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A New Antiproliferative Sulfated alkene From The Mediterranean Tunicate *Microcosmus vulgaris*

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Abstract: A new antiproliferative C11 alkyl sulfate, (3Z)-4,8-dimethylnon-3-en-1- sodium (or potassium) sulfate (**1a**) has been isolated from the Mediterranean ascidian *Microcosmus vulgaris*. Its structure determination was performed by spectroscopic and chemical methods.
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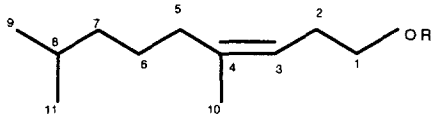
Sulfated metabolites, although relatively unusual, occur in marine organisms. The sulfate group is a common feature of echinoderm metabolites, such as sterol glycosides (saponins) and sterol sulfates[1]. It has also been encountered among fish metabolites in the form of sulfated sterols[2] and from marine sponges, the most frequently found sulfated esters, being steroidal and phenolic sulfates[3].

Only a few examples of sulfated alkanes/alkenes have been reported from marine sources. Findlay and coworkers have reported the isolation of two sulfated hydrocarbons from a starfish[4] and from a sea cucumber[5]. Recently, simple sulfate compounds are increasingly turning out to be common metabolites of marine Ascidians. The first report of a sulfated hydrocarbon from a tunicate was the finding of sodium (or potassium) 2,6-dimethylheptyl sulfate from *Policitor adriaticus*[6] in 1994; almost in the same time, four new antibacterial and antifungal alkane and alkenes sulfate have been isolated from the hepatopancreas of the Japanese ascidian *Halocynthia roretzi*[7].

In 1996 we reported the isolation of two antiproliferative alkyl sulfates from the ascidian *Ascidia mentula*[8] collected along the southern Italy coasts. In our ongoing search for bioactive metabolites of Mediterranean Ascidians, we have now encountered a new sulfated alkene (**1a**) in the Tunicate *Microcosmus vulgaris* Heller (Fam. Pyuridae), whose isolation and structure determination are here described. The antiproliferative activity of compound **1a** on GM7373 (bovine endothelial), J774 (murine monocyte/macrophage), WEHI 164 (murine fibrosarcoma) and P388 (murine leukemia) tumor cell lines is also reported.

Compound **1a** is a quite simple molecule which arouses the problem of its biogenetic origin. Its carbon skeleton, referable to a monoterpene possessing a terminal extra carbon atom, led to the hypothesis that it could originate from a higher terpenoid by oxidative cleavage.



1a R = $SO_3^- Na^+ (K^+)$

1b R = H

The tunic was removed from several specimens of *Microcosmus vulgaris* and the whole bodies were extracted, after homogenization, with MeOH. The EtOAc extract from the MeOH soluble material was subjected to a bioassay-guided fractionation by MPLC on a Silica gel column. The portion eluted with MeOH, which showed antiproliferative activity on WHEI 164 cell line, was further chromatographed on a Silica gel column using a linear gradient of MeOH (from 10 to 100%) in $CHCl_3$ as the eluent to obtain the pure sulfate **1a**.

The negative ion FAB mass spectrum of compound **1a** exhibited a quasi-molecular anion peak at *m/z* 249 corresponding to $C_{11}H_{21}SO_4$, while, the positive ion FAB mass spectrum in glycerol matrix exhibited two prominent ions at *m/z* 365 and 381 corresponding to $[M(Na)+G+H]^+$ and $[M(K)+G+H]^+$. The presence of a sulfate group was inferred by IR spectrum of compound **1a**, which displayed absorption bands at ν_{max} 1210 and 1110 cm^{-1} and substantiated by solvolysis of **1a**, carried out in a dioxane-pyridine mixture at 130°C (3h), which gave the alcohol **1b** (EIMS *m/z* 170, appropriate for the molecular formula $C_{11}H_{22}O$).

The ^{1}H-NMR spectrum of **1a** showed methyl signals at δ 1.72 (3H, s), typical of a CH_3 linked to an sp^2 carbon atom, and 0.91 (6H, d), this latter signal being part of an isopropyl group, as deduced from the ^{1}H-^{1}H COSY spectrum. Additional features of the ^{1}H-NMR spectrum were a series of well separated signals in the high field region of the spectrum and two triplet signals, one at δ 5.19 (1H), due to an olefinic proton, and one at δ 3.99 (2H), attributable to the protons of a methylene group bearing the sulfate moiety. The observed upfield shift (δ 3.65) for these latter protons in the ^{1}H-NMR spectrum of the alcohol **1b** strongly corroborated this hypothesis.

The ^{1}H-^{1}H COSY spectrum of **1a** provided useful information enough to delineate the whole spin sequence H1-H11 and allowed us to build up the gross structure of **1a**, that was 4,8-dimethylnon-3-en-1- sulfate (see Table 1).

The ^{13}C NMR spectrum and DEPT analysis of **1a** were consistent with the proposed structure. Particularly, the olefinic region of the spectrum showed only two signals at δ 139.4 (s) and 121.0 (d), thus confirming the presence of a trisubstituted double bond; furthermore, the chemical shift of a solitary methylene signal at δ 69.0 gave a further support to the presence of a Na^+ (K^+) sulfate functionality linked to a methylene group. All the resonances of the ^{13}C-NMR spectrum of **1a** were assigned on the basis of a reverse heteronuclear correlation experiment (HMQC) and they are reported in Table 1.

Table 1. NMR Data of Compound **1a** (CD_3OD)

Pos.	δC.	δH (mult., *J* in Hz)
1	69.0	3.99 (t, 7)
2	29.1	2.40 (bq, 7)
3	121.0	5.19 (bt, 7)
4	139.4	
5	32.9	2.06 (t, 7.5)
6	26.8	1.43 (m)
7	39.9	1.21 (m)
8	29.0	1.57 (m)
9	23.0	0.91 (d, 6.5)
10	23.6	1.72 (bs)
11	23.0	0.91 (d, 6.5)

The geometry of the double bond was deduced to be Z from the ROESY spectrum of **1a**, which displayed correlation peaks between the Me-10 (δ 1.72) and H-3 (δ 5.19), and between H-5 (δ 2.06) and H-2 (δ 2.40).

Compound **1a** was tested for its antiproliferative activity on GM7373 (bovine endothelial), J774 (murine monocyte/macrophage), WEHI 164 (murine fibrosarcoma) and P388 (murine leukemia) cell line *in vitro*. It inhibited the growth of all cell lines evaluated at 96 h and this effect is reported in Figure 1 as IC_{50} (μg/ml). These preliminary data show that compound **1a** is more active on endothelial GM7373 cells and fibrosarcoma WHEI cells than on both J774 and P388 cells, monocyte-macrophages cell-type.

EXPERIMENTAL SECTION

Instrumentation

IR (KBr) spectrum was recorded on a Bruker model IFS-48 spectrophotometer. Low and high resolution FAB mass spectra (CsI ions, glycerol matrix) and EI mass spectrum (40eV) were performed on a VG Prospec (FISONS) mass spectrometer. ^1H (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: δ_H = 3.34, δ_C = 49.0). Methyl, methylene, and methine carbons were distinguished by DEPT experiments. Homonuclear ^1H connectivities were determined by ^1H-^1H COSY experiment. One bond heteronuclear ^1H-^{13}C connectivities were determined with a 2D HMQC[9] experiment, interpulse delays were adjusted for an average $^1J_{CH}$ of 135 Hz. Nuclear Overhauser effect (nOe) measurements were performed by 2D ROESY experiment. Medium-pressure liquid chromatography (MPLC) was performed using a Büchi 861 apparatus with an SiO_2 (70-230 and 230-400 mesh) column.

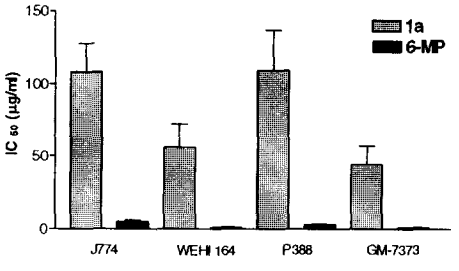


Fig.1.*In vitro* antiproliferative activity (IC_{50}) of compound **1a**. Results are expressed as mean ± S.E.M. of three separate experiments in triplicate.

Extraction and isolation of compound 1a.

Specimens of *Microcosmus vulgaris* were collected in the Bay of Naples (Procida, Punta Pizzaco) at a depth of 40 m (a voucher specimen is deposited at the Dipartimento di Chimica delle Sostanze Naturali, Napoli, Italy). The tunic was removed from the animals and the whole bodies (40 g dry weight after extraction) were extracted at room temperature with MeOH (4 x 300 ml). The concentrated aqueous combined residue was partitioned between EtOAc and H_2O. Separation of the EtOAc soluble material (1.5 g) was achieved by gradient silica gel (230-400 mesh) MPLC (hexane→EtOAc→MeOH). A bioactive fraction (400 mg), eluted with MeOH, was rechromatograped on a silica gel column (70-230 mesh) with a linear gradient of MeOH in $CHCl_3$ (10%→100%) as the eluent. Fractions eluted with MeOH/$CHCl_3$ 1:1 contained pure compound **1a** (120 mg).

Compound Ia. Colorless amorphous solid; IR (KBr) ν_{max} 1110, 1210, 1240 cm^{-1}; ^1H and ^{13}C NMR (CD_3OD): see Table 1. Negative FAB-MS *m/z* 249 $[M - Na(K)]^-$; positive FAB-MS *m/z* 381 (100) $[M(K) + G + H]^+$ and 365 (80) $[M(Na) + G + H]^+$; HRFABMS *m/z* 249.1162 $[M-Na(K)]^-$, $C_{11}H_{21}SO_4$ requires 249.1155.

Solvolysis of 1a.

Compound **1a** (10mg) was dissolved in a dioxane-pyridine mixture 1:1 (5ml) and heated at 130°C (3h). H_2O (10 ml) was added to the cooled solution before extraction with $CHCl_3$ (3 x 5ml). The organic phase was evaporated *in vacuo* to give the alchol **1b** (5mg): EIMS *m/z* 170, 152. ^1H NMR (CD_3OD): δ 3.65 (2H, t, *J*=7Hz, H-1), 2.28 (2H, q, *J*=7Hz, H-2), 4.95 (1H, t, *J*=7Hz, H-3), 1.72 (3H, s, H-10) and 0.91 (6H, d, *J*= 6.5 Hz, H-9 and H-11).

Bioassays.

Cells. 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% heat-inactivated fetal bovine serum (FBS), 25 mM HEPES, penicillin (100 U/ml) and streptomycin (100 μg/ml). J774 cells (murine monocyte/macrophage cell line) were grown in suspension culture, in Techne stirrer bottles, spun at 25 rpm and incubated at 37°C in DMEM medium supplemented with 10% FBS, 25 mM Hepes, glutamine (2mM), penicillin (100U/ml) and streptomycin (100 μg/m). GM7373 cells (bovine endothelial cell line) were grown in adhesion on Petri dishes with Minimum Essential Medium Eagle (MEM) supplemented with 10% FBS, 25 mM HEPES, penicillin (100 U/ml) and streptomycin (100 μg/ml). P388 cells (murine

leukemia cell line) was grown in adhesion on Petri dishes with L-15 (Leibovitz) medium supplemented with 10% FBS, 25 mM HEPES, penicillin (100 U/ml) and streptomycin (100 µg/ml).

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

Antiproliferative activity. WEHI 164, J774, GM7373, P 388 ($3.5x10^3$ cells) were plated on 96-well plates in 50 µl and allowed to adhere at 37 °C in 5% CO_2/95% air for 2 h. Thereafter 50 µl of 1:4 v/v serial dilution of the test compound **1a** was added and then the cells incubated for 96 h. In some experiments 6-mercaptopurine (6-MP) was added as standard compound for antiproliferative activity. The cells viability was assessed through an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-phenyl-2H-tetrazolium bromide] conversion assay[10]. Briefly, after 96 h 25 µl of MTT (5 mg/ml) was added and the cells were incubated for additional 3 h. Following this time the cells were lysed and the dark blue crystals solubilized with 100 µl of a solution containing 50% (v:v) N,N-dimethylformamide, 20% (w:v) SDS with an adjusted pH of 4.5[11]. The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCC/340) equipped with a 620 nm filter. The viability of each cell line in response to treatment with compounds **1a** and 6-MP was calculated as: % dead cells = 100 - (OD treated/OD control) x 100. Fig.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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