Semisynthesis and Cytotoxicity of Styryl-Lactone Derivatives

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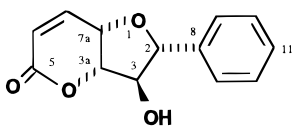
The cytotoxicity and the cell-cycle action of altholactone (**1**), goniofufurone (**2**), and eight altholactone derivatives (**5**−**12**), were determined in vitro on L-1210 cells. Semisyntheses and structure−activity relationships of these compounds are described. The results of this study suggest that the cytotoxicity of altholactone (**1**), 11-nitro-altholactone (**8**), and 7-chloro-6,7-dihydroaltholactone (**10**) is due to the accumulation of the cells in the G2 + M phase of the cell cycle.

The styryl-lactones found abundantly in *Goniothalamus* species (Annonaceae) are an interesting group of cytotoxic and antitumor agents.[1] Altholactone (**1**), a furano-pyrone first isolated in 1977[2] and recently identified as the major compound from the stem bark of *Goniothalamus arvensis*,[3] is known to possess significant cytotoxicity against several tumor cell lines,[4,5] and stereoselective syntheses of **1** were carried out.[6,7] Goniofufurone (**2**), a known furano-furone also isolated from *G. arvensis*[8] and other furano or pyrano styryl-lactone derivatives, showed interesting biological activities.[9,10] The cytotoxic activities previously reported for eight-membered-ring ζ-lactones (heptolides)[11,12] were recently explained by a noncompetitive mechanism of action as inhibitors of the mammalian respiratory chain complex I for almuheptolide A (**3**), a 8-phenyl-2-oxocanone isolated from *G. arvensis* and also synthesized from **1** *via* the 6,7-dihydro-7-ethoxy-altholactone (etharvensin, **4**).[13]



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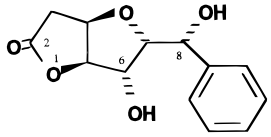

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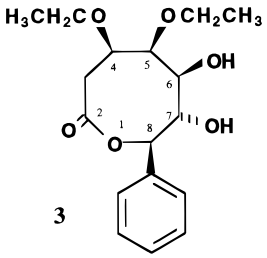

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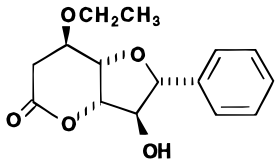

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Herein we describe the semisynthesis of eight new styryl-lactone derivatives in the altholactone series (**5**−**12**). The cytotoxicity of these and the two natural compounds **1** and **2** are also reported.

Results and Discussion

Altholactone (**1**), a 2-phenyl-3-hydroxy-6,7-dihydro-furano-pyrone, represents a good starting material because it is available in large quantity from *G. arvensis*.[3] Treatment of **1** (previously 3-acetylated) with osmium tetroxide (OsO_4) in water, and *N*-methylmorpholine *N*-oxide monohydrate (NMO) to regenerate the oxidizing agent,[14] afforded the corresponding *cis*-dihydroxylated derivative **5**. The 3-acetyl-6,7-diacetoxy-6,7-dihydro-altholactone (**6**) was obtained by treatment of **1** with NMO and OsO_4, followed by acetic anhydride in dry pyridine. When a solution of **5** was treated with an excess of triethylamine and a precipitate obtained from Me_3SiCl and Me_2SO,[15] the 6,7-methylendioxy derivative was afforded (**7**) (see Scheme 1).

The methylendioxy moiety that was formed in **7** connects the diol of **5** but does not change the stereochemistry of their carbinol centers. A *cis*-configuration among the 6, 7, and 7a centers in these three last compounds (**5**−**7**) can be explained as in etharvensin (**4**)[16] by observation of their ^{1}H NMR coupling constants ($J_{7a,7}$ and $J_{7,6}$ between 3.5 and 4 Hz). On the other hand, when an organic solution of **1** was added to a mixture (1:1) of concentrated nitric acid and sulfuric acid, two nitro-altholactone derivatives, **8** (11-nitro-altholactone) and **9** (9,13-dinitro-altholactone), were obtained in similar yields. The mechanism of aromatic nitration involves an electrophilic attack of the nitronium ion at the ortho- or para-position in the monosubstituted aromatic ring of **1**.

An original stereospecific chlorination at C-7 of **1** (β to the lactonic carbonyl) was carried out under acidic conditions using $POCl_3$ at room temperature. An EIMS fragment ion at *m*/*z* 232 $[M - HCl]^+$, as well as a dd at δ 4.54 in ^{1}H NMR (H-7) with its characteristic coupling constant values corresponding to a *cis*-relationship between the neighbor protons ($J_{6a,7}$ = 3.7 Hz and $J_{7a,7}$ = 4 Hz) and a signal at δ 50.5 in ^{13}C NMR (C-7), were in agreement with a β-7-chloro-6,7-dihydro-altholactone (**10**).

A method for the preparation of both 7-alkoxylated-6,7-dihydro-δ-lactone (**11**) and 4,5-dialkoxylated eight-membered-ring ζ-lactones with a heptolide skeleton (**12**), has been successfully achieved from **1**.[13] The enantiospecific syntheses of compounds **11** and **12** from **1** was carried out in a one-step reaction, using MeOH and concentrated H_2SO_4. Formation of 7-methoxy-6,7-dihydro-altholactone (**11**) is postulated to be a direct Michael-type addition of MeOH from the less electron-dense position (β to the C=O).[16] Subsequent nucleophilic attack with a second MeOH molecule by a S_N2 mechanism would lead to inversion of the configuration, followed by a concerted opening of the α-pyrone and the tetrahydrofuran ring and a subsequent intramolecular eight-ring closure.[13]

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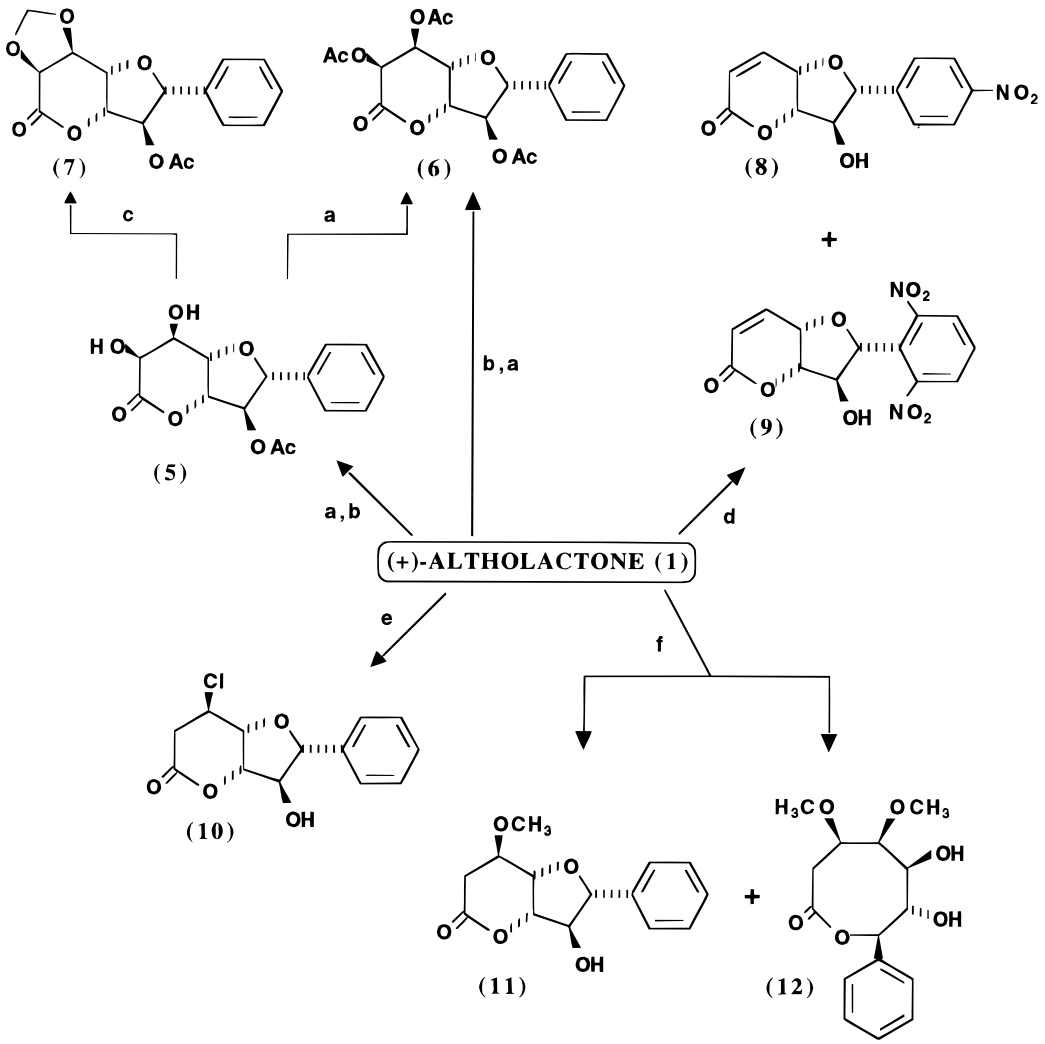
Scheme 1[a]



[a] Reagents and conditions: (a) Ac_2O/Pyr, room temperature; (b) NMO/OsO_4 room temperature, followed by 2% $NaHSO_3$, room temperature; (c) Me_3SiCl/Me_2SO, followed by $(C_2H_5)_3N$, room temperature; (d) HNO_3/H_2SO_4, 0 °C; (e) $POCl_3/CH_2Cl_2$, room temperature, (f) $MeOH/H_2SO_4$, reflux.

Table 1. Cytotoxic Activity of Styryl−Lactone Derivatives[a]

compounds	IC_{50} (μg/mL)
1	2.9
8	3.2
9	5.0
10	3.7
12	5.2

[a] Inhibition of L-1210 cell proliferation measured by the MMT assay. IC_{50} of compounds **2**, **5**−**7**, and **11** with IC_{50} > 10 μg/mL.

In view of the biological interest focused on this class of compounds, a study of the cytotoxic activities of the known natural (**1** and **2**) and the new synthetic (**5**−**12**) styryl-lactone derivatives was carried out in vitro using L-1210 leukemia cells. Altholactone (**1**), some of its furano-pyrone derivatives (**8**−**10**) and the heptolide **12**, were more cytotoxic than the remaining derivatives (**2**, **5**−**7**, and **11**) inhibiting L-1210 cell proliferation (see Table 1). Structure−activity relationships in the styryl-lactone series indicate that the 6,7-double bond on the pyrone ring or the chloration at 7 position is the more important structural requirement to increase the cytotoxicity. So, altholactone (**1**), the two nitro-derivatives on the aromatic ring (**8**,**9**), and the chloro dihydro-altholactone (**10**) were more cytotoxic than the furano-furone (**2**) or the 6,7-dihydro derivatives of altholactone, whether dihydroxylated (**5**), triacetylated (**6**), methylendioxy (**7**), or methoxylated (**11**). Moreover, the heptolide **12** shows a very important cytotoxicity, explained by the inhibition of the mammalian respiratory chain complex I.[13]

Perturbation of the cell cycle induced by compounds **1**, **8**, **9**, **10**, and **12** was studied using the same cell line. Altholactone (**1**), 11-nitro-altholactone (**8**), and 7-chloro-6,7-dihydro-altholactone (**10**) induced a partial accumulation (35−45%) of the cells in the G2 + M phase on the cell cycle. However, the 9,13-dinitro-altholactone (**9**) and the heptolide (**12**) showed a nonselective accumulation in the cell cycle.

Experimental Section

General Experimental Procedure. Optical rotations were determined on a Perkin−Elmer 241 polarimeter. IR spectra (film) were run on a Perkin−Elmer 1750 FTIR spectrometer. MS were recorded with a VG Auto Spec Fisons spectrometer, and the liquid secondary ion mass spectrum (LSIMS) was obtained by fast ion bombardment. ^{1}H NMR (250, 300 and 400 MHz), ^{13}C NMR, and DEPT (62.5, 75 and 100 MHz) spectra were recorded on Bruker AC-250, Varian Unity-300, and Varian Unity-400 instruments. The signals of ^{1}H NMR of all the compounds were assigned by COSY 45. Si gel TLC plates were observed under UV light (254 nm) and after spraying with anisaldehyde in H_2SO_4.

Plant Material. *Goniothalamus arvensis* Scheff. (Annonaceae) was collected in the National Park of Varirata, located

in the Central Province of Papua New Guinea. A voucher specimen was deposited in the herbarium of the University of Papua New Guinea.

Extraction and Isolation. Dried and powdered stem bark of *G. arvensis* (368 g) was macerated with MeOH at room temperature. The concentrated MeOH extract was partitioned between hexane and 50% aqueous MeOH. The aqueous MeOH extract was fractionated successively into CH_2Cl_2 and EtOAc. The CH_2Cl_2 extract (7.0 g) was applied by Si gel 60 H column chromatography to afford altholactone (**1**, 2.3 g) and goniofufurone (**2**, 165 mg).

(+)-Altholactone (1): $C_{13}H_{12}O_4$; $[\alpha]_D$ +174.2° (*c* 1.0, EtOH); EIMS *m/z* $[M]^+$ 232.[3,17,18]

(+)-Goniofufurone (2): $C_{13}H_{14}O_5$; $[\alpha]_D$ +12° (*c* 1.1, EtOH); mp 154−155 °C (EtOH−hexane); EIMS *m/z* $[M]^+$ 250.[8,17,18]

(−)-3-Acetyl-6,7-dihydroxy-6,7-dihydro-altholactone (5). Treatment of **1** (150 mg, 0.64 mmol) with dry pyridine (1 mL) and Ac_2O (2 mL) afforded 3-acetyl-altholactone (170 mg; 0.62 mmol; 96%) after stirring for 1 h at room temperature. To a solution of 3-acetyl-altholactone (55 mg; 0.20 mmol) and *N*-methylmorpholine *N*-oxide monohydrate (NMO, 27.1 mg; 0.20 mmol) in 3 mL of Me_2CO-H_2O (2:1) was added a 4% solution of OsO_4 in H_2O (0.3 mL; 0.05 mmol) dropwise. The reaction mixture was stirred at room temperature for 1.5 h, and then a 2% solution of $NaHSO_3$ in H_2O (2 mL) was added. After being stirred for 30 min, the reaction mixture was extracted with EtOAc, which was washed with H_2O and brine, and concentrated to dryness. The reaction residue was subjected to column chromatography on Si gel 60 H (eluted with CH_2Cl_2) to afford 46.5 mg of **5** (75% from 3-acetyl-altholactone): $C_{15}H_{16}O_7$; $[\alpha]_D$ −12.2° (*c* 1.8, EtOH); IR (dry film) ν_{max} 3432 (OH), 2920, 1752 (C=O), 1373, 1235, 1117, 1047, 950, 914, 864, 763, 735, 700 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.38−7.30 (m, H-9 to H-13), 5.31 (dd, H-3), 5.08 (dd, H-3a), 4.88 (d, H-2), 4.62 and 4.60 (m, 2H, H-6 and H-7), 4.53 (m, H-7a), 2.13 (s, 3H, 3-OCOC*H*$_3$); $J_{2,3}$ = 5.1 Hz; $J_{3,3a}$ = 1.4 Hz; $J_{3a,7a}$ = 3.9 Hz; $J_{7a,7}$ = 3.2 Hz; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.0 (C-5), 169.4 (3-O*C*OCH$_3$), 136.9 (C-8), 128.8 (3 CH), 126.1 (2 CH), 85.0 (C-3a), 84.8 (C-2), 82.6 (C-3), 77.0 (C-7a), 68.1 (C-6 and C-7), 20.7 (3-OCO*C*H$_3$); ^1H and ^{13}C spectra were unambiguously assigned by using 2D NMR techniques (COSY 45 and HMQC); EIMS *m/z* (%) $[M]^+$ 308 (5), 266 $[M - COCH_2]^+$ (17), 248 $[M - HOCOCH_3]^+$ (74), 232 $[M - OCOCH_3 - OH]^+$ (3), 215 $[M - OCOCH_3 - 2\ OH]^+$ (1), 144 (100), 107 (78), 91 (49), 77 (32).

3-Acetyl-6,7-diacetoxy-6,7-dihydro-altholactone (6). To a solution of altholactone (**1**, 50 mg; 0.21 mmol) in 3 mL of Me_2CO-H_2O (2:1) was added NMO (27.1 mg; 0.20 mmol) and catalytic 4% solution of OsO_4 in H_2O (0.3 mL; 0.05 mmol) dropwise, and the resulting solution was stirred at room temperature for 6 h. The reaction mixture was concentrated to dryness by blowing air over the solution, and the residue was dissolved in EtOAc and filtered through a plug of Si gel. The solution was concentrated to dryness, and the residue was dissolved in dry pyridine (1 mL) and Ac_2O (2 mL). The resulting solution was stirred at room temperature for 4 h. The residue obtained was extracted with CH_2Cl_2, washed with H_2O, and dried. The dry extract was subjected to column chromatography on Si gel 60 H (eluted with CH_2Cl_2−EtOAc 100:1) to give 39 mg of **6** (46%): $C_{19}H_{20}O_9$; IR (dry film) ν_{max} 2927, 1752 (C=O), 1372, 1218, 1047, 702 cm^{-1}; ^1H NMR ($CDCl_3$, 250 MHz) δ 7.37−7.28 (m, H-9 to H-13), 5.90 (d, H-6), 5.78 (t, H-7), 5.35 (dd, H-3), 5.02 (dd, H-3a), 4.94 (d, H-2), 4.49 (t, H-7a), 2.17, 2.15 and 2.13 (3s, 9H, 3-, 6-, and 7-OCOC*H*$_3$); $J_{2,3}$ = 4.7 Hz; $J_{3,3a}$ = 1.3 Hz; $J_{3a,7a}$ = 4.0 Hz; $J_{7a,7}$ = 3.8 Hz; $J_{7,6}$ = 3.4 Hz; ^{13}C NMR ($CDCl_3$, 62.5 MHz) δ 169.2 (3 C, 3-, 6-, and 7-O*C*OCH$_3$), 164.3 (C-5), 136.4 (C-8), 128.9 (3 CH), 126.0 (2 CH), 85.4 (C-3a), 84.4 (C-2), 82.5 (C-3), 75.1 (C-7a), 68.2 and 65.8 (C-6 and C-7), 20.7, and 20.4 (3 C, 3-, 6-, and 7-OCO*C*H$_3$); EIMS *m/z* (%) $[MH]^+$ 393 (5), 334 $[M - OCOCH_3]^+$ (2), 274 $[M - 2\ OCOCH_3]^+$ (3), 215 $[M - 3\ OCOCH_3]^+$ (50), 185 (100), 155 (75), 105 (94), 91 (70), 77 (24).

3-Acetyl-6,7-dihydro-6,7-methylendioxy-altholactone (7). To Me_3SiCl (2 mL) was added Me_2SO (2 mL) and dry CH_2Cl_2 (2 mL), and the mixture was allowed to stand at room temperature for about 1 h until a white precipitate appeared. The excess of unreacted reagents was decanted, and the white precipitate was quickly washed with CH_2Cl_2 (1 mL). A solution of **5** (25 mg; 0.08 mmol) in CH_2Cl_2 (2 mL), and an excess of $(C_2H_5)_3N$ was added to this precipitate and stirred at room temperature for 15 h. When the reaction was complete as estimated by TLC, the mixture was washed using 1% $NaHCO_3$ (5 mL) and H_2O (2 × 5 mL), and the CH_2Cl_2 layer was evaporated in vacuo. The crude product was subjected to column chromatography on Si gel 60 H (eluted with CH_2Cl_2−EtOAc 60:40) to afford 14 mg of **7** (54%): $C_{16}H_{16}O_7$; IR (dry film) ν_{max} 2800, 1752 (C=O), 1238, 1155 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.35−7.30 (m, H-9 to H-13), 5.33 (dd, H-3), 5.11 and 5. 06 (2d, 2 H, OCH_2O), 4.99 (dd, H-3a), 4.91 (d, H-2), 4.87 (d, H-6), 4.68 (dd, H-7), 4.54 (t, H-7a), 2.14 (s, 3 H, OCOC*H*$_3$); $J_{2,3}$ = 4.2 Hz; $J_{3,3a}$ = 1.2 Hz; $J_{3a,7a}$ = 3.9 Hz; $J_{7a,7}$ = 4.1 Hz; $J_{7,6}$ = 2.5 Hz; J_{OCH2O} = 7.3 Hz;^{13}C NMR ($CDCl_3$, 75 MHz) δ 169.2 (3-*C*OCH$_3$), 167.0 (C-5), 136.9 (C-8), 128.9 (3 CH), 126.2 (2 CH), 94.8 (O*C*H$_2$O), 85.8, 83.3 and 82.5 (3C, C-2, C-3 and C-3a), 74.6 and 72.4 (2C, C-6 and C-7), 20.8 (3-OCO*C*H$_3$); EIMS *m/z* (%) $[M]^+$ 320 (3), 261 $[M - OCOCH_3]^+$ (20), 233 (12), 145 (64), 133 (85).

Semisyntheses of 8 and 9. To 1.5 mL of concentrated HNO_3 was added dropwise at 0 °C concentrated H_2SO_4 (0.5 mL). To a CH_2Cl_2 solution (0.5 mL) of altholactone (**1**, 150 mg, 0.64 mmol) was added dropwise at 0 °C concentrated H_2SO_4 (2.0 mL). Both solutions were mixed and stirred at room temperature for 30 min, then H_2O was added and the reaction products extracted into CH_2Cl_2. The residue was subjected to column chromatography on Si gel 60 H (eluted with CH_2Cl_2−EtOAc 90:10) to afford 39 mg of **8** (22%) and 55 mg of **9** (27%).

(+)-11-Nitro-altholactone (8): $C_{13}H_{11}NO_6$; $[\alpha]_D$ +184° (*c* 0.7, EtOH); mp 178−180 °C ($CHCl_3$−MeOH); IR (dry film) ν_{max} 3356 (OH), 1714 (C=O), 1529 (NO_2), 1339, 1243, 1089 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.16 (d, 2 H, H-10 and H-12), 7.51 (d, 2 H, H-9 and H-13), 7.05 (dd, H-7), 6.25 (d, H-6), 4.94 (dd, H-3a), 4.87 (d, H-2), 4.70 (t, H-7a), 4.38 (dd, H-3); $J_{2,3}$ = 5.3 Hz; $J_{3,3a}$ = 2.0 Hz; $J_{3a,7a}$ = 4.9 Hz; $J_{7,7a}$ = 5.0 Hz; $J_{7,6}$ = 10.0 Hz; $J_{9,10}$ = $J_{12,13}$ = 8.7 Hz; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 161.2 (C-5), 147.6 (C-8), 145.7 (C-11), 140.1 (C-7), 126.4 (2 CH, C-9 and C-13), 123.9 (C-6), 123.8 (2 CH, C-10 and C-12), 86.3 (C-3a), 84.8 (C-2), 83.5 (C-3), 68.4 (C-7a); LSIMS *m/z* 300 $[M + Na]^+$, 278 $[MH]^+$; EIMS *m/z* (%) $[M]^+$ 277 (7), 232 $[MH - NO_2]^+$ (10), 213 $[M - NO_2 - H_2O]^+$ (20), 122 (16), 97 (100).

(+)-9,13-Dinitro-altholactone (9): $C_{13}H_{10}N_2O_8$; $[\alpha]_D$ +155° (*c* 0.8, EtOH); mp 103−106 °C ($CHCl_3$−MeOH); IR (dry film) ν_{max} 3382 (OH), 1718 (C=O), 1529 (NO_2), 1340, 1244, 1102, 1052 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.23 (d, 2 H, H-10 and H-12), 7.53 (d, H-11), 7.07 (dd, H-7), 6.35 (d, H-6), 5.46 (dd, H-3a), 5.12 (d, H-2), 5.08 (br t, H-3), 4.63 (dd, H-7a); $J_{2,3}$ = 3.7 Hz; $J_{3,3a} \approx$ 1.0 Hz; $J_{3a,7a}$ = 3.9 Hz; $J_{7,7a}$ = 5.4 Hz; $J_{7,6}$ = 9.8 Hz; $J_{10,11}$ = $J_{11,12}$ = 9.0 Hz; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 159.5 (C-5), 148.1 (C-8), 143.6 (2 C, C-9 and C-13), 138.1 (C-7), 126.9 (2 CH, C-10 and C-12), 125.2 (C-11), 124.1 (C-6), 90.8 (C-3a), 83.0 (C-2), 82.5 (C-3), 69.5 (C-7a); LSIMS *m/z* 345 $[M + Na]^+$, 323 $[MH]^+$; EIMS *m/z* (%) $[MH]^+$ 323 (10), 277 $[MH - NO_2]^+$ (40), 231 $[MH - 2\ NO_2]^+$ (19), 213 $[M - OH - 2\ NO_2]^+$ (10), 167 (87), 165 (100).

(+)-6,7-Dihydro-7-chloro-altholactone (10). To a dry CH_2Cl_2 solution (10 mL) of altholactone (**1**, 50 mg, 0.21 mmol) was added $POCl_3$ (1.0 mL; 0.5 mmol) dropwise. After stirring for 4 h at room temperature, the reagents were removed under reduced pressure, then H_2O was added and the reaction mixture extracted into CH_2Cl_2. The organic solution after usual workup was purified by column chromatography on Si gel 60 H (eluted with CH_2Cl_2−EtOAc 90:10), to afford 30 mg of **10** (52%). $C_{13}H_{13}O_4Cl$; $[\alpha]_D$ +22.3° (*c* 2.8, EtOH); IR (dry film) ν_{max} 3421 (OH), 3035, 2920, 1733 (C=O), 1386, 1055, 762, 700 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.37−7.28 (m, H-9 to H-13), 5.04 (dd, H-3a), 4.78 (d, H-2), 4.54 (dd, H-7), 4.51 (t, H-7a), 4.34 (dd, H-3), 3.21 (dd, H-6b), 2.93 (dd, H-6a); $J_{2,3}$ = 5.6 Hz; $J_{3,3a}$ = 1.4 Hz; $J_{3a,7a}$ = 4.0 Hz; $J_{7a,7}$ = 4.1 Hz; $J_{7,6b}$ = $J_{7,6a}$ = 3.7

Hz; $J_{6a,6b}$ = 17.5 Hz; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 167.1 (C-5), 137.7 (C-8), 128.8 (2 CH), 128.5 (CH), 125.9 (2 CH), 86.9 (C-3a), 86.8 (C-2), 83.4 (C-3), 75.4 (C-7a), 50.6 (C-7), 34.9 (6-CH_2); ^{1}H and ^{13}C spectra were unambiguously assigned by using 2D NMR techniques (COSY 45 and HMQC); EIMS *m/z* (%) $[M]^+$ 268.5 (14), 251 $[M - OH]^+$ (15), 232 $[M - HCl]^+$ (64), 215 $[M - OH - HCl]^+$ (5), 107 (83), 97 (100), 91 (62), 77 (60).

(−)-6,7-Dihydro-7-methoxy-altholactone (11): $C_{14}H_{16}O_5$; $[\alpha]_D$ -69.2° (*c* 1.3, EtOH); EIMS *m/z* $[M]^+$ 264; proposed according to literature.[13]

(+)-4,5-Methoxy-6,7-hydroxy-8-phenyl-oxocanone (12): $C_{15}H_{20}O_6$; $[\alpha]_D$ +15° (*c* 0.8, EtOH); LSIMS *m/z* $[MH]^+$ 297; proposed according to literature.[13]

Cytotoxicity and Cell Culture. L-1210 cells were cultivated in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 units/mL of penicillin, 50 μg/mL of streptomycin, and 10 mM of Hepes buffer (pH = 7.4). Cells were exposed to graded concentrations of drug for 48 h. Cytotoxicity was measured by the microculture tetrazolium assay.[19] Results are expressed as IC_{50}, the concentration needed to reduce by 50% the optical density of treated cells with respect to the optical density of untreated controls.

For the cell-cycle analysis, L-1210 cells were incubated for 21 h at 37 °C with various concentrations of drugs. Cells were then fixed by 70% EtOH (v/v), washed, and incubated with PBS containing 100 μg/mL RNAse and 25 μg/mL propidium iodide for 30 min at 20 °C. Results are expressed as percentage of accumulated cells in the G2 + M phase after 21 h compared with the control.

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