Topsentinols, 24-Isopropyl Steroids from the Marine Sponge *Topsentia* sp.

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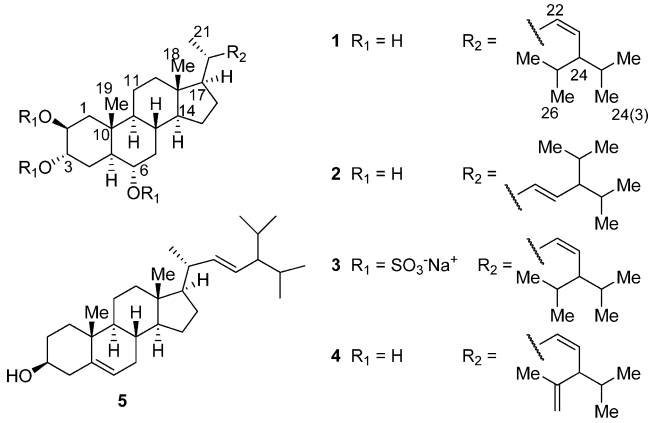
Three isopropyl steroids, topsentinols K, L, and K trisulfate (**1**−**3**), were isolated from an undescribed species of *Topsentia*. The structures of the new compounds were determined by extensive 1D and 2D NMR experiments and mass spectrometry measurements. Topsentinol K trisulfate (**3**) inhibited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

Marine sponges are a rich source of polyoxygenated and polysulfated steroids with unusual side chains.[1] According to Djerassi and co-workers, 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 *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 H_2O-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**), and 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 $C_{30}H_{52}O_3$. This formula was deduced after analysis of the HREIMS ion at *m*/*z* 460.3926 ($[M]^+$, Δ 2.1 ppm) and the ^{13}C 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 sp^3 carbons ($\delta_{C\text{-}2}$ 71.6, $\delta_{C\text{-}3}$ 70.1, and $\delta_{C\text{-}6}$ 70.0). Initial inspection of the ^1H and ^{13}C NMR data (Tables 1 and 2) indicated that **1** contained two methyl singlets ($\delta_{H\text{-}18}$ 0.71, $\delta_{H\text{-}19}$ 0.98), five methyl doublets ($\delta_{H\text{-}26}$ 0.80, $\delta_{H\text{-}24(2)}$ 0.84, $\delta_{H\text{-}24(3)}$ 0.89, $\delta_{H\text{-}27}$ 0.91, $\delta_{H\text{-}21}$ 0.98), and three oxygenated methines ($\delta_{H\text{-}6}$ 3.35, $\delta_{H\text{-}2}$ 3.79, $\delta_{H\text{-}3}$ 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 H_2-24(2) resonances observed in **4** for the terminal olefin (δ_H 4.86, 4.80) were replaced by a H_3-24(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 ^{13}C NMR spectrum of **1** and allowed the structure to be assigned as depicted.

The relative configuration of **1** was deduced through coupling constant and ROE 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 ($^3J_{H\text{-}22,H\text{-}23}$ = 10.8 Hz). A typical sterol configuration was suggested by the series of ROE cross-peaks (Figure 1) observed from H-8, -15, and -20 to the axial methyl H_3-18 and from H-4β, -8, and -11β to H_3-19. A β-configuration for H-6 in the B ring was assigned on the basis of the observed ROE correlation between H_3-19/H-6 and the proton−proton couplings (in pyridine-d_5) observed at this stereogenic center ($^3J_{H,H}$ = 10.2, 10.2, 4.3 Hz). Finally, the 17β-orientation of the side chain was assigned on the basis of ROESY cross-peaks between H-12β/H_3-21 and H_3-18/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 $C_{30}H_{52}O_3$ on the basis of the HREIMS ion at *m*/*z* 460.3920 ($[M]^+$, Δ 0.8 ppm) and analysis of the ^{13}C NMR spectroscopic data. As **2** was an isomer of **1**, the spectroscopic data were quite similar. Comparison of the ^1H and ^{13}C NMR spectra of **2** (Tables 1 and 2) with those of **1** indicated that the steroidal cores were the same.

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Table 1. ^{1}H NMR Spectroscopic Data (500 MHz, δ_H (*J* in Hz)) for Compounds **1**−**3** in CD_3OD

	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 2. ^{13}C NMR Spectroscopic Data (125 MHz, δ_C) for Compounds **1**−**3** in CD_3OD

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

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 ($^3J_{H\text{-}22,H\text{-}23}$ = 15.3 Hz).

Topsentinol K trisulfate (**3**) provided a molecular formula of $C_{30}H_{49}O_{12}S_3Na_3$, as deduced from the negative HRESIMS ion at *m*/*z* 743.2181 ([M − Na]$^-$, Δ −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** $\delta_{H\text{-}2}$ 3.79 vs **3** $\delta_{H\text{-}2}$ 4.79; **1** $\delta_{H\text{-}3}$ 3.81 vs **3** $\delta_{H\text{-}3}$ 4.75; **1** $\delta_{H\text{-}6}$ 3.35 to **3** $\delta_{H\text{-}6}$ 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 ^{1}H and ^{13}C NMR and LC-MS spectra with those reported in the literature.[7,4]

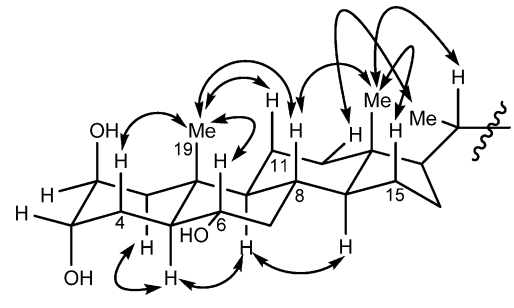

Figure 1. Key ROESY correlations used to establish the relative configuration of the ABCD rings of **1**.

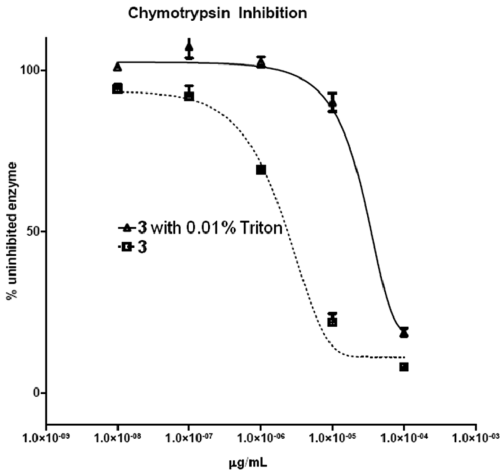

Figure 2. Detergent-dependent inhibition of chymotrypsin by **3** indicative of an aggregation mechanism.

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 IC_{50} 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 analogue (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 nonspecific inhibitor. Recently, Shoichet et al. have demonstrated that many promiscuous inhibitors aggregate with enzymes in a nonstoichiometric 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 nonspecific 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 IC_{50} value of **3** was strongly affected by the addition of this additive (2.7 vs 34.6 μM 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 nonspecific fashion.[10]

Although there are reports of alkaloids,[11] terpenes,[12] fatty acids,[13] and adenine bases[14] isolated from *Topsentia* species, the majority of compounds from this genus are steroids[15] such as described here. Interestingly, many of these steroids contain highly modified side chains, incorporating for example heteroaromatic rings[16] or halogenation.[17] Modifications involving iterative biomethylations are characteristic as well, but steroids containing side chains modified to include C-24 isopropyl groups have been reported in only two instances from this genus.[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 CaF_2 disk using a PerkinElmer 1600 series FTIR. NMR spectra were acquired on a Varian Inova 500 MHz spectrometer operating at 500 (^{1}H) or 125 (^{13}C) MHz using the residual solvent signals as an internal reference (CD_3OD 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 43 m off the Lighthouse Reef dive site (2°16′03″ N, 118°14′22″ E), Derawan, Indonesia, on March 24, 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 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 × 1 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 C_8, 250 × 10 mm, a linear gradient from 90 to 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 × 25 mm) eluting with $CHCl_3$−MeOH (1:1, flow rate 2 mL/min), and the resulting samples were 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% MeOH(aq)) 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 C_8, 250 × 10 mm, a linear gradient from 10 to 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; $[\alpha]^{22}_D$ −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 NMR spectroscopic data; HREIMS *m*/*z* 460.3926 $[M]^+$ (calcd for $C_{30}H_{52}O_3^+$, 460.3926; Δ = 2.1 ppm).

Topsentinol L (2): white powder; $[\alpha]^{22}_D$ +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 NMR spectroscopic data; HREIMS *m*/*z* 460.3920 $[M]^+$ (calcd for $C_{30}H_{52}O_3^+$, 460.3920; Δ = 0.8 ppm).

Topsentinol K Trisulfate (3): white powder; $[\alpha]^{22}_D$ +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 NMR spectroscopic data; HRESIMS *m*/*z* 743.2181 $[M - Na]^-$ (calcd for $C_{30}H_{49}O_{12}S_3Na_2^-$, 743.2180; Δ = 0.1 ppm).

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

BACE1 Assay. The proteolytic cleavage of amyloid precursor protein 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 μL) 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.

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

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