Inhibition of Hepatitis B Surface Antigen Secretion on Human Hepatoma Cells. Components from *Rubia cordifolia*

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The antiviral activity in the roots of *Rubia cordifolia* was examined, and three naphthohydroquinones, furomollugin (**1**), mollugin (**2**), and rubilactone (**3**), were isolated from it. Compounds **1** and **2** strongly suppressed the secretion of hepatitis B surface antigen (HBsAg), both with $IC_{50} = 2.0$ μg/mL, in human hepatoma Hep3B cells while having little effect on the viability of the cells. Evaluation of structurally related derivatives of **1** and **2** revealed that a 6-hydroxy group and a pyran or furan ring contribute to this suppressive effect.

Hepatitis B virus (HBV) infection commonly results in chronic and acute hepatitis. Males over 40 years of age infected with HBV have high chances of developing primary hepatocellular carcinoma.[1−4] Immunization against HBV has helped somewhat in preventing chronic infection cases,[5] but still not enough to solve this problem. Therefore, new drugs to eradicate HBV in chronic carriers[6−9] are still needed.

Recently, it was demonstrated that human hepatoma Hep3B cells, a cell line containing HBV genomes which continually secretes HBsAg into the culture medium, can serve as a quick assay system for screening agents from natural resources[10] for anti-HBV activity.

During our continuing screening for anti-HBV activity in traditional drugs and herbal materials, we found that the $CHCl_3$ fraction of the MeOH extract obtained from the roots of *Rubia cordifolia* L. (Rubiaceae), a Chinese herbal medicine with antitumor and other activities,[11,12] suppressed HBsAg secretion in human hepatoma Hep3B cells. From this fraction we isolated three known naphthohydroquinones: furomollugin (**1**), mollugin (**2**), and rubilactone (**3**).[13,14] Compounds **1** and **2** showed strong suppressive activity on HBsAg secretion in human hepatoma Hep3B cells. From these results, furomollugin methyl ether (**4**), mollugin methyl ether (**5**), dihydromollugin (**6**), acetylmollugin (**7**), and structurally related derivatives of naphtho[1,2-*b*]pyran (**8**−**12**) were prepared and evaluated. The activities of **1** and **2** are also reported. Chromatography separation on Si gel of the active $CHCl_3$ fraction resulted in furomollugin (**1**), mollugin (**2**), rubilactone (**3**), and two anthraquinones: 1-hydroxy-2-methyl-9,10-anthraquinone and 2-methyl-1,3,6-trihydroxy-9,10-anthraquinone. The identities of these compounds were confirmed spectroscopically and/or compared with authentic samples.[15−18] Experimental results (Table 1) showed that furomollugin (**1**) and mollugin (**2**) were the major principles responsible for the suppressive effect since the treated cells were still viable and continued to proliferate during the 48 h incubation period (Figure 1); they did not show cytotoxicity. 2-Methyl-1,3,6-trihydroxy-9,10-anthraquinone 3-*O*-(3′-*O*-acetyl)-α-rhamnosyl(1→2)glucoside and 2-methyl-1,3,6-trihydroxy-9,10-anthraquinone 3-*O*-α-rhamnosyl-(1→2)glucoside obtained from the *n*-butanol fraction as well as two anthraquinones from the $CHCl_3$ fraction did not give significant results ($IC_{50} > 5$ μg/mL).

Nine derivatives **4**−**12** were synthesized and then assayed for their biological activity. The results are depicted in Table 1. In general, the presence of substituted groups on carbon positions 5 and 6 of 2,2-dimethyl-2*H*-naphtho[1,2-*b*]pyran affected the suppression of HBsAg secretion. A 6-methoxy group in compounds **4**, **5**, **10**, and **12** caused the activity to diminish. A large substituent such as isopentyl or benzyl on **8** and **9** at C-5 and C-6, respectively, increased the activity, indicating that the binding pocket on carbons 5 and 6 is essentially hydrophobic. The presence of a lactone ring, as in **3** and **11**, abolished the activity. This showed that pyran or furan rings are also important for activity. In summary, the above results showed that minor changes in substitution patterns can cause observable changes in anti-HBV activities. Further studies are needed to determine the mechanism of anti-HBV activity by these compounds.

Experimental Section

General Experimental Procedures. Melting points were taken on a Yanaco micromelting point apparatus and were uncorrected. Infrared spectra (potassium bromide unless otherwise noted) were determined on a Bio-Rad FTIR FTS-7 spectrophotometer. NMR spectra were recorded on either a Bruker AC-300 or Varian

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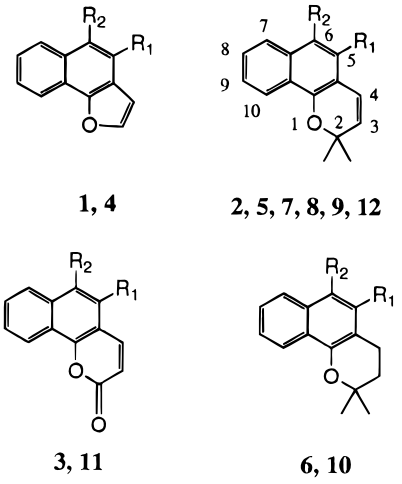
Table 1. Biological Testing Results of Furomollugin, Mollugin and Their Derivatives for Suppression of HBsAg Secretion in Hep3B Cells[a]



no.	R_1	R_2	IC_{50}[b] (μg/mL)	CC_{50}[c] (μg/mL)
1	COOMe	OH	2.0	11.5
2	COOMe	OH	2.0	*e*
3	COOMe	OH	*d*	4.0
4	COOMe	OMe	*d*	*e*
5	COOMe	OMe	*d*	*e*
6	COOMe	OH	2.0	8.2
7	COOMe	OAc	2.0	10.0
8	$COOCH_2CH_2CH(CH_3)_2$	OMe	0.8	10.0
9	COOMe	OCH_2Ph	1.0	*e*
10	COOMe	OMe	*d*	7.0
11	COOMe	OMe	*d*	12.5
12	COOH	OMe	4.5	*e*

[a] All compounds at different concentrations were tested in triplicate for their effect on the secretionof HBsAg by Hep3B cells in 48 h.[10,19] [b] IC_{50} is the concentration of tested compounds which exerted 50% reduction on HBsAg secretion in Hep3B cells. [c] CC_{50} is the concentration of tested compounds which showed 50% cellular cytotoxicity in Hep3B cells using MTT assay. [d] Suppression of HBsAg secretion was not detected relative to nontreated cells or which the concentration was greater than 5 μg/mL. [e] The cytotoxicity was not detected relative to nontreated cells or which the concentration was greater than 20 μg/mL.[20]

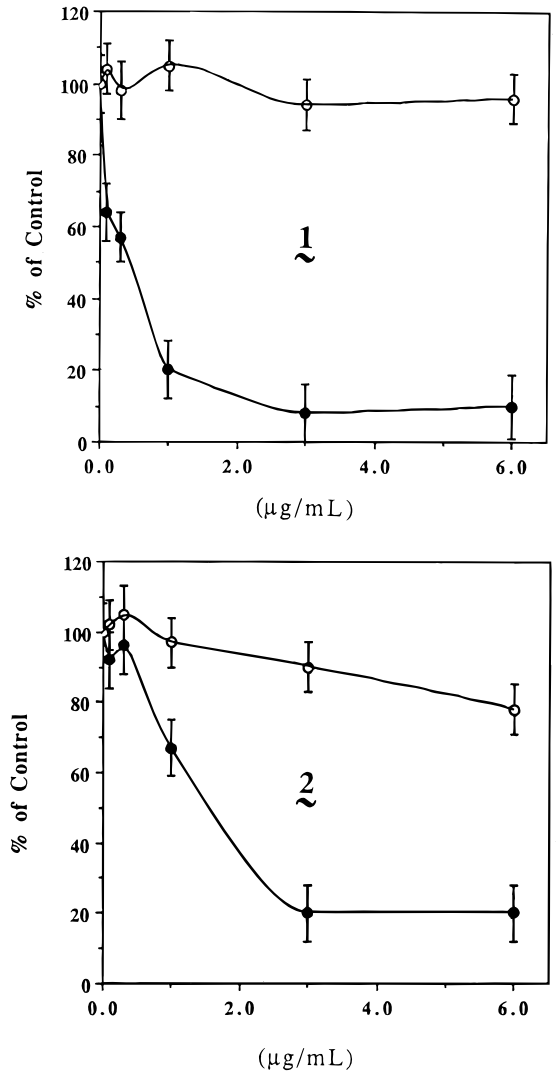


Figure 1. Effect of furomollugin (**1**) and mollugin (**2**) on the cell growth and secretion of HBsAg by human hepatoma Hep3B cells. Viable cells (○) and HBsAg (●) titers were measured in Hep3B cell cultures after 48 h of growth in serum-free DMEM containing furomollugin (**1**) and mollugin (**2**). The titers obtained without **1** or **2** were set at 100%.

Gemini-200 spectrometer. Chemical shifts are reported in δ units relative to internal tetramethylsilane. Mass measurements were measured with a Finnigan MAT TSQ-46C mass spectrometer. THF and dioxane were distilled from Na/benzophenone, CH_2Cl_2 was distilled from calcium hydride, and all other solvents were used without further purification. Si gel column chromatography was effected using Kieselgel 60 (70−230 or 230−400 mesh, E. Merck). HBsAg enzyme immunoassay (EIA) kits were purchased from Ever New Corp. (Taipei, Taiwan). Fetal calf serum was obtained from Hyclone (Logan, UT). Dulbecco's modified Eagle's medium (DMEM) balanced salt solutions were obtained from Gibco/BRL (Gaithersburg, MD).

Plant Material. The roots of *R. cordifolia* were purchased from a local Chinese drug market and identified by Mr. Jun-Chih Ou, National Research Institute of Chinese Medicine. A voucher specimen is deposited at the herbarium of this institute.

Isolation of Furomollugin (1), Mollugin (2), and Rubilactone (3). The MeOH extract of chopped roots of *R. cordifolia* (3 kg) was partitioned successively with *n*-hexane, $CHCl_3$, *n*-butanol, and water. The $CHCl_3$ layer was subjected to column chromatography over Si gel and eluted with *n*-hexane−$CHCl_3$ (9:1−1:1) to give furomollugin, mollugin, rubilactone, 1-hydroxy-2-methyl-9,10-anthraquinone, and 2-methyl-1,3,6-trihydroxy-9,10-anthraquinone after recrystallization with *n*-hexane/CH_2Cl_2. 2-Methyl-1,3,6-trihydroxy-9,10-anthraquinone 3-*O*-(3′-*O*-acetyl)-α-rhamnosyl(1→2)glucoside and 2-methyl-1,3,6-trihydroxy-9,10-anthraquinone 3-*O*-α-rhamnosyl-(1→2)glucoside were obtained from the *n*-butanol layer after column chromatography over Si gel eluting with $CHCl_3$−MeOH (9:1−1:1). These known compounds were characterized by spectroscopic analysis.

Methylfuromollugin (4). To a solution of furomollugin (100 mg, 0.41 mmol) in acetone (20 mL) was added K_2CO_3 (200 mg, 1.45 mmol). After the reaction solution was stirred at rt for 1 h, MeI (0.1 mL, 1.6 mmol) was added. The reaction mixture was then stirred at rt for overnight. After removal of the solvent *in vacuo*, Et_2O (15 mL) was added. The etheral solution was washed with saturated NaCl solution, dried over $MgSO_4$, and concentrated to give a light yellow residue which was purified by column chromatography on Si gel using 5% EtOAc/*n*-hexane as eluent to yield methylfuromollugin (92 mg, 87%): mp 42 °C; ^1H NMR ($CDCl_3$) δ 8.30−8.23

(2H, m), 7.75 (1H, d, J = 2.0 Hz), 7.70−7.49 (2H, m), 7.21 (1H, d, J = 2.0 Hz), 4.05 (3H, s), 4.03 (3H, s); IR ν_{max} 1723 cm^{-1}; EIMS m/z 256 [M^+].

Mollugin Methyl Ether (5): yellow oil (198 mg, 95%); ^1H NMR ($CDCl_3$) δ 8.17−8.20 (1H, m), 7.99−8.04 (1H, m), 7.46−7.50 (2H, m), 6.40 (1H, d, J = 9.9 Hz), 5.66 (1H, d, J = 9.9 Hz), 3.98 (3H, s), 3.95 (3H, s), 1.50 (6H, s); IR (CH_2Cl_2) ν_{max} 1726 cm^{-1}; EIMS m/z 298 [M^+] (30), 283 (100), 210 (12).

Dihydromollugin (6). Pt/C (5%, 25 mg) was added to a solution of mollugin (100 mg, 0.35 mmol) in methanol (15 mL), and the mixture was stirred in a H_2 atmosphere for 5 h. The solution was then filtered through Celite 545. Evaporation of the solvent followed by Si gel chromatography gave dihydromollugin as a yellow solid (87 mg, 90%): mp 96−97 °C; ^1H NMR ($CDCl_3$) δ 12.1 (1H, s), 8.35 (1H, d, J = 8.1 Hz), 8.16 (1H, d, J = 8.1 Hz), 7.58 (1H, t, J = 7.8 Hz), 7.47 (1H, t, J = 7.8 Hz), 3.97 (3H, s), 3.04 (2H, t, J = 6.6 Hz), 1.82 (2H, t, J = 6.6 Hz), 1.39 (6H, s); EIMS m/z 286 [M^+].

Acetylmollugin (7). To a solution of mollugin (100 mg, 0.35 mmol) in pyridine (5 mL) was added acetic anhydride (5 mL). After the mixture was stirred overnight at rt, ice-cold water (30 mL) was added. The mixture was then extracted with Et_2O (20 mL) twice, dried over $MgSO_4$, and concentrated to give an oil residue which was purified by chromatography on Si gel using 10% CH_2Cl_2/hexane as eluent to give acetymollugin (110 mg, 96%): ^1H NMR ($CDCl_3$) δ 8.25−8.18 (1H, m), 7.76−7.68 (1H, m), 7.51−7.43 (2H, m), 6.59 (1H, d, J = 10.0 Hz), 5.66 (1H, d, J = 10.0 Hz), 3.92 (3H, s), 2.39 (3H, s), 1.50 (6H, s); IR (CH_2Cl_2) ν_{max} 1770 and 1725 cm^{-1}; EIMS m/z 326 [M^+].

Isopentyl 2,2-Dimethyl-6-methoxy-2*H*-naphtho-[1,2-*b*]pyran-5-carboxylate (8). To a solution of mollugin (50 mg, 0.18 mmol) in 4-methyl-1-butanol was added a few drops of H_2SO_4, and the mixture was refluxed for 15 h. The solvent was removed by evaporation, and the residue was then extracted with diethyl ether, washed with water, dried over $MgSO_4$, and concentrated to give an oil residue which was purified by chromatography on Si gel using 10% CH_2Cl_2/*n*-hexane as eluent to give **8** (18 mg, 28%) as a colorless oil along with recovered starting material: ^1H NMR ($CDCl_3$) δ 8.16−8.22 (1H, m) 7.99−8.04 (1H, m), 7.43−7.51 (2H, m), 6.42 (1H, d, J = 9.9 Hz), 5.67 (1H, d, J = 9.9 Hz), 4.43 (2H, t, J = 6.9 Hz), 4.00 (3H, s), 1.82 (1H, m), 1.70 (2H, q, J = 6.9 Hz), 1.50 (6H, s), 0.97 (6H, d, J = 6.5 Hz); IR (CH_2Cl_2) ν_{max}: 1722 cm^{-1}; EIMS m/z 354 [M^+] (32), 339 (100), 269 (28).

Methyl 2,2-dimethyl-6-benzoxy-2*H*-naphtho[1,2-*b*]pyran-5-carboxylate (9): yellow oil (172 mg, 46%); ^1H NMR ($CDCl_3$) δ 8.21−8.24 (1H, m), 8.06−8.09 (1H, m), 7.35−7.53 (7H, m), 6.45 (1H, d, J = 10.2 Hz), 5.69 (1H, d, J = 10.2 Hz), 5.11 (2H, s), 3.90 (3H, s), 1.52 (6H, s); IR (CH_2Cl_2) ν_{max} 1729 cm^{-1}; EIMS m/z 374 [M^+] (28), 283 (94), 251 (100), 223 (52), 91 (46).

Dihydromollugin methyl ether (10): light yellow oil (93 mg, 92%); ^1H NMR ($CDCl_3$) δ 8.18−8.24 (1H, m), 7.99−8.05 (1H, m), 7.44−7.50 (2H, m), 3.96 (3H, s), 3.95 (3H, s), 2.78 (3H, t, J = 6.7 Hz), 1.85 (3H, t, J = 6.7 Hz), 1.39 (6H, s); IR (CH_2Cl_2) ν_{max} 1728 cm^{-1}; EIMS m/z 300 [M^+] (27), 253 (18), 244 (30), 229 (100), 213 (30) 83 (93).

Methylrubilactone (11). To a solution of rubilactone (100 mg, 0.37 mmol) in acetone (20 mL) was added K_2CO_3 (200 mg, 1.45 mmol). After the reaction solution was stirred at rt for 1 h, MeI (0.1 mL, 1.6 mmol) was added. The reaction mixture was then stirred at 35−40 °C for 4 h. The solvent was then removed in vacuo, and Et_2O (15 mL) was added. The etheral solution was washed with water (15 mL), dried over $MgSO_4$, and concentrated to give a light yellow residue which was purified by chromatography on Si gel using 5% EtOAc/*n*-hexane as eluent to yield methylrubilactone (84 mg, 80%): mp 146 °C; ^1H NMR ($CDCl_3$) δ 8.47−8.42 (1H, m), 8.13−8.09 (1H, m), 7.88 (1H, d, J = 9.8 Hz), 7.69−7.59 (2H, m), 6.47 (1H, d, J = 9.8 Hz), 4.01 (3H, s), 3.98 (3H, s); IR ν_{max} 1737 and 1718 cm^{-1}; EIMS m/z 284 [M^+].

2,2-Dimethyl-6-methoxy-2*H*-naphtho[1,2-*b*]pyran-5-carboxylic Acid (12). To a solution of mollugin methyl ether (300 mg, 1.0 mmol) in 1:1 methanol/water solution was added KOH (1 g, 17.8 mmol), and the mixture was refluxed for 6 h. The solution was then acidified with dilute aqueous 0.1 N HCl, extracted with Et_2O, and dried over $MgSO_4$. Evaporation of the solvent followed by Si gel chromatography gave **12** (200 mg, 70%) as a white solid: mp 147−148 °C; ^1H NMR ($CDCl_3$) δ 8.97 (1H, br), 8.17−8.22 (1H, m), 7.99−8.05 (1H, m), 7.45−7.52 (2H, m), 6.76 (1H, d, J = 10.0 Hz), 5.67 (1H, d, J = 10.0 Hz), 4.00 (3H, s), 1.49 (6H, s); IR ν_{max} 1678 cm^{-1}; EIMS m/z 284 [M^+] (25), 269 (100), 210 (14).

Determination of HBsAg. Human hepatoma cells, Hep3B, were seeded in 24-well plates at a density of 1 × 10^5 cells/well in DMEM medium containing 10% fetal calf serum. After 24 h incubation, cells were washed three times with phosphate-buffered saline (pH 7.0) and treated with various concentration of drugs in serum-free DMEM for 48 h. The HBsAg in culture medium were measured by enzyme immunoassay (EIA) kits. Control cells produced 15 ng of HBsAg/10^6 cells/48 h. Cell numbers were determined by hemocytometer and trypan blue exclusion[10,19] and/or MTT staining.[20]

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