$CONH_2$ + H). Anal. ($C_7H_7ClN_6O_2 \cdot 0.5H_2O$) C, H, N.

A silica TLC plate (EtOAc) examination of the product revealed the presence of two minor spots in addition to the main spot (R_f 0.60). One of the minor spots did not move from the original place of application. It was found to be the residual pyrazolotriazine **9**. The other faint spot had an R_f value of 0.85 (compound 8, formed by the condensation of **9** and $ClCH_2CH_2N{=}C{=}O$). Compound 8 has the same molecular weight and molecular formula as that of pyraoncozine, **6a**.

A slurry of 200 g of silica gel (60–200 mesh, J. T. Baker 2405-5) in EtOAc was packed into a 4.5 × 60 cm^2 column. To the wet-packed column was carefully added a well-mixed and finely ground solid powder coctaining 1.6 g of the crude pyraoncozine, **6a**, and 4 g of silica gel in EtOAc. The column was eluted with EtOAc. No TLC spots were detected in the initial 500 mL of EtOAc eluted. The TLC spot of the isomeric compound 8 was observed in the subsequent eluants; the fading of the undesired spot and the appearance of the desired one were monitored by continuous TLC examination from each of the eluted fractions. The fractions containing only the desired compound **6a** were collected (ca. 1500 mL) and evaporated to dryness. The resulting white fluffy crystals, mp 198–198.5 °C (eff), were recrystallized from 120 mL of EtOAc to give white fluffy needles, mp 200 °C (eff). The column purification was repeated and, from a total of 3.2 g of crude product, 1.6 g (13% yield) of chromatographically pure **6a** was obtained: UV λ_{max} ($CHCl_3$) 314 nm (ϵ 9000). Anal. ($C_7H_7ClN_6O_2$) C, H, N.

The entire operation was conducted under ordinary laboratory illumination. The product is stable under light even after 6 months. Pyraoncozine was found to be stable in $CHCl_3$ but unstable in MeOH. The ultraviolet absorption maximum of pyraoncozine in MeOH, on standing, gradually shifted from 314 to 265 nm.

Attempted use of less quantity of the isocyanate or less volume of EtOAc in subsequent trials resulted in less pure product.

3-Methyl-3,4-dihydro-4-oxopyrazolo[5,1-*d*]-1,2,3,5-tetrazine-8-carboxamide (6b). To a stirred suspension of 10 g (0.073 mol) of finely powdered **7** in 950 mL of EtOAc was added, at room temperature, 25 g (0.44 mol) of $CH_3N{=}C{=}O$. The suspension was stirred at room temperature for 4 days. To the resulting mixture (still a suspension) was added another 25 g (0.44 mol) of $CH_3N{=}C{=}O$, and the mixture was heated at 35–40 °C for an additional 4 days. The reaction mixture was filtered while hot, and the solid was washed repeatedly with EtOAc and dried. The light-brown solid product **6b** weighed 12 g (85% yield): mp 265–267 °C (dec). This compound was found to be much less soluble in EtOAc or $CHCl_3$ than the corresponding chloroethyl analogue: UV λ_{max} ($CHCl_3$) 310 nm (ϵ = 7200); mass spectrum, 194 (M^+), 137 ($M^+ - CH_3N_3$), 94 ($M^+ - CH_3N_3 - CONH_2$), 57 (CH_3N_3). Anal. ($C_6H_6N_6O_2$) C, H, N.

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Registry No. **6a**, 90521-23-6; **6b**, 90521-24-7; **7**, 102613-59-2; **8**, 102587-14-4; **9**, 19818-50-9; $Cl(CH_2)_2NCO$, 1943-83-5; CH_3NCO, 624-83-9; 3-amino-4-pyrazole carboxamide hemisulfate, 102587-13-3.

Antitumor Agents. 78.[1] Inhibition of Human DNA Topoisomerase II by Podophyllotoxin and α-Peltatin Analogues

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It has been reported that the action of etoposide (VP-16) (**14**) as an antitumor agent is mediated through its interaction with DNA topoisomerase II which results in DNA breakage inside the cell. In order to understand the mechanism of action as well as structure–activity relationships of **14**, several novel, synthetic and some naturally occurring analogues related to podophyllotoxin were examined for inhibition of the DNA topoisomerase II activity. Compound **2** exhibited enhanced activity and compound **5** slightly diminished activity relative to **14**. A 4β-substituted ether at the C ring and O-demethylation at the E ring appear to enhance activity.

The class of 4′-demethylated epipodophyllotoxins, exemplified by clinical prototypes **14** and teniposide (VM-26), has emerged into the forefront of antitumor drug development. It is probable that type II DNA topoisomerase is required for the action of these drugs, which have been found to inhibit the DNA catenation activity of rat-derived enzyme.[2] The inhibition of this strand-passing function is likely the result of stabilization of the cleavable complex between DNA and type II DNA topoisomerase. These drugs produce single- and double-strand breaks in DNA in vitro in the presence of purified calf-thymus type II DNA topoisomerase,[19] as well as in vivo,[20] which are likely responsible for the cytotoxicity.[3] As part of our continuing investigation on the synthesis of novel potential antitumor podophyllotoxin analogues,[4] this paper describes the use of a rapid in vitro screening procedure for the evaluation of inhibition of the unknotting activity of human DNA topoisomerase II by these agents.

Chemistry

An adaptation of the method used by Kuhn et al.[5] was used to regioselectively O-demethylate the 4′-position of podophyllotoxin (**9**) and to invert stereochemistry of the C-4 hydroxyl group. The major product 4′-demethylepipodophyllotoxin (**1**) was accompanied by four other com-

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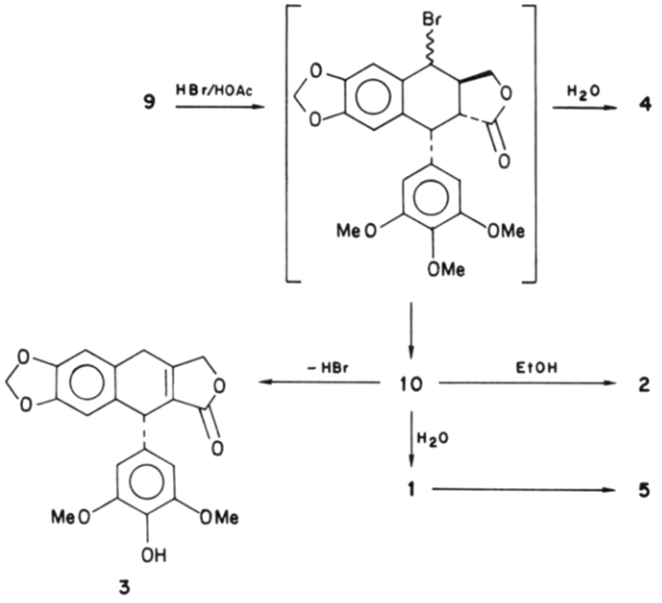
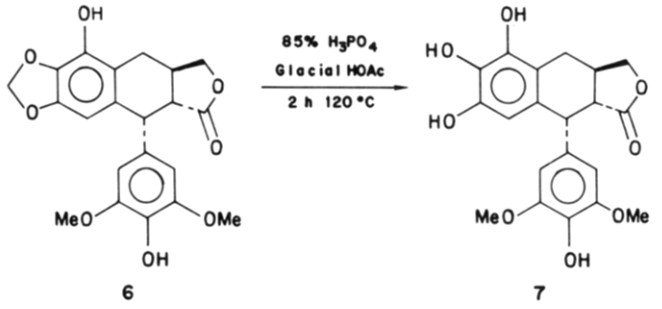
0022-2623/86/1829-1547$01.50/0

Scheme I



Scheme II



pounds, 4′-demethylepipodophyllotoxin 4-ethyl ether (**2**), 4′-demethyl-β-apopicropodophyllin (**3**), epipodophyllotoxin (**4**), and 3′,4′-didemethylepipodophyllotoxin (**5**). An examination of the reaction mixture by TLC showed the presence of four ferric chloride positive spots which indicate phenols and one negative spot. The mixture was fractionated on a silica gel column with a chloroform–methanol mobile phase. Eluting first was **2**, the 4′-O-demethylated congener of the known compound epipodophyllotoxin ethyl ether.[6] Presumably, **2** was formed via the bromide **10** (see Scheme I) by reaction with trace quantities of ethanol present in the chloroform. Participation of the 4β-bromo intermediates led to formation of a benzylic carbonium ion, which underwent nucleophilic attack by ethanol in the case of **2** and by water in the case of **1**, **4**, and **5**. The next eluant was **3**, which was likely formed by a dehydration or dehydrobromination followed by ethylenic double bond isomerization to the more stable α,β-unsaturated enone system. The compound exhibits a positive Legal's test for α,β-unsaturated lactones.[7] The ultraviolet spectrum was identical with that reported in the literature[8] and the infrared spectrum indicates a lactone carbonyl absorbance shift to a lower frequency due to conjugation, an effect seen for β-apopicropodophyllin.[9] The proton NMR shows a downfield shift of the C-1

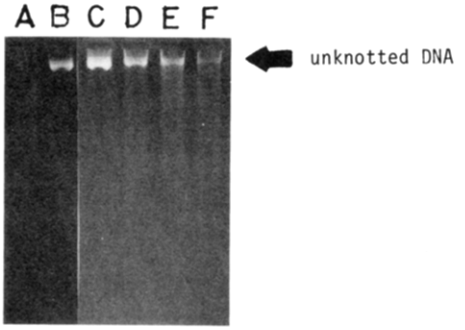
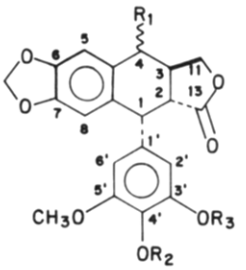


Figure 1. Gel electrophoresis of enzyme inhibition assay products, showing a dose–response relationship. Lane A: DNA without enzyme. Lane B: DNA with enzyme. Lane C–F: DNA, enzyme and **1** at 0.05, 0.1,. 0.2, and 0.4 mM, respectively.

proton from 4.6 to 5.4 ppm due to coplanarity with the α,β enone system that was created by the bulky pendant E ring, which assumed an axial conformation to avoid steric interference with the carbonyl. The previously characterized **4**[6] and **1**[15] eluted next, respectively. The last reaction product to elute was the bis-O-demethylated **5**. The foregoing chemical transformations are illustrated in Scheme I.



1: R_1 = β-OH, R_2 = H, R_3 = Me
2: R_1 = β-OCH_2CH_3, R_2 = H, R_3 = Me
4: R_1 = β-OH, R_2 = R_3 = Me
5: R_1 = β-OH, R_2 = R_3 = H
9: R_1 = α-OH, R_2 = R_3 = Me
10: R_1 = β-Br, R_2 = H, R_3 = Me
12: R_1 = α-OH, R_2 = H, R_3 = Me

The acid-catalyzed cleavage of the methylenedioxy ring of α-peltatin (**6**) according to Schrier's method[10] afforded the novel 6,7-de-*O*-methylene-α-peltatin (**7**) (Scheme II). The 6,7-de-*O*-methylene-α-peltatin tetraacetate (**8**) was made to confirm structure.

Results and Discussion

The action of agents **1**–**9** and **11**–**15** against human type II DNA topoisomerase was studied. They were screened at 0.05, 0.1, 0.2, and 0.4 mM concentrations. Only compounds **2**, **5**, and **14** were active at each of these concentrations. Inhibition was observed in a dose-dependent manner as seen in Figure 1. Table I presents the active C- and E-ring-modified congeners in descending order of activity. Compound **14** was about as potent as **2** and slightly more potent than **5**. Activity was seen for **4** at 0.2 and 0.4 mM levels only, while it was previously reported as inactive against a rat type II DNA topoisomerase.[2] These findings indicate that a free 4′-hydroxyl is required for good activity and that there may be species heterogeneity with respect to the enzyme. The two most potent compounds, **2** and **14**, both possess a 4β ether linkage, which may be an important structural feature to enhanced activity. The enhancement of activity of **5** compared to **1** is due to the additional free hydroxyl group at the C-3′

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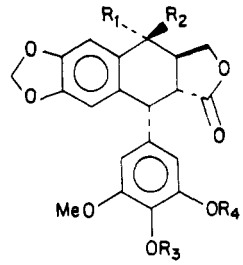
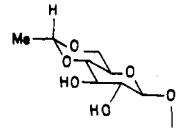
Table I. Inhibition of DNA Topoisomerase II by Podophyllotoxin Analogues

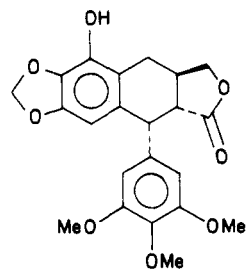


compd	R_1	R_2	R_3	R_4	% inhibn of DNA topoisomerase II[a]
2 (4′-demethylepipodophyllotoxin 4-ethyl ether)	H	OEt	H	CH_3	89
14 (etoposide (VP-16))	H		H	CH_3	85
5 (3′,4′-demethylepipodophylotoxin)	H	OH	H	H	75
1 (4′-demethylepipodophyllotoxin)	H	OH	H	CH_3	60
4 (epipodophyllotoxin)	H	OH	CH_3	CH_3	30

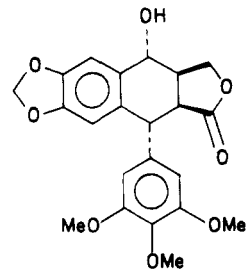
[a] Equal to $100 - (D_{drug} - D_{-E})/(D_{+E} - D_{-E})$, where D = density of P_4 unknotted DNA form.

position on the pendant E ring. This effect is possibly due to availability of an additional hydrogen-bonding site at this region.

Table II presents the active A- and E-ring-modified congeners. Cleavage of the methylenedioxy A ring afforded the novel **7**, which showed better activity than **6** in preliminary DNA topoisomerase II inhibition studies. The tetraacetate **8** exhibited reduced activity relative to that of **6**. The diacetate **15** of α-peltatin also showed diminished activity. These results indicate that the opening of the methylenedioxy ring enhances activity slightly and that the 4′-hydroxyl group enhances activity. The other compounds screened, which included podophyllotoxin (**9**), β-peltatin (**11**), 4′-demethylpodophyllotoxin (**12**), and picropodophylllotoxin (**13**), were virtually inactive. The comparison of **11** with **6** again illustrates the significance of a free 4′-hydroxyl group to activity. Placement and configuration of the hydroxyl group on the B or C ring effects activity also. An epi-substituted hydroxyl at the C-4 position as in **1** is better than a C-5 substitution as in **6**, which in turn is better than C-4 α-substitution as in **12**.



11 (β-peltatin)

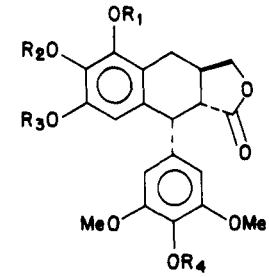


13 (picropodophyllotoxin)

Experimental Section

All melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected. Infrared spectra were recorded on a Perkin Elmer 1320 spectrophotometer, UV spectra were taken on a Varian 2200 UV–vis spectrophotometer, and ^1H NMR spectra were obtained from either a JEOL FX-60 or a Bruker-250 NMR spectrometer; all chemical shifts are reported in parts per million from $(CH_3)_4Si$. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN. Mass spectral analyses were determined on a V.G. Micromass 70-70 instrument at 70 eV with a direct inlet system. Analytical thin-layer chromatography (TLC) was carried out on Merck precoated silica gel 60 F-254. EM Kieselgel 60 (230–400 mesh ASTM) was used for column chromatography. Preparative TLC was performed on Analtech precoated silica gel GF (500 μm, 20 × 20 cm). All new compounds were characterized by melting point, ^1H NMR and IR spectral, and elemental analysis.

Table II. Inhibition of DNA Topoisomerase II by α-Peltatin Analogues



compd	R_1	R_2	R_3	R_4	% inhibn of DNA topoisomerase II
7 (6,7-de-*O*-methylene-α-peltatin)	H	H	H	H	45
8 (6,7-de-*O*-methylene-α-peltatin tetraacetate)	Ac	Ac	Ac	Ac	25
6 (α-peltatin)	H	CH_2	H		20
15 (α-peltatin diacetate)	Ac	CH_2	Ac		10

Natural Product Origin. Podophyllotoxin (**9**), α-peltatin (**6**), β-peltatin (**11**), and 4′-demethylpodophyllotoxin (**12**) were isolated from the ethanolic extract of the roots and rhizomes of *Podophyllum peltatum* by using modified literature procedures,[11,12] which include fractionation of the extract on a silical gel column with a 2:1 chloroform–ethyl acetate mobile phase. Total separation of the podophyllotoxin and α-peltatin components was difficult, so a 1% aqueous NaOH partition was performed to remove the phenolic α-peltatin.

Synthesis of Compounds 1–5. One gram (2.42 mmol) of **9** was dissolved into 20 mL of glacial acetic acid by gentle warming. Anhydrous hydrogen bromide gas was bubbled through the solution until saturated (about 15 min or until gas was seen escaping from vessel). The reaction mixture was capped and allowed to stand for about 20 h at room temperature wherein the color darkened from golden to dark brown. Evaporation of acetic acid in vacuo left a residue, which was solubilized in $CHCl_3$ and ex-

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tracted with 3% aqueous $NaHCO_3$ three times and H_2O once. Then the organic phase was dried with anhydrous $MgSO_4$ and evaporated to dryness (residue weight 0.74 g). This residue was resolubilized in $CHCl_3$ and applied to a silica gel column and fractionated with use of $CHCl_3$ and increasing percent of MeOH. The compounds 1–5 isolated were characterized and shown as follows:

4′-Demethylepipodophyllotoxin Ethyl Ether (2). Crystals were obtained from MeOH: mp 242 °C; ^1H NMR ($(CD_3)_2CO$) δ 6.98 (s, 1 H, 5-H), 6.56 (s, 1 H, 8-H), 6.33 (s, 2 H, 2′,6′-H), 6.20 (s, 2 H, OCH_2O), 4.2–4.7 (m, 4 H), 3.7 (s, 6 H, 3′,5′-OCH_3), 1.2 (t, 3 H, CH_3); IR (KBr) ν 3370 (phenol), 1760 (γ-lactone), 1608, 1515, 1480, and 1455 (arom C=C) cm^{-1}; MS, m/z 428 (M^+). Anal ($C_{23}H_{24}O_8$) C, H.

4′-Demethyl-β-apopicropodophyllin (3): mp 256–257 °C; IR (KBr) ν 3390 (phenol), 1730 (γ-lactone), 1685 (ethylenic C=C), 1608 (arom C=C) cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.72 (s, 1 H, 5-H), 6.62 (s, 1 H, 8-H), 6.40 (s, 2 H, 2′,5′-H), 5.95 (q, 2 H, OCH_2O), 5.40 (s, 1 H, 1-H), 4.83 (m, 3 H), 3.82 (s, 6 H, 3′,5′-OCH_3), 3.7 (s, 1 H), 1.60 (s, H_2O); UV (0.001 N in 95% EtOH) ν_{max} 288 nm, ν_{min} 262.5 nm; MS, m/z 382 (M^+). Anal ($C_{21}H_{18}O_7 \cdot {}^1/_2H_2O$) C, H.

3′,4′-Didemethylepipodophyllotoxin (5): crystallized from CH_2Cl_2, mp 264–266 °C; IR (KBr) ν 3540, 3480 (phenol), 1745 (γ-lactone), 1612 (arom C=C) cm^{-1}; ^1H NMR ($(CD_3)_3CO$) δ 7.5 (s, 1 H, exchangeable), 6.96 (s, 1 H, 5-H), 6.52 (s, 1 H, 8-H), 6.49 (d, J = 2.5 Hz, 1 H, 2′-H), 6.01 (s, 2 H, OCH_2O), 5.98 (overlapped d, J = 2.5 Hz, 1 H, 6′-H), 4.87 (m, 1 H), 4.80 (s, 1 H, exchangeable), 4.2–4.6 (m, 4 H), 3.75 (s, 3 H, OCH_3), 2.89 (s, H_2O); MS, m/z 388 (M^+). Anal ($C_{20}H_{20}O_8 \cdot {}^3/_4H_2O$) C; H: calcd, 4.61; found, 5.21.

4′-Demethylepipodophyllotoxin (1): crystallized from ethyl ether, mp 224–226 °C; IR (KBr) ν 3400 (phenol), 1749 (γ-lactone), 1608 (arom C=C); ^1H NMR (pyridine-d_5) δ 7.24 (s, 1 H, 5-H), 6.83 (s, 2 H, 2′,6′-H), 6.79 (s, 1 H, 8-H), 5.97 (s, 2 H, OCH_2O), 4.99 (s, H_2O), 4.86 (d, J = 5 Hz, 1 H, H-1), 4.7 (m, 1 H), 4.3 (t, 1 H), 3.83 (m, 1 H), 3.27 (m, 1 H, 3-H); MS, m/z 400 (M^+). Anal ($C_{21}H_{20}O_8 \cdot {}^1/_3H_2O$) C, H.

Epipodophyllotoxin (4). The ^1H NMR spectral data was identical with that reported in the literature.[13]

6,7-De-*O*-methylene-α-peltatin (7). A solution of **6** (0.5 g, 1.25 mmol) and phenol (0.475 g, 5.05 mmol) in 15 mL of H_3PO_4 and 5 mL of glacial AcOH was heated for 3 h at 120 °C at which time the reaction mixture was diluted with water and extracted with EtOAc. The organic phase was washed with 5% aqueous $NaHCO_3$ and water and then evaporated in vacuo and fractionated on a silica gel column to afford a red oil (180 mg, 36% yield). Crystals were obtained from ether–EtOH–EtOAc; mp 260–262 °C; IR (KBr) ν 3520–3280 (phenol), 1760 (γ-lactone), 1610 (arom C=C) cm^{-1}, ^1H NMR (pyridine-d_5) δ 6.95 (s, 2 H, arom), 6.82 (s, 1 H, arom), 4.90 (s, H_2O), 4.88 (m, 2 H), 4.40 (m, 2 H), 4.04 (m, 1 H), 3.68 (s, 6 H, OCH_3), 2.95 (m, 1 H), 2.65 (m, 1 H); MS m/z 388 (M^+). Anal ($C_{20}H_{20}O_8 \cdot H_2O$) C, H.

6,7-De-*O*-methylene-α-peltatin Tetraacetate (8). Compound **7** was acetylated in pyridine with Ac_2O: mp 226–229 °C; IR (KBr) ν 1770 br (γ-lactone), 1600 (arom C=C) cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.98 (s, 1 H, 8-H), 6.35 (s, 2 H, 2′,6′-H), 4.8 (m, 1 H), 4.5–3.8 (m, 4 H), 3.71 (s, 6 H, OCH_3), 2.7 (m, 2 H), 2.37, 2.32, 2.25 (s, 12 H, $COCH_3$), 1.58 (s, H_2O). Anal ($C_{28}H_{28}O_{12} \cdot {}^1/_2H_2O$) C, H.

Picropodophyllotoxin (13). A solution of **9** (0.1 g, 0.242 mmol) in $CHCl_3$ was washed with 1% aqueous NaOH in a separatory funnel and allowed to stand for 12 h. The organic layer was washed with water, dried, filtered, and evaporated in vacuo to give a residue, which crystallized from benzene to afford **13** in nearly quantitative yield.

α-Peltatin Diacetate (15). The melting point agrees with that reported in the literature.[14] ^1H NMR ($CDCl_3$) δ 6.51 (s, 1 H, 8-H), 6.38 (s, 2 H, 2′,6′-H), 6.02 (s, 2 H, OCH_2O), 3.74 (s, 6 H, OCH_3), 2.39, 2.32 (s, 6 H, $COCH_3$).

Isolation of Human DNA Topoisomerase II. Human DNA topoisomerase II was isolated from peripheral blast cells of a patient with chronic lymphocytic leukemia. The enzyme was purufied by polyethylene glycol (PEG) 8000, precipitation, hydroxylapatite column, heparin–Sepharose column, and hexylamine agarose affinity chromatography, to near homogeneity. Three bands (142 000, 132 000, and 114 000 daltons) could be seen with Coomassie blue staining. The detailed procedure, which is a partial combination of Wang's[15] and Liu's[16] procedures, will be published elsewhere.

Preparation of Drugs. Drugs were dissolved in 1/10 volume of Me_2SO and 9/10 volume of 0.1 M Hepes buffer, pH 6.7, was added to make a 2 mM final concentration of each drug.

DNA Topoisomerase II Assay. The P4 knotted DNA unknotting reaction was a modification of the Hsieh[18] procedure.

The reaction mixture (20 μL), which contained 50 mM Hepes, pH 6.7, 50 mM KCl, 100 mM NaCl, 0.1 mM EDTA, 10 mM $HgCl_2$, 0.1 mP ATP, 50 μg/mL bovine serum albumin, 0.26 μg P4 knotted DNA, and enzyme, was incubated with or without drugs.

The reaction mixture was incubated at 37 °C for 30 min and terminated by adding a stop solution (2% sodium dodecyl sulfate, 20% glycerol, 0.05% bromphenol blue). These samples were loaded onto a 1% agarose gel and electrophoresed at 50 V overnight with an electrophoresis buffer that contained 90 mM Tris–boric acid, pH 8.3, and 2.5 mM EDTA. At completion, the gel was stained in 0.5 μg/mL of ethidium bromide. Then a photograph was taken of the DNA bands visualized with fluorescence induced by a long-wavelength UV lamp. One unit was defined as the amount of enzyme that converts 50% of 0.26 μg of knotted DNA on the gel to the unknotted form in 30 min as judged by densitometer tracing of the formation of unknotted DNA. Two units of enzyme were used per assay. The data reported in Tables I and II reflect a 0.4 mM drug concentration.

Acknowledgment. This work was supported in part by NIH Grants No. CA17625 (K.-H.L.) and CA27448 (Y.-C.C.). We thank Dr. David Millington for mass spectral analysis.

Registry No. 1, 6559-91-7; 2, 102306-95-6; 3, 102306-96-7; 4, 4375-07-9; 5, 102306-97-8; 6, 568-53-6; 7, 20594-85-8; 8, 20407-98-1; 9, 518-28-5; 11, 518-29-6; 12, 40505-27-9; 13, 477-47-4; 14, 33419-42-0; 15, 102306-98-9; DNA topoisomerase II, 80449-01-0.

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