.0 ± 2.1	0	33.0 ± 1.0	25
	5	3.0 ± 0.2	0	19.0 ± 3.0	0
4	40	36.0 ± 5.6	65.0 ± 7.7	37.0 ± 2.7	75
	20	12.0 ± 3.0	37.0 ± 2.1	29.0 ± 4.9	50
	10	0	8.0 ± 2.0	21.0 ± 1.3	0
	5	0	0	10.0 ± 0.5	0

[a] For HSV-1, polio, and Junin virus the antiviral activity is expressed as percent reduction of virus plaque formation ± standard deviation (results are the average of two experiments). For RSV results are expressed as percent reduction of CPE as detailed in the Experimental Section.

The viral replication was inhibited in a concentration-dependent manner, showing a specific effect of the compounds on viral growth. Negligible antiviral activity was seen at a concentration of 5μg/mL. On the other hand, cell viability was 100% even when the highest concentration (40 μg/mL) of the compounds was tested. Because compounds **1**$-$**4** differ only in the side chain, results obtained in the antiviral tests indicate the importance of the Δ^{22} double bond (compounds **2** and **4**) in the inhibitory effect on the replication of RSV as well as the $\Delta^{24(28)}$ double bond (compound **3**) on JV replication.

Experimental Section

General Experimental Procedures. ^1H- and ^{13}C-NMR spectra were recorded on a Bruker ACE-200 instrument. FABMS were obtained on a VG-ZAB mass spectrometer. 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 C_{18} Bondclone 10-μ column (30 cm × 7.8 mm i.d.); flow rate 2 mL/min. TLC was performed on precoated Si gel F_{254} and C_{18} reversed-phase plates.

Animal Material. Specimens of *O. januarii* (1.1 kg) were collected in 1993 off Bajo Oliveira near San Antonio Oeste on the Argentine Patagonian coast. The animals were identified by Dr. Alejandro Tablado of the Museo de Ciencias Naturales "Bernardino Rivadavia", where a voucher specimen of the organism is preserved (MACN no. 31239).

Extraction and Isolation. The animals, frozen prior to storage, were homogenized in MeOH (1 L) and centrifuged. The MeOH was evaporated, and the residue was partitioned between H_2O and *n*-hexane. This aqueous residue was then extracted twice with *n*-BuOH. The glassy material obtained after the evaporation of the *n*-BuOH extract was chromatographed on

a Sephadex LH 20 column (80 cm × 4 cm i.d., MeOH). Fractions (10 mL) were analyzed by TLC on SiO_2 in *n*-BuOH−HOAc−H_2O (4:5:1) (upper layer) and by C_{18} reversed-phase TLC [MeOH−H_2O (65:35)] and detected by spraying with H_2SO_4. Fractions 30−50 contained the polar sulfated steroids. Final purification of this last fraction was accomplished by HPLC on a C_{18} Bondclone column with MeOH−H_2O (50:50), to give the pure compounds **1** (8.2 mg), **2** (6.4 mg), **3** (6.3 mg), and **4** (3.5 mg).

(22*E*)-5*β*-24-Norcholesta-22-ene-3α,4α,11*β*,21-tetrol 3,21-disulfate (4): obtained as a white powder; $[\alpha]^{25}_D$ +10.9° [*c* 0.62, MeOH]; ^{1}H-NMR (CD_3OD, 200.1 MHz) δ 0.93 (3H, s, Me-18), 0.96 (6H, d, J = 6.6 Hz, Me-26 and Me-27), 1.15 (3H, s, Me-19), 3.90 (1H, dd, J = 9.8, 6.2 Hz, H-21), 4.17 (4H, m, H-3*β*, H-4*β*, H-11α, H-21), 5.25 (1H, dd, J = 15.1, 7.8 Hz, H-22), 5.43 (1H, dd, J = 15.1, 6.2 Hz, H-23); ^{13}C-NMR (CD_3OD, 50.3 MHz) δ 139.3 (d, C-23), 129.8 (d, C-22), 82.5 (d, C-3), 75.3 (d, C-4), 71.5 (t, C-21), 68.8 (d, C-11), 59.5 (d, C-14), 52.7 (d, C-17), 49.6 (t, C-12), 48.8 (d, C-5), 46.6 (d, C-20), 45.8 (d, C-9), 42.6 (s, C-13), 36.4 (s, C-10), 36.1 (t, C-1), 31.6 (d, C-8), 30.7 (d, C-25), 29.9 (t, C-16), 28.8 (t, C-7), 27.4 (q, C-19), 27.1 (t, C-6), 25.1 (t, C-15), 23.4 (t, C-2), 23.2 (q, C-26), 23.0 (q, C-27), 15.4 (q, C-18).

Solvolysis of Compound 1. A solution of **1** (4.5 mg) in pyridine (0.25 mL) and dioxane (0.25 mL) was heated at 130 °C for 3 h in a stoppered reaction vial. After removal of the solvent mixture, the residue was suspended in H_2O and purified by passage through a C_{18} cartridge and eluted with 50% MeOH−H_2O and MeOH. The tetrol **1a** was recovered from the MeOH fraction and analyzed by TLC and ^{1}H- and ^{13}C-NMR spectroscopy.

5*β*-Cholestane-3α,4α,11*β*,21-tetrol (1a): ^{1}H-NMR ($CDCl_3$) δ 0.87 (6H, d, J = 6.6 Hz, Me-26 and Me-27), 0.91 (3H, s, Me-18), 1.16 (3H, s, Me-19), 3.60 (1H, dd, J = 11.3, 4.4 Hz, H-3*β*), 3.69 (1H, dd, J = 11.2, 4.2 Hz, H-21), 3.74 (1H, dd, J = 11.2, 3.2 Hz, H-21), 3.90 (1H, t, J = 3.1, H-4*β*), 4.22 (1H, q, J = 2.9 Hz, H-11α); ^{13}C-NMR (CD_3OD) δ 77.1 (C-4), 74.1 (C-3), 69.0 (C-11), 63.0 (C-21), 59.5 (C-14), 52.5 (C-17), 49.8 (C-12), 48.1 (C-5), 45.9 (C-9), 43.5 (C-20), 42.7 (C-13), 40.7 (C-24), 36.4 (C-10), 35.9 (C-1), 31.7 (C-8), 30.7 (C-22), 30.5 (C-16), 29.2 (C-25), 28.3 (C-7), 27.4 (C-19), 27.0 (C-6), 25.6 (C-2), 25.2 (C-15), 24.8 (C-23), 23.1 (C-26), 23.0 (C-27), 15.2 (C-18).

Viruses and Cells. The virus strains used were as follows: HSV-1 (strain F), JV (strain IV 4454), PV type 3 (strain Sabin), and RSV (strain Long). Vero and Hep-2 cells were grown in 24-well culture plates with Eagle Minimal Essential Medium (MEM) containing 5% of bovine serum. Maintenance medium (MM) consisted of MEM with 1.5% of bovine serum. Stocks of HSV-1, JV, and PV were prepared in Vero cells, whereas RSV was propagated in Hep-2 cell cultures.

Compound Solutions. Stock solutions of the compounds at a concentration of 2 mg/mL were prepared in MeOH and sterilized. The solutions were kept at −20 °C until use.

Cytotoxicity Test. The cytotoxic activity was determined by incubating monolayers of Vero cells with 5, 10, 20, or 40 μg/mL of the compounds in MM. The cytotoxicity measurements were achieved by determining the number of viable cells by trypan blue exclusion after an incubation time of 7 days at 37 °C.

Antiviral Assays. Antiviral activity was evaluated by two methods: reduction of virus plaque formation and inhibition of cytopathic effect (CPE). In the plaque reduction test, Vero cell monolayers grown in 24-well plates were infected with about 50 PFU of virus/well in the absence or presence of various concentrations of the compounds. After 1 h of adsorption, residual inoculum was replaced by MM containing 0.7% methylcellulose and the corresponding dose of each compound. Plaques were counted after 1 day of incubation at 37 °C for PV, 2 days for HSV-1, and 7 days post-infection for JV. The antiviral activity was calculated as the percent reduction of virus plaque formation. All determinations were performed in duplicate. To evaluate the cytopathic effect, Hep-2 monolayers were infected in quadruplicate with RSV at a MOI of 0.1 in the absence or presence of various concentrations of the compounds. Cell controls were included in each experiment. After 48 h of incubation at 37 °C, the CPE was examined under an inverted microscope. Viral CPE was graded on a progressive scale of 0 (= normal cells) to 4 (= complete destruction of the cell monolayer) (0 = 100% inhibition, 1 = 75% inhibition, 2 = 50% inhibition, 3 = 25% inhibition, 4 = 0% inhibition).

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