Inhibition of Topoisomerase I by Anthracycline Antibiotics: Evidence for General Inhibition of Topoisomerase I by DNA-Binding Agents

Robert T. Crow and Donald M. Crothers*

Sterling Laboratory of Chemistry, Yale University, P.O. Box 6666, New Haven, Connecticut 06511

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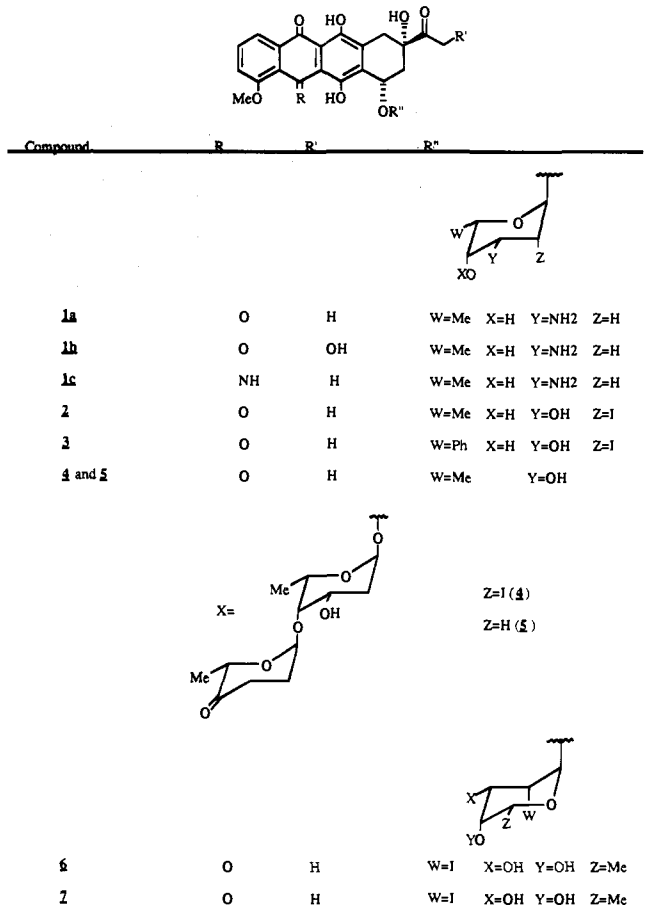
Inhibition of eukaryotic topoisomerase I by daunomycin and structurally modified daunomycin analogues is demonstrated. These studies suggest that binding of the drug to DNA, at low ratios of drug to base pairs of DNA, is sufficient for inhibition that is nonspecific, i.e., inhibition that does not involve the trapping of covalent DNA-topoisomerase I cleavable complexes. Inhibition of numerous DNA-processing enzymes by DNA-binding agents is also implicated.

Introduction

The anthracycline family of antibiotics, of which daunomycin (**1a**, Chart 1) is a member, has been known for nearly three decades[1] and they have been used clinically in the treatment of a variety of cancers for many of those years.[2] It was not until 1984, however, that Liu and co-workers discovered the putative target of these antitumor agents, topoisomerase II (topo II).[3,4] Daunomycin and the related compounds adriamycin (**1b**) and 5-iminodaunorubicin (**1c**) were found to stabilize the cleavable complex **C** (Figure 1), such that treatment of reaction mixtures with protein denaturant and a protease revealed double-stranded DNA breaks.[3] Thus, the anthracyclines joined a growing class of anticancer agents whose principle target seemed to be topo II.[5]

As part of our interest in the areas of anthracycline chemistry and topoisomerase inhibitors, the modified anthracyclines **2**–**7** were synthesized.[6,7] While the original intent was to investigate possible inhibition of topo II by these reagents, screening for activity against topoisomerase I (topo I) seemed appropriate. This report demonstrates the ability of these drugs to interfere with the relaxation of supercoiled DNA catalyzed by topo I, as has been previously seen with adriamycin (**1b**),[8] and presents data which imply that this inhibition of topo I is nonspecific, i.e., arising solely from DNA binding.

Chart 1



Results

Preliminary Investigations. Compounds **2**–**7** are known to bind DNA.[9] Analogues **2** and **3** have the highest affinities, which are slightly lower than that of **1a**, and analogues **5** and **7** have the poorest affinities (R.T.C., unpublished observations). High drug concentrations (1.4 bp DNA:1 molecule drug) completely inhibited DNA relaxation by topo I (Figure 2, Table 1), presumably by saturating the DNA, thereby preventing the enzyme from binding the plasmid. Similar behavior has been seen in anthracycline-mediated topo II inhibition.[3] At lower drug concentrations (2 μM, 14:1 bp:drug), the strongest DNA binders **1a**, **2**, **3**, and **6** decreased the rate of DNA relaxation (Figure 3). The amount of relaxed DNA is plotted against time (see Experimental Section), and a time at which 63% (1/e) of the DNA is relaxed can be calculated. The ratios of these relaxation times, in the presence and absence of drug, are presented in Table 1. At 2 μM, the best intercalators inhibit topo I approximately 5-fold more than a similar concentration of camptothecin (R.T.C. and D.M.C., unpublished; see also ref 10), the only known specific inhibitor of topo I (see below).

Topo I Inhibition by Anthracyclines Does Not Involve Stabilization of Cleavable Complexes. Having established the ability of these anthracyclines to inhibit topo I, efforts were directed toward elucidating the mechanism of inhibition. Presumably, inhibition arises either through stabilization of nicked intermediates **F** (Figure 1), a mechanism analogous to the competitive inhibition of topo II by anthracyclines, or through binding of the drugs to DNA, which would prevent the enzyme from binding DNA. These effects

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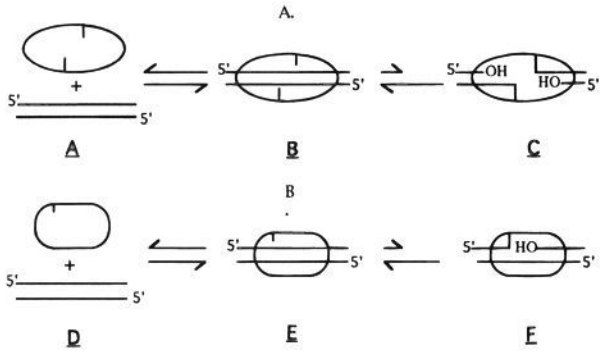


Figure 1. Mechanism of DNA relaxation by topoisomerases II (A) and I (B). Free DNA and enzyme (**A** and **D**) are in equilibrium with noncovalently bound binary complex **B** (**E**). **B** (**E**) leads, through enzyme-mediated double-stranded breakage to **C** (**F**) which, upon twisting of the DNA strand, gives a topoisomer of **B** (**E**).

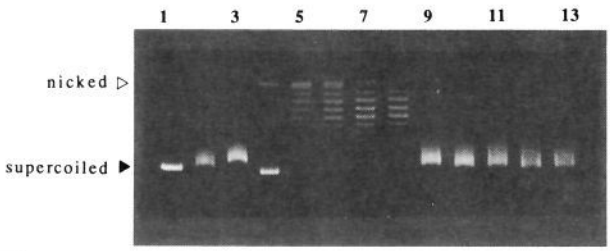


Figure 2. Inhibition of Topo I by high concentrations of anthracycline **3**. Reaction conditions are described in Experimental Section. Briefly, DNA and topo I were incubated at 30 °C in the presence and absence of 12 μM **3**. Aliquots of the reaction were stopped and analyzed on a 1% agarose gel in TPE: lane 1, control DNA; lanes 2 & 3, DNA + drug incubated for 2 and 20 min, respectively; lanes 4–8, DNA + topo I incubated for 1, 2.5, 5, 10, and 20 min, respectively; lanes 9–13, same as 4–8, except containing 12 μM **3**.

Table 1. Interaction of Anthracyclines **1a** and **2–7** with the Topoisomerase I Reaction[a]

drug	concentration used in assay, μM	$K_{a1} \times 10^5$ (ref 9)	$\frac{t_{1/e}\,(+\text{drug})}{t_{1/e}\,(-\text{drug})}$
1a	20	980	complete inhibition
	2		1.4 ± 0.3
2	20	120	complete inhibition
	2		2.0 ± 0.2
3	20	400	complete inhibition
	2		3.5 ± 0.6
4	20	nd	initial complete inhibition; effect is diminished over time
	2		1.4 ± 0.1
5	9	nd	1.4
	2		0.92
6	17	5	initial complete inhibition, effect is diminished over time
	2		1.5 ± 0.2
7	18	nd	1.0
	2		0.76 ± 0.2

[a] Reaction were performed as described in the Experimental Section and the caption of Figure 2.

can be differentiated by assaying for the presence of elevated levels of nicked DNA. Nicked DNA and relaxed (closed circular) DNA, however, comigrate in the agarose gel matrix, rendering one-dimensional gel electrophoresis useless in this experiment. Two-dimensional gel electrophoresis technology,[11] therefore, was used to analyze the components of the reaction. A

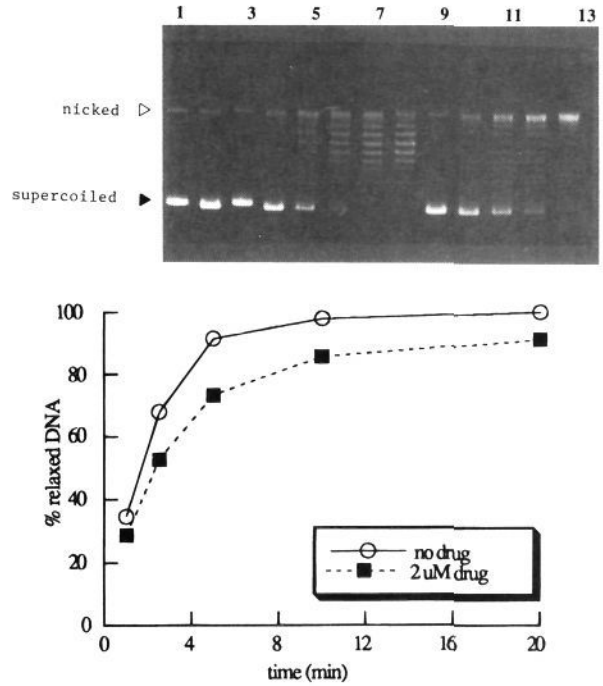


Figure 3. Decrease in the rate of topo I-mediated DNA relaxation induced by anthracycline **2**. Reaction conditions are described in the Experimental Section and the legend to Figure 2, except that the drug concentration was 2 μM. Densitometric analysis of the gel was performed as described in the Experimental Section: lane 1, control DNA, lanes 2 and 3, DNA + drug incubated for 2 and 20 min, respectively; lanes 4–8, DNA + topo I incubated for 1, 25, 5, 10, and 20 min, respectively; lanes 9–13, same as 4–8, except containing 2 μM **2**.

typical gel is shown in Figure 4, and the experimental results are presented in Table 2. No significant increase in the amount of nicked DNA, compared to control reactions, was observed for any of the drugs. It should also be noted that experiments performed on ^{32}P-labeled linearized pBR322 similar to those used to demonstrate stabilization of topo I-generated cleavable complexes by camptothecin[10] failed to show any enhancement in the level of nicked DNA (Figure 5). It is interesting to note, though, that compounds **1a**, **2**, and **3** all show an increased number of highly negatively supercoiled DNA species. This was previously interpreted as a change from processive to distributive kinetics induced by intercalating agents.[12] In addition, the equilibrium distribution in the presence of strongly binding intercalators is expected to be shifted toward negative topoisomers because of DNA unwinding effects.

Discussion

Clearly, inhibition of topoisomerase I by anthracycline antibiotics readily occurs,[8] although there seems to be no strict correlation between the binding constant and topo I inhibition (see, for example, data for compounds **1** and **2** in Table 1). Failure of the anthracyclines to stabilize the covalent complexes of the topo I reaction suggests that the inhibition is nonspecific, arising solely from DNA binding rather than from interaction between drug and covalent enzyme–DNA species. DNA binding is sufficient for inhibition of topo I, and, presumably, other DNA processing enzymes. Indeed, inhibition of topo I by both intercalators and nonintercalative minor groove binding drugs has been observed,[8,12,13] and

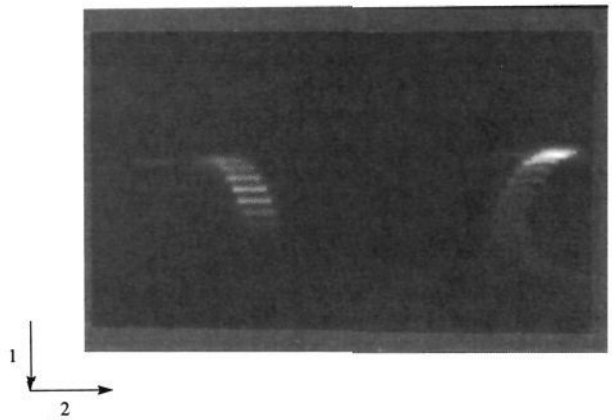


Figure 4. Two-dimensional agarose gel electrophoresis to topo I reaction products. Reaction conditions were as described in the Experimental Section. DNA and topo I were incubated at 30 °C in the presence and absence of 2 μM **2** for 20 min, followed by quenching and electrophoresis in TPE in the first dimension. The gel was then equilibrated in TPE + 10 μM chloroquine and turned 90°, and electrophoresis was reinitiated: lane 1, DNA + topo I; lane 2, DNA + topo I + 2 μM **2**.

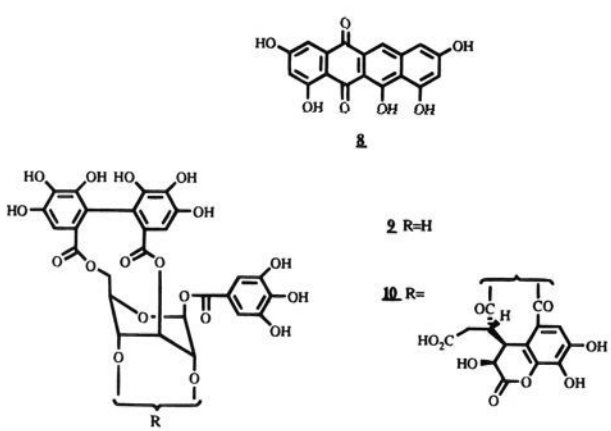
Table 2. Summary of Data from the Two-Dimensional Gel Analyses[a]

drug	incubation time, min	distribution of topoisomers produced
none (control)	10–30	narrow; positive supercoils
1a	5	wide; negative supercoils
1a	20	wide; negative supercoils
2	10	wide; negative supercoils
2	30	wide; negative supercoils
3	30	wide; negative supercoils
4	30	identical to control
5	30	identical to control
6	30	identical to control
7	30	identical to control

[a] Conditions are described in the Experimental Section and in the legend of Figure 4.

daunomycin and actinomycin have been shown to inhibit the various stages of transcription initiation.[14,15]

The idea of general enzymatic inhibition through DNA binding should be considered with respect to recently discovered topoisomerase inhibitors saintopin (**8**),[16] corilagin (**9**), and chebulagic acid (**10**).[17] Saintopin



was found to be a weak intercalator and inhibited both topoisomerases; this may be a reflection of its DNA binding affinity. Corilagins **9** and **10**, on the other hand, may be selective toward topo I, despite the fact that their structures suggest that they may be intercalators

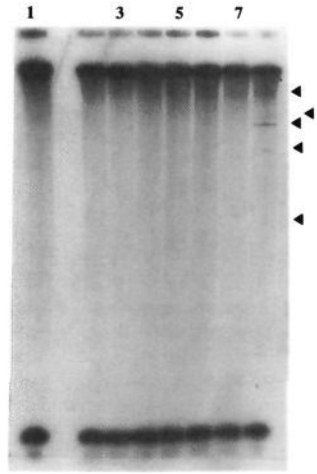


Figure 5. Detection of topo I cleavage products. Reaction conditions are as described in the Experimental Section: lane 1, control DNA; lanes 2 and 3, DNA + topo I, incubated for 0.5 and 5 min, respectively; lanes 4–7, DNA + topo I + 2 μM daunomycin, incubated for 0.5, 1, 2.5, and 5 min, respectively; lane 8, DNA + topo I + 100 μM camptothecin, incubated for 5 min.

or, possibly, nucleic acid mimics (no rigorous assay for DNA binding or protein binding by **9** or **10** was reported[17]). Chebulagic acid was found to inhibit topo II 40-fold less than it inhibited topo I, and this may be related to as yet undiscovered DNA binding activity.

In conclusion, it is of great potential interest to see what other nonspecific effects of known DNA binding agents may be uncovered. While drugs like the anthracyclines may *prefer* to target topo II–DNA complexes, their ability to inhibit other enzymatic processes may contribute to their overall antitumor activity and cytotoxicity. Clearly, *in vivo* effects of chemotherapeutic agents may not be as specific as one would have hoped they would be.

Experimental Section

Regents. ΦX 174 R_fI DNA was purchased from New England Biolabs. Calf thymus DNA topoisomerase I was purchased from Bethesda Research Labs. Compounds **2**–**7** were synthesized as described,[6,7] dissolved in absolute ethanol (Aaper Alcohol and Chemical Co.) in submillimolar concentrations, and stored at −20 °C (in the dark). All other solutions were either autoclaved or sterile filtered using 0.2 or 0.45 μm filterware (Nalgene).

Topo I Inhibition Assays. Reactions contained 0.5 μg ΦX 174 R_fI DNA, 10 units topo I, 50 mM Tris (pH 7.4), 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, 50 μg/mL BSA, 10% (v/v) ethanol, and drug (where present) in a final volume of 30 μL.[10] Reactions were incubated at 30 °C with 6 μL aliquots removed at specific time points (see the figures). The aliquots were added to 10% sodium dodecyl sulfate (SDS, 1 μL), followed by addition of 1.2 μL of 1 mg/mL Proteinase K (Boehringer) and reincubation at 37 °C for 1 h. Loading dye [1 μL, 40% (w/v) sucrose, 0.025% bromophenol blue] was added, followed by electrophoresis as described below.

Agarose Gel Electrophoresis. All gels contained 1% agarose in TPE (0.08 M Tris, 0.08 M phosphate, 0.008 M EDTA) buffer and were run at 3 V/cm, with careful attention that the resistance did not exceed 3.6 mA/cm, as to prevent heating of the gel, which broadens the bands and complicates densitometric analysis. Gels of samples containing daunomycin contained 0.03% SDS, to prevent smearing of the bands, and were soaked in water prior to staining. Gels were stained with ethidium bromide for 15 min, followed by destaining for

45 min. Negatives of the gel were made, using Polaroid 665 film, followed by scanning with a densitometer. All nonsupercoiled DNA was added together as "% relaxed DNA" and plotted as a function of time (see the figures).

Two-Dimensional Gel Electrophoresis Experiments. Reaction mixtures were identical to those listed above. Samples were incubated at 30 °C for 20 min, followed by addition of 3 μL of 10% SDS and 3 μL of 1 mg/mL Proteinase K and reincubation at 37 °C for 1 h. Loading dye (3 μL) was added and 15 mL of the reaction mixture was loaded onto a 30 cm × 30 cm × 6 mm 1% agarose gel, the wells of which were 1 mm × 5 mm and spaced 8 cm apart (two sets of wells, spaced 15 cm apart, per gel). Electrophoresis was engaged (95 V) for 4 h in TPE buffer. The gel was then soaked, with gentle shaking, in TPE buffer containing 10 μM chloroquine for 3 h.[11] The gel was then rotated 90°, and electrophoresis in the chloroquine containing buffer was engaged for 4 h. The gel was then washed with three changes of water, stained with ethidium bromide for 25 min, followed by destaining in water at 4 °C, with three to four changes of water, over a period of 1 h. The gel was then photographed.

Denaturing Polyacrylamide Gel Electrophoresis. Reaction mixtures contained 0.5 μg of 3' labeled linearlized pBR322 DNA,[3] 25 units of topo I, drug (anthracycline or camptothecin) where present, and buffer as described above. The samples were incubated at 30 °C. Five microliter aliquots were removed and stopped/treated as described above; 6 μL of 8 M urea/loading dye was then added, and the samples were boiled and loaded immediately on an 8% (20:1) polyacrylamide gel containing 8 M urea.

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References

(1) DiMarco, A.; Gaetani, M.; Orezzi, P.; Scarpinato, B.; Silvestrini, R.; Soldati, M.; Dasdia, T.; Valentini, L. 'Daunomycin', a New Antibiotic of the Rhodomycin Group. *Nature* **1964**, *201*, 706–707.

(2) Myers, C. E.; Chabner, B. A. Anthracyclines. In *Cancer Chemotherapy: Principles and Practice*. Chabner, B. A., Collins, J. M., Eds.; Lippincott, Philadelphia, 1990; pp 356–381.

(3) Tewey, K. M.; Rowe, T. C.; Yang, L.; Halligan, B. D.; Liu, L. F. Andriamycin-induced DNA Damage Mediated by Mammalian DNA Topoisomerase II. *Science* **1984**, *226*, 466–468.

(4) Review: Cozzarelli, N. R.; Wang, J. C., Eds. *DNA Topology and its Biological Effects*; Cold Spring Harbor Laboratory Press, 1990; New York.

(5) Review: D'Arpa, P.; Liu, L. F. Topoisomerase-Targeting Antitumor Drugs. *Biochim. Biophys. Acta* **1989**, *989*, 163–177.

(6) Suzuki, K.; Sulikowski, G. A.; Friesen, R. W.; Danishefsky, S. J. Application of Substituent-Controlled Coupling of Glycals in a Synthesis and Structural Corraboration of Ciclamycin O: New Possibilities for the Construction of Hybrid Anthracyclines. *J. Am. Chem. Soc.* **1990**, *112*, 8895–8902.

(7) Berkowitz, D. B.; Danishefsky, S. J.; Shulte, G. K. A Route to Artificial Glycoconjugates and Oligosaccharides via Enzymatically Resolved Glycals: Dramatic Effects of the Handedness of the Sugar Domain Upon the Properties of an Anthracycline Drug. *J. Am. Chem. Soc.* **1992**, *114*, 4518–4529.

(8) Fogelsong, P. D.; Reckford, C.; Swink, S. Doxorubicin Inhibits Human DNA Topoisomerase I. *Cancer Chemother. Pharmacol.* **1992**, *30*, 123–125.

(9) Roche, C. J.; Berkowitz, D.; Sulikowski, G. A.; Danishefsky, S. J.; Crothers, D. M. Binding Affinity and Site Selectivity of Daunomycin Analogues, *Biochemistry* **1994**, *33*, 936–942.

(10) Hsiang, Y.-H.; Hertzberg, R.; Hecht, S.; Liu, L. F. Camptothecin Induces Protein-Linked DNA Damage via Mammalian DNA Topoisomerase I. *J. Biol. Chem.* **1985**, *260*, 14873–14878.

(11) Peck, L. J.; Wang, J. C. Energetics of B-to-Z Transition in DNA. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *80*, 6206–6210.

(12) Pommier, Y.; Covey, J. M.; Kerrigan, D.; Markovits, J.; Pham, R. DNA Unwinding and Inhibition of Mouse Leukemia L1210 DNA Topoisomerase I by Intercalators. *Nucleic Acids Res.* **1987**, *15*, 6713–6731.

(13) McHugh, M. M.; Woynarowski, J. M.; Sigmund, R. D.; Beerman, T. A. Effect of Minor Groove Binding Drugs on Mammalian Topoisomerase I Activity. *Biochem. Pharm.* **1989**, *38*, 2323–2328.

(14) Straney, D. C.; Crothers, D. M. Effect of Drug-DNA Interactions Upon Transcription Initiation at the *lac* Promoter. *Biochemistry* **1987**, *26*, 1987–1995.

(15) Phillips, D. R.; White, R. J.; Trist, H.; Cullinane, C.; Dean, D.; Crother, D. M. New Insight into Drug-DNA Interactions at Individual Drug Binding Sites Probed by RNA Polymerase During Active Transcription of the DNA. *Anti-Cancer Drug Design* **1990**, *5*, 21–29.

(16) Yamashita, Y.; Kawada, S.-Z.; Fujii, N.; Nakano, H. Induction of Mammalian DNA Topoisomerase I and II Mediated DNA Cleavage by Saintopin, a New Antitumor Agent from Fungus. *Biochemistry* **1991**, *30*, 5838.

(17) Berry, D. E.; MacKenzie, L.; Shultis, E. A.; Chan, J. A.; Hecht, S. M. Naturally Occurring Inhibitors of Topoisomerase I Mediated DNA Relaxation. *J. Org. Chem.* **1992**, *57*, 420–421.