Antitumor Agents. 164.† Podophenazine, 2″,3″-Dichloropodophenazine, Benzopodophenazine, and Their 4*β*-*p*-Nitroaniline Derivatives as Novel DNA Topoisomerase II Inhibitors

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We report here the synthesis and biological evaluation of novel DNA topoisomerase II inhibitors, podophenazine (**8**), 2″,3″-dichloropodophenazine (**9**), and benzopodophenazine (**10**), and their 4*β*-*p*-nitroaniline derivatives **13**−**15**. Among these, 4′-*O*-demethyl-4*β*-(4‴-nitroanilino)-4-desoxypodophenazine (**13**) and 4′-*O*-demethyl-2″,3″-dichloro-4*β*-(4‴-nitroanilino)-4-desoxypodophenazine (**14**) were found to inhibit KB cells at sub-micromolar concentrations (IC_{50} = 0.11 ± 0.03 and 0.48 ± 0.17 *μ*M, respectively). Against KB/7d cells (a pleiotrophic multiple drug-resistant subclone selected with etoposide which has reduced level of topoisomerase II), only compound **13** out of a target series maintained activity in the sub-micromolar concentration range with a IC_{50} value of 0.56 ± 0.13 *μ*M. The differential toxicity ratio for **13** [IC_{50}(KB/7d)/IC_{50}(KB)] was ∼5. Unlike etoposide and its congeners, compounds **13** and **14** were found to be weak inhibitors of the catalytic activity of topoisomerase II (IC_{100} = >100 and >150 *μ*M, respectively). *In vitro* protein-linked DNA complex formation assay revealed that **13** and **14**, respectively, induced marginal response (**13** at 1 *μ*M, 320.3 ± 124.5 cpm; **13** at 50 *μ*M, 308.8 ± 139.9 cpm; **13** at 100 *μ*M, 446.0 ± 153.5 cpm) and no response (**14** at 1 *μ*M, 104.9 ± 52.6 cpm; **14** at 50 *μ*M, 103.3 ± 42.6 cpm; **14** at 100 *μ*M, 101.4 ± 35.2 cpm) compared to the enzyme control. On the basis of these results, we conclude that the mechanism of enzyme inhibition of these compounds is distinct from that of etoposide and its congeners. We are currently investigating the mechanism(s) of action of compounds **13** and **14** as well as synthesizing other derivatives in order to better characterize structure−activity relationships of this series of compounds.

Introduction

Topoisomerases are ubiquitous enzymes that play an important role in the modulation of DNA topology. Since the DNA topology dictates how genetic information is transcribed and expressed, a proper DNA structure must be maintained. The importance of topoisomerases has been implicated in many essential cell functions such as transcription, replication, and chromosome segregation at mitosis, DNA recombination, and ribosomal DNA recombination.[2,3] Both prokaryotic and eukaryotic topoisomerases are classified as type I or II based on their ability to break one strand (type I) or both strands (type II) of the DNA double helix in their catalytic cycles. Their enzymatic properties also differ from each other in relaxing pattern of supercoils, ability to catenate/decatenate and knot/unknot, cofactor requirement, active site tyrosine−DNA adduct, and subunit structure.[4] These differences in biochemical properties probably separate their roles in relieving the mechanical and torsional stresses during different stages of the cell life cycle. In the eukaryotic cell, DNA topoisomerase II must be present for segregating daughter chromosomes,[5,6] as well as maintaining chromosome structure,[7,8] and a normal function of this enzyme is vital to cell survival.[9,10] Selective inhibition of DNA topoisomerase II, therefore, can be very useful in the area of chemotherapy, and the enzyme is the target for a number of clinically useful and experimental antineoplastic agents such as etoposide and its congeners (Figure 1; teniposide, **1**, and **2**), daunorubicin, doxorubicin, menogaril, nogalamycin, actinomycin D, acridinecarboxamide, pyrazoloacridine, amsacrine, crisnatol, ellipticine, acodazole, 2-phenylquinolinecarboxamide, nitracrine, and mitonoafide.[11−14]

On the basis of molecular modeling studies, MacDonald *et al.* proposed a common binding mode for intercalating (*e.g.*, daunorubicin and amsacrine) and nonintercalating (*e.g.*, etoposide) topoisomerase II inhibitors.[15] Superimposition of these compounds revealed three domains that are thought to be important for DNA topoisomerase II inhibitory activity: the DNA intercalating (or "intercalation-like" in ternary complex) moiety, the minor groove binding site, and the molecular region that can accommodate a number of structurally diverse substituents, which might also bind to the minor groove (Figure 2).[16] Among the three domains of this composite pharmacophore, only the minor groove binding region[16,17] and variable substituent accommodating region[1] have been studied, and our understanding of the DNA intercalating (or "intercalation-like" in ternary complex) domain is limited.

As a part of our ongoing effort to understand the mechanism of enzyme inhibition and design inhibitors with clinical potential, we investigated this unexplored domain of the pharmacophore model. To do this, podophyllotoxin (**3**) was selected as the starting material

† For Part 163, see ref 1.
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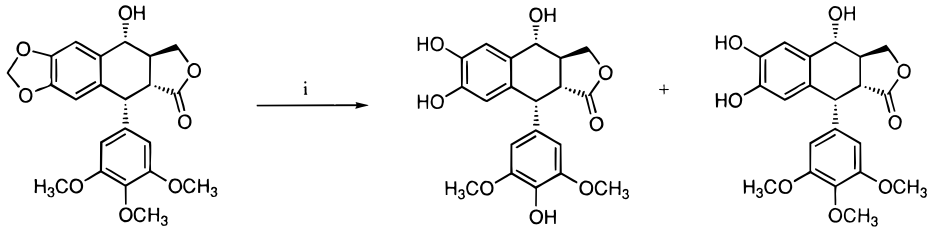
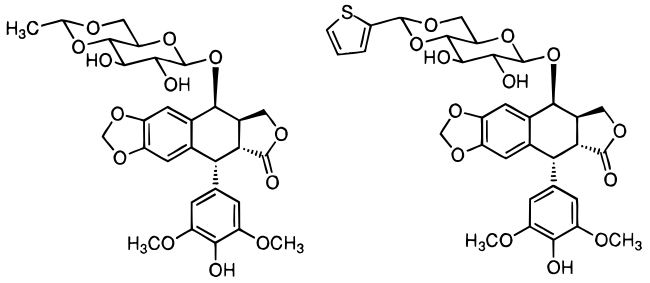
Scheme 1[a]

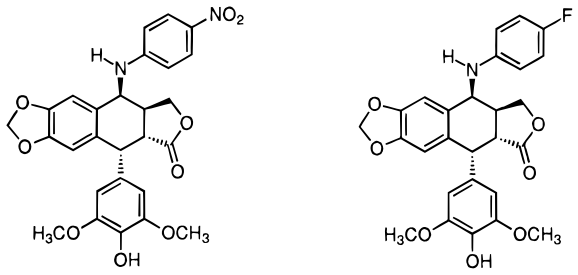


3 **4** **5**

[a] Reagents: (i) BCl_3, CH_2Cl_2, −70 °C, 2 h.



Etoposide Teniposide



1 **2**

Figure 1. Etoposide and its congeners.

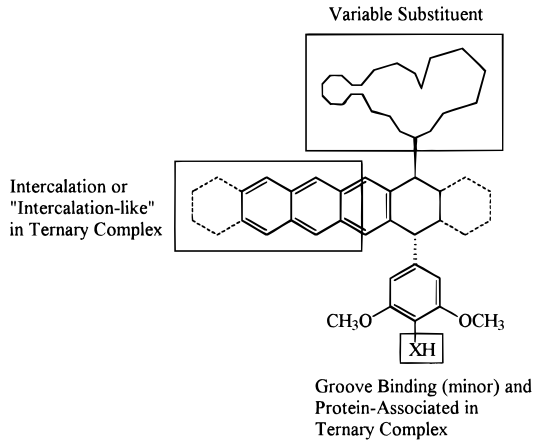


Figure 2. Composite pharmacophore model for expression of topoisomerase II activity (adapted from ref 15).

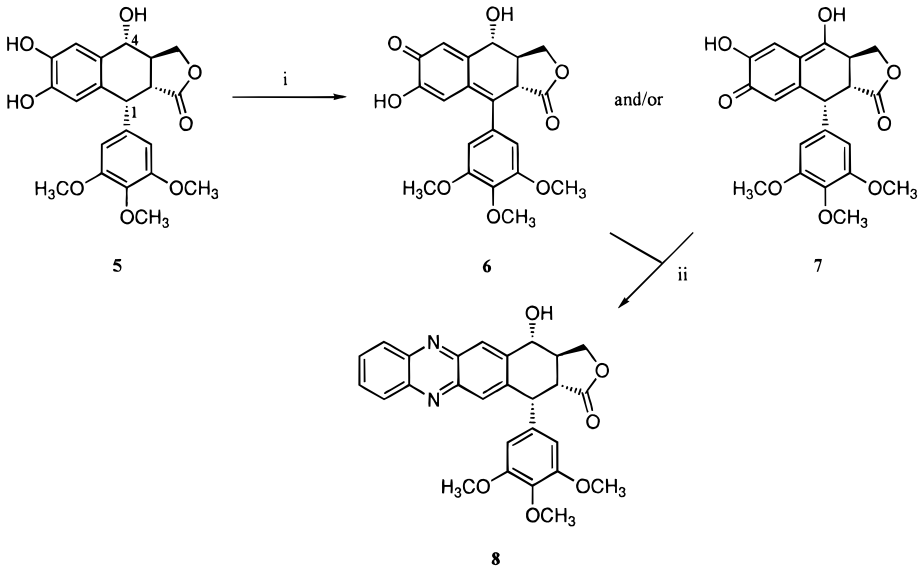
because 4′-*O*-demethylepipodophyllotoxin analogs of **3**, such as **1** and **2**, are potent topoisomerase II inhibitors with substructures that can be potentially modified to interact with all three domains. In this paper, we report the synthesis and biological evaluation of novel DNA topoisomerase II inhibitors, podophenazine (**8**), 2″,3″-dichloropodophenazine (**9**), and benzopodophenazine (**10**), and their 4β-*p*-nitroaniline derivatives **13**−**15**. We found that the mechanism of enzyme inhibition of target compounds is distinct from that of etoposide and its congeners; cytotoxic activity is retained, and spectrum of cytotoxic activity (against drug-resistant cells) can be improved.

Chemistry

Phenazines are generally prepared by the reaction of catechol and 1,2-phenylenediamine.[18] The key step in this reaction is the formation of *o*-benzoquinone, which is normally achieved by oxidizing catechol. Consequently, **3** was treated with BCl_3 to form 4′-demethyl-6,7-*O*,*O*-demethylenepodophyllotoxin (**4**) and 7-*O*,*O*-demethylenepodophyllotoxin (**5**) (Scheme 1).[19] Numerous reagents to convert catechol to *o*-benzoquinone are known. These include $NaIO_4$,[20] tetrachloro-1,2-benzoquinone,[21] $NaNO_2$,[21] PbO_2,[19] Ag_2O,[22] and Ag_2CO_3/Celite.[23] In an attempt to synthesize 6,7-benzoquinone derivatives of podophyllotoxin, we tried a number of these oxidizing reagents. Since $NaIO_4$, tetrachloro-1,2-benzoquinone, and $NaNO_2$ are known to oxidize the E-ring of 4′-demethylpodophyllotoxin derivatives,[20,21] we decided to use **5** rather than **4** as the starting material in the synthesis of 6,7-benzoquinone derivatives to protect the E-ring oxidation.

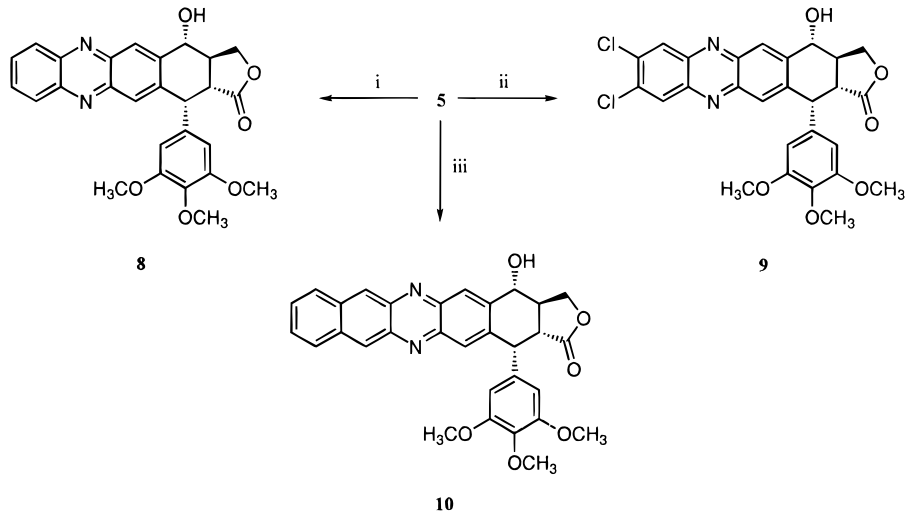
Our initial oxidation reactions using $NaIO_4$ and $NaNO_2$ resulted in complicated TLC patterns indicating the instability of **5** toward these oxidizing reagents. In addition, none of the TLC spots were bright red, which is the characteristic color of an *o*-benzoquinone. We then used milder oxidizing reagents, such as Ag_2O and Ag_2CO_3/Celite. When Ag_2O was used to oxidize **5**, a simpler TLC pattern was observed. The major product in this reaction was less polar than **5**. However, like previous reactions, it did not show the characteristic red color of an *o*-benzoquinone. Spectral examination of this pale yellow product revealed the absence of either 1- or 4-H. On the basis of these results, we hypothesized that one of the carbonyl groups of 6,7-benzoquinone derivative may be enolized to form compounds **6** and/or **7** due to the acidity of the 1- and 4-H, respectively. We then considered using the unstable enolized product(s) of **5** (**6** and/or **7**) directly in the synthesis of compound **8**. Addition of 1,2-phenylenediamine to an ethanol/acetic acid solution containing the Ag_2O-oxidized product of **5** produced a pale yellow product (Scheme 2). The structure of this product was assigned as **8** by spectral examination. To improve the reaction yield, Ag_2O was introduced to the solution containing both **5** and 1,2-phenylenediamine; this approach allows the continuous oxidation of **5**. This procedure, however, did not improve the overall reaction yield due to the susceptibility of 1,2-phenylenediamine to Ag_2O oxidation. We, therefore, decided to use Ag_2CO_3/Celite, which is a milder oxidizing agent than Ag_2O, to limit the oxidation of 1,2-

Scheme 2[a]



[a] Reagents: (i) Ag_2O, dioxane, room temperature, 3 h; (ii) 1,2-phenylenediamine, EtOH/AcOH, room temperature, overnight.

Scheme 3[a]



[a] Reagents: (i) 1,2-phenylenediamine, Ag_2CO_3/Celite, EtOH, room temperature, 7 h; (ii) 4,5-dichloro-1,2-phenylenediamine, Ag_2CO_3/Celite, EtOH, room temperature, 4 h; (iii) 2,3-diaminonaphthalene, Ag_2CO_3/Celite, EtOH, room temperature, overnight.

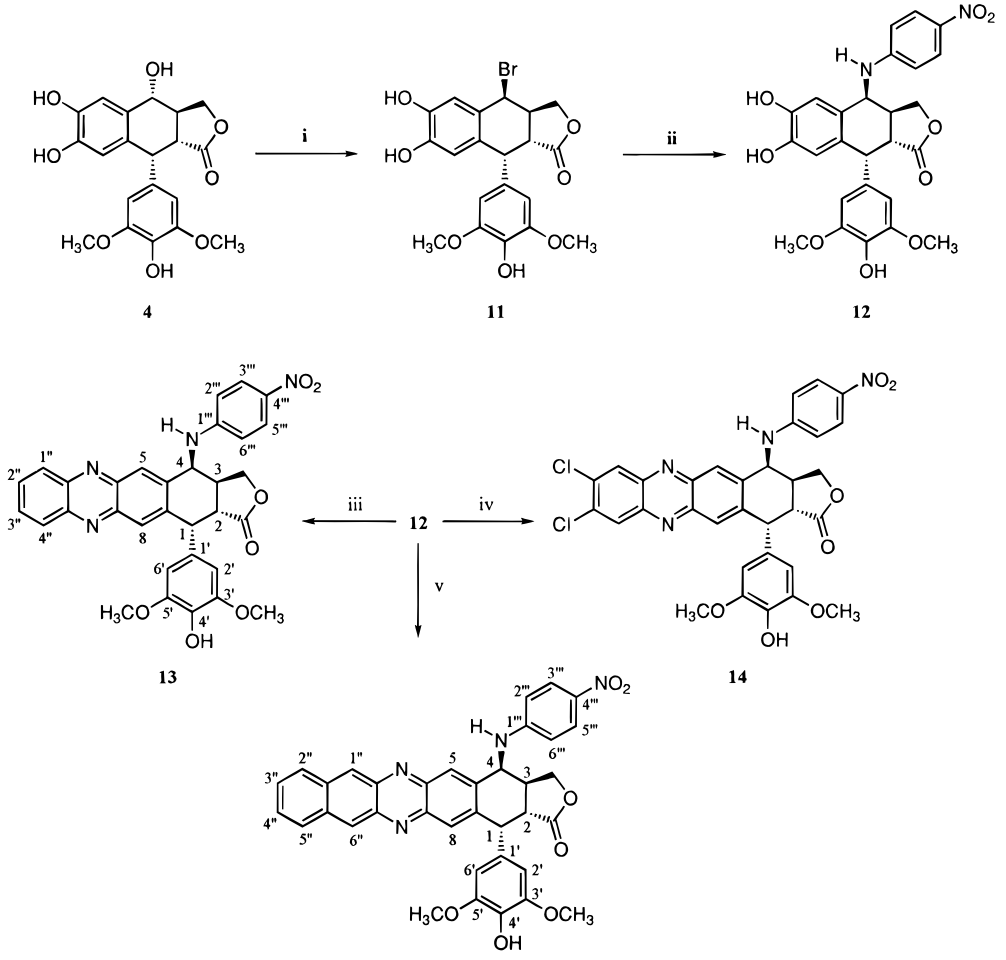
phenylenediamine and found that this oxidizing reagent improved the reaction yield. The preparation of compounds **8**−**10** from **5** using a freshly prepared Ag_2CO_3/Celite reagent and 1,2-phenylenediamine, 4,5-dichloro-1,2-phenylenediamine, or 2,3-diaminonaphthalene is shown in Scheme 3.

Initially, we planned to synthesize 4′-demethylated nitroaniline derivatives from compounds **8**−**10** using previously described methods.[19,24,25] However, we found that selective demethylation of 4′-OCH_3 was very difficult, and a low yield and complex TLC pattern further complicated the problem. We, then, began with compound **4** to avoid the demethylation step. Compound **4** was converted first to the nitroaniline derivative **12** prior to oxidation and phenazine formation because the nitroaniline addition reaction is cleaner and produces a higher yield than the oxidation reaction (Scheme 4). Since the Ag_2CO_3/Celite reagent is a weak oxidizing reagent, we postulated that a phenazine formation would be preferred over the E-ring oxidation. Compound **12** was successfully used in the synthesis of compounds **13**−**15** (Scheme 4). However, the reaction yields were very low, reflecting the susceptibility of the 4′-OH and possibly of the nitroaniline group of **12** to Ag_2CO_3/Celite oxidation.

Results and Discussions

The ability of etoposide, **1**, **3**−**5**, **8**, **9**, and **12**−**15** to inhibit KB cell growth was initially tested by microscopical examination of cultures treated for 2 days at 0.1, 1, and 10 μM drug concentrations (data not shown); on the basis of these studies, **10** was excluded from further consideration because of its poor solubility in DMSO, and the remaining compounds were re-evaluated over a narrow dose range. After 2 days of drug treatment, viable cells were manually enumerated using a hemocytometer and IC_{50} (the drug concentrations that inhibit 50% of the control) values were graphically determined. The obtained growth inhibition profiles were used to determine the appropriate IC_{50} value ranges for compounds **1**, **8**, **9**, and **12**−**15**. Similarly, IC_{50} value ranges for those compounds against KB/7d

Scheme 4[a]



[a] Reagents: (i) HBr, dioxane, room temperature, 1.5 h; (ii) 4-nitroaniline, $BaCO_3$, dioxane, room temperature, overnight; (iii) 1,2-phenylenediamine, Ag_2CO_3/Celite, EtOH, room temperature, 8 h; (iv) 4,5-dichloro-1,2-phenylenediamine, Ag_2CO_3/Celite, EtOH, room temperature, overnight; (v) 2,3-diaminonaphthalene, Ag_2CO_3/Celite, EtOH, room temperature, overnight.

Table 1. Growth Inhibitory Activities against KB and KB/7d (VP-16-Resistant) Cells

	IC_{50} (μM)[a]		
	KB	KB/7d	IC_{50}(KB/7d)/IC_{50}(KB)
etoposide	0.164 ± 0.044[b]	23.8 ± 1.5[b]	~145
1	0.032 ± 0.008	0.55 ± 0.26	~17
12	0.42 ± 0.05	16.58 ± 13.79	~39
13	0.11 ± 0.03	0.56 ± 0.13	~5
14	0.48 ± 0.17	10.59 ± 3.98	~22
15	6.63 ± 3.30	ND[c]	ND[c]
8	23.42 ± 0.35[d]	6.21 ± 0.88[d]	ND[e]
9	13.30 ± 6.09[d]	9.20 ± 1.13[d]	ND[e]

[a] The drug concentrations that inhibit 50% of the control. [b] IC_{50} values were obtained from ref 26. [c] Not determined due to poor solubility (see text). [d] Formed microscopic precipitation (see text). [e] Not determined due to formation of microscopic precipitation (see text).

cells (a pleiotrophic multiple drug-resistant subclone selected with etoposide which has reduced level of topoisomerase II)[26] were obtained. The IC_{50} values for etoposide, **1**, **8**, **9**, and **12**−**15** against KB and KB/7d cells are shown in Table 1. Reproducible IC_{50} values for **8** and **9** against KB and KB/7d cell lines were obtained despite the fact that both compounds formed microscopic precipitates over time; however, the IC_{50} value for **15** against KB/7d cell line was not determined due to its poor solubility at higher concentrations. IC_{50} values for compounds **1** and **12** were obtained for comparison. The results of the growth inhibition assay show that compounds **13** and **14** inhibit KB cells at submicromolar concentrations (IC_{50} = 0.11 ± 0.03 and 0.48 ± 0.17 μM, respectively, Table 1). As the phenazine ring system is extended further (*i.e.*, **15**), a decrease in the growth inhibitory activity was observed (IC_{50} = 6.63 ± 3.30 μM, Table 1). Against KB/7d cells, only compound **13** out of the target series maintained activity in the sub-micromolar concentration range with an IC_{50} value of 0.56 ± 0.13 μM. The differential toxicity ratio for **13** [IC_{50}(KB/7d)/IC_{50}(KB)] was ~5 (Table 1). By comparison, differential toxicity ratios for etoposide, **1**, **12**, and **14** were ~145, ~17, ~39, and ~22, respectively (Table 1). Although a wide range of differential toxicity ratios were observed, all compounds generally exhibited lower growth inhibitory activities against KB/7d cells. This is consistent with the fact that these cells contain a reduced level of topoisomerase II. The growth inhibitory activity of compound **13**, however, was intriguing because it exhibited the lowest differential toxicity profile. This prompted an examination of compounds **13** and **14** as inhibitors of topoisomerase II in order to determine if their mechanism of action is fundamentally different from that of etoposide and its congeners (*e.g.*, **1**, **2**, and **12**).

The results of *in vitro* topoisomerase II inhibition assay showed that at 50 μM drug concentration, etopo-

Table 2. Stimulation of *in Vitro* Topoisomerase II-Linked DNA Complex Formation and Interference with Etoposide-Induced Cleavage

	counts per min (cpm) at various drug concentrations			
	0 μM	1 μM	50 μM	100 μM
DNA control	39.9 ± 7.1[a]	NA[b]	NA[b]	NA[b]
enzyme control	95.3 ± 17.7[a]	NA[b]	NA[b]	NA[b]
etoposide	NA[b]	216.4 ± 78.4[c]	2464.9 ± 420.5[c]	1840.3 ± 208.3[c]
1	NA[b]	ND[d]	2930.8 ± 827.6[c]	ND[d]
12	NA[b]	ND[d]	661.3 ± 213.9[c]	ND[d]
13	NA[b]	320.3 ± 124.5[c]	308.8 ± 139.9[c]	446.0 ± 153.5[c]
14	NA[b]	104.9 ± 52.6[c]	103.3 ± 42.6[c]	101.4 ± 35.2[c]
etoposide + **14**	NA[b]	ND[d]	2633.5 ± 35.5[e]	ND[d]

[a] Mean ± measured standard deviation for six determinations. [b] Not applicable. [c] Mean ± measured standard deviation for four determinations. [d] Not determined. [e] Mean ± measured standard deviation for two determinations.

side, **1**, and **12** (known topoisomerase II inhibitors) were the only compounds which inhibit enzyme-catalyzed DNA unknotting (data not shown). Topoisomerase II inhibition assay was repeated for **13** and **14** at various concentrations. In order to measure the percent inhibition of topoisomerase II catalytic activity, the catalytic activity of an enzyme control was terminated at 15, 30, and 60 min, which roughly translate into 25%, 50%, and 100% completion of catalytic activity, respectively. Compounds **13** and **14** were found to inhibit topoisomerase II at higher concentrations (IC_{100} = >100 and >150 μM, respectively). These results indicate that **13** and **14** are weak catalytic inhibitors of topoisomerase II *in vitro*.

The ability of compounds **13** and **14** to stabilize the *in vitro* cleavable complex was also examined. Protein-linked radiolabeled DNA−enzyme complexes were precipitated and quantified at 1, 50, and 100 μM drug concentrations (Table 2). For comparison, etoposide, **1**, and **12** were also evaluated. As expected, etoposide and **1** produced a significant amount of protein-linked DNA complexes at 50 and 100 μM drug concentrations (Table 2; etoposide at 50 μM, 2464.9 ± 420.5 cpm; etoposide at 100 μM, 1840.3 ± 208.3 cpm; **1** at 50 μM, 2930.8 ± 827.6 cpm). In contrast, compounds **12**−**14**, respectively induced marginal response (Table 2; **12** at 50 μM, 661.3 ± 213.9 cpm; **13** at 1 μM, 320.3 ± 124.5 cpm; **13** at 50 μM, 308.8 ± 139.9 cpm; **13** at 100 μM, 446.0 ± 153.5 cpm) and no response (Table 2; **14** at 1 μM, 104.9 ± 52.6 cpm; **14** at 50 μM, 103.3 ± 42.6 cpm; **14** at 100 μM, 101.4 ± 35.2 cpm) compared to the enzyme control; the activities of compounds **12** and **13** were statistically different from the enzyme control at the 99% confidence level, whereas compound **14** was not a cleavage complex-forming inhibitor. In addition, the ability of **14** to interfere with etoposide-induced protein-linked DNA complex formation was examined at 50 μM drug concentration. Compound **14** was chosen because it clearly did not stimulate cleavable complex formation (Table 2), and therefore reduction of etoposide-induced protein-linked DNA complex formation would be more clearly detected. No statistically significant reduction of etoposide-induced cleavage complexes was observed at the 99% confidence level (Table 2; etoposide at 50 μM, 2464.9 ± 420.5 cpm; etoposide + **14** at 50 μM, 2633.5 ± 35.5 cpm).

Gel analyses of etoposide-, **1**-, **12**-, **13**-, and **14**-induced protein-linked DNA cleavages from a representative experiment are shown in Figure 3. The cleavage activities are consistent with the results shown in Table 2. Etoposide and **1** induced significant amounts of topoisomerase II-stimulated DNA cleavage; however, the cleavage pattern for etoposide differed from that of compound **1** (Figure 3).

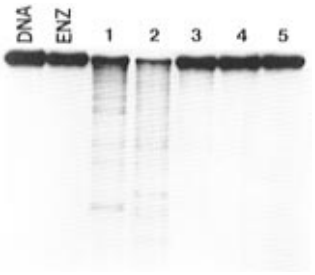


Figure 3. Gel analysis of protein-linked DNA complex formation. *In vitro* protein-linked DNA complex formation assay was performed as described in the Experimental Section. An autoradiogram of a dried agarose gel from a reproducible experiment is shown. Cleavage products shown in lanes 1−5 are from reactions containing etoposide, **1**, **12**, **13**, and **14**, respectively, at 50 μM concentration.

In summary, the intercalating (or "intercalation-like" in ternary complex) domain of the composite pharmacophore model (Figure 2) was probed by introducing various phenazine ring systems into 4′-*O*-demethylepipodophyllotoxin analogs, and activities of compounds before and after the chemical modifications were compared. The data obtained from growth inhibition and *in vitro* protein-linked DNA complex formation assays revealed that compounds **13** and **14** exhibit novel mechanism(s) of action, and compound **13** exhibits an improved cytotoxic profile compared to that of etoposide and its congener **1**, which is currently undergoing a clinical trial. We are currently investigating mechanism(s) of action of compounds **13** and **14** as well as synthesizing other derivatives in order to better characterize the intercalating (or "intercalation-like" in ternary complex) domain.

Experimental Section

All melting points were taken on a Fischer-Johns melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer 1320 spectrophotometer. ^{1}H NMR spectra were obtained using Bruker AC-300 and WM 250 NMR spectrometers with TMS as the internal standard. All chemical shifts are reported in ppm. FABMS and HRFABMS spectral analyses were determined on a JOEL HX-110 instrument. Analytical thin-layer chromatography (TLC) was carried out on Merck precoated aluminum silica gel sheets (Kieselgel 60 F-254). Optical rotations were measured with a Rudolph Research Autopol III polarimeter. All new target compounds were characterized by ^{1}H and ^{13}C NMR and IR spectral analyses and MS analyses.

Podophenazine (8). Commercially available 1,2-phenylenediamine (1.09 g, 9.94 mmol) and **5** (2 g, 4.97 mmol) were dissolved in dry ethanol (75 mL). To this solution was added

a fresh Ag_2CO_3/Celite reagent (14.2 g, 24.9 mmol). The reaction mixture was stirred for 7 h at room temperature. The reaction mixture was filtered, and the filtrate was evaporated under reduced pressure. The desired product was purified by column chromatography ($CHCl_3$:MeOH = 50:1) to give 370 mg (16% yield) as yellow prisms crystallized from methanol: mp 240 °C dec; $[\alpha]^{18}_D = +101.7°$ (c = 0.12, $CHCl_3$); negative FABMS m/z 472 M^-; positive FABMS m/z 473 $(M + H)^+$; HRFABMS calcd for $C_{27}H_{25}O_6N_2$ 473.1713, found m/z 473.1712; IR (KBr) 3400, 3200, 1770, 1570, 1485, 1440, 1410 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 3.05 (dd, 1H, J = 4, 14 Hz, 2-H), 3.10−3.25 (m, 1H, 3-H), 3.68 (s, 6H, 3′,5′-OCH_3), 3.79 (s, 3H, 4′-OCH_3), 4.22 (t, 1H, J = 9 Hz, 11-H), 4.75 (t, 1H, J = 8.5 Hz, 11-H), 5.14 (d, 1H, J = 4 Hz, 1-H), 5.17 (d, 1H, J = 9.5 Hz, 4-H), 6.33 (s, 2H, 2′,6′-H), 7.93 (m, 2H, 2″,3″-H), 8.14 (s, 1H, 8-H), 8.27 (br d, 1H, J = 9.5 Hz, 1″-H), 8.35 (br d, 1H, J = 9.5 Hz, 4″-H), 8.84 (s, 1H, 5-H). Anal. ($C_{27}H_{24}O_6N_2$) C, H, N.

2″,3″-Dichloropodophenazine (9). Commercially available 4,5-dichloro-1,2-phenylenediamine (44 mg, 0.25 mmol) and **5** (50 mg, 0.12 mmol) were dissolved in dry ethanol (5 mL). To this solution was added a freshly prepared Ag_2CO_3/Celite reagent (357 mg, 0.62 mmol). The reaction mixture was stirred for 4 h at room temperature. The reaction mixture was filtered, and the filtrate was evaporated under reduced pressure. The desired product was purified by silica gel column chromatography ($CHCl_3$:MeOH = 60:1) to give 30 mg (45% yield) as yellow granules crystallized from methanol: mp 270 °C dec; $[\alpha]^{18}_D = +90.8°$ (c = 0.105, $CHCl_3$); negative FABMS m/z 540 $(M - H)^-$; positive FABMS m/z 541 M^+; HRFABMS calcd for $C_{27}H_{22}O_6N_2Cl_2$ 540.0855, found m/z 540.0854; IR (KBr) 3400, 3200, 1775, 1575, 1495, 1445, 1410 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 2.95−2.98 (m, 1H, 3-H), 3.03 (dd, 1H, J = 4, 14 Hz, 2-H), 3.69 (s, 6H, 3′,5′-OCH_3), 3.80 (s, 3H, 4′-OCH_3), 4.23 (t, 1H, J = 9 Hz, 11-H), 4.75 (t, 1H, J = 8 Hz, 11-H), 5.15 (br d, 2H, J = 3.5 Hz, 1,4-H), 6.32 (s, 2H, 2′,6′-H), 8.08, 8.34, 8.39, and 8.64 (each s, 1H, 5,8,1″,4″-H). Anal. ($C_{27}H_{22}O_6N_2Cl_2$) C, H, N.

Benzopodophenazine (10). Commercially available 2,3-diaminonaphthalene (39.3 mg, 0.25 mmol) and **5** (50 mg, 0.12 mmol) were dissolved in dry ethanol (5 mL). To this solution was added a freshly prepared Ag_2CO_3/Celite reagent (177 mg, 0.31 mmol). The reaction mixture was stirred overnight at room temperature. The reaction mixture was filtered, and the filtrate was evaporated under reduced pressure. The desired product was purified by silica gel column chromatography (hexane:acetone = 2:1) to give 20 mg (31% yield) as red granules crystallized from methanol: mp 290 °C dec; $[\alpha]^{18}_D = +223.1°$ (c = 0.125, $CHCl_3$); negative FABMS m/z 523 $(M + H)^-$; positive FABMS m/z 523 $(M + H)^+$; HRFABMS calcd for $C_{31}H_{27}O_6N_2$ 523.1869, found m/z 523.1865; IR (KBr) 3360, 1765, 1570, 1490, 1440, 1400 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 3.03 (dd, 1H, J = 4.5, 14 Hz, 2-H), 3.13−3.18 (m, 1H, 3-H), 3.68 (s, 6H, 3′,5′-OCH_3), 3.79 (s, 3H, 4′-OCH_3), 4.22 (t, 1H, J = 9.5 Hz, 11-H), 4.78 (t, 1H, J = 8.5 Hz, 11-H), 5.15 (br d, 2H, J = 5 Hz, 1,4-H), 6.36 (s, 2H, 2′,6′-OCH_3), 7.57 and 8.13 (each m, 2H, 2″,3″,4″,5″-H), 8.11 (s, 1H, 8-H), 8.75, 8.88, and 8.92 (each s, 1H, 5,1″,6″-H). Anal. ($C_{31}H_{27}O_6N_2$) C, H, N.

4′-*O*-Demethyl-4*β*-(4‴-nitroanilino)-4-desoxypodophenazine (13). Commercially available 1,2-phenylenediamine (85.2 mg, 0.79 mmol) and **12** (200 mg, 0.39 mmol) were dissolved in dry ethanol (20 mL). To this solution was added a freshly prepared Ag_2CO_3/Celite reagent (1.36 g, 2.36 mmol). After 1 h of stirring, 85.2 mg of phenylenediamine was added to the reaction mixture. An additional 85.2 mg of phenylenediamine was added after 4 h. The reaction mixture was stirred for a total of 8 h at room temperature. The reaction mixture was filtered, and the filtrate was evaporated under reduced pressure. The desired product was purified by silica gel column chromatography (hexane:acetone = 2:1; preparative TLC, $CHCl_3$:MeOH = 20:1) to give 8 mg (3% yield) as yellow needles crystallized from methanol: mp 272−273 °C; $[\alpha]^{18}_D = -80.6°$ (c = 0.09, acetone); negative FABMS m/z 578 M^-; positive FABMS m/z 579 $(M + H)^+$; HRFABMS calcd for $C_{32}H_{27}O_7N_4$ 579.1879, found m/z 579.1877; IR (KBr) 3370, 1765, 1590, 1500, 1310, 825 cm^{-1}; ^1H NMR (300 MHz, acetone-d_6) δ 3.57−3.62 (m, 2H, 2,3-H), 3.64 (s, 6H, 3′,5′-OCH_3), 4.03 (dd, 1H, J = 10, 13 Hz, 11-H), 4.60 (dd, 1H, J = 8.5, 10 Hz, 11-H), 5.23 and 5.85 (each br s, 1H, 1,4-H), 6.41 (s, 2H, 3′,5′-OCH_3), 7.00 and 8.14 (each d, 2H, J = 11 Hz, 2‴,3‴,5‴,6‴-H), 7.93 and 8.19 (each m, 2H, 1″,2″,3″,4″-H), 8.14 (s, 1H, 8-H), 8.44 (s, 1H, 5-H). Anal. ($C_{32}H_{26}O_7N_4 \cdot {}^1/_2H_2O$) C, H, N.

4′-*O*-Demethyl-2″,3″-dichloro-4*β*-(4‴-nitroanilino)-4-desoxypodophenazine (14). Commercially available 4,5-dichloro-1,2-phenylenediamine (140 mg, 0.79 mmol) and **12** (200 mg, 0.39 mmol) were dissolved in dry ethanol (20 mL). To this solution was added a freshly prepared Ag_2CO_3/Celite reagent (1.36 g, 2.36 mmol). The reaction mixture was stirred for 1 h at room temperature. The reaction mixture was filtered, and the filtrate was evaporated under reduced pressure. The desired product was purified by silica gel column chromatography (hexane:acetone = 2:1; preparative TLC, $CHCl_3$:MeOH = 10:1) to give 10 mg (4% yield) as light brown granules crystallized from methanol/acetone: mp 263−270 °C; $[\alpha]^{18}_D = -94.3°$ (c = 0.1, acetone); negative FABMS m/z 646 M^-; positive FABMS m/z 647 $(M + H)^+$; HRFABMS calcd for $C_{32}H_{25}O_7N_4Cl_2$ 647.1100, found m/z 647.1090; IR (KBr) 3320, 1770, 1590, 1500, 1310, 825 cm^{-1}; ^1H NMR (300 MHz, acetone-d_6) δ 3.61 (br s, 2H, 2,3-H), 3.65 (s, 6H, 3′,5′-OCH_3), 4.04 (br t, 1H, J = 8 Hz, 11-H), 4.58 (br t, 1H, J = 7 Hz, 11-H), 5.25 and 5.87 (each br s, 1H, 1,4-H), 6.42 (s, 2H), 7.01 and 8.14 (each d, 2H, J = 9 Hz, 2‴,3‴,5‴,6‴-H), 8.15 (s, 1H, 8-H), 8.41, 8.44, and 8.46 (each s, 1H, 5, 1″,4″-H). Anal. ($C_{32}H_{24}O_7N_4Cl_2 \cdot {}^1/_2H_2O$) C, H, N.

4′-*O*-Demethyl-4*β*-(4‴-nitroanilino)-4-desoxybenzopodophenazine (15). Commercially available 2,3-diaminonaphthalene (125 mg, 0.79 mmol) and **12** (200 mg, 0.39 mmol) were dissolved in dry ethanol (20 mL). To this solution was added a freshly prepared Ag_2CO_3/Celite reagent (1.36 g, 2.36 mmol). The reaction mixture was stirred overnight at room temperature. The reaction mixture was filtered, and the filtrate was evaporated under reduced pressure. The desired product was purified by silica gel column chromatography ($CHCl_3$:MeOH = 60:1; hexane:acetone = 2:1) to give 11 mg (4% yield) as a dark red amorphous powder from methanol/acetone: mp >300 °C; $[\alpha]^{18}_D = +114.58°$ (c = 0.125, acetone); negative FABMS m/z 628 M^-; positive FABMS m/z 629 $(M + H)^+$; HRFABMS calcd for $C_{36}H_{29}O_7N_4$ 629.2036, found m/z 629.2035; IR (KBr) 3360, 1770, 1590, 1500, 1310, 820 cm^{-1}; ^1H NMR (300 MHz, acetone-d_6) δ 3.59−3.63 (m, 2H, 2,3-H), 3.65 (s, 6H, 3′,5′-OCH_3), 4.04 (dd, 1H, J = 10, 13 Hz, 11-H), 4.60 (dd, 1H, J = 8, 10 Hz, 11-H), 5.21 and 5.85 (each br s, 1H, 1,4-H), 6.45 (s, 2H, 2′,6′-H), 7.01 and 8.14 (each d, 2H, J = 11 Hz, 2‴,3‴,5‴,6‴-H), 7.60 and 8.23 (each m, 2H, 2″,3″,4″,5″-H), 8.11 (s, 1H, 8-H), 8.42, 8.91, and 8.93 (each s, 1H, 5,1″,6″-H). Anal. ($C_{36}H_{28}O_7N_4 \cdot {}^1/_2H_2O$) C, H, N.

Biological Assays. KB and 7D Cell Growth Inhibition Assay. The human nasopharyngeal carcinoma KB and KB/7d cell lines were provided by M. Fisher (Pharmacology, UNC-CH). Antiproliferative effects were evaluated by direct cell counts following a 2 day treatment. One milliliter of cell suspension (50 000 or 5000 cells) was seeded per 3.8 cm^2 well and incubated overnight. Compounds from DMSO-diluted stocks were added to media at 2 times the desired final concentration, and 1 mL was then added to cultures in duplicate. Cultures were observed microscopically for toxic and/or growth inhibitory effects at day 1 and immediately before harvesting. Cells were trypsinized and resuspended in phosphate-buffered saline containing 0.5% (w/v) trypan blue before being enumerated manually using a hemocytometer. Blue-staining cells were scored as being nonviable.

DNA Topoisomerase II Catalytic Assay. Inhibition of topoisomerase II catalytic activity was examined using the standard P4 DNA unknotting assay.[27] The reaction mixtures contained 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, nuclease-free bovine serum albumin (30 μg/mL), 0.5 mM DTT, 0.5 mM EDTA, 1 mM ATP, P4 DNA substrate (10 μg/mL), 1.25 U of enzyme, and various amounts of test compounds. The dimethyl sulfoxide carrier did not interfere with catalytic activity at the highest concentration used. After 1 h at 37 °C, the reactions were terminated by the addition of SDS-sucrose stop solution and the mixtures analyzed after horizontal agarose (0.7%, v/v) gel electrophoresis by staining

with ethidium bromide and photography (Polaroid type 667 film). Inhibition was quantified by eye, by comparing catalytic activity in test reactions to the DNA substrate and enzyme control reactions.

***In Vitro* Protein-Linked DNA Complex Formation Assay**. The method used to assess interference with drug-induced topoisomerase-mediated DNA cleavage *in vitro* was based on the published procedure.[28] The *Hin*dIII-cut PBR322 DNA was end-labeled with [α-^{32}P]dCTP (600 Ci/mmol; ICN) using a commercial T4 polymerase-labeling system. Cleavage reaction mixtures 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-mercaptoethanol, and 30 μg/mL nuclease-free BSA. Topoisomerase II (4 U) was added to the enzyme control reaction and drug treatments. After a 30 min incubation at 37 °C, reaction mixtures were divided equally and prepared for gel analysis with autoradiography and for quantifying protein-linked DNA breaks using K^+/SDS precipitation.

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References

(1) Cho, S. J.; Tropsha, A.; Suffness, M.; Cheng, Y. C.; Lee, K. H. Antitumor Agents. 163. Three-Dimensional Quantitative Structure−Activity Relationship Study of 4′-*O*-Demethylepipodophyllotoxin Analogs Using the Modified CoMFA/q^2-GRS Approach. *J. Med. Chem.* **1996**, *39*, 1383−1395.

(2) Osheroff, N.; Zechiedrich, E. L.; Gale, K. C. Catalytic Function of DNA Topoisomerase II. *BioEssays* **1991**, *13*, 269−275.

(3) Alton, P. A.; Harris, A. L. Annotation. *Br. J. Haematol.* **1993**, *85*, 241−245.

(4) Liu, L. F.; Wang, J. C. Biochemistry of DNA Topoisomerases and Their Poisons. In *DNA Topoisomerases in Cancer*; Potmesil, M., Kohn, K. W., Eds.; Oxford University Press: New York, 1991; pp 13−22.

(5) Dinardo, S.; Voelkel, K.; Sternglanz, R. DNA Topoisomerase II Mutant of *Saccharomyces Cerevisiae*: Topoisomerase II Is Required for Segregation of Daughter Molecules at the Termination of DNA Replication. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 2616−2620.

(6) Uemura, T.; Ohkura, H.; Adachi, Y.; Morino, K.; Shiozaki, K.; Yanagida, M. DNA Topoisomerase II Is Required for Condensation and Separation of Mitotic Chromosomes in S. Pombe. *Cell* **1987**, *50*, 917−925.

(7) Earnshaw, W. C.; Halligan, B.; Cooke, C. A.; Heck, M. M. S.; Liu, L. F. Topoisomerase II Is a Structural Component of Mitotic Chromosome Scaffolds. *J. Cell Biol.* **1985**, *100*, 1706−1715.

(8) Gasser, S. M.; Laemmli, U. K. The Organization of Chromatin Loops: Characterization of a Scaffold Attachment Site. *EMBO J.* **1986**, *5*, 511−518.

(9) Uemura, T.; Yanagida, M. Isolation of Type I and II DNA Topoisomerase Mutants from Fission Yeast: Single and Double Mutants Show Different Phenotypes in Cell Growth and Chromatin Organization. *EMBO J.* **1984**, *3*, 1737−1744.

(10) Holm, C.; Goto, T.; Wang, J. C.; Botstein, D. DNA Topoisomerase II Is Required at the Time of Mitosis in Yeast. *Cell* **1985**, *41*, 553−563.

(11) Zhang, Y. L.; Tropsha, A.; McPhail, A. T.; Lee, K. H. Antitumor Agents. 152. *In Vitro* Inhibitory Activity of Etoposide Derivative NPF Against Human Tumor Cell Lines and a Study of Its Conformation by X-ray Crystallography, Molecular Modeling, and NMR Spectroscopy. *J. Med. Chem.* **1994**, *37*, 1460−1464.

(12) Zhang, Y. L.; Guo, X.; Cheng, Y. C.; Lee, K. H. Antitumor Agents. 148. Synthesis and Biological Evaluation of Novel 4β-Amino Derivatives of Etoposide with Better Pharmacological Profiles. *J. Med. Chem.* **1994**, *37*, 446−452.

(13) Jehn, U.; Heinemann, V. New Drugs in the Treatment of Acute and Chronic Leukemia with Some Emphasis on m-AMSA. *Anticancer Res.* **1991**, *11*, 705−712.

(14) Baguley, B. C. DNA Intercalating Anti-Tumour Agents. *Anti-Cancer Drug Des.* **1991**, *6*, 1−35.

(15) MacDonald, T. L.; Lehnert, E. K.; Loper, J. T.; Chow, K. C.; Ross, W. E. On the Mechanism of Interaction of DNA Topoisomerase II with Chemotherapeutic Agents. In *DNA Topoisomerase in Cancer*; Potmesil, M., Kohn, K. W., Eds.; Oxford University Press: New York, 1991; pp 119−214.

(16) Sackett, D. L. Podophyllotoxin, Steganacin, and Combretastatin: Natural Products That Bind at the Colchicine Site of Tubulin. *Pharmacol. Ther.* **1993**, *59*, 163−228.

(17) Liu, S. Y.; Hwang, B. D.; Haruna, M.; Imakura, Y.; Lee, K. H.; Cheng, Y. C. Podophyllotoxin Analogs: Effects on DNA Topoisomerase II, Tubulin Polymerization, Human Tumor KB Cells, and Their VP-16-Resistant Variants. *Mol. Pharmacol.* **1989**, *36*, 78−82.

(18) Fieser, L. F.; Fieser, M. *Reagents for Organic Synthesis*; John Wiley and Sons, Inc.: New York, 1976; pp 533−536.

(19) Wang, Z. Q.; Hu, H.; Chen, H. X.; Cheng, Y. C.; Lee, K, H. Antitumor Agents. 124. New 4β-Substituted Aniline Derivatives of 6,7-*O*,*O*-Demethylene-4′-*O*-demethylpodophyllotoxin and Related Compounds as Potent Inhibitors of Human DNA Topoisomerase II. *J. Med. Chem.* **1992**, *35*, 871−877.

(20) Zhang, Y. Z.; Shen, Y. C.; Wang, Z. Q.; Chen, H. X.; Guo, X.; Cheng, Y. C.; Lee, K. H. Antitumor Agents, 130. Novel 4β-Arylamino Derivatives of 3′,4′-Didemethoxy-3′,4′-dioxo-4-deoxypodophyllotoxin as Potent Inhibitors of Human DNA Topoisomerase II. *J. Nat. Prod.* **1992**, *55*, 1100−1111.

(21) Miyahara, M.; Kashiwada, Y.; Guo, X.; Chen, H. X.; Cheng, Y. C.; Lee, K. H. Nitrosourea Derivatives of 3′, 4′-Didemethoxy-3′, 4′-dioxo-4-deoxypodophyllotoxin and Urea Derivatives of 4′-*O*-Demethylpodophyllotoxin as Potent Inhibitors of Human DNA Topoisomerase II. *Heterocycles* **1994**, *39*, 361−369.

(22) Fieser, L. F.; Fieser, M. *Reagents for Organic Synthesis*; John Wiley and Sons, Inc.: New York, 1976; pp 1011−1015.

(23) Balogh, V.; Fetizon, M.; Golfier, M. Oxidations with Silver Carbonate/Celite. V. Oxidation of Phenols and Related Compounds. *J. Org. Chem.* **1971**, *36*, 1339−1341.

(24) Zhang, Y. L.; Guo, X.; Cheng, Y. C.; Lee, K. H. Antitumor Agents. 148. Synthesis and Biological Evaluation of Novel 4β-Amino Derivatives of Etoposide with Better Pharmacological Profiles. *J. Med. Chem.* **1994**, *37*, 446−452.

(25) Klein, L. L.; Yeung, C. M.; Chu, D. T.; McDonald, E. J.; Clement, J. J.; Plattner, J. J. Synthesis and Antitumor Activity of Structural Analogues of the Epipodophyllotoxins. *J. Med. Chem.* **1991**, *34*, 984−992.

(26) Ferguson, P. J.; Fisher, M. H.; Stephenson, J.; Li, D. H.; Zhou, B. S.; Cheng, Y. C. Combined Modalities of Resistance in Etoposide-Resistant Human KB Cell lines. *Cancer Res.* **1988**, *48*, 5956−5964.

(27) Liu, L. F.; Davis, J. L.; Calendar, R. Novel Topologically Knotted DNA from Bacteriophage P4 Capsids: Studies with DNA Topoisomerases. *Nucleic Acid Res.* **1981**, *9*, 3979−3989.

(28) Harker, W. G.; Slade, D. L.; Drake, F. H.; Parr, R. L. Mitoxantrone Resistance in HL-60 Leukemia Cells: Reduced Nuclear Topoisomerase II Catalytic Activity and Drug-Induced DNA Cleavage in Association with Reduced Expression of the Topoisomerase II Beta Isoform. *Biochemistry* **1991**, *30*, 9953−9961.

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