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Novel Antiproliferative Alkyl Sulfates From The Mediterranean Tunicate *Ascidia mentula*

Anna Aiello, Ernesto Fattorusso*, Marialuisa Menna

Dipartimento di Chimica delle Sostanze Naturali, Università degli Studi di Napoli Federico II, Via D. Montesano 49, I-80131 Napoli, Italy

Rosa Carnuccio, Fulvio D'Acquisto

Dipartimento di Farmacologia Sperimentale, Università degli Studi di Napoli Federico II, Via D. Montesano 49, I-80131 Napoli, Italy

Abstract: A bioassay guided approach was used to isolate two antiproliferative sulfated metabolites, the 3,7,11,15-tetramethyl-hexadecan-1,19-sodium disulfate (**1a**) and the heneicosane-1,21-sodium disulfate (**2a**) from the Mediterranean tunicate *Ascidia mentula*. Their structures were determined by an extensive spectroscopic analysis and chemical methods. © 1997 Elsevier Science Ltd.

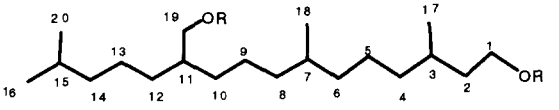
During the last 20 years ascidians, sessile filter feeders invertebrates, have increasingly become the target of natural products research. The interest in their chemistry has been stimulated by the peculiarity of their secondary metabolites, having promising biological activity, which have attracted the attention of both chemists and pharmacologists[1,2]. In this regard it seems quite significant that didemnin B, an ascidian metabolite, is the first marine natural product evaluated in clinical trials as an anticancer agent[3]. The increased understanding of ascidians secondary metabolism has also provided a solid foundation for the investigation of several interesting biological aspects of their chemistry, including the role of the microalgal symbiosis and that of secondary metabolites in the chemical defense of the producing organism.

Surprisingly, the chemistry of ascidians species present in the Mediterranean sea has until now subjected to a very limited number of investigations and this appears to be in striking contrast to the extensive chemical analysis carried out in the last thirty years on other Mediterranean invertebrates, like Porifera and Echinoderms.

As a part of our search to isolate and characterize biologically active substances of marine origin we have focused our interest on the natural constituents of ascidians from the Mediterranean coastal region[4]. In the

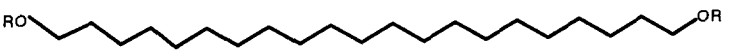
frame of this program we have investigated the chemistry and the pharmacological properties of the solitary tunicate *Ascidia mentula* Müller (Fam. Ascididae) collected in the Ionian Sea, in Corigliano Gulf, Southern Italy. This investigation resulted in the isolation of two novel alkyl sulfates **1a** and **2a** whose isolation and structure characterization are described in this paper. The antiproliferative activity of compounds **1a** and **2a**, estimated on IGR-1 (human melanoma), J774 (murine monocyte/macrophage), WEHI 164 (murine fibrosarcoma) and P388 (murine leukemia) cell line, is also reported.

Specimens of *A. mentula* were extracted, after homogenization, with MeOH. Combined extracts were concentrated and then partitioned between *n*-BuOH and water. The *n*-BuOH soluble material in a preliminary screening exhibited an antiproliferative activity on WHEI 164 cell line. Bioactivity-directed fractionation of the *n*-BuOH extract was performed by gel filtration on Sephadex LH-20 eluting with MeOH in $CHCl_3$ (from 10% to 100%). The bioactive fraction, eluted with $CHCl_3$/MeOH 2:8 was a mixture of highly polar compounds; HPLC separation on an RP18 column eluting isocratically with MeOH/H_2O (6:4) provided the resolution of the mixture in the individual components **1a** ($[\alpha]_D$ +7) and **2a** (optically inactive).



1a R = $SO_3^-Na^+$

1b R = H



2a R = $SO_3^-Na^+$

2b R = H

Negative ion FAB mass spectrum of compound **1a** exhibited a prominent quasi-molecular ion peak at *m/z* 495 corresponding to $C_{20}H_{40}S_2O_8Na$. An intense fragment at *m/z* 393 was interpreted as resulting from the loss of SO_3 Na (+H) from *m/z* 495. The presence of Na sulfate groups was also substantiated by IR spectrum with bands at 1240, 1210 and 1110 cm^{-1}. The solvolysis of **1a**, carried out in a dioxane-pyridine mixture at 130°C (3h), which gave the corresponding diol **1b** (EIMS *m/z* 314, appropriate for the molecular formula $C_{20}H_{42}O_2$), confirmed this hypothesis, also defining that the molecule contains two sulfate groups.

The ^{13}C NMR spectrum and DEPT analysis of **1a** showed three methyls, eight methylenes and four methines in the high field region, and two methylene signals at δ 71.6 and 67.5. These latter chemical shifts, together with the presence in the ^1H NMR spectrum of a multiplet at δ 4.07 (2H) and a doublet at δ 3.94 (2H), suggested that the two sulfate functionalities were both linked to methylene groups. This was further confirmed by ^1H NMR spectrum of **1b**, clearly indicating an upfield shift for the signals relative to the above methylene protons (δ 3.68 and 3.54).

Going on with the analysis of the ^1H NMR spectrum, its general appearance suggested that **1a** possesses a linear saturated diterpene skeleton. It contained three doublets at δ 0.90 (3H, J=6.5Hz), 0.91 (6H, J=6.5Hz) and 0.96 (3H, J=6.5Hz) with the second doublet being part of an isopropyl group as deduced from the ^1H- ^1H

COSY spectrum of **1a**. Further connectivity patterns (H1-H4, H18-H6/H8, H19-H10/H12, H16,20-H13) were deduced from ^{1}H-^{1}H COSY spectrum, but the severe overlapping of some methylene signals prevented the delineation of the whole spin-sequence H1-H20. Fortunately, the ^{1}H- ^{13}C 2D NMR experiments (HMQC, HMBC) allowed us to connect almost all the NMR resonances leading to the structure **1a** especially on the basis of some diagnostic HMBC correlations (Table 1). In particular, the HMBC correlation of 2H-14 (δ 1.22) with the carbon signal at δ 32.3, which in turn was long range coupled with the methylene group at δ 3.94, proved that the oxymethylene sulfate group was linked at C-11. This permitted also to assign the resonance at δ 32.3 unambiguously at C-12, whose protons resonated in the complex region at δ 1.33-1.40.

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

Pos.	δC.	δH, mult., *J* in Hz	HMBC (H to C)
1	67.5	4.07 (m)	2, 3
2	37.6	1.48[b] (Ha)	17, 3, 4, 1
3	30.8	1.65[b]	5, 4
4	38.6	1.35[b]	17, 3, 5
5	25.3	1.33-1.40[b]	
6	38.7[a]	1.32[b]	18
7	34.1	1.45[b]	18, 6, 8
8	38.6[a]	1.14 (m, Ha)	18, 9
		1.35[b] (Hb)	18
9	25.6	1.33-1.40[b]	
10	32.5	1.31[b]	
11	39.2	1.67[b]	10, 12
12	32.3	1.35[b]	
13	25.6	1.33-1.40[b]	
14	40.5	1.22 (m)	12, 13, 15, 16, 20
15	29.1	1.58 (m)	13, 14, 16, 20
16	23.1	0.91 (d, 6.5)	14, 15
17	19.7	0.96 (d, 6.5)	2, 3, 4
18	20.0	0.90 (d, 6.5)	6, 7, 8
19	71.6	3.94 (d, 5)	10, 11, 12
20	23.1	0.91 (d, 6.5)	14, 15

a. Values with the same superscript may be interchanged

b. Signals overlapped by other resonances.

Other informative long range couplings were those observed between H-3 (δ 1.65) and the signal at δ 25.3, which permitted to assign this resonance at C-5 (whose protons were in the 1.33-1.40 complex region) and between the signal at δ 1.14 (H-8) and the ^{13}C resonances at δ 20.0 (C-18) and δ 25.6 (C-9).

These latter correlations allowed to define the sequence C18-C9 and fully established the structure of **1a**. As confirm of the proposed structure, the observed ^{13}C chemical shifts for **1a** are in excellent agreement with those calculated using the additivity relationship and the shift parameters reported for branched chain alkanes[5].

The FAB spectrum (negative ion mode) of compound **2a** displayed a quasi-molecular ion at *m/z* 509 $[M - Na]^-$ ($C_{21}H_{42}O_8S_2Na$) and an additional fragment ion at *m/z* 407 $[509 - NaSO_3 + H]^-$ which attested to the presence also in **2a** of two $NaOSO_3$ moieties. This was further substantiated by IR spectrum which showed bands at 1240, 1215 and 1110 cm^{-1}.

The quite simple structure of **2a** was deduced by its ^{13}C NMR spectrum and DEPT experiment. They evidenced the methylene nature of all the carbon atoms which resonated in the high field region of the spectrum apart from a signal, due to two carbon atoms, at δ 69.1. This clearly indicated that structure **2a** is a linear alkyl chain in which the two terminal carbon atoms are functionalized. These functions, as deduced from mass data, must be two sulfate groups. This hypothesis is in a good agreement with the ^{1}H NMR spectrum, whose distinguishing features comprise a triplet (4H, J=6 Hz) at δ 4.02, attributable to the equivalent protons at C1 and C21, a multiplet (4H) at δ 1.69, assigned to the equivalent 2H-2 and 2H-20 protons through an ^{1}H-^{1}H COSY experiment, and a large methylene signal at δ 1.35-1.42.

Further support for the proposed structure **2a** was provided by its solvolysis in the same system employed for **1a** which afforded the heneicosane-1, 21-diol **2b**; it displayed the predictable upfield shift (δ 3.57) for the 1 and 21 protons and exhibited a molecular ion at *m/z* 328 in the EIMS. Thus, compound **2a** was formulated as the heneicosane-1,21-disulfate.

Compounds **1a** and **2a** were tested for their antiproliferative activity on IGR-1 (human melanoma), J774 (murine monocyte/macrophage), WEHI 164 (murine fibrosarcoma) and P388 (murine leukemia) cell line *in vitro*. Both compounds **1a** and **2a** inhibited the growth of all cell lines evaluated at 96 h. The effects of these compounds are reported in Figure 1 as IC_{50} (μg/ml).

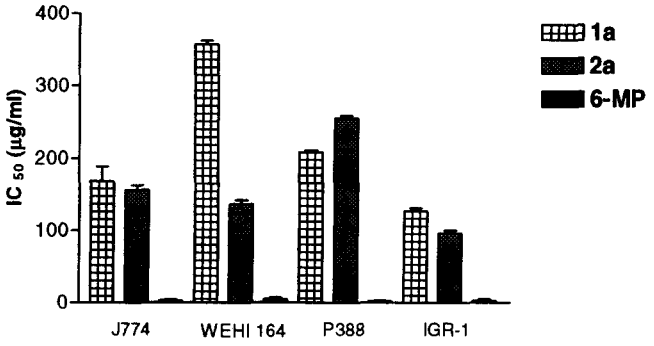


Fig.1. *In vitro* antiproliferative activity (IC_{50}) of compounds **1a** and **2a** on J774 (murine monocyte/macrophage), WEHI 164 (murine fibrosarcoma), P388 (murine leukemia) and IGR-1 (human melanoma), cell line. Results are expressed as mean ± S.E.M. of tree separate experiments in triplicate.

EXPERIMENTAL SECTION

Instrumentation

Optical rotation was measured in MeOH on a Perkin-Elmer 192 polarimeter equipped with a sodium lamp (λ = 589 nm) and a 10 cm microcell. IR (KBr) spectra were recorded on a Bruker model IFS-48 spectrophotometer. Low and high resolution FAB mass spectra (CsI ions, glycerol matrix) and EI mass spectra (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 using COSY experiments. One bond heteronuclear ^1H-^{13}C connectivities were determined with the Bax-Subramanian[6] HMQC pulse sequence using a BIRD pulse 0.50 s before each scan. Two and three bond ^1H-^{13}C connectivities were determined by HMBC experiments optimized for a $^{2,3}J_{CH}$ of 10 Hz. Medium-pressure liquid chromatography (MPLC) was performed using a Büchi 861 apparatus with an SiO_2 (230-400 mesh) column. High performance liquid chromatography (HPLC) separations were achieved on a Waters 501 apparatus equipped with an RI detector and with a RP18 LiChrospher (250 x 4 mm) column.

Extraction and isolation of compounds 1a and 2a.

Specimens of *Ascidia mentula* were collected in Corigliano Gulf (Ionian Sea, Southern Italy) at a depth of 52 m. A voucher specimen is deposited at the Dipartimento di Chimica delle Sostanze Naturali, Napoli, Italy. The frozen animals (90g dry weight after extraction) were homogenized and extracted at room temperature with methanol (4 x 300 ml). The solvent was removed and the combined residues were partitioned between EtOAc and H_2O and, subsequently, between *n*-BuOH and H_2O. Separation of the *n*-

BuOH soluble material (500 mg) was achieved by gel filtration chromatography on a Sephadex LH-20 column eluting with a linear gradient of CH_3OH in $CHCl_3$ (10%→100%). The fraction eluted with $CH_3OH/CHCl_3$ 8:2 was further chromatographed by HPLC using a LiChrosper RP18 column 5 μm (4x250mm) with the mobile phase CH_3OH/H_2O 6:4. to give compounds **1a** (30 mg) and **2a** (15 mg)

Compound 1a. Colorless amorphous solid $[\alpha]_D^{25}$ +7 (c = 0.004, MeOH); IR (Kbr) ν_{max} 1110, 1210, 1240 cm^{-1}; ^1H and ^{13}C NMR (CD_3OD): see Table 1. FAB-MS (negative ion mode) *m/z* 495, 393; HRFABMS *m/z* 495.2063 $[M-Na]^-$, $C_{20}H_{40}S_2O_8Na$ requires 495.2052.

Compound 2a. IR (KBr) ν_{max} 1210, 1245 cm^{-1}; ^1H NMR (CD_3OD): δ 4.02 (4H, t, J= 6 Hz, H-1 and H-21), 1.69 (4H, m, H-2 and H-20), 1.42 (4H, m, H-3 and H-19) 1.32 (large signal, H-5/H-18); ^{13}C NMR (CD_3OD): δ 69.1 (C-1 and C-21), 30.5 (C-2 and C-20), 30.1 (C-4 /C-18) and 26.8 (C-3 and C-19). FAB-MS (negative ion mode) *m/z* 509, 407. HRFABMS *m/z* 509.2220 $[M-Na]^-$, $C_{21}H_{42}S_2O_8Na$ requires 509.2208.

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 diol **1b** (5mg): EIMS *m/z* 314, 296 and 278; ^1H NMR (CD_3OD): δ 3.68 (2H, m, H-1), 1.60 (1H, submerged, H-2a), 1.38 (1H, subm., H-2b), 1.55 (1H, subm., H-3), 1.28 (1H, subm., H-4a), 1.16 (1H, m, H-4b), 1.27 (2H, subm., H-5), 1.39 (2H, subm., H-7), 1.30 (2H, subm., H-10), 1.48 (2H, m, H-11), 1.30 (2H, subm., H-12), 1.53 (1H, m, H-15), 0.87 (6H, d, *J*= 6.5 Hz, H-16 and H-20), 0.89 (3H, d, *J*=6.5 Hz, H-17), 0.84 (3H, d, *J*=6.5 Hz, H-18) and 3.54 (2H, d, *J*=5Hz, H-19); ^{13}C NMR (CD_3OD): δ 65.8 (CH_2), 61.1 (CH_2), 41.7 (CH), 40.9 (CH_2), 40.6 (CH_2), 38.6 (CH_2, 2C), 38.4 (CH_2), 33.9 (CH), 32.4 (CH_2), 32.3 (CH_2), 30.7 (CH), 29.1 (CH), 25.7 (CH_2), 25.3 (CH_2), 25.4 (CH_2), 23.1 (CH_3, 2C), 20.2 (CH_3), 20.1 (CH_3).

Solvolysis of 2a.

Compound **2a** (5 mg) was desulfated using the same procedure reported for **1a** affording the diol derivative **2b** (2 mg): EIMS *m/z* 328, 310 and 292; ^1H NMR (CD_3OD): δ 3.63 (4H, t, J= 6 Hz, H-1 and H-21), ^{13}C NMR (CD_3OD): δ 63.0 (CH_2, C-1, C-21), 33.7 (CH_2, C-2, C-20), 26.9 (CH_2, C-3, C-19), 30.7 (CH_2, C-4/C-18).

Determination of biological activity. .

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). IGR-1 cells (human melanoma 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.

Proliferation and cytotoxicity assay. WEHI 164, J774, IGR-1, P 388 ($1x10^4$ 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 the medium was replaced with 50 µl of fresh medium and 75 µl aliquot of 1.2 v/v serial dilution of each test compound **1a**, **2a** was added and then the cells incubated for 96 h. In some experiments 6-mercaptopurine (6-MP) was added. The cells viability was assessed through an MTT conversion assay[7]. Briefly, 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[8]. 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**, **2a**, 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%).

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