Anticancer Constituents from the Roots of *Rubia cordifolia* L.

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Activity-directed isolation of the methylene chloride fraction of the roots of *Rubia cordifolia* L. resulted in the identification of a new epoxymollugin (3) and eight known compounds (1, 2, 4—9). The structures of the compounds were elucidated from chemical and spectroscopic evidence. In addition, their topoisomerase I and II inhibitory activities and cytotoxicities were measured.

Key words *Rubia cordifolia* L.; epoxymollugin; topoisomerase; cytotoxicity

The roots of *Rubia cordifolia* L. have been used as a traditional Korean medicine to treat cough, bladder and kidney stones, joint inflammation, uterine hemorrhage and uteritis. Anthraquinone, anthraquinone glycoside, naphthoquinone, naphthoquinone glycoside, furomollugin, mollugin, alizarin, lucidin pimeveroside, ruberythric acid, purpurin, xanthopurpurin, cyclohexapeptide, alkaloid and lignan have been reported from *Rubia* species.1—4)

In the course of screening tests for topoisomerase I and II inhibitors and cytotoxicity of 40 species of Korean traditional anticancer plants, we found that the methylene chloride fraction of the roots of *Rubia cordifolia* L. (the methanol extract of this root was subsequently fractionated into four fractions; methylene chloride, ethyl acetate, n-butanol and water) showed strong cytotoxicity against HT-29 and MCF-7 cell lines, as well as DNA topoisomerase I and II inhibitory activities. In our previous studies of the methylene chloride fraction of this plant, we isolated seven compounds belonging to the lignan, anthraquinone, triterpenoid and sterol groups.5) As part of our continuing efforts to find anticancer constituents from the methylene chloride fraction of this plant, we have isolated compounds 1—9 on column chromatography over silica gel and RP C18, and investigated their DNA topoisomerase I and II inhibitory activities and cytotoxicities. Compound 3 was identified as epoxymollugin, and this marked its first isolation from a natural source. Compounds 1, 2 and 4—9 were known compounds: furomollugin (1),4) mollugin (2),1) 3,3’/H11032, bis(3,4-dihydro-4-hydroxy-6-methoxy-2H-1-benzopyran) (4)4) 2-carbomethoxy-2,3-epoxy-3-prenyl-1,4-naphthoquinone (5),4) 1-acetoxy-3-methoxy-9,10-anthraquinone (6),7) 1,6-dihydroxy-2-methyl-9,10-anthraquinone (soranjidiol, 7),8—10) 1-hydroxy-2-methoxy-9,10-anthraquinone (alizarin 2-methyl ether, 8)10) and oleanolic acid (9).11) These compounds were identified by direct comparison with authentic samples or by comparing their physical and spectral data with those in the literature. Compound 6 had been reported on 13C-NMR chemical shifts through the synthetic processes,7) but this marked its first isolation from

Fig. 1. Chemical Structures of Compounds 1—8
natural sources. Compounds 4, 7 and 9 were isolated for the first time from this plant.

HR-FAB-MS spectral analysis of compound 3 showed the [M+H]+ at m/z 301.0772 (Calcd 301.0776) which corresponded to the molecular formula C17H17O5. Its IR (ν \text{ max} 3433, 1653, 1238 cm−1) data suggested the presence of a hydroxyl group and an ester band.

The 1H- and 13C-NMR data of compound 3 were identified by comparison to those of 2. The 1H-NMR spectrum (Table 1) of compound 3 showed doublets at δ 3.82 (1H, J = 5.0 Hz) and δ 5.19 (1H, J = 5.0 Hz), which were attributed to H-3 and H-4 of a pyran ring epoxidized between C-3 and C-4. There was also a multiplet at δ 7.57 (2H, H-8, 9) and doublets at δ 8.17 (1H, J = 7.5 Hz, H-7) and δ 8.33 (1H, J = 7.5 Hz, H-10). Accordingly, H-7, 8, 9 and H-10 of aromatic protons were closed, which was also confirmed from the HMBC spectrum.

There were three singlets at δ 1.48 (6H), 4.03 (3H) and δ 11.15 (1H) for two methyl protons bonded at C-2, methyl proton of a methyl ester and a phenolic hydroxyl proton, respectively.

The 13C-NMR and DEPT spectra showed three methyl, six methine and eight quaternary carbon signals including characteristic peaks of different methyl groups and ester group. In the 13C-NMR spectrum of 3, C-3 and C-4 signals were observed at δ 72.1 and δ 64.3 instead of δ 129.0 and δ 122.3 as in compound 2 due to the epoxidation of the double bond. That spectrum also indicated one aromatic carbon (δ 155.6) bearing hydroxyl group, and methyl carbon (δ 52.8) and carbonyl carbon (δ 171.2) of a methyl ester. The correlations in the 1H–1H correlation spectroscopy (COSY) spectrum displayed connectivities between H-4 (δ 5.19) and H-3 (δ 3.82), between H-7 (δ 8.17) and H-9 (δ 7.57), and between H-10 (δ 8.33) and H-8 (δ 7.57). In the HMBC spectrum of 3, connectivities were observed between H-3 and C-2, and between H-4, C-3, C-2, C-4a and C-10b, respectively.

Accordingly, 3 was identified as epoxymollugin, the first isolation from a natural source.

Compound 6 had the molecular formula C_{17}H_{12}O_{5} as determined from El-MS and NMR data. Its UV (λ\text{ max} (CHCl_{3}) 461.0 nm (ε 1639)) and IR (ν\text{ max} 1737, 1677, 1594, 1026 cm−1) data suggested the presence of carbonyl and aromatic double bonds and an ester band. The 1H-NMR spectrum of compound 6 showed singlets at δ 2.36 and δ 4.01, which were attributed to methyl protons of acetyl and methoxy groups. Multiplets were seen at δ 7.79 (2H, H-6 and 7), and δ 8.09 (2H, H-5 and 8), respectively. Accordingly, H-5, 6, 7 and H-8 of aromatic protons were closed, which was confirmed in the HMBC spectrum. The 13C-NMR and DEPT spectra showed two methyl, six methine and nine quaternary carbon signals including characteristic peaks of ketonic carbonyl carbons and methoxy carbon. In the 13C-NMR spectrum of 6, ketonic carbonyl carbon signals and carbonyl carbon of acetyl group signals were shown at δ 183.2 and δ 182.0, and δ 157.6, respectively. The 1H–13C COSY spectrum displayed connectivities between H-2 (δ 6.26) and methyl proton of acetyl group (δ 2.36), between H-4 (δ 7.66), and H-2 (δ 6.26) and methoxyl proton (δ 4.01), respectively. This compound was reported on 13C-NMR chemical shifts through the synthetic processes,7) but was first isolated from natural source, and spectroscopic data such as UV, IR, 1H-NMR and MS have never been reported.

Topoisomerase I and II inhibitory activities were measured by assessing the relaxation of supercoiled pBR 322 plasmid DNA. As shown in Table 2, 1, 6 and 8 strongly inhibited DNA topoisomerase I at 100 μM, and when these three compounds were assayed at 20 μM, 1 and 6 showed 42 and 33% inhibition, respectively. In DNA topoisomerase II assays, 1, 4 and 9 showed 65, 54 and 70% inhibition at 100 μM, respectively. The new compound, 3, showed 42 and 23% inhibition for topoisomerases I and II at 100 μM, respectively. Camptothecin and etoposide for the positive control assays of topoisomerase I and II inhibited 81 and 86% at 100 μM and 70 and 58% at 20 μM, respectively.

The tetrazolium-based colorimetric assay (MTT assay) was used to assess cytotoxicity towards human colon carcinoma (HT-29), human breast carcinoma (MCF-7) and human liver carcinoma (HepG2) cell lines. As shown in Table 2, IC_{50} values of 66.9, 51.6 and 61.4 μM were obtained for 1, 8 and 9 in the HT-29 cell line, 41.3 μM for 3 in the MCF-7 cell
line, and 60.2 and 69.3 $\mu m$ for 2 and 6 in the HepG2 cell line. Notably, the IC$_{50}$ values for 5 were 14.3, 25.0 and 40.5 $\mu m$ in the HT-29, MCF-7 and HepG2 cell lines, respectively.

At 100 $\mu m$, 6 showed the strongest inhibitory activity toward DNA topoisomerase I, and 8, the second most effective inhibitor, has selective inhibitory activity against DNA topoisomerase I compared with DNA topoisomerase II. Against DNA topoisomerase II, the first and second most effective inhibitors, 9 and 1, had selective inhibitory activity against DNA topoisomerase II compared with DNA topoisomerase I. However, these four compounds showed weak cytotoxicity against DNA topoisomerase II compared with DNA topoisomerase I.

However, these four compounds showed weak cytotoxicity against HT-29, MCF-7 and HepG2 cell lines. Compound 5 showed strong cytotoxicity against HT-29 and MCF-7 cell lines, but weak inhibitory activity against DNA topoisomerases I and II. These results indicate no obvious correlation between the cytotoxicity of these compounds and their inhibitory activity against DNA relaxation and decatenation by DNA topoisomerases I and II. Compound 1, with its naphthofuran moiety, exhibited stronger inhibitory activity against DNA topoisomerase I and II than 2, with its naphthopyran ring. The DNA topoisomerase I and II inhibitory activities and cytotoxicity against MCF-7 cell line of 3 (epoxymollugin) were stronger than those of 2 (mollugin) due to epoxidation. Based on the inhibitory activities against DNA topoisomerases I and II of anthraquinones (6–8), the acetyl group on C-1 and the methoxyl group on C-2 or C-3 are considered to enhance the inhibition of DNA topoisomerases I and II.

Experimental

**Plant Material** The roots of *Rubia cordifolia* L. were purchased in November 2002 from a folk medicine market, "Yak-ryong-si", in Daegu, Republic of Korea. These materials were confirmed taxonomically by Professor Chong Won Kim, College of Pharmacy, Catholic University of Daegu, Korea. A voucher specimen has been deposited at the College of Pharmacy, Catholic University of Daegu, Korea.

**General Procedures** Melting points were measured using a Yanaco micro melting point apparatus. Optical rotation was measured using a JASCO DIP-370 digital polarimeter. UV spectra were measured on a Shimadzu UV-160A spectrometer. IR (KBr disk) spectra were measured on a Mattson Genesis II and JASCO-300E FT-IR spectrophotometers, EI-MS and HR-FAB-MS were performed with a Quattro II spectrometer. The NMR spectra were recorded in CD$_3$OD, CDCl$_3$, and C$_6$D$_5$N on Varian Unity INOVA-500 and Bruker DMX 250.

**Extraction and Isolation** Roots of *Rubia cordifolia* L. (10kg) were cut into small pieces and extracted four times with MeOH at 60°C. MeOH extract (1048.4 g) was dissolved in H$_2$O (2.2 l) and fractionated with CH$_2$Cl$_2$ (21 l, 360.0 g). The H$_2$O layer was extracted sequentially with EtOAc (21 l, 214.2 g) and n-butanol (21 l, 117.6 g). One hundred and eighty grams of CH$_2$Cl$_2$ extract were subjected to flash column chromatography over 4 kg silica gel using 100% hexane to 100% CH$_2$Cl$_2$ in 2–15% stepwise elutions. The authentic samples were confirmed by UV, IR, $^{1}$H and $^{13}$C-NMR data that follows in the Results and Discussion and by comparison with authentic samples.

**Compound 3** (Epoxymollugin): Brown powder, mp 135.2–137.1°C, $\left[\alpha\right]_D^{22}$ +33.3 $^{(c}=0.02,\text{CHCl}_3\right)$, UV $\lambda_{max}$ (CHCl$_3$) nm (log e): 369.5 (3.57), $\nu_{max}$ (KBr) cm$^{-1}$: 3433 (OH), 1653 (C=O), 1447 (C=C), 1238 (ester C–O). EI-MS m/z: 318 [M+H$^+$]), 300 [M$^+$], 286, 215, 214, 158. HR-FAB-MS (pos.) m/z: 301.1072 for C$_{16}$H$_{16}$O$_4$ (Calcd 301.1076). $^{1}$H- and $^{13}$C-NMR data are shown in Table 1.

**Compound 6** (1-Acetoxy-3-methoxy-9,10-anthraquinone): Yellowish powder, mp 101.2–102.9°C, UV $\lambda_{max}$ (CHCl$_3$) nm (log e): 461.0 (3.21), IR $\nu_{max}$ (KBr) cm$^{-1}$: 1737, 1570, 1514 (C=C–O), 1584 (C=C–O). EI-MS m/z: 296 [M$^+$], 281 [M–CH$_3$], 265 [M–OCH$_3$]$,^2$ 253 [M–COCH$_3$]$^2$, 238. $^{1}$H-NMR ($\delta$, CDCl$_3$): 2.36 (3H, s, –OCOMe), 4.01 (3H, s, –OCH$_3$), 6.26 (1H, d, J=3.5 Hz, H-2), 7.66 (1H, d, J=3.5 Hz, H-4). 7.69–7.89 (2H, m, H$_3$-2, 3). 22.7 mg), methyl 2,2-dimethyl-3,4-epoxy-6-hydroxy-2-fl-naphth[1,2-b]pyran-5-carboxylate (epoxymollugin, 3, 16.3 mg), 1,6-di-hydroxy-2-methyl-9,10-anthraquinone (sarosanidol, 7, 13.3 mg) and 1-hydroxy-2-methoxy-9,10-anthraquinone (alizarin 2-methyl ether, 8, 18.9 mg).

The structures of these compounds were elucidated on the basis of the data that follows in the Results and Discussion and by comparison with authentic samples.

## Table 2. Inhibitory Effects of Compounds 1—9 on DNA Topoisomerases I and II (% Inhibition Ratio of Relaxation) and Their IC$_{50}$ Values against HT-29, MCF-7 and HepG2 Cell Lines

<table>
<thead>
<tr>
<th>Compd.</th>
<th>Topo I (%)</th>
<th>Topo II (%)</th>
<th>IC$_{50}$ ($\mu m$)</th>
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<td>20 (µm)</td>
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<tr>
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<td>CPT$^{(d)}$</td>
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<td>70</td>
<td>NA$^{(e)}$</td>
</tr>
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6, 7), 8.06—8.12 (2H, m, \textit{H}-5, 8). \textsuperscript{13}C-NMR (\delta, \text{CDCl}_3): 14.0 (–OCOMe), 52.7 (–OMe), 111.0 (C-2), 124.1 (C-4), 126.2 (C-5), 126.8 (C-8), 129.7 (C-4a), 130.2 (C-9a), 131.3 (C-10a), 132.0 (C-8a), 133.9 (C-6), 134.3 (C-7), 144.0 (C-1), 157.6 (–OCOMe), 166.0 (C-3), 182.0 (C-10), 183.2 (C-9).

\textbf{Assay for DNA Topoisomerase I Inhibition in Vitro} DNA topoisomerase I inhibition assays were carried out according to the method reported by Fukuda et al.\textsuperscript{12} with minor modifications. DNA topoisomerase I activity was measured by assessing the relaxation of supercoiled pBR 322 plasmid DNA. The reaction mixture contained 35 mM Tris–HCl (pH 8.0), 72 mM KCl, 5 mM MgCl\textsubscript{2}, 5 mM dithiothreitol, 2 mM spermidine, 0.01% bovine serum albumin (BSA), 250 ng pBR 322 plasmid DNA, and 0.3 U calf thymus DNA topoisomerase I. This reaction mixture was used to measure the inhibition of DNA relaxation by the DNA topoisomerase I, by a test compound solution (less than 0.25% DMSO) in a final volume of 10 \textmu l. The reaction mixtures were incubated for 30 min at 37 °C, and terminated by adding a dye solution containing 2.5% SDS, 15% ficoll-400, 0.05% bromophenol blue, 0.05% xylene cyanole and 25 mM EDTA (pH 8.0). The reaction products were determined by electrophoresis on 1% agarose gels in TBE (Tris-borate-EDTA) running buffer at 1.5 V/cm for 10 h. The gels were stained with ethidium bromide (0.5 g/ml) for 30 min, and then destained in water for 30 min. For visualization and quantitative analyses of DNA topoisomerase I activities, the gels were directly scanned with an image analyzer, and the area representing supercoiled DNA was calculated.

\textbf{Assay for DNA Topoisomerase II Inhibition in Vitro} DNA topoisomerase II activity was measured by assessing relaxation of supercoiled pBR 322 plasmid DNA. The reaction mixtures contained 50 mM Tris–HCl (pH 8.0), 120 mM KCl, 10 mM MgCl\textsubscript{2}, 0.5 mM ATP, 0.5 mM dithiothreitol, 300 ng pBR 322 plasmid DNA, 0.3 U human DNA topoisomerase II, and the indicated concentrations of test compounds (less than 0.25% DMSO) in a final volume of 20 \textmu l. The reaction mixtures were incubated for 30 min at 37 °C and terminated by addition of 5 \textmu l of a mixture containing 0.77% SDS, 77 mM EDTA (pH 8.0), 30% sucrose, 0.5% bromophenol blue and 0.5% xylene cyanole. The reaction products were determined by electrophoresis on 1% agarose buffer running at 1.5 V/cm for 10 h. The gels were stained with 0.5 \mu g/ml ethidium bromide for 30 min and destained in water for 30 min. For visualization and quantitative analyses of the DNA topoisomerase II activity, the gels were directly scanned with an image analyzer, and the area representing supercoiled DNA was calculated.

\textbf{Cytotoxicity Bioassays} The tetrazolium-based colorimetric assay (MTT assay) was used as an \textit{in vitro} assay of cytotoxicity against human colon carcinoma (HT-29), human breast carcinoma (MCF-7) and human liver carcinoma (HepG2) cells.\textsuperscript{13}

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\textbf{References}