Isolation and Identification of Acetylenic Spiroketal Enol Ethers from *Artemisia lactiflora* as Inhibitors of Superoxide Generation Induced by a Tumor Promoter in Differentiated HL-60 Cells

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Six acetylenic compounds were isolated from the leaves of *Artemisia lactiflora* (Compositae), an edible plant of Thailand. Four of them were identified as the stereoisomers of 3,4-epoxy-2-(2,4-hexadiynylidene)-1,6-dioxaspiro[4.5]decane and its derivatives, whose planar structures have already been reported. The other two were identified as novel chlorohydrin derivatives of the corresponding diacetylene spiroketal enol ethers. The inhibitory effects of the polyacetylenes isolated in the present study together with genistein on TPA-induced O_2^- generation were examined. It was revealed that an acyloxyl group at the C-2 position enhanced the inhibitory effect, while the absolute configurations at C-5, -6, and -7 were not important. While polyacetylenes are known to possess several biological roles in various ecosystems, we first found inhibitory effects of the diacetylenes on TPA-induced O_2^- generation in differentiated HL-60 cells.

Keywords: *Artemisia lactiflora; superoxide; HL-60; diacetylene spiroketal enol ether; tumor promoter; cancer chemoprevention*

INTRODUCTION

Tumor promoter-induced reactive oxygen species (ROS) generation is considered to play some important roles in tumor promotion (Perchellet et al., 1995). In particular, 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-type tumor promoters are reported to trigger superoxide (O_2^-) generation in epithelial cells and leukocytes through the xanthine/xanthine oxidase (XA/XOD) and NADPH oxidase systems, respectively. Yoon et al. (1993) advocated a close relationship between the generation of ROS including O_2^- by phagocytic cells in inflammatory processes and tumor promotion. Kensler et al. (1989) have proposed a hypothesis that the first treatment of mouse skin with TPA application causes a chemotactic action, i.e., recruitment of neutrophils responsible for ROS generation induced by the second TPA treatment. Moreover, ROS production by double or multiple TPA treatments is closely associated with the metabolic activation of proximate carcinogens (Ji et al., 1992; Kensler et al., 1987) and increased levels of oxidized DNA bases (Wei and Frenkel, 1992, 1993).

In our continuing studies of food phytochemicals for chemoprevention (Ohigashi et al., 1997), we have focused on O_2^- generation inhibitors rather than radical scavengers as effective and promising candidates for prevention of oxidative stress-related diseases including cancer, because O_2^- is one of the precursors of several types of ROS. It is well-known that O_2^- is converted to H_2O_2 nonenzymatically or by a function of superoxide dismutase in biological systems. The hydroxyl radical (•OH), formed subsequently from H_2O_2, randomly reacts with biological components such as lipids or DNA bases within the cell. Takeuchi et al. (1996) reported that •OH may directly induce formation of 8-hydroxydeoxyguanosine in DMSO-differentiated HL-60 cells. Therefore O_2^- generation inhibitor effectively suppresses H_2O_2 and lipid peroxidation in vitro and in vivo (Murakami et al., 1997; Nakamura et al., 1998a,c). Moreover we and other groups have recently reported that some natural chemopreventers inhibited O_2^- generation by leukocytes, suggesting, at least in part, this inhibition to be an important action mechanism for antitumor promotion (Murakami et al., 1996, 1997; Nakamura et al., 1996, 1998a,c; Wei and Frenkel, 1993).

Based upon this, exploration of food phytochemicals with O_2^- generation inhibition in leukocytes in vitro was conducted. In this paper, a chemical study on the active constituents containing both diacetylene and spiroketal moieties from *Artemisia lactiflora* (Compositae), an edible plant in southeast Asia, is described. Next, their marked inhibitory activities against tumor promoter TPA-induced O_2^- generation in differentiated HL-60 cells are reported. The structure−activity relationships of this series of compounds on O_2^- generation inhibition are also discussed.

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MATERIALS AND METHODS

General Procedure. Analytical instruments used were as follows: HPLC, HITACHI 655A-11; $[\alpha]_D$, Jasco DIP-1000; UV, Shimadzu UV 200 and UV 2200AI; IR, Shimadzu IR-435; MS,

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Table 1. ^{1}H NMR Data for Compounds 4−6 ($CDCl_3$, TMS, 500 MHz)

	4	5	6
position	δ (ppm), m,[a] *J* (Hz)	δ (ppm), m,[a] *J* (Hz)	δ (ppm), m,[a] *J* (Hz)
1*ax*	3.90, m	4.03, dd, 12.8, 1.4	3.91, m
1*eq*	3.86, m	3.94, d, 12.8	3.86, m
2		4.90, s	
3, 4		2.08−2.18, m	
2, 3, 4	1.60−1.98, m		1.60−1.98, m
6	3.79, d, 2.8	4.09, dd, 7.5, 4.5	4.03, dd, 7.5, 4.5
6-OH		2.87, d, 7.5	2.87, d, 7.5
7	4.28, d, 2.8	4.78, dd, 4.5, 1.4	4.76, dd, 4.5, 1.4
9	5.15, brs	5.21, s	5.19, s
14	1.98, s	1.99, s	1.98, s
2′		2.24, d, 7.0	
3′		2.10, m	
4′, 5′		0.97, d, 6.7	

[a] m, multiplicity.

JEOL JMS-DX 300 and JMS600; ^{1}H NMR, Bruker ARX500 and AC300 (reference TMS). Chromatographic materials used were as follows: Wako gel C-100 and Wako gel C-200 from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan), YMC I-40/64 gel from Yamamura Chemical Laboratory (Kyoto, Japan), and Nova Pak C_{18} (3.9-mm i.d. × 150 mm; Waters Associates, Milford, MA).

Chemicals and Cells. TPA was obtained from Research Biochemicals International, Natick, MA. RPMI 1640 medium and fetal bovine serum were purchased from Gibco RBL, Grand Island, NY. Cytochrome *c* was obtained from Sigma, St. Louis, MO. All other chemicals were purchased from Wako Pure Chemical Industries, Osaka, Japan. Human promyelocytic leukemia HL-60 cells was obtained from the Health Science Research Resources Bank (Collins et al., 1977).

Isolation of Compounds 1−6 from *A. lactiflora*. Fresh leaves of *A. lactiflora* (1 kg) were extracted with MeOH at room temperature, and the filtrate was concentrated in vacuo to give a green oily syrup. Further fractionation was carried out by monitoring the inhibitory activity of TPA-induced O_2^- generation in differentiated HL-60 cells. The extract (30 g) was partitioned between EtOAc and water. The active EtOAc layer was chromatographed on silica gel (Wako gel C-100) to give active 2.5−5% MeOH in $CHCl_3$ eluates. The combined active eluate was further separated on silica gel (Wako gel C-200, *n*-hexane/EtOAc, stepwise) and then on ODS gel (MeOH/H_2O, stepwise). The final purification was done by preparative HPLC on Nova Pak C_{18} (acetonitrile:H_2O = 7:3) to afford six colorless compounds (**1**, 23 mg; **2**, 72 mg; **3**, 10 mg; **4**, 7 mg; **5**, 72 mg; **6**, 5 mg). Compounds **1**−**3** were identical with a diacetylenic spiroketal enol ether: 3,4-epoxy-2-(2,4-hexadiynylidene)-1,6-dioxaspiro[4.5]decane (**3**) and its 2-isovaleroyloxy (**1**) and 2-acetoxy (**2**) derivatives, respectively (Bohlmann et al., 1982; Birnecker et al., 1988).

Compound 4: colorless oil; $[\alpha]^{21}_{D}$ −259.8° (*c* 0.08, $CHCl_3$); UV λ_{max} (EtOH) nm (log ε) 216 (4.17), 225 (4.15), 265 (3.94), 278 (4.11), 293 (4.01); IR ν_{max} (KBr) cm^{-1} 2900, 2140, 1640; ^{1}H NMR see Table 1; ^{13}C NMR (75 MHz, $CDCl_3$) δ 4.7, 18.6, 24.4, 31.2, 58.9, 63.9, 64.7, 68.9, 80.1, 80.8, 82.4, 85.4, 105.5, 166.0, 172.5; EI-MS (probe, 70 eV) *m*/*z* 230 ($[M]^+$, $C_{14}H_{14}O_3$).

Compound 5: colorless oil; $[\alpha]^{24}_{D}$ +290.2° (*c* 0.10, $CHCl_3$); UV λ_{max} (EtOH) nm (log ε) 204 (4.15), 219 (4.27), 261 (4.04), 276 (4.21), 289 (4.17); IR ν_{max} (KBr) cm^{-1} 3400, 2950, 2140, 1720, 1640; ^{1}H NMR see Table 1; ^{13}C NMR (75 MHz, $CDCl_3$) δ 4.7, 22.3, 22.8, 25.9, 43.5, 58.6, 64.6, 65.4, 68.5, 80.4, 81.1, 82.3, 86.1, 105.0, 165.6, 172.5; HR-EI-MS (probe, 70 eV) *m*/*z* 366.1227 ($[M]^+$, calcd for $C_{19}H_{23}O_5Cl$, 366.1234).

Compound 6: colorless oil; $[\alpha]^{24}_{D}$ +342.9° (*c* 0.70, $CHCl_3$); UV λ_{max} (EtOH) nm (log ε) 205 (4.18), 225 (4.28), 265 (4.07), 280 (4.23), 294 (4.20); IR ν_{max} (KBr) cm^{-1} 3450, 2950, 2140, 1640; ^{1}H NMR (500 MHz, $CDCl_3$) see Table 1; ^{13}C NMR (75 MHz, $CDCl_3$) δ 4.7, 19.6, 25.4, 32.2, 59.8, 64.8, 83.4, 86.4, 106.5, 167.0; HR-EI-MS (probe, 70 eV) *m*/*z* 266.0708 ($[M]^+$, calcd for $C_{14}H_{15}O_3Cl$, 266.0710).

LAH Reduction of 3 and 4. Compound **3** (6.1 mg, 26.5 μmol) in dry diethyl ether (Et_2O) was treated with LAH (1 mg, 26.4 μmol). After 1 h, a small amount of water was added, and the mixture was filtered and extracted with Et_2O. The organic layer was dried over Na_2SO_4 and concentrated. The residue was chromatographed on silica gel with *n*-hexane−EtOAc (4:1) to give **7** (3.0 mg, 12.9 μmol, 49% yield): colorless oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.58−2.02 (6H, m), 1.97 (3H, d, 14-H), 2.77 (1H, d, *J* = 17.7 Hz, 7*trans*-H), 3.14 (1H, ddd, *J* = 17.7, 5.5, 2.6 Hz, 7*cis*-H), 3.68 (1H, ddd, *J* = 11.0, 2.5, 2.5 Hz, 1*eq*-H), 3.78 (1H, ddd, *J* = 11.0, 11.0, 3.8 Hz, 1*ax*-H), 4.02 (1H, t, *J* = 5.5 Hz, 6-H), 5.00 (1H, brs, 9-H); EI-MS (probe, 70 eV) *m*/*z* 232 ($[M]^+$, $C_{14}H_{16}O_3$).

A solution of **4** (8.9 mg, 38.7 μmol) in dry Et_2O was treated with LAH (1.5 mg, 39.6 μmol). After 40 min, a small amount of water was added, and the mixture was filtered and extracted with Et_2O. The organic layer was dried over Na_2SO_4, concentrated, and chromatographed on silica gel to give **8** (5.2 mg, 22.4 μmol, 58% yield): colorless oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.60−1.68 (6H, m), 1.97 (3H, d, *J* = 1.0 Hz, 14-H), 2.67 (1H, ddd, *J* = 15.2, 8.8, 2.5 Hz, 7*trans*-H), 3.16 (1H, ddd, *J* = 16.9, 7.7, 1.4 Hz, 7*cis*-H), 3.82−3.86 (2H, m, 1-H), 3.90 (1H, brd, *J* = 7.0 Hz, 6-H), 4.91 (1H, brs, 9-H); EIMS (probe, 70 eV) *m*/*z* 232 ($[M]^+$, $C_{14}H_{16}O_3$). 7*cis* and 7*trans* (compounds **7** and **8**) refer to the positions relative to the proton at C-6.

Preparation of MTPA Esters. Compound **7** (2.6 mg, 11.2 μmol) was dissolved in dry pyridine (50 μL), and (*R*)-(−)-MTPA chloride (5 μL) was added. After 30 min, *N*,*N*-dimethyl-1,3-propanediamine (5 μL) was added and then incubated another 10 min. The mixture was extracted with EtOAc. The organic layer was dried over Na_2SO_4 and concentrated. The product was chromatographed on silica gel with *n*-hexane−EtOAc (9:1) to give the (*S*)-(−)-MTPA ester of **7** (3.8 mg, 8.0 μmol, 71% yield). In the same way, the (*R*)-(+)-MTPA ester of **7** (5.2 mg, 11.9 μmol, 92% yield) from **7** (3.0 mg, 12.9 μmol), the (*S*)-(+)-MTPA ester of **8** (2.5 mg, 5.3 μmol, 88% yield) from **8** (1.4 mg, 6.0 μmol), and the (*R*)-(+)-MTPA ester of **8** (2.6 mg, 6.0 μmol, 92% yield) from **8** (1.5 mg, 6.5 μmol) were prepared, respectively.

NMR Experiments. For the determination of the LIS values, increasing amounts of $Eu(fod)_3$ were added to a solution of **5** or **7** (3 mg in 0.5 mL of $CDCl_3$). The LIS for a concentration ratio of **5** or **7**:$Eu(fod)_3$ = 1:1 was obtained by extrapolation of four different reagent conditions.

Isomerization of 3 to 9. Isomerization of **3** was done by the method previously reported (Birnecker, 1988). Compound **3** (4.2 mg, 17.8 μmol) was dissolved in MeOH/Et_2O = 3:1, to which *p*-toluenesulfonic acid (1 mg, 5.26 μmol) was added. After 6 h, the products were purified by preparative TLC (*n*-hexane/EtOAc = 4:1) to give **9** (0.6 mg, 2.60 μmol, 14.6% yield): colorless oil; $[\alpha]^{23}_{D}$ +250.7° (*c* 0.08, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.61−1.94 (6H, m), 1.98 (3H, s, 14-H), 3.79 (1H, d, *J* = 2.8 Hz, 6-H), 3.86−3.91 (2H, m, 1-H), 4.29 (1H, d, *J* = 2.8 Hz, 7-H), 5.15 (1H, brs, 9-H); EI-MS (probe, 70 eV) *m*/*z* 230 ($[M]^+$, $C_{14}H_{14}O_3$).

Δ^6-Olefin Formation of 7 and 8. Compound **7** (2.6 mg, 11.2 μmol) and *p*-toluenesulfonyl chloride (20 mg, 105.3 μmol) in pyridine were stirred at room temperature for 3 h. The solution was poured into ice-cold water and extracted with EtOAc. The organic layer was washed with water and dried over Na_2SO_4. The reaction mixture was purified by preparative TLC (*n*-hexane/EtOAc = 4:1) to give *p*-toluenesulfonyl ester of **7** (1.9 mg, 2.60 μmol). To a DMSO solution of the *p*-toluenesulfonyl ester was added potassium *tert*-butoxide (1.5 mg, 13.4 μmol), and the mixture was stirred at room temperature for 24 h. The reaction mixture was separated by preparative TLC (*n*-hexane/EtOAc = 9:1) to give **10** (0.4 mg, 1.89 μmol, 17% yield) and **11** (0.4 mg, 1.89 μmol, 17% yield).

Compound 10: colorless oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.42−1.89 (6H, m), 1.98 (3H, d, *J* = 1.0 Hz, 14-H), 3.82−4.02 (2H, m, 7-H), 4.97 (1H, brs, 9-H), 6.22 (1H, dd, *J* = 5.9, 1.7 Hz, 6-H), 6.65 (1H, d, *J* = 5.9 Hz, 7-H); EI-MS (probe, 70 eV) *m*/*z* 214 ($[M]^+$, $C_{14}H_{14}O_3$).

Compound 11: colorless oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.42−1.89 (6H, m), 1.98 (3H, d, *J* = 1.0 Hz, 14-H), 3.82−4.02

(2H, m, 1-H), 4.61 (1H, brs, 9-H), 6.17 (1H, d, J = 5.6 Hz, 6-H), 6.19 (1H, d, J = 5.6 Hz, 7-H); EIMS (probe, 70 eV) m/z 214 ([M]$^+$, $C_{14}H_{14}O_3$).

In the same manner, compound **8** (3.0 mg, 12.9 μmol) was treated with *p*-toluenesulfonyl chloride and potassium *tert*-butoxide to give **10** (0.6 mg, 2.84 μmol, 22% yield) and **11** (0.5 mg, 2.37 μmol, 18% yield). Analytical HPLC of **10** from both **7** and **8** on YMC ODS(A) (mobile phase, 60% acetonitrile in water; flow rate, 1.0 mL/min, detected by UV 254 nm) showed a peak at t_R 12.5 min.

Acetylation of 5 and 6. Compound **5** (2.40 mg, 6.6 μmol) in dry pyridine (0.3 mL) and acetic anhydride (0.02 mL) was allowed to stand at room temperature for 3 h. The solution was poured onto ice-cold water and extracted with EtOAc. The organic layer was washed with water, dried over Na_2SO_4, and purified by preparative HPLC on μBondasphere C_{18} (80% acetonitrile in water) to give an acetate of **5** (1.8 mg, 4.4 μmol, 67% yield): colorless oil; IR ν_{max} (KBr) cm^{-1} 2950, 2140, 1735, 1640; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.96 (6H, d, 6.6 Hz, 4′,5′-H), 1.85−1.92 (6H, m), 1.99 (3H, d, J = 1.1 Hz, 14-H), 2.16 (3H, s, 6-OAc), 2.22 (2H, d, J = 7.0 Hz, 2′-H), 3.88 (1H, d, J = 12.9 Hz, 1*eq*-H), 3.96 (1H, dd, J = 1.6, 12.8 Hz, 1*ax*-H), 4.85 (1H, brs, 2-H), 5.23 (1H, s, 9-H), 5.30 (1H, d, J = 5.2 Hz, 6-H); EIMS (probe, 70 eV) m/z 408 ([M]$^+$, $C_{21}H_{25}O_6Cl$).

Compound **6** (4.72 mg, 17.7 μmol) in dry pyridine (0.5 mL) and acetic anhydride (0.05 mL) was allowed to stand at room temperature for 4 h. The solution was poured onto ice-cold water and extracted with EtOAc. The organic layer was washed with water, dried over Na_2SO_4, and purified by preparative HPLC on μBondasphere C_{18} (75% acetonitrile in water) to give an acetate of **6** (3.8 mg, 12.3 μmol, 69% yield): colorless oil; IR ν_{max} (KBr) cm^{-1} 2950, 2140, 1735, 1640; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.56−1.87 (6H, m), 1.99 (3H, d, J = 1.2 Hz, 14-H), 2.17 (3H, s, 6-OAc), 3.80−3.85 (2H, m, 1-H), 4.97 (1H, dd, J = 3.5, 1.7 Hz, 7-H), 5.22 (1H, s, 9-H), 5.26 (1H, d, J = 5.2 Hz, 6-H); EIMS (probe, 70 eV) m/z 308 ([M]$^+$, $C_{16}H_{17}O_4Cl$).

Alkaline Treatment of 5 and 6. To a MeOH (1 mL) solution of **5** (2.6 mg, 7.1 μmol) was added 0.01% NaOH (0.2 mL). The mixture was stirred at 40 °C for 4 h, then diluted with water, and extracted with EtOAc. The organic layer was washed with water, dried over Na_2SO_4, and purified by preparative TLC (*n*-hexane/EtOAc = 4:1) to give **1** (1.2 mg, 3.6 μmol, 51% yield). Compound **6** (2.5 mg, 9.4 μmol) was treated in the same manner to give **4** (1.0 mg, 4.3 μmol, 46% yield).

Acidic Treatment of 1 and 4. To a MeOH (0.2 mL) solution of **1** (0.5 mg, 1.5 μmol) or **4** (0.5 mg, 2.2 μmol) was added 10% HCl (80 μL). The mixture was stirred at room temperature for 10 min, then diluted with water, and extracted with EtOAc. The organic layer was washed with a saturated $NaHCO_3$ solution, dried over Na_2SO_4, and concentrated. Analytical HPLC of the products from **1** on YMC ODS(A) (mobile phase, 65% acetonitrile in water; flow rate, 1.0 mL/min, detected by UV 254 nm) gave a main peak at t_R 12.5 min, which was in good agreement with that of **5**. In the same way, analytical HPLC of the products from **4** on YMC ODS(A) (mobile phase, 60% acetonitrile in water; flow rate, 1.0 mL/min, detected by UV 254 nm) showed a main peak at t_R 9.5 min in accordance with that of **6**.

Inhibitory Test of TPA-Induced O_2^- Generation in Differentiated HL-60 Cells. Inhibitory test of TPA-induced O_2^- generation in DMSO-differentiated HL-60 cells was done as previously reported (Markert et al., 1984; Murakami et al., 1997). Briefly, to determine the inhibitory effect of O_2^- generation, a test compound dissolved in 5 μL of DMSO was added to DMSO-induced differentiated HL-60 cell suspension in PBS (1 mL) and incubated at 37 °C for 15 min. The cells were washed with PBS twice for removal of extracellular test compound to omit O_2^- scavenging effect. TPA (100 nM) and cytochrome *c* solution (1 μg/mL) were added to the reaction mixture, which was incubated for another 15 min. The reaction was terminated by placing it on ice. After centrifugation at 250*g*, the visible absorption at 550 nm was measured. Inhibitory effects are expressed by a relative decreasing ratio of absorbance of test compounds to a control experiment. No significant decrease in cell viability, assessed by the trypan blue-exclusion test, was observed at concentrations up to 100 μM (data not shown).

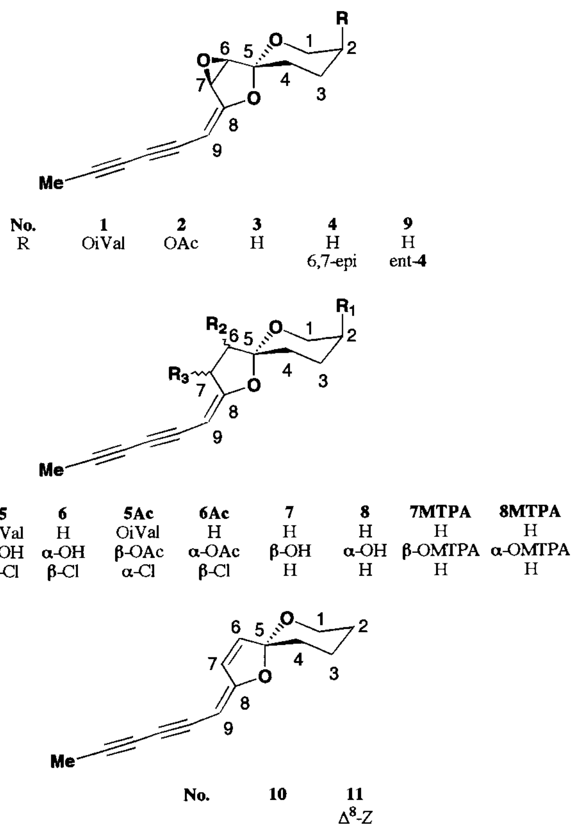

Figure 1. Chemical structures of the acetylenic spiroketal enol ethers (**1**−**6**) from *A. lactiflora* and their derivatives.

RESULTS AND DISCUSSION

Isolation of O_2^- Generation Inhibitors. In the screening test of a variety of edible plants for O_2^- generation inhibition in differentiated HL-60 cells, the methanol (MeOH) extract of *A. lactiflora* showed the highest inhibitory activity. The active constituents were traced by the inhibition test of TPA-induced O_2^- generation in differentiated HL-60 cells. Fresh leaves of *A. lactiflora* (1 kg) were extracted with MeOH, and the extract (30 g) was partitioned between ethyl acetate (EtOAc) and water. The active EtOAc layer was chromatographed on silica gel to give active fractions eluted with 2.5−5% MeOH in $CHCl_3$. The active combined fraction was further separated on silica gel (*n*-hexane/EtOAc, stepwise) and then on ODS gel (MeOH/H_2O, stepwise). The final purification was done by preparative HPLC on Nova Pak C_{18} (acetonitrile:H_2O = 7:3) to afford six colorless compounds (**1**, 23 mg; **2**, 5 mg; **3**, 72 mg; **4**, 10 mg; **5**, 7 mg; **6**, 72 mg).

Structure Elucidation. Three (**1**−**3**) of the six compounds purified have already been isolated from this species by Bohlmann et al. (1982) and Birnecker et al. (1988). Also their structures including absolute stereochemistry, except for the configuration of the epoxide moiety in **1**, were confirmed as shown in Figure 1. On the other hand, the three remaining compounds (**4**−**6**) were concluded to be new on the basis of their spectral data.

Compound **4** ($C_{14}H_{14}O_3$; $[\alpha]^{21}_D$ −276.0°, *c* 0.10, $CHCl_3$) was suggested to be a diastereomer of **3** ($C_{14}H_{14}O_3$; $[\alpha]^{28}_D$

Table 2. ^1H NMR LIS Data for Compounds 5 and 7[a]

	5	5 + Eu(fod)$_3$		7	7 + Eu(fod)$_3$	
position	δ ^1H	δ ^1H	$\Delta\delta$ ^1H	δ ^1H	δ ^1H	$\Delta\delta$ ^1H
1*ax*	4.03	4.21	0.18	3.78	3.88	0.10
1*eq*	3.94	4.16	0.22	3.68	3.82	0.14
4*eq*	2.11	2.41	0.30	2.00	2.43	0.43
6	4.09	4.43	0.34	4.02	4.63	0.61
7*trans*[b]	4.78	5.04	0.26	2.77	3.20	0.43
7*cis*[b]				3.14	3.37	0.23
9	5.21	5.26	0.05	5.00	5.15	0.15

[a] In ppm from internal TMS in $CDCl_3$. [b] 7*cis* and 7*trans* refer to the positions relative to the proton at C-6.

−26.5°, *c* 0.50, $CHCl_3$), on the basis of the MS, ^1H NMR (Table 1), and optical rotation data. The absolute configurations at C-6 of **3** and **4** were examined by the advanced Mosher's method (Fontana et al., 1996). Lithium aluminum hydride (LAH) reductions of **3** and **4** gave the corresponding 6-carbinols (**7** and **8**, respectively) which were then converted to both (*R*)- and (*S*)-α-methoxy-α-(trifluoromethyl)phenylacetate (MTPA) esters. Chemical shift differences ($\Delta\delta$; $\delta S - \delta R$) between *S*- and *R*-esters of **7** showed positive values on H-7 ($\Delta\delta$ = 0.02 ppm) and H-9 ($\Delta\delta$ = 0.07 ppm), while a negative value was shown for H-1 ($\Delta\delta$ = −0.01 ppm). On the other hand, the data from the esters of **4** were reversed (H-7, negative, $\Delta\delta$ = −0.10 ppm; H-1, positive, $\Delta\delta$ = 0.04 ppm). These ^1H NMR analyses clearly indicated that the absolute configurations at C-6 of **3** and **4** are *R* and *S*, respectively. The ^1H NMR data of **7** using lanthanide-induced shift (LIS) reagent allowed us to determine the relative configuration concerning *anti* or *syn* between the hydroxyl group at C-6 and the oxygen of tetrahydropyran. As for **7**, the signals at H-6, H-4 equatorial, and H-7α were significantly shifted downfield ($\Delta\delta$ = 0.43−0.61 ppm). The LIS value for H-4 equatorial ($\Delta\delta$ = 0.43 ppm) was larger than those of H_2-1 ($\Delta\delta$ = 0.14, 0.10 ppm) (Table 2). This result suggested that the oxygen of the tetrahydropyran is oriented in the *anti*-direction to the hydroxyl at C-6. On the basis of these data, the absolute configurations at C-5, -6, and -7 of **3** were determined to be *R*, *R*, and *S*, respectively, as previously reported (Birnecker et al., 1988). The LIS study of **8** failed since all signals were too broad to analyze, suggesting that the oxygen of the tetrahydropyran in **8** is *not* oriented in the *anti*-direction to the hydroxyl at C-6 (Birnecker et al., 1988).

Acid treatment of **3** gave **9**, an isomerized product at C-5 (Birnecker et al., 1988), whose optical rotation (+250.7°) showed the opposite sign to that of **4** (−259.8°), indicating evidently that **4** is the diastereomeric isomer of **3** only in the epoxide orientation. This was further supported by a double-bond formation reaction of **7** and **8**. The tosylates of 6-carbinols **7** and **8** were treated with base to give the corresponding diene spiroketal **10**. The retention time on HPLC as well as ^1H NMR data of **10** from **8** were in good agreement with those of **10** from **7**. Thus the stereochemistry of **4** (5*R*, 6*S*, and 7*R*) was first established. Interestingly, easy stereoisomerization of the exo double bond at C-8 under light was detected. Therefore, it was necessary to carefully treat the diene spiroketal in the dark.

The NMR data of **5** and **6** were similar to those of **1** and **3** (or **4**), respectively, except for the chemical shifts of H-6 and H-7 (Table 1). The chemical shifts of both H-6 and H-7 of **5** (δ 4.09 and 4.78, respectively) and **6** (δ 4.03 and 4.76, respectively) were significantly shifted downfield by 0.2−0.4 ppm as compared with those of **1** and **3** (or **4**). The acetylation of **6** afforded a monoacetate whose ^1H NMR showed a downfield shift of C-6 (δ 5.26), suggestive of the existence of a hydroxyl group at C-6 in **6**. The molecular formulas suggested **5** and **6** ($C_{19}H_{23}O_5Cl$ and $C_{14}H_{15}O_3Cl$, respectively) to be chlorohydrin forms of **1** and **3** (or **4**), respectively.

Alkaline treatment of **5** and **6** afforded **1** and **4**, respectively, and inversely, treatment of **1** and **4** with hydrochloric acid again gave the corresponding chlorohydrins **5** and **6** as main products, respectively. Since chloride ion attacks the carbon on the opposite side of the epoxide at C-7, the absolute configurations of **6** at C-5, -6, and -7 were revealed as *R*, *R*, and *S*. On the other hand, although the absolute configuration at C-5 in **1** had already been confirmed to be *R* by X-ray analysis and CD measurement, the absolute configurations of the epoxide-forming carbons in **1** have not been confirmed yet (Bohlmann et al., 1982). Next the ^1H NMR LIS data of **5** allowed us to determine the relative configuration concerning *anti* or *syn* between the hydroxyl group at C-6 and the oxygen of tetrahydropyran in the same manner as in the case of **7** described above. The signals at H-6, H-4 equatorial, and H-7 were significantly shifted downfield ($\Delta\delta$ = 0.26−0.34 ppm). The LIS value for H-4 equatorial ($\Delta\delta$ = 0.30 ppm) was larger than those of H_2-1 ($\Delta\delta$ = 0.18, 0.22 ppm) (Table 2). This result suggested that the oxygen of the tetrahydropyran is oriented in the *anti*-direction to the hydroxyl at C-6 of **5**. Thus, the absolute configurations of **5** and **6** at C-5, -6, and -7 were confirmed as *R*, *S*, and *R* and *R*, *R*, and *S*, respectively. On the basis of the stereochemistry of **5**, it is thus evident that the absolute configurations of **1** at C-6 and -7 are the same as those of **3** as shown in Figure 1 (*R* and *S*, respectively).

There is some doubt that these chlorohydrins are naturally occurring compounds although the related chlorohydrins are reported to be present in other families of Compositae plants, e.g., *Carthamus*, *Eclipta*, *Echinops*, etc., but not in the *Artemisia* genera (Bohlmann et al., 1973). However, it is important to note that they were detectable in the methanol extract of the fresh leaves by HPLC analysis (data not shown).

Table 3. Inhibitory Effects of the Acetylenes on TPA-Induced O_2^- Generation[a]

	% inhibition at concn of (μM)					
compd	5	10	20	50	100	IC$_{50}$ (μM)
1	28	60	81	>99	NT[b]	7.6
2	NT	32	NT	61	80	29
3	13	15	23	51	77	47
4	NT	NT	23	55	78	43
5	NT	29	NT	67	75	28
6	NT	19	NT	47	60	56
7	NT	5	NT	14	24	>100
9	NT	19	NT	50	61	50
10	NT	6	NT	27	43	>100
11	NT	7	NT	28	40	>100
genistein	NT	0	NT	30	49	102

[a] The maximal SD for each experiment was 5% from at least duplicate tests. [b] NT, not tested.

Inhibitory Activities against TPA-Induced O_2^- Generation. The inhibitory effects of the natural (**1**−**6**) and the related synthesized (**7**, **9**−**11**) compounds on TPA-induced O_2^- generation are summarized in Table 3 together with the effect of genistein, a well-known antitumor promoter from soybean. Compound **1** (IC$_{50}$ = 7.6 μM) was determined to be a quite potent O_2^-

generation inhibitor, whose activity was much stronger than that of genistein ($IC_{50} = 102\ \mu M$). On the other hand, the activities of **3**, **4**, and **9** ($IC_{50} = 47$, 43, and 50 μM, respectively) were 5 times weaker than that of **1**. These results suggested that an acyloxyl group at the C-2 position may be an enhancing factor for inhibition, while the absolute configurations are not important. Inhibitory effects of **1** and its analogues on O_2^- generation were not due to their O_2^- scavenging effect since these compounds were washed out before TPA stimulation in the experimental conditions. Ascorbic acid, which is a water-soluble scavenger of O_2^-, showed no inhibitory effect on O_2^- generation in this system (Nakamura at al., 1998b), suggesting that the substances in the medium but not in the cells could be removed by washing twice with PBS. Significant cellular uptake was observed for compound **1** ($23 \pm 2\%$ during 15 min, total 10 μM **1**; Nakamura et al., unpublished data) using HPLC for monitoring the intra- and extracellular levels. NADPH oxidase is known to play a major role in O_2^- generation in leukocytes such as macrophages, neutrophils, or granulocytes (Cross et al., 1991). Indeed compound **1** showed no O_2^- scavenging potential in the XA/XOD system (data not shown). These results suggested that the polyacetylenes isolated in the present study may act as a generation inhibitor such as enzyme activity inhibitor and/or signal transduction modifier but not just as a scavenger. The inhibitory activities of **10** ($IC_{50} > 100\ \mu M$) and **11** ($IC_{50} > 100\ \mu M$) were significantly lower than those of the corresponding epoxide **3**. Moreover, 6-carbinol **7** ($IC_{50} > 100\ \mu M$) also showed much less inhibitory activity than **3**. These results clearly showed that the reductive ring opening of the epoxy group reduces inhibitory activity. In addition, the stereochemistry of the exo double bond at C-8 is not important for the activity. The evaluation of the presence of the triple bonds for activity and the exact mechanism of action of O_2^- generation inhibition remains to be elucidated.

Conclusion. Three already-known acetylenic compounds and three novel derivatives were isolated and identified from the leaves of *A. lactiflora*, an edible plant in Thailand. In any case, while polyacetylenes are known to possess several biological roles in various ecosystems (nematicidal, antibiotic, insect repellent, etc.) (Yano, 1983), we first described inhibitory effects of the diacetylenes against TPA-induced O_2^- generation in differentiated HL-60 cells. Thus these inhibitors were evaluated as an effective and promising candidate for antitumor-promoting agents since some natural chemopreventers inhibited O_2^- generation by leukocytes as mentioned above. Recently we found these compounds indeed inhibited TPA-induced H_2O_2 generation by leukocytes in mouse skin (Nakamura et al., 1998c). The chemopreventive effect in mouse skin should be examined in the future.

ABBREVIATIONS USED

ROS, reactive oxygen species; XA/XOD, xanthine/xanthine oxidase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; LIS, lanthanide-induced shift.

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