# Novel Bioactive Sulfated Alkene and Alkanes from the Mediterranean Ascidian *Halocynthia papillosa*

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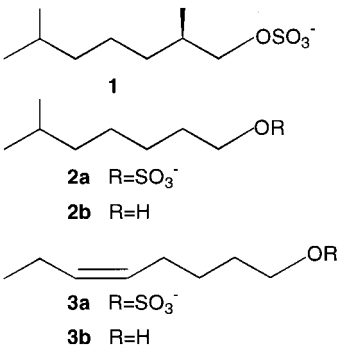
Three sulfated alkene and alkanes—(*R*)-2,6-dimethylheptyl sulfate (**1**), 6-methylheptyl sulfate (**2a**), and (*E*)-5-octenyl sulfate (**3a**)—with cytotoxic activity in vitro, have been isolated from the Mediterranean ascidian *Halocynthia papillosa*. The structures of the new compounds **2a** and **3a** have been elucidated by spectroscopic analysis.

Sulfated alkanes and alkenes have been isolated recently from several marine sources, most of them exhibiting pharmacological effects, such as antibacterial, antifungal, and antiproliferative activity.[1−6] Some of these compounds were shown to be present in relatively remarkable quantities in ascidians, thus indicating that they could play a biological role in these organisms.[3−6]

In the course of our search for bioactive metabolites of Mediterranean ascidians, we found that the MeOH extracts of the hepatopancreas, gonads, body muscle, and tunic of the species *Halocynthia papillosa* Hartmeyer 1904 (family Pyuridae) were toxic to WEHI 164 (murine fibrosarcoma) cells. A bioassay-guided fractionation of each extract revealed that the activity was mainly due to the presence of a mixture of sulfated alkanes and alkenes; it was separated and purified, thus affording compounds **1**, **2a**, and **3a**; they showed cytotoxic activity, which has been estimated on WEHI 164 and C6 (rat glioma) cell lines (Table 1).

The normonoterpenoid **1** was previously found in the Mediterranean ascidian *Policitor adriaticus*;[3] it was also isolated, as a racemate, from another species of *Halocynthia, H. roretzi*,[4] but it was shown to be present only in the hepatopancreas. Alkyl sulfates **2a** and **3a** are new compounds, and their isolation and structure elucidation are described in this paper.




The tunics were removed from several specimens of *H. papillosa* and, then, the hepatopancreas and the gonads were dissected from the body muscle. Each tissue, after homogenization, was separately extracted with methanol; the concentrated aqueous residues were partitioned between water and ethyl acetate and, subsequently, the polar layers were re-extracted with *n*-BuOH. The *n*-BuOH extracts, all showing cytotoxic activity, were subjected to reversed-phase $C_{18}$ chromatography, using a linear gradient of MeOH (from 0 to 100%) in $H_2O$. The bioactive fractions, eluted with MeOH/$H_2O$ (3:7), were further separated and purified by $RP_{18}$ HPLC (MeOH/$H_2O$, 1:1) to give compounds **1**, **2a**, and **3a** in the pure state.

**Table 1.** $IC_{50}$ ($\mu$g/mL) of Compounds **1**, **2a**, and **3a**[a]

| Cell line | **1** | **2a** | **3a** | 6-MP |
|---|---|---|---|---|
| WEHI | 20.9 ± 2 | 15.0 ± 1 | 12.2 ± 0.9 | 1.3 ± 0.03 |
| C6 | inactive | 545.4 ± 7.5 | 515.2 ± 5.2 | 30.2 ± 1.5 |

[a] Results are expressed as mean ± S. E. M. of three experiments in quadruplicate.

Compound **1**, which was the major alkyl sulfate in each extract, was identified as (*R*)-2,6-dimethylheptyl sulfate by comparison of its spectral properties (negative FABMS, $^1$H and $^{13}$C NMR, $[\alpha]^{25}_D$) with those reported in the literature.[3,7]

The FABMS (negative ion mode) of **2a** showed a $[M]^-$ ion peak at *m*/*z* 209, corresponding to the formula $C_8H_{17}SO_4$. The presence of a sulfate group was inferred from the IR absorption band at 1210 cm$^{-1}$. The main features of its $^1$H NMR spectrum were a doublet in the high-field region at $\delta$ 0.92 (6H, $J$ = 6.5), due to the methyl protons of an isopropyl group, and a triplet signal in the midfield region at $\delta$ 4.02 (2H, $J$ = 6.8), attributable to the protons of a methylene bearing the sulfate group. Solvolysis of compound **2a** gave the corresponding alcohol **2b**, which displayed the predictable upfield shift ($\delta$ 3.63) for this latter signal, further proving the presence in **2a** of the sulfate moiety. Interpretation of the $^1$H−$^1$H COSY allowed delineation of the whole spin sequence H1−H7/H8 and the identification of compound **2a** as 6-methylheptyl sulfate. The $^{13}$C NMR spectrum and DEPT analysis were consistent with the proposed structure (Table 2).

Compound **3a** had the molecular formula $C_8H_{15}SO_4$ as deduced from the $[M]^-$ ion peak at *m*/*z* 207 in the negative ion FABMS. Mass data and the IR absorption at 1210 cm$^{-1}$ attested to the presence of a sulfate function. The $^1$H NMR spectrum of **3a** contained two double triplets at $\delta$ 5.33 and 5.40, respectively, corresponding to two olefinic protons,

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**Table 2.** $^1$H and $^{13}$C NMR Data of Compounds **2a** and **3a** in $CD_3OD$[a]

| | **2a** | | **3a** | |
|---|---|---|---|---|
| position | $^1$H (mult, J) | $^{13}$C | $^1$H (mult, J) | $^{13}$C |
| 1 | 4.02 (t, 6.8) | 68.9 | 4.01 (t, 6.8) | 68.9 |
| 2 | 1.69 (m) | 30.6 | 1.65 (m) | 29.6 |
| 3 | 1.43 (m) | 26.9 | 1.45 (m) | 26.5 |
| 4 | 1.32 (m) | 30.0 | 2.15 (m) | 27.0 |
| 5 | 1.23 (m) | 40.3 | 5.23 (dt, 11, 6.8) | 129.1 |
| 6 | 1.58 (m) | 28.8 | 5.40 (dt, 11, 6.8) | 132.0 |
| 7 | 0.92 (t, 6.5) | 23.0 | 2.08 (m) | 20.9 |
| 8 | 0.92 (t, 6.5) | 23.0 | 0.99 (t, 7) | 14.7 |

[a] Assignments were based on $^1$H−$^1$H COSY and HMQC experiments.

and two triplets, one at $\delta$ 0.99 (3H) due to a methyl group and one at $\delta$ 4.02 (2H) assigned to a sulfated methylene group; a series of well-separated methylene signals in the high-field region of the spectrum ($\delta$ 1.45−2.15) were also present. This good dispersion of proton signals allowed us to easily establish the gross structure of **3a** as 5-octenyl sulfate, from the connectivities observed in the $^1$H−$^1$H COSY spectrum. The Z configuration of the double bond was assigned on the basis of the value of the H-5/H-6 coupling constant (11 Hz) and of the $^{13}$C NMR chemical shifts (Table 1) of the allylic methylenes C-4 and C-7.[4,8]

As hypothesized for a previously isolated sulfated alkene, whose structure displayed a recognizable isoprenoid portion,[6] normonoterpenoid **1** could originate by oxidative cleavage from a higher mevalonate precursor. An analogous process, starting from higher acetate-derived precursors (e.g., fatty acids), could account for the biogenesis of the co-occurring sulfates **2a** and **3a**.

The cytotoxic activity of compounds **1**, **2a**, and **3a** was evaluated at 96 h on WEHI 164 and C6 cell lines in vitro, and the effect on both cell lines is reported in Table 1 as $IC_{50}$ (the concentration that inhibited the cell growth by 50%). These data show that the compounds act selectively on fibrosarcoma rather than glioma cells, which are described in the literature as very resistant also to chemotherapy.[9]

## Experimental Section

**General Experimental Procedures.** FABMS (CsI ions, glycerol matrix) and EIMS (40 eV) were performed on a VG Prospec (FISONS) mass spectrometer. Optical rotation was measured in MeOH on a Perkin-Elmer 192 polarimeter equipped with a sodium lamp ($\lambda$ = 589 nm) and a 10-cm microcell. IR (KBr) spectra were recorded on a Bruker model IFS-48 spectrophotometer. $^1$H (500.14 MHz) and $^{13}$C (125.03 MHz) NMR spectra were determined on a Bruker AMX-500 spectrometer; chemical shifts were referred to the residual solvent signal ($CD_3OD$: $\delta_H$ = 3.34, $\delta_C$ = 49.0; $CDCl_3$: $\delta_H$ = 7.26;). Methyl, methylene, and methine carbons were distinguished by DEPT experiments. Homonuclear $^1$H connectivities were determined using COSY experiments. One-bond heteronuclear $^1$H−$^{13}$C connectivities were determined with the Bax-Subramanian[6] HMQC pulse sequence using a BIRD pulse 0.50 s before each scan in order to suppress the signals originating from protons not directly bound to $^{13}$C (interpulse delay set for $^1J_{CH}$ = 140 Hz). Medium-pressure liquid chromatography (MPLC) was performed using a Büchi 861 apparatus with an $RP_{18}$ Si gel (particle size 40−63 $\mu$m) packed column. High performance liquid chromatography (HPLC) separations were achieved on a Waters 501 apparatus equipped with an RI detector and with an $RP_{18}$ LiChrospher (250 × 4 mm) column.

**Animal Material.** *Halocynthia papillosa* was collected in the Corigliano Gulf (Ionian Sea, southern Italy) at a depth of 40 m. A voucher specimen is deposited at the Dipartimento di Chimica delle Sostanze Naturali, Napoli, Italy.

**Extraction and Isolation of Compounds 1−3.** The tunic was removed from the specimens of *H. papillosa*, and then hepatopancreas and gonads were dissected from the bodies. Each tissue (hepatopancreas, 11 g dry wt after extraction; gonads, 4 g; bodies, 16 g; tunic, 170 g) was homogenized and extracted at room temperature with MeOH. The solvent was removed, and the concentrated aqueous residues were partitioned between EtOAc and $H_2O$ and, subsequently, between *n*-BuOH and $H_2O$. The *n*-BuOH extracts were fractionated by $RP_{18}$ MPLC eluting with a linear gradient of MeOH in $H_2O$ (0 → 100%). In each case, bioactive fractions, eluted with $H_2O$/MeOH (7:3), were further chromatographed by HPLC using a LUNA 3 $\mu$m $C_{18}$ column (4.60 × 150 mm) with MeOH/$H_2O$ (1:1) as the eluent to give compounds **1** (100 mg), **2a** (50 mg), and **3a** (5 mg) in the pure state.

**6-Methylheptyl sulfate (2a)**: colorless amorphous solid; IR (KBr) 1242, 1210, and 1110 cm$^{-1}$; $^1$H and $^{13}$C NMR data, see Table 2; negative FABMS *m*/*z* 209.

**(*E*)-5-Octenyl sulfate (3a)**: colorless amorphous solid; IR (KBr) 1240, 1210, and 1110 cm$^{-1}$; $^1$H and $^{13}$C NMR data, see Table 2; negative FABMS *m*/*z* 207.

**Solvolysis of 2a and 3a.** Compound **2a** (10 mg) was dissolved in a dioxane−pyridine mixture, 1:1 (5 mL) and heated at 130 °C (3 h). Water (10 mL) was added to the cooled solution before extraction with $CHCl_3$ (3 × 5 mL). The organic phase was evaporated in vacuo to give the alcohol **2b** (5 mg): $^1$H NMR ($CDCl_3$) $\delta$ 3.63 (2H, t, $J$ = 6.8 Hz, H-1); EIMS *m*/*z* 130, 112. An identical procedure was used for the solvolysis of **3a** (2 mg), which gave the alcohol **3b** (0.6 mg): $^1$H NMR ($CDCl_3$), $\delta$ 3.64 (2H, t, $J$ = 6 Hz, H-1); EIMS *m*/*z* 128, 110.

**Cell Cultures and Biological Activity.** WEHI 164 cells (murine fibrosarcoma cell line) were maintained in adhesion on Petri dishes with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v:v) heat-inactivated fetal bovine serum (FBS), 25 mM HEPES, penicillin (100 U/mL), and streptomycin (100 $\mu$g/mL). C6 (rat glioma cell line) cells were maintained in adhesion on Petri dishes with DMEM medium supplemented with 5% FBS, 25 mM HEPES, glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 $\mu$g/mL).

All reagents for cell culture were from Biowhittaker. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-phenyl-2H-tetrazolium bromide] and 6-mercaptopurine (6-MP) were from Sigma.

WEHI 164 and C6 ($3.5 \times 10^3$ cells) were plated on 96-well microliter plates and allowed to adhere at 37 °C in 5% $CO_2$/95% air for 2 h. Thereafter, 50 $\mu$L of 1:4 (v/v) serial dilution of compounds **1**, **2a**, and **3a** (2.5 mg/mL) were added and the cells incubated for 96 h. In some experiments 6-MP was added as standard compound for antiproliferative activity. Cell viability was assessed through an MTT conversion assay as previously described.[6] The viability of each cell line in response to treatment with compounds **1**, **2a**, **3a**, and 6-MP was calculated as % dead cells = 100 − (OD treated/OD control) × 100. Table 1 shows the results expressed as $IC_{50}$ (the concentration that inhibited the cell growth by 50%). Statistical analysis was made by paired two-tailed Student's *t*-test: The level of statistically significant difference was defined as $p < 0.01$.

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