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New Antiproliferative and Antiinflammatory 9,11-Secosterols from the Gorgonian *Pseudopterogorgia* sp.

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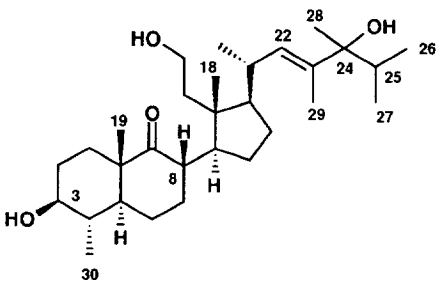
Abstract. Protein kinase C assay-guided study of the extracts of *Pseudopterogorgia* sp. led to the isolation of three new 9,11-secosterols **1-3**, in addition to a known secosterol **4**. The latter was converted to semi-synthetic sterols **6** and **7**, and the structures of these were determined by the analysis of NMR and MS data. Secosterols **1-3**, **6** and **7** exhibited moderate inhibitory activity against protein kinase C, and **6** showed potent antiproliferative and antiinflammatory activity.

Although marine invertebrates have proven to be prolific producers of novel sterols, relatively few secosterols have been isolated so far.[1-9] For the several examples of the known secosterols, only *in vitro* cytotoxicity[8,9] and ichthyotoxicity[3] have been reported. As part of a continuing program to discover novel and potent protein kinase C (PKC) modulators from natural sources[10] as potential leads for cancer and inflammatory diseases, we have examined a number of marine invertebrates collected in the Florida Keys.[11] In this article, we report the isolation and structure elucidation of three new 9,11-secosterols **1-3** and a known secosterol **4** from the gorgonian *Pseudopterogorgia* sp. collected in the channel between Islemorada and Long Keys. The conversion of secosterol **4** to **6** and **7** and the biological activity of the above mentioned secosterols are also presented.



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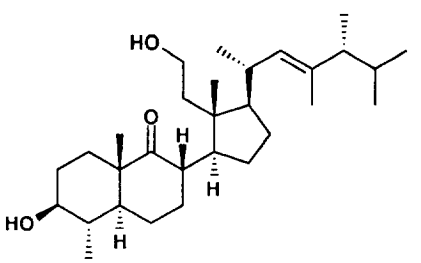

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RESULTS AND DISCUSSION

A lyophilized sample of the gorgonian was extracted exhaustively with MeOH, and the extract was partitioned between water and 2:1 $CHCl_3$-MeOH. The organic-soluble fraction was subjected to gel filtration on Sephadex LH-20 (MeOH), followed by silica gel column chromatography and reversed-phase HPLC to afford three new secosterols **1-3** and the known secogorgosterol **4**.

The molecular formula of secosterol **1** was deduced as $C_{30}H_{52}O_4$ by HRFABMS (*m/z* 499.3763 $(M+Na)^+$ calcd for $C_{30}H_{52}O_4Na$, Δ - 0.8 mmu). The IR spectrum showed absorptions due to hydroxyl (3380 cm^{-1}) and carbonyl (1697 cm^{-1}) groups. Consistent with the FABMS data, the ^{13}C NMR spectrum revealed signals due to 1 ketone, 1 trisubstituted olefin, 1 oxyquaternary, 2 quaternaries, 1 oxymethine, 7 methines, 1 oxymethylene, 7 methylenes and 8 methyls. The presence of a secodinosterane skeleton[12] was discerned from the ^1H, ^{13}C, COSY and HMBC data (Table 1). Thus, in the COSY spectrum connectivities from H_2-1 through H-5 and H-4 to H_3-30 (secondary methyl at C-4) were clearly observed. The proton at the hydroxyl-bearing C-3 (δ_H 3.05 and δ_C 75.6 ppm) showed correlations to a methine proton at δ 1.46 (H-4), in addition to methylene protons at δ 1.91 and 1.47 (H_2-2), which in turn was correlated to methylene protons at δ 1.70 and 1.54 (H_2-1) adjacent to a quaternary carbon. The methine proton at δ 1.46 (H-4) showed additional correlations to the secondary methyl protons at δ 0.97 (H_3-30) and a methine proton at δ 1.08 (H-5). The presence of this spin system was corroborated by the HMBC spectrum which showed long-range correlations from C-3, C-4 and C-5 to H_3-30. The 9,11-seco skeleton was evident from the diagnostic resonances of an oxymethylene (δ 3.77 and 3.65 each m, H_2-11; δ 59.1, C-11) and a saturated ketone (δ 217.9). The ketone functionality at C-9 was further established by its long-range HMBC correlations to quaternary methyl protons (δ 1.18 s, H_3-19) and a sp^3 methine proton (δ 2.80, H-8). The structure of the side chain resembled that of dinosterol,[12] except for the hydroxylation at C-24 as implied by the chemical shifts of H_3-28 (δ 1.18) and C-24 (δ 77.4). Several HMBC correlations (Figure 1) provided unambiguous evidence for the side chain.

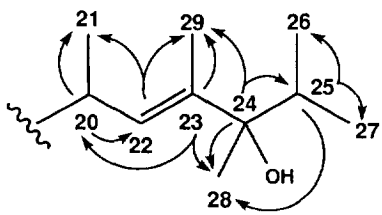


Figure 1. HMBC correlations ($^{13}C \rightarrow {}^1H$) of side chain of **1**.

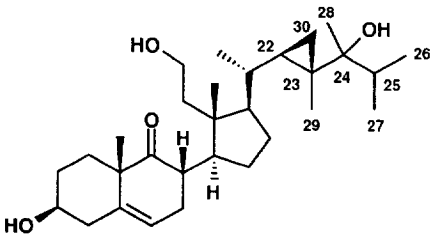
The stereochemistry of **1** was determined using coupling constant analysis and difference NOE spectra as detailed below. The two large coupling constants (diaxial) of H-3 to one of the diastereotopic H-2 and H-4 suggested β orientation of the C-3 hydroxyl and α orientation of the C-4 methyl group. The significant NOEs between H_3-19 and H-8 and H-4 indicated that they were *cis* to each other and oriented β. Likewise, the NOEs between H-3, H-5 and one of the H-1 (δ 1.70) indicated that they were *cis* to each other and oriented α. The relative configurations at C-13, C-14, C-17 and C-20 were assumed to have the normal steroidal configurations.

The nearly identical chemical shifts of C-13, C-14 and C-17 when compared to the related 9,11-secosterols[3,7,8] and the co-occurrence of **1**with **4** whose absolute configuration was established by X-ray analysis[1] support this assumption. The geometry of the C-22 double bond was assumed to be *E* by analogy with dinosterol. The stereochemistry at C-24, however, remains to be determined.

The structure of the other new secosterol **2**, which also possessed the secodinosterone skeleton and bore the same side chain as that of dinosterol, was apparent from the ^{1}H, ^{13}C NMR and FABMS data (see experimental and Table 2).

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data of Secosterols **1** ($CDCl_3$) and **3** ($CDCl_3$ + 3 drops of CD_3OD).

	1			**3**		
Position	C	H (*J*, Hz)	HMBC (^{1}H)	C	H (*J*, Hz)	HMBC (^{1}H)
1	30.9	1.70 m 1.54 m	19	31.1	1.77 m 1.43 m	19
2	30.5	1.91 dq (13.5, 4) 1.47 m		30.5	1.87 m 1.48 m	4
3	75.6	3.05 dt (5, 10.5)	2, 30	71.0	3.42 m	4
4	38.8	1.46 m	30	40.4	2.35 m 2.20 tq (12, 2)	6
5	51.8	1.08 ddd (12.5, 12, 3.5)	6, 19, 30	140.4	—	7, 19
6	24.5	1.80 dq (14, 3.5) 1.50 m		121.2	5.44 br d (5.5)	4
7	32.7	2.05 m 1.21 dq (13.5, 3.5)	8	32.5	2.39 m 2.00 m	6, 8
8	44.3	2.80 dt (13, 6)		43.0	3.00 dt (12, 6.5)	6, 7, 15
9	217.9	—	8, 19	217.7	—	7, 8, 19
10	49.0	—	19	48.3	—	4, 6, 19
11	59.1	3.77 m 3.65 m	12	58.8	3.77 m 3.66 m	12
12	40.6	1.66 m 1.36 m	18	40.3	1.72 m 1.27 m	18
13	45.3	—	8, 12, 17, 18	45.6	—	8, 12, 18
14	42.2	2.51 m	8, 12, 18	41.6	2.55 m	8, 12, 15, 18
15	23.9	1.48 m 1.28 m	14	24.1	1.49 m 1.28 m	14
16	24.8	1.71 m 1.68 m	15, 17, 20	27.8	1.96 m 1.28 m	15, 17
17	50.3	1.72 m	12, 18, 20, 21	50.2	1.67 br q (9)	16, 18, 20
18	17.6	0.63 s		17.3	0.66 s	12
19	16.6	1.18 s		22.9	1.33 s	1
20	33.0	2.44 m	21, 22	34.6	1.06 m	21, 30
21	21.0	0.95 d (6.5)	20, 22	20.4	1.02 br d (6)	
22	128.6	5.39 br d (10)	17, 20, 21, 29	26.0	0.68 dd (9, 6.5)	29, 30
23	136.8	—	20, 28, 29	28.6	—	28, 29, 30
24	77.4	—	22, 25, 28, 29	74.5	—	26, 27, 28, 29, 30
25	33.9	1.86 septet (6.5)	26, 27, 28	34.7	1.86 septet (6.5)	26, 27, 28
26	16.9	0.85 d (6.5)	25	17.2	0.86 d (6.5)	25
27	16.9	0.78 d (6.5)		17.0	0.86 d (6.5)	25
28	23.8	1.18 s	25	23.9	1.14 s	
29	13.1	1.58 br s	22	16.9	0.95 s	30
30	15.1	0.97 d (6.5)		16.1	0.81 dd (9, 4.5) -0.27 br dd (6.5, 4.5)	22, 29



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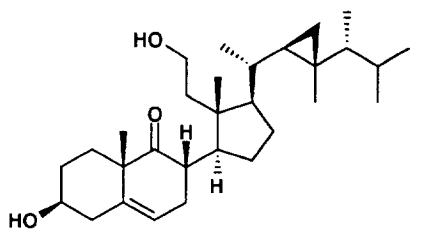


4

The cyclopropane-containing sterol was identified as 3β, 11, 24- trihydroxy, 9,11-secogorgost-5-en-9-one (**3**) on the basis of HRFABMS and 1D and 2D NMR data. The molecular formula $C_{30}H_{50}O_4$ indicated one additional oxygen when compared to the known secogorgosterol **4**. The striking differences in the ^1H NMR spectra of **3** and **4** were the resonances due to the cyclopropane ring protons H-22 and H_2-30 (δ 0.68 in **3**; 0.25 in **4** and δ 0.81 and -0.27 in **3**; 0.48 and -0.12 in **4**, respectively) and the absence of H-24 in **3** (δ 0.25 in **4**). Thus, the above data and the presence of the quaternary carbon resonating at δ 74.5 suggested hydroxylation at C-24 in **3**. This was further verified by the long-range HMBC correlations as shown in Figure 2. The C-24 stereochemistry remains to be determined. Complete ^1H and ^{13}C NMR chemical shifts together with the ^{13}C-^1H correlations observed in the HMBC spectrum are given in Table 2.

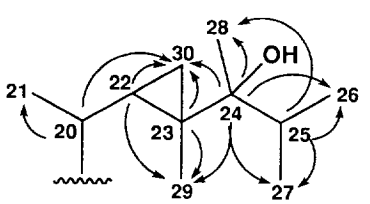


Figure 2. HMBC correlations ($^{13}C \rightarrow ^1H$) of side chain of **3**.

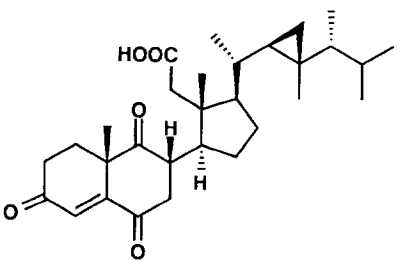
To our knowledge, the occurrence of **1** and **2** in *Pseudopterogorgia* sp. represents the first example of the 9,11-secosterols bearing the dinosterane skeleton. The side chain hydroxylation at C-24 is very rare among marine sterols, and is observed in two different side chains in the present work.

Table 2. ^{13}C (75 MHz) NMR Data of Secosterols **2**, and **4** ($CDCl_3$), **6** and **7** (CD_3OD).

Position	2[a]	4[b]	6	7
1	30.8	31.1	30.9	36.8
2	30.4	30.7	34.6	34.6
3	75.6	71.3	200.0	202.7
4	38.8	40.6	128.2	121.3
5	51.8	140.3	154.6	173.5
6	24.5	121.4	187.2	81.6
7	32.8	32.8	139.6	38.2
8	44.4	43.2	156.0	36.4
9	218.2	217.6	200.5	69.0
10	48.9	48.3	48.6	43.2
11	59.2	59.2	174.6	177.2
12	40.5	40.4	43.3	44.2
13	45.2	45.6	48.3	47.5
14	42.2	41.7	46.2	46.6
15	23.8	24.2	28.7	22.5
16	24.5	27.5	28.9	29.2
17	50.2	50.3	53.2	53.5
18	17.5	17.0	17.6	17.9
19	16.6	22.9	25.7	16.7
20	32.5	35.0	36.1	35.8
21	20.2	20.6	21.3	21.6
22	130.0	32.0	33.3	33.5
23	125.5	25.8	27.0	27.0
24	50.5	50.6	52.1	52.3
25	30.8	31.7	33.3	33.4
26	21.7	22.2	22.6	22.8
27	21.3	21.4	21.9	22.1
28	16.7	15.3	15.9	16.0
29	12.7	14.2	14.7	14.8
30	15.1	21.2	22.3	22.1

[a] Assignments made by comparison with **1**.
[b] ^{13}C NMR shifts not reported previously.[1,3]

The known secogorgosterol **4** was isolated in relatively large amounts, and we prepared from **4** the semi-synthetic sterols **6** and **7**. Oxidation of **4** with Jones reagent afforded unstable **5** which readily air oxidized to **6**. Reduction of **5** with $NaBH_4$ furnished a major diol **7**. The stereochemistry at C-6 and C-9 of **7** was determined by difference NOE experiments and coupling constant analysis. Significant NOEs were observed for H_3-19 and H-8 when H-6 was irradiated, implying that they were *cis* to each other, and the C-6 hydroxyl was oriented α. The large diaxial coupling (11 Hz) between H-8 and H-9 indicated β orientation of the C-9 hydroxyl group.



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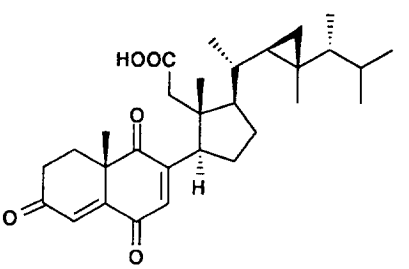

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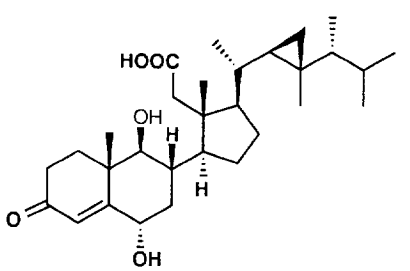

7

Secosterols **1-3**, **6** and **7** inhibited the human PKC enzymes α, βI, βII, γ, δ, ε, η, and ζ with IC_{50} values in the range 12->50 μM. Since PKC has been implicated in both inflammatory and proliferative processes, the above compounds were tested in cells related to these processes and the results are summarized in Table 3. All the compounds inhibited MCF-7 human breast carcinoma proliferation as measured by tritiated thymidine (^{3}H-T) incorporation in the range 3-13 μM and were even more potent against proliferation of normal human epidermal keratinocytes (NHEK) in the 0.6-5.0 μM range. Direct cytolethality as measured by MTT formazan dye reduction was 10 to 20 fold higher than antiproliferative activity for all compounds, suggesting cellular activity may be due to a block in cell cycle progression rather than to a directly cytolethal event. These data are consistent with those reported for other secosterols[9] and with the antiproliferative effect of 1,25-dihydroxyvitamin D_3 on NHEK cells (IC_{50}=0.2 μM) shown in our laboratory. At even lower concentrations (0.01 μM), 1,25-dihydroxyvitamin D_3 induced terminal differentiation of NHEK cells (data not shown). Whether the secosterols may also induce terminal differentiation of tumor cells or psoriatic keratinocytes remains to be determined.

Secosterols **2** and **6** exhibited potent inhibition of isolated human peripheral blood neutrophil superoxide production, suggesting that these compounds may also possess antiinflammatory activity. Compound **6** was the most potent and inhibited free radical production in whole blood as well, implying that it may retain activity *in vivo*. Compound **6** was also the most potent antiproliferative agent with no cytolethality in NHEK cells.

Table 3. Cellular Activity of Secosterols **1**-**3**, **6**, and **7**.[a]

Compound	MCF-7		NHEK		Neutrophil	Whole Blood
	^{3}H-T	MTT	^{3}H-T	MTT		
1	6.1 ± 1.2 (n=3)	76.3 ± 5.1 (n=3)	2.2 ± 1.1 (n=3)	33.9 ± 1.8 (n=3)	>10 (n=3)	NT
2	13.1 ± 1.6 (n=3)	47.3 ± 5.3 (n=3)	5.4 ± 1.4 (n=3)	>100 (n=3)	3.9 ± 0.5 (n=3)	>100 (n=3)
3	7.8 ± 1.1 (n=3)	79.3 ±2.5 (n=3)	1.1 ± 0.2 (n=3)	12.8 ±0.9 (n=3)	>10 (n=3)	NT
6	2.6 ± 0.2 (n=3)	>100 (n=3)	0.6 ± 0.2 (n=4)	>100 (n=3)	0.5 ±0.1 (n=3)	15.3 ± 2.7 (n=4)
7	7.2 ± 1.3 (n=3)	>100 (n=3)	0.9 ± 0.2 (n=3)	26.2 ± 4.4 (n=3)	>10 (n=3)	NT

[a] IC_{50} in μM; the data represent means ± S.E. NT = not tested

EXPERIMENTAL

General methods.

TLC analysis was performed on silica gel plates (Merck HPTLC Silica Gel 60 F_{254}, 0.25 mm). HPLC was performed on a Waters 600 E system with a Waters 486 tunable absorbance detector (λ=254 nm) using Waters μBondapak C_{18} column (7.8 x 300 mm, 10 μm). Optical rotations were measured with a Perkin-Elmer Model 241 Polarimeter at ambient temperatures. Spectra were measured on the following instruments: IR, Mattson 2020 Galaxy series FT-IR spectrophotometer; FAB-MS, VG Analytical ZAB 2-SE high-field mass spectrometer; NMR, Varian Gemini 300 and Unity 500 spectrometers.

Animal collection.

The specimen of *Pseudopterogorgia* sp. was collected on August 15, 1992, in the channel between Islemorada and Long Keys, Florida, in 2 m of water. A voucher specimen is deposited at the Florida Institute of Technology, Melbourne, FL (voucher No. FIT 92-268).

Extraction and isolation of secosterols.

The freeze dried gorgonian (290 g) was ground and extracted with MeOH (2 L) at room temperature. Evaporation of MeOH gave 47 g of a greenish oil. The oil was then partitioned between water and 2:1 $CHCl_3$-MeOH. The organic layer was separated and evaporated to yield 18.5 g of gum. A portion (11 g) of this gum was

chromatographed over a Sephadex LH-20 (Pharmacia) column with MeOH as solvent. Five consecutive fractions (20 mL each) which showed PKC inhibitory activity (IC_{50} = ~40 μg/mL) were combined (9.7 g) and rechromatographed over silica gel (100 g) with hexanes-ethyl acetate in order of increasing polarity to afford fractions 4-8 (hexanes-EtOAc 3:1, 500 mL, 240 mg, fraction A), 9-22 (hexanes-EtOAc 1:1, 1400 mL, 1.2 g, fraction B) and 23-28 (hexanes-EtOAc 1:3, 600 mL, 380 mg, fraction C).

Purification of fraction A by HPLC (85% MeOH, flow rate 4 mL/min, 4 injections) yielded 28 mg of **2**. A portion (240 mg) of fraction B was purified by HPLC (78% MeOH) to give 102 mg of **4**. Similar purification of fraction C (370 mg) gave 51 mg of **1**, 18 mg of **3** and 25 mg of **4**.

1 White powder; $[\alpha]_D$ = +6.8° (*c* 2.7, MeOH); IR (neat): 3380, 2958, and 1697 cm^{-1}; HRFABMS: Obsd *m/z* 499.3755. Calcd for $C_{30}H_{52}O_4Na$ *m/z* 499.3763 ($M+Na^+$) and obsd *m/z* 459.3850. Calcd for $C_{30}H_{51}O_3$ *m/z* 459.3839 (MH^+-H_2O); ^1H and ^{13}C NMR see Table 1.

2 White powder; $[\alpha]_D$ = -11.5° (*c* 1.7, MeOH); IR (neat) 3280, 2957 and 1697 cm^{-1}; HRFABMS: Obsd *m/z* 461.3987. Calcd for $C_{30}H_{53}O_3$ *m/z* 461.3994 (MH^+); ^1H NMR (300 MHz, $CDCl_3$): δ 5.01 (br d, 10, H-22), 3.76, 3.66 (m, H_2-12), 3.06 (ddd, 11, 11, 5.3, H-3), 2.80 (ddd, 13.3, 5.7, 5.7, H-8), 1.47 (3H, d, 1.5, H_3-29), 1.18 (3H, s, H_3-19), 0.90 (3H, d, 6.4, H_3-28), 0.93 (3H, d, 7.5, H_3-21), 0.97 (3H, d, 6.8, H_3-30), 0.83, 0.75 (each 3H, d, 6.6, H_3-26 and H_3-27), 0.64 (3H, s, H_3-18); ^{13}C NMR see Table 2.

3 White powder; $[\alpha]_D$ = +1.5° (*c* 1.2, MeOH); IR (neat): 3390, 2965 and 1707 cm^{-1}; HRFABMS: Obsd *m/z* 497.3615. Calcd for $C_{30}H_{50}O_4Na$ *m/z* 497.3607 ($M+Na^+$) and obsd *m/z* 457.3685. Calcd for $C_{30}H_{49}O_3$ *m/z* 457.3681 (MH^+-H_2O). ^1H and ^{13}C NMR see Table 1.

Preparation of ***6***. To a stirred solution of **4** (78 mg) in acetone (3 mL) was added 10 equivalents of Jones reagent. The reaction mixture was stirred at rt for 30 min, and 2-propanol (1.5 mL) was added. The mixture was loaded on a silica gel column (60 g) and eluted with 1:1 EtOAc-hexanes containing 0.1% acetic acid to yield 55 mg of unstable **5** which air oxidized readily to **6**. Yellow powder; $[\alpha]_D$ = -42.6° (*c* 1.4, MeOH); UV (MeOH): λ_{max} 367 (ε = 3236), 271 (8155) and 221 (7439) nm; IR (neat): 3320, 1688, and 1665 cm^{-1}; HRFABMS: Obsd *m/z* 483.3103. Calcd for $C_{30}H_{43}O_5$ *m/z* 483.3110 (MH^+); ^1H NMR (500 MHz, $CDCl_3$): δ 2.60, 2.38 (each m, H_2-1), 2.60, 2.27 (each m, H_2-2), 6.53 (s, H-4), 6.88 (s, H-7), 2.62, 2.25 (AB, 16.5, H_2-12), 3.85 (t, 9.5, H-14), 1.85, 1.68 (each m, H_2-15), 2.22, 1.58 (each m, H_2-16), 2.37 (m, H-17), 0.84 (s, H_3-18), 1.56 (s, H_3-19), 1.08 (m, H-20), 1.08 (br s, H_3-21), 0.28 (m, H-22), 0.30 (m, H-24), 1.59 (m, H-25), 0.96, 0.87 (each d, 6.5, H_3-26, H_3-27), 0.95 (d, 7.5, H_3-28), 0.93 (s, H_3-29), 0.51 (dd, 8.5, 4.5, H-30), and -0.09 (dd, 7, 4.5, H-30); ^{13}C NMR see Table 2.

Preparation of ***7***. Freshly prepared **5** (80 mg) in MeOH (1.5 mL) was cooled to -78°C. A solution of $NaBH_4$ (8 mg) in MeOH (0.5 mL) was added slowly and the mixture was stirred at -78°C for 15 min. The reaction was quenched with water (50 mL) containing 2% acetic acid and extracted with EtOAc (3 x 50 mL). The crude product mixture was purified by HPLC (78-100% MeOH) to yield **7** as the major product. White powder; UV (MeOH): λ_{max} 240 (ε = 9740) nm; IR (neat): 3360, 1698, and 1652 cm^{-1}; HRFABMS: Obsd *m/z* 489.3579.

Calcd for $C_{30}H_{49}O_5$ *m/z* 489.3579 (MH^+); ^1H NMR (500 MHz, CD_3OD): δ 2.46 (m, H-1), 2.16 (ddd, 14, 4, 4, H-1), 2.32 (ddd, 13, 4, 4, H-2), 1.87 (m, H-2), 6.10 (br s, H-4), 4.38 (dd, 12.5, 4, H-6), 2.07 (m, H-7), 1.21 (t, 13, H-7), 2.07 (m, H-8), 3.06 (d, 11, H-9), 2.58, 2.53 (AB, 13.5, H_2-12), 2.47 (m, H-14), 1.55, 1.48 (each m, H_2-15), 2.03, 1.46 (each m, H_2-16), 2.09 (m, H-17), 0.91 (s, H_3-18), 1.25 (s, H_3-19), 1.13 (m, H-20), 1.13 (br s, H_3-21), 0.28 (m, H-22), 0.25 (m, H-24), 1.58 (m, H-25), 0.97, 0.87 (each d, 6.5, H_3-26, H_3-27), 0.96 (d, 7.5, H_3-28), 0.93 (s, H_3-29), 0.50 (dd, 9.5, 4.5, H-30), and -0.07 (dd, 6.5, 4.5, H-30); ^{13}C NMR see Table 2.

Biological assays

The PKC and cellular assays are as described in reference 10 (b).

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