Calothrixins, a New Class of Human DNA Topoisomerase I Poisons[⊥]

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Calothrixins A (**1**) and B (**2**) were converted to their O- and N-methylated derivatives, respectively. All four compounds were found to act as poisons of DNA topoisomerase I and to do so reversibly. Three of the calothrixins (**1**−**3**) were tested for their cytotoxicity toward cultured (p53 proficient) CEM leukemia cells and found to exhibit IC_{50} values ranging from 0.20 to 5.13 μM. The cell cycle effects of calothrixins **1**−**3** were also studied. Calothrixin B (**2**) produced G_1 arrest at 0.1 μM concentration, while higher concentrations of calothrixins **1** and **3** resulted in cell accumulation in both the S and G_2/M phases of the cell cycle. The cell cycle effects produced by the calothrixins were more readily reversible upon removal of the compounds than those produced by camptothecin.

Human DNA topoisomerase I catalyzes the relaxation of supercoiled DNA by the transient breakage and religation of the DNA phosphodiester backbone.[1] The enzyme participates in DNA replication, transcription, and possibly also recombination.[2] Topoisomerase I-mediated DNA strand scission is accompanied by the formation of a covalent topoisomerase I−DNA binary complex, which is a validated target for antitumor therapy.[3] The camptothecins are the prototype topoisomerase I poisons.[2b,3] Two camptothecins have been approved for use clinically, and a number of other camptothecins are of current interest for (pre)clinical development.[2b,4]

In addition to the camptothecins, several other structural classes of topoisomerase I poisons have been reported, including aporphine alkaloids,[5] indolocarbazoles,[6] indenoisoquinolines,[7] terbenzimidazoles,[8] and topopyrones.[9,10] The calothrixins are cytotoxic agents initially isolated from *Cyanobacter calothrix*.[11] The calothrixins have also been reported to inhibit the growth of a chloroquine-resistant strain of the human malaria parasite *Plasmodium falciparum*.[12] Given the cytotoxicity of the calothrixins and their structural similarity to known topoisomerase I poisons,[3–10] it seemed of interest to determine whether the calothrixins are DNA topoisomerase I poisons, capable of stabilizing the enzyme−DNA covalent binary complex and mediating topoisomerase I-dependent cell death.[13] Presently, we demonstrate that calothrixins A (**1**) and B (**2**), as well as two methylated synthetic analogues (**3** and **4**) (Figure 1), do reversibly stabilize the topoisomerase I−DNA binary complex, leading to cell killing.

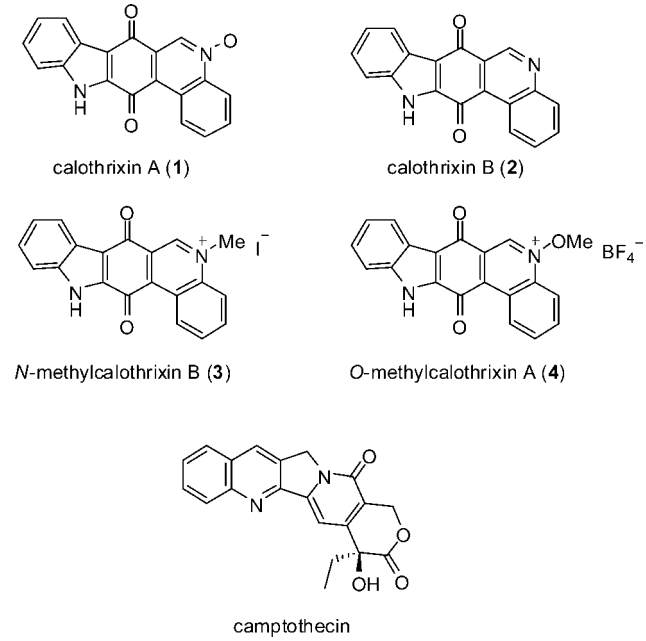
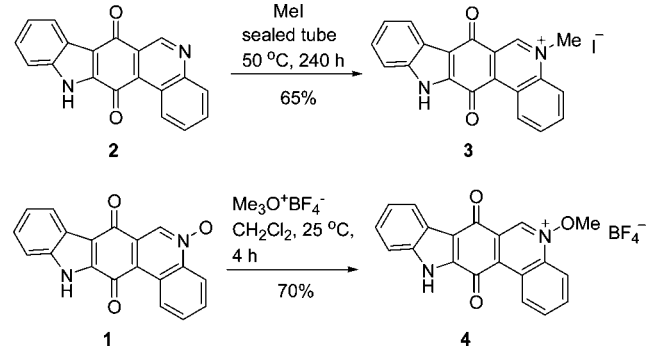


Figure 1. Structures of calothrixins A (**1**) and B (**2**), the two structural analogues *N*-methylcalothrixin B and (**3**) *O*-methylcalothrixin A (**4**), and camptothecin.

Results and Discussion

The calothrixins were synthesized as described previously.[14] Calothrixin B was N-methylated to afford **3** in 65% yield by treatment with methyl iodide (Scheme 1). Calothrixin A was O-methylated in 70% yield by treatment with trimethyloxonium tetrafluoroborate (Scheme 1).

Scheme 1



To study the possible topoisomerase I-mediated DNA cleavage induced by calothrixins and their methylated analogues, we employed a 23 bp double-stranded oligonucleotide substrate derived from the *Tetrahymena thermophilus* rDNA spacer sequence[15] (Figure 2). This sequence contains a strong topoisomerase I cleavage site having G at the +1 position of the 5′-terminus of the break.[16] All of the calothrixins produced cleavage well above that produced by topoisomerase I alone when employed at 1−10 μM concentrations, ranging from a maximum of 11−12% for calothrixin analogues **3** and **4** to 18% for calothrixin A and 24% for calothrixin B, in comparison to an arbitrary 100% value for 1 μM camptothecin. However, the extent of cleavage did not increase steadily with increasing concentration for any of the four calothrixins, likely reflecting direct DNA binding (with inhibition of topoisomerase-mediated cleavage) at the higher concentrations of test compounds.[10,17]

[⊥] Dedicated to Dr. David G. I. Kingston of Virginia Polytechnic Institute and State University for his pioneering work on bioactive natural products.

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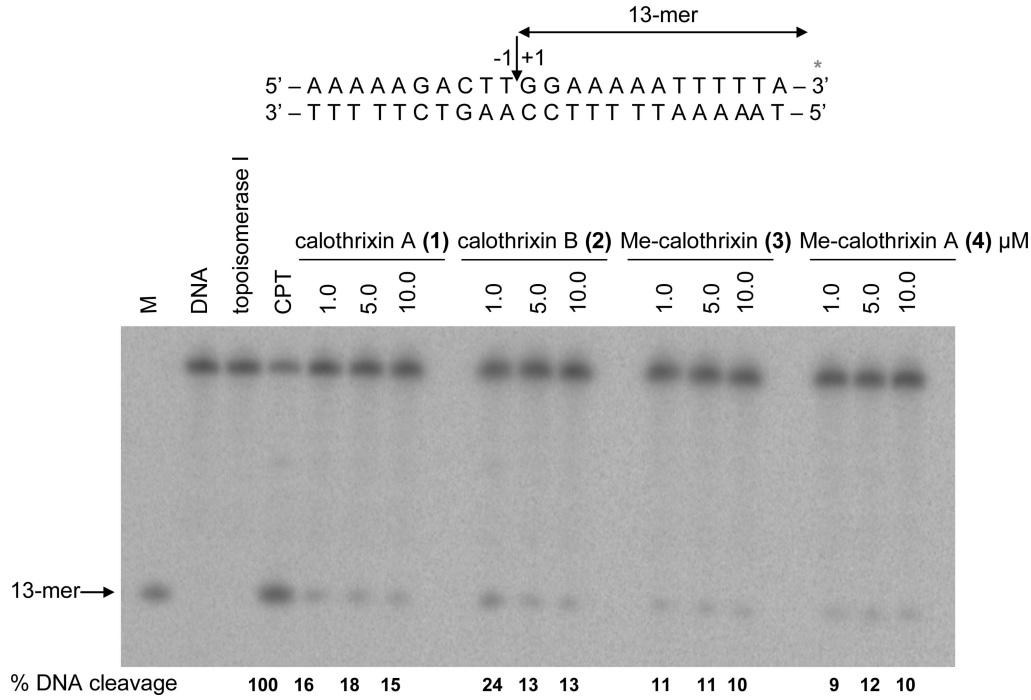

Figure 2. Effect of four calothrixins on human topoisomerase I-mediated DNA cleavage/religation.

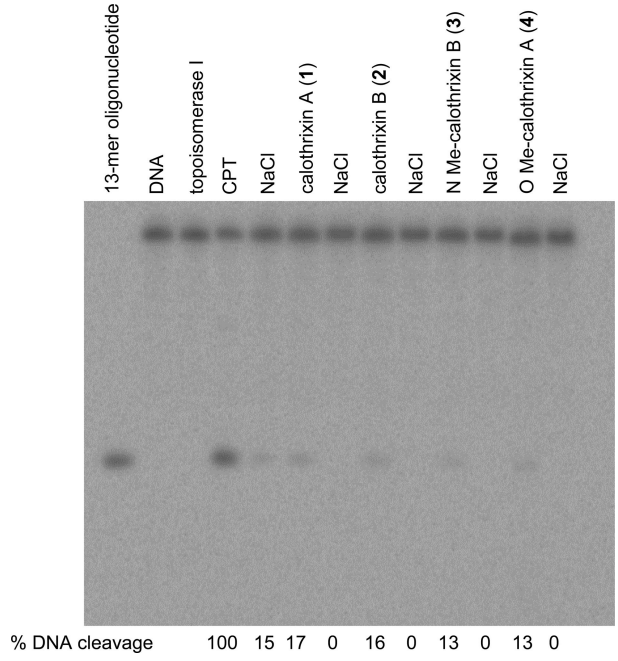

Figure 3. Effect of NaCl on the stability of the topoisomerase I−DNA complexes induced by the calothrixins. The calothrixins were employed at 5 μM concentrations.

Also determined was the stability of the calothrixin-induced topoisomerase I covalent binary complexes; this was done by examining the effect of added salt. NaCl has been shown to initiate the reversal of drug-stabilized topoisomerase I−DNA binding complexes.[18] Figure 3 demonstrates the effect of 0.35 M NaCl on the reversibility of the calothrixin-induced topoisomerase I covalent binary complexes. As shown, the reversal of topoisomerase I DNA cleavage complexes for the calothrixins and their analogues was complete within 5 min. This result indicates that the calothrixins do reversibly stabilize the enzyme−DNA complexes, albeit less efficiently than camptothecin.

p53-Mediated cell cycle arrest is of central importance in understanding the mechanism of action of anticancer agents and in developing new strategies for the treatment of cancer.[19] Delay of cells in the S phase following DNA damage is associated with a block of replication initiation as well as suppression of DNA elongation.[20]

Table 1. Cytotoxicity of the Calothrixins toward Cultured CEM Leukemia Cells

compound	IC_{50} (μM)[a]
CPT	0.04 ± 0.01
calothrixin A (**1**)	0.20 ± 0.02
calothrixin B (**2**)	1.05 ± 0.30
N-methylcalothrixin B (**3**)	5.13 ± 0.72

[a] Determined by MTT assay.

Previous work on the cytotoxicity of camptothecin during the DNA replication phase of the cell cycle has suggested that CPT causes irreversible DNA damage during the S phase.[21] Given that the majority of cancer cells are undergoing DNA synthesis compared to normal cells, tumors can be highly susceptible to the action of such anticancer agents. Calothrixins **1**−**3** were tested for cytotoxicity toward CEM human leukemia cells, which are p53 proficient (Table 1). In comparison with camptothecin, calothrixin A had an IC_{50} value that was 5-fold greater (40 vs 200 nM). Calothrixins **2** and **3** were less potent, still having IC_{50} values of ~1 and 5 μM, respectively (Table 1). Although the calothrixins exhibited significantly greater IC_{50} values compared to camptothecin in p53 proficient CEM human leukemia cells, we were interested in determining how these molecules affect the distribution of cells through the cell cycle.

To understand which phase of the cell cycle was most affected by the action of the calothrixins, nocodazole (a mitotic inhibitor, which blocks re-entry of cells into the G_1 phase) was added following treatment with calothrixins. Figure 4 shows that treatment with low (0.1 μM) calothrixin concentrations produced G_1 arrest in response to calothrixin B (**2**), but no cell cycle perturbations were observed in response to treatment with 0.1 μM calothrixin A (**1**) or *N*-methylcalothrixin B (**3**). However, at higher concentrations, cells were accumulated in both the S and G_2/M phases in response to treatment with calothrixin A (**1**) (Figure 4d,e) and *N*-methylcalothrixin B (**3**) (Figure 4k). No significant changes in the

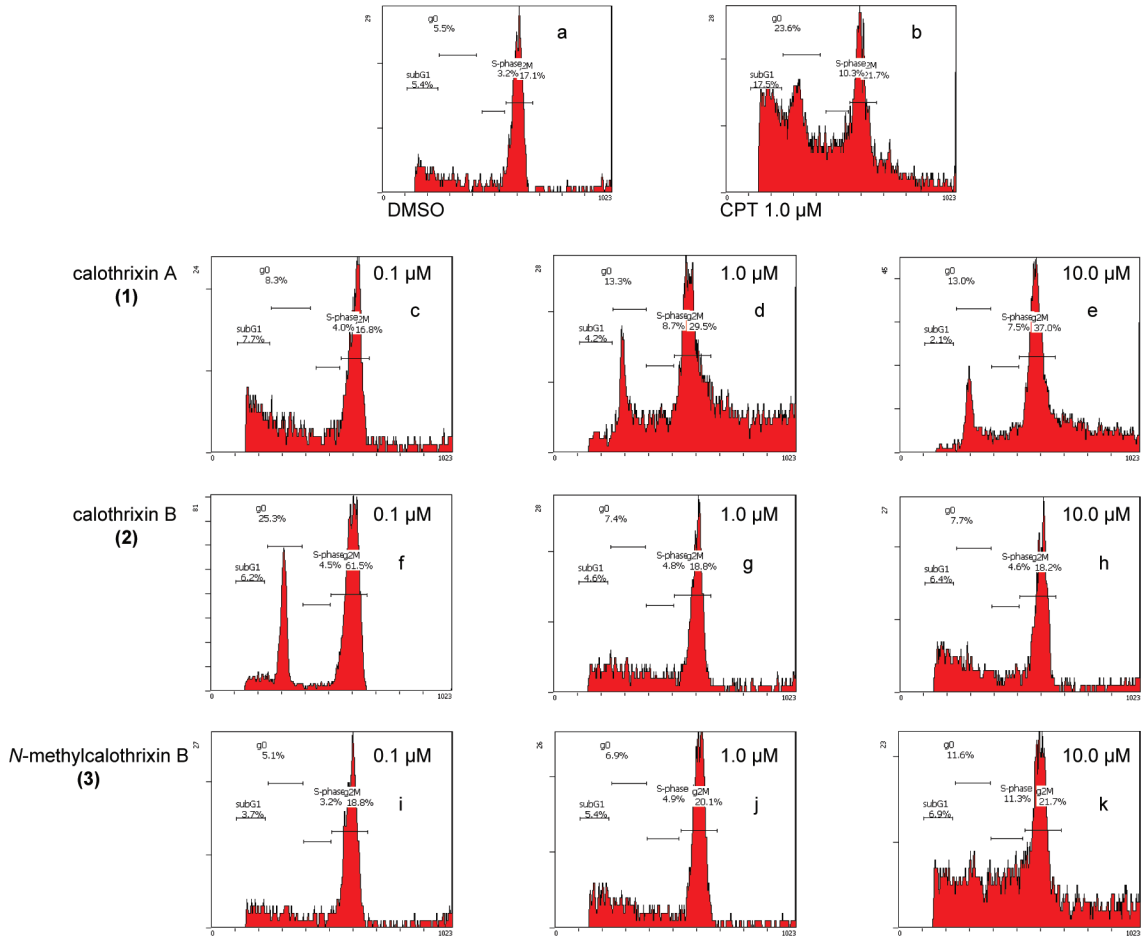

Figure 4. Effect of calothrixins **1**−**3** on progression of cells through the cell cycle in the presence of nocodazole.

progression of the first cell cycle were observed at higher concentrations of calothrixin B (**2**) (Figure 4g,h).

To investigate if the observed calothrixin-induced cell cycle perturbation was reversible, the CEM human leukemia cells were treated with calothrixins for 3 h followed by removal of the drug by washing the cells twice with PBS (pH 7.4). The cells were released in fresh culture medium in the absence of any drug and were allowed to grow for an additional 15 h. Figure 5b shows that the profile of CPT-treated cells was barely changed under these conditions, and the cells remained arrested in the S and G_2/M phases of the cell cycle. However, the effect of calothrixin was reversed upon removal of the drug, as reflected by the cell cycle profiles that were similar to that of the control (Figure 5c,e−k). The exception was for treatment with 1.0 μM calothrixin A (Figure 5d), for which a low degree of dead cells accumulated in the sub-G_1 phase. These observations indicate that the cell cycle perturbations induced by calothrixins were reversed upon removal of the drug. By contrast, the CPT-induced inhibition of DNA synthesis persists for several hours.

In summary, four calothrixins have been shown to act as poisons of DNA topoisomerase I, and calothrixins **1**−**3** exhibited cytotoxicity toward cultured CEM leukemia cells. Calothrixin **2** produced G_1 arrest at 0.1 μM concentration. Higher concentrations of calothrixins **1** and **3** caused cell accumulation in the S and G_2/M cell cycle phases. In comparison with camptothecin, the cell cycle effects of the calothrixins were readily reversed upon removal of the compounds.

Experimental Section

General Experimental Procedures. Chemicals and solvents were of reagent grade and were used without further purification. Anhydrous grade solvents were purchased from VWR. All reactions involving air- or moisture-sensitive reagents or intermediates were performed under an argon atmosphere. Flash chromatography was carried out using Silicycle 200−400 mesh silica gel. Analytical TLC was carried out using 0.25 mm EM silica gel 60 F_{250} plates that were visualized by UV irradiation (254 nm). ^{1}H NMR spectra were obtained using an Inova 500 Varian instrument. Chemical shifts are reported in parts per million (ppm, δ) referenced to the residual ^{1}H resonance of the solvent (DMSO-d_6, 2.50 ppm; CD_3OD, 3.34 ppm). Splitting patterns are designated as follows: s, singlet; br, broad; d, doublet; t, triplet; m, multiplet. High-resolution mass spectra were obtained at the Ohio State University Mass Spectrometry Facility.

Biochemical Reagents. Terminal deoxynucleotidyl transferase (TdTase) was purchased from Roche Applied Science (Indianapolis, IN). Human topoisomerase I was obtained from Topogen, Inc. (Access Alley, FL). Phosphate-buffered saline (PBS) was from Invitrogen (Carlsbad, CA). All synthetic oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Radiolabeled nucleotides were purchased from Perkin-Elmer Life Sciences (Waltham, MA).

Cell Lines. The CEM cell line was obtained from Dr. Yves Pommier (Laboratory of Molecular Pharmacology, NCI, Bethesda, MD). The human cell line was cultured in RPMI 1640 (Life Technologies, Inc.) containing 10% heat-inactivated FBS and 2 mM glutamine in a humidified incubator maintained at a temperature of 37 °C and containing 5% CO_2.

Calothrixin A (1) and Calothrixin B (2). These two compounds were synthesized as described previously.[14]

5-Methyl-7,13-dioxo-12,13-dihydro-7*H*-12-aza-5-azonia-indeno[1,2-*b*]phenanthrene Iodide (*N*-methylcalothrixin B, 3). A solution of 6.0 mg (20 μmol) of calothrixin B (**2**) in 3.0 mL of iodomethane was stirred at 50 °C in a sealed tube for 10 days, then allowed to cool to room temperature. The solvent was removed under diminished pressure, and the residue was transferred to a test tube with a small amount of acetone. After centrifugation, the product (**3**) was collected as a dark colored solid: yield 5.8 mg (65%); ^{1}H NMR (DMSO-d_6) δ 4.85 (3H, s), 7.55 (1H, t, J = 7.5 Hz), 7.65−7.72 (2H, m), 8.22 (1H, d, J = 7.5 Hz),

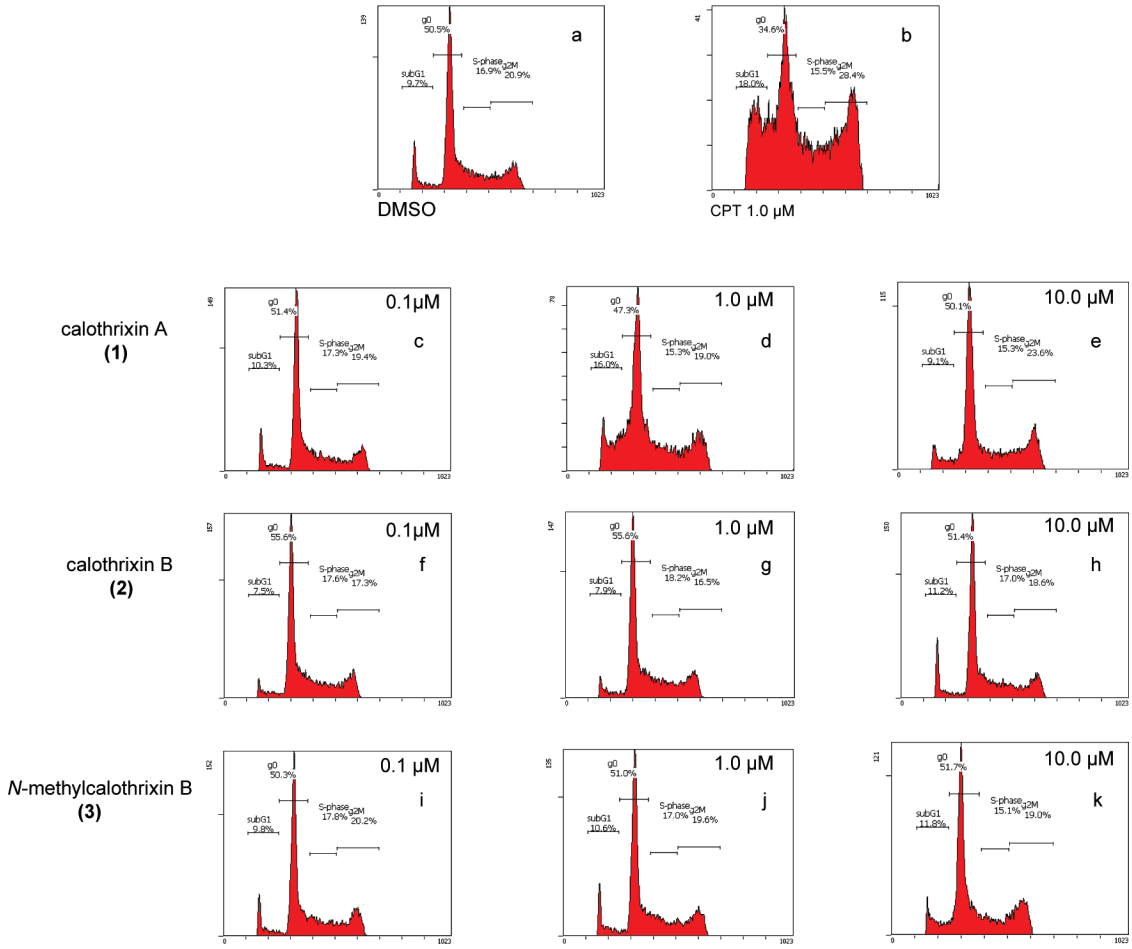

Figure 5. Reversibility of the cell cycle effects of calothrixins **1**−**3**.

8.27 (1H, t, J = 8.0 Hz), 8.39−8.44 (1H, m), 8.64 (1H, d, J = 8.5 Hz), 9.85 (1H, d, J = 7.0 Hz), 10.19 (1H, s), and 13.54 (1H, br s); ESIMS m/z 313.0970 (M^+) ($C_{20}H_{13}N_2O_2$ requires m/z 313.0977).

5-Methoxy-7,13-dioxo-12,13-dihydro-7*H*-12-aza-5-azonia-indeno[1,2-*b*]phenanthrene tetrafloroborate (*O*-methylcalothrixin A, 4). To a solution of 5.5 mg (17.0 μmol) of calothrixin A (**1**) in 2.0 mL of anhydrous dichloromethane was added 2.6 mg (17.5 μmol) of trimethyloxonium tetrafluoroborate.[22] The reaction mixture was stirred at room temperature for 4 h. An orange-red solid precipitated from the reaction mixture. The reaction mixture was transferred to a test tube and centrifuged to afford the product (**4**): yield 5.8 mg (70%); ^{1}H NMR (CD_3OD) δ 5.50 (3H, s), 7.46 (1H, t, J = 7.5 Hz), 7.55 (1H, t, J = 8.0 Hz), 7.68 (1H, d, J = 8.5 Hz), 8.09 (1H, s), 8.26 (1H, t, J = 8.0 Hz), 8.31 (1H, d, J = 8.0 Hz), 8.41 (1H, t, J = 8.5 Hz), 8.65 (1H, d, J = 9.0 Hz), 10.10 (1H, d, J = 8.0 Hz), and 10.14 (1H, s); ESIMS m/z 329.0919 (M^+) ($C_{20}H_{13}N_2O_3$ requires m/z 329.0926).

Cytotoxicity Assay. Cytotoxicity was measured by using a MTT assay[23] after continuous treatment with the drug for 3 days. Three thousand cells/100 μL culture medium/well were seeded in a 96-well microtiter plate. A 100 μL amount of culture medium containing varying drug concentrations was added to the wells. Controls were added containing cells only and medium only. The cells were incubated for 72 h in a humidified atmosphere in an incubator maintained at 37 °C and supplemented with 5% CO_2. At the end of the drug treatment period, 20 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (5 mg/mL) was added to each well including the blank. Treated microtiter plates were incubated at 37 °C for an additional 4 h. MTT was removed using a vacuum device, and 200 μL of DMSO was added to each well to achieve cell lysis. Microtiter plates were kept on a shaker for 30 min. Absorbance was recorded at 595 nm on a spectrophotometer (Spectramax 190, Molecular Devices).

Flow Cytometry Analysis. CEM cells (10^6/sample) were treated with increasing concentrations of each compound for 3 h, and then cells were maintained at 37 °C for an additional 15 h in the presence or absence of 0.4 μg/mL of nocodazole. Cells were harvested, and the cell pellets were washed twice with PBS (1.06 M KH_2PO_4, 0.155 M NaCl, 2.96 M $Na_2HPO_4 \cdot 7H_2O$), pH 7.4, and fixed with 70% ethanol. Fixed cells were washed with PBS and treated with RNase A solution (3 U/mL) at 37 °C for 15 min. Cells were stained with 50 μg/mL propidium iodide for 20 min.[24] The DNA content of 10 000 cells/analysis was monitored on a Beckman-Coulter flow cytometer.

3′-End Labeling and Purification of Oligonucleotides. 3′-End labeling of oligonucleotides was performed according to the protocols provided by the manufacturer (Roche Applied Science). The labeled oligonucleotides were purified using Sephadex G-25 mini quick Spin Oligo columns (Roche Applied Science). Briefly, 3′-^{32}P end labeling was done by adding 10 pmol of template DNA, 50 μCi [α-^{32}P] cordycepin 5′-triphosphate, 400 units of recombinant terminal deoxytransferase in the reaction buffer, and distilled H_2O to a final volume of 40 μL. The reaction mixture was incubated at 37 °C for 1 h. Reactions were terminated by addition of sodium dodecyl sulfate (SDS) to a final concentration of 0.5%.

Topoisomerase I-Mediated DNA Cleavage Assay. Single-stranded DNA oligonucleotides were labeled at their 3′ ends with [α-^{32}P] cordycepin as described above. The labeled DNA strands were annealed with their complementary strands in 10 μL (total volume) of 10 mM Tris-HCl, pH 7.8, containing 100 mM NaCl and 1 mM EDTA. The annealing process involved heating the reaction mixture at 95 °C for 5 min, followed by slow cooling to room temperature. Duplex DNA substrates (approximately 100 fmol per reaction), 5 units of topoisomerase I, and either CPT or a calothrixin at the indicated concentrations were incubated at room temperature for 30 min. The reaction was carried out in 10 μL (total volume) of 10 mM Tris-HCl, pH 7.9, containing 50 mM KCl, 1.5 M NaCl, 0.1 mM EDTA, 15 μg/mL bovine serum albumin (BSA), and 1 mM spermidine. The reactions were stopped by the addition of SDS to a final concentration of 0.5%. For the NaCl-induced religation reactions, the SDS addition was preceded by addition of NaCl for the indicated times. The reaction mixtures were then diluted in 3.3 vol of buffer containing 98% (v/v) formamide, 10 mM EDTA, 10 mM NaOH, 1 mg/mL xylene cyanol, and 1 mg/mL

bromophenol blue.[8] Then 5 μL of each sample were loaded onto a denaturing (7 M urea) 16% (w/v) polyacrylamide gel and electrophoresed at 40 V/cm and 55 °C for 3 h. Imaging was performed with a phosphorimager (Molecular Dynamics).

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