Catalytic Inhibition of Topoisomerase IIα by Demethylzeylasterone, a 6-Oxophenolic Triterpenoid from *Kokoona zeylanica*

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In a study to evaluate celastroloids as potential anticancer agents, demethylzeylasterone (**5**), a 6-oxophenolic triterpenoid from *Kokoona zeylanica*, was found to be an inhibitor of the enzyme topoisomerase IIα (IC_{50} = 17.6 μM). Studies of the relationship of this inhibitor to both DNA and the enzyme resulted in **5** being classified as a "catalytic inhibitor" of topoisomerase II. Demethylzeylasterone selectively inhibits the growth of the breast cancer cell line MCF-7 (IC_{50} = 12.5 μM) without inhibiting the growth of non-small cell lung cancer (NCI-H460) and CNS glioma (SF-268) cell lines. This is the first report of topoisomerase II inhibitory activity in a celastroloid.

In our search for potential anticancer agents from natural sources we have employed two approaches: one utilizing random screening of extracts followed by bioassay-guided fractionation of active extracts and the other involving the screening of pure natural products and their derivatives obtained from other studies. This paper reports an extension of our second approach in which we have evaluated six celastroloids and two celastroloid derivatives in mechanism-based anticancer bioassays employing genetically engineered yeast strains and the topoisomerase II enzyme.

Celastroloids constitute a minor class of unsaturated and oxygenated D:A-*friedo-nor*-oleanane triterpenoids with a wide variety of biological (anticancer, antimicrobial, antimalarial, and spermicidal) activities and are confined to the plant families Celastraceae and Hippocrateaceae.[1] In vitro and in vivo anticancer activities shown by a number of celastroloids and their derivatives[2] have led to clinical evaluations[3] of tingenone (**1**) and efforts to understand its possible mechanism of anticancer action. These mechanistic studies have indicated that some celastroloids exert marked inhibitory effects on in vitro protein and RNA synthesis.[4] Reports of their anticancer activity together with the availability of a series of celastroloids and structurally related 6-oxophenolic triterpenoids isolated from several Sri Lankan Celastraceae prompted us to screen these and some of their derivatives for topoisomerase II inhibitory activity in genetically engineered yeast and enzyme assays.

Topoisomerase II (topo II) is an enzyme that has the ability to relieve torsional strain in eukaryotic DNA. The enzyme acts through the cleaving of double-stranded DNA, allowing passage of another helix through the break, and then finally re-ligating the broken DNA strands.[5] The important role of topoisomerase II in eukaryotic cell viability has made it an effective target for anticancer drug discovery, and a number of topo II inhibitors are already in clinical use (e.g., doxorubicin, daunorubicin, etoposide).[6] Topo II inhibitors are generally grouped into one of two classes determined by the stage at which the enzymatic process is blocked. Those compounds that allow the cleavage of the DNA but prevent its re-ligation through the stabilization of the DNA−enzyme complex are collectively known as topo II "poisons". Most of the compounds used clinically fall into this class. Catalytic inhibitors comprise the second class, and these inhibitors may stop the initial binding of the topoisomerase enzyme to the DNA (e.g., aclarubicin),[7] halt the cleavage event itself (e.g., novobiocin),[7] or prevent the release of the enzyme from the religated complex (e.g., ICRF-187).[7,8]

Table 1. Bioactivity Data of Tingenone (**1**), 20α-Hydroxytingenone (**2**), Balaenonol (**11**), Camptothecin, and Streptonigrin[a]

compound	*RAD+*	*rad 52Y*	RS321N
tingenone (**1**)	NA	NA	325
20α-hydroxytingenone (**2**)	NA	NA	185
balaenonol (**11**)	NA	NA	220
streptonigrin		0.4	<0.4
camptothecin	110	0.6	>20

[a] Results expressed as IC_{12} (μg/mL); NA = not active at 200 μg/mL.

Due to the large number of compounds and extracts that required testing, a prescreen using strains of topoisomerase-deficient *Saccharomyces cerevisiae* (*rad52Y* and RS321N) was conducted utilizing an agar well diffusion assay.[9] Provided there is no inhibition of growth of the wild type (*RAD+*) strain, differential inhibition of growth in any of the mutant strains (*rad52Y* or RS321N) acts as an indicator of DNA-damaging activity. However, inhibition of the RS321N strain in excess of 3 times that of *rad52Y* indicates potential topoisomerase II inhibitory activity.[10] Compounds tested included tingenone (**1**), 20α-hydroxytingenone (**2**), isoiguesterin (**3**), pristimerin (**4**), demethylzeylasterone (**5**), balaenonol (**11**), and the semisynthetic derivatives pristimerol diacetate (**6**) and pristimerol dimethyl ether (**7**). Our yeast assay indicated weak topo II activity in some celastroloids tested. All eight celastroloids were then subjected to a confirmatory enzymatic assay with human topoisomerase IIα. Of those tested, demethylzeylasterone (**5**) was found to inhibit topo II in a dose-dependent manner. Its mode of action was also investigated.

Results and Discussion

Compounds **1**−**7** and **11** were tested in the yeast mutant assay. Three of the celastroloids exhibited weak RS321N activity (Table 1). While this assay did not suggest a strong

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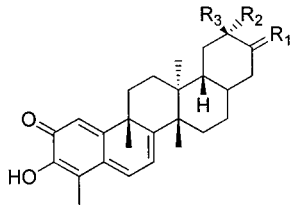
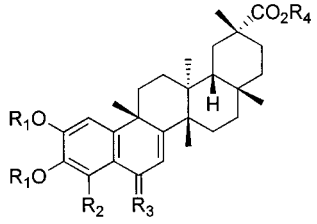
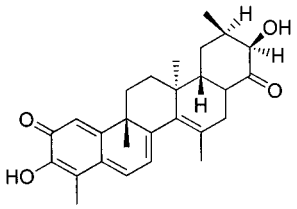


1 $R_1 = O$; $R_2 = H$; $R_3 = Me$
2 $R_1 = O$; $R_2 = OH$; $R_3 = Me$
3 $R_1 = H_2$; $R_2 = R_3 = CH_2$
4 $R_1 = H_2$; $R_2 = CO_2Me$; $R_3 = Me$



5 $R_1 = R_4 = H$; $R_2 = CO_2H$; $R_3 = O$
6 $R_1 = Ac$; $R_2 = R_4 = Me$; $R_3 = H_2$
7 $R_1 = R_2 = R_4 = Me$; $R_3 = H_2$
8 $R_1 = H$; $R_2 = CO_2H$; $R_3 = O$; $R_4 = Me$
9 $R_1 = H$; $R_2 = CHO$; $R_3 = O$; $R_4 = Me$
10 $R_1 = H$; $R_2 = R_4 = Me$; $R_3 = H_2$



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topoisomerase II inhibitory activity for any of the compounds, testing these against purified human topoisomerase IIα at a concentration of 100 μM demonstrated strong activity for demethylzeylasterone (**5**). Unlike the positive control etoposide (100 μM), demethylzeylasterone did not yield any linear DNA (see Figure 1). The absence of DNA that has been cleaved by the enzyme or DNA that has been relaxed suggests that this compound is not acting as a topoisomerase poison but as a catalytic inhibitor.[11] The IC_{50} of demethylzeylasterone (**5**) was determined to be 17.6 μM through a dilution series performed in triplicate (see Figure 1). Topo II activity inhibited by **5** prompted us to test some of its structural analogues, zeylasterone (**8**), zeylasteral (**9**), and pristimerol (**10**), in the enzyme assay. Interestingly, none of these were found to be active at 100 μM. The inability of the C-29 methyl ester of **5**, namely, zeylasterone (**8**), to inhibit topo II enzyme even at a dose of 100 μM suggests the requirement of the C-29 free carboxylic acid group in 6-oxophenolic triterpenoids for topo II inhibitory activity.

Having determined that demethylzeylasterone (**5**) belongs to the catalytic inhibitor group, we proceeded to determine its mode of topo II inhibition. The methyl green displacement assay much like ethidium bromide has been used to identify intercalators of DNA.[12] Since no displacement of methyl green from DNA occurred, it is possible that **5** either binds to the enzyme and prevents DNA from interacting with the topoisomerase II or allows binding but prevents the cleavage event. The electrophoretic mobility shift assay (EMSA) has the ability to detect protein−DNA interactions.[13] When run at the ideal pH, the topo II enzyme will retard the movement of the protein-associated DNA through the agarose gel. In our experiment, the control containing only topo II and DNA, along with the control containing topo II, DNA, and 50 μM etoposide, exhibited retardation of supercoiled DNA migration (see Figure 2). Demethylzeylasterone (**5**) sample was identical in migration pattern to the supercoiled DNA control. It appears therefore that demethylzeylasterone (**5**) is preventing the topo II from associating with the DNA. This mode of action is not unique. Another pentacyclic triterpenoid, acetylboswellic acid from *Boswellia serrata*, is reported to be a catalytic inhibitor of topoisomerse II as well as acting as an inhibitor of topoisomerase I.[14] This same study had demonstrated weak topo II inhibitory activity in several other pentacyclic triterpenoid acids.

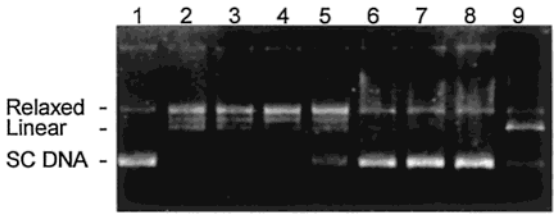

Figure 1. Inhibitory effects of demethylzeylasterone (**5**) on human p170α topoisomerase II on pBluescript KS(+) plasmid DNA. Lane 1, DNA alone; lane 2, DNA + topo II; lanes 3−8, DNA, topo II + 1, 5, 10, 25, 50, and 100 μM demethylzeylasterone (**5**); lane 9, DNA + topo II + 100 μM etoposide.

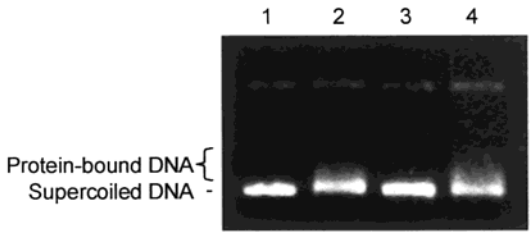

Figure 2. Electrophoretic mobility shift assay (EMSA) of pBluescript KS(+) plasmid DNA against demethylzeylasterone (**5**) for the detection of compound−DNA interaction. Lane 1, pBluescript KS(+) plasmid DNA alone; lane 2, DNA + topo II; lane 3, DNA + topo II + demethylzeylasterone (100 μM); lane 4, DNA + topo II + etoposide (50 μM).

Demethylzeylasterone (**5**) was evaluated for cytotoxic activity against several cancer cell lines. It is noteworthy that **5** inhibited the growth of breast cancer cell line (MCF-7; $IC_{50} = 12.5$ μM) but had no effect on non-small cell lung cancer (NCI-H460) and CNS glioma (SF-268) cell lines at concentrations of up to 10 μM.

Experimental Section

General Experimental Procedures. Gels were visualized using a BioRad Gel Doc 2000 system and DNA bands analyzed with the BioRad Quantity One quantitation software (v.4.1.1.). A Molecular Devices Thermomax 96-well plate reader with SOFTmax Pro (v.2.4.1) analysis software measured MTT and methyl green absorbances. Agarose gels were run on the BioRad Sub-Cell GT horizontal electrophoresis system. Topoisomerase IIα and etoposide were purchased from TopoGEN Inc. (Columbus, OH). All buffer and additional assay components were obtained from Sigma-Aldrich. pBlueScript is a product of Stratagene.

Celastroloids. The isolation and characterization of tingenone (**1**), 20α-hydroxytingenone (**2**), pristimerin (**4**), and balaenonol (**11**) from *Cassine balae*,[15] isoiguesterin (**3**) from *Salacia reticulata* var. *diandra*,[16] and demethylzeylasterone (**5**), zeylasterone (**8**), and zeylasteral (**9**) from *Kokoona zeylanica*,[17] and the semisynthesis of pristimerol (**10**),[18] pristimerol diacetate (**6**),[18] and pristimerol dimethyl ether (**7**)[19] have been reported elsewhere.

Yeast Assay. The RS321N, *rad52Y*, and *RAD*+ strains of *Saccharomyces cerevisiae* were obtained through kind donations from Dr. David G. I. Kingston (Virginia Polytechnic Institute and State University) and Dr. Randall K. Johnson (SmithKline Beecham Pharmaceuticals). Individual strains of yeast were seeded onto 1% nutrient agar plates. Samples were solublized in 1:1 DMSO−MeOH to a concentration of 200 μg/mL and placed in agar wells made through the removal of 7 mm plugs from the media. Activity was measured as the zone (mm) surrounding the well where no yeast growth was visible. For active compounds, multiple concentrations were tested, and the dose responsible for an inhibition zone of 12 mm was calculated (IC_{12}) through regression analysis. Camptothecin and streptonigrin were used at 5 μg/mL as controls.

Assay for Topoisomerase II Inhibition in Vitro. The supercoiled plasmid pBlueScript KS(+) was used for the measurement of relaxation through the action of human topoisomerase II. Reaction volumes were 20 μL in an assay buffer (50 mM Tris-HCl, pH 8.0, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol) containing 4 units of human topoisomerase II (p170 form) and approximately 200 ng of DNA. Samples were solublized using Cremophor EL (0.5% final concentration). Compounds were tested at 100 μM to identify inhibitory activity. Reaction tubes were incubated for 30 min at 37 °C. Reactions were stopped through the addition of SDS (1%). Enzyme was separated from DNA using proteinase K (50 μg/mL final concentration). Upon addition of loading buffer, samples were clarified of remaining DNA−enzyme complexes through a chloroform−isoamyl alcohol (24:1) partitioning. The aqueous phases were applied to a 1% TAE agarose gel. Bands were visualized post-electrophoresis using 0.5 μg/mL ethidium bromide.

Methyl Green Assay. The methyl green assay was performed as described by Burres et al.[12] Daunomycin (50 μg/mL) was used as a positive control.

Electrophoretic Mobility Shift Assay (EMSA). The assay is described in detail elsewhere.[13,14] The assay differed from the typical topo II assay in that 6 units of human topoisomerase II (p170 form) were used in each sample and ATP is absent from the buffer. The pH of the assay buffer, running buffer, and gel was 6.4. The lower pH is designed to improve the degree of shift between free plasmid and topo II-associated plasmid. Reactions were not stopped with SDS and proteinase K but loaded directly onto a TAE gel containing 1.0 μg/mL ethidium bromide.

Cytotoxicity Assays. The NCI-H460 (non-small cell lung carcinoma), MCF-7 (breast carcinoma), and SF-268 (CNS glioma) cell lines were maintained in RPMI culture medium with 10% fetal bovine serum (FBS). All cell lines were cultured at 37 °C in an atmosphere of 5% CO_2 in air (100% humidity). The cells, at log phase of their growth cycle, were treated in triplicate with various concentrations of the test compounds (0−10 μM) and incubated for 48 h at 37 °C in a humidified atmosphere of 5% CO_2. Endpoint determinations of growth were made using MTT.[20]

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