Structures and Absolute Configurations of Sulfate-Conjugated Triterpenoids Including an Antifungal Chemical Defense of the Green Macroalga *Tydemania expeditionis*

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Cytotoxicity-guided fractionation of the green macroalga *Tydemania expeditionis* led to isolation of four sulfate-conjugated triterpenoids including one new lanostane-type triterpenoid disulfate, lanosta-8-en-3,29-diol-23-oxo-3,29-disodium sulfate (**1**), and three known cycloartane-type triterpenoid disulfates, cycloartan-3,29-diol-23-one 3,29-disodium sulfate (**2**), cycloart-24-en-3,29-diol-23-one 3,29-disodium sulfate (**3**), and cycloartan-3,23,29-triol 3,29-disodium sulfate (**4**). Extensive 1D and 2D NMR analyses in combination with X-ray crystallography established the structure and absolute configuration of **1** and allowed determination of the absolute configurations of **2**−**4** with a revision of previously assigned configuration at C-5. Each natural product was moderately cytotoxic in tumor cell and invertebrate toxicity assays. Of the natural products, only **4** exhibited significant antifungal activity at whole-tissue natural concentrations against the marine pathogen *Lindra thalassiae*. Comparison of the biological activities of natural products with their desulfated derivatives indicated that sulfation does not appear to confer cytotoxicity or antifungal activity.

Tydemania expeditionis Weber-van Bosse (Udoteaceae) is a weakly calcified green alga distributed in the tropical Pacific and Indian Oceans. Previous studies on this species from Guam led to the isolation of three norcycloartene triterpenoids[1] and a linear diterpenoid,[2] while investigation of another population from Micronesia revealed three cycloartanol sulfates with inhibitory activity against the pp60^{v-rsc} protein tyrosine kinase.[3] However, none of these studies were related to chemical ecology. In our ongoing effort to isolate and identify ecological leads for drug discovery from marine algae, we investigated the chemical components of *T. expeditionis* collected in Fiji. Herein, we report the structure elucidation and absolute configuration of triterpenoid sulfates (**1**−**4**) from *T. expeditionis* and their ecological effects on *Lindra thalassiae*, a marine fungal pathogen known to infect some macroalgae.[4]

The high-resolution ESI mass spectrum of lanosta-8-en-3,29-diol-23-oxo-3,29-disodium sulfate (**1**) showed a quasimolecular ion $[M - Na]^-$ at *m/z* 639.2651, corresponding to $C_{30}H_{48}O_9S_2Na$ with six degrees of unsaturation. The presence of sulfur was suggested by the low density mass offset of +2 amu (^{34}S) for both parent and fragment ions and the presence of sulfate confirmed by the characteristic loss of 102 amu at *m/z* 537.3262 $[M - SO_3Na + H - Na]^-$.

The ^{13}C NMR spectrum displayed 30 signals consistent with a triterpenoid skeleton and, in combination with DEPT analysis, suggested one carbonyl, seven methyls, 11 methylenes, five methines, and six quaternary carbon atoms (Supporting Information). The low-field region of the ^{13}C NMR spectrum contained resonances for an oxo group at δ 213.0 (s, C-23), a tetrasubstituted olefin at δ 135.1 (s, C-8) and 134.4 (s, C-9), one oxymethine at δ 79.6 (d, C-3), and one oxymethylene at δ 69.0 (t, C-29). Inspection of the ^1H NMR spectrum revealed three secondary methyl groups at δ 0.89 (d, $J = 6.5$ Hz, H_3-21), 0.90 (d, $J = 7.0$ Hz, H_3-27), and 0.91 (d, $J = 7.0$ Hz, H_3-26) and four quaternary methyl groups at δ 0.77 (s, H_3-18), 0.81 (s, H_3-28), 0.92 (s, H_3-30), and 1.06 (s, H_3-19). The resonances for one oxygenated methine and one isolated oxygenated methylene were observed at δ 4.37 (dd, $J = 4.5$, 11.5 Hz, H-3), 3.96 (d, $J = 9.5$ Hz, H-29β), and 3.83 (d, $J = 9.5$ Hz, H-29α). The downfield-shifted resonances of H-3/C-3 and H_2-29/C-29 relative to typical hydroxylated methines and methylenes,[5] respectively, suggested sulfate groups at these positions.

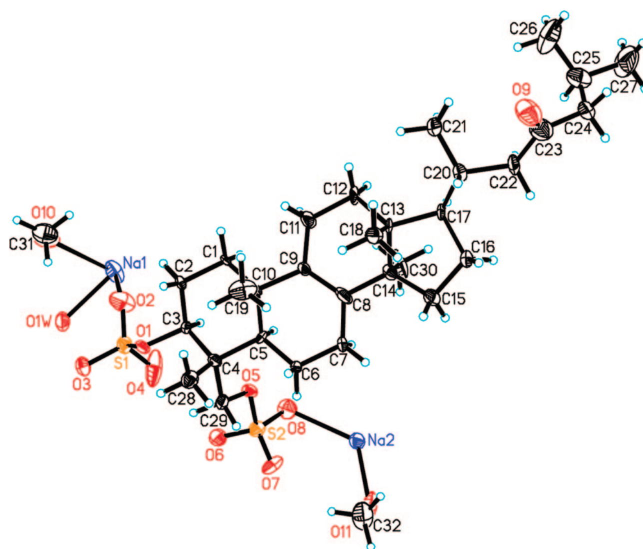

Figure 1. ORTEP view of **1**·2MeOH·H_2O from X-ray diffraction data. Atoms are shown as 30% thermal ellipsoids. Selected interatomic distances and angles: Na1−O2 2.328 Å, Na2−O8 2.525 Å, Na1−O2−S1 131.3°, Na2−O8−S2 121.4°.

The assignment and connectivity of **1** were established by $^1H-^1H$ COSY, HSQC, and HMBC spectra. The $^1H-^1H$ COSY data indicated two spin systems within the side chain, H-21↔H-20↔H-22 and H-24↔H-25↔H-26 (↔H-27). The HMBC cross-peaks H-22→C-23 and H-24→C-23 indicated that these two spin systems were connected by a carbonyl carbon (C-23), supporting a 2-methylheptan-4-one side chain. The HMBC correlation H-17→C-20

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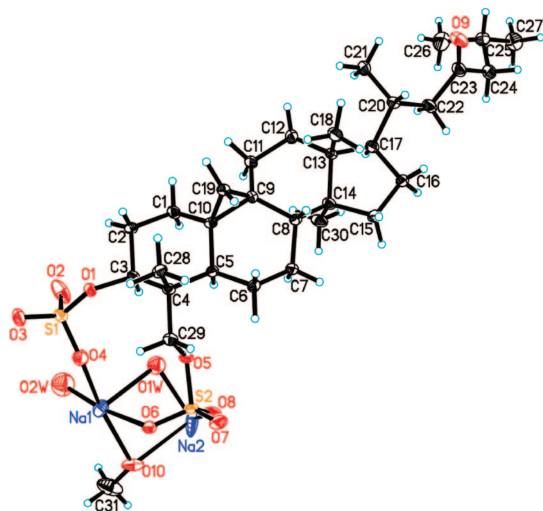


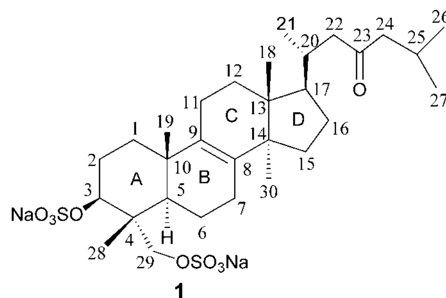
Figure 2. ORTEP view of $2 \cdot MeOH \cdot 2H_2O$ from X-ray diffraction data.

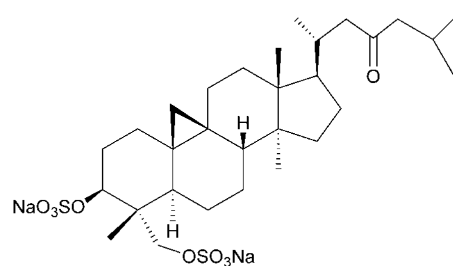
and the $^1H-^1H$ COSY cross-peak H-17↔H-20 revealed C-17↔C-20 connectivity. The location of the $\Delta^{8,9}$ double bond was confirmed by HMBC correlations H-19→C-9 and H-30→C-8. The HSQC correlation H-3 (δ 4.37)→C-3 (δ 79.6) in combination with the HMBC correlations H-3→C-4, H-3→C-28, H-1→C-3, and H-2→C-3 indicated the location of a sulfate moiety at C-3. Similarly, the HSQC correlation H_2-29 (δ 3.96 and δ 3.83)→C-29 (δ 69.0) in combination with the HMBC correlations H-29→C-3, C-4, C-5, and C-28 confirmed another sulfate moiety at C-29.

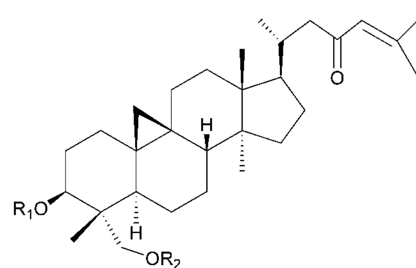
The absolute configuration of **1** was established by single-crystal X-ray analysis,[6] which revealed a stable complex constructed from two fundamental units: a triterpenoid sulfate anion and a sodium cation coordinated by one water and two methanol molecules (Figure 1 and Supporting Information). In the crystalline state, the sodium sulfate moiety formed an inorganic layer of a face-sharing NaO_6 coordination network along the (110) plane and the triterpenoid units of **1** were extended, at the junctions of O-1 and O-5, on both sides of inorganic layers to form a scaffold-like structure. Because **1** contained two sodium and two sulfur atoms exhibiting sufficient anomalous dispersion, the final refinement resulted in a Flack parameter of 0.18(5), allowing an unambiguous assignment of the absolute configuration (3*S*, 4*R*, 5*R*, 10*S*, 13*R*, 14*R*, 17*R*, 20*R*).

Triterpenoid sulfates, previously reported primarily from marine macroalgae, vary in both their triterpene cores and patterns of sulfation. Examination of previous natural product structures reveal four common types: (i) cycloartane or norcycloartane skeletons bearing one sulfate group at C-3, e.g., methyl 3β,23-dihydroxycycloart-24-en-28-oate-3-sulfate from the red alga *Tricleocarpa fragilis*;[7] (ii) cycloartane skeletons bearing one sulfate group at C-29, e.g., capisterone A from the green alga *Penicillus capitatus*;[8] (iii) cycloartane skeletons bearing two sulfate groups at C-3 and C-29, e.g., sulfated triterpenoids from green algae of the *Tuemoya* genus[9] and from *Tydemania expeditionis*[3] (also see below), and (iv) norlanostane skeletons bearing one sulfate group at C-3, e.g., methyl 3β,23-dihydroxy-29-norlanosta-8,24-dien-28-oate 3-sulfate from *Tricleocarpa fragilis*.[7] The discovery of **1** represents a fifth type, the first lanostane-type triterpenoid bearing 3,29-disodium sulfate moieties.

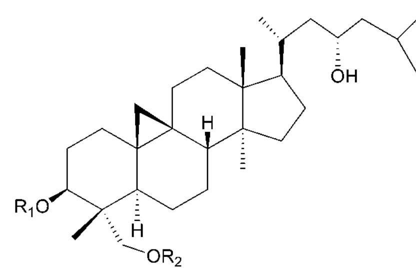
The structures of three additional compounds were identified by comparing their spectroscopic data (UV, ESIMS, ^1H and ^{13}C NMR) with a published report on the chemistry of *Tydemania expeditionis*,[3] leading to identification of cycloart-24-en-3,29-diol-23-one 3,29-disodium sulfate (**2**), cycloartan-3,29-diol-23-one 3,29-disodium sulfate (**3**), and cycloartan-3,23,29-triol 3,29-disodium sulfate (**4**). H-5 in **2**–**4** was previously reported to be β-oriented,[3] despite its characteristic axial coupling (dd, $J = 2, 7$ Hz) at δ 1.83 resulting







3 $R_1 = SO_3Na$, $R_2 = SO_3Na$
3a $R_1 = H$, $R_2 = SO_3Na$
3b $R_1 = SO_3Na$, $R_2 = H$
3c $R_1 = H$, $R_2 = H$



4 $R_1 = SO_3Na$, $R_2 = SO_3Na$
4a $R_1 = H$, $R_2 = SO_3Na$
4b $R_1 = SO_3Na$, $R_2 = H$
4c $R_1 = H$, $R_2 = H$

from interactions with two vicinal protons, one axial (H-6α) and the other equatorial (H-6β), with dihedral angles close to 180° and 60°, respectively. Accordingly, H-5 is α-oriented in compounds **2**–**4**.

X-ray diffraction analysis of **2** confirmed an α-oriented H-5 and revealed the presence of a three-membered ring at C-9 and C-10 with an average bond distance of 1.514 Å and bond angle of 60°, characteristic of cycloartanes (Figure 2 and Supporting Information). Similar to **1**, **2** contained two sodium and two sulfur atoms, and the final refinement resulted in a suitably low Flack parameter,[10] permitting assignment of its absolute configuration as 3*S*, 4*R*, 5*R*, 8*S*, 9*S*, 10*R*, 13*R*, 14*S*, 17*R*, 20*R*. We predict that **3** and **4**, which share a carbon skeleton with **2**, possess the same absolute configuration. The α-oriented hydroxy group at C-23 in cycloartanol sulfates was previously established by Mosher's method.[7] The 23-

hydroxy group in **4** was inferred to share the same α-orientation by considering the biogenetic relationship in this class of compounds.

With LC-MS, using prominent MS ion signals at discrete retention times, linear calibration curves were obtained for pure samples of **1**−**4** (r^2 = 0.99 for all, n = 6 concentrations each). Comparison of standard curve data with ion intensities for each compound within the crude extract resulted in the following estimate of natural whole-tissue concentrations: 1.38 mM (**1**), 1.20 mM (**2**), 1.07 mM (**3**), and 1.59 mM (**4**). Since this measurement was taken from only one crude extract, an assessment of variation among algal specimens was not possible.

In order to investigate the biological role of sulfation, PTSA-catalyzed sulfate ester hydrolysis was undertaken on **3** and **4** (Supporting Information). The ^{1}H NMR spectra of **3a**−**3c** were similar to that of **3** except for the high-field shifts of H-3 in **3a** (δ 3.76, dd, J = 5.3, 10.8 Hz), H-29 in **3b** (H-29β: δ 3.58, d, J = 12.0 Hz; H-29α: δ 3.34, d, J = 12.0 Hz), and both H-3 and H-29 in **3c** (H-3: δ 3.71, dd, J = 5.0, 11.5 Hz; H-29β: δ 3.58, d, J = 11.0 Hz; H-29α: δ 3.34, d, J = 11.0 Hz), which indicated that 3-*O*-sulfate, 29-*O*-sulfate, and 3,29-*O*-disulfate were converted to hydroxy groups in **3a**, **3b**, and **3c**, respectively. Accordingly, these three compounds were identified as cycloart-24-en-3,29-diol-23-one 29-sodium sulfate (**3a**), cycloart-24-en-3,29-diol-23-one 3-sodium sulfate (**3b**), and cycloart-24-en-3,29-diol-23-one (**3c**). Pseudomolecular ions with m/z 535 [M − Na]$^-$, 535 [M − Na]$^-$, and 457 [M + H]$^+$ for **3a**, **3b**, and **3c**, respectively, confirmed their structures. Similarly, monosulfates from the hydrolysis of **4** were identified as cycloartan-3,23,29-triol 29-sodium sulfate (**4a**) and cycloartan-3,23,29-triol 3-sodium sulfate (**4b**). A small amount of desulfate **4c** was suggested by LC-MS, but was not purified nor fully characterized.

When **1**, **2**, **3**, **3a**, **3b**, **3c**, and **4** were tested for inhibitory activity against a panel of 12 breast, colon, lung, prostate, and ovarian tumor cell lines, monosulfated derivatives **3a** and **3b** and desulfated **3c** showed moderate activities with mean IC_{50} values of 6.0 to 11 μM. Disulfated natural products **1**−**4** exhibited weaker antitumor effects with mean IC_{50} values ranging from 31 to 38 μM. Inhibition of invertebrate (rotifer) feeding, a proxy for cytotoxicity,[12] ranged from 9−87% when each compound was tested at 10 ppm.

In contrast to fairly uniform cytotoxic effects of **1**−**4** described above, only **4** exhibited significant antifungal activity against the marine pathogen *Lindra thalassiae* when tested at 1 mM, a concentration close to the whole-tissue natural concentrations of **1**−**4** of 1.07−1.59 mM (Figure 3A). This suggested the C-23 hydroxy group (as in **4**) was essential to antifungal activity since **2**, with a carbonyl group in this position, was not significantly active. In contrast, capisterones A and B, bearing C-23 carbonyls and differing from **2**−**4** in A-ring substitution and sulfation patterns, were active against *L. thalassiae* with low micromolar IC_{50} values.[8]

An antifungal IC_{50} value of 26 μM (17 μg/mL) was determined for **4**, indicating that *Tydemania expeditionis* contains approximately 60 times more **4** than required to suppress growth of this pathogen (Figure 3B). Monosulfates **4a** and **4b** exhibited antifungal IC_{50} values of 55 and 30 μM, respectively, similar to the disulfated parent compound **4**. As with cytotoxic activities described above, sulfation did not appear to confer antifungal activity.

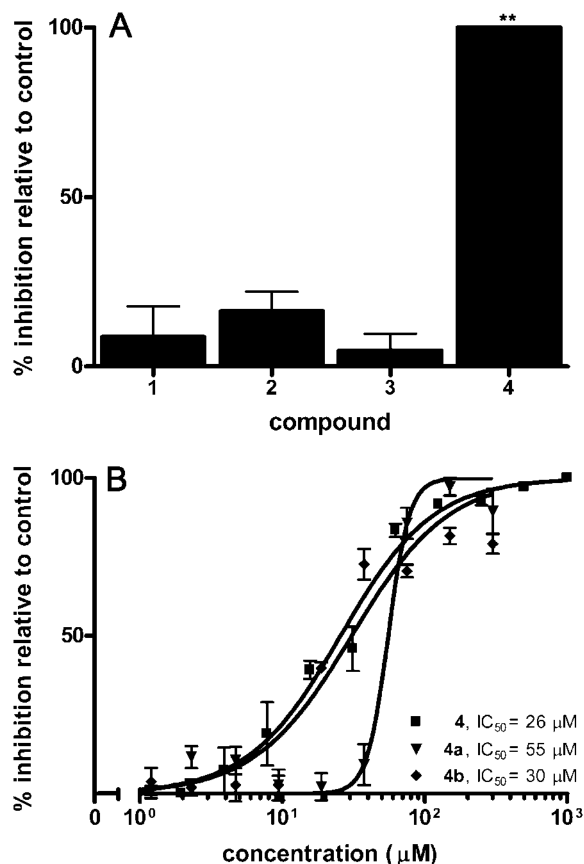


Figure 3. Ecological antifungal activity: (A) growth inhibitory effects of **1**−**4** at 0.67 mg/mL (1 mM) on *Lindra thalassiae*, showing that **4** is the only significantly active antifungal agent (bars represent one standard deviation; ** denotes p < 0.01, 1-way ANOVA with Dunnett's post test); (b) dose−response curves for **4**, **4a**, and **4b**.

Experimental Section

General Experimental Procedures. HPLC separations were completed and UV spectra recorded with a Waters 2695 HPLC system and Waters 2996 diode-array detector. Optical rotations were measured with a Jasco P-1010 spectropolarimeter. NMR spectra (^{1}H, ^{13}C, DEPT, COSY, HSQC, HMBC, and NOESY) were acquired on a Bruker DRX-500 instrument, using a 5 mm broadband or inverse detection probe. HRMS were generated by electrospray ionization with an Applied Biosystems QSTAR-XL hybrid quadrupole time-of-flight tandem mass spectrometer and Analyst QS software.

Collection. *T. expeditionis* (tuft form) was collected from depths of 7−20 m at Herald Pass, Kadavu Province, Fiji (18°46.370′ S, 178°27.746′ E) and frozen at −20 °C until extraction (collection G-2004-06-45). A voucher specimen was identified by Dr. Posa Skelton at the University of the South Pacific by comparison with previously described morphological traits,[11] stored in aqueous formaldehyde, and deposited at the University of the South Pacific.

Isolation Guided by Rotifer Ingestion Toxicity Assay. The whole plant (500 g, wet mass) was exhaustively extracted with MeOH and MeOH−CH_2Cl_2 to afford a crude extract, which was partitioned between MeOH−H_2O (9:1) and hexanes. The aqueous fraction was successively partitioned against EtOAc and *n*-BuOH to afford hexanes-, EtOAc-, *n*-BuOH- and H_2O-soluble fractions. The ethyl acetate-soluble fraction showed the most potent toxicity. Further fractionation was accomplished by semipreparative reversed-phase HPLC, yielding four compounds: **1** (15 mg), **2** (9 mg), **3** (12 mg), and **4** (15 mg). Crystals of **1** and **2** were obtained by recrystallization from MeOH (for details see Supporting Information).

Determination of Natural Concentrations of 1−4. Pure **1**−**4** were solubilized in MeOH, diluted to six concentration levels, and analyzed by LC-MS (Supporting Information). Calibration curves were constructed from peak areas of appropriate molecular ions. Concentrations of **1**−**4** in the crude extract were calculated from corresponding standard curves.

Rotifer Ingestion Toxicity Assay. Experiments were performed in 24-well microtiter plates using previously described procedures.[12]

Antitumor Assay. Compounds were tested against a panel of tumor cell lines: breast cancer (BT-549, DU4475, MDA-MB-468, MDA-MB-231), colon cancer (HCT116), lung cancer (NCI-H446 and SHP-77), prostate cancer (PC-3, LNCaP-FGC, Du145), ovarian cancer (A2780/DDP-S), and leukemia (CCRF-CEM). Cells were seeded in 96-well plates and compounds added 24 h later. After 72 h of exposure, cells were stained with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium inner salt and quantified spectrophotometrically. Conversion of the MTS dye in the mito-

chondria was directly proportional to live cells. Dose−response curves were generated and IC_{50} values calculated for each compound and cell line, with a mean IC_{50} value determined for the 12 cell line panel.

Antifungal Assay. The antifungal activity of isolated compounds was evaluated against a marine pathogen, *Lindra thalassiae* (ATCC 56663), using a procedure similar to that described previously.[13]

Lanosta-8-en-3,29-diol-23-oxo-3,29-disodium sulfate (1): Colorless powder; $[\alpha]^{20}_{D}$ +50.0 (*c* 0.02 in CH_3OH); UV δ_{max}^{MeOH} 210 nm; ^{1}H NMR (CD_3OD) 4.37 (1H, dd, J = 4.5, 11.5 Hz, H-3), 3.96 (1H, d, J = 9.5 Hz, H-29β), 3.83 (1H, d, J = 9.5 Hz, H-29α), 2.48 (1H, dd, J = 2.5, 16 Hz, H-22β), 2.30 (2H, d, J = 7 Hz, H-24), 2.21 (1H, m, H-2α), 2.17 (1H, dd, J = 1.0, 16 Hz, H-22α), 2.09 (1H, m, H-25), 2.07 (1H, m, H-11α), 2.01 (2H, m, H-7), 1.98 (1H, m, H-20), 1.93 (1H, m, H-16α), 1.82 (1H, m, H-6β), 1.79 (1H, m, H-11β), 1.78 (1H, m, H-2β), 1.77 (2H, m, H-12), 1.74 (1H, m, H-1α), 1.70 (1H, dd, J = 2, 10.5 Hz, H-5), 1.65 (1H, m, H-15β), 1.56 (1H, m, H-17), 1.52 (1H, m, H-6α), 1.30 (1H, m, H-16β), 1.22 (1H, m, H-1β), 1.19 (1H, m, H-15α), 1.06 (3H, s, H-19), 0.92 (3H, s, H-30), 0.91 (3H, d, J = 7.0 Hz, H-26), 0.90 (3H, d, J = 7.5 Hz, H-27), 0.89 (3H, d, J = 7.0 Hz, H-21), 0.81 (3H, s, H-28), 0.77 (3H, s, H-18); ^{13}C NMR (CH_3OH-d_4) 213.0 (s, C-23), 135.1 (s, C-8), 134.4 (s, C-9), 79.6 (d, C-3), 69.0 (t, C-29), 52.3 (t, C-24), 50.7 (d, C-17), 50.5 (t, C-22), 50.2 (s, C-14), 44.8 (s, C-13), 43.2 (d, C-5), 41.8 (s, C-4), 36.9 (s, C-10), 35.2 (t, C-1), 33.4 (d, C-20), 31.2 (t, C-12), 30.9 (t, C-15), 28.4 (t, C-16), 26.0 (t, C-7), 24.6 (d, C-25), 24.2 (t, C-2), 23.5 (q, C-30), 21.9 (q, C-26), 21.8 (q, C-27), 21.0 (t, C-6), 19.2 (q, C-21), 18.9 (q, C-19), 17.8 (t, C-11), 15.4 (q, C-18), 12.4 (q, C-28); ESI-MS *m/z* 639 $[M - Na]^-$, 617 $[M - 2Na + H]^-$, 537 $[M - SO_3Na + H - Na]^-$, 519 $[M - SO_3Na + H - Na - H_2O]^-$, 308 $[M - 2Na]^{2-}$; HRESIMS *m/z* $[M - Na]^-$ 639.2651, calcd for $C_{30}H_{48}O_9S_2Na$ 639.2643.

***p*-Toluenesulfonic Acid (PTSA)-Catalyzed Sulfate Ester Hydrolysis.** Compounds **3** and **4** were hydrolyzed according to the reported procedures,[14] as reported in the Supporting Information.

Crystal data of **1** and **2** in standard CIF format have been deposited with the Cambridge Crystallographic Data Centre with CCDC numbers as shown in refs 6 and 10. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-1223-336033; e-mail: deposit@ccdc.cam.ac.uk).

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Supporting Information Available: Overview of natural sulfate bioconjugates; isolation details; X-ray analysis; derivatization of natural products; LC-MS analysis; ^{1}H and ^{13}C NMR spectra for **1**; X-ray crystal structure diagrams; mass spectrum of **3**; and proposed fragmentation pathway. This material is available free of charge via the Internet at http://pubs.acs.org.

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(6) Crystal data for compound **1**. $C_{30}H_{48}O_9S_2Na_2 \cdot 2CH_3OH \cdot H_2O$, colorless plates (0.28 × 0.21 × 0.11 mm) were recrystallized from MeOH solution, orthorhombic, $P2_12_12_1$, a = 7.1495(5) Å, b = 10.4548(7) Å, c = 49.990(3) Å, V = 3736.6(4) Å^3, Z = 4, d_x = 1.317 g cm^{-3}, $F(000)$ = 1584, λ(CuKα) 1.54178 Å, Flack parameter 0.18(5). Data collection was performed on a SMART 1000 CCD; 5594 [R(int) 0.0409] unique reflections were collected to θ_{max} = 65.9°, in which 5110 reflections were observed [$F^2 > 4\sigma(F^2)$]. The crystal structure was resolved by direct methods using SHELXS-97. In the final stage, R = 0.105 and S = 1.094. CCDC no. 686956.
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(10) Crystal data for compound **2**. $C_{30}H_{46}O_9S_2Na_2 \cdot CH_3OH \cdot 2H_2O$, colorless plates (0.25 × 0.21 × 0.08 mm) were recrystallized from MeOH solution, monoclinic, $P2_1$, a = 6.899(1) Å, b = 9.952(1) Å, c = 26.382(3) Å, β = 91.73(1)°, V = 1810.4(4) Å^3, Z = 2, d_x = 1.331 g cm^{-3}, $F(000)$ = 774, λ(Cu Kα) 1.54178 Å, Flack parameter 0.05(3). Data collection was performed on a SMART 1000 CCD; 4376 [R(int)) 0.0348] unique reflections were collected to θ_{max} = 66.11°, in which 4069 reflections were observed [$F^2 > 4\sigma(F^2)$]. The crystal structure was resolved by direct methods using SHELXS-97. In the final stage, R = 0.061 and S = 1.080. CCDC no. 686957.
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