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# *In Vitro* Inhibitory Effects of DNA Topoisomerase II by Fernane-Type Triterpenoids Isolated from a *Euphorbia* genus

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**Abstract:** Several kinds of naturally occurring fernane-type triterpenoids isolated from a *Euphorbia* genus were tested on the inhibitory effects of DNA Topoisomerases I (Topo I) and II (Topo II) activities. A-ring cleaved 3,4-*seco*-8β*H*-ferna-4(23),9(11)-dien-3-oic acid and its 3-hydroxyl derivative were found to be selective inhibitors of Topo II activity without the stabilization of a DNA/Topo II cleavable complex. 

DNA topoisomerases (Topos) are important enzymes for essential cellular events such as replication, recombination and transcription. Topos are classified into two types: the type I enzymes (Topo I) mediate the transient single-strand breakage of duplex DNA, and the type II topoisomerases (Topo II) break both strands of the duplex.[1] In recent years, many antitumor drugs have been shown to be Topo inhibiting agents, and Topos are identified as the target in the search for potent antitumor agents.[2]

We have focused on the bioactivities of triterpenoids from plant sources, and reported that derivatives of abieslactone, a natural triterpenoid isolated from several *Abies* genus, exhibited antitumor-promoting activities.[3] At this time, in our search for antitumor compounds, the inhibitory effects of Topo activities *in vitro* were used as the primary screening, and several kinds of naturally occurring fernane-type triterpenoids isolated from a *Euphorbia* genus were tested on the activity. To the best of our knowledge, there are no reports on the significant Topo II inhibitory activities of triterpenoids.

The conversion of supercoiled plasmid DNA to relaxed DNA by eukaryotic Topo I was examined in the presence of five compounds, fern-8-en-3β-ol (**1**), 3β,7α-dihydroxyfern-8-en-11-one (**2**), 3β,11β-dihydroxyfern-8-en-7-one (**3**), 3β-hydroxyfern-8-en-7,11-dione (**4**)[4] and 3,4-*seco*-8β*H*-ferna-4(23),9(11)-dien-3-oic acid (**5**).[5] Among these compounds, potent inhibitory effects were not observed, even though the concentrations were increased up to 200 μM.

Decatenation assays with eukaryotic Topo II were made on the detection of Topo II inhibitors.[6] The inhibitory effects of the five compunds on the decatenation of kinetoplast DNA are summarized in Table 1.

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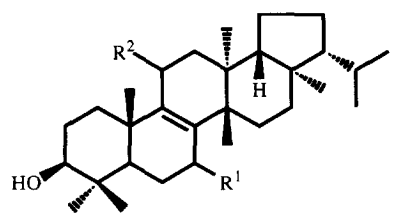


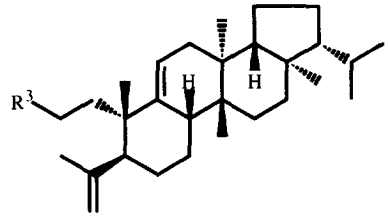


| | $R^1$ | $R^2$ | | $R^3$ |
|---|---|---|---|---|
| 1 | $H_2$ | $H_2$ | 5 | COOH |
| 2 | α-OH | =O | 5a | $COOCH_3$ |
| 3 | =O | β-OH | 5b | $COOCH_2C_6H_5$ |
| 4 | =O | =O | 5c | $CH_2OH$ |

Compounds **1** ~ **4** showed no significant Topo II inhibitory activities at the concentrations of 100 μM, while the inhibition of reactions by **5** was observed when different concentrations of **5** were added to the incubation mixture (Fig. 1). Compound **5** completely inhibited the decatenation activity of Topo II at concentrations up to 25 μM and the inhibitiory effects were seen to be in a dose-dependent manner. Compound **5** exhibited a minimum effective dose of 6.25 μM. The inhibitory effect of **5** was much higher than that of a Topo II inhibitor, etoposide.[7]

The effects of the carboxylic acid in the molecule of **5** on the activity were investigated by the conversion to the methyl ester (**5a**), the benzyl ester (**5b**) and the hydroxymethyl (**5c**) derivatives. The preparations of **5a** and **5b** were carried out by the reactions of **5** with trimethylsilyldiazomethane and **5** with lithium aluminium hydride, respectively, and compound **5b** was synthesized by the couple of **5** and benzylalcohol with 4-dimethylaminopyridine and *N,N'*-dicyclohexylcarbodiimide. Compound **5c** showd a comparable Topo II inhibitory activity with **5** and the inhibitory effect of **5b** was much lower as compared with that of **5**, while compound **5a** was inactive at the concentration of 100 μM (Table 1). These results suggest that a hydrophillic functional group such as a carboxylic acid and a hydroxyl group at the C-3 position in the A-ring cleaved fernane molecule plays an important role in the activity.

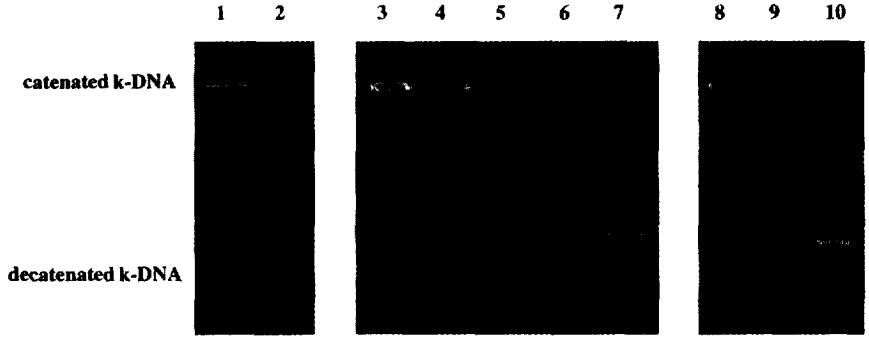


Fig. 1. Effects of Compound **5** and Etoposide on Decatenation Activities by DNA Topoisomerase II
Lane 1, catenated k-DNA alone; Lane 2, DNA/Topoisomerase II; Lane 3, 50 μM compound **5**; Lane 4, 25 μM; Lane 5, 12.5 μM; Lane 6, 6.3 μM, Lane 7, 3.2 μM; Lane 8, 100 μM etoposide, Lane 9, 50 μM; Lane 10, 25 μM

Table 1. Inhibitory Effects of DNA Topoisomerase II Activity by Fernane-Type Triterpenoids and Etoposide

| Conc. (μM) | 1 | 2 | 3 | 4 | 5 | 5a | 5b | 5c | Etoposide |
|---|---|---|---|---|---|---|---|---|---|
| 100 | – | – | – | – | +++ | – | ++ | +++ | ++ |
| 50 | | | | | +++ | | + | +++ | + |
| 25 | | | | | +++ | | – | +++ | – |
| 12.5 | | | | | ++ | | – | ++ | – |
| 6.3 | | | | | + | | – | + | – |
| 3.2 | | | | | – | | – | – | – |

+++, strong inhibition; ++, evidence inhibition; +, slight inhibition; –, no inhibition

Some Topo II inhibiting agents, including etoposide, are known to stimulate the stabilization of a cleavable complex as the inhibiting mechanism.[7] To examine whether compound **5** stimulates the stabilization of the cleavable complex, supercoiled pBR 322 DNA was incubated with 6 units of Topo II in the presence of **5** or etoposide as a control compound. Treatment of the reaction mixture containing etoposide with a denaturant and proteinase K showed a double-stranded DNA breakage, *i.e.*, the formation of linear DNA in an agarose gel (Fig. 2).[8] In contrast, compound **5** did not show the double-stranded DNA breakage. The results indicate that compound **5** inhibits the enzyme reaction without the formation of the cleavable complex, similar to Topo II inhibitors, merbarone,[9] aclarubicin,[10] fostriecin [11] and bis(2,6-dioxopiperidine) derivatives.[12]

To investigate the interaction of **5** with Topo II or with substrate DNA, Topo II or substrate DNA was increased in the reaction mixture and the inhibitory profiles were observed. When the amount of the enzyme was increased from 2 to 6 units, recovery of the enzyme activity was observed. In contrast, increasing the amount of DNA showed no recovery of the enzyme activity. The results suggest that compound **5** primarily interacts with Topo II and not with DNA.

We postulate that both a bulky tetracyclic part and a carboxylic acid or a hydroxyl group in the molecule of **5** or **5c** are necessary for Topo II inactivation, and the former part in the molecule seems to exert spatially-specific functions close to the active sites and the latter function groups seem to occupy the catalytic site of the enzyme. We conclude that these triterpenoids have potency as antitumor agents and are a different kind of Topo II inhibitor from those so far reported.

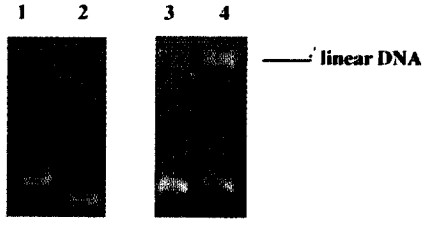


Fig. 2. Effects on the Formation of a DNA/Topo II Cleavable Complex by Compound **5** and Etoposide
Lane 1, pBR 322 DNA alone; Lane 2, DNA/6 units Topoisomerase II; Lane 3, 100 μM compound **5**; Lane 4, 100 μM etoposide

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[6] Topo II activity was measured by assessing decatenation of catenated kinetoplast DNA (0.2 μg). Reaction buffer contained 50 mM Tris-HCl, pH 8, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol and 30 μg/ml BSA. Reactions were incubated with 2 units of Topo II in the presence or absence of test compounds for 30 min at 37 °C. One unit of Topo II can decatenate 0.2 μg kinetoplast DNA for 30 min at 37 °C. The reactions were stopped by adding a mixture of sarkosyl, bromophenol blue and glycerol, and electrophoresed in 1 % agarose gel with 0.5 μg/ml ethidium bromide in Tris-acetate buffer containing 1 mM EDTA. The DNA bands were visualized over UV light and photographed.

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[8] Topo II cleavage reactions contained 6 units of the enzyme and 100 ng supercoiled pBR 322 DNA in a cleavage buffer (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 50 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$ and 0.5 mM ATP). After incubation at 37 °C for 30 min, the reaction mixture was treated with SDS and proteinase K and electrophoresed in 1 % agarose gel.

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