The Journal of
Organic
Chemistry

VOLUME 58, NUMBER 19 SEPTEMBER 10, 1993

Communications

Inhibitors of DNA Topoisomerase I Isolated from the Roots of *Zanthoxylum nitidum*

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Received May 25, 1993

Summary: A methanol extract of *Zanthoxylum nitidum* found to inhibit topoisomerase I-mediated DNA relaxation and stabilize the covalent binary complex between the enzyme and DNA was subjected to bioassay-guided fractionation; three strongly inhibitory principles were identified including nitidine, chelerythrine, and a new alkaloid for which the name isofagaridine is suggested.

The inhibition of DNA topoisomerases is of great interest at present because they play a critical cellular role in that they alter the topological state of DNA and are thereby required for processes such as DNA replication and transcription.[1] DNA topoisomerase I changes the DNA linking number [1a,b,2] by mediating a transient break on one strand of DNA; a tyrosine OH group at the enzyme active site becomes covalently bound to the broken DNA strand via a phosphate ester linkage (Figure 1). Topoisomerase II functions in an analogous fashion, but creates breaks on both DNA strands to permit the requisite "strand passage"[1b,3] that results in a change in linking number.

Consistent with their ability to inhibit a mediator of essential cell functions, several inhibitors shown to stabilize the covalent binary complex between topoisomerase II and DNA have useful antitumor activity.[4] Camptothecin is an alkaloid that inhibits topoisomerase I-mediated DNA relaxation by analogous stabilization of the topoisomerase I–DNA covalent binary complex.[5] Camptothecin has strong antitumor activity in animal tumor models,[6] and

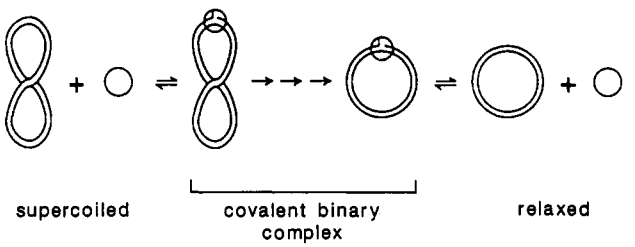

Figure 1. Mechanism of topoisomerase I-mediated plasmid DNA relaxation. Topoisomerase I (O) becomes covalently attached to the DNA through a 3′-*O*-phosphorotyrosine bond with concomitant (reversible) breakage of one DNA strand. DNA relaxation occurs via DNA strand passage, after which the open circular form is resealed, affording a relaxed circular DNA and free enzyme. Compounds 1–3 all inhibited the overall process of plasmid DNA relaxation, but only nitidine efficiently stabilized the enzyme–DNA covalent binary complex.

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three derivatives of camptothecin are in clinical trials as antitumor agents.[7]

In contrast with topoisomerase II, there are few specific inhibitors of topoisomerase I-mediated DNA relaxation,[8,9] and the camptothecins are the only agents known that specifically stabilize the topoisomerase I–DNA covalent binary complex without binding to the enzyme or DNA alone.[5] As part of a continuing search for novel topoisomerase I inhibitors, we investigated the properties of a methanol extract of *Zanthoxylum nitidum*, a climbing shrub 1–3 m in height that is widely distributed in the south of China.[10] We found that the extract strongly inhibited the relaxation of supercoiled plasmid pSP64 DNA by calf thymus DNA topoisomerase I and stabilized the covalent enzyme–DNA complex (Figure 1). Three structurally related benzophenanthridine alkaloids responsible for the observed activities were isolated by bioassay-guided fractionation and characterized structurally as **1**–**3**. Remarkably, while all three compounds exhibited comparable inhibition of DNA relaxation, only nitidine (**1**) efficiently stabilized the covalent topoisomerase I–DNA binary complex. Chelerythrine (**2**) failed to stabilize the complex at any tested concentration, while a new alkaloid (**3**, for which the name isofagaridine is proposed) effected binary complex stabilization only at concentrations >100-fold greater than nitidine (**1**).

A methanol extract prepared from the roots of *Zanthoxylum nitidum* was found to inhibit topoisomerase I-mediated DNA relaxation[11] and to stabilize the enzyme–DNA covalent binary complex;[12] both assays were used to guide the isolation of the active principles. The methanol extract (32 g) was dissolved in aqueous solution containing 5% HOAc. After filtration, the pH was adjusted (to 8–9) with NH_4OH, and the solution was extracted with CH_2Cl_2, affording 1.6 g of an active fraction. Following treatment with HCl, compound **1** crystallized from methanol as yellow needles (96 mg).[13] The mother liquor deposited an additional 128 mg of yellow crystals from acetone; fractional crystallization of this material afforded **2**[15] (31 mg) and **3**[17] (27 mg), each of which was purified by preparative silica gel TLC as the hydroxide and then crystallized as the chloride.

The structures of **1**[13] and **2**[15] were established as nitidine and chelerythrine, respectively, based on comparison of

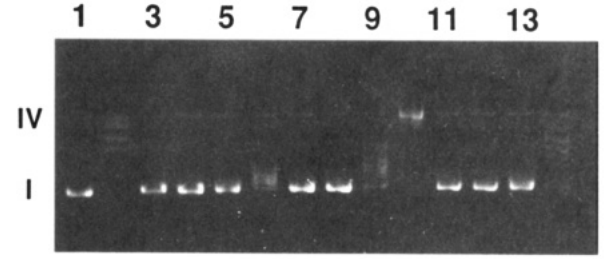
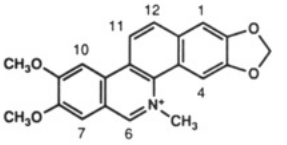
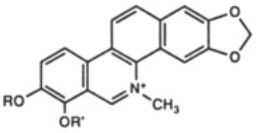


Figure 2. Inhibition of DNA relaxation by nitidine (**1**), chelerythrine (**2**), and isofagaridine (**3**). Supercoiled (form I) plasmid DNA was incubated alone (lane 1), in the presence of topoisomerase I (lane 2), or in the presence of enzyme + inhibitor. Lanes 3–6: 500, 167, 55, and 18 μM nitidine (**1**), respectively. Lanes 7–10: 500, 167, 55, and 18 μM chelerythrine (**2**), respectively. Lanes 11–14: 500, 167, 55, and 18 μM isofagaridine (**3**), respectively. Form IV DNA was relaxed circular DNA.



1 nitidine



2 R , R' = CH_3 chelerythrine

3 R = H ; R' = CH_3 isofagaridine

4 R = CH_3 ; R' = H fagaridine

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(8) Numerous compounds have been reported to act as topoisomerase I inhibitors,[9] although most appear to be simple DNA binding agents or to act nonspecifically, a conclusion reinforced by the effects on topoisomerase I of agents known to interact strongly with DNA. See, e.g.: (a) Yoshida, T.; Habuka, N.; Takeuchi, M.; Ichishima, E. *Agric. Biol Chem.* **1986**, *50*, 515. (b) McHugh, M. M.; Woynarowski, J. M.; Sigmund, R. D.; Beerman, T. A. *Biochem. Pharmacol.* **1989**, *38*, 2323. (c) Mortensen, U. H.; Stevnsner, T.; Krogh, S.; Olesen, K.; Westergaard, O.; Bonven, B. J. *Nucleic Acids Res.* **1990**, *18*, 1983.

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(10) *Zanthoxylum nitidum* was collected in southern China; a voucher specimen is preserved at the Herbarium, Shanghai Second Military Medical University, Shanghai.

(11) In a typical assay, the incubation mixture consisted of 20 μL of 50 mM Tris–HCl, pH 7.5, containing 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, 50 μg/mL of bovine serum albumin, 6% polyethyleneglycol, 250 ng of supercoiled plasmid pSP64 DNA, and 0.4–2 μg of crude extract. The reaction was initiated by the addition of 2.2 ng of calf thymus topoisomerase I,[5d] incubated at 37 °C for 30 min, and analyzed by electrophoresis on a 1% agarose gel. The DNA bands were visualized by staining with 0.5 μg/mL ethidium bromide following electrophoresis.

(12) Stabilization of the covalent binary complex was measured by modification of the procedure of Hsiang et al.[5a] using 68 ng of topoisomerase I and 200 ng of supercoiled pSP64 DNA.

(13) Isolated nitidine chloride had mp 274–6 °C (lit.[14] mp 272–5 °C); λ_{max} (CH_3OH) 224 nm (log ε 4.80), 232 (4.76), 274 (4.83), 280 (4.82), 302 (4.65), 328 (4.60) and 386 (3.90); ^{1}H-NMR (DMSO-d_6) δ 4.00 (s, 3), 4.18 (s, 3), 4.85 (s, 3), 6.30 (s, 2), 7.74 (s, 1), 7.86 (s, 1) 8.26 (d, 1, J = 8.7 Hz), 8.27 (s, 1), 8.33 (s, 1), 8.87 (d, 1, J = 8.7 Hz) and 9.78 (s, 1); mass spectrum m/z (rel intensity) 348 (M^+, 38), 333 (100), 317 (20), 304 (8) and 290 (20).

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(15) Chelerythrine chloride was isolated as yellow needles, mp 202–4 °C (lit.[16] mp 202–3 °C); λ_{max} (CH_3OH) 228 nm (log ε 4.48), 2.74 (sh, 4.54), 282 (4.63), 320 (4.16) and 346 (sh, 3.54); ^{1}H-NMR (DMSO-d_6) δ 4.07 (s, 3), 4.13 (s, 3), 4.94 (s, 3), 6.30 (s, 2), 7.75 (s, 1), 8.24 (d, 1, J = 9 Hz), 8.26 (s, 1), 8.28 (d, 1, J = 9 Hz), 8.80 (d, 2, J = 9 Hz) and 10.04 (s, 1); mass spectrum m/z (rel intensity) 348 (M^+, 42), 333 (100), 318 (20), 304 (5) and 290 (35).

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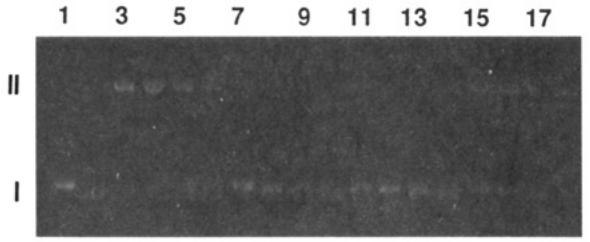
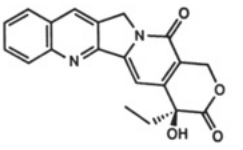


Figure 3. Effect of alkaloids 1–3 on the stability of the topoisomerase I–DNA covalent complex. Supercoiled (form I) plasmid DNA was incubated alone (lane 1), in the presence of topoisomerase I (lane 2), or in the presence of enzyme + an alkaloid. Lanes 3–6: 100, 20, 4, and 0.8 μM camptothecin, respectively. Lanes 7–10: 100, 20, 4, and 0.8 μM chelerythrine, respectively. Lanes 11–14: 100, 20, 4, and 0.8 μM isofagaridine, respectively. Lanes 15–18: 100, 20, 4, and 0.8 μM nitidine, respectively. Form II DNA was nicked circular DNA, putatively formed by digestion of the topoisomerase I–DNA covalent complex with SDS-proteinase K prior to agarose gel electrophoresis.

their physical and spectral properties with those of the authentic materials.[14,16,18] Compound **3** was also believed to be a benzophenanthridine alkaloid based on its UV spectral characteristics.[19] The mass spectrum of this compound indicated M_r 334, consistent with its formulation as the known alkaloid fagaridine (**4**), and treatment with diazomethane gave a product identical with chelerythrine as would be expected for authentic fagaridine. However, this third compound (isolated as the chloride) had mp 226–8 °C[17] and failed to give a color with $FeCl_3$ reagent, whereas fagaridine chloride had a mp of 206–8 °C[20] and gave a red coloration in the presence of $FeCl_3$ reagent.[21] The logical alternative structure for the newly isolated compound, i.e., an isomer of fagaridine having 7-OCH_3 and 8-OH substituents, was supported by nuclear Overhauser experiments.[22] Isofagaridine is suggested as a name for this species.

As shown in Figure 2, compounds **1–3** effected complete inhibition of pSP64 DNA relaxation by 2.2 ng of calf thymus DNA topoisomerase I when employed at 167 or 500 μM concentrations for 30 min; nitidine (**1**) and isofagaridine (**3**) were also effective at 55 μM concentration, while chelerythrine gave partial inhibition at this concentration. These results compared favorably with those obtained for camptothecin, which inhibited relaxation



camptothecin

partially at 167 μM and completely at 500 μM concentrations.[5] As shown in Figure 3, nitidine stabilized the covalent binary complex between topoisomerase I and DNA (cf. Figure 1) to a somewhat lesser extent than camptothecin. Densitometric analysis of the agarose gel indicated 56% covalent binary complex formation at 100 μM nitidine (vs 69% at 100 μM camptothecin) and 44% covalent complex at 4 μM nitidine (vs 58% for camptothecin). Isofagaridine was only weakly active in this assay, while chelerythrine had no detectable activity.

Nitidine and other 8,9-substituted benzophenanthridine alkaloids such as fagaronine have been shown to have antitumor activity in animal tumor models,[23] an activity which could be related to inhibition of DNA topoisomerase I. Interestingly, 7,8-substituted benzophenanthridine alkaloids such as chelerythrine, have not generally been found to have antitumor activity.[23a,c,e] The present results are of importance in that they provide a potential lead structure for the development of new antitumor agents. Less obvious, but no less important, is the finding of differential activities for **1–3** in inhibiting DNA relaxation vs binary complex stabilization, since this should provide an important tool for determining the actual molecular event(s) whose alteration leads to cell death in the presence of compounds that mediate cytotoxicity at the locus of topoisomerase I.

Acknowledgment. We thank Dr. Matthew Suffness, National Cancer Institute, for an authentic sample of nitidine and Dr. Brian Rogers for helpful discussions during the course of this work. This work was supported by NIH Research Grant CA50771 from the National Cancer Institute.

Supplementary Material Available: Procedural flow chart and spectra (12 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(17) Isofagaridine chloride was obtained as yellow needles: mp 226–8 °C; λ_{max} (CH_3OH) 228 nm (log ε 4.20), 284 (4.29) and 322 (sh, 3.82); λ_{min} 252 (3.85); ^{1}H-NMR (DMSO-d_6) δ 4.12 (s, 3), 4.94 (s, 3), 6.31 (s, 2), 7.74 (s, 1), 7.99 (d, 1, J = 9 Hz), 8.25 (d, 1, J = 9 Hz), 8.27 (s, 1) 8.69 (d, 1, J = 9 Hz), 8.72 (d, 1, J = 9 Hz), 9.98 (s, 1) and 11.04 (s, 1, ex D_2O); mass spectrum m/z (rel intensity) 334 (M^+, 15), 333 (32), 319 (100), 304 (60), 290 (20) and 276 (30).

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(22) Irradiation of the NCH_3 resonance gave enhanced signals corresponding to H-6 (16.0%) and H-4 (21.0%), as expected. Irradiation of (putative) 7-OCH_3 also resulted in enhancement of H-6 (5.2%) as would be expected for a compound having structure **3**.

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