

## Spheciosterol Sulfates, PKC $\zeta$ Inhibitors from a Philippine Sponge *Spheciospongia* sp.

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Three new sterol sulfates, spheciosterol sulfates A–C (**1–3**), and the known sterol sulfate topsentiasterol sulfate E (**4**) have been isolated from the sponge *Spheciospongia* sp., collected in the Philippines. Structures were assigned on the basis of extensive 1D and 2D NMR studies as well as analysis by HRESIMS. Compounds **1–4** inhibited PKC $\zeta$  with IC<sub>50</sub> values of 1.59, 0.53, 0.11, and 1.21  $\mu$ M, respectively. In a cell-based assay, **1–4** also inhibited NF- $\kappa$ B activation with EC<sub>50</sub> values of 12–64  $\mu$ M.

Marine organisms are widely known for producing a variety of polyoxygenated<sup>1</sup> and polysulfated steroids with unusual, modified side chains.<sup>2</sup> Sulfated sterols isolated from sponges have received a considerable amount of attention due to their attractive biological activities, particularly against HIV-1.<sup>3–5</sup>

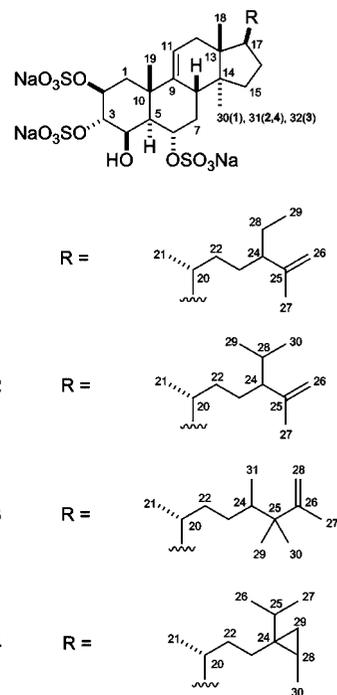
Members of the protein kinase C (PKC) family of serine/threonine kinases are involved in a wide range of cellular functions including proliferation, gene expression, cell cycle control, cell migration, mitogenic signaling, cytoskeleton function, glucose metabolism, differentiation, and regulation of cell survival and apoptosis.<sup>6,7</sup> PKCs also play a central role in coordination of cross talk between major signaling pathways.<sup>6</sup> PKC has been implicated in the progression of numerous diseases, which makes PKC inhibitors attractive therapeutic agents.<sup>8</sup> In fact, inhibitors of PKCs are currently being tested in clinical trials for various disorders.<sup>9</sup> At least 11 closely related PKC isoforms have been reported that differ in structure, biochemical properties, tissue distribution, subcellular localization, and substrate specificity. PKC isoforms are classified as conventional ( $\alpha$ ,  $\beta$ 1,  $\beta$ 2,  $\gamma$ ), novel ( $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\theta$ ,  $\mu$ ), and atypical ( $\zeta$ ,  $\lambda$ /i).<sup>7</sup> Conventional PKCs are regulated by diacylglycerol, phosphatidylserine, and calcium. Novel PKCs are regulated by diacylglycerol and phosphatidylserine, but are calcium independent. Atypical PKC isoforms are phosphatidylserine-dependent, but are not affected by calcium, diacylglycerols, or phorbol esters.<sup>6</sup>

Atypical PKCs have been implicated in the establishment of cell polarity, cell proliferation, and cell survival.<sup>10</sup> PKC $\zeta$  and PKC $\iota$  exhibit 72% sequence homology at the amino acid level, but recent data suggest their functions are not overlapped.<sup>10</sup> Expression profiling demonstrated that PKC $\zeta$  and PKC $\iota$  exhibit distinct patterns of expression in various tissues and cell types; PKC $\iota$  is ubiquitously expressed, whereas PKC $\zeta$  exhibits a much more restricted pattern of expression.<sup>11</sup> Also, genetic disruption of the PKC $\zeta$  and PKC $\iota$ / $\lambda$  genes has very different effects on the embryonic development of the mouse. Knockouts of PKC $\iota$ / $\lambda$  are embryonically lethal, whereas knockouts of PKC $\zeta$  result in viable mice that exhibit only subtle immunological deficiencies.<sup>10</sup> Finally, PKC $\zeta$  and PKC $\iota$ / $\lambda$  preferentially couple to distinct downstream signaling pathways. Using mouse embryo fibroblasts generated from either PKC $\zeta$  or PKC $\iota$  knockout mice, it has been shown that PKC $\zeta$  couples more efficiently to the NF- $\kappa$ B pathway, a well-characterized downstream effector pathway of atypical PKCs, than does PKC $\iota$ / $\lambda$ . Thus PKC $\zeta$ -

deficient fibroblasts exhibit defects in NF- $\kappa$ B signaling, whereas PKC $\iota$ / $\lambda$ -deficient fibroblasts do not.<sup>10</sup>

PKC $\zeta$  alone has been implicated as an important factor in several types of cancer<sup>12–15</sup> as well as osteoarthritis.<sup>7,16</sup> Therefore, PKC $\zeta$  inhibitors could be beneficial in a number of diseases and disorders.

As part of an ongoing search for bioactive marine metabolites, crude extracts from our marine invertebrate library were screened for PKC $\zeta$  activity. The methanol extracts of a *Spheciospongia* sp., collected in Cagayan de Oro, Philippines, showed promising activity in the initial screen. Three new sulfated sterols, spheciosterol sulfates A–C (**1–3**), as well as the known sulfated sterol topsentiasterol sulfate E (**4**), were isolated from this sponge. The pure compounds, spheciosterol sulfates A–C (**1–3**) and topsentiasterol sulfate E (**4**), were active against PKC $\zeta$  and inhibited NF- $\kappa$ B activation.



## Results and Discussion

The specimen (CDO-00-12-141) of *Spheciospongia* sp. was exhaustively extracted with MeOH. The crude extract was separated on an HP20SS resin using a step gradient of H<sub>2</sub>O to IPA (25% steps, 5 fractions). Bioassay-guided fractionation of the third fraction

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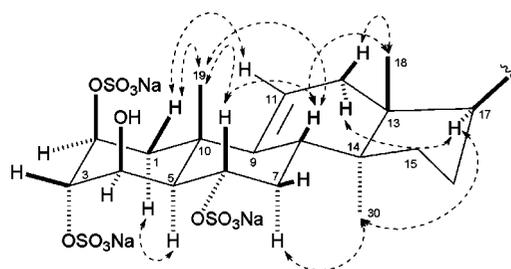
**Table 1.** NMR Data for Spheciosterol Sulfates A–C (**1–3**) (500 MHz, CD<sub>3</sub>OD)

position	spheciosterol sulfate A ( <b>1</b> )		spheciosterol sulfate B ( <b>2</b> )		spheciosterol sulfate C ( <b>3</b> )	
	$\delta_{\text{H}}$ mult ( <i>J</i> , Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ mult ( <i>J</i> , Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ mult ( <i>J</i> , Hz)	$\delta_{\text{C}}$
1 $\alpha$	1.85, br dd (14.6, 4.0)	37.2	1.86, br dd (14.7, 3.9)	37.3	1.83 br dd (14.6, 3.9)	37.2
1 $\beta$	2.33, br d (14.6)		2.31, br d (14.7)		2.37, br d (14.6)	
2	4.96, m	75.4	4.96, m	75.4	4.99, m	75.5
3	4.83 <sup>a</sup>	75.8	4.82 <sup>a</sup>	75.8	4.82 <sup>a</sup>	76.1
4	4.46, dt (3.1, 1.0)	68.4	4.46, dt (2.8, 1.0)	68.4	4.47, dt (3.0, 1.0)	68.3
5	1.48, dd (11.2, 3.1)	47.7	1.48, dd (11.4, 2.8)	47.7	1.50, dd (11.3, 3.0)	47.8
6	4.84 <sup>a</sup>	75.7	4.84 <sup>a</sup>	75.7	4.84 <sup>a</sup>	76.0
7 $\alpha$	1.55, m	35.3	1.55, m	35.2	1.57, m	35.0
7 $\beta$	2.23, dt (12.0, 4.6)		2.23, dt (11.7, 4.5)		2.24, dt (11.8, 4.6)	
8	2.50, br d (12.5)	41.1	2.49, br d (12.9)	41.1	2.51, br d (12.9)	41.1
9		146.5		146.4		146.5
10		39.4		39.7		39.4
11	5.35, br d (5.5)	117.5	5.35, br d (5.2)	117.4	5.35, br d (5.4)	117.3
12 $\alpha$	2.12, br d (17.3)	38.2	2.11, br d (16.8)	38.1	2.10, br d (17.1)	38.2
12 $\beta$	1.94, dd (17.3, 5.5)		1.95, dd (16.8, 5.2)		1.98, dd (16.9, 5.5)	
13		45.4		45.4		45.3
14		47.9		47.9		47.9
15 $\alpha$	1.37, m	34.5	1.36, m	34.5	1.39, m	34.7
15 $\beta$	1.43, m		1.45, m		1.47, m	
16 $\alpha$	1.89, m	28.6	1.89, m	28.7	1.92, m	28.9
16 $\beta$	1.32, m		1.31, m		1.33, m	
17	1.63, m	52.1	1.63, m	52.0	1.68, m	51.9
18	0.70, s	14.7	0.68, s	14.7	0.70, s	14.7
19	1.42, s	25.2	1.42, s	25.2	1.45, s	25.2
20	1.42, m	36.8	1.34, m	37.6	1.38, m	37.5
21	0.89, d (6.6)	18.5	0.91, d (6.2)	18.9	0.90, br d (6.5)	18.9
22a	1.36, m	34.7	1.38, m	34.6	1.43, m	34.3
22b	0.97, m		0.97, m		1.07, dd (13.3, 6.6)	
23a	1.40, m	30.2	1.53, m	30.3	1.57, m	29.2
23b	1.29, m		1.08, m		1.32, m	
24	1.87, m	50.6	1.52, m	56.8	1.39, m	40.5
25		148.5		148.4		43.0
26a	4.75, dd (2.5, 1.3)	112.1	4.77, dd (2.5, 1.4)	112.6		154.6
26b	4.66, br d (2.5)		4.62, br d (2.5)			
27	1.57, br s	17.6	1.57, br s	18.8	1.70, br s	19.4
28a	1.34, m	27.4	1.52, m	31.1	4.74, dd (2.4, 1.3)	110.0
28b					4.72, br d (2.4)	
29	0.82, t (7.3)	12.2	0.81, d (6.2)	21.6	0.97, s	22.8
30	0.80, s	18.5	0.92, d (6.2)	20.9	0.96, s	24.2
31			0.80, s	18.6	0.82, d (6.7)	14.5
32					0.81, s	18.5

<sup>a</sup> Overlapped with HOD signal.

(50/50 H<sub>2</sub>O/IPA), utilizing several rounds of reversed-phase HPLC, resulted in the isolation of spheciosterol sulfates A–C (**1–3**) and topsentiasterol sulfate E (**4**).

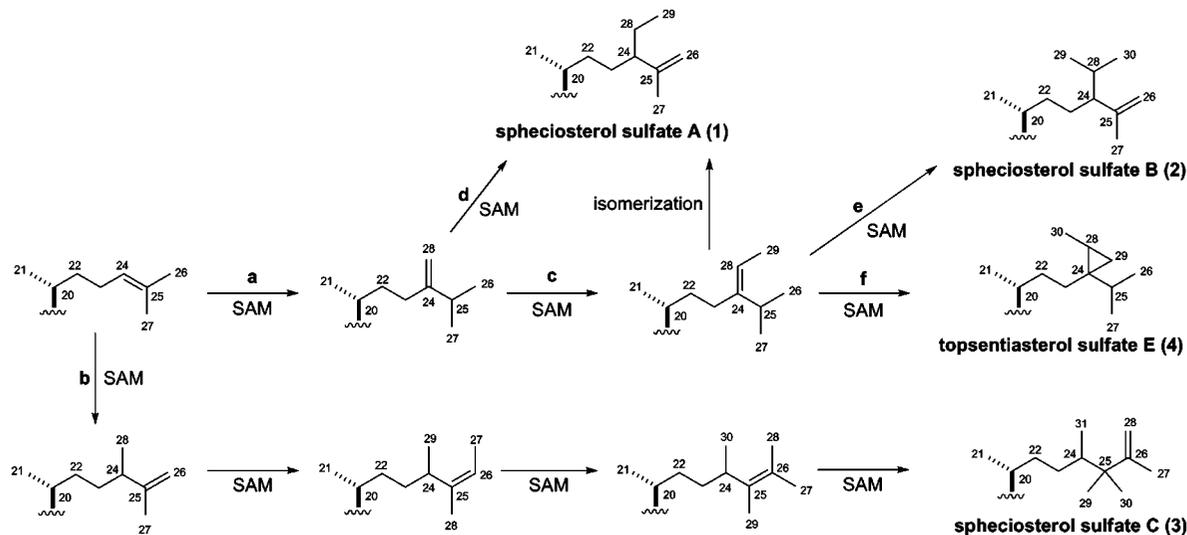
The molecular formula for spheciosterol sulfate A (**1**), C<sub>30</sub>H<sub>47</sub>O<sub>13</sub>S<sub>3</sub>Na<sub>3</sub>, was derived from NMR data and the HRESIMS ion at *m/z* 757.1984 ([M – Na]<sup>–</sup>,  $\Delta$  +1.3 ppm). The presence of multiply charged ions and characteristic losses of 80 Da in MS/MS experiments indicated the presence of sulfate esters in **1**. The structure of spheciosterol sulfate A (**1**) was established on the basis of extensive 1D and 2D NMR studies. Initial interpretation of the <sup>1</sup>H and <sup>13</sup>C NMR data (Table 1) indicated that **1** contained four methyl singlets ( $\delta$  0.70, 0.80, 1.42, 1.57), one methyl doublet ( $\delta$  0.89), one methyl triplet ( $\delta$  0.82), four oxygenated methines ( $\delta$  4.96, 4.84, 4.83, 4.46), a terminal olefin ( $\delta$  4.75, 4.66), and a trisubstituted olefin ( $\delta$  5.35). The data also indicated that spheciosterol sulfate A (**1**) contained five quaternary carbons, 10 methines, and nine methylenes. Interpretation of COSY and HMBC data allowed the assignment of the A–D steroid ring system. Rings A and B were assembled on the basis of COSY correlations among all adjacent protons between H-1 $\alpha$  and H-1 $\beta$  and H-8, and *w*-coupling correlations from H-8 to H-11. HMBC correlations from Me-19 to C-1, C-5, C-9, and C-10 completed the structural assignment of the A and B rings. Rings C and D were assigned on the basis of HMBC correlations from Me-18 to C-12, C-13, C-14, and C-17 and from Me-30 to C-8, C-13, C-14, and C-15. HMBC correlations from H-11 to C-8 and C-12 confirmed the position of



**Figure 1.** Key ROE correlations supporting the relative configuration of spheciosterol sulfate A.

the 9,11-double bond. COSY correlations from H-15 to H-16 and from H-16 to H-17 completed the structural assignment of the C and D rings. The structure of the side chain was established using HMBC correlations from Me-21 to C-17, C-20, and C-22; Me-27 to C-24, C-25, and C-26; and Me-29 to C-24 and C-28. COSY correlations from H-22a and H-22b to H-23; H-23 to H-24; and H-24 to H-28 completed the assignment of the molecule.

The relative configuration of spheciosterol sulfate A (**1**), as drawn, was determined on the basis of coupling constants and ROESY data (Figure 1). H-2 and H-3 were deemed equatorial on the basis of the small coupling between H-1 $\alpha$  and H-2 (*J* = 4.0 Hz), as well as the *w*-coupling between H-1 $\beta$  and H-3 observed in the COSY data. A large coupling constant between H-5 and H-6



**Figure 2.** Proposed biosynthesis for the side chains present in spheciosterol sulfates A–C (**1–3**) and topsentiasterol sulfate E (**4**).

( $J = 11.2$  Hz) suggested that H-5 and H-6 were both axial, while a small coupling constant between H-4 and H-5 ( $J = 3.1$  Hz) suggested that H-4 was equatorial. The large vicinal coupling between H-5 and H-6 and the ROE effect observed between Me-19 and H-6 suggested a trans A/B ring juncture. ROE effects observed from Me-18 to H-8 and H-12 $\beta$  and from Me-30 to H-7 $\alpha$  and H-17 supported a trans-fused C/D ring juncture. Biosynthetic precedent and ROE effects for H-8 to H-6 and Me-19; Me-19 to H-1 $\beta$  and H-6; and H-17 to H-12 $\alpha$  led to the assignment of spheciosterol sulfate A (**1**) as 2*S*, 3*S*, 4*R*, 5*R*, 6*S*, 8*S*, 10*S*, 13*R*, 14*S*, 17*R*, 20*R*. The relative configuration of C-24 was not determined. These data were consistent with the data reported for topsentiasterol sulfate E (**4**)<sup>17</sup> and Sch 575867,<sup>18</sup> which contain an identical steroid nucleus.

The molecular formula for spheciosterol sulfate B (**2**), C<sub>31</sub>H<sub>49</sub>O<sub>13</sub>S<sub>3</sub>Na<sub>3</sub>, was derived from NMR data and the HRESIMS ion at  $m/z$  363.12123 ( $[M - 3Na + H]^{-2}$ ,  $\Delta$  0.01 ppm). Compounds **1**, **3**, and **4** exhibited sequential losses of 3 Na<sup>+</sup> ions on a Micromass Q-tof micro, supporting the presence of 3 Na in all of the spheciosterol sulfates' molecular formulas. Losses of 80 Da in MS/MS experiments and the presence of multiply charged ions indicated **2** contained three sulfate esters. Initial interpretation of the <sup>1</sup>H and <sup>13</sup>C NMR data (Table 1) indicated that **2** contained four methyl singlets ( $\delta$  0.68, 0.80, 1.42, 1.57), three methyl doublets ( $\delta$  0.81, 0.91, 0.92), four oxygenated methines ( $\delta$  4.46, 4.82, 4.84, 4.96), a terminal olefin ( $\delta$  4.77, 4.62), and a trisubstituted olefin ( $\delta$  5.35). The data also indicated that spheciosterol sulfate B (**2**) contained five quaternary carbons, 11 methines, and eight methylenes. After comparing the NMR data of **1** and **2**, it was apparent that spheciosterol sulfate B (**2**) contained the same steroid nucleus as spheciosterol sulfate A (**1**) with an isopropyl group instead of the ethyl group present in **1**.

The molecular formula for spheciosterol sulfate C (**3**), C<sub>32</sub>H<sub>51</sub>O<sub>13</sub>S<sub>3</sub>Na<sub>3</sub>, was derived from NMR data and the HRESIMS ion at  $m/z$  370.12910 ( $[M - 3Na + H]^{-2}$ ,  $\Delta$  + 0.5 ppm). **3** also exhibited losses of 80 Da and multiply charged ions, signifying sulfate esters in the molecule. Initial interpretation of the <sup>1</sup>H and <sup>13</sup>C NMR data (Table 1) indicated that **3** contained six methyl singlets ( $\delta$  0.70, 0.81, 0.96, 0.97, 1.45, 1.70), two methyl doublets ( $\delta$  0.82, 0.90), four oxygenated methines ( $\delta$  4.47, 4.82, 4.84, 4.99), a terminal olefin ( $\delta$  4.74, 4.72), and a trisubstituted olefin ( $\delta$  5.35). The data also indicated that spheciosterol sulfate C (**3**) contained six quaternary carbons, 10 methines, and eight methylenes. Comparison of the NMR data between **1**, **2**, and **3** showed that the same steroid nucleus was present in **3**, along with a slightly different side chain. The structure of the side chain was established using

HMBC correlations from Me-21 to C-17, C-20, and C-22; Me-27 to C-25, C-26, and C-28; Me-29 and Me-30 to C-24, C-25, C-26, and to each other; and Me-31 to C-23, C-24, and C-25, which completed the assignment of spheciosterol sulfate C (**3**).

The molecular formula for **4**, C<sub>31</sub>H<sub>49</sub>O<sub>13</sub>S<sub>3</sub>Na<sub>3</sub>, was derived from NMR data and the HRESIMS ion at  $m/z$  363.12124 ( $[M - 3Na + H]^{-2}$ ,  $\Delta$  +0.1 ppm). Interpretation of COSY, HSQC, and HMBC data allowed for the assignments of all <sup>1</sup>H and <sup>13</sup>C signals, which verified that **4** was the known compound topsentiasterol sulfate E (**4**).<sup>17</sup>

Spheciosterol sulfates A–C (**1–3**) and topsentiasterol sulfate E (**4**) inhibited PKC $\zeta$  with IC<sub>50</sub> values of 1.59, 0.53, 0.11, and 1.21  $\mu$ M, respectively. In a cell-based assay using primary human chondrocytes, **1–4** also inhibited NF- $\kappa$ B activation with EC<sub>50</sub> values of 12–64  $\mu$ M. These sterols exhibited potent activity against PKC $\zeta$ , but displayed less of an effect on NF- $\kappa$ B. The side chain appears to be important for activity against PKC $\zeta$ ; spheciosterol sulfate C (**3**) is 10-fold more active than spheciosterol sulfate A (**1**) and topsentiasterol sulfate E (**4**).

The spheciosterols are examples of a small group of 4 $\beta$ -hydroxy-14 $\alpha$ -methyl sterols that occur among the wealth of steroids isolated from marine organisms. Topsentiasterols A–E<sup>17</sup> and Sch 575867<sup>18</sup> are the only previously reported members of the 4 $\beta$ -hydroxy-14 $\alpha$ -methyl sterol group. The  $\Delta^{9(11)}$ -4,4-dimethyl-4-deoxy-14 $\alpha$ -methyl substitution pattern is also rare in marine sterols, observed only in ibisterol sulfates A–C<sup>4,19</sup> and lembeherols A and B.<sup>20</sup> In holothurians, the  $\Delta^{9(11)}$ -14 $\alpha$ -methyl substitution pattern is biosynthesized directly from parkeol (the  $\Delta^{9(11)}$  isomer of lanosterol) rather than from lanosterol or cycloartenol.<sup>21</sup> It is likely that sponges utilize the same pathway.

The proposed biosynthesis for the side chains present in spheciosterol sulfates A–C (**1–3**) and topsentiasterol sulfate E (**4**) is shown in Figure 2. The side chains present in **1–4** are likely formed via methylations by *S*-adenosylmethionine (SAM) to the side chain present in parkeol, along with other modifications. Following pathway **a**, an electrophilic methyl equivalent from SAM is added to the 24,25-double bond, and a Wagner–Meerwein 1,2-hydride shift generates the 24,28-double bond that can follow pathway **c** or **d**. Pathway **c** illustrates the addition of another methyl from SAM to C-28, a loss of a proton to generate the 24,28-double bond, and isomerization to form the side chain in **1**. Pathway **d** illustrates the addition of a methyl from SAM to C-28 and a Wagner–Meerwein 1,2-hydride shift to form the 25,26-double bond present in the side chain of **1**. Pathway **c** is more likely than **d**, as it generates the side chains of **2** and **4**. Pathway **e** illustrates the addition of a methyl from SAM to C-28 and a Wagner–Meerwein

1,2-hydride shift to form the 25,26-double bond present in the side chain of **2**. Pathway **f** illustrates the addition of a methyl from SAM to C-28, nucleophilic attack by the methyl group to close the cyclopropane ring, and simultaneous loss of a proton to generate the side chain in **4**. Pathway **b** illustrates the addition of a methyl from SAM to C-24 and a loss of a proton to form the 25,26-double bond. Then, SAM adds a methyl twice to C-26, and a loss of a proton re-forms the 25,26-double bond. Finally, SAM adds a methyl to C-25, and a loss of a proton generates the 26,28-double bond present in the side chain of **3**.

The side chain present in spheciosterol sulfate A (**1**) has been reported from several marine sources, including a few species of the green alga *Codium*,<sup>22–25</sup> the green alga *Ulva lactuca*,<sup>26</sup> the sponge *Echinoclathria subhispidia*,<sup>27</sup> and an unidentified chrysophyte.<sup>28</sup> The side chain present in spheciosterol sulfate B (**2**) has been reported in two different sponges, *Dysidea herbacea*<sup>29</sup> and *Verongia cauliformis*,<sup>30</sup> and an unidentified chrysophyte.<sup>28</sup> The side chain present in spheciosterol sulfate C (**3**) has never been reported. However, side chains with different olefin patterns have been reported from sponges of the family Halichondriidae<sup>31–33</sup> and an unidentified chrysophyte.<sup>28</sup> The side chain present in topsentiasterol sulfate E (**4**) has been reported from the sponges *Topsentia* sp.<sup>17,19</sup> and *Petrosia weinbergi*<sup>5</sup> and an unidentified chrysophyte.<sup>28</sup> Interestingly, the side chains present in **1**, **2**, and **4** and an isomer of the side chain in **3** are all present in an unidentified chrysophyte.<sup>28</sup> In contrast, the steroid nucleus among the two organisms is different.

## Experimental Section

**General Experimental Procedures.** Optical rotations were measured on a JASCO DIP-370 digital polarimeter. UV spectra were acquired in spectroscopy grade MeOH using a Hewlett-Packard 8452A diode array spectrophotometer. IR spectra were recorded on a JASCO FT/IR-420 spectrometer. NMR data for **1–4** were collected using a Varian INOVA 500 (<sup>1</sup>H 500 MHz, <sup>13</sup>C 125 MHz) NMR spectrometer with a 3 mm Nalorac MDBG probe and referenced to residual solvent ( $\delta_{\text{H}}$  3.31,  $\delta_{\text{C}}$  49.15 for CD<sub>3</sub>OD). High-resolution ESIMS analyses were performed on either a ThermoFinnigan LTQ-FT or a Micromass Q-tof micro. Initial purification was performed on HP20SS resin. HPLC was performed on an Agilent 1100 system.

**Biological Material.** *Spheciospongia* sp. (Hadromerida, Clionaidae), sample CDO-00-12-141, was collected by scuba in Cagayan de Oro, Philippines (09°39.385' N, 125°23.449' E); a voucher specimen is maintained at the University of Utah. This gray olive-green *Spheciospongia* sp. is a massive sponge with small fistules projecting through the substrate, with no indication of a boring habit. The ectosome consists of a moderately dense palisade of discrete, erect bundles of tylostyles forming a nearly continuous surface skeleton and a thin layer of spiraster microscleres lying on or near the surface. The choanosomal skeleton is disorganized, criss-crossed bundles of tylostyles becoming slightly denser in the center of the sponge. Heavy concentrations of spirasters are scattered throughout the choanosome and lining of aquiferous canals. Megascleres: tylostyles not apparently divided into more than one size category but with size range of 240–720 × 8–17  $\mu\text{m}$ , with moderately large tylole, occasionally subtyle bases, and tapering to sharp points. Microscleres: single size class of spiraster, 13–52  $\mu\text{m}$ . This species is similar in habit and skeletal structure to *Spheciospongia vagabunda* (Ridley, 1884) but with significantly larger spicule sizes.

**Extraction and Isolation.** The *Spheciospongia* sp. specimen (CDO-00-12-141) was exhaustively extracted with MeOH to yield 1.2 g of crude extract. The crude extract was separated on HP20SS resin using a gradient of H<sub>2</sub>O to IPA in 25% steps, and a final wash of 100% MeOH, to yield 5 fractions, 15 mL each. The third fraction (50/50 H<sub>2</sub>O/IPA, 35.4 mg) was chromatographed by HPLC using a Phenomenex Onyx C<sub>18</sub> monolithic column (100 × 10 mm) employing a gradient of 20% CH<sub>3</sub>CN/H<sub>2</sub>O to 65% CH<sub>3</sub>CN/H<sub>2</sub>O at 5 mL/min over 30 min to yield 6 fractions, 187A–187F. Fraction 187E was further chromatographed by HPLC using an Agilent ZORBAX Eclipse XDB C<sub>8</sub> column and employing a gradient of 2% CH<sub>3</sub>CN/H<sub>2</sub>O to 100% CH<sub>3</sub>CN at 4 mL/min over 45 min to yield 11 fractions, 191A–191K. Fraction 191F was purified by HPLC using an Agilent ZORBAX Eclipse XDB C<sub>8</sub> column employing a gradient of 30% CH<sub>3</sub>CN/20 mM NaCl in H<sub>2</sub>O to 50% CH<sub>3</sub>CN/20 mM NaCl in H<sub>2</sub>O at 4.5 mL/min over 45 min to

provide spheciosterol sulfate A (**1**, 0.4 mg) eluting at 25.7 min. Fraction 191G was purified by HPLC using an Agilent ZORBAX Eclipse XDB C<sub>8</sub> column employing a gradient of 30% CH<sub>3</sub>CN/20 mM NaCl in H<sub>2</sub>O to 50% CH<sub>3</sub>CN/20 mM NaCl in H<sub>2</sub>O at 4.5 mL/min over 45 min to provide spheciosterol sulfate B (**2**, 1.0 mg) eluting at 28.4 min. Fraction 191H was purified by HPLC using an Agilent ZORBAX Eclipse XDB C<sub>8</sub> column employing a gradient of 2% CH<sub>3</sub>CN/0.2 M NaCl in H<sub>2</sub>O to 50% CH<sub>3</sub>CN/0.2 M NaCl in H<sub>2</sub>O at 4.5 mL/min over 48 min to provide topsentiasterol sulfate E (**4**, 1.3 mg) eluting at 44.9 min and spheciosterol sulfate C (**3**, 1.0 mg) eluting at 45.7 min.

Desalting of HPLC fractions was achieved by extracting the fractions with 1:1 EtOAc/MeOH, followed by concentration *in vacuo*. The samples were then filtered through C<sub>18</sub> Sep-Pak cartridges; salts were removed by washing with 100% H<sub>2</sub>O, and individual compounds were eluted with 100% MeOH.

**Spheciosterol sulfate A (1):** amorphous, white solid;  $[\alpha]_{\text{D}}^{25}$  –34.2 (c 0.02, MeOH); UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 206 (3.66) nm; IR (cell)  $\nu_{\text{max}}$  2648, 1662, 1242, 933 cm<sup>-1</sup>; <sup>1</sup>H NMR and <sup>13</sup>C NMR data, see Table 1; HRESIMS  $m/z$  757.1984 [M – Na]<sup>-</sup> (calcd for C<sub>30</sub>H<sub>47</sub>O<sub>13</sub>S<sub>3</sub>Na<sub>2</sub>, 757.1974).

**Spheciosterol sulfate B (2):** amorphous, white solid;  $[\alpha]_{\text{D}}^{25}$  –31.6 (c 0.07, MeOH); UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 206 (3.70) nm; IR (cell)  $\nu_{\text{max}}$  2644, 1662, 1248, 935 cm<sup>-1</sup>; <sup>1</sup>H NMR and <sup>13</sup>C NMR data, see Table 1; HRESIMS  $m/z$  363.12123 [M – 3Na + H]<sup>-2</sup> (calcd for C<sub>31</sub>H<sub>50</sub>O<sub>13</sub>S<sub>3</sub>, 363.12123).

**Spheciosterol sulfate C (3):** amorphous, white solid;  $[\alpha]_{\text{D}}^{25}$  –36.0 (c 0.07, MeOH); UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 204 (3.62) nm; IR (cell)  $\nu_{\text{max}}$  2648, 1662, 1244, 931 cm<sup>-1</sup>; <sup>1</sup>H NMR and <sup>13</sup>C NMR data, see Table 1; HRESIMS  $m/z$  370.12910 [M – 3Na + H]<sup>-2</sup> (calcd for C<sub>32</sub>H<sub>52</sub>O<sub>13</sub>S<sub>3</sub>, 370.12905).

**Topsentiasterol sulfate E (4):** amorphous, white solid;  $[\alpha]_{\text{D}}^{25}$  +58.3 (c 0.10, MeOH); UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 204 (3.60) nm; IR (film)  $\nu_{\text{max}}$  3460, 2930, 1650, 1220 cm<sup>-1</sup>; <sup>1</sup>H NMR and <sup>13</sup>C NMR data see Supplemental Table 1; HRESIMS  $m/z$  363.12124 [M – 3Na + H]<sup>-2</sup> (calcd for C<sub>31</sub>H<sub>50</sub>O<sub>13</sub>S<sub>3</sub>, 363.12123).

**PKC $\zeta$  LanthaScreen Assay.** The compounds were dissolved in 100% DMSO and tested at different concentrations in an *in vitro* PKC $\zeta$  TR-FRET-based LanthaScreen assay (Invitrogen) to determine IC<sub>50</sub> values. The assay consisted of full-length PKC $\zeta$  (Invitrogen), a fluorophore-labeled custom PKC $\zeta$  substrate peptide (SynPep), and ATP (Sigma), which were added sequentially to the serially diluted compounds in assay plates. The optimized final concentrations of the constituents were 1 × compound, 6.25% DMSO, 0.2  $\mu\text{M}$  peptide, 15  $\mu\text{M}$  ATP (at 1 × K<sub>m</sub>), and 80 pM PKC $\zeta$ . The final volume of the enzyme reaction was 10  $\mu\text{L}$  performed in black, low-volume, 384-well polypropylene MatriPlates (Matrical). The enzyme reaction was stopped with 5  $\mu\text{L}$  of 60 mM EDTA after 1 h incubation at rt. Tb-pSer-PKC antibody (Invitrogen) made up in TR-FRET buffer (Invitrogen) was used for detection. The final concentrations of EDTA and Tb-antibody were 15 mM and 1 nM, respectively. The plates were read using Envision after 30 min of incubation at rt. Staurosporine was used as the positive control for the assay. Signal:background noise was determined, and a dose–response curve was generated for the compounds from which the IC<sub>50</sub> was determined using an XLFit inhibition model.

**NF- $\kappa$ B Assay.** Fresh human cartilage samples from OA patients undergoing knee replacement surgeries were obtained from New England Baptist Hospital, Boston, MA, with patient consent. Harvested cartilage pieces were sliced into 2 mm pieces in growth medium consisting of D-MEM/F12 (Gibco, Carlsbad, CA) + 1% antimycotic antibiotic solution (Sigma, St. Louis, MO) + 10% FBS (Sigma), washed twice with PBS, and treated with 1 mg/mL Pronase (Roche, Indianapolis, IN) at 37 °C for 1 h in growth media. Cartilage pieces were washed with media twice and treated with 0.25 mg/mL collagenase (Roche) at 37 °C for 14–15 h in serum-free media. The cell suspension was filtered through a 70  $\mu\text{m}$  nylon cell strainer (BD Falcon, Bedford, MA), centrifuged, washed, and resuspended in growth media. Cells were counted on a hemocytometer. Cell viability was determined by trypan blue dye exclusion and was found to be  $\geq 95\%$ .

NF- $\kappa$ B activation was tested by the commercial FACE NF- $\kappa$ B p65 profiler (Active Motif, Carlsbad, CA) following the manufacturer's protocol for primary cells. Optimal conditions for using primary chondrocytes to test NF- $\kappa$ B activation in response to cytokine  $\pm$  inhibitors were determined empirically. The conditions found to be optimal were plating density of 30 000 cells/well, 10 ng/mL IL-1 $\beta$

concentration, 2 h preincubation with inhibitors prior to cytokine induction, and 3 h incubation with cytokine before performing the assay. Briefly, human osteoarthritic primary chondrocytes were harvested and seeded into the 96-well ELISA plate from the kit. Inhibitors were added to the wells at various concentrations and incubated for 2 h at 37 °C. After this incubation, IL-1 was added and allowed to incubate for 3 h at 37 °C. After stimulation of the NF- $\kappa$ B pathway, the cells were fixed in order to preserve activation-specific protein modifications followed by running the NF- $\kappa$ B p65 ELISA.

Cytotoxicity testing was performed for each of the inhibitors at the concentrations used in the NF- $\kappa$ B assay either by measuring lactate levels in culture media as a measure of cellular metabolism and viability using a kit from Sigma or by directly measuring cellular proliferation using the WST-1 assay (Roche). The EC<sub>50</sub> values were determined from the dose–response curves of the signal:background ratio in an XLFit inhibition model.

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**Supporting Information Available:** <sup>1</sup>H NMR spectra of **1–4**, <sup>13</sup>C NMR spectra for **1** and **4**, and gHSQC NMR spectra for **2** and **3**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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