

New Bioactive Sulfated Metabolites from the Mediterranean Tunicate *Sidnyum turbinatum*

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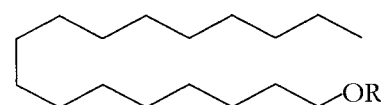
In addition to the known sodium 3,7,11,15-tetramethylhexadeca-1,19-diyl sulfate (**4**), the BuOH extract of the Mediterranean tunicate *Sidnyum turbinatum* was shown to contain four new metabolites: 1-heptadecanyl sulfate (**1**), 1-octadecanyl sulfate (**2**), sodium (2*S*)-2,6,10,14-tetramethylpentadeca-1,18-diyl sulfate (**3**), and 1-hexyl sulfate (**5**). Their structures were determined by spectroscopic and chemical methods. Compounds **1–5** exhibited in vitro antiproliferative activity estimated on the WEHI 164 cell line.

Although nitrogenous compounds constitute the majority of natural products from ascidians, a small but significant number of non-nitrogenous compounds have also been isolated.¹ Among these, terpenoid compounds are quite rare, while, during the last five years, simple sulfated alkanes/alkenes are increasingly turning out to be common metabolites of these organisms.^{2,3} In our continuous search for bioactive metabolites from Mediterranean ascidians, we have investigated the MeOH extract of the tunicate *Sidnyum turbinatum* Savigny (Polyclinidae) collected in the Bay of Naples. A bioactivity-directed fractionation of the extract afforded, in addition to the known diterpene **4** previously isolated from *Ascidia mentula*, four new alkyl sulfates (**1–3** and **5**), whose isolation and structural elucidation are here described. Interestingly, compound **3** is a lower homologue of the diterpene **4**; the co-occurrence of both these compounds confirms our earlier hypothesis that a variety of alkyl sulfates could originate in ascidians from a higher terpenoid precursor by sequential structural degradation. Compounds **1–5** exhibited in vitro antiproliferative activity estimated on the WEHI 164 (murine fibrosarcoma) cell line.

Specimens of *S. turbinatum* were extracted, after homogenization, with MeOH. The *n*-BuOH extract from the MeOH-soluble material, which exhibited in a preliminary screening an antiproliferative activity on the WEHI 164 (murine fibrosarcoma) cell line, was subjected to a bioassay-guided fractionation by MPLC on a RP-18 column. The bioactive fractions were separated and purified by HPLC on LUNA RP-18(2) columns, thus yielding compounds **1–5**.

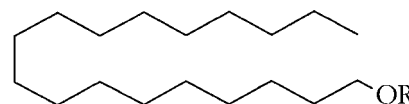
Compound **4** was identified as 3,7,11,15-tetramethylhexadeca-1,19-diyl sulfate by comparison of its spectral data and optical rotation with those reported for the sulfated diterpene previously isolated by us from *A. mentula*.²

Compounds **1** and **2** were two homologues (C₁₇H₃₅O₄S and C₁₈H₃₇O₄S, respectively), as suggested by their HR-FABMS. IR data (ν_{\max} 1110, 1240 cm⁻¹) supported the presence of a sulfate function, further confirmed by treatment of both compounds with pyridine–dioxane (1:1) at 130 °C (3 h), which gave the corresponding alcohols (**1a**



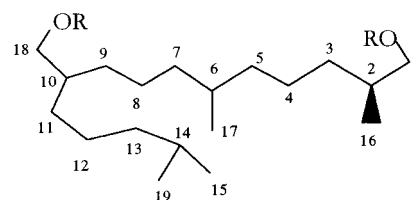
1 R=SO₃⁻Na⁺

1a R=H



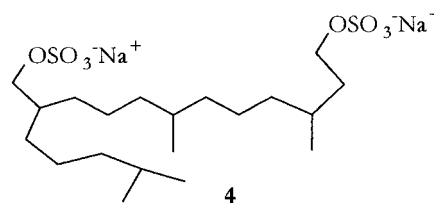
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2a R=H

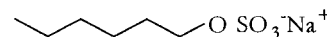


3 R=SO₃⁻Na⁺

3a R=H



4



5

and **2a**, respectively). The ¹H and ¹³C NMR spectra of **1** and **2** were virtually identical. They showed the presence in both molecules of a terminal methyl group [¹³C δ 13.2; ¹H δ 0.98 (3H, t, *J* = 6.7 Hz)] and the methylene nature of the remaining carbon atoms, which resonated in the upfield region of the ¹³C NMR spectra apart from a signal at δ 67.4

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(C-1). This latter chemical shift together with the presence in their ^1H NMR spectra of a triplet at δ 3.99 (2H, t, $J = 6$ Hz) clearly indicated that structures **1** and **2** are linear alkyl chains with a terminal methylene functionalized with a sulfate group.

The negative ion FAB mass spectrum of compound **3** exhibited two prominent ion peaks at m/z 481 and 379, corresponding to $\text{C}_{19}\text{H}_{38}\text{S}_2\text{O}_8\text{Na} [\text{M} - \text{Na}]^-$ and $\text{C}_{19}\text{H}_{39}\text{SO}_5 [\text{M} - \text{SO}_3\text{Na} (+\text{H})]^-$, respectively. Mass and IR (absorptions at ν_{max} 1210 and 1110 cm^{-1}) data suggested the presence of two sulfate groups. This hypothesis was confirmed by solvolysis of **3**, performed in the same system as employed for **1** and **2**, which afforded the envisaged diol **3a** (EIMS: $[\text{M}]^+$ at m/z 300, appropriate for the molecular formula $\text{C}_{19}\text{H}_{40}\text{O}_2$).

The ^1H and ^{13}C NMR spectra of compound **3** displayed a close resemblance to those of **4**, and the observed differences were due to the initial segment of the two compounds (C1–C3/C16 in **3** and C1–C4/C17 in **4**). The sequence C1–C3/C16 in **3** was established from COSY and HMBC data. In particular, the long-range coupling (HMBC) between the AB protons of the ABX system at δ 3.80 (1H, dd, $J = 9.5$ and 6.6 Hz, H-1a) and 3.90 (1H, dd, $J = 9.5$ and 6.6 Hz, H-1b) and the carbon signals at δ 36.8 (C-2) and 19.5 (C-16) permitted the location of the methyl group (Me-16) at C-2. A detailed analysis of the COSY, HMQC, and HMBC data of **3** allowed us to assign all proton and carbon resonances corresponding to the segment C5–C15/C17–19 and established the presence of an identical structural moiety in **3** and **4**.

The substructures C1–C3 and C5–C15/C17–C19 in **3** can be connected only through the methylene C-4, which lacks correlations in the HMBC spectrum and whose proton resonances were assigned from COSY data. The proposed structure **3** was further confirmed by the good consistency between all ^{13}C NMR resonances with those calculated using the additivity relationship rule.⁴ Thus, compound **3** was formulated as the 1-nor-derivative of **4**.

As far as the stereochemistry is concerned, the absolute configuration *S* at C-2 was deduced by the high-field FT NMR application of Mosher's method.⁵ This method is generally used to define the absolute configuration of secondary alcohols. Recently, however, it has been applied to determine the absolute stereochemistry at C-25 of 26-hydroxysteroids evaluating the chemical shift differences of the 26-methylene protons in the spectra of the 26-(+)-MTPA and 26-(–)-MTPA esters.⁶ The apparent structural analogy between the 26-hydroxysteroids side chain and the initial segment of compound **3** allowed us to apply this method to determine the chirality at C-2. The diol **3a**, obtained by solvolysis of **3**, was treated with an excess of (+)- and (–)- α -methoxy- α -(trifluoromethyl)phenylacetyl chloride (MTPA-Cl) to yield 1,18-(+)-MTPA and 1,18-(–)-MTPA diesters, respectively. The methylene proton signals of C-1 in the (–)-MTPA diester appeared as well-separated double doublets at δ 4.08 ($J = 10.3$ and 6.6) and 4.23 ($J = 10.3$ and 6.0), while in the spectrum of (+)-MTPA diester they appeared as a doublet at δ 4.15 ($J = 6.3$). These data were consistent with the *S* configuration at C-2.

IR and negative FABMS data together with ^1H NMR features (see Experimental Section) of the most polar compound, **5**, easily allowed us to identify it as a further linear sulfated alkane, the sulfuric ester of 1-hexanol. The ^1H NMR spectrum exhibited one triplet methyl signal at δ 0.98 ($J = 6.7$ Hz), four methylene signals in the region δ 1.32–1.68, and a 2H triplet, at δ 4.00 ($J = 6$ Hz), attributable to a methylene bearing the sulfate functional-

ity. All resonances of the ^1H NMR spectrum of **5** were assigned on the basis of a COSY experiment.

The antiproliferative activity of compounds **1–5** was estimated on WEHI 164 (murine fibrosarcoma) cells in vitro. All new metabolites isolated from *S. turbinatum* inhibited the growth of this cell line, and these effects, evaluated at 96 h and reported as IC_{50} ($\mu\text{g}/\text{mL}$), were the following: **1** (400 ± 1), **2** (410 ± 1), **3** (230 ± 5), **4** (300 ± 1), **5** (150 ± 2), and **6-MP** (6-mercaptopurine: 1.30 ± 0.02). Results are expressed as mean \pm SEM of three separate experiments in triplicate.

Experimental Section

General Experimental Procedures. 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 (40 eV) 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 are referred to the residual solvent signal (CD_3OD : $\delta_{\text{H}} = 3.34$, $\delta_{\text{C}} = 49.0$). Methyl, methylene, and methine carbons were distinguished by DEPT experiments. Homonuclear ^1H connectivities were determined by a ^1H – ^1H COSY experiment. One-bond heteronuclear ^1H – ^{13}C connectivities were determined with a 2D HMQC experiment, and interpulse delays were adjusted for an average $^1J_{\text{CH}}$ of 135 Hz. Two- and three-bond ^1H – ^{13}C connectivities were determined by HMBC experiments optimized for a $^2,3J_{\text{CH}}$ 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 two Luna RP-18(2) columns (5 μm , 4×250 mm; 3 μm , 4×150 mm).

Extraction and Isolation of Compound 1–5. Specimens of *S. turbinatum* 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 organisms (29 g dry weight after extraction) were extracted at room temperature with MeOH (4×200 mL). The concentrated aqueous combined residue was partitioned between *n*-BuOH and H_2O . Separation of the *n*-BuOH-soluble material (2 g) was achieved by gradient RP-18 Si gel (230–400 mesh) MPLC ($\text{H}_2\text{O} \rightarrow \text{MeOH} \rightarrow \text{CHCl}_3$). Two bioactive fractions A (100 mg) and B (70 mg) were eluted with H_2O –MeOH, 7:3 and 4:6, respectively. The fraction A was rechromatographed by HPLC using a LUNA RP-18(2) 5 μm column (4×250 mm) with the mobile phase H_2O –MeOH, 1:1, to give compounds **1** (2 mg), **2** (2 mg), **3** (7 mg), and **4** (5 mg). The less polar fraction B was purified on a Luna RP-18 (2) 3 μm column (4×150 mm) using 2:3 H_2O –MeOH as eluent, affording pure **5** (1.2 mg).

Compound 1: colorless amorphous solid; IR (KBr) ν_{max} 1110, 1240 cm^{-1} ; ^1H NMR (CD_3OD) δ 3.99 (2H, t, $J = 6$ Hz, H-1), 1.68 (2H, m, H-2), 1.41 (2H, m, H-3) 1.35–1.42 (large signal, H-4/H-15), 1.36 (2H, m, H-16), 0.98 (3H, t, $J = 6.7$ Hz, H-17); ^{13}C NMR (CD_3OD): δ 67.4 (C-1), 30.2 (C-2), 26.6 (C-3), 30.0 (C-4 /C-16), and 13.2 (C-17); negative FABMS m/z 335; HRFABMS m/z 335.2244 $[\text{M} - \text{Na}(\text{K})]^-$, $\text{C}_{17}\text{H}_{35}\text{O}_4\text{S}$ requires 335.2256.

Compound 2: colorless amorphous solid; IR (KBr) ν_{max} 1110, 1210, 1240 cm^{-1} ; ^1H NMR (CD_3OD) δ 3.99 (2H, t, $J = 6$ Hz, H-1), 1.68 (2H, m, H-2), 1.41 (2H, m, H-3) 1.35–1.42 (large signal, H-4/H-16), 1.36 (2H, m, H-17), 0.98 (3H, t, $J = 6.7$ Hz, H-18); ^{13}C NMR (CD_3OD) δ 67.4 (C-1), 30.2 (C-2), 26.6 (C-3), 30.0 (C-4 /C-17), and 13.2 (C-18); negative FAB-MS m/z 349; HRFABMS m/z 349.2400 $[\text{M} - \text{Na}(\text{K})]^-$, $\text{C}_{18}\text{H}_{37}\text{O}_4\text{S}$ requires 349.2413.

Compound 3: colorless amorphous solid, $[\alpha]_{\text{D}}^{25} +5^\circ$ (*c* 0.004, MeOH); IR (KBr) ν_{max} 1110, 1210, 1240 cm^{-1} ; ^1H and ^{13}C NMR (CD_3OD), see Table 1; negative FABMS m/z 481, 379; HRFABMS m/z 481.1894 $[\text{M} - \text{Na}(\text{K})]^-$, $\text{C}_{19}\text{H}_{38}\text{O}_8\text{S}_2$ requires 481.1906.

Table 1. NMR Data of Compound **3** (CD₃OD)

position	δ H (mult., J in Hz)	δ C	HMBC (¹ H to C)
1a	3.80 (dd, 6.6-9.5)	76.4	2, 16
1b	3.90 (dd, 6.6-9.5)		2, 16
2	1.82 (m)	36.8	
3a	1.19 (m)	37.0	
3b	1.46 ^a		
4	1.35-1.40 ^a	27.6	
5	1.32	41.1	17
6	1.45 ^a	36.6	5, 7, 17
7a	1.14 (m)	41.1	
7b	1.34 (m)		17
8	1.35-1.40 ^a	27.6	
9	1.32 ^{a,b}	34.7	7a, 7b, 11
10	1.68	41.7	9, 11
11	1.40 ^{a,b}	34.7	9
12	1.35-1.40 ^a	27.6	
13	1.21 (m)	43.0	11, 12, 14, 15, 19
14	1.57 (m)	31.3	15, 19
15	0.91 (3H, d, 6.3)	25.3	13, 14, 19
16	0.98 (3H, d, 6.3)	19.5	1, 2
17	0.89 (3H, d, 6.3)	22.4	5, 6, 7
18	3.94 (3H, d, 6.0)	73.9	9, 10, 11
19	0.91 (3H, d, 6.3)	25.3	13, 14, 15

^a Signals overlapped by other resonances. ^b Values with the same superscript may be interchanged.

Compound 5: colorless amorphous solid; IR (KBr) ν_{\max} 1110, 1240 cm⁻¹; ¹H NMR δ 4.00 (2H, t, $J = 6$ Hz, H-1), 1.68 (2H, m, H-2), 1.45 (2H, m, H-3), 1.37 (H-4), 1.36 (2H, m, H-5), 0.98 (3H, t, $J = 6.7$ Hz, H-6); ¹³C NMR (CD₃OD) δ 66.0 (C-1), 33.7 (C-2), 26.8 (C-3), 24.5 (C-4), 20.5 (C-5), and 13.5 (C-6); negative FABMS m/z 181; HRFABMS m/z 181.0520 [M - Na(K)]⁻, C₆H₁₃O₅S requires 181.0535.

Solvolysis of Compounds 1–3. Compounds **1** (1.5 mg), **2** (1.5 mg), and **3** (5 mg) were desulfated using the same procedure. Each compound was dissolved in a dioxane–pyridine mixture (1:1, 3 mL) and heated at 130 °C (3 h). H₂O (5 mL) was added to the cooled solution before extraction with CHCl₃ (3 × 4 mL). The organic phase was evaporated in vacuo to give the respective alcohols **1a** (0.8 mg), **2a** (1 mg), and **3a** (4 mg).

Compound 1a: EIMS m/z 256; ¹H NMR (CD₃OD) δ 3.63 (2H, t, $J = 6$ Hz, H-1), 0.94 (3H, t, $J = 6.7$ Hz, H-17).

Compound 2a: EIMS m/z 270; ¹H NMR (CD₃OD) δ 3.63 (2H, t, $J = 6$ Hz, H-1), 0.94 (3H, t, $J = 6.7$ Hz, H-18).

Compound 3a: EIMS m/z 300; ¹H NMR (CDCl₃) δ 3.42 (1H, dd, $J = 6.6$ and 10.3 Hz, H-1a), 3.50 (1H, dd, $J = 5.9$ and 10.3 Hz, H-1b), 1.61 (1H, overlapped, H-2), 1.09 (1H, overlapped, H-3a), 1.37 (1H, overlapped, H-3b), 1.28 (2H, overlapped, H-4), 1.29 (1H, overlapped, H-5a), 1.09 (1H, overlapped, H-5b), 1.39 (1H, m, H-6), 1.31 (2H, overlapped, H-9 or H-11), 1.25 (2H, overlapped, H-9 or H-11), 1.46 (1H, m, H-10), 1.16 (2H, m, H-13), 1.54 (1H, m, H-14), 0.87 (3H, d, $J = 6.6$ Hz, H-15), 0.91 (3H, d, $J = 6.6$ Hz, H-16), 0.84 (3H, d, $J = 6.6$ Hz, H-17), 3.54 (2H, d, $J = 5.15$ Hz, H-18) and 0.87 (3H, d, $J = 6.6$ Hz, H-19); ¹³C NMR (CDCl₃) δ 68.5 (C-1), 35.7 (C-2), 33.2 (C-3), 24.3 (C-4), 37.3 (C-5), 32.5 (C-6), 37.3 (C-7), 24.3 (C-8), 31.2 (C-9), 40.6 (C-10), 31.2 (C-11), 24.3 (C-12), 39.1 (C-13), 27.8 (C-14), 22.6 (C-15), 16.4 (C-16), 19.5 (C-17), 65.7 (C-18), 22.6 (C-19).

MTPA Esters of the Compound 3a. Compound **3a** (2 mg) was esterified with (–)- α -methoxy- α -(trifluoromethyl)phenylacetyl chloride (5 μ L) in dry pyridine (0.2 mL) for 2 h at room temperature to give, after removal of the solvent, the 1,18-di-(–)-MTPA ester: ¹H NMR (CDCl₃) δ 4.08 (1H, dd, $J = 10.3$ and 6.6 Hz, H-1a) and 4.23 (1H, dd, $J = 10.3$ and 6.0 Hz, H-1b), 1.83 (1H, m, H-2), 1.14 (1H, m, H-3a), 1.29 (1H, overlapped, H-3b), 1.35 (1H, overlapped, H-6), 1.68 (1H, m, H-10), 4.19- (1H, dd, $J = 10.3$ and 5.88 Hz, H-18a), 4.27 (1H, dd, $J = 10.3$ and 5.88 Hz, H-18b), 1.12 (2H, m, H-13), 1.50 (1H, m, H-14), 0.90 (3H, d, $J = 6.6$ Hz, H-16), 0.84 (3H, d, $J = 6.6$ Hz, H-15), 0.81 (3H, d, $J = 6.6$ Hz, H-17), 0.84 (3H, d, $J = 6.6$ Hz, H-19).

The 1,18-di-(+)-MTPA ester of **3a** was prepared using (+)- α -methoxy- α -(trifluoromethyl)phenylacetyl chloride (5 μ L) in an identical manner. ¹H NMR (CDCl₃) values were identical with those reported for the (–)-MTPA diester except for the signal of the C-1 protons: δ 4.15 (2H, d, $J = 6.3$ Hz, H-1).

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 μ 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 were from Sigma.

WEHI 164 (3.5 × 10³) cells were plated on 96-well plates in 50 μ L and allowed to adhere at 37 °C in 5% CO₂/95% air for 2 h. Thereafter, 50 μ L of a 1:4 v/v serial dilution of the test compounds **1–5** were added, and then the cells were 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 conversion assay as previously described.³ The viability of each cell line in response to treatment with compounds **1–5** and 6-MP was calculated as % dead cells = 100 – (OD treated/OD control) × 100. The results are expressed as IC₅₀ (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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References and Notes

- Davidson, B. S. *Chem. Rev.* **1993**, *93*, 1771–1791.
- Aiello, A.; Fattorusso, E.; Menna, M.; Carnuccio, R.; D'Acquisto, F. *Tetrahedron* **1997**, *53*, 5877–5882.
- Aiello, A.; Fattorusso, E.; Menna, M.; Carnuccio, R.; Iuvone, T. *Tetrahedron* **1997**, *53*, 11489–11492.
- Breitmaier, E.; Woelter, W. In *Carbon-13 NMR Spectroscopy*, 3rd ed.; VCH: Weinheim, 1987.
- Ohtani, I.; Kusumi, T.; Kasham, Y.; Kakisawa, H. *J. Am. Chem. Soc.* **1991**, *113*, 4092–4096.
- De Riccardis, F.; Minale, L.; Riccio, R.; Giovannitti, B.; Iorizzi, M.; Debitus, C. *Gazz. Chim. Ital.* **1993**, *123*, 79–86.

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