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Topsentinols, 24-Isopropyl steroids from the marine sponge *Topsentia* sp.

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Abstract

Three isopropyl steroids, topsentinols K, L, and K trisulfate (**1–3**) were isolated from an undescribed species of *Topsentia* (Demospongiae: Halichondrida: Halichondriidae). The structures of the new compounds were determined by extensive 1D and 2D NMR experiments and mass spectrometry measurements. Topsentinol K trisulfate (**3**) inhibited the aspartic protease BACE1 although in a detergent-dependent manner suggestive of non-specific aggregation.

Graphical Abstract

Marine sponges are a rich source of polyoxygenated and polysulfated steroids with unusual side chains.¹ According to Djerassi and coworkers, the structural diversity inherent to steroids produced by sponges may actually be greater than that found in any other organism in the entire animal kingdom.² Despite this impressive structural diversity, steroids with an additional isopropyl group appended at C-24 are relatively rare. The first such compounds were reported in 1979 from marine sponges belonging to the genera *Pseudaxinyssa* and *Verongia*.^{3,4} Almost 20 years later, Ishibashi *et al.* described topsentinols A–J and Umeyama *et al.* reported 24-isopropylcholesterol derivatives from marine sponges of the genera

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Supporting Information Available: Copies of the ¹H, ¹³C and 2D spectroscopic data for **1** and **2**, NMR data in pyridine for **1**, the ¹H and the ¹³C NMR spectra of **3** and a photo of the organism are available free of charge via the Internet at <http://pubs.acs.org>.

*Topsentia*⁵ and *Epipolasis*,⁶ respectively. During our search for biologically active marine natural products for the treatment of neurological disorders and cancers, an extract derived from an undescribed species of *Topsentia* (Demospongiae: Halichondrida: Halichondriidae) was identified in our Alzheimer's screen as active. Bioassay-guided fractionation of this extract has now led to the isolation of three new 24-isopropyl steroids along with two known compounds. In this paper, we describe that research and the biological activity of this series of rare C-24 isopropyl derivatives.

The sponge sample was exhaustively extracted with MeOH. The combined extract was then partitioned into EtOAc-, *n*-BuOH-, and H₂O-soluble portions. The *n*-BuOH-soluble portion displayed potent activity in our primary screen, a BACE1 enzyme-fragment complementation assay. This fraction was separated by chromatography on Sephadex LH-20 and Si columns. Final purification required several rounds of reversed-phase HPLC before topsentinols K, L (**1**, **2**), K trisulfate (**3**) and polasterol B (**4**)⁷ were obtained in pure form. The EtOAc-soluble material was separated by repeated reversed-phase HPLC to afford 22-dehydro-24-isopropylcholesterol (**5**).⁴

Compound **1** was isolated as a white powder that provided a molecular formula of C₃₀H₅₂O₃. This formula was deduced after analysis of the HREIMS ion at *m/z* 460.3926 ([M]⁺, 2.1 ppm) and the ¹³C NMR spectroscopic data. All three of the oxygens present were in the form of alcohols as indicated by the strong IR absorptions at 3341 cm⁻¹ and the presence of three oxygenated sp³ carbons (δ_{C-2} 71.6, δ_{C-3} 70.1, and δ_{C-6} 70.0). Initial inspection of the ¹H and ¹³C NMR data (Tables 1 and 2) indicated that **1** contained two methyl singlets (δ_{H-18} 0.71, δ_{H-19} 0.98), five methyl doublets (δ_{H-26} 0.80, δ_{H-24(2)} 0.84, δ_{H-24(3)} 0.89, δ_{H-27} 0.91, δ_{H-21} 0.98), and three oxygenated methines (δ_{H-6} 3.35, δ_{H-2} 3.79, δ_{H-3} 3.81). These data also indicated that topsentinol K contained two quaternary carbons, 14 methines, and 7 methylenes.

The spectroscopic data for **1** were similar to those of polasterol B (**4**), a 24-isopropylcholesterol steroid previously isolated from a marine sponge (*Epipolasis* sp.).⁶ The major differences between the NMR spectra of these two compounds were that the H₂-24(2) resonances observed in **4** for the terminal olefin (δ_H 4.86, 4.80) were replaced by a H₃-24(2) methyl group in **1**. These changes produced corresponding upfield shifts of both the C-24(3) and C-24(1) resonances that were observed in the ¹³C NMR spectrum of **1** and allowed the structure to be assigned as depicted.

The relative configuration of **1** was deduced through coupling constant and NOE analyses. The *Z* geometry of the acyclic olefin in **1** was proven by the magnitude of the three-bond proton-proton coupling constant for H-22 ($^3J_{\text{H-22,H-23}} = 10.8$ Hz). A typical sterol configuration was suggested by the series of NOE cross-peaks (Figure 1) observed from H-8, -15, and -20 to the axial methyl H₃-18, and from H-4 β , -8, and -11 β to H₃-19. A β -configuration for H-6 in the B ring was assigned on the basis of the observed NOE correlation between H₃-19/H-6 and the proton-proton couplings (in pyridine-*d*₅) observed at this stereogenic center ($^3J_{\text{H,H}} = 10.2, 10.2, 4.3$ Hz). Finally, the 17 β -orientation of the side chain was assigned on the basis of ROESY cross-peaks between H-12 β /H₃-21 and H₃-18/H-20. Comparison of the NMR spectra of this compound in pyridine-*d*₅ with the data for **4** supported the relative stereochemical assignment deduced above and assigned the configuration of C-20 as shown.

The empirical formula of compound **2** was assigned as C₃₀H₅₂O₃ on the basis of the HREIMS ion at *m/z* 460.3920 ([M]⁺, 0.8 ppm) and analysis of the ¹³C NMR spectroscopic data. As **2** was an isomer of **1**, the spectroscopic data were quite similar. Comparison of the ¹H and ¹³C NMR spectra of **2** (Tables 1 and 2) with those of **1** indicated that the steroidal cores were the same. Analysis of the NMR data indicated a change in the configuration of the C-22/C-23 double bond from a *Z* to an *E* geometry in **2**. This conclusion was supported by the larger magnitude of the proton-proton coupling observed for the vinyl protons ($^3J_{\text{H-22,H-23}} = 15.3$ Hz).

Topsentinol K trisulfate (**3**) provided a molecular formula of C₃₀H₄₉O₁₂S₃Na₃, as deduced from the negative HRESIMS ion at *m/z* 743.2181 ([M-Na]⁻, -0.1 ppm). The presence of the three sulfur atoms in the molecular formula for **3** and the IR vibrations at 1206 and 1250 cm⁻¹ strongly suggested that **3** possessed sulfate groups. The NMR spectra of **1** and **3** were very similar, with the significant differences localized to C-2, -3 and -6. The chemical shifts observed for the H-2, -3 and -6 resonances were shifted downfield by approximately 1 ppm (1 $\delta_{\text{H-2}}$ 3.79 vs. **3** $\delta_{\text{H-2}}$ 4.79; **1** $\delta_{\text{H-3}}$ 3.81 vs. **3** $\delta_{\text{H-3}}$ 4.75; **1** $\delta_{\text{H-6}}$ 3.35 to **3** $\delta_{\text{H-6}}$ 4.18) consistent with sulfonation at these positions. Compound **3** was therefore topsentinol K trisulfate.

Two previously reported steroids were also identified in the extract as polasterol B (**4**) and 22-dehydro-24-isopropylcholesterol (**5**) by comparison of their ¹H, ¹³C NMR and LC-MS spectra with those reported in the literature.^{7, 4}

All three new compounds were screened against the aspartic protease BACE1 (memapsin-2). Topsentinol K trisulfate (**3**) inhibited BACE1-mediated cleavage of amyloid precursor protein (APP) in a dose dependent manner with an IC₅₀ value of 1.2 μM . Neurosteroid sulfates are well known in vertebrates, suggesting this class of marine compounds might be of interest for further study as leads in neurodegenerative disorders.⁷ Sulfonic acids have also been previously tested in clinical trials as Alzheimer's therapeutics. Alzhemed, 3-aminopropanesulfonic acid, was recently evaluated as an amyloid- β agonist in Phase III clinic trials. Phase I clinical studies on a pro-drug analog (NRM8499) with improved pharmacodynamic properties are now underway as well.⁸

Interestingly, compounds **1**, **2**, **4**, and **5** were inactive which indicates that the inhibition observed for **3** was due to the presence of the sulfate esters. The inactivity observed for the desulfated compounds raised concerns that **3** was a non-specific inhibitor. Recently, Shoichet *et al.* have demonstrated that many promiscuous inhibitors aggregate with enzymes in a non-stoichiometric fashion, which disrupts protein folding.⁹ To determine if the observed inhibitory effect for **3** was due to aggregation caused by the stronger hydrogen bonding of the sulfate moiety, our BACE1 assay was repeated with the addition of detergent to disrupt these non-specific interactions. Unfortunately, our original assay was unable to tolerate the suggested conditions (0.01 or 0.1% Triton X-100).¹⁰ Presumably, this additive was also disrupting the crucial protein aggregation step in our complementation assay. The activity of **3** was therefore investigated using a surrogate system. Compound **3** was assayed against the serine protease chymotrypsin in a standard chemiluminescent assay with and without the addition of detergent. As shown in Figure 2, the IC₅₀ value of **3** was strongly affected by the addition of this additive (2.7 vs. 34.6 μ M with 0.01% Triton X-100). This behavior was similar to that observed for rottlerin, a known aggregator, suggesting that **3** was interacting with both enzymes in a similar non-specific fashion.¹⁰

Although there are reports of alkaloids,¹¹ terpenes,¹² fatty acids¹³ and adenine bases¹⁴ isolated from *Topsentia* species, the majority of compounds from this genus are steroids¹⁵ such as described here. Interestingly, many of these steroids contain highly modified side chains, incorporating for example heteroaromatics rings¹⁶ or halogenation.¹⁷ Modifications involving iterative biomethylations are characteristic as well, but steroids containing side chains modified to include C-24 isopropyl groups have only been reported in two instances from this genus.^{5,18} This unusual side chain containing the C-24 isopropyl residue is derived through successive SAM methylations of a C-24 terminal alkene.¹⁹

Experimental Section

General Experimental Procedures

Optical rotations were measured on a Jasco-DIP-700 polarimeter at the sodium line (589 nm). UV spectra were obtained on a Hewlett-Packard 8453 spectrophotometer and IR spectra were measured as a thin film on a CaF₂ disk using a PerkinElmer 1600 series FTIR. NMR spectra were acquired on a Varian Inova 500 MHz spectrometer operating at 500 (¹H) or 125 (¹³C) MHz using the residual solvent signals as an internal reference (CD₃OD δ _H 3.30 ppm, δ _C 49.0 ppm). Samples were in 3 mm Shigemi tubes during NMR analyses. High-resolution mass spectrometry data were obtained on an Agilent LC-MSTOF with ES ionization in the negative mode or on a VG Analytical 70SE Mass Spectrometer (EI). Gradient separations used a Shimadzu system consisting of LC-20AT Solvent Delivery Modules, a SPD-M20A VP Diode Photodiode Array Detector, and a SCL-20A VP System Controller. The flow rate was 3 mL/min for all HPLC separations unless noted otherwise.

Biological Material

The sponge was collected from a deep coral reef slope at 43 m off the Lighthouse Reef dive site (2° 16'03" N, 118° 14' 22"E), Derawan, Indonesia on the 24th of March, 1996. The sponge forms a bulbous mass with broad mounds on the surface. Oscules are scattered over

the upper surface. The surface is hispid with projecting spicules, and the texture is solid and very tough. The area directly beneath the surface is cavernous. The external color in life is beige with a greenish tone, the interior is paler. The internal skeleton is utterly confused, but at the ectosome vague confused tracts of oxeas emerge to align paratangentially to the surface. The megascleres are large robust angular oxeas in two size categories, roughly 1000–1400 μm and 600–800 μm in overall length. The sponge is a species of *Topsentia*, in the strict sense of *Trachyopsis* (= *Topsentia*) *halichondrioides* Dendy, 1905 from Sri Lanka, but is not this species. The sponge is an undescribed species of *Topsentia* (Order Halichondrida: Family Halichondriidae). A voucher specimen has been deposited in the Natural History Museum, London (BMNH 2010.3.31.1).

Extraction and Isolation

The freeze-dried sponge (83.7 g) was exhaustively extracted with MeOH ($5 \times 1 \text{ L}$) at room temperature to afford 8.6 g of lipophilic extract. The residue was suspended in H_2O then partitioned with hexane, EtOAc and *n*-BuOH.

The residue from the *n*-BuOH partition (600 mg) was separated on a silica gel flash column eluting with a gradient of MeOH in CH_2Cl_2 to afford eight fractions. Fraction 6 (50 mg) was separated by RP-HPLC (Luna C₈, $250 \times 10 \text{ mm}$, a linear gradient from 90–100% MeCN in H_2O over 40 min) to afford compounds **1** (t_{R} 11.4 min, 2.0 mg, 0.0023% yield), **2** (t_{R} 12.6 min, 1.8 mg, 0.0022% yield) and **4** (t_{R} 9.9 min, 1.5 mg, 0.0017% yield). Active fraction 8 (100 mg) was separated on a Sephadex LH-20 column ($900 \times 25 \text{ mm}$) eluting with CHCl_3 :MeOH (1:1, flow rate 2 mL/min) and the resulting samples pooled into three fractions (a–c) on the basis of TLC analyses. Fraction 8c (40.0 mg) was further separated by successive Si (CHCl_3 :MeOH; 1:1) and RP column chromatography (10% aq. MeOH) to yield pure **3** (3.0 mg, 0.0036% yield).

The residue from the initial EtOAc partition (400 mg) was subjected to column chromatography on a silica flash column eluting with a gradient of MeOH in CH_2Cl_2 to afford 12 fractions. Fraction 2 (25 mg) was separated by RP-HPLC (Luna C₈, $250 \times 10 \text{ mm}$, a linear gradient from 10–100% MeCN in H_2O over 40 min) to afford compound **5** (t_{R} 20.5 min, 5.0 mg, 0.058% yield).

Topsentinol K (1)—white powder; $[\alpha]_{\text{D}}^{22} -6$ (c 0.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 205 (3.2) nm; IR (film) ν_{max} 3341 cm^{-1} ; see Tables 1 and 2 for tabulated NMR spectroscopic data; HREIMS m/z 460.3926 $[\text{M}]^+$ (calcd for $\text{C}_{30}\text{H}_{52}\text{O}_3^+$, 460.3926; $\delta = 2.1$ ppm).

Topsentinol L (2)—white powder; $[\alpha]_{\text{D}}^{22} +27$ (c 0.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 207 (4.0) nm; IR (film) ν_{max} 3359 cm^{-1} ; see Tables 1 and 2 for tabulated NMR spectroscopic data; HREIMS m/z 460.3920 $[\text{M}]^+$ (calcd for $\text{C}_{30}\text{H}_{52}\text{O}_3^+$, 460.3920; $\delta = 0.8$ ppm).

Topsentinol K trisulfate (3)—white powder; $[\alpha]_{\text{D}}^{22} +14$ (c 0.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 203 (3.7) nm; IR (film) ν_{max} 3417, 1250, 1206 cm^{-1} ; see Tables 1 and 2 for

tabulated NMR spectroscopic data; HRESIMS m/z 743.2181 [M-Na]⁺ (calcd for C₃₀H₄₉O₁₂S₃Na₂⁺, 743.2180; δ = 0.1 ppm).

Polasterol B (4) and 22-dehydro-24-isopropylcholesterol (5)—¹H NMR, ¹³C NMR, and MS data for these compounds matched those previously published.^{4,6}

BACE1 Assay

The proteolytic cleavage of amyloid precursor protein (APP) was assayed as described by Naqvi.²⁰ Test compounds were solubilized in DMSO at the desired concentration and incubated in triplicate with the enzyme for 16 h in 96-well plates. A DMSO control (1.5 μ L) and an inhibitor standard were also tested in triplicate. The chemiluminescence signal was read using a Fluostar Optima spectrophotometer. Data were analyzed using GraphPad Prism. BACE1 activity was calculated as a percent of the positive control using a nonlinear regression analysis function that corresponded to a best one-fit model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Key ROESY correlations used to establish the relative configuration of the ABCD rings of **1**.

Figure 2.
Detergent-dependent inhibition of chymotrypsin by **3** indicative of an aggregation mechanism.

Table 1¹H NMR Spectroscopic Data (500 MHz, δ_{H} (*J* in Hz)) for Compounds **1–3** in MeOH-*d*₄

	1	2	3
1	1.70, dd (13.6, 2.0)	1.68, dd (13.5, 2.5)	2.07, br d (14.5)
	1.40, dd (13.6, 3.4)	1.41, dd (13.5, 3.7)	1.46, dd (15.0, 2.5)
2	3.79, br s	3.79, br s	4.79, br s
3	3.81, br d (2.3)	3.81, br d (2.5)	4.75, br s
4	1.69, m	1.68, br s	2.28, br d (15.0)
	1.93, ddd (12.5, 8.5, 3.0)	1.93, ddd (12.6, 9.2, 3.5)	1.79, ddd (15.0, 13.0, 2.0)
5	1.42, m	1.44, m	1.62, ddd (14.0, 10.5, 2.0)
6	3.35, m	3.35, ddd (10.9, 10.9, 4.5)	4.18, ddd (10.5, 10.5, 4.5)
7	1.93, m	1.94, m	2.35, ddd (12.0, 4.5, 4.5)
	0.87, m	0.86, m	1.02, m
8	1.48, m	1.47, m	1.52, m
9	0.69, m	0.68, m	0.75, m
11	1.51, m	1.53, m	1.54, m
	1.30, m	1.30, m	1.32, m
12	1.99, m	1.99, m	2.00, m
	1.16, m	1.16, m	1.17, m
14	1.08, m	1.09, m	1.11, m
15	1.58, m	1.58, m	1.57, m
	1.06, m	1.08, m	1.10, m
16	1.70, m	1.70, m	1.71, m
	1.30, m	1.29, m	1.21, m
17	1.24, m	1.20, m	1.20, m
18	0.71, s	0.70, s	0.72, s
19	0.98, s	0.99, s	1.04, s
20	2.41, m	2.07, m	2.42, m
21	0.98, d (6.7)	1.02, d (6.8)	0.98, d (6.5)
22	5.28, t (10.8)	5.14, dd (15.3, 8.4)	5.28, t (11.0)
23	4.97, t (10.8)	4.97, dd (15.3, 9.5)	4.98, t (11.0)
24	1.86, m	1.44, m	1.91, m
25	1.76, m	1.70, m	1.63, m
26	0.80, d (6.6)	0.78, d (6.7)	0.88, d (6.5)
27	0.91, d (6.6)	0.86, d (6.7)	0.92, d (6.5)
24(1)	1.73, m	1.70, m	1.61, m
24(2)	0.84, d (6.6)	0.79, d (6.8)	0.80, d (6.6)
24(3)	0.89, d (6.6)	0.85, d (6.8)	0.90, d (6.6)

Table 2¹³C NMR Spectroscopic Data (125 MHz, δ_C) for Compounds **1–3** in MeOH-*d*₄

Position	1	2	3
C-1	41.1	41.2	39.9
C-2	71.6	71.7	75.5
C-3	70.1	70.8	75.6
C-4	26.3	26.4	25.0
C-5	47.0	47.0	45.3
C-6	70.0	70.0	78.7
C-7	42.5	42.6	39.0
C-8	35.1	35.2	35.1
C-9	56.1	56.2	55.8
C-10	37.5	37.5	37.6
C-11	21.9	21.9	21.8
C-12	41.3	41.3	41.1
C-13	43.8	43.7	43.7
C-14	57.7	57.2	57.5
C-15	25.1	25.2	25.1
C-16	29.5	29.8	29.1
C-17	58.3	57.8	58.1
C-18	12.9	12.7	12.8
C-19	15.7	15.7	15.3
C-20	35.5	41.9	35.4
C-21	21.2	21.2	21.2
C-22	139.4	140.6	139.3
C-23	128.0	128.4	128.0
C-24	51.0	57.7	50.9
C-25	30.2	29.8	30.1
C-26	19.3	19.4	19.3
C-27	22.3	22.3	22.3
C-24(1)	30.8	30.2	30.8
C-24(2)	20.1	19.6	20.1
C-24(3)	22.5	22.5	22.5