# Structural Modifications of Camptothecin and Effects on Topoisomerase I Inhibition

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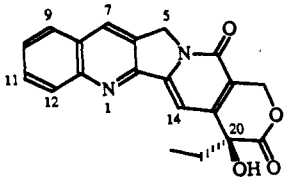
Camptothecin (1), a potent antitumor alkaloid, is known to inhibit topoisomerase I, an enzyme that relaxes supercoiled DNA. Modifications have been made to the B, D, and E rings of this natural product. Specifically, compounds **2–10** either have an ester moiety in place of the E ring lactone, a methyl ester attached to position 14, a saturated (or nonexistent) deaza B ring, or contain a combination of these permutations. We have conducted in vitro assays against the topoisomerase I relaxation reaction which verify the necessity for a lactone in the E ring. Furthermore, steric requirements at position 14 are shown to be crucial for activity, and planarity of the A and B rings of camptothecin is also implicated in the ability of the drug to inhibit topoisomerase I. Speculation on the nature of the drug binding pocket is presented.

Camptothecin (1), an alkaloid present in extracts from the Chinese tree *Camptotheca acuminata*, has been known for over a quarter of a century.[1] The antitumor activity



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of this compound eventually resulted in Phase I clinical trials, which uncovered a variety of unacceptable side effects, and the studies were subsequently dropped.[2] Interest in the drug was revived in 1985, when Liu and co-workers reported that unlike many other antitumor antibiotics that were known to inhibit topoisomerase II, camptothecin was found to inhibit topoisomerase I,[3] an enzyme that relaxes supercoiled DNA by a nicking/resealing mechanism.[4]

Despite an enormous amount of research in this area,[4,5] many aspects of the cytotoxicity and antitumor activity of camptothecin remain a mystery. This is, for the most part, due to a lack of insight into the mechanistic details of topoisomerase–DNA interactions. Topoisomerase I is known to associate with DNA in a noncovalent fashion, followed by a nicking of the phosphodiester backbone by a tyrosine residue,[4,6] as shown in Figure 1. These steps

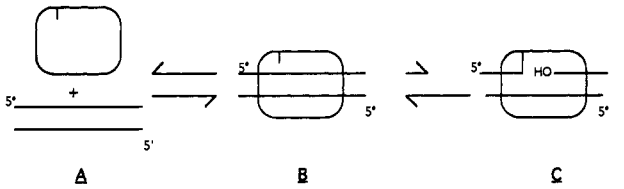


**Figure 1.** Mechanism of relaxation of DNA by topoisomerase I. Free topo I and DNA (A) first bind in a noncovalent fashion to form intermediate B. The enzyme then nicks the DNA, leaving a free 5′ hydroxyl (C), and twists the broken strand around the intact strand and religates to give a relaxed isomer of B.

are then followed by a twisting of the ends of the broken strand around the intact strand, and concomitant resealing of the backbone, thus altering the linking number of the DNA in increments of ±1.[7]

The ability of camptothecin to inhibit this process is also poorly understood. It is known that the drug does not affect DNA in the absence of protein, nor does it affect the enzyme if no DNA is present.[3] The drug is believed to stabilize the "cleavable complex", species C in Figure 1, such that subsequent inactivation and removal of the protein shows elevated levels of nicked DNA.[3] This inhibitory process has clearly been demonstrated to be a three-body event, through a series of experiments conducted by Hertzberg and co-workers, wherein equilibrium dialysis measurements gave strong support to the importance of the ternary drug–DNA–enzyme complex.[8]

The initially promising clinical outlook of camptothecin has resulted in a number of synthetic efforts toward both the parent drug[9] and a substantial number of analogs, many of which have been evaluated biologically.[2,8,10–14] In the hope of deducing other structural parameters critical

(1) Wall, M. E.; Wani, M. C.; Cook, C. E.; Palmer, K. H.; McPhail, A. T.; Sim, G. A. Plant Antitumor Agents. I. The Isolation and Structure of Camptothecin, a Novel Alkaloidal and Tumor Inhibitor from *Camptotheca acuminata*. *J. Am. Chem. Soc.* **1966**, *88*, 3888–3890.

(2) Giovanella, B. C.; Stehlin, J. S.; Wall, M. E.; Wani, M. C.; Nicholas, A. W.; Liu, L. F.; Silber, R.; Potmesil, M. DNA Topoisomerase I-Targeted Chemotherapy of Human Colon Cancer in Xenografts. *Science* **1989**, *246*, 1046–1048, and references therein.

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

(4) For an excellent review on the topoisomerases, see Osherhoff, N. Biochemical Basis for the Interaction of Type I and Type II Topoisomerases with DNA. *Pharmacol. Ther.* **1989**, *41*, 223–241.

(5) For an additional review, see D'Arpa, P.; Liu, L. F. Topoisomerase-targeting Antitumor Drugs. *Biochim. Biophys. Acta* **1989**, *989*, 163–177.

(6) Champoux, J. DNA is Linked to the Rat Liver DNA Nicking-Closing Enzyme by a Phosphodiester Bond to Tyrosine. *J. Biol. Chem.* **1981**, *256*, 4805–4809.

(7) For an overview of DNA supercoiling and its biological effects, see Wu, H.-Y.; Shyy, S.; Wang, J. C.; Liu, L. F. Transcription Generates Positively and Negatively Supercoiled Domains in the Template. *Cell* **1988**, *53*, 433–440.

(8) Hertzberg, R. P.; Caranfa, M. J.; Hecht, S. M. On the Mechanism of Topoisomerase I Inhibition by Camptothecin: Evidence for Binding to an Enzyme–DNA Complex. *Biochemistry* **1989**, *28*, 4629–4638.

(9) For a review of the synthetic approaches, see Cai, J.-C.; Hutchinson, C. R. *The Alkaloids. Chemistry and Pharmacology*; Brossi, A., Ed.; Academic Press: New York, 1983; Vol. 21, p 101.

(10) Kingsbury, W. D.; Boehm, J. C.; Jakas, D. R.; Holden, K. G.; Hecht, S. M.; Gallagher, G.; Caranfa, M. J.; McCabe, F. L.; Faucette, L. F.; Johnson, R. K.; Hertzberg, R. P. Synthesis of Water-Soluble (Aminoalkyl)camptothecin Analogues: Inhibition of Topisomerase I and Antitumor Activity. *J. Med. Chem.* **1991**, *34*, 98–107, and refs 7–12 therein.

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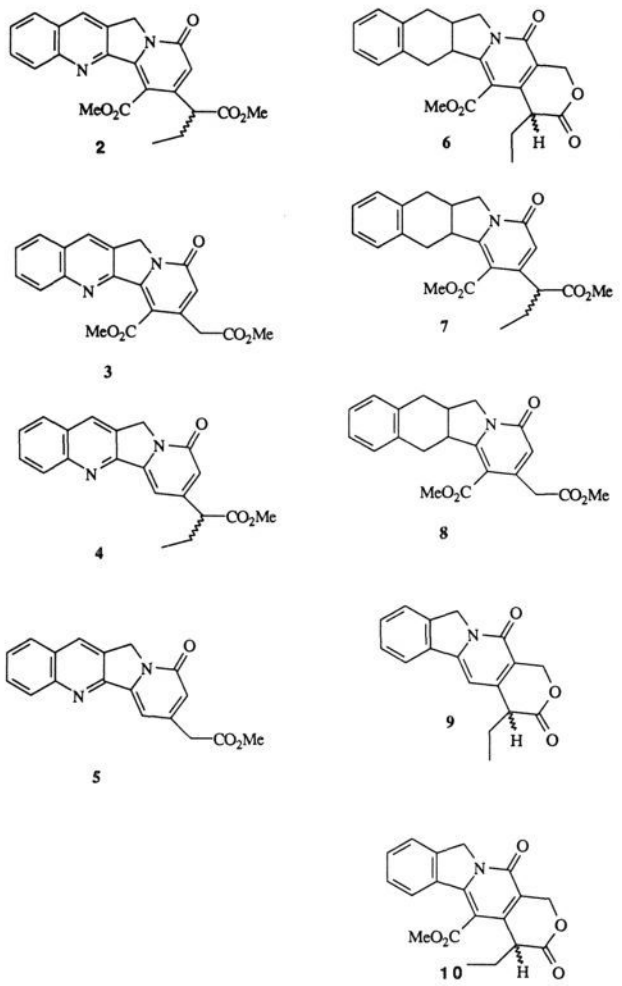
**Chart I**




to the ability of camptothecin to recognize the binary enzyme–DNA complex, a series of analogs, presented in Chart I, were screened for topoisomerase I inhibition. Compounds **2–5** were synthetic precursors to camptothecin itself[15,16] and were screened to test the importance of both the E ring lactone and steric encumbrance at position 14. Analogs **6–8** were precursors to the aromatic, all-carbon analog of camptothecin; however, aromatization was never successfully achieved.[17] These compounds would hopefully relay information about the relevance of planarity of the left hand portion of the molecule to biological activity. Finally, analogs **9** and **10** were designed to test the importance of the B ring to the antitumor efficacy of the drug.[17]

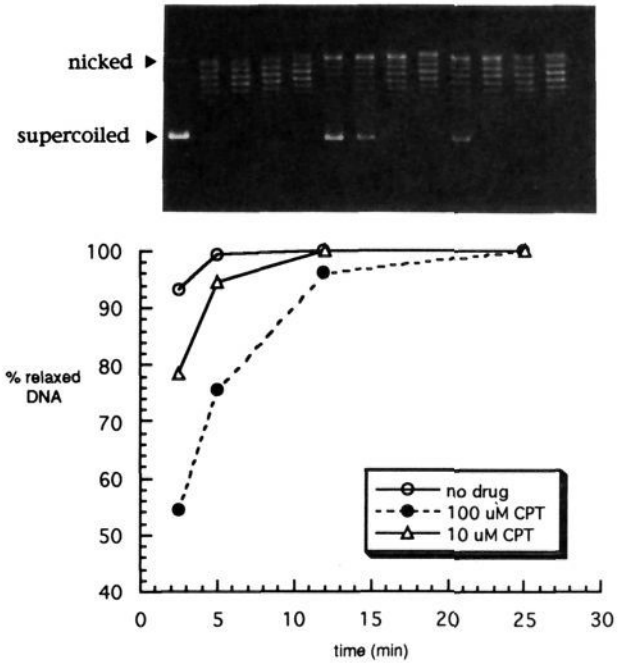



**Figure 2.** Time course of camptothecin inhibition of topo I (see Experimental Section for the gel conditions and method of analysis). Lane 1, control $\phi$X 174 $R_f$ I DNA. Lanes 2–5, DNA + topoisomerase I, incubated for 2.5, 5, 12, and 20 min, respectively. Lanes 6–9 are identical to lnes 2–5, except they contain 100 $\mu$M camptothecin. Lanes 10–13 are also identical to lanes 2–5, except they contain 10 $\mu$M camptothecin.

## Biological Testing

Inhibitory activity of these compounds toward the topoisomerase I relaxation reaction was evaluated using a time course experiment. Briefly, aliquots from the reaction mixture were added to protein denaturant, and the protein was removed from the DNA by treatment with proteinase K.[18] Agarose gel electrophoresis was then initiated to separate the nicked, supercoiled, and topoisomeric forms of DNA. The amount of relaxed closed circular DNA and the intermediate topoisomers were added together as "relaxed DNA" and plotted as a function of time, as shown in the sample gel and plot in Figure 2.

Like the parent drug, none of the analogs had any effect on the DNA in the absence of protein (data not shown). Disappointingly, the majority of analogs exhibited little, if any, activity relative to camptothecin, as shown in Figure 3 and summarized in Table I. Those analogs with only one concentration listed were inactive at and below that concentration; assays at higher concentrations were not possible, due to limited analog solubility.[19] As can be seen, blockage of position 14 with a methyl ester results in a decrease in activity in the camptothecin precursors

(11) Hertzberg, R. P.; Busby, R. W.; Caranfa, M. J.; Holden, K. G.; Johnson, R. K.; Hecht, S. M.; Kingsbury, W. D. Irreversible Trapping of the DNA-Topoisomerase I Covalent Complex. *J. Biol. Chem.* **1990**, *265*, 19287–19295.

(12) Hsiang, Y.-H., Liu, L. F., Wall, M. E., Wani, M. C., Nicholas, A. W., Manikumar, G., Kirshenbaum, S., Silber, R., Potmesil, M. DNA Topoisomerase I-mediated DNA Cleavage and Cytotoxicity of Camptothecin Analogues. *Cancer Res.* **1989**, *49*, 4385–4389, and references therein.

(13) Jaxel, C., Kohn, K. W., Wani, M. C., Wall, M. E., Pommier, Y. Structure–Activity Study of the Actions of Camptothecin Derivatives on Mammalian Topoisomerase I: Evidence for a Specific Receptor Site and a Relation to Antitumor Activity. *Cancer Res.* **1989**, *49*, 1465–1469.

(14) Danishefsky, S.; Etheredge, S. J. Synthesis and Biological Evaluation of De-AB-camptothecin. *J. Org. Chem.* **1974**, *39*, 3430–3432.

(15) Volkmann, R.; Danishefsky, S.; Eggler, J.; Solomon, D. M. A Total Synthesis of *dl*-Camptothecin. *J. Am. Chem. Soc.* **1971**, *93*, 5576–5577.

(16) Volkmann, R. A. A Total Synthesis of dl-Camptothecin. Ph.D. Thesis, University of Pittsburgh, 1972. All compounds were fully characterized by IR, proton NMR, mass spectroscopy, and combustion analysis, and were reevaluated by proton NMR and TLC analysis before use.

(17) Zimmer, A. Synthesis of Camptothecin Analogs. Ph.D. Thesis, University of Pittsburgh, 1978. All compounds were fully characterized by IR, proton NMR, mass spectroscopy, and combustion analysis, and were reevaluated by proton NMR and TLC analysis before use.

(18) This is a slight modification of the procedure in ref 3.

(19) The maximum concentrations listed in Table I are approximately 10-fold less than the maximum solubility of the analogs in DMSO.

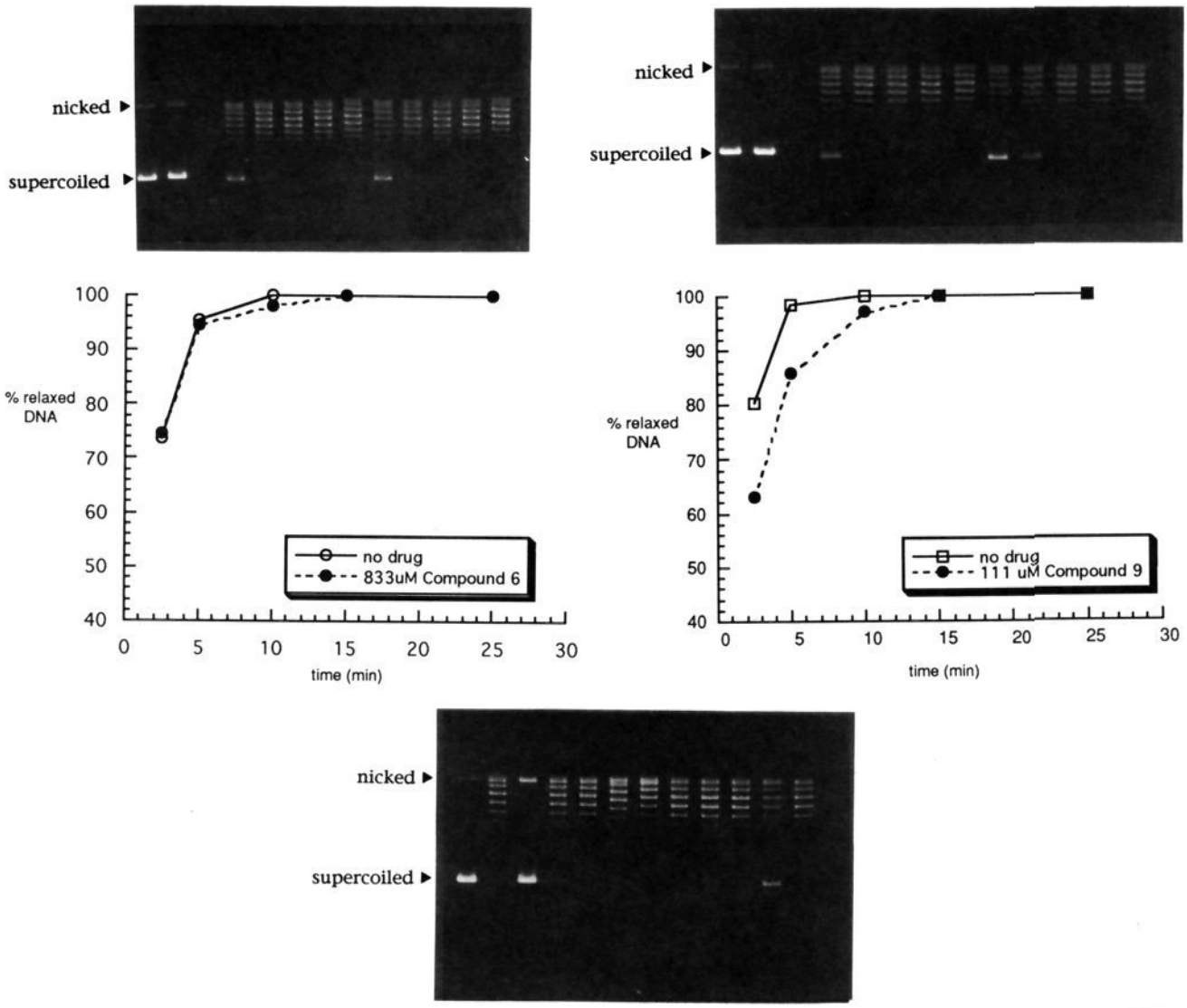



**Figure 3.** Time course experiments for analog **6** (top left) and analog **9** (top right). Lane 1, control $\phi$X 174 $R_f$ I DNA. Lane 2, DNA + drug, at the last time point of the experiment. Lanes 4–8, DNA + enzyme, incubated at 2.5, 5, 10, 15, and 25 min, respectively. Lanes 9–13 are identical to lanes 4–8, except they contain 833 $\mu$M **6** (top left) or 111 $\mu$M **9** (top right). Reaction of topoisomerase I with DNA in the presence of camptothecin and analogs (bottom; see ref 3 for experimental protocol). All reactions contained 0.5 $\mu$g $\phi$X 175 $R_f$ I DNA, 10 units topoisomerase I, 13% (v/v) DMSO, and drug, when present. Each was incubated for 7 min at 30 °C followed by the usual quenching protocol.[3] Lane 1, control $\phi$X $R_f$ I DNA. Lane 2, DNA + topo I. Lanes 3–12, DNA, enzyme, 100 $\mu$M camptothecin, 400 $\mu$M **4**, 500 $\mu$M **5**, 500 $\mu$M **2**, 100 $\mu$M **3**, 500 $\mu$M **6**, 500 $\mu$M **7**, 500 $\mu$M **8**, 100 $\mu$M **9**, and 350 $\mu$M **10**, respectively.

(compare compounds **2** and **4**, and **3** and **5** in Table I). In the saturated B ring compounds, position 14 is again blocked, and no activity is observed, even at high concentrations. The des-B ring analogs, though, are the most interesting. Racemic **9** is by far the most active of the analogs, having an activity roughly one order of magnitude less than camptothecin itself. As with the other compounds, replacement of hydrogen with a methyl ester at position "14" (analog **10**) leads to decreased activity, although some inhibition is observed. It should be noted that analysis of the DNA species generated by incubation of topo I with $^{32}$P-labeled linearized pBR322, under conditions similar to those of Hsiang, et al.,[3] showed that analogs **4**, **5**, **9**, and **10** did not stabilize cleavage at any sites other than those stabilized by camptothecin; however, the stabilization of nicked intermediates by these analogs was weaker than was seen for camptothecin (data not shown).

## Discussion

Several structural elements that are important to the topoisomerase I inhibitory activity of camptothecin can be derived from the results shown above. Clearly, blockage of position 14 with a methyl ester results in lowered activity. This is intriguing, as the proposed mechanism of action of camptothecin involves attack of a nucleophile (contained within the enzyme–DNA complex) on the lactone carbonyl of the drug (Figure 4).[8] Presumably, an additional ester is an additional electrophile and could be attacked as well. Clearly, steric considerations override any inherent electrophilicity of the additional ester.

Planarity of the left hand portion of the drug also appears to be a critical factor in topoisomerase inhibition. Unfortunately, compounds **6**–**8** are blocked at position 14, thus prohibiting direct assessment of the contribution of planarity to biological activity. The des-B ring analogs, on the other hand, imply that planarity, when coupled

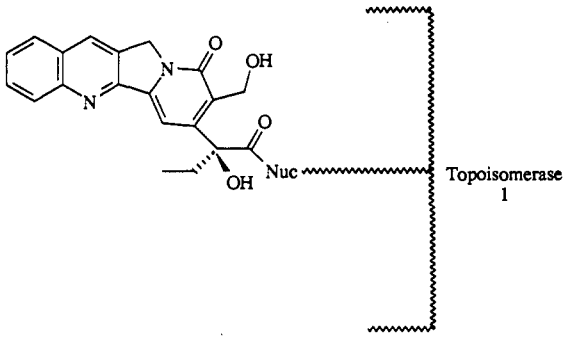



**Figure 4.** Proposed covalent adduct between camptothecin and topoisomerase I.

**Table I.**[a] Relative Activities of Camptothecin Analogs

| compd | concn in assay, μM | experimental observation |
|---|---|---|
| 2 | 625 | inactive |
| 3 | 166 | inactive |
| 4 | 418 | active, 40-fold less than parent |
| | 200 | inactive |
| 5 | 554 | active, 50-fold less than parent |
| | 277 | inactive |
| 6 | 833 | inactive |
| 7 | 833 | inactive |
| 8 | 833 | inactive |
| 9 | 83 | active, 10-fold less than parent |
| | 25 | inactive |
| 10 | 360 | active, 35-fold less than parent |
| | 12 | inactive |

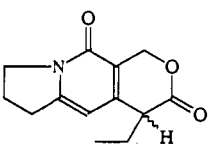
[a] Activities are based on comparison of a time course experiment of an analog with a similar experiment done with camptothecin (see Experimental Section); i.e., an analog listed as 20-fold less active than the parent compound would have an inhibitory effect at 200 μM similar to that seen for camptothecin at 10 μM.

with an intact lactone ring, is sufficient for activity, even if the lactone carbonyl is not activated by a "C-20" hydroxy group (i.e., 9 and 10). This is a provocative result as this internal activation has been shown to be critical to the activity of camptothecin.[20] This discovery, coupled with the observation that the "C-14" blocked analog 10 retains some activity, could be an indication that smaller compounds may fit more easily into a binding pocket on the binary enzyme–DNA complex, but this is merely conjecture. It should be noted that autooxidation of 20-deoxycamptothecin is known to give small amounts of camptothecin.[15] TLC analysis of reagents 4, 5, 9, and 10 before and after being subjected to the reaction conditions shown that a small amount (<3%) of a byproduct of lower $R_f$ was produced from 9 and 10 (but not 4 or 5; data not shown). Given the chemistry of the parent compound, these byproducts are, most likely, "C-20" oxidation products; however, considering the minute quantities produced during the assay, it is doubtful whether or not these byproducts are responsible for the observed biochemical effects.

To date, the major observations regarding functionalization of the camptothecin chromophore have been that substitution at positions 11 and 12 are detrimental to activity, while positions 7, 9, and 10 are acceptable sites for functionalization.[10-13] In addition, the present study has suggested that position 14 requires a substituent of minimal steric bulk (Table I). Finally, it was recently disclosed that incubation of a camptothecin derivative bearing a bromoacetyl group at position 10 with DNA and topoisomerase I resulted in alkylation of the enzyme in an irreversible fashion.[11] Analysis of these experimental observations allows for some speculation on the nature of the camptothecin binding pocket on the enzyme–DNA complex. On the basis of the fact that substituents on positions 11, 12, and 14 deactivate the drug, one can assume that these atoms are on the "inside" of the complex; i.e., that region of camptothecin recognizes a surface on the binary complex.[21] This supposition is further supported by the hypothesis that attack at the E ring carbonyl by a nucleophilic residue of the binary complex is an important part of the inhibitory activity of camptothecin (Figure 4).[8] If this is indeed the case, then this "southern" side of the molecule must be close to the complex, otherwise attack would not be facile.

A further observation is that this sensitivity toward functionalization of the A ring may lend some explanation as to why compounds 9 and 10, which lack the "C-20" hydroxyl and are also blocked at "C-14", respectively, retain some activity (Table I). It has been reported that substituents on positions 9, 10, or 10 and 11 enhance the antitumor properties of the drug, whereas functionalization of positions 11 and 12 is not readily tolerated.[10-13] This implies that the A ring may be required to fit in a rather narrow cavity on the enzyme–DNA complex. Omission of this ring altogether, as in compounds 9 and 10, may have created an analog with more freedom of movement within the binding pocket, resulting in activity despite apparent structural deficiencies.

One is tempted to conclude that if the B ring is not necessary, the A ring might also be discarded. This issue was addressed some years ago, when analogs 11 and 12



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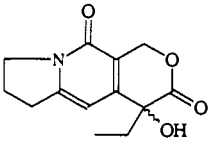

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were synthesized and screened for activity against L1210 cells, showing little activity.[14] Clearly, antitumor activity and, in all likelihood, topoisomerase I inhibitory activity, requires at least one flat, aromatic appendage on the drug, in addition to the other requirements previously reported,[10-14] which are summarized in schematic form in Figure 5. The reasons, however, for the necessity of an aromatic section on the drug remain, for the moment, unknown.

In summary, studies on the series of analogs 2–10 confirmed the necessity for a lactone in the E ring, accessibility of position 14, and planarity in the western sector of the alkaloid camptothecin. Hopefully, this study has provided some insight into the nature of drug–DNA–enzyme interactions, as the need for information in this area cannot be overstated.

## Experimental Section

**General.** Calf thymus topoisomerase I was purchased from Bethesda Research Laboratories and stored at −20 °C. φX 174 $R_f$ I DNA (double stranded) was purchased from New England Biolabs, and was stored at 4 °C. Proteinase K was purchased from Boehringer Mannheim Biochemicals, suspended in water, and either frozen at −20 °C for long term storage or kept at 4 °C

(20) Reference 8, and references therein.

(21) Some of these ideas have been previously proposed. See ref 13.

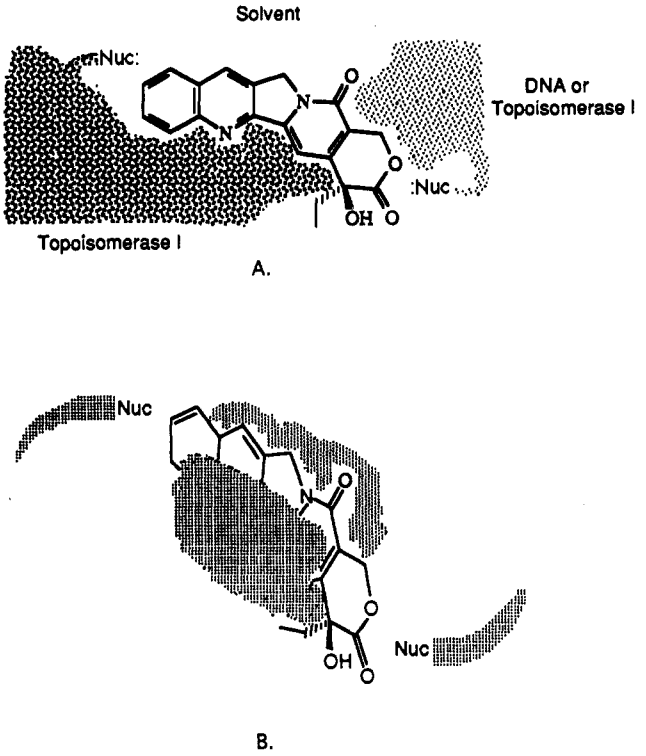


**Figure 5.** Two views of the proposed interaction of camptothecin with the topoisomerase I–DNA complex. The nucleophile adjacent to the A ring is a nucleophilic residue on topoisomerase I that has previously been shown to attack 10-(bromoacetyl)-camptothecin.[11] The second nucleophile has previously been proposed to be a part of the mechanism of inhibition of topoisomerase I by camptothecin.[3] Part A (top) shows a cross-section of the ternary complex in the plane of the drug. Part B (bottom) shows a 45° view of the drug within the proposed binding pocket (see Discussion).

when needed. Bovine serum albumin (BSA) was purchased from IBI and stored at 4 °C. Camptothecin was purchased from Sigma. All drugs were dissolved in DMSO (10 mM for camptothecin, otherwise maximum possible concentrations, based on maximum solubility of the analog) and kept at 4 °C.

**Standard Time Course Assay (as displayed in Figure 3, top right).** All reaction mixtures (30 μL total volume) were 50 mM Tris (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA (pH 8.0), 0.5 mM DTT, 0.25 μg/mL BSA, and contained 0.5 μg φX 174 DNA, 10 units topoisomerase I, and 111 μM compound 9 (in DMSO, final concentration of DMSO, 13% v/v), with the order of addition being buffer, DNA, drug, enzyme. All of the reaction mixtures were gently vortex mixed immediately before incubation.[22] The tubes were placed in a 30 °C water bath, with gentle agitation. At the times indicated on the graph in Figure 3, 6-μL aliquots were removed from the tube and added to 1 μL of SDS that had previously been pipetted into another Eppendorf tube. 1.2 μL of 1 mg/mL proteinase K was then added to the denatured aliquot, followed by gentle vortex mixing and reincubation at 37 °C for 1 h.[18]

**Gel Electrophoresis.** Samples were electrophoresed using a 1% agarose gel in standard 1X TPE buffer (0.08 M Tris, 0.08 M phosphate, 8 mM EDTA). The aliquots that have been denatured and have been treated with proteinase were then charged with 1 μL of loading buffer (0.25% bromophenol blue, 40% sucrose), and loaded into the gel. Electrophoresis was conducted at 3 V/cm, with special attention that the resistance of the gel does not exceed 3.6 mA/cm.[23]

**Gel Processing and Densitometric Analysis.** After electrophoresis, gels were stained with ethidium bromide and then destained in water for 30–60 min. The gel was then placed on a UV box, and a negative photographic image was made of the gel, using Polaroid 665 film. The negative was scanned and analyzed with a BioRad (Hoefler Scientific) Model 1650 densitometer. All of the intermediate topoisomers and the relaxed closed circular DNA were added together as "relaxed DNA" and plotted as a function of time (see Figure 3). The activity of each analog was based, in a qualitative sense, by comparison of the plot from an analog assay with a series of plots from camptothecin assays of variable concentrations (e.g., Figure 2).[24]

**Acknowledgment.** We thank Prof. Samuel J. Danishefsky for providing the compounds used in this study. We also thank Prof. Sidney Altman for the use of his densitometer. R.T.C. would like to thank Dr. Jacqueline Drak for helpful advice and discussions. Support for this work was provided by the National Institutes of Health (GM21966).

(22) Vortex mixing of the sample has been found to be critical for obtaining repeatable results. Crow, R. T., unpublished observations.

(23) Gels whose resistance exceeds this value exhibit band broadening, which leads to difficulty in densitometric analysis. Crow, R. T., unpublished observations.

(24) Assays ranging from 100 μM to 0.1 μM camptothecin have been performed in this laboratory, as a comparison. Crow, R. T., unpublished.