Notes

Screening of Triterpenoids Isolated from *Phyllanthus flexuosus* for DNA Topoisomerase Inhibitory Activity

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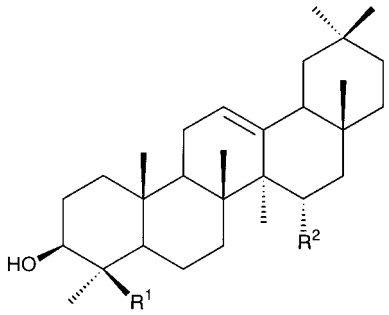
DNA topoisomerases (Topos) are enzymes that play a crucial role in DNA metabolism events such as replication, transcription, recombination, and chromosome segregation at mitosis. Thus, Topo inhibitors could be expected to have antitumor effects. Naturally occurring lupane- and oleanane-type triterpenoids isolated from the bark of *Phyllanthus flexuosus* were screened for human Topos I and II inhibitory activities. Olean-12-en-3β,15α-diol (**1**), olean-12-en-3β,15α,24-triol (**3**), lupeol (**4**), and betulin (**6**) were found to be selective catalytic inhibitors of human Topo II activity with IC_{50} values in the range of 10−39 μM.

DNA topoisomerases (Topos) are nuclear enzymes that play crucial roles in DNA metabolism events such as replication, transcription, recombination, and chromosome segregation at mitosis.[1] Topos are mechanistically divided into two main classes: the type I enzymes (Topo I) catalyze an ATP-independent relaxation of DNA supercoils by transiently breaking and religating single-stranded DNA, and the type II topoisomerases (Topo II) relax supercoiled DNA through catalysis of a transient breakage of double-stranded DNA in an ATP-dependent manner. To date, many Topo inhibitors, e.g., camptothecin[2] for Topo I, aclarubicin,[3] merbarone,[4] etoposide,[5] and ICRF-193[6] for Topo II, have been developed, and Topos have been identified as a target for anticancer chemotherapeutic drug development.[7]

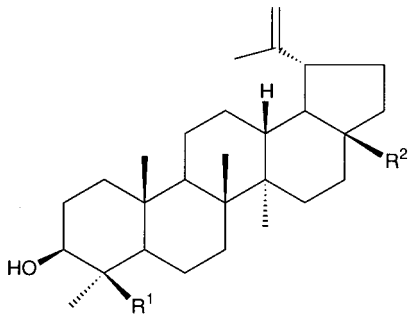
We have an interest in the discovery of novel inhibitors as lead structures for drug development by isolating active constituents from plants. In our continuing studies toward Topo inhibitors, we have previously reported that 3,4-*seco*-8β*H*-ferna-4(23),9(11)-dien-3-oic acid isolated from *Euphorbia chamaesyce* was a selective catalytic inhibitor of Topo II activity without the stabilization of a DNA/Topo II cleavable complex.[8] Recently, it has been reported that triterpenoid compounds lupeol,[9] 3α,27-dihydroxylup-20(29)-en-28-oic acid methyl ester,[10] fomitellic acid A, and ursolic acid[11] inhibit significantly *Drosophila melanogaster* or human Topo II activity. Lupeol also exhibited cytotoxic activity, and the mechanism was thought to be inhibition of Topo II activity.[9] A detailed examination of the inhibitory activity exhibited by lupeol has not been performed.

Continuously, we have screened naturally occurring constituents for Topo inhibitory activity to discover more potent inhibitors. We report herein the result of screening of lupane- and oleanane-type triterpenoids isolated from the bark of *Phyllanthus flexuosus*[12] for Topos I and II inhibitory activities.

The conversion of supercoiled plasmid DNA to relaxed DNA by human Topos I and II was examined in the presence of six triterpenoids, olean-12-en-3β,15α-diol (**1**), olean-12-en-3β,24-diol (**2**), olean-12-en-3β,15α,24-triol (**3**), lupeol (**4**), lup-20(29)-en-3β,24-diol (**5**), and betulin (**6**).[12]



	R^1	R^2
1	Me	OH
2	CH_2OH	H
3	CH_2OH	OH



	R^1	R^2
4	Me	Me
5	CH_2OH	Me
6	Me	CH_2OH

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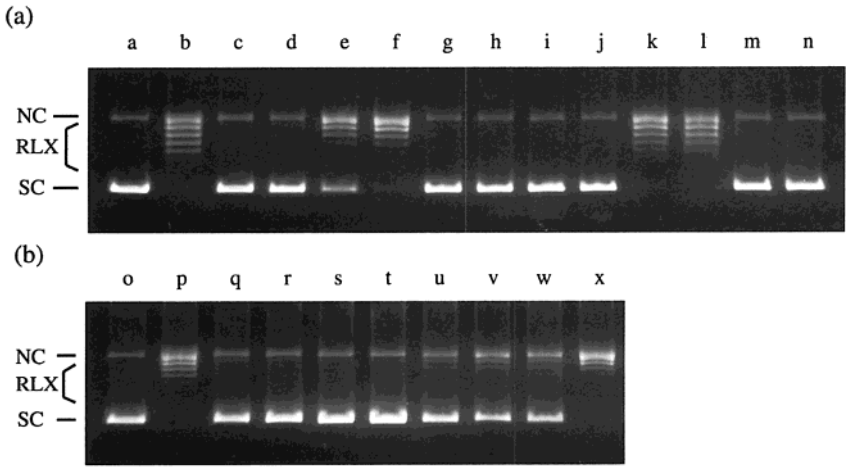

Figure 1. Effects of triterpenoids **1**−**6** on the DNA relaxation activity by DNA topoisomerase II (a) and those of DNA and enzyme concentrations on topoisomerase II inhibition by compound **4** (b). (a) Lane a, supercoiled DNA alone (no drug, no enzyme); lane b, control (no drug); lanes c, e, g, i, k, and m, 200 μM of **1**, **2**, **3**, **4**, **5**, and **6**, respectively; lanes d, f, h, j, l, and n, 100 μM of **1**, **2**, **3**, **4**, **5**, and **6**, respectively. SC: supercoiled DNA. RLX: relaxed DNA topoisomers. NC: nicked circle DNA. (b) Lane o, DNA alone (198 ng of DNA); lane p, control (198 ng of DNA and 1 unit of enzyme); lane q, same as lane p but 50 μM of compound **4** was added; lanes r−t, same as lane q except that the amount of DNA was increased to 264, 330, and 396 ng, respectively; lanes u−x, same as lane q except that the amount of the enzyme was increased to 2, 3, 4, and 5 units, respectively.

Table 1. IC_{50} Values of Triterpenoids and Etoposide on Topoisomerase II Catalytic Activity

	IC_{50} (μM)[a]
1	17.5
2	>200
3	38.7
4	10.4
5	N.I.[b]
6	38.6
etoposide	50.0

[a] IC_{50} values were determined by interpolation from plots of enzyme activity versus inhibitor concentration. The IC_{50} values are means from at least three independent experiments. [b] N.I.: no inhibition at 200 μM.

None of these compounds inhibited DNA relaxation by Topo I, even though the concentrations were increased up to 200 μM (data not shown here). However, among these compounds, **1**, **3**, **4**, and **6** showed complete inhibition of the catalytic reaction by Topo II at concentrations of 200 and 100 μM, respectively (Figure 1a). Compounds **2** and **5** showed 33% and no inhibition of Topo II relaxation activity at the concentration of 200 μM, respectively (Figure 1a). The inhibitory effects of **1**, **3**, **4**, and **6** were dose-dependent. Compounds **1**, **3**, **4**, and **6** have IC_{50} values of 17.5, 38.7, 10.4, and 38.6 μM for the Topo II catalytic activity, respectively (Table 1). These facts indicate that compounds **1**, **3**, **4**, and **6** are selective inhibitors against Topo II catalytic activity and that the triterpenoids having a hydroxymethyl group at position 4 in the A-ring of the molecule, compounds **2** and **5**, are likely to have weaker inhibition against Topo II, with the exception of **3**.

To investigate the interaction of the most potent inhibitor, **4**, with Topo II or with substrate DNA, Topo II or substrate DNA was increased in the reaction mixture and the inhibitory profiles were observed (Figure 1b). When the amount of DNA was increased from 264 to 396 ng (lanes r−t), the compound-mediated inhibition was not overcome. Further increasing the amount of DNA up to 600 ng did not influence the reaction. In contrast, increasing the amount of the enzyme from 2 to 5 units (lanes u−x) showed recovery of the enzyme activity. These results suggest that compound **4** primarily interacts with Topo II and not with DNA.

The dose-dependent inhibition and its recovery by increasing enzyme concentration indicate that the compound interferes with binding of the enzyme to DNA and prevents binary complex formation between Topo II and DNA. Topo II inhibitors such as etoposide[5] are known to stimulate the stabilization of covalent complexes between topo II and DNA, the so-called "cleavable complex", thus generating DNA double-strand breaks and consequently causing cytotoxicity. Compound **4** did not stabilize the cleavable complex (data not shown here), similar to 3,4-*seco*-8β*H*-ferna-4(23),9(11)-dien-3-oic acid.[8] This result indicates that compound **4** inhibits the enzyme activity in a different manner from etoposide.

In recent years, a diverse drug group of the cleavable complex forming type has been found in natural and synthetic compounds including aclarubicin,[3] merbarone,[4] and ICRF-193.[6,13] These compounds inhibit Topo II catalytic activity and show cytotoxic activity, but do not stabilize the cleavable complex. The triterpenoids are thought to be catalytic inhibitors. Lupeol is available in abundant supply from common natural sources. Therefore, as a novel inhibitor, it could serve as a lead structure for drug development. Further evaluation of the inhibition mechanism by triterpenoids is now in progress.

Experimental Section

Chemicals and Reagents. The isolation and structural elucidation of test triterpenoids **1**−**6** has been reported previously.[12] Human Topos I (2 U/μL) and IIα (p170 form, 2 U/μL) were purchased from TopoGen, Inc. (Columbus, OH). Supercoiled pBR 322 plasmid DNA was obtained from Toyobo (Osaka). Etoposide was purchased from SIGMA Chemicals. Test compounds were dissolved in DMSO at 20 mM as a stock solution.

Topo II Assay. Topo II activity was measured by assessing relaxation of supercoiled pBR 322 plasmid DNA. The reaction mixture contained 50 mM Tris-HCl (pH 8), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, pBR 322 plasmid DNA (198 ng), the indicated drug concentrations (less than 10% DMSO), and 1 U of Topo II in a final volume of 20 μL. Reaction mixtures were incubated for 30 min at 37 °C and stopped by addition of 5 μL of a mixture of 5% sarkosyl, 0.0025% bromophenol blue, and 25% glycerol. Reaction products were electrophoresed on a 1% agarose gel in TAE (Tris-acetate-EDTA) running buffer at 1.5 V/cm for 3.5 h. Gels were stained with ethidium bromide (0.5 μg/mL) for 60 min and

destained in water for 30 min. For the visualizing and quantitative analyses of Topo II activity, the gels were directly scanned with a FLA-2000 fluoroimage analyzer (Fuji Photo Film Co., Ltd.), and the area representing supercoiled DNA was calculated. Concentrations for 50% inhibition (IC_{50}) were determined by interpolation from plots of Topo II activity versus inhibitor concentration. The IC_{50} values are means from at least three independent experiments.

Topo I Assay. Reactions were carried out in the same manner as described for the Topo II relaxation assay except that reaction mixtures contained 10 mM Tris-HCl (pH 7.9), 1 mM EDTA, 150 mM NaCl, 0.1% bovine serum albumin (BSA), 0.1 mM spermidine, 5% glycerol, and pBR 322 plasmid DNA (198 ng).

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