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Synthesis and Biological Evaluation of Tetrademethyl Isocolchicine Derivatives as Inhibitors of DNA Topoisomerase Action *In Vitro*†

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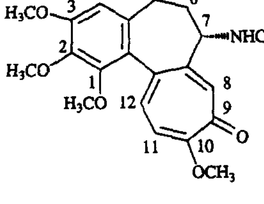
Abstract—Four tetrademethyl isocolchicine analogs were prepared and evaluated as inhibitors of mammalian DNA topoisomerases *in vitro*. All compounds inhibited topoisomerase II-dependent DNA unknotting by a mechanism which did not involve "cleavable-complex" formation. *N*-Deacetylation as well as *N*-substitution with the (3',4',5'-trihydroxybenzoyl)-group afforded compounds which were less selective, based on their added ability to inhibit topoisomerase I-mediated DNA relaxation.

Introduction

Colchicine (**1**), a major alkaloid present in *Colchicum autumnale* has been extensively investigated, and the principal biological action of this drug as a microtubule spindle toxin is well established (the stereochemistry of all compounds are given in Figure 1).[2,3] Recently, we prepared the tetrademethyl derivatives of *N*-trifluoroacetyldeacetylcolchicine (**2**) and *N*-(3',4',5'-trihydroxybenzoyl)deacetylcolchicine (**3**) and found that these cytocidal analogs exhibited interesting biological activities not shared by **1**, including the inhibition of DNA unknotting by purified mammalian DNA topoisomerase II.[4] This enzyme is one of two general types capable of modulating DNA topology, which it does by passing DNA strands through transient double-stranded DNA breaks.[5]



1 (colchicine)

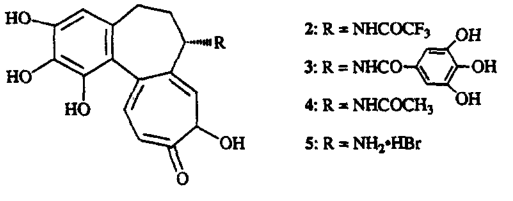
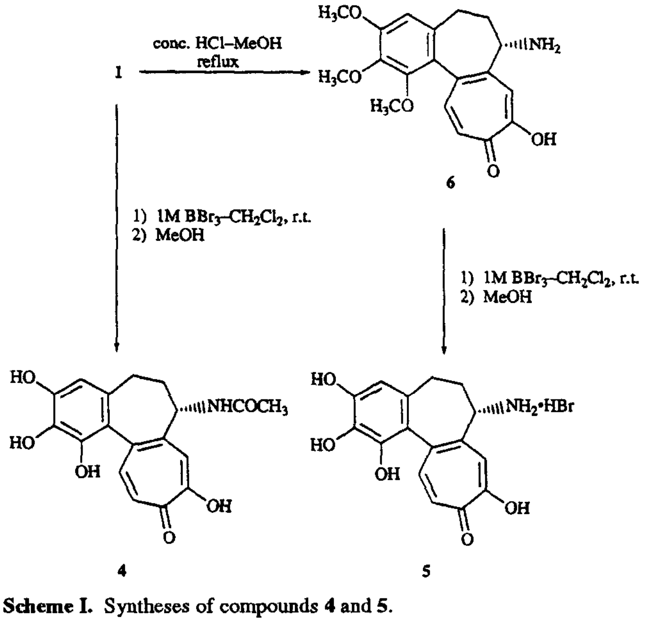


Figure 1. The structures of colchicine (**1**) and tetrademethyl isocolchicine analogs (**2–5**)

Most current inhibitors of DNA topoisomerases are natural products or their derivatives and several are efficacious antitumor agents. The majority of these drugs act though a common inhibitory mechanism, by stabilizing covalent reaction intermediates between topoisomerases and DNA known as "cleavable-complexes".[6] Such a mechanism could account for the cytotoxic action of **2** and **3** since these compounds, unlike **1**, were found to induce intracellular protein-associated DNA breaks.[4] Thus, tetrademethyl derivatives of **1** may be a novel chemical class of topoisomerase poison with potential for drug development. In order to explore these possibilities, two additional analogs (**4** and **5**) were prepared, then all four derivatives were evaluated as inhibitors using *in vitro* assays for topoisomerase-dependent catalytic and non-catalytic action.[7]



Scheme I. Syntheses of compounds **4** and **5**.

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†Antitumor Agents 146. For paper 145 in this series, see Ref. 1.

Chemistry

The general procedures used for preparing target compounds **4** and **5** are outlined in Scheme I. Hydrolysis of **1** with concentrated hydrochloric acid in methanol afforded 10-demethyldeacetylcolchicine (**6**).[8] Exhaustive demethylation of compounds **1** and **6** with boron tribromide afforded compounds **4** and **5**.[9] The structures of all intermediates and final products were secured by spectroscopic and analytical data.

Biological Results

Inhibitory activities of the four **1** analogs toward HeLa topoisomerase II were first evaluated using the DNA unknotting assay. The result for **4** is shown in Figure 2. Enzyme action was completely inhibited at 50 µM (compare lane 4 to the control reaction in lane 1). In similar tests, complete inhibition by compounds **2**, **3**, and **5** occurred at 25, 6, and 12 µM, respectively.[10]

Compounds were examined for stimulation of topoisomerase II-dependent DNA cleavage because we anticipated this mode of inhibition based on an earlier study.[4] Suprisingly, none of the analogs behaved in the expected fashion. A typical experimental result for compound **2** illustrates this finding (Figure 3). Incubation with VP-16 (etoposide),[11] produced dose-dependent DNA fragmentation, a characteristic property of inhibitors which act by stabilizing "cleavable-complexes" (compare lane 1 through 4 to lane 10).[6,7] In contrast, incubating enzyme with compound **2** from 50 to 400 µM had no impact on DNA integrity under cleavage reaction conditions (lanes 5 through 8).

The mode of enzyme inhibition was examined further using the DNA cleavage method by co-incubating compounds with VP-16. This approach identifies agents which prevent the formation of drug-stabilized "cleavable-complexes", a known property of certain inhibitors that do not themselves stabilize covalent reaction intermediates.[12] The **1** analogs were found to be active as shown by reversal of DNA fragmentation (Figure 4, lanes 5–10 compared to lane 3) and by the reduced amounts of "cleavable-complexes" recovered from reactions following potassium/sodium dodecylsulfate (SDS) precipitation. Compounds **3** and **5** altered the gel-mobility of ^{32}P-labeled linearized plasmid DNA (Figure 4, compare lanes 6–7 and 10 to lane 1). This effect was subsequently shown to be dependent on the dose of compound and occurred in the absence of enzyme (data not shown), possibly indicating compounds directly precipitate DNA in the reaction mixture. Based on these results, DNA binding was evaluated with the ethidium bromide displacement method and compounds **2**, **4**, and **5** were found to be inactive using this assay.[13]

DNA topoisomerase I was also investigated as a potential biochemical target for **1** analogs. Two derivatives, compounds **3** and **5** inhibited enzyme-dependent DNA relaxation (Figure 5, compare lanes 4 and 6 to lane 2) with **3** alone giving detectable inhibition at 100 µM (Figure 5, lane 6). Subsequent studies using DNA cleavage assays showed that the mode of topoisomerase I inhibition also involved a mechanism which blocked formation of drug-stimulated "cleavable-complexes" (data not shown).[14]

Discussion

The inhibition of DNA topoisomerase II by **1** analogs resulted in a blockade of VP-16 stimulated "cleavable-complexes". This suggests a common mode of inhibition involving binding to the enzyme, the non-covalent enzyme–DNA intermediate or both. Our studies do not permit resolution of these possible modes. Other data presented in this paper indicate that the ultimate inhibitory mechanism involved could be different for **3** and **5**. Both compounds exhibited additional activity toward topoisomerase I which could be due to interactions between **3**, **5**, and DNA. This possibility was revealed by the decreased gel-mobility of linear plasmid DNA in cleavage experiments. However, DNA binding by **5** was not detected using the ethidium displacement procedure. Additional studies are required to reconcile these results.

In an earlier study first identifying **2** and **3** as topoisomerase II inhibitors, the compounds were also shown to induce intracellular protein-linked DNA breaks.[4] This effect was dose-dependent, reversible, and correlated with cytotoxicity, all properties compatible with the action of cleavable complex-forming type inhibitors.[6] The current observations on the mode of enzyme inhibition by **1** analogs *in vitro* appear to conflict with our earlier study. A recent comparison of protein-associated DNA break production by caffeine derivatives and topoisomerase II inhibitors revealed functional similarities, but discrepancies were noted between effects observed in nuclei versus those found using purified enzyme.[15] The authors suggested that methylated oxopurines may only be active on natural chromatins templates or act on a particular topoisomerase II isozyme. Similar explanations could account for the conflicting behavior of **1** analogs, alternatively, other proteins capable of covalent interaction with DNA could be the intracellular target(s), or these compounds could be bioactivated in cells to form topoisomerase poisons. The spectrum of cell cytotoxicity for **2** and **3** found earlier,[4] suggests that further mode of action studies with compounds of this type will be worthwhile.

The information obtained from this study concerning the structural requirements of **1** derivatives for topoisomerase II inhibition is limited. The N-substituent was found to modulate inhibitory activity but in the case of **3** and **5**, a favorable effect occurred at the expense of selectivity. Although demethylation at the 1, 2, 3, and 9-positions is a common structural feature of the four active **1** analogs, the question whether exhaustive demethylation is a critical requirement was not addressed in the present work. Such studies are in progress.

Experimental Section

Chemistry

Optical rotations were determined using Rudolph Research Autopol III polarimeter. IR spectra were recorded on a

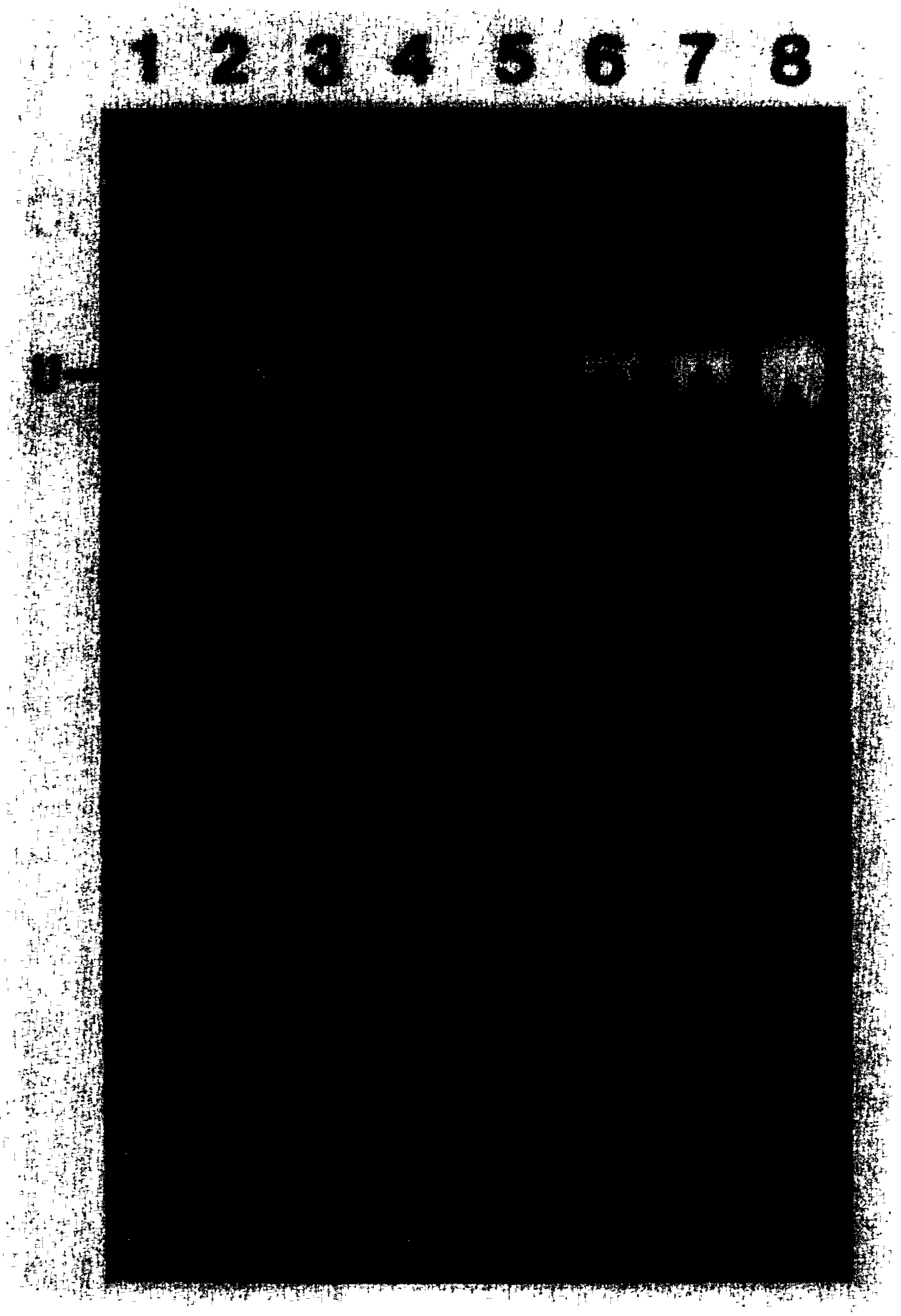

Figure 2. Inhibition of topoisomerase II-dependent DNA unknotting. Inhibition of topoisomerase II catalytic activity was assayed as described in the "Experimental Section". A photograph of an ethidium bromide-stained gel is shown. Lanes 1 and 2 contain the DNA and enzyme controls respectively. Lanes 3 to 8 contain serial dilutions of compound 4 in two-fold increments to give final concentrations from 100 μM to 3 μM respectively. "U" in the left margin indicates mobility of the unknotted DNA reaction product

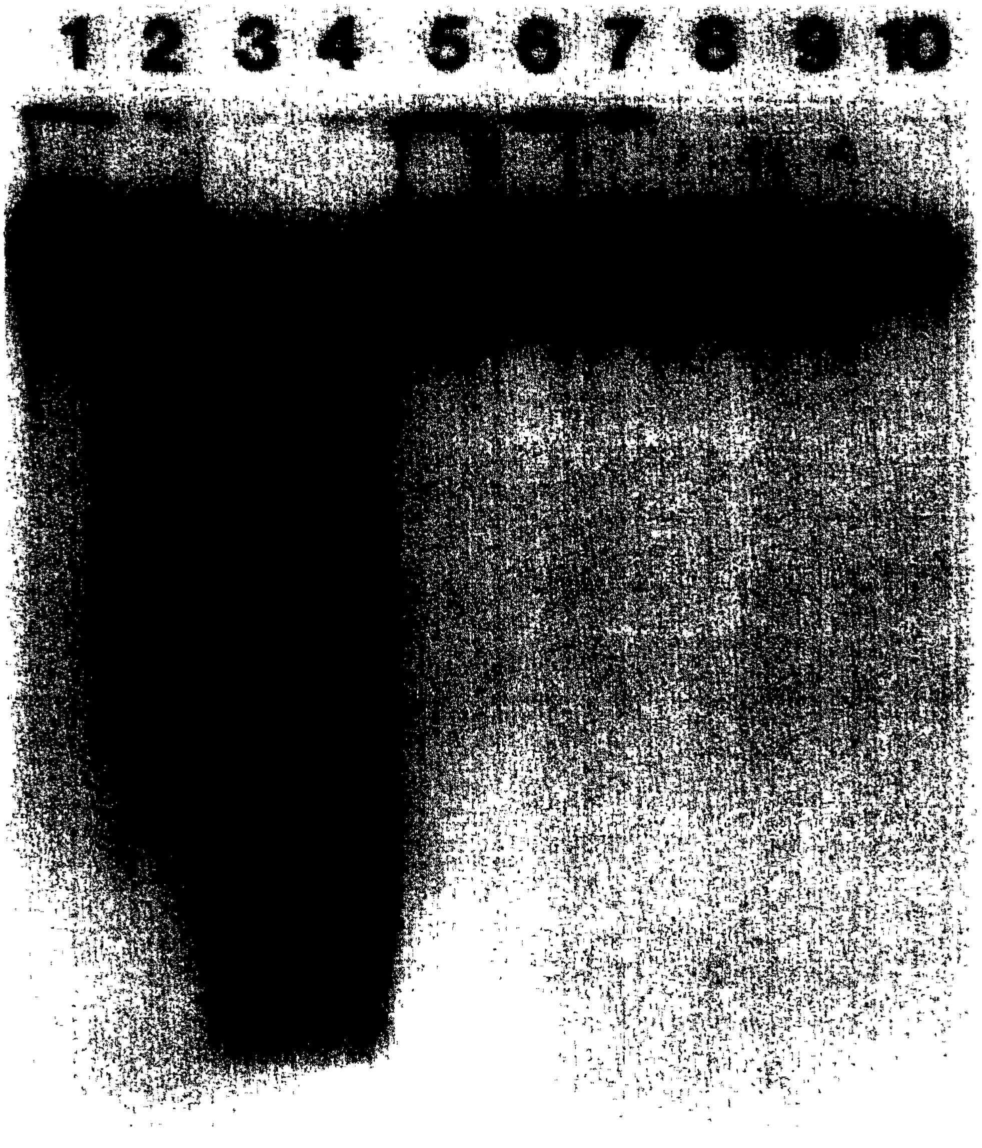

Figure 3. Impact of VP-16 and compound **2** on topoisomerase-II-dependent DNA cleavage. The effects of VP-16 and 2 were evaluated using the *in vitro* cleavage assay method described in the "Experimental Section". An autoradiogram of a dried agarose gel is shown. Lanes 9 and 10 contain the enzyme and DNA controls respectively. VP-16 was tested at 1, 10, 50, and 100 μM (lanes 1 to 4). Lanes 5 to 8 contain compound **2** in two-fold increments from 50 to 400 μM respectively

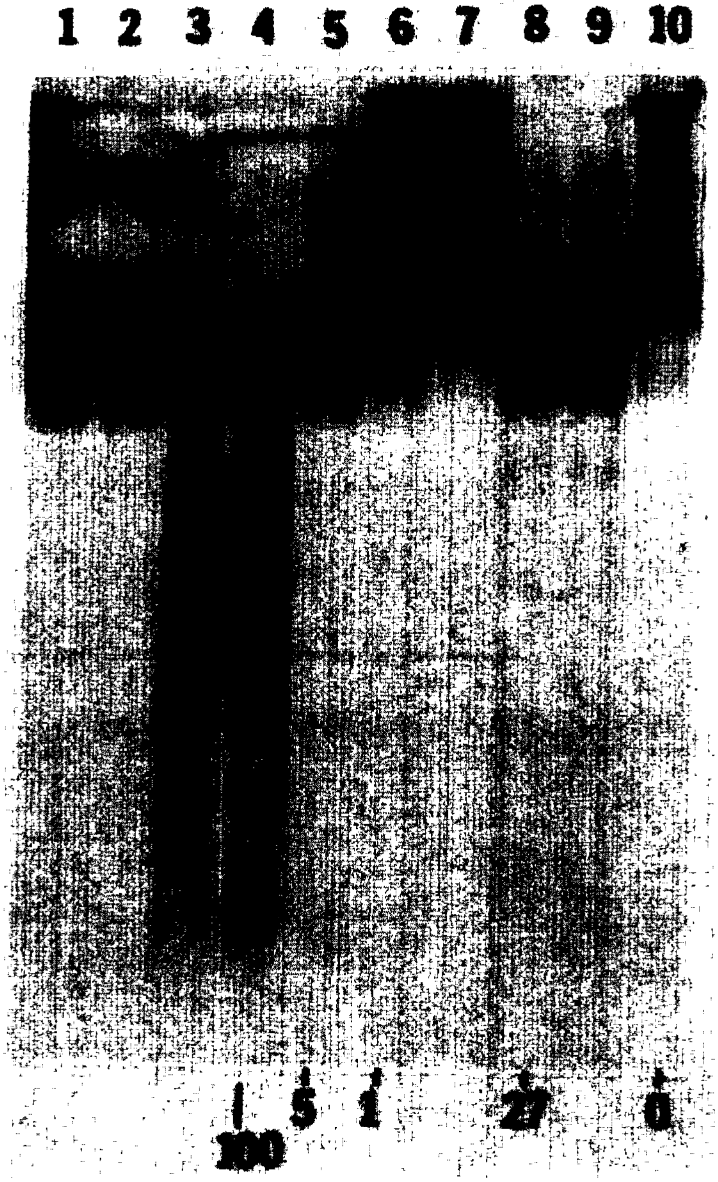

Figure 4. Interference with VP-16 stimulated topoisomerase II-mediated DNA cleavage. The effects of **1–5** on drug-stimulated DNA cleavage was evaluated by co-incubating compounds with VP-16 under standard cleavage reaction condition. Cleavable-complex formation was estimated from half of each reaction by potassium/SDS precipitation as described in the "Experimental Section". An autoradiogram of a dried agarose gel is shown. Lanes 1–3 contain DNA, enzyme, and VP-16 (20 μM) controls respectively. Compounds **2–5** were tested for interference at 10 μM. Co-treatments with **1** (100 μM) **4**, **5**, **2**, and **3** are in lanes 4, 5, 6–7, 8–9, and 10 respectively. Percent values under each lane represent cleavable complex formation relative to the VP-16 control level (1200 ^{32}P-CPM)

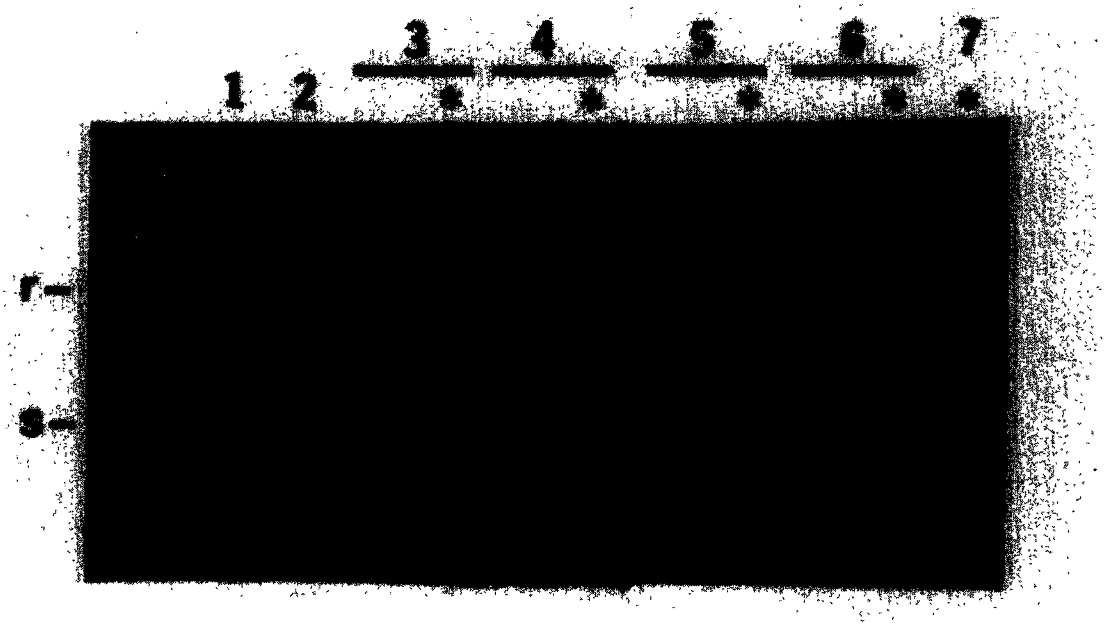

Figure 5. Inhibition of topoisomerase I-mediated DNA relaxation. Inhibition of topoisomerase I catalytic activity was assayed as described in the "Experimental Section". Lanes 1 and 2 contain the DNA and enzyme control respectively. Compounds **2–5** were tested at 100 μM and 300 μM (lanes denoted by *). A photograph of the ethidium bromide-stained gel is shown. Lane 3, compound **4**; lane 4, compound **5**; lane 5, compound **2**; lane 6, compound **3**, and lane 7, compound **1**. "*R*" and "*S*" denote the relaxed DNA product and supercoiled DNA substrate respectively. Non-adjacent wells from a single gel were juxtaposed to prepare this figure

Perkin Elmer 1320 Infrared spectrophotometer. ^{1}H NMR spectra were recorded on a Bruker AC-300 spectrometer.The chemical shift was presented in terms of ppm with Me_4Si as the internal reference. Elemental analyses were performed by Atlantic Microlab, Inc., (Norcross, GA). Toyopearl (HW-40F) from TOSOH Corp., (Tokyo, Japan) was utilized to purify **1** analogs by column chromatography. Compound **1** was purchased from Aldrich, Inc. (Milwaukee, WI)

General procedures for exhaustive demethylation of 1 analogs

To a solution of **1** analogs in anhydrous CH_2Cl_2 was added dropwise 1M of borontribromide in CH_2Cl_2 under ice cooling. The reaction mixture was maintained at 0 °C for 1 h and then stirred at room temperature overnight. The reaction was cooled using an ice bath and methanol was then added dropwise to work up for 1 h. The solvent was evaporated under reduced pressure, and the residue was purified by Toyopearl HW-40F column chromatography using water and then methanol as eluents.

Tetrademethyl-isocolchicine (4)

Yield 71%; amorphous; $[\alpha]_D^{20}$ 357° (c 0.2, EtOH); IR (KBr) 3340, 3320, and 3070 (br, OH and NH), 2930 and 2850 (aliphatic C–H), 1640 (C=O, amide), and 1620 (C=O, tropolone) cm^{-1}; ^{1}HNMR (Acetone-d_6) δ 2.10–2.45 (m, 4H, H-5,6), 4.61 (m, 1H, H-7), 6.41 (s, 1H, H-4), 7.37 (d, J = 12.0 Hz, 1H, H-11), 7.66 (s, 1H, H-8), 7.75 (d, J = 12.0 Hz, 1H, H-12), and 8.31 (d, J = 7.06 Hz, 1H, N*H*COCH$_3$-7). Anal. ($C_{18}H_{17}O_6N{\bullet}5/4H_2O$) C, H, N.

Tetrademethyl-deacetyl-isocolchicine bromide (5)

Yield 30%; amorphous; $[\alpha]_D^{20}$ -265° (c 0.12, EtOH); IR (KBr) 3350, 3200, and 3050 (br, OH and NH), 2920 and 2860 (aliphatic C–H), and 1600 (C=O, tropolone) cm^{-1}; ^{1}HNMR (Acetone-d_6) δ 2.25–2.70 (m, 4H, H-5,6), 4.29 (m, 1H, H-7), 6.45 (s, 1H, H-4), 7.34 (d, J = 11.8 Hz, 1H, H-11), 7.65 (s, 1H, H-8), and 7.72 (d, J = 11.8 Hz, 1H, H-12). Anal. ($C_{16}H_{15}O_5N{\bullet}HBr{\bullet}H_2O$) C, H, N, Br.

Biology: enzyme and reagents

Topoisomerase II prepared from HeLa cells by a method described previously[16] was used for the unknotting and *in vitro* cleavage assays. Studies with topoisomerase I used the calf thymus enzyme purchased from BRL Inc. (Bethesda, MD). A kit containing T4 DNA polymerase and the restriction endonuclease Hind III both from BRL Inc., were used to prepare plasmid DNA for cleavage assays. Proteinase K was from BMB Corp. (Indianapolis, IN). For biological testing, compounds were prepared in DMSO as 50 mM stocks and diluted in water prior to use. VP-16 and camptothecin were from the Natural Products Laboratory, School of Pharmacy, University of North Carolina at Chapel Hill. DNA substrates for topoisomerase assays were prepared using standard procedures.[17] All other chemicals were reagent grade.

Topoisomerase assays

Inhibition of DNA strand-passing activity was determined using the standard relaxation and unknotting assay procedures developed for DNA topoisomerases.[7,18] Assay conditions for topoisomerase II were described previously.[16] For topoisomerase I, reactions contained 14 μg/ml supercoiled PBR322 DNA substrate and 33 U/ml topoisomerase I with other conditions as specified by the enzyme supplier. After 30 min at 37 °C, reactions were terminated by the addition of SDS–sucrose stop solution and analyzed after horizontal agarose (1% w/v) gel electrophoresis by staining with ethidium bromide and photography (Polaroid Type 667 film).

Cleavage–interference assay method

The method used to assess drug-induced topoisomerase-mediated DNA cleavage and interference with cleavage were based on a published procedure.[19] Hind III-cut PBR322 DNA was end-labeled with [α-^{32}P] dCTP (600 Ci/mMol, ICN) using a commercial T4 polymerase labeling system. Cleavage reactions of 50 μL contained 20 ng/ml labeled DNA, 30 mM Tris-HCL [pH 7.6], 60 mM KCl, 8 mM $MgCl_2$, 3 mM ATP, 15 mM β-mercatoethanol and 30 μg/ml nuclease-free BSA. Topoisomerases I or II (1U and 4U respectively) were added to the enzyme control reaction and drug treatments and after 30 min of incubation at 37 °C, reactions were divided equally for analysis. Half was adjusted to 1% SDS (w/v), then digested with 100 μg/ml proteinase K for 1 h before agarose (1.5% w/v) gel analysis and autoradiography. Protein-linked DNA breaks in the remaining half were determined using potassium/SDS precipitation[20] and scintillation spectrometry.

Ethidium bromide displacement assay

Displacement was measured with a Perkin-Elmer MPF-3 fluorocolorimeter using a method based on published studies.[21] At an excitation wavelength of 546 nm, the emitted fluorescence was adjusted to 100% relative intensity against a buffer containing 5 μM ethidium bromide, 2 mM Hepes [pH 7.0], 9.4 mM NaCl, 20 mM EDTA, and 2 μM calf thymus DNA. Relative intensity readings were taken after additions of compounds in DMSO or DMSO alone. Compounds were also evaluated for interference with fluorescence measurements in buffer without ethidium bromide.

Acknowledgement

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13. This assay which measures competition of added drug with ethidium for DNA intercalation sites, is thoroughly described in Ref. 20. Adriamycin, a known DNA intercalater used as a positive control in the experiment reduced the relative fluorescence by 50% at 0.1 μg/ml. Compounds **2**, **4**, and **5** tested at 50 μM did not themselves interfere with fluorescent measurements and did not alter relative fluorescence in the displacement assay. Compound **3** interfered with fluorescence measurements and consequently could not be successfully evaluated using the method.

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