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DNA topoisomerase I inhibitors from *Rinorea anguifera*

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Abstract—An organic extract prepared from *Rinorea anguifera* was investigated in order to identify the natural principle(s) responsible for stabilization of a topoisomerase I–DNA covalent binary complex. Bioassay-guided fractionation resulted in the isolation of mauritianin and (+)-syringaresinol as new topoisomerase I inhibitors, and also of the known inhibitor camptothecin.

DNA topoisomerases are a ubiquitous class of enzymes essential for a number of cellular processes that involve DNA unwinding including DNA replication, transcription, recombination, and chromatin remodeling.[1] Topoisomerases are classified as type I and type II on the basis of distinct differences in enzyme sequence, subunit composition and function. Type II enzymes are dimeric, transiently break both strands of duplex DNA (using a catalytic tyrosine residue in each subunit), and pass an intact DNA duplex through this transient double-stranded break in an ATP-dependent manner. In contrast, type I topoisomerases are monomeric, require no energy source, and relax superhelical DNA by cleaving one strand of duplex DNA.[2]

Inhibitors of DNA topoisomerases include a number of compounds having clinically useful activity as antitumor agents. These include etoposide, teniposide and *m*-AMSA, all of which stabilize the covalent binary complex formed between topoisomerase II and DNA.[3] There are fewer known inhibitors of DNA topoisomerase I, but the camptothecin derivatives topotecan and irinotecan are used clinically and function at this locus.[4]

In an effort to identify additional inhibitors of DNA topoisomerase I, we have surveyed a number of plant-derived extracts for their ability to stabilize the covalent enzyme–DNA intermediate involved in topoisomerase I-mediated DNA relaxation. Samples of *Rinorea anguifera* were collected in Satan, Thailand in April 1987. (Voucher speciment Q66O0980 is stored at the U.S. National Arboretum Herbarium, Washington, DC.) An organic extract prepared from *R. anguifera* stabilized the covalent binary complex and was fractionated in an effort to isolate the active principle(s). Presently we report the isolation of the flavonol glycoside mauritianin (**1**) and of the lignan (+)-syringaresinol (**2**) recognized for the first time as inhibitors of topoisomerase I, as well as the known topoisomerase I inhibitor camptothecin (**3**).

The crude extract of *R. anguifera* was first applied to a polyamide 6S column, which was washed successively with H_2O, 1:1 MeOH–H_2O, 4:1 MeOH–CH_2Cl_2, 1:1 MeOH–CH_2Cl_2, and then 9:1 MeOH–NH_4OH. The 1:1 CH_3OH–H_2O fraction exhibited the most potent stabilization of the covalent topoisomerase I–DNA binary complex. Further fractionation of the active fraction on a C_{18} reversed phase column provided two active fractions in the 2:3 and 3:2 CH_3OH–H_2O washes. The active fraction that had eluted with 2:3 CH_3OH–H_2O was applied to a 5 μM C_{18} reversed-phase HPLC column (250 × 10 mm) and washed with a linear gradient of 1:19 → 3:2 CH_3CN–H_2O over a period of 55 min at a flow rate of 4.0 mL/min (UV monitoring at 265 nm). This resulted in the isolation of one active principle (**1**). Further fractionation of the 3:2 CH_3OH–H_2O fraction from the C_{18} reversed-phase column on a C_8 reversed-phase column, and then by C_{18} reversed-phase HPLC using conditions similar to those noted for **1**, led to the isolation of compounds **2** and **3**.

Compound **1** was obtained as a pale yellow amorphous powder, and displayed a peak at *m*/*z* 741 $(M+H)^+$ in the chemical ionization (positive ion) mass spectrum. Signals typical of a 3,5,7,4′-tetrahydroxy flavonol were

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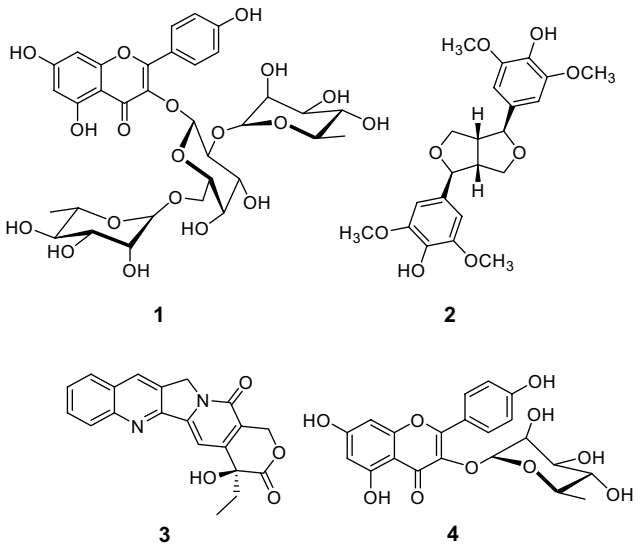

Table 1. Stabilization of human topoisomerase I–DNA covalent complex by compounds **1**–**3**

Inhibitor	Concentration (μM)	Topoisomerase I	Covalent binary complex[a] (%)
None		–	2
None		+	9
Mauritianin (**1**)	100	–	10
Mauritianin (**1**)	100	+	75
Mauritianin (**1**)	1	+	31
Syringaresinol (**2**)	100	–	2
Syringaresinol (**2**)	100	+	15
Syringaresinol (**2**)	1	+	8
Camptothecin (**3**)	2	–	2
Camptothecin (**3**)	2	+	96
Camptothecin (**3**)	0.25	+	84

[a] DNA present as Form II (nicked, circular) DNA as a percentage of all DNA.

observed in the ^{1}H NMR spectrum at δ 6.18, 6.37, 6.89, and 8.02, and in the ^{13}C NMR spectrum at 94.9, 100.1, 104.2, 116.1, 123.2, 132.1, 134.3, 156.5, 159.7, 161.3, 164.7, and 179.0.[5] Three anomeric signals were also observed in the ^{1}H NMR (^{13}C NMR) spectra at δ 4.50 (102.3), 5.23 (102.7), and 5.60 (100.5), along with several other signals attributed to sugars. This data, in comparison with published reports,[5–7] established the structure of **1** as that of mauritianin, a known flavonol glycoside.

Compound **2** was obtained as a colorless amorphous powder. The chemical ionization mass spectrum indicated a molecular weight of 418; the ^{1}H NMR spectrum included resonances at δ 6.66, 5.49, and 3.87. This was consistent with the structure of syringaresinol, one of the most commonly distributed lignans in terrestrial plants.[8] This assignment was confirmed by ^{13}C NMR spectroscopy.

Compound **3** was identified readily as camptothecin by its spectroscopic properties (UV, fluorescence, ^{1}H NMR, and mass spectra) in direct comparison with those of an authentic sample.[9] This compound has been shown previously to be an inhibitor of topoisomerase I.[10]

The ability of mauritianin (**1**), (+)-syringaresinol (**2**) and camptothecin (**3**) to stabilize the formation of the DNA–topoisomerase I covalent binary complex was studied using purified recombinant human topoisomerase I as described before.[10,11] Supercoiled pBR322 plasmid DNA was incubated in the presence of topoisomerase I and varying concentrations of **1**–**3**. After 60 min at 37 °C the reaction was terminated by the addition of sodium dodecylsulfate, and the denatured topoisomerase I was digested with proteinase K. As shown in Figure 1, in the presence of increasing concentrations of compounds **1**–**3**, the amount of topoisomerase I–DNA binary complex (assayed as nicked (Form II) DNA after proteolytic digestion of the topoisomerase) was observed. Densitometric analysis of the agarose gel from a similar experiment (Table 1) demonstrated that human topoisomerase I-dependent DNA breakage increased from 9% to 75% in the presence of 100 μM mauritianin (**1**), and that compound **1** stabilized the covalent binary complex detectably at a concentration as low as 1 μM. In a human topoisomerase I-mediated DNA relaxation assay, mauritianin (**1**) showed no inhibition of DNA relaxation at any inhibitor concentration tested. Camptothecin itself is only weakly active in this assay, and only at high (e.g., 500 μM) concentration. It is interesting that flavonol glycoside **4**, which is closely related structurally to mauritianin, did not stabilize the topoisomerase I–DNA binary complex.

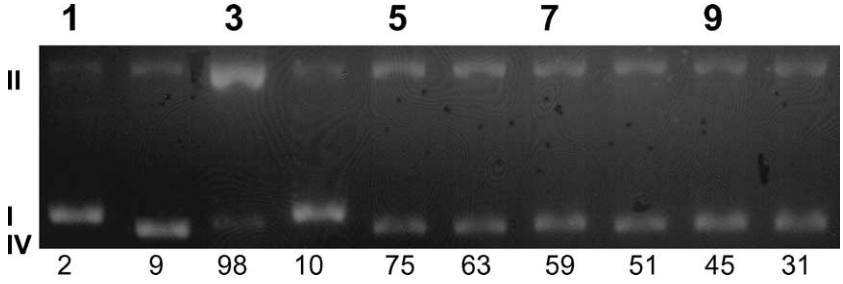

Figure 1. Stabilization of recombinant human topoisomerase I–DNA covalent binary complex by mauritianin (**1**), measured after agarose gel electrophoresis. Lane 1, pBR322 plasmid DNA alone; lane 2, recombinant human topoisomerase I; lane 3, topoisomerase I + 20 μM camptothecin (**3**); lane 4, 100 μM **1**; lanes 5–10, topoisomerase I + 100, 50, 25, 10, 5, and 1 μM **1**, respectively. The percent Form II DNA present is shown below each lane.

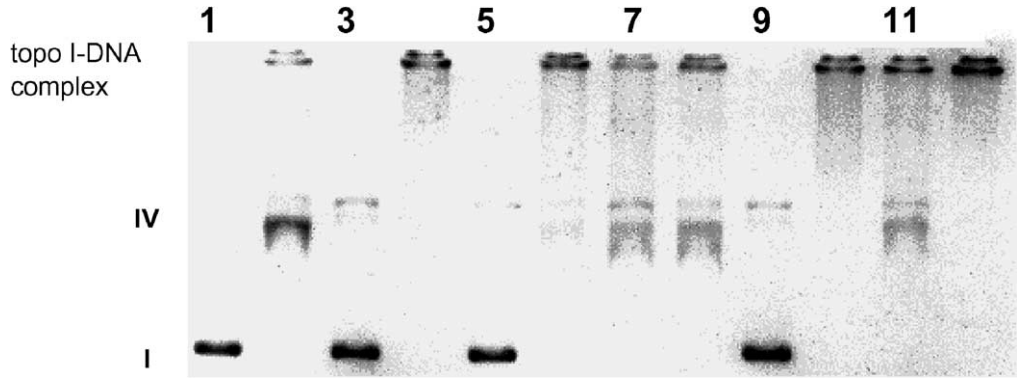

Figure 2. Stabilization of recombinant human topoisomerase I–DNA covalent complex by (+)-syringaresinol (**2**) and the racemic synthetic **2**, analyzed by agarose gel electrophoresis. Instead of digestion of recombinant human topoisomerase I with proteinase K after a 60 min incubation, the reaction product was mixed with 5 μL of 30% glycerol–0.01% bromophenol blue and was analyzed by electrophoresis in a 1.0% agarose gel. Lane 1, pBR322 plasmid DNA alone; lane 2, recombinant human topoisomerase I; lane 3, 20 μM camptothecin (**3**), lane 4, topoisomerase I + 20 μM camptothecin (**3**); lane 5, 500 μM **2**; lanes 6–8, topoisomerase I + 500, 200, and 100 μM **2**, respectively, lane 9, 500 μM racemic synthetic **2**; lanes 10–12, topoisomerase I + 500, 200, and 100 μM racemic synthetic **2**, respectively.

(+)-Syringaresinol (**2**) exhibited weak but reproducible activity in stabilization of the DNA–topoisomerase I covalent binary complex (Fig. 2 and Table 1). Because (+)-syringaresinol and camptothecin had similar elution properties on the C_{18} reversed phase HPLC column employed for their purification, a sample of racemic syringaresinol was prepared synthetically[12] to permit verification of DNA–topoisomerase I stabilization. As shown in Figure 2, racemic syringaresinol also exhibited stabilization of the covalent enzyme–DNA binary complex. The racemic material was shown to be less potent as a topoisomerase I inhibitor than (+)-syringaresinol. In common with mauritianin, (+)-syringaresinol had no effect on topoisomerase I-mediated DNA relaxation at any tested concentration.

In comparison with **1** and **2**, camptothecin (**3**) stabilized the covalent enzyme–DNA covalent binary to a greater extent, even at much lower concentration. For example, in the presence of 0.25 μM camptothecin, 84% of the DNA substrate was stabilized as the enzyme–DNA binary complex (Table 1).

A few interesting flavonoids have been identified previously as topoisomerase inhibitors. Genistein, an isoflavone, has been reported to stabilize the covalent topoisomerase II–DNA binary complex and thus functions as a topoisomerase II poison.[13] Velutin, a flavone derivative from the New Caledonian tree *Lethedon tannaensis* Forst., not only stabilized the binary complex between calf thymus DNA topoisomerase I, but also inhibited topoisomerase I catalytic activity in the low micromolar range.[14] Recently, Constantinou et al. reported their studies of 20 representative flavonoids as potential DNA topoisomerase inhibitors.[15] In this study, quercetin, myricetin, fisetin, and morin were found to inhibit the catalytic activities of both DNA topoisomerases I and II, while phloretin, kaempferol, and 4′,6,7-trihydroxyisoflavone specifically inhibited topoisomerase II. Boege et al. reported that quercetin and related natural flavone derivatives, such as acacetin, apigenin, kaempferol, and morin, stabilize the covalent DNA topoisomerase I–DNA post-cleavage complex by a mechanism different from camptothecin.[16] In contrast to camptothecin, these compounds were reported not to act directly on the catalytic intermediate and also not to interfere with DNA cleavage. However, formation of a ternary complex with topoisomerase I and DNA during cleavage reaction inhibited the subsequent DNA religation step.

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