Full Papers

Tetrahydrofuran Acetogenins from *Laurencia glandulifera*

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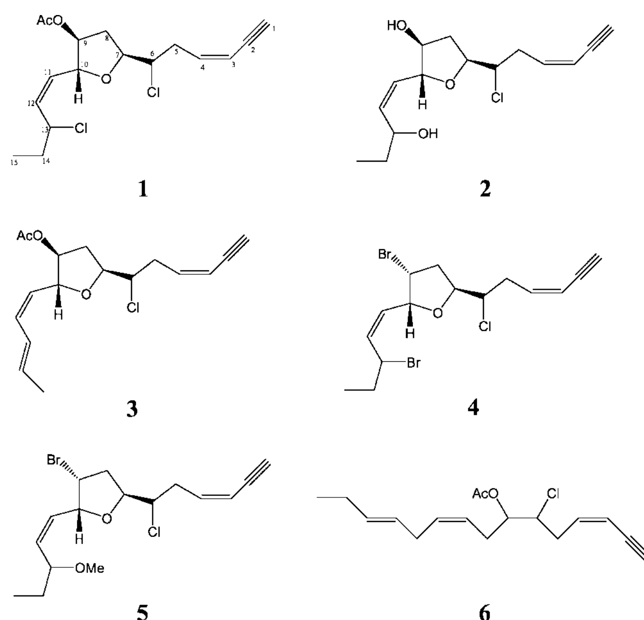
Five new C_{15} acetogenin en-ynes (**1**−**5**) with a rare tetrahydrofuran moiety and a linear biosynthetic precursor (**6**) were isolated from an organic extract of *Laurencia glandulifera*, collected from the island of Crete in the south Aegean Sea. The structures of the new natural products, as well as their relative configuration, were established by means of spectroscopic data analysis. The cytotoxicity of the isolated natural products was evaluated against five human tumor cell lines.

Red algae of the genus *Laurencia* (family Rhodomelaceae, order Ceramiales) are distributed widely in different geographical areas around the world.[1] Species of the alga *Laurencia* biosynthesize a wide variety of secondary metabolites with diverse structural features.[2] Most *Laurencia* species accumulate a characteristic major metabolite or a class of compounds not widely distributed within the genus.[3] The majority of these metabolites fall into one of the following categories: sesquiterpenes,[4,5] diterpenes,[6] triterpenes,[7] and C_{15}-acetogenins.[8] A number of these compounds have exhibited significant antibacterial,[9] insecticidal,[10] antifungal,[11] and antiviral effects,[12] as well as cytotoxicity against mammalian cells.[13]

In the course of our research on the isolation of bioactive secondary metabolites from marine organisms,[14−16] specimens of *Laurencia glandulifera* collected from the island of Crete were investigated. In this report, the isolation and structure elucidation of six new metabolites (**1**−**6**) from the nonpolar fractions of the organic extract of this species are described. The cytotoxicity of those metabolites isolated in adequate amounts to enable successful testing was assessed toward the HT-29 (colorectal adenocarcinoma), MCF-7 (mammary adenocarcinoma), PC-3 (prostate adenocarcinoma), HeLa (cervical adenocarcinoma), and A431 (epidermoid carcinoma) human tumor cell lines.[17]



Results and Discussion

The CH_2Cl_2/MeOH extract of the fresh alga *L. glandulifera* was subjected to a series of vacuum-column chromatographic steps over silica gel and normal-phase HPLC, using mixtures of cyclohexane/EtOAc and hexane/2-propanol as mobile phases, to yield compounds **1**−**6**.

Compound **1** was isolated as a colorless oil. Its molecular formula was deduced as $C_{17}H_{22}Cl_2O_3$ from the HRFABMS and NMR spectroscopic data (Tables 1 and 2). The EIMS of **1** exhibited $[M - Cl]^+$ fragment ions at *m/z* 311 and 309 (3:1), characteristic for the presence of one chlorine atom in this molecular fragment. The IR spectrum of the metabolite displayed absorptions for a terminal alkyne moiety (3293, 2326 cm^{-1}), an ether functionality (1050 cm^{-1}), and one ester carbonyl (1733 cm^{-1}). The ^{13}C NMR spectrum of **1** (Table 2) exhibited signals for 17 carbons: two quaternary, 10 methine, three methylene, and two methyl carbon atoms. Among these carbons, one was assigned as a carbonyl (δ 170.5 ppm), two as halogenated [δ 58.2 (CH, C-13) and 62.9 (CH, C-6)], three bonded to oxygens [δ 78.1 (CH, C-9), 79.6 (CH, C-10), and 79.8 (CH, C-7)], and four olefinic [δ 111.1 (CH, C-3), 127.8 (CH, C-11), 134.5 (CH, C-12), and 140.4 (CH, C-4) ppm]. The presence of a terminal en-yne moiety, which is frequently encountered in *Laurencia* C_{15} acetogenins,[18] was evident from the tertiary carbon resonances at δ 82.7, 111.1, and 140.4 ppm and the quaternary carbon at δ 79.7 ppm. Furthermore, the ^1H NMR spectrum (Table 1) of **1** showed signals at 6.16 (1H, dt, J = 10.8, 6.8 Hz, H-4), 5.61 (1H, dd, J = 10.8, 2.0 Hz, H-3), and 3.13 (1H, d, J = 2.0 Hz, H-1) ppm, supporting the presence of the terminal ene-yne moiety. The coupling (J = 10.8 Hz) between H-3 and H-4 as well as the chemical shift of the acetylenic proton (δ 3.13) showed Z geometry of the carbon−carbon double bond. Moreover, the ^1H NMR spectrum exhibited signals for a terminal methyl group [δ 0.99 ppm (t, J = 7.3 Hz, H-15)] and five protons on methines bonded with either a halogen or oxygen atom [δ 4.01 (ddd, J = 9.3, 4.9, 4.4

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Table 1. ^{1}H NMR Data (400 MHz, $CDCl_3$) of Compounds **1**−**6**

	1	2	3	4	5	6
proton	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)
1	3.13 (d, 2.0)	3.13 (d, 1.8)	3.12 (d, 1.9)	3.14 (d, 2.0)	3.12 (d, 1.5)	3.12 (d, 2.0)
2						
3	5.61 (dd, 10.8, 2.0)	5.60 (dd, 10.8, 1.8)	5.60 (dd, 10.5, 1.9)	5.61 (m)	5.60 (dd, 10.2, 1.5)	5.59 (dd, 10.8, 2.0)
4	6.16 (dt, 10.8, 6.8)	6.14 (dt, 10.8, 7.0)	6.16 (dt, 10.5, 6.7)	6.16 (dt, 10.5, 7.0)	6.15 (dt, 10.2, 7.0)	6.06 (dt, 10.8, 7.3)
5a	2.81 (m)	2.85 (m)	2.79 (m)	2.91 (m)	2.90 (m)	2.70 (m)
5b	2.88 (m)		2.89 (m)			2.82 (m)
6	4.01 (ddd, 9.3, 4.9, 4.4)	4.02 (ddd, 8.2, 5.0, 3.2)	4.01 (ddd, 9.3, 5.0, 4.4)	4.00 (m)	3.99 (m)	4.04 (ddd, 10.3, 6.9, 2.9)
7	4.32 (ddd, 7.3, 4.9, 2.0)	4.23 (ddd, 7.3, 5.0, 2.6)	4.27 (ddd, 7.6, 5.0, 2.0)	4.62 (m)	4.60 (m)	5.01 (ddd, 10.3, 6.8, 3.4)
8α	2.53 (ddd, 14.2, 7.3, 6.8)	2.44 (ddd, 13.2, 7.3, 6.6)	2.52 (ddd, 14.0, 7.6, 6.7)	2.51 (dd, 14.0, 6.4)	2.53 (dd, 13.4, 6.1)	2.49 (ddd, 14.7, 7.8, 6.8)
8β	2.02 (m)	1.96 (m)	2.05 (m)	2.72 (m)	2.73 (m)	
9	4.91 (ddd, 6.8, 4.4, 2.4)	4.10 (m)	4.98 (m)	4.47 (m)	4.46 (m)	5.32 (m)
10	4.82 (m)	4.69 (dd, 7.9, 6.4)	4.94 (m)	4.82 (dd, 6.7, 3.5)	4.80 (dd, 7.9, 3.8)	5.53 (ddd, 10.8, 7.3, 1.5)
11	5.42 (dd, 10.8, 7.3)	5.46 (dd, 11.4, 7.9)	5.19 (dd, 10.5, 8.2)	5.58 (m)	5.75 (dd, 10.5, 7.9)	2.77 (m)
12	5.66 (m)	5.66 (dd, 11.4, 8.0)	6.10 (m)	5.70 (dd, 10.2, 10.2)	5.50 (dd, 10.5, 9.9)	5.37 (m)
13	4.78 (m)	4.41 (m)	6.40 (m)	4.57 (m)	3.80 (m)	5.46 (m)
14a	1.75 (m)	1.52 (m)	5.76 (ddd, 13.7, 7.3, 7.0)	1.77 (ddd, 14.3, 7.3, 7.0)	1.45 (m)	1.98 (m)
14b	1.82 (m)	1.61 (m)		1.86 (ddd, 14.3, 7.3, 6.7)	1.63 (m)	
15	0.99 (t, 7.3)	0.92 (t, 7.6)	1.78 (d, 7.0)	0.98 (t, 7.3)	0.87 (t, 7.3)	0.94 (t, 7.3)
CH_3 (Ac)	2.06 (s)		2.06 (s)			2.08 (s)
OMe					3.28 (s)	

Table 2. ^{13}C NMR Data (50.3 MHz, $CDCl_3$) of Compounds **1**−**6**

	1	2	3	4	5	6
carbon	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C
1	82.7	82.8	82.6	82.5	82.7	82.8
2	79.7	79.8	79.7	79.8	79.8	79.7
3	111.1	111.1	111.0	111.1	111.1	111.3
4	140.4	140.3	140.5	140.3	140.2	139.9
5	34.9	35.2	34.9	35.7	35.8	35.2
6	62.9	63.9	63.2	63.2	63.3	61.3
7	79.8	79.2	79.5	79.3	79.2	74.1
8	34.0	37.4	34.3	40.3	40.3	29.3
9	78.1	76.3	78.9	54.1	54.6	123.2
10	79.6	81.4	79.4	78.5	78.2	132.2
11	127.8	128.8	124.2	129.1	130.0	30.5
12	134.5	137.3	132.9	133.9	135.3	126.7
13	58.2	69.6	126.5	58.6	78.4	132.4
14	32.1	30.0	133.0	32.0	28.0	25.5
15	10.8	9.7	18.4	10.8	9.6	13.8
CH_3 (Ac)	20.9		21.0			20.9
CO (Ac)	170.5		170.3			170.3
OMe					56.3	

Hz, H-6), 4.32 (ddd, J = 7.3, 4.9, 2.0, H-7), 4.78 (m, H-13), 4.82 (m, H-10), and 4.91 (ddd, J = 6.8, 4.4, 2.4 Hz, H-9) ppm]. With an unsaturation degree of six, the structure was predicted to contain one carbonyl group, two carbon−carbon double bonds, one carbon−carbon triple bond, an ether bridge, and one ring.

Interpretation of the correlations observed in the $^{1}H-^{1}H$ COSY spectrum of **1** indicated the position of the isolated double bond as being between C-11 and C-12, as well as the positions of the heteroatoms on carbons C-6, C-7, C-9, C-10, and C-13. On the basis of long-range $^{1}H-^{13}C$ correlations observed in the HMBC spectrum between H-7 (δ_H 4.32) and C-10 (δ_C 79.6), and H-10 (δ_H 4.82) with C-7 (δ_C 79.8), the ether bridge was placed between C-7 and C-10, indicating an oxolane skeleton for **1**. Moreover, the HMBC correlation between H-9 (δ_H 4.91) and the carbonyl carbon (δ_C 170.5) confirmed the position of the acetoxy group at C-9. The correlations between H-6/C-7 and H-13/C-12 were used to secure the positions of the halogens at C-6 and C-13.

The relative configurations at C-7, C-9, and C-10 were proposed on the basis of NOE results. NOE effects of H-7/H-9, H-7/H-8α, and H-8α/H-9 revealed their *cis* orientation, while a cross-peak of H-8β/H-10 indicated their cofacial arrangement. Accordingly, metabolite **1** is (3Z,11Z,7*S**,9*S**,10*R**)-9-acetoxy-6,13-dichloro-7:10-epoxypentadeca-3,11-dien-1-yne.

Compound **2** was isolated as a colorless oil. The molecular formula of **2** was deduced to be $C_{15}H_{21}ClO_3$ from the HRFABMS and ^{13}C NMR data. The EIMS exhibited fragment ions at m/z 257 and 255 (3:1), indicating the presence of one chlorine atom. Comparison of the spectroscopic data of **2** with those of **1** revealed great similarity in the structures of these two compounds.The chemical shift of C-13 (δ_C 69.6) suggested the presence of an oxygen on this carbon. The relative stereochemistry of **2** was assigned on the basis of NOESY experiments and was found to be identical to that of **1**. Accordingly, metabolite **2** is (3Z,11Z,7*S**,9*S**,10*R**)-6-chloro-7:10-epoxypentadeca-3,11-dien-1-yne-9,13-diol.

Compound **3** was isolated as a colorless oil. Both the ^{13}C NMR and HRFABMS data supported the molecular formula, $C_{17}H_{21}ClO_3$. The EIMS exhibited fragment ions $[M - Ac]^+$ at m/z 267 and 265 (3:1), characteristic for the presence of one chlorine atom in the molecule. The ^{13}C NMR spectrum of **3** (Table 2) exhibited signals for 17 carbons. Out of these carbons, one was a carbonyl, one was halogenated, three were bonded to oxygens, and six were olefinic. All protonated carbons and their protons were matched precisely by the HSQC experiment. Interpretation of the correlations observed in the $^{1}H-^{1}H$ COSY spectrum indicated the position of the additional double bond to be between C-13 and C-14. The relative configuration of **3** was assigned on the basis of NOESY experiments and was found identical to that of **1** and **2**. Accordingly, metabolite **3** is (3Z,11Z,13*E*,7*S**,9*S**,10*R**)-9-acetoxy-6-chloro-7:10-epoxypentadeca-3,11,13-trien-1-yne.

Compound **4** was isolated as a colorless oil. Combination of its ^{13}C NMR data and HRFABMS suggested a molecular formula of $C_{15}H_{19}Br_2ClO$. The CIMS exhibited a peak cluster at m/z 409/411/413/415 with a ratio of 1.2/2.8/2.0/0.4, characteristic for the presence of one chlorine and two bromine atoms in the molecule. The ^{13}C NMR spectrum showed the presence of 15 carbons. Among these carbons, three were halogenated, two were bonded to oxygens, and four were olefinic. Interpretation of the correlations observed in the $^{1}H-^{1}H$ COSY spectrum indicated the position of the double bond to be between C-11 and C-12, as well as the position of the heteroatoms at C-6, C-7, C-9, C-10, and C-13. The relative configuration of the asymmetric centers was proposed on the basis of NOE enhancements. The strong NOE effects of H-9/H-10, H-8β/H-9, and H-8β/H-10 revealed their *cis* orientation. Accordingly, metabolite **4** is (3Z,11Z,7*S**,9*R**,10*R**)-6-chloro-9,13-dibromo-7:10-epoxypentadeca-3,11-dien-1-yne.

Compound **5** was isolated as a colorless oil. Both the ^{13}C NMR data and HRFABMS supported a molecular formula of $C_{16}H_{22}BrClO_2$. Comparison of the spectroscopic data of **5** with those of **4** revealed a great similarity in the structures of these two compounds. The most significant difference was observed at the ^{13}C NMR chemical shift of C-13 at δ 78.4, indicating oxygenation.

Furthermore, the presence of a singlet at δ_H 3.28 and the absence of an IR absorption attributable to a hydroxyl indicated a methoxy substituent at C-13. The relative stereochemistry of **5** was assigned on the basis of NOESY experiments and was found identical to that of **4**. Accordingly, metabolite **5** is (3*Z*,11*Z*,7*S**,9*R**,10*R**)-13-methoxy-6-chloro-9-bromo-7:10-epoxypentadeca-3,11-dien-1-yne.

Compound **6** was isolated as a colorless oil. The molecular formula of **6** was deduced as $C_{17}H_{23}ClO_2$ from the HRFABMS and ^{13}C NMR data. The EIMS exhibited fragment ions $[M - Ac]^+$ at *m/z* 253 and 251 (3:1), characteristic for the presence of one chlorine atom in the molecule. The ^{13}C NMR spectrum of **6** (Table 2) exhibited signals for 17 carbons: two quaternary, nine methine, four methylene, and two methyl carbon atoms. The ^1H NMR spectrum showed signals for four olefinic protons [δ 5.32 (m, H-9), 5.37 (m, H-12), 5.46 (m, H-13), and 5.53 (ddd, $J = 10.8$, 7.3, 1.5 Hz, H-10)] and two methine protons adjacent to a halogen or oxygen atom [δ 4.04 (ddd, $J = 10.3$, 6.9, 2.9 Hz, H-6) and 5.01 (ddd, $J = 10.3$, 6.8, 3.4 Hz, H-7) ppm]. With an unsaturation degree of six, the structure was suggested to contain one carbonyl group, three carbon−carbon double bonds, and one carbon−carbon triple bond. Careful examination of the $^1H-^1H$ COSY spectrum revealed all proton−proton connectivities, the heteroatom positions to be at C-6 and C-7, and the additional double bond positions at C-9 and C-12. The geometry of the Δ^9 bond was established as *Z* from the H-10 coupling constant ($J = 10.8$ Hz), whereas the assignment of the *E* configuration of the Δ^{12} bond was based on the deshielded ^{13}C NMR shifts of the bis-allylic methylene (C-11: 30.5 ppm) and the allylic methylene (C-14: 25.5 ppm).[19,20] In view of the above-mentioned data, the proposed structure for metabolite **6** is (3*Z*,9*Z*,12*E*)-7-acetoxy-6-chloropentadeca-3,9,12-trien-1-yne, which might be the biosynthetic precursor of metabolites **1**−**5**.

Metabolites **1**, **2**, **5**, and **6** were evaluated for their cytotoxicity toward HT-29, MCF-7, PC-3, HeLa, and A431 human tumor cell lines. After 48 h of incubation none of the metabolites showed any significant activity ($IC_{50} \geq 10\ \mu M$) toward the above-mentioned cancer cell lines.

The need for the development of a computational (*in silico*) prescreening method for activity prediction, so only potentially active compounds would be committed to the appropriate pharmacological evaluation, becomes apparent in studies like the present one, where only small quantities of metabolites were isolated. In this direction, the creation of a pharmacophore, which is a drug−target interaction model, has been attempted. A ligand-based pharmacophore for MCF-7 cells was built using the Catalyst software[21] and is able to predict with accuracy the cytotoxicity levels for an array of compounds against this cell line. Full details of the pharmacophore modeling for compounds **1**−**6** will be reported in a more specialized journal.

Experimental Section

General Experimental Procedures. The general experimental procedures were carried out as described previously.[14]

Plant Material. *Laurencia glandulifera* was collected by hand from a depth of 0.5−1 m, in Loutraki Bay, located on the northwestern part of the island of Crete, Greece, in November 2005 and authenticated by Professor G. Furnari of University of Catania, Italy. A specimen is kept at the Herbarium of the Laboratory of Pharmacognosy and Chemistry of Natural Products, University of Athens (ATPH/MO/153).

Extraction and Isolation. Fresh algal tissue was exhaustively extracted with a mixture of CH_2Cl_2/MeOH (3:1) at room temperature. The combined extracts were concentrated to afford a dark green, oily residue (12.9 g), which was subjected subsequently to vacuum-column chromatography (VCC) on silica gel, using as mobile phase cyclohexane with increasing amounts (5%) of EtOAc and finally MeOH. Fraction I (40% EtOAc in cyclohexane) (187.0 mg) was further purified by passage over a gravity column containing silica gel using cyclohexane with increasing amounts (2%) of EtOAc. Fraction I-II (2% EtOAc) (20.7 mg) was subjected to normal-phase HPLC, using as mobile phase hexane/2-propanol (99:1), to yield pure compounds **4** (1.0 mg) and **6** (2.3 mg). Compound **1** (5.0 mg) was isolated in pure form in fraction I-IIID (4% EtOAc) (5.0 mg). Fraction I-IIIA (4% EtOAc) (11.0 mg) was subjected to normal-phase HPLC, using hexane/2-propanol (99:1) as mobile phase, to yield pure compounds **3** (1.4 mg) and **5** (1.0 mg). Fraction N (65% EtOAc in cyclohexane) (302.7 mg) was further purified by gravity column chromatography on silica gel using cyclohexane/EtOAc (87:13). Compound **2** (4.8 mg) was isolated in pure form in fraction N21 (30% EtOAc).

Compound 1: colorless oil; $[\alpha]^{20}_D$ +30 (*c* 0.05, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 297.6 (1.7) nm; IR ($CHCl_3$) ν_{max} 3293, 3244, 2967, 2930, 2877, 2367, 2326, 1733, 1234, 1050 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; EIMS 70 eV *m/z* 309:311 $[M - Cl]^+$ (10:3), 301:303:305 (2:1.6:0.4), 267:269 (6:2), 249:251 (31:10), 213 (40), 171 (42), 135 (92), 105 (100), 77 (96); HRFABMS *m/z* 345.1023 $[M + H]^+$ (calcd for $C_{17}H_{23}{}^{35}Cl_2O_3$, 345.1026).

Compound 2: colorless oil; $[\alpha]^{20}_D$ −15.0 (*c* 0.04, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 298.5 (1.9) nm; IR ($CHCl_3$) ν_{max} 3378, 3302, 2962, 2928, 2361, 2327, 1717, 1454, 1243 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; EIMS 70 eV *m/z* 255:257 (1:0.3), 237:239 (2:0.8), 231:233 (4:1.5), 213:215 (2.8:0.8), 187 (4), 173 (3), 153 (8), 145 (15), 133 (22), 105 (68), 79 (63), 57 (100); HRFABMS *m/z* 285.1251 $[M + H]^+$ (calcd for $C_{15}H_{22}{}^{35}ClO_3$, 285.1256).

Compound 3: colorless oil; $[\alpha]^{20}_D$ −10.0 (*c* 0.07, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 284.0 (1.6) nm; IR ($CHCl_3$) ν_{max} 2914, 2848, 2355, 2329, 1738, 1237, 1053 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; EIMS 70 eV *m/z* 265:267 $[M - Ac]^+$ (1:0.3), 233:235 (2:0.7), 213 (95), 173 (20), 157 (17), 145 (37), 133 (47), 105 (82), 95 (100), 79 (89), 65 (74); HRFABMS *m/z* 309.1236 $[M + H]^+$ (calcd for $C_{17}H_{22}{}^{35}ClO_3$, 309.1259).

Compound 4: colorless oil; $[\alpha]^{20}_D$ +18.0 (*c* 0.10, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 284.0 (1.7) nm; IR ($CHCl_3$) ν_{max} 3029, 2968, 2927, 2858, 2361, 2334, 1738, 1724, 1653, 1459, 1248, 1067 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; PCIMS (CH_4) *m/z* 409:411:413:415 (1.2:2.8:2:0.4), 365:367:369 (2:3:1), 329:331:333 (34:26:8), 249:251:253 (100:64:18), 213 (54), 117 (32), 107 (49), 79 (40); HRFABMS *m/z* 407.9461 $[M]^+$ (calcd for $C_{15}H_{19}{}^{79}Br_2{}^{35}ClO$, 407.9492).

Compound 5: colorless oil; $[\alpha]^{20}_D$ +14.0 (*c* 0.05, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 287.0 (2.8) nm; IR ($CHCl_3$) ν_{max} 2964, 2927, 2872, 2360, 1725, 1458, 1195, 1092, 1074 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; PCIMS (CH_4) *m/z* 361:363:365 (8:8:3), 307:309:311 (16:22:12), 243 (20), 227 (22), 191 (38), 181 (24), 113 (42), 81 (72), 73 (98), 55 (100); HRFABMS *m/z* 361.0558 $[M + H]^+$ (calcd for $C_{16}H_{23}{}^{79}Br^{35}ClO_2$, 361.0571).

Compound 6: colorless oil; $[\alpha]^{20}_D$ +2.5 (*c* 0.08, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 294.6 (1.5), 307.8 (1.4) nm; IR ($CHCl_3$) ν_{max} 3295, 3015, 2966, 2931, 2876, 2364, 2341, 1746, 1735, 1375, 1234, 1022 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; EIMS 70 eV *m/z* 251:253 $[M - Ac]^+$ (1:0.3), 233:235 (1:0.3), 205:207 (10:3.3), 169:171 (40:14), 129 (88), 91 (86), 79 (100); HRFABMS *m/z* 295.1477 $[M + H]^+$ (calcd for $C_{17}H_{24}{}^{35}ClO_2$, 295.1466).

Determination of Cytotoxicity. The cytotoxicity assays were performed as previously described.[14,22,23]

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