Antitumor Agents. 194. Synthesis and Biological Evaluations of 4-β-Mono-, -Di-, and -Trisubstituted Aniline-4′-*O*-demethyl-podophyllotoxin and Related Compounds with Improved Pharmacological Profiles[1]

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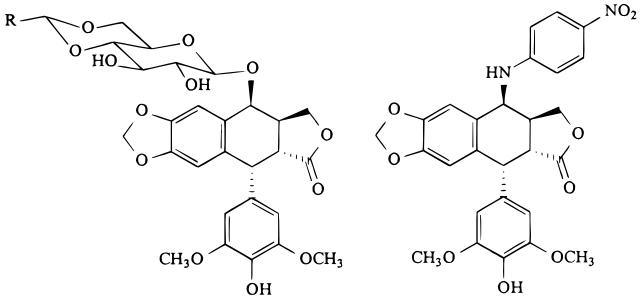
As a continuation of our structure−activity relationship studies, several new 4-β-substituted 4′-*O*-demethyl-4-desoxypodophyllotoxins bearing mono-, di-, or trisubstituted anilines have been synthesized and evaluated as inhibitors of DNA topoisomerase II and tumor cell growth in tissue culture. Selected compounds were further evaluated as cytotoxic agents using a clonogenic survival assay. The target compounds include 4′-*O*-demethyl-4β-[(4″-(benzimidazol-2″-yl)-anilino]-4-desoxypodophyllotoxin (**21**), 4′-*O*-demethyl-4β-(−)-(4″-camphanamido-anilino)-4-desoxypodophyllotoxin (**25**), 4-β-disubstituted-anilino-4′-demethyl-4-desoxypodophyllotoxins (**18**−**20, 26**), 4-α-disubstituted-anilino-4′-demethyl-4-desoxypodophyllotoxin (**27**), 4-β-trisubstituted-anilino-4′-demethyl-desoxypodophyllotoxin (**22**, **23**), and 4′-*O*-demethyl-4β-[4″-(benzimidazol-2″-yl)amino]-4-desoxypodophyllotoxin (**24**). Among the target series, **19, 21,** and **24** displayed significant growth inhibitory action against a panel of tumor cell lines including human epidermoid carcinoma of the nasopharynx (KB) and its etoposide-resistant (KB7B) and vincristine-resistant (vin20c KB) subclones, lung carcinoma (A549), human ileocecal carcinoma (HCT-8), human kidney carcinoma (CAKI-1), breast adenocarcinoma (MCF-7), and human malignant melanoma (SK-MEL-2) cells. Compounds **19**, **21**, **24**, and **25** were "cleavable-complex"-forming DNA topoisomerase II inhibitors with either improved or similar activity compared with the prototype drug etoposide (VP-16). Compound **21** was the most active analogue, being 10-fold more potent than etoposide in both cell killing and topoisomerase II inhibition in vitro assays. Using mouse models of antitumor activity, **21** was effective against (P388/0) leukemia but not against the growth of a (MCF7) mammary tumor.

Introduction

Etoposide (VP-16, **1**) and teniposide (VM-26, **2**), two semisynthetic podophyllotoxin derivatives, are important drugs currently used in the treatment of small-cell lung cancer, testicular carcinoma, lymphoma, and Kaposi's sarcoma.[2,3] Another podophyllotoxin derivative, GL-331 (**3**), discovered and developed in our laboratory, is currently undergoing phase II clinical trials for treatment of various cancers.[4]

In a previous study,[5] a comparative molecular field analysis (CoMFA) and a novel CoMFA/q^2-GRS technique were developed to identify the essential structural requirements for podophyllotoxin derivatives to form topoisomerase II−DNA complexes. In the derived model, steric and electrostatic contour plots were compared with the DNA phosphate backbone environment of the DNA−4′-*O*-demethylepipodophyllotoxin analogue complex. This comparison revealed that the CoMFA steric and electrostatic fields are compatible with stereochemical properties of the DNA backbone. Examination of the contour plots suggested that topoisomerase II inhibitory activity was related to three structurally distinct pharmacophoric domains: the DNA intercalating moiety, the minor groove binding site, and a molecular region that can accommodate a number of structurally diverse substituents, which might also interact with the minor groove (Figure 1). Active compounds do not have to interact with all three domains.[6] However, based on the stated analysis, the functional groups have to be sterically and electrostatically compatible with the environment of the DNA minor groove (i.e., inactive compounds exhibit detrimental steric interactions with the DNA backbone, and a positive charge around the 4-β-substituent is optimal



Etoposide (VP-16 **1**): R=CH_3 GL-331 (**3**)

Teniposide (VM-26, **2**): R=

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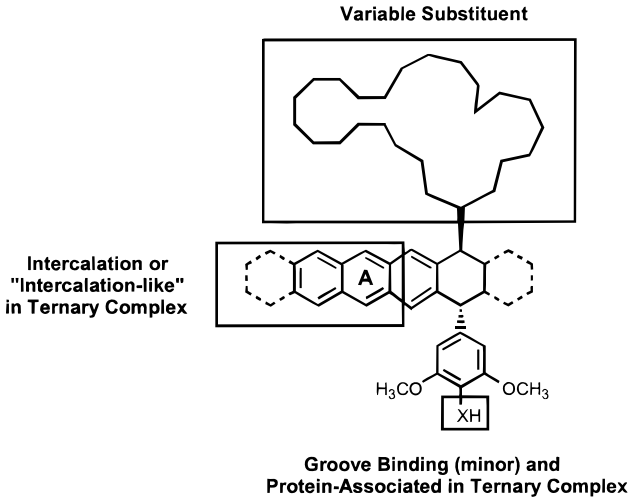
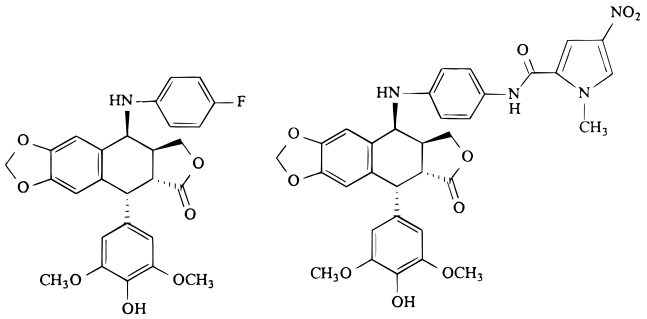


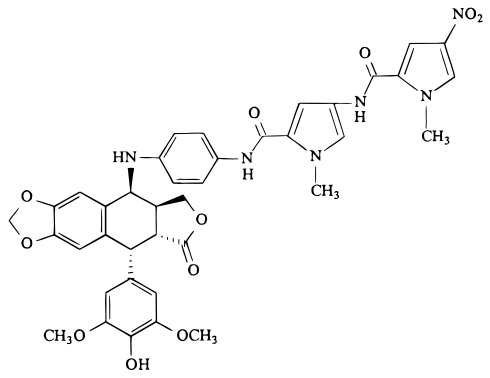
Figure 1. Composite pharmacophore model for etoposide-like analogues expressing topoisomerase II activity (adapted from ref 6).

to complement the negative rich environment of the DNA backbone).

As part of a continuing search for potential anticancer drug candidates, we have synthesized a series of substituted 4β-anilino-4′-demethyl-4-desoxypodophyllotoxins.[7] Among them, GL-331, **4**, **5**, and **6** were potent topoisomerase II inhibitors with activities comparable to those of the antitumor drugs VP-16 and VM-26. Most



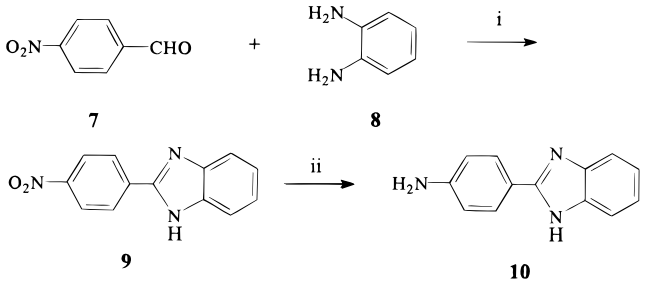
4 **5**



6

of the analogues studied to date bear a monosubstituted aniline component at the 4β-position of podophyllotoxin. In this paper, we report the synthesis and biological evaluation of analogues with di- and trisubstituted anilines at the 4β-position as well as an aniline bearing a benzimidazole group. Benzimidazole is a structural component of the cytotoxic and DNA minor groove binding agent pibenzimol (Hoechst 33258)[8−10] and, thus, is an ideal target to increase DNA topoisomerase II activity based on the ComFA analysis.

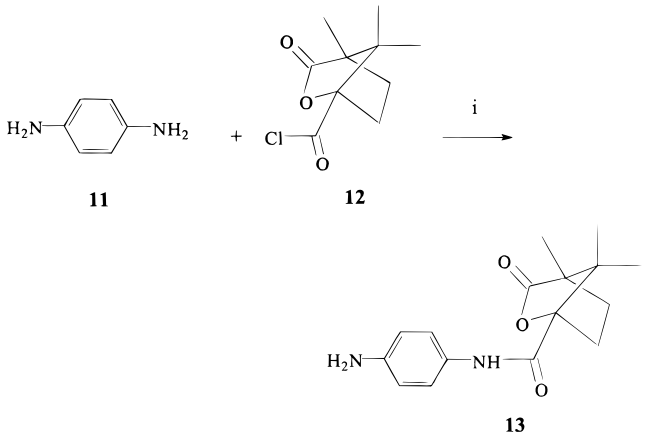
Scheme 1[a]



7 **8** **9** **10**

[a] Reagents and conditions: (i) $PhNO_2$ (nitrobenzene), DMF, 150 °C, 24 h; (ii) $NaBH_4$, MeOH.

Scheme 2[a]



11 **12** **13**

[a] Reagents: (i) pyridine, $(CH_2Cl)_2$.

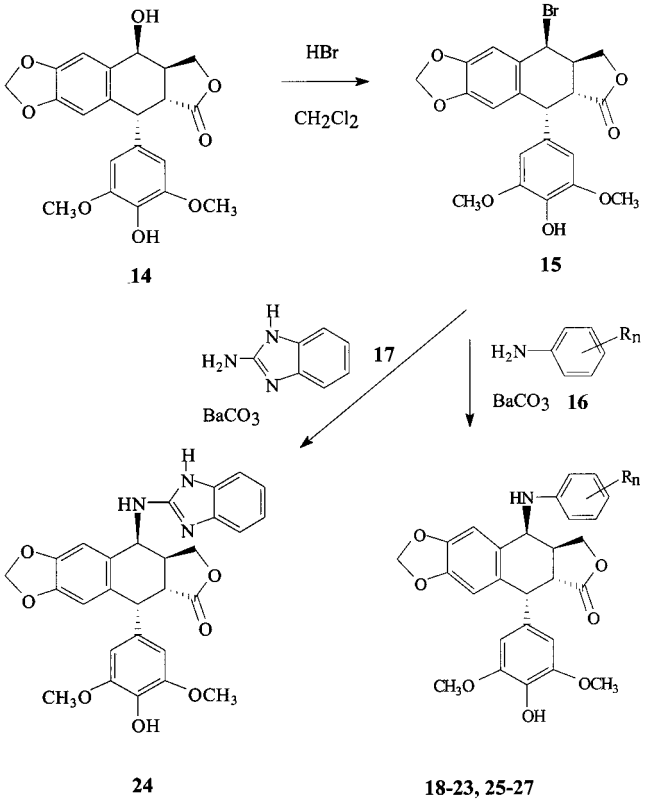
Chemistry

2-(*p*-Aminophenyl)-benzimidazole (**10**) was synthesized in two steps: oxidative condensation[11] of *p*-nitrobenzaldehyde (**7**) and 1,2-phenylenediamine (**8**) in DMF and nitrobenzene to produce intermediate **9**, and reduction of the nitro group of **9** by $NaBH_4$ in MeOH in the presence of CuCl (Scheme 1). The strong electron-withdrawing effect of the *p*-nitro group increases basicity of the imidazole functional group. Thus, the solubility of **9** decreased dramatically in most commonly used organic solvents, and therefore, this compound was quite difficult to handle and separate.

p-Camphanoylaminoaniline (**13**) was synthesized by monoacylation of 1,4-diaminoaniline with camphanic chloride (Scheme 2). Although diacylation does lower the yield, this procedure is simple, easy to process, and needs no protective groups.

The intermediate **15** was synthesized by reacting 4′-demethyl-epipodophyllotoxin (**14**) and HBr.[12] As shown in Scheme 3, the preparation of **18**−**24**, **26**, and **27** was achieved by treatment of **15** with appropriately substituted arylamines. Although this reaction occurred via a S_N1 mechanism,[13] the bulky C-1α pendent aromatic ring probably directed the stereoselective substitution, resulting in preferential formation of C-4β-oriented products, accompanied by trace amounts of the C-4α-oriented isomers (*i.e.*, **27**).

Scheme 3



Biological Results and Discussion

As illustrated in Table 1, compared to etoposide and GL-331, most 4*β*-substituted-anilino-podophyllotoxin compounds bearing di- and trisubstituted anilines (**18**−**20**, **22**, **23**, **26**, **27**) showed significantly reduced or comparable activity in inhibiting human DNA topoisomerase II and causing cellular protein−DNA breakage. The most active compounds, **21** and **25**, were almost 10-fold more potent than etoposide in inhibiting human DNA topoisomerase II and induced similar (**25**) or more (**21**) cellular protein−DNA strand breaks than etoposide under similar testing conditions. Removing the phenyl ring linking the amino aryl benzimidazole group of **21** afforded a less active compound (**24**). Generally, inhibition of tumor cell growth correlated with activity against topoisomerase II, with **21** showing the highest activity in the series in both assay types (Table 2). Compound **21** showed promise for further development since growth inhibitory activity varied no more than 7-fold against a panel of human tumor cell lines and drug-resistant KB subclones (Table 2). Furthermore, **21** was 5-fold more cytotoxic than GL-331 in a clonogenic cell survival assay (Table 1). On the basis of the in vitro testing results, **21** was compared to etoposide against implanted tumor cell growth in mouse models: P388/00, an etoposide-sensitive tumor, and MCF-7 mammary tumor, an etoposide-resistant tumor (Tables 3 and 4, respectively). Compound **21**, at highest tolerated dose, caused substantially less tumor reduction than etoposide in (P388/00) leukemia growth at treatment end (Table 3). The discrepancy between in vitro and in vivo results is not yet explained, but may be related to metabolic factors. Compound **21** gave no significant antitumor effect (results not shown). Thus, **21**, a novel and potent antitumor epipodophyllotoxin analogue, may warrant further preclinical evaluation in other species.

Experimental Section

All melting points were taken on Fisher-Johns and Mel-Temp II 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. FAB MS and HR-FAB MS spectral analyses were determined on a JEOL 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 JASCO DIP-1000 polarimeter. All new target compounds were characterized by ^{1}H and IR spectral analyses and MS analyses.

2-(*p*-Nitrophenyl)-benzimidazole (9). A mixture of 2.42 g (16 mmol) of 4-nitrobenzaldehyde, 1.78 g (16 mmol) of 1,2-diaminobenzene, 2.5 mL of nitrobenzene, and 20 mL of DMF was stirred at 150 °C under N_2 for 24 h. After the mixture cooled to room temperature, the crude product was purified by column chromatography to give 2.72 g (58.8%), mp 318−321 °C (DMF).

2-(*p*-Aminophenyl)-benzimidazole (10). To a mixture of 1.82 g (7.6 mmol) of **9**, 151 mg (1.5 mmol) of CuCl, and 30 mL of MeOH was added 575 mg (15.2 mmol) of $NaBH_4$ in portions at room temperature. After the reaction was completed as checked by TLC, the reaction mixture was filtered and evaporated to dryness. The residue was purified via chromatotron to give product (727 mg) in a 45.8% yield. The structure was verified by spectroscopic and analytical data.

Compounds **18**−**27** were prepared in an analogous manner. NMR data were similar, and complete data are given only for **18**, **21**, and **25**.

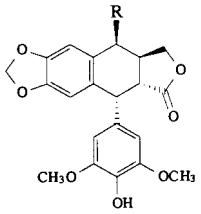
Synthesis of 4*β*-Polysubstituted-anilino-4′-demethyl-desoxypodophyllotoxins (18−27). A solution containing 4′-*O*-demethyl-4*β*-bromo-4-desoxypodophyllotoxin (**15**) (695 g, 1.5 mmol), anhydrous barium carbonate (590 g, 3.0 mmol), and the appropriate arylamine (1.65 mmol) in 15 mL of dry dichloroethane under nitrogen was stirred overnight at room temperature. The reaction mixture was filtered, diluted with EtOAc, washed with water, dried over anhydrous magnesium sulfate, and purified via silica gel column chromatography using CH_2Cl_2−acetone−EtOAc (100:5:5) as an eluent. NMR data for **18**−**27** were similar, and complete data are given only for **18**, **21**, and **25**.

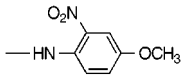
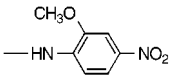
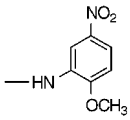
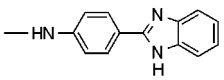
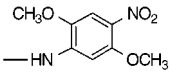
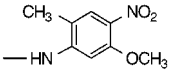
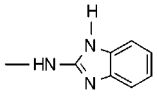
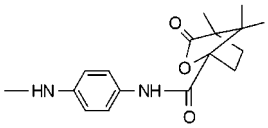
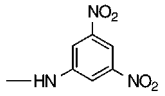
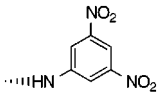
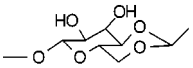
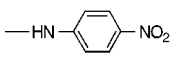
4′-*O*-Demethyl-4*β*-(2″-nitro-4″-methoxyanilino)-4-desoxypodophyllotoxin (18): yield 85.5%; crystals from acetone; mp 233−235 °C; $[\alpha]^{25}_D$ −87.2 (*c* = 0.11, $CHCl_3$); IR (KBr) 3350 (OH), 2900 (aliphatic C−H), 1758 (lactone), 1600, 1510, 1480 (aromatic C=C) cm^{-1}; MS *m/e* 550 [M]$^+$; ^{1}H NMR (DMSO-d_6, D_2O exchange) *δ* 8.32 (b, 1 H, exchangeable, 4′-OH), 7.89 (d, *J* = 6.0 Hz, exchangeable, 4-NH), 7.56 (d, *J* = 2.91 Hz, 1 H, 3″-H), 7.30 (dd, *J* = 2.91, 9.31 Hz, 1 H, 5″-H), 7.24 (d, *J* = 9.31 Hz, 1 H, 6″-H), 6.95 (s, 1 H, 5-H), 6.59 (s, 1 H, 8-H), 6.25 (s, 2 H, 2′,6′-H), 6.02 (d, 2 H, 12-H), 5.23 (dd, *J* = 4.7, 12.1, 1 H, 4-H), 4.56 (d, *J* = 5.06 Hz, 1 H, 1-H), 4.38 (t, *J* = 7.6 Hz, 1 H, 11-H), 3.79 (s, 3 H, 4″-OCH_3), 3.65 (s, 6 H, 3′,5′-OCH_3), 3.61 (t, *J* = 7.6 Hz, 1 H, 11-H), 3.28 (m, 1 H, 2-H), 3.07 (m, 1 H, 3-H); Anal. ($C_{28}H_{26}N_2O_{10}\cdot 1^1/_2H_2O$) C, H, N.

4′-*O*-Demethyl-4*β*-(2″-methoxy-4″-nitroanilino)-4-desoxypodophyllotoxin (19): yield 61.8%; crystals from MeOH; mp 199−200.5 °C; $[\alpha]^{25}_D$ −117 (*c* = 0.07, $CHCl_3$); IR (KBr) 3390 (OH), 2900 (aliphatic C−H), 1760 (lactone), 1590, 1500, 1475 (aromatic C=C) cm^{-1}; MS *m/e* 551 [M + 1]$^+$; Anal. ($C_{28}H_{26}N_2O_{10}$) C, H, N.

4′-*O*-Demethyl-4*β*-(2″-methoxy-5″-nitroanilino)-4-desoxypodophyllotoxin (20): yield 77.1%; crystals from MeOH; mp 223−224 °C; $[\alpha]^{25}_D$ −97 (*c* = 0.225, $CHCl_3$); IR (KBr) 3390 (OH), 2900 (aliphatic C−H), 1770 (lactone), 1610, 1580, 1475 (aromatic C=C) cm^{-1}; MS *m/e* 550 [M]$^+$; Anal. ($C_{28}H_{26}N_2O_{10}\cdot 1/_2H_2O$) C, H, N.

Table 1. Cytotoxic Activities and Topoisomerase II Inhibition



compd	R	cytotoxicity[a] LD_{50}	inhibition of topo II[b] IC_{100}	cellular protein−DNA[c] complex formn (%)
18		>20	>100	15 ± 6
19		>20	>100	83 ± 25
20		>20	>100	12
21		0.2	10	128 ± 12
22		ND[d]	>100	4.4 ± 0.5
23		ND	>100	3.5 ± 0.7
24		ND	100	58 ± 15
25		ND	10	88 ± 22
26		ND	>100	20 ± 1
27		ND	>100	8 ± 0.5
VP-16		20	100	100
GL-331		1	10	122 ± 7

[a] Cytotoxicity was determined by a clonogenic "cell survival" assay under conditions described in the Experimental Section. The LD_{50} is the drug concentration (μM) that reduced plating efficiency by 50% relative to control. [b] Inhibition of human DNA topoisomerase II was determined by the P4 DNA unknotting assay described in the Experimental Section. IC_{100} is the drug concentration (μM) that completely inhibited enzyme activity relative to the DNA control reaction without enzyme. [c] Cellular protein−DNA complex formation was determined by the SDS/potassium precipitation method (Experimental Section). VP-16 at 50 μm induced DNA complexes 50−56-fold over control levels. Percent values are levels induced by drug treatment (50 μm) relative to the VP-16 control set arbitrarily at 100%. [d] Not determined.

4′-*O*-Demethyl-4*β*-[4″-(benzimidazol-2″-yl)anilino]-4-desoxypodophyllotoxin (21): yield 2.1%; crystals from EtOAc; mp 82−84 °C; $[\alpha]^{25}_D$ −75 (c = 0.02, $CHCl_3$); IR (KBr) 3360 (OH), 2905 (aliphatic C−H), 1760 (lactone), 1600, 1490, 1470 (aromatic C=C) cm^{-1}; FAB MS *m/e* 592 $[M + 1]^+$; ^1H NMR (CD_3OD) δ 7.90 (d, J = 8.7 Hz, 2H, 3″,5″-H), 7.53 (m, 2H, 3″,6″-H), 7.19 (m, 2 H, 4″,5″-H), 6.83 (d, J = 8.7 Hz, 2 H, 2″,6″-H), 6.79 (s, 1 H, 5-H), 6.51 (s, 1 H, 8-H), 6.38 (s, 2 H, 2′,6′-H), 5.93 (s, 2 H, 12-H), 4.97 (d, J = 3.8 Hz, 1 H, 4-H), 4.59 (d, J = 5.1 Hz, 1 H, 1-H), 4.45 (t, J = 7.7 Hz, 1 H, 11-H), 3.91 (t, J = 7.7 Hz, 1 H, 11-H), 3.74 (s, 6 H, 3′,5′-OCH_3), 3.12 (m, 2 H, 2,3-H); HR-FAB MS (M + H; obtained in glycerol matrix) calcd for $C_{34}H_{30}N_3O_7$ 592.2084, found 592.2073.

4′-*O*-Demethyl-4*β*-(2″,5″-dimethoxy-4″-nitroanilino)-4-desoxypodophyllotoxin (22): yield 45.8%; crystals from acetone; mp 174−177 °C; $[\alpha]^{25.4}_D$ −123.3 (c = 0.15, $CHCl_3$); IR (KBr) 3350 (OH), 2900 (aliphatic C−H), 1750 (lactone), 1600, 1575, 1510, 1475 (aromatic C=C) cm^{-1}; MS *m/e* 580 $[M]^+$; Anal. ($C_{29}H_{28}N_2O_{11}$) C, H, N.

4′-*O*-Demethyl-4*β*-(2″-methyl-4″-nitro-5″-methoxyanilino)-4-desoxypodophylloto-xin (23): yield 79.7%; crystals from CH_2Cl_2; mp 199−201 °C; $[\alpha]^{26}_D$ −136.7 (c = 0.06, $CHCl_3$); IR (KBr) 3400 (OH), 2900 (aliphatic C−H), 1770 (lactone), 1610, 1570, 1520 (aromatic C=C) cm^{-1}; MS *m/e* 565 $[M + 1]^+$; Anal. ($C_{29}H_{28}N_2O_{10}$) C, H, N.

Table 2. Tumor Cell Growth Inhibition

	cell line/ED_{50}[a]							
compd	KB	KB-7d	KB-vin20c	A-549	HCT-8	CAKI-1	MCF-7	SK-MEL-2
18	0.85	0.74	0.40	1.70	2.00	0.63(25−30)[b]	2.50	3.00
19	0.32	4.00	6.70	0.63	>10(46)[c]	1.10	5.00	4.80
20	1.90	1.80	1.30	2.25	4.80	2.5(34−39)[b]	2.40	2.40
21	0.20	0.45	1.40	0.23	0.63	0.35	0.85	0.45
22	18.50	37.00	20.00	21.50	>50(38)[c]	45.00	ND	>50(46)[c]
23	44.50	ND	ND	>50(18)[c]	>50(0)[c]	>50(26)[c]	ND	>50(7)[c]
24	2.00	0.90	1.00	3.50	3.30	17.50	ND	>25(38)[c]
25	1.90	5.60	0.80	2.20	20.00	>1.25	>5.00	ND
26	10.00	17.50	>40(47)[c]	>40(46)[c]	26	10.00	>5.00(33−42)[b]	ND
27	10.00	7.60	>40(42)[c]	>5.00(20−29)[b]	>5.00(27−30)[b]	>10.00(20−21)[b]	>2.50(36−42)b	ND
VP-16	0.200[d]	23.80[d]	30.60[d]	1.95	>5(47)[c]	2.20	>5(34)[c]	ND
GL331	0.49[e]	ND	ND	ND	8.50	1.50	28.50	16.5

[a] Cytotoxicity as ED_{50} for each cell line is the concentration of compound that causes a 50% reduction in adsorbance at 562 nm relative to the untreated cell using the SRB assay (Experimental Section). [b] Survival curves plateaued at percentage values indicated in parentheses (up to 10 mM). [c] When the inhibition was below 50% at the highest test concentration, the growth inhibition value observed is indicated in the parentheses. [d] See ref 14. [e] See ref 15.

Table 3. Antitumor Activity of Compound **21** against (P388/0) Leukemia in Mice

		therapeutic response		
agent	dosage (mg/kg/dose)[a]	median day of death	%ILS	net log change in tumor burden[b]
control (saline)		10		
etoposide	40.0	26	+160	−5.5
	27.0	23	+130	−3.4
	10.0	19	+90	−0.7
21	5.0	17	+70	+0.7
	2.5	12	+20	+1.9

[a] Treatment: IV, Q4DX3. [b] Measured at end of treatment, e.g., a −6 log change means that there was 99.9999% reduction and +3 log change means there was a 1000-fold increase in tumor burden.

Table 4. Antitumor Activity of Compound **21** against Subcutaneous Mammary Tumor MCF-7

agent	dosage (mg/kg/dose)	nonspecific deaths/total	tumor-free survival	days to tumor doubling
control (saline)			1/15	17.1
etoposide	40.0	2/8	0/8	>24.1
	27.0	0/8	0/8	18.5
	10.0	8/8	0/8	
21	5.0	2/8	0/8	21.5
	2.5	8/8	1/8	18.7

4′-*O*-Demethyl-4β-[4″-benzimidazol-2″-yl)amino]-4-desoxypodophyllotoxin (24): yield 46.7%; crystals from EtOAc; mp 273−276 °C; $[\alpha]^{25}_D$ -75 (c = 0.02, $CHCl_3$); IR (KBr) 3340 (OH), 2910 (aliphatic C−H), 1770, 1700 (lactone), 1620, 1595, 1575, 1500, 1475 (aromatic C═C) cm^{-1}; MS m/e 516 $[M + 1]^+$; Anal. ($C_{28}H_{25}N_3O_7 \cdot 1/_2EtOAc$) C, H, N.

4′-*O*-Demethyl-4β-(4″-camphanamido-anilino)-4-desoxypodophyllotoxin (25): yield 69.6%; crystals from MeOH; mp 210−212 °C; $[\alpha]^{24.7}_D$ −134.0 (c = 0.19, $CHCl_3$); IR (KBr) 3350 (OH), 2950 (aliphatic C−H), 1760, 1650 (lactone), 1610, 1500, 1475 (aromatic C═C) cm^{-1}; ^1H NMR ($CDCl_3$, D_2O exchange) δ 7.43 (d, J = 8.7 Hz, 2 H, 3″,5″-H), 6.75 (s, 1 H, 5-H), 6.54 (d, J = 8.7 Hz, 2H, 2″,6″-H), 6.53 (s, 1 H, 8-H), 6.33 (s, 2 H, 2′,6′-H), 5.97 (d, J = 5.3 Hz, 2 H, 12-H), 5.45 (b, 1 H, exchangeable, 4-NH), 4.66 (d, J = 4.9 Hz, 1 H, 4-H), 4.60 (d, J = 4.8 Hz, 1 H, 1-H), 4.38 (t, J = 8.0 Hz, 1 H, 11-H), 3.98 (t, J = 8.0 Hz, 1 H, 11-H), 3.80 (s, 6 H, 3′,5′-OCH_3), 3.14 (d,d, J = 4.7, 14 Hz, 1 H, 2-H), 3.0 (m, 1 H, 3-H), 2.6, 2.05, 1.75 (each m, 4 H in total, camphanoyl-CH_2), 1.18 (s, 3 H, camphanoyl-CH_3), 1.16 (s, 3 H, camphanoyl-CH_3), 1.0 (s, 3 H, camphanoyl-CH_3); Anal. ($C_{27}H_{23}N_3O_{11}$) C, H, N·$^1/_2H_2O$.

4′-*O*-Demethyl-4β-(3″,5″-dinitro-anilino)-4-desoxypodophyllotoxin (26): yield 67%; crystals from EtOAc; mp 206−208 °C; $[\alpha]^{24}_D$ −96.9 (c = 0.23, $CHCl_3$); IR (KBr) 3350 (OH), 2950 (aliphatic C−H), 1750 (lactone), 1600, 1500, 1475 (aromatic C═C) cm^{-1}; Anal. ($C_{27}H_{23}N_3O_{11} \cdot 1^3/_4MeOH$) C, H, N.

4′-*O*-Demethyl-4β-(3″,5″-dinitro-anilino)-4-desoxypodophyllotoxin (27): yield 3%; crystals from EtOAc; mp 192−194 °C; $[\alpha]^{24.4}_D$ −34.0 (c = 0.62, $CHCl_3$); IR (KBr) 3350 (OH), 2910 (aliphatic C−H), 1760 (lactone), 1600, 1530, 1500 (aromatic C═C) cm^{-1}; Anal. ($C_{27}H_{23}N_3O_{11} \cdot {}^3/_4EtOAc$) C, H, N.

Biological Assays. The cell growth inhibition assay using sulforhodamine B (SRB) was carried out according to procedures described in Rubinstein et al.[16] Drug stock solutions were prepared in DMSO and the final solvent concentration was ≤2% DMSO (v/v), a concentration without effect on cell replication. The human tumor cell line panel consisted of epiderimoid carcinoma of the nasopharynx (KB), two subclones resistant to etoposide (KB7B) and vincristine (KB vin20c), lung carcinoma (A549), ileocecal carcinoma (HCT-8), renal cancer (CAKI-1), breast cancer (CF-7), and melanoma (SKMEL-2). Cells were cultured at 37 °C in RPMI-1640 with 100 mm/mL kanamycin and 10% (v/v) fetal bovine serum in a humidified atmosphere containing 5% CO_2. Initial seeding densities varied among the cell lines to ensure a final absorbance reading in control (untreated) cultures in the range 1−2.5 A_{562} units. Drug exposure was for 3 days, and the ED_{50} value, the drug concentration that reduced the absorbance by 50%, was interpolated from dose−response data. Each test was performed in triplicate, and absorbance readings varied no more than 5%. ED_{50} values determined in independent tests varied no more than 30%.

Cytotoxicity Assay. LD_{50} concentrations were determined by treating cells for 3 h with selected compounds at various concentrations and then replating cells to measure colony formation and plating efficiency, the percentage of total cells forming colonies. Compounds were evaluated in three independent experiments. The plating efficiencies of untreated cells varied between experiments with values ranging from 14 to 31% for KB cells. The concentration of compound that reduced plating efficiency by 50% relative to control, the LD_{50}, was determined graphically as stated in the preceding section. LD_{50} values determined in independent tests varied by no more than 15%.

Enzyme Assay. Inhibition of topoisomerase II activity was examined using the P4 DNA unknotting assay.[17] Reactions contained 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, nuclease-free bovine serum albumin (30 mg/mL), 0.5 mM DTT, 0.5 mM EDTA, 1 mM ATP, P4 DNA substrate (10 mg/mL), 0.25 U enzyme (human p170 isoform; TopoGen, Columbus, OH), 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, reactions were terminated by the addition of SDS-sucrose stop solution and analyzed after horizontal agarose (0.7%, v/v) gel

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

Cellular Protein−DNA Complex Formation. Stimulation of intracellular protein-associated DNA breaks was measured using a standard assay method.[18] Briefly, KB cells were labeled overnight with tritiated thymidine (0.5 mCi/mL, 60 Ci/mmol), chased for 2 h, and then treated in triplicate with test compounds at 15 mm. After 1 h, samples were processed, and protein−DNA complexes were measured as potassium-SDS precipitable radioactivity.

Activity in Implanted Tumor Bearing Mice. Compounds were tested for antitumor activity in mouse models by Southern Research Institute (Birmingham, AL) under a contracted agreement. Briefly, female CD 2F1 or athymic nude mice were implanted (ip) with P388/0 leukemia or (sc) with MCF-7 human mammary tumor cell, respectively. Implanted mice were treated (iv) with either saline (control), etoposide (in saline), or compound **21** in 10% DMSO + 35% PEG 300 + 5% Tween 80 + 50% D5W. Injection volume was 0.1 cm^3/10 g body weight, and treatment schedule was Q4D × 3.

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