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Eunicidiol, an Anti-inflammatory Dilophol Diterpene from *Eunicea fusca*

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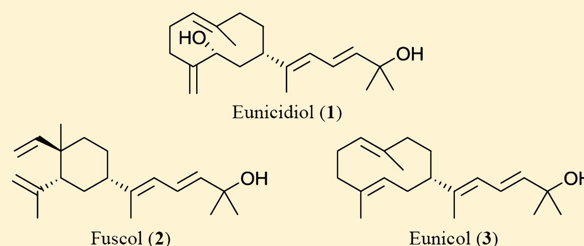
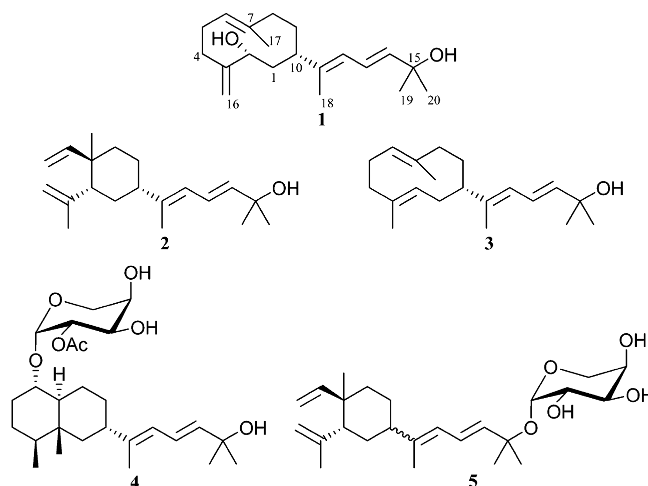
Supporting Information

ABSTRACT: A new dilophol diterpene, eunicidiol (**1**), has been isolated from the crude extract of *Eunicea fusca*, a gorgonian coral collected from Hillsboro Ledge, Florida. This compound was purified, along with fuscol (**2**) and eunicol (**3**), using a combination of normal- and reversed-phase chromatography methods. The structure of eunicidiol (**1**) was elucidated by 1D and 2D NMR spectroscopic analysis, and the absolute configuration was assigned using Mosher's method. The anti-inflammatory activity of **1**−**3** was evaluated by measuring their ability to reduce phorbol myristate acetate (PMA)-induced edema in a mouse ear model. Topical application of a 100 μg/ear dose of diterpenes **1**−**3** significantly reduced edema by 44%, 46%, and 54%, respectively. This activity was superior to indomethacin, a known anti-inflammatory used as a control.



Marine corals belonging to the order Gorgonacea have provided a wealth of structurally diverse diterpenes with potential application as therapeutic agents.[1] The Caribbean gorgonian *Eunicea fusca* is a source of the anti-inflammatory fuscosides A (**4**) and B (**5**), diterpene arabinose glycosides that reduce phorbol myristate acetate (PMA)-induced mouse ear edema with activities comparable to indomethacin.[2,3] Fuscoside B is known for its selective inhibition of 5-lipoxygenase and negligible effect on cyclooxygenases and prostaglandin biosynthesis.[4] As part of our interest in this glycoside, we recently synthesized a small library of 1,2-*trans* glycoside analogues of fuscoside B, including a novel class of dilophol glycosides called the eunicosides, by glycosylating the tertiary hydroxy groups of fuscol (**2**) and eunicol (**3**).[5] In addition we have also investigated the biosynthesis of **2**−**4** by examining the metabolism of ^{3}H-geranylgeranyl diphosphate by *E. fusca* cell-free extracts.[6]

We have continued our search for new anti-inflammatory compounds from *E. fusca* by examining minor constituents displaying structural homology to **2**, **4**, and **5**. We report herein the characterization of eunicidiol (**1**) using NMR spectroscopy and mass spectrometry. This diterpene is composed of an uncommon dilophol carbon skeleton that consists of a cyclodecane ring as well as an eight-carbon side chain, which is analogous to the structure of fuscol (**2**) and eunicol (**3**). While the relative configuration of **1** was obtained by NOESY correlations, the absolute configuration was solved by Mosher's method using α-methoxyphenylacetic acid (MPA). Furthermore, an enantiospecific Cope rearrangement of the cyclodecadiene moiety of **3** to produce **2** has firmly established the absolute configuration of **3**. Diterpenes **1**−**3** were evaluated for in vivo anti-inflammatory activity by assessing their ability to reduce PMA-induced edema in a mouse ear model.



RESULTS AND DISCUSSION

Eunicidiol (**1**) was obtained, along with known compounds fuscol (**2**) and eunicol (**3**), from the crude extract of *E. fusca* and purified by semipreparative reversed-phase high-performance liquid chromatography (RP-HPLC) using a combination of C_{18} and phenylhexyl stationary phases. The molecular formula of **1** was established by HRESIMS analysis (m/z 327.2286 $[M + Na]^{+}$),

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indicating the molecular formula $C_{20}H_{32}O_2$ and an index of hydrogen deficiency of five. The existence of hydroxy groups was reflected by the IR spectrum (3357 cm^{-1}) and the two oxygen-bearing carbon resonances (δ_C 76.2, 70.4). The NMR spectroscopic data for **1** were acquired using benzene-d_6, as other solvents, including methanol-d_4, dichloromethane-d_2, and acetone-d_6, produced poorly resolved NMR spectra with broader and weaker signals. Notably, such signal broadening was also observed in the ^{1}H and ^{13}C NMR spectra of **3**, as a consequence of conformational isomerism of the cyclodecane ring (Figures S17, S18).

The ^{1}H NMR spectrum of **1** indicated a conjugated diene system, identical to that of **2** and **3**, comprising the olefinic resonances of H-13 [δ_H 6.60 (1H, dd, J = 15.1, 10.8 Hz)], H-12 [δ_H 5.99 (1H, d, J = 10.8 Hz)], and H-14 [δ_H 5.72 (1H, d, J = 15.1 Hz)] (Table 1). The HMBC spectrum showed correlations between H-14 and C-19/20 (δ_C 30.2) and C-15 (δ_C 70.4), indicating the presence of geminal dimethyl groups and a tertiary hydroxy group. The C-20 methyl protons (δ_H 1.24) displayed HMBC correlations with C-19 and C-15 to further establish this moiety. The olefinic proton H-12 exhibited HMBC coupling with C-10 (δ_C 44.8) and C-18 (δ_C 13.9), confirming that the side chain of **1** was the same as that of **2** and **3**. The C-10 methine proton showed COSY correlations with the C-1 methylene protons (δ_H 1.60), thus providing partial structure A (Figure 1).

Table 1. NMR Spectroscopic Data (600 MHz, Benzene-d_6) for Eunicidiol (1) [δ in ppm Relative to Residual Solvent Signal][a]

position	δ_C, type	δ_H, multiplicity (J in Hz)	COSY[b]	HMBC[c]	NOESY[b]
1	36.9, CH_2	1.60, br m	10		2
2	76.2, CH	3.87, br d (7.7)			1, 10, 12, 16a
3	150.0, C				
4a	25.7, CH_2	2.20, app. td (12.3, 3.9)	4b, 16b	2, 3, 5, 16	16b, 17
4b		2.05, m	4a, 5a, 16b		16b
5a	25.8, CH_2	2.29, m	4b, 5b, 6		
5b		2.08, m	5a		
6	125.9, CH	5.45, m	5a		8a, 10
7	133.5, C				
8a	40.6, CH_2	1.93, m	9		6
8b		2.01, m			9
9	31.2, CH_2	1.29, m	8a, 10		8b
10	44.8, CH	1.86, app. t (8.5)	1, 9	2, 8, 9, 11, 12, 18	2, 6, 12
11	141.9, C				
12	124.8, CH	5.99, d (10.8)	13, 18	10, 14, 18	1, 10, 14
13	123.2, CH	6.60, dd (15.1, 10.8)	12, 14	11, 15, 19, 20	18, 19, 20
14	140.1, CH	5.72, d (15.1)	13	12, 15, 19, 20	12, 19, 20
15	70.4, C				
16a	114.7, CH_2	5.04, br s	4a, 16b		2
16b		4.94, s	4b, 16a	2, 3, 4	4a, 4b
17	15.7, CH_3	1.53, s		5, 6, 7, 8, 9	4a
18	13.9, CH_3	1.67, s	12	10, 11, 12	13
19/20	30.2, CH_3	1.24, s		13, 14, 15, 19, 20	13, 14

[a]See Figures S1−S8 for NMR spectra. [b]COSY and NOESY correlations are from proton(s) stated to the indicated proton. [c]HMBC correlations are from proton(s) stated to the indicated carbon.

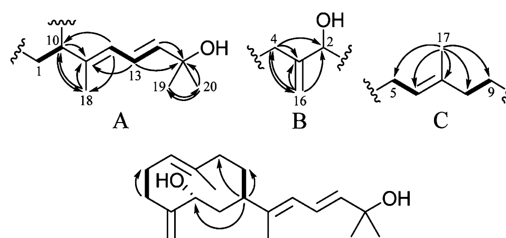

Figure 1. Selected HMBC (^{1}H → ^{13}C) and COSY correlations (bold bonds) of partial structures A−C as well as the assembled structure of eunicidiol (**1**).

Owing to the lack of unambiguous coupling of partial structure A to other nuclei, additional partial structures were elucidated independently.

The C-3 (δ_C 150.0) and C-16 (δ_C 114.7) resonances were assigned by HMBC and HSQC coupling with terminal olefinic methylene protons H-16a/b (δ_H 5.04, 4.94). Long-range heteronuclear correlations between H-16b and C-4 (δ_C 25.7) and C-2 (δ_C 76.2) established the 1,1-disubstituted double bond. The incorporation of a secondary hydroxy group at C-2 was established on the basis of the deshielded proton (δ_H 3.87) and carbon chemical shifts. This analysis provided a four-carbon segment referred to as partial structure B. Partial structure C was composed of a trisubstituted double bond according to long-range HMBC correlations between the remaining olefinic C-17 methyl group (δ_H 1.53) and multiple carbon resonances [C-5 (δ_C 25.8), C-9 (δ_C 31.2), C-6 (δ_C 125.9), C-7 (δ_C 133.5), and C-8 (δ_C 40.6)]. The COSY spectrum indicated a vicinal correlation between the C-6 olefinic methine (δ_H 5.45) and C-5 methylene (δ_H 2.29) as well as between H-8a (δ_H 1.93) and H-9 (δ_H 1.29).

The three partial structures must assemble to provide a 10-membered ring to satisfy the required index of hydrogen deficiency of five. The HMBC coupling between H-10 and C-2 provided the basis for connecting partial structures A and B at C-1 and C-2. In addition, the C-10 methine proton of partial structure A displayed a COSY correlation with the C-9 methylene protons of partial structure C to signify the attachment of C-9 and C-10. Moreover, the HMBC correlations between H-10 and C-8 and C-9 confirmed this assembly. It was not possible to establish the attachment of C-4 and C-5 via COSY correlations as a result of the inability to discern between cross-peaks caused by the vicinal correlation between H-4a/H-5b and the geminal correlation between H-4a/H-4b. Instead, this assignment was made on the basis of an HMBC correlation between H-4 and C-5. The results of a TOCSY experiment agreed with the assignment of the methylene proton resonances to the two separate spin systems of the cyclodecane ring (Figure S8).

The large vicinal coupling constant (J = 15.1 Hz) across the $\Delta^{13,14}$ double bond indicated the E configuration. The $\Delta^{11,12}$ and $\Delta^{6,7}$ double bonds were also assigned E configurations given the observed NOESY correlations between H-18/H-13 and H-6/H-8a. The determination of relative configuration was also enabled by interpretation of NOESY correlations. The observed NOESY correlation between H-6 and H-10 suggested that both protons occupied the same side of the average plane of the ring. Meanwhile, the apparent lack of NOESY correlations between H-2/H-4b and H-2/H-6 implied the opposite orientation for H-2. This configuration was upheld by the NOESY correlation between H-2/H-12. The observed NOESY correlations were in agreement with the MM2-minimized model of eunicidiol (**1**)

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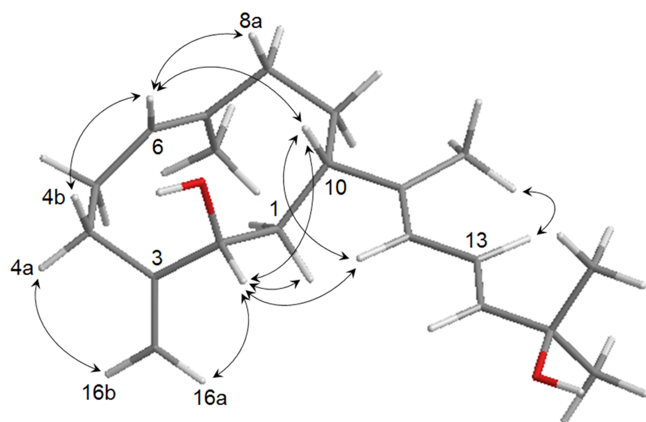
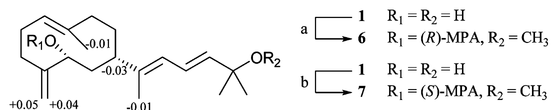


Figure 2. Key NOESY correlations ($^{1}H \leftrightarrow ^{1}H$) for eunicidiol (**1**) [ChemBioDraw 3D MM2-minimized model].

(Figure 2). Compound **1** and fuscol (**2**) likely have a common biosynthetic precursor and, therefore, presumably have the same configuration at C-10. As a result, the relative configuration of **1** was determined to be 2*R**,10*S**.

The absolute configuration of eunicidiol (**1**) was unambiguously assigned by modifying the asymmetric secondary hydroxy group with (*R*)- and (*S*)-α-methoxyphenylacetic acid (Scheme 1).

Scheme 1. Synthesis of the (*R*)- and (*S*)-MPA Esters of Eunicidiol and ^{1}H NMR Chemical Shift Differences $\Delta\delta^{R-S}$ ($\delta^{R} - \delta^{S}$) in ppm[a]

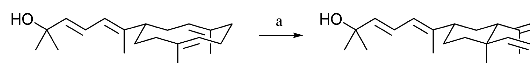


[a]Reagents and conditions: (a) (i) (*R*)-MPA, DCC, DMAP, DCM, rt, (ii) MeOH. (b) (i) (*S*)-MPA, DCC, DMAP, DCM, rt, (ii) MeOH. See Figures S11, S12 for ^{1}H NMR spectra.

While the (*R*)- and (*S*)-MPA esters of eunicidiol (**6** and **7**) were successfully synthesized using submilligram quantities of **1**, the tertiary allylic hydroxy group was transformed to its methyl ether during the reaction workup. The scarcity of **1** precluded any effort to synthesize MPA esters without methyl ethers; however such efforts were deemed unnecessary, as substitution of the achiral tertiary hydroxy group was not expected to affect the analysis. Compared to **7**, H-10, H-17, and H-18 of **6** experienced greater shielding from the phenyl ring, while H-16a/b were less shielded. As a result, the $\Delta\delta^{R-S}$ values indicated the 2*R* configuration, providing the absolute configuration of 2*R*,10*S*.

Cope rearrangements of germacrenes and germacrenolides to elemenes and elemenolides, respectively, are known to proceed with high stereospecificity and retention of the configuration at C-10.[7] While the absolute configuration of fuscol (**2**) has been established,[8] the configuration of eunicol (**3**) was assumed to be 10*S* given its role as a biosynthetic precursor to **2**.[6] To unambiguously assign the absolute configuration of **3**, we enabled an enantiospecific Cope rearrangement of the cyclodecadiene moiety of **3** (Scheme 2). The ^{1}H and ^{13}C NMR spectra of the rearranged product were identical to those of **2** (Figure S13−S16), indicating the 2*R*,7*R*,10*S* configuration or its enantiomer. The absolute configuration of 2*R*,7*R*,10*S* was ascertained by comparing the specific rotation of the rearranged product with that of **2**. This observation is in agreement with eunicol having the 10*S* configuration, and for that reason the absolute configuration of eunicol was established as such. We also suggest that the configurations of fuscoside A (**4**) and eunicene A (**8**) are consistent with their biosynthetic relatives **2** and **3** and therefore have the absolute configurations displayed in Scheme 3.

Scheme 2. Semisynthesis of Fuscol (2) by Cope Rearrangement of Eunicol (3)[a]



[a]Reagents and conditions: (a) Δ toluene, 2.5 h (38%).

We have a long-standing interest in the biosynthesis of marine diterpenes and have previously shown that in *E. fusca* the diterpene cyclase product is eunicene A (**8**) and the first oxidation product is eunicol (**3**).[6] Compound **3** leads to both fuscoside A (**4**) and fuscol (**2**). It seems reasonable to suggest that eunicidiol (**1**) is derived from an oxidation of **3** as outlined in Scheme 3.

Fuscosides A (**4**) and B (**5**) are known to exhibit topical anti-inflammatory activity, as they reduce PMA-induced mouse ear edema by 85% and 52%, respectively, and are comparable to the known anti-inflammatory agent indomethacin.[3] Fuscoside B (**5**) is further distinguished as a selective 5-lipoxygenase (5-LO) inhibitor, having a negligible effect on cyclooxygenase and other targets in the inflammatory cascade.[4]

Diterpenes **1**−**3** were evaluated for in vivo anti-inflammatory activity in the mouse ear edema assay at a single dose (100 μg/ear) (Table 2). Indomethacin was administered as a reference anti-inflammatory agent (3 mg/ear). Topical application of diterpenes **1**−**3** significantly reduced mouse ear edema at 24 h post-treatment. Although comparable to indomethacin, the reduction of edema by **1**−**3** was notably achieved with much smaller doses. Eunicol (**3**) displayed a modest increase in potency over fuscol (**2**) and eunicidiol (**1**) at 24 h; however the difference between the diterpenes was not statistically significant. In addition, compounds **1**−**3** displayed no signs of toxicity and otherwise appeared to be safe at the administered dose. An evaluation of structure−activity relationships has implicated the tertiary hydroxy group and conjugated diene moiety as being important for the observed anti-inflammatory activity, as this is the most common structural feature of the diterpenes tested. It remains to be determined whether diterpenes **1**−**3** operate through a similar mechanism of action as fuscoside B (**5**) as inhibitors of 5-LO.

■ EXPERIMENTAL SECTION

General Experimental Procedures. Medium-pressure liquid chromatography (MPLC) was performed over RediSep columns using a Teledyne Combiflash Rf, while all flash chromatography was carried out on silica gel (230−400 mesh, Fisher). Separation of products by HPLC was carried out using Phenomenex Luna Phenyl-Hexyl (250 × 10 mm, 5 μm) or Phenomenex Gemini C_{18} (250 × 10 mm, 5 μm) reversed-phase semipreparative columns. All ^{1}H and ^{13}C NMR spectra were acquired on a Bruker 600 MHz NMR spectrometer operating at 600 and 150 MHz, respectively. All chemical shifts are reported in δ units and were relative to the residual solvent signal of benzene-d_6 (^{1}H, 7.15 ppm; ^{13}C, 128.02 ppm). Coupling constants are reported with the abbreviations (s) singlet, (d) doublet, (t) triplet, (m) multiplet, (br) broad, (app.) apparent. High-resolution mass spectra were measured on a Thermo Scientific Exactive mass spectrometer with an electrospray ionization source. Optical rotations were measured on a Rudolph Autopol III polarimeter. Infrared spectra were acquired using attenuated total reflectance on a Thermo Nicolet 6700 FT-IR spectrometer.

Scheme 3. Biosynthesis of Diterpenes in *E. fusca* with Proposed Origin of Eunicidiol (1)

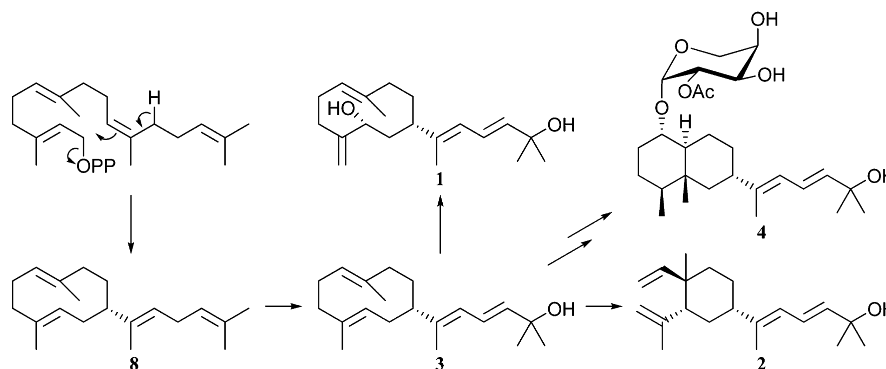


Table 2. Reduction of PMA-Induced Mouse Ear Edema by Diterpenes 1−3

group	dosage (mg/ear)	reduction of ear edema (%)[a,b]
indomethacin	3.0	51.4 ± 2.7[c]
eunicidiol (1)	0.1	44.4 ± 3.2[c]
fuscol (2)	0.1	46.3 ± 3.7[c]
eunicol (3)	0.1	53.7 ± 4.9[c]

[a]Data are presented as the mean ± SEM. [b]Percent reduction of ear edema is relative to the control group. [c]Statistically significant difference relative to the control group, unpaired Student's *t*-test ($p < 0.05$).

Analysis of samples by LC with hyphenated MS-ELSD-UV was performed using a Finnigan LXQ ion trap mass spectrometer and an analytical C_{18} RP-HPLC column.

Isolation of Fuscol (2) and Eunicol (3). *E. fusca* was collected from Hillsboro Ledge, Florida by scuba, air-dried at the surface, and kept frozen during transportation. The identity of each coral was confirmed by comparing the TLC profile to that of a pure sample of **2**. After lyophilization, the gorgonians (124.6 g) were exhaustively extracted with DCM, and the combined extracts were concentrated to provide a viscous oil (11.47 g). The crude extract was initially partitioned between hexanes and MeOH/H_2O (9:1); afterward the hexanes layer was evaporated (6.319 g) and separated by vacuum flash chromatography using a stepwise gradient elution with hexanes/EtOAc. The hexanes/EtOAc (4:1) fraction (592.0 mg) contained **2** and **3** according to LC-MS analysis. This fraction was separated by C_{18} RP-MPLC (MeOH/H_2O gradient) to provide a mixture of **2** and **3** (289.9 mg, 2.3% of dry coral weight). The diterpenes were separated by phenylhexyl RP-HPLC (MeOH/H_2O, 87:13, 3.0 mL/min, **2** retention time (t_R) 24.8 min, **3** t_R 26.7 min) and further purified by C_{18} RP-HPLC (MeOH/H_2O, 83:17, 3.0 mL/min, **2** t_R 37.6 min, **3** t_R 39.6 min). For **2**: HRESIMS *m/z* 311.2346 $[M + Na]^+$ (calcd for $C_{20}H_{32}ONa$, 311.2345). For **3**: HRESIMS *m/z* 311.2346 $[M + Na]^+$ (calcd for $C_{20}H_{32}ONa$, 311.2345). All ^1H and ^{13}C NMR spectroscopic data were in total agreement with the literature (Figures S13, S15, S17, and S18).[6,9]

Isolation of Eunicidiol (1). Eunicidiol (**1**) was present in the hexanes/EtOAc (4:1) fraction and collected alongside **2** and **3** during the above-mentioned C_{18} RP-MPLC separation. The compound was purified by a combination of phenylhexyl RP-HPLC (MeOH/H_2O, 77:23, 3.0 mL/min, t_R 31.1 min) and C_{18} RP-HPLC (MeOH/H_2O, 80:20, 3.0 mL/min, t_R 21.5 min) to provide a colorless oil (**1**, 1.5 mg). Eunicidiol (**1**): $[\alpha]^{25}_D = -33.4$ (*c* 0.02 in MeOH); IR ν_{max} 3357, 3064, 3039, 2971, 2925, 2857, 1645, 1450, 1385, 1371, 1149, 1021, 968, 899; HRESIMS *m/z* 327.2286 $[M + Na]^+$ (calcd for $C_{20}H_{32}O_2Na$, 327.2295). NMR spectroscopic data (Figures S1−S8) are summarized in Table 1.

Synthesis of (*R*)- and (*S*)-MPA Esters of Eunicidiol (6 and 7). A solution containing eunicidiol (**1**) (100 μg, 0.33 μmol), DMAP (3.8 mg, 31.1 μmol), DCC (20.0 mg, 96.9 μmol), and 3 Å molecular sieves was stirred in freshly distilled DCM (3 mL) under an N_2 atmosphere for 20 min. Subsequently, an excess of (*R*)- or (*S*)-MPA (13.8 mg, 83.0 μmol) was added and the reaction was left to stir overnight at room temperature. The reaction mixture was concentrated under a stream of N_2 and eluted through a plug of C_{18} using MeOH. The filtrate was evaporated in vacuo to provide the reaction crude, which was separated by C_{18} RP-HPLC using a gradient of MeOH/H_2O (90:10 for 5 min, increasing linearly to 100:0 at 30 min, 3.0 mL/min, **6**/**7** t_R 15.2 min). For **6**: HRESIMS *m/z* 489.2980 $[M + Na]^+$ (calcd for $C_{30}H_{42}O_4Na$, 489.2975). For **7**: HRESIMS *m/z* 489.2973 $[M + Na]^+$ (calcd for $C_{30}H_{42}O_4Na$, 489.2975). See Figures S11 and S12 for ^1H NMR spectra of **6** and **7**. Product yields could not be determined with accuracy owing to the small scale of the reactions.

Semisynthesis of Fuscol (2). A purified sample of eunicol (**3**) (1.01 mg, 3.49 μmol) was refluxed in toluene for 2.5 h and evaporated in vacuo. Analysis of the reaction crude by NMR revealed the formation of fuscol (**2**) (1.28 μmol, 38%), which was later purified by C_{18} RP-HPLC (MeOH/H_2O, 88:12, 3.0 mL/min, t_R 25.6 min). Semisynthetic fuscol (**2**): $[\alpha]^{25}_D = +28.8$ (*c* 0.02 in $CHCl_3$). Isolated fuscol (**2**): $[\alpha]^{25}_D = +20.4$ (*c* 0.06 in $CHCl_3$). Fuscol (lit.): $[\alpha]_D = +21.0$ (*c* 0.9 in $CHCl_3$).[8b] See Figures S13−S16 for a comparison of ^1H and ^{13}C NMR spectra.

In Vivo Mouse Ear Edema Assay. The assay was completed by Amplia PharmaTek Inc. as previously described by Martinez et al.[10] The 6−7-week-old female CD-1 mice (Charles River Canada Inc.) were provided with food and water ad libitum and acclimatized for at least 5 days. PMA (100 μg/mL, $n = 6$), indomethacin (150 mg/mL, $n = 6$), and diterpenes **1**−**3** (5 mg/mL, $n = 3$) were formulated in acetone (PMA) and DMSO (indomethacin and diterpenes). Initially, the right ears were treated with DMSO (20 μL, vehicle control), indomethacin (3 mg in 20 μL of DMSO), or diterpene (100 μg in 20 μL of DMSO), while the left ears received vehicle (20 μL of DMSO). One hour later, the phlogistic agent, PMA (2 μg, 20 μL of acetone), was administered by topical application to the right ears and the left ears received vehicle (20 μL of acetone). Edema was measured at 6 and 24 h post-PMA treatment using a digital caliper and calculated by subtracting the thickness of the left ear from the right ear. The data are given as the mean ± SEM in terms of percent reduction of edema. The statistical significance of the comparison between test groups and the PMA control was assessed by Student's unpaired *t*-test. A *p* value < 0.05 was considered to indicate statistical significance.

■ ASSOCIATED CONTENT

Supporting Information

NMR spectra and MS data for diterpenes **1**−**3** and MPA esters **6** and **7** are available. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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