Thiocamptothecin†

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The synthesis and the X-ray structure of 16a-thiocamptothecin (TCPT), the thiopyridone analog of camptothecin (CPT), are accomplished. The crystal contains two structurally identical, yet independent molecules. Both of them are connected to other molecules via two intermolecular hydrogen bonds. S-Methylation of TCPT leads to the cleavage of the C-ring. The cytotoxic activity of TCPT was evaluated against different human tumor cell lines using CPT as reference compound.

Introduction

Camptothecin (CPT, **1** of Figure 1) exhibits an excellent antineoplastic activity against a broad spectrum of tumors, by inhibition of the human DNA topoisomerase. Such inhibition prevents the religation step of the enzyme action, which results in the accumulation of a covalent ternary Topo I−CPT−DNA complex.[1] Enormous progress has been made concerning the elucidation of the structural elements required for the camptothecin activity expression. These efforts have led to the approval of topotecan (Hycamtin; **2**) and irinotecan (Camptosar; **3**) for colon and ovarian cancers treatment, respectively,[2] and to the synthesis of several novel CPT derivatives that are currently in various stages of clinical trials. High expectations meet the next generation of CPTs, and research continues on several important fields. Remarkably, only few studies have been focused on the D ring modification, which involves the 14 and 4 positions. These studies led to the discovery of the 14-chloro (**4**),[3] the 14-aza (**5**),[4] and the 4-deaza (**6**) analogues.[5] However, their poor antitumor activity, strengthened by molecular modeling results,[5,6] led researchers to the conclusion that the unsubstituted pyridone D ring is a key structural feature for optimal drug binding to the cleavable enzyme−DNA complex. The introduction of substituents in the D ring may produce steric hindrance, or may affect the possible involvement of the pyridone carbonyl in stabilizing the drug−enzyme−DNA ternary complex.[5a,7]

Despite these findings, we wondered that CPT's isosteres of the pyridone pharmacophore were never explored, although its replacement, for example with a thiopyridone moiety, could have led advantages in terms of pharmacological or pharmacokinetic drug profile. In fact, an effect on the biological activity deriving from the isosteric replacement of an oxygen atom with sulfur is exhaustively documented for several compounds.[8]

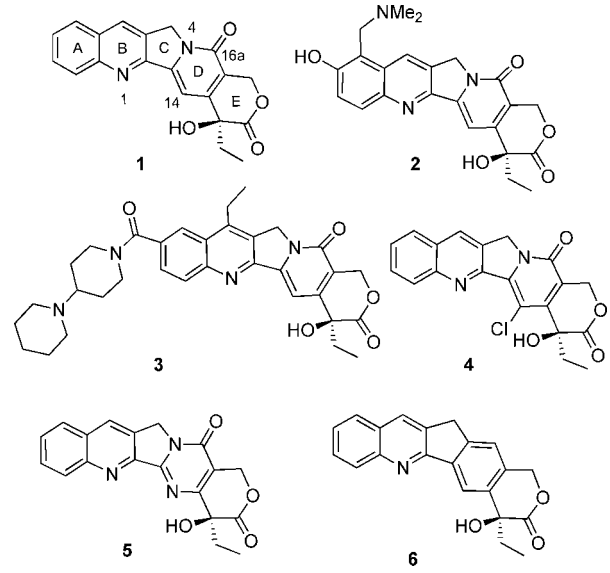

Figure 1. Camptothecin (**1**), topotecan (**2**), irinotecan (**3**), and D-ring modified analogues (compounds **4**−**6**).

Results and Discussion

Concerning the synthesis of 16a-thio-CPT, we considered that Lawesson's reagent (LR[a])[9] could have in principle afforded amide thionation. However, it has been reported that treatment of CPT with LR only yields small amounts of 20-mercapto camptothecin, along with 20,21-bis-mercaptocamptothecin.[10] Nevertheless, we resolved to repeat the thionation reaction under milder conditions and quite surprisingly, we exclusively obtained the 20-deoxy-16a-tiocamptothecin (**7**) in 68% yields (Scheme 1).

The isolation of **7** suggested that the C=S formation on the pyridone ring was feasible, once the 20-OH group of **1** was suitably protected. For this aim, CPT was treated with triethylsilyl chloride (TESCl) in DMF to afford the 20(*S*)-*O*-triethylsilyl-camptothecin **8** in 98% yield (Scheme 2). Thionation of **8** with LR in xylene provided the 20(*S*)-OTES-16a-thiocamptothecin **9** in 84% yield. Desilylation of **9** with $Et_3N \cdot 3HF$ yielded the expected 20(*S*)-thiocamptothecin **10** in 98%.

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† Dedicated to professor Angelo Mangini: A pioneer of the Italian sulfur chemistry.

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[a] Abbreviations: TCTP, thiocamptothecin; LR, Lawesson's reagent; TESCl, triethyl silyl chloride; IGROV-1, human ovary carcinoma; H460, non small lung cancer cell line.

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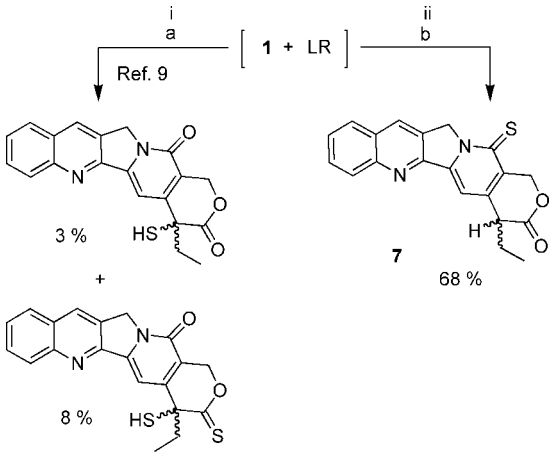
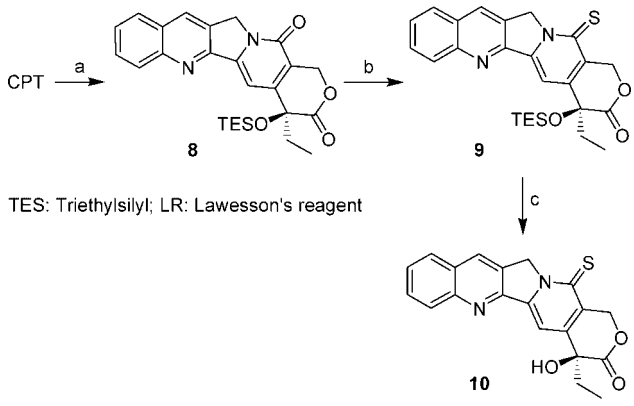
Scheme 1. Reactions of Camptothecin with Lawesson's Reagent[a]



[a] Reagents and conditions: (a) pyridine, reflux, LR (5.0 equiv), 30 h [ref 10]; (b) xylene, reflux, LR (1.4 equiv), 3 h.

Scheme 2. Synthesis of 16a-Thiocamptothecin **10**[a]



[a] Reagents and conditions: (a) dimethylformamide, TESCl, imidazole/DMAP; (b) LR (0.9 equiv), xylene; (c) $Et_3N \cdot 3HF$ (2.6 equiv), THF.

The structure of thiocamptothecin, determined by single crystal X-ray analysis,[11] is similar to that of camptothecin, the only difference being the sulfur substituting the oxygen atom ring. The crystal packing is rather peculiar and deserves a further comment. The asymmetric unit contains two structurally identical, yet independent molecules, rotated about 180 degrees one with respect to the other along a C2 axis perpendicular to the planes of the condensed rings. Both of them are connected to other molecules via two intermolecular hydrogen bonds. The difference lies in the atoms involved in such bonds and, as a consequence, in their chemical neighborhood. These interactions are very important in the solid state and significantly drive the whole crystal packing.

One set of molecules is connected to one another via both their 17-OH and 21-carbonyl groups, giving O···O intermolecular distances of 2.908 Å (**10a**, Figure 2). The second set is made of molecules that, instead, involve their hydroxyl oxygen and sulfur atoms in the hydrogen bonds, entailing longer S···O distances of 3.241 Å (**10b**, Figure 3). The value fits perfectly with the lower electronegativity of the sulfur with respect to the oxygen atom, which results in weaker hydrogen bonds. The fact that the two thio-camptothecins in the asymmetric unit exploit different atoms for the hydrogen interactions is also reflected in some of the intramolecular bond lengths. More specifically, in **10a**, where only oxygen atoms are involved, the S−C bond is 1.669 (4) Å long, while in **10b**, where the sulfur is involved, the bond length goes up to 1.682 Å. This prefigures that the thiocarbonyl π-bonding electrons of **10b** are transferred to the sulfur atom, so that the C(16a-S) bond adopts a single bond character due to an increase of the negative charge at the heteroatom, which favors the 20-OH−sulfur interaction.

Significant differences were observed in the chemical behavior of camptothecin and its 16a-thio analogue **10** in the presence of bases due to the sulfur polarizability. This probably affects the stereoelectronic proprieties of thiocamptothecin even in solution since the contribution of the tautomeric enol form is increased (Scheme 3).[12] In fact, thiocamptothecin incorporated deuterium at the C-5 position with a 85% uptake after 15 min at 20 °C when treated with 2.0 equiv of the moderate organic base imidazole in a 12:1 mixed solvent DMSO/D_2O, while only a 70% uptake was observed for camptothecin after 3 days treatment with 5.0 equiv of imidazole at 65 °C. Moreover, the treatment of camptothecin with NaOH yielded the corresponding E-ring opened sodium salt,[13] while thiocamptothecin rapidly decomposed.

The enol form was further evidenced by ^{1}H NMR spectroscopy monitoring the *t*-BuOK induced deprotonation of the C5-H of **9** in the inert solvent tetrahydrofuran-d8.

The corresponding thiolate was stable in solution for several hours. Next, the base-induced *S*-alkylation of the thiolate was performed in the presence of MeI, which afforded the methyl sulfide **11** (Scheme 4). This compound decomposed in a few days only by standing in solution. Moreover, treatment of **11** with saturated aqueous NH_4Cl yielded the opened compound **12** (Scheme 4).

Cytotoxic Activity. Cytotoxic activity of thiocamptothecin **10** was evaluated against different human tumor cell lines using CPT as reference compound (Table 1).

After a 1 h exposure, thiocamptothecin was more cytotoxic than CPT in all the tumor cell models. Following a prolonged exposure (72 h), the cytotoxic effect of both camptothecins was substantially increased. This effect was more evident for **10** in IGROV-1 cells. The increase of cytotoxic potency of **10** versus CPT was even more evident in HT29 colon carcinoma cells and in the subline HT29/mit, selected for resistance to mitoxantrone and characterized by overexpression of the membrane efflux pump BCRP.[14] The ability of **10** to overcome the BCRP-mediated resistance supports that, in contrast to CPT and other conventional analogue, thiocamptothecin was not a substrate for BCRP.

Topoisomerase I-mediated DNA cleavage assays with purified human topoisomerase I[15] were performed to investigate the capability of thiocamptothecin to stimulate the DNA damage. Compound **10** revealed an intensity of DNA damage slightly superior to that observed for CPT (Figure 4). Cleavage pattern of **10** was found identical to that produced by **1**.

Conclusions

Several reasons could account for the higher cytotoxic activity of **10** with respect to camptothecin, such as an enhanced hydrogen bond interaction of the sulfur atom with the DNA favored by the increased negative charge at the heteroatom, or a greater lipophilicity of **10** which could favor a better absorption into the tumor cell nucleus, or to a greater lactone stabilization. In conclusion, this study provides evidence for the key role of the pyridone pharmacophore on the antitumor activity of CPT

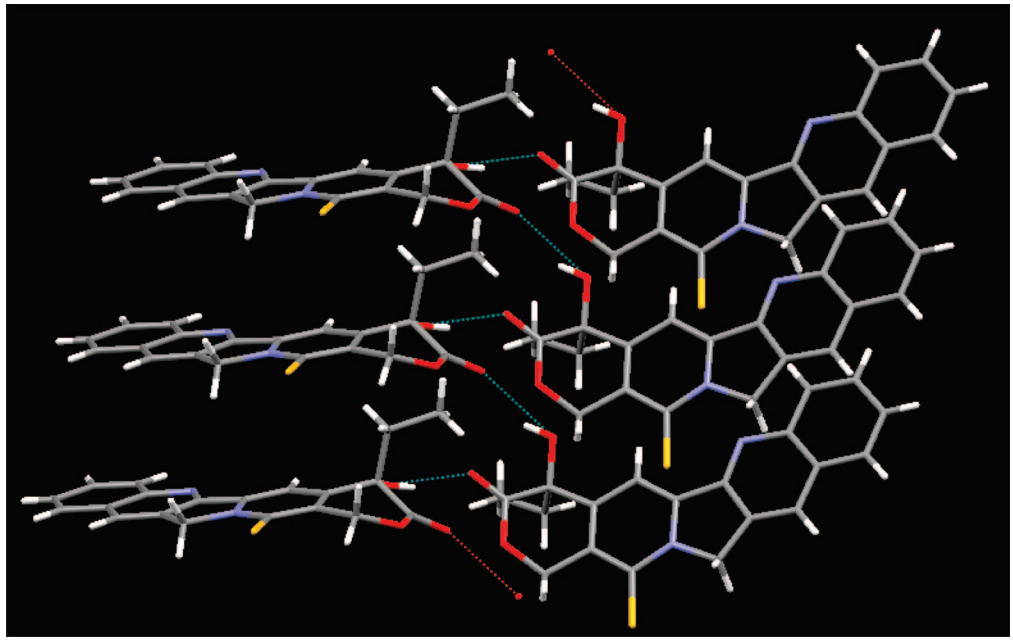


Figure 2. Stereoview of **10a**. Dotted lines represent intramolecular hydrogen bonds.

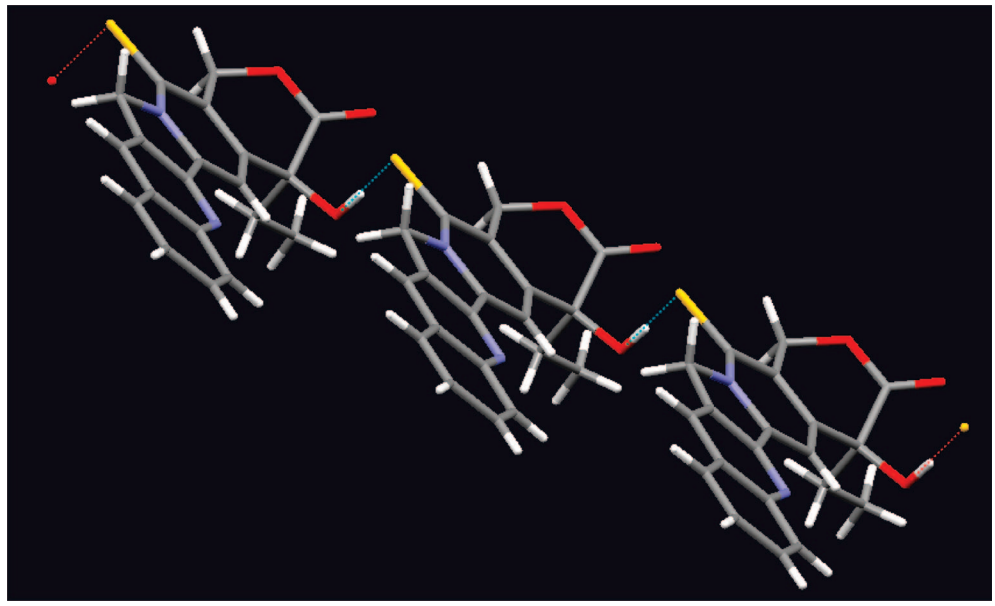
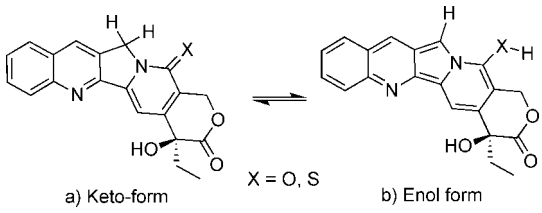


Figure 3. Stereoview of **10b**. Dotted lines represent intramolecular hydrogen bonds.

Scheme 3. Molecular Structures of Camptothecin (X═O) and Thiocamptothecin (X═S): (a) Keto Form; (b) Enol Form

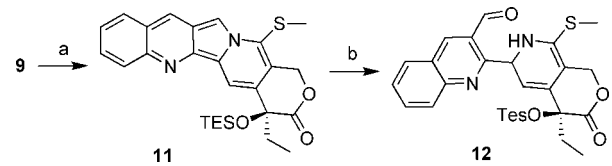


since the isosteric replacement of the oxygen atom with sulfur could provide potential useful camptothecin analogues.

Experimental Section

Chemistry. Synthesis of 20-OTES-Camptothecin (8). To a suspension of camptothecin (**1**) (100 mg, 0.29 mmol) in dry DMF (3 mL), imidazole (98 mg, 1.44 mmol) was added. After 10 min, triethylsilyl chloride (0.19 mL, 1.15 mmol) was introduced dropwise, followed by DMAP (40 mg, 0.29 mmol). The reaction was stirred at RT until TLC analysis CH_2Cl_2−MeOH (30:1) showed the complete conversion (60 h). Excess of solvents was removed under diminished pressure and water was added to the residue. The

Scheme 4. *S*-Alkylation of Thiocamptothecin with MeI and its Hydrolysis[a]



[a] Reagents and conditions: (a) MeI, *t*-BuOK, THF-d_8; (b) H_2O/H^+.

Table 1. Cytotoxic Activity (IC_{50}, μM) of CPT **1** and Thiocamptothecin **10** against Human Tumor Cell Lines[a]

		IC_{50} (μM)	
		compound	
cell line	exposure time (h)	**1**	**10**
H460	1	0.33 ± 0.05	0.14 ± 0.058
	72	0.0062 ± 0.000204	0.0074 ± 0.00038
IGROV-1	1	1.65 ± 0.10	0.22 ± 0.041
	72	0.012 ± 0.0004	0.00467 ± 0.00077
HT29	1	3.16 ± 1.2	0.17 ± 0.091
HT29/mit	1	8.81 ± 2.35	0.11 ± 0.03

[a] The activity was determined by the antiproliferative assay (cell count) at 72 h following 1 and 72 h exposure. IC_{50}, drug concentration required for 50% reduction of cell growth as compared with untreated controls. Means ± SD are reported from at least three experiments.

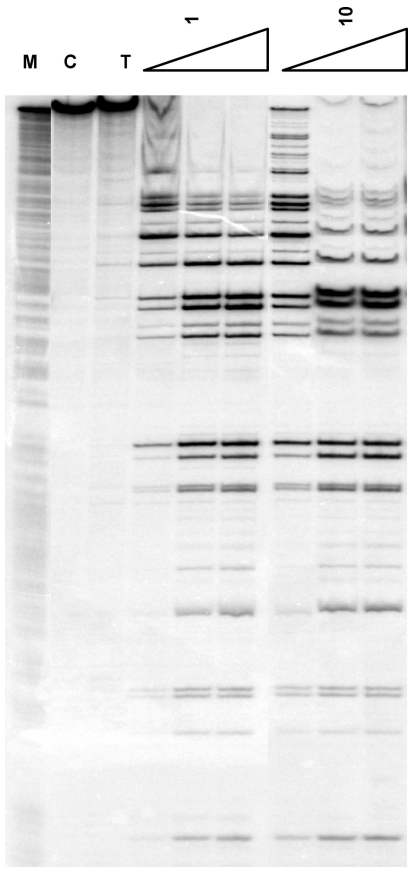


Figure 4. Topoisomerase I-mediated DNA cleavage assay of compounds **1** and **10**. Samples were reacted with 1, 10, and 50 μM drug at 37 °C for 30 min. Reaction was than stopped by adding 0.5% SDS, 0.3 mg/mL of proteinase K, and incubating for 45 min at 42 °C before loading on a denaturing 8% polyacrylamide gel: C, control DNA; T, reaction without drug; M, purine markers. The experiment was repeated three times and the results of a representative value are reported.

aqueous layer was extracted with CH_2Cl_2 (20 mL × 3). The organic layer was dried over Na_2SO_4, filtered, and concentrated in vacuo. The resulting solid residue was purified by silica gel flash chromatography CH_2Cl_2−MeOH (30:1) to afford (**8**; 130 mg, 98%) as a pale yellow solid: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.37 (s, 1 H, Ar, H-7), 8.25 (d, 1 H, J = 8.4 Hz, Ar), 7.92 (d, 1 H, J = 8.0 Hz, Ar), 7.82 (t, 1 H, J = 8.0 Hz, Ar), 7.65 (t, 1 H, J = 8.4 Hz, Ar), 7.57 (s, 1 H, H-14), 5.67 (d, 1 H, J = 16.4 Hz, H-17), 5.29 (s, 2 H, H-5), 5.25 (d, 1 H, J = 16.4 Hz, H-17), 2.00–1.84 (m, 2 H, H-19), 1.03–0.93 (m, 12 H), 0.80–0.71 (m, 6 H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.7, 157.6, 152.5, 151.5, 149.0, 145.9, 130.9, 130.4, 130.0, 128.4, 128.1, 128.0, 127.9, 118.9, 94.4, 75.3, 66.0, 50.0, 33.2, 7.9, 7.2, 6.4. MS (70 eV): m/z (rel intensity), 433 [$M^+ - 29$] (89), 390 [$M^+ - 72$] (100) HR-MS: calcd mass, 462.197; mass found, 462.193.

Synthesis of 20-OTES-Thiocamptothecin (9). To a solution of 20-OTES-camptothecin (**8**; 664 mg, 1.44 mmol) in dry xylene (20 mL), Lawesson's reagent (LR) (523 mg, 1.29 mmol) was added. The reaction was heated at 90–100 °C and TLC analysis (*n*-hexane−AcOEt, 1:1) showed that after 18 h conversion was not proceeding. The solvent was removed in vacuo and the residue was purified by silica gel flash chromatography, *n*-hexane−AcOEt (step gradient elution 4:1, 7:2), to give (**9**; 578 mg, 84%) as a yellow solid: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.46 (s, 1 H, Ar, H-7) 8.29 (d, 1 H, J = 8.4 Hz, Ar), 8.03 (s, 1 H, H-14), 7.97 (d, 1 H, J = 8.4 Hz, Ar), 7.86 (t, 1 H, J = 8.4 Hz, Ar), 7.69 (t, 1 H, J = 8.4 Hz, Ar), 6.15 (d, 1 H, J = 16.9 Hz, H-17), 5.62 (d, 1 H, J = 21.0 Hz, H-5), 5.57 (d, 1 H, J = 21.0 Hz, H-5), 5.34 (d, 1 H, J = 16.9 Hz, H-17), 1.92 (q, 2 H, H-19), 1.05–0.91 (m, 12 H), 0.82–0.71 (m, 6 H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.3, 171.5, 151.9, 149.1, 148.3, 147.2, 130.8, 130.6, 130.6, 130.1, 128.3, 128.2, 128.2, 128.0, 104.5, 75.0, 68.8, 56.3, 33.5, 7.7, 7.2, 6.4. MS (70 eV): m/z (rel. intensity), 479 [$M^+ + 1$] (10), 450 [$M^+ - 28$] (100). HR-MS: calcd mass, 478.175; mass found, 478.169.

Synthesis of Thiocamptothecin (10). To a solution of 20-OTES-thiocamptothecin (**9**; 150 mg, 0.31 mmol) in dry THF (10 mL), $Et_3N \cdot 3HF$ (0.14 mL, 0.82 mmol) was added dropwise. The mixture was reacted for 48 h at RT and TLC analysis (*n*-hexane−AcOEt, 1:1) showed complete conversion. The solvent was removed under diminished pressure and the residue was purified by flash chromatography CH_2Cl_2−MeOH (30:1) to afford (**10**; 112 mg, 98%) as yellow solid: $[\alpha]^{20}_D$ = 137.7 (c = 0.47 g cm^{-3} in THF); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.46 (s, 1 H, Ar, H-7), 8.27 (d, 1 H, J = 8.4 Hz, Ar), 8.13 (s, 1 H, H-14), 7.97 (d, 1 H, J = 8.4 Hz, Ar), 7.86 (t, 1 H, J = 8.4 Hz, Ar), 7.70 (t, 1 H, J = 8.4 Hz, Ar), 6.25 (d, 1 H, J = 16.9 Hz, H-17), 5.62 (d, 1 H, J = 21.0 Hz, H-5), 5.58 (d, 1 H, J = 21.0 Hz, H-5), 5.37 (d, 1 H, J = 16.9 Hz, H-17), 3.80 (s, 1 H, OH), 1.90 (q, 2 H, H-19), 1.03 (t, 3 H, J = 7.2 Hz, Me). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.5, 172.6, 151.8, 149.1, 148.7, 145.5, 130.9, 130.8, 130.5, 129.9, 128.3 (2 C), 128.2, 128.0, 104.3, 72.3, 69.2, 56.3, 32.0, 7.8. MS (70 eV): m/z (rel. intensity), 364 [M^+] (100). IR (NaCl): 1752, 1631, 1537 cm^{-1}. HR-MS: calcd mass, 364.088; mass found, 364.081. Elem. Anal. Calcd for $C_{20}H_{16}N_2O_3S$: C, 65.92; H, 4.43; N, 7.69. Found: C, 65.96; H, 4.38, N, 7.72.

Growth Inhibition Assay. The human cell lines used were the nonsmall cell lung cancer cell line NCI-H460 (ATCC, HTB-177), the ovarian carcinoma cell line IGROV-1, the colorectal adenocarcinoma cell line HT29 (ATCC, HTB-38), and the corresponding mitoxantrone resistant variant HT29/mit (Perego et al., 2001). Cells were cultured in RPMI-1640 containing 10% fetal calf serum. Cytotoxicity was assessed by growth inhibition assay after 1 and 72 h drug exposure. Cells in the logarithmic phase of growth were harvested and seeded in duplicates into six-well plates. A total of 24 hours after seeding, cells were exposed to the drug for 1 or 72 h. Cells were counted 72 h after drug exposure for 1 h of treatment and at the end of drug exposure in the case of 72 h of treatment. IC_{50} is defined as the inhibitory drug concentration causing a 50% decrease of cell growth over that of untreated control.

Topoisomerase I-Dependent DNA Cleavage Assay. A 3′-end labeled gel purified 751-bp *Bam*HI-*Eco*RI fragment of SV40 DNA was used for the cleavage assay. SV40 plasmid was first linearized with *Bam*HI enzyme and then 3′-labeled by using DNA polymerase I large (klenow) fragment (Invitrogen, Paisley, U.K.) in presence of dGTP and α^{32}P ddATP. The labeled DNA was then restricted with *Eco*RI enzyme and the corresponding 751-bp was purified on agarose gel. Topoisomerase I DNA cleavage reactions (20000 cpm/sample) were performed in 20 μL of 10 mM Tris-HCl (pH 7.6), 150 mM KCl, 5 mM $MgCl_2$, 15 μg/mL BSA, 0.1 mM dithiotritol, and 640 ng of human recombinant enzyme (full length topoisomerase I; Beretta et al. 1999) for 30 min at 37 °C. Reactions were stopped by 0.5% SDS and 0.3 mg/mL of proteinase K for 45 min at 42 °C. After precipitation DNA was resuspended in denaturing buffer (80% formamide, 10 mM NaOH, 0.01 M EDTA

and 1 mg/mL dyes) before loading on a denaturing 8% polyacrylamide gel in TBE buffer.

Supporting Information Available: Synthetic procedures for compounds **7** and **12** and all NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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