Antitumor Agents. 234.† Design, Synthesis, and Biological Evaluation of Novel 4β-[(4′′-Benzamido)-Amino]-4′-*O*-Demethyl-Epipodophyllotoxin Derivatives

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A series of 4β-[(4′′-benzamido)-amino]-4′-*O*-demethyl-epipodophyllotoxin derivatives (**11**−**23**) were designed to enhance DNA topoisomerase II inhibition, overcome drug resistance, and modulate water solubility of etoposide (**1**) analogues. The target compounds were synthesized and evaluated for their effects against DNA topoisomerase II and KB or **1**-resistant KB-7d tumor cells in tissue culture. As compared with **1**, most compounds showed superior inhibition against both KB and KB-7d cells. Nine compounds (**13**−**18**, **20**−**22**) induced higher levels of cellular protein-linked DNA breaks than did **1**. Ten compounds selected from these and related derivatives were further examined for their antitumor spectra and drug-resistance profiles. Like **1**, these compounds selectively inhibited the growth of KB (nasopharyngeal) and 1A9 (ovarian) tumor cells. More notably, they retained inhibitory activity against etoposide-, camptothecin-, and paclitaxel-resistant KB or 1A9 subclones. In general, these C_4-modified new derivatives exhibited superior activity profiles, particularly against drug-resistant cell lines, to those of **1**. Preliminary metabolism studies on compounds **16** and **20** revealed that **20** was relatively resistant to metabolism by rat serum and liver enzymes, while **16** was metabolically unstable.

Introduction

Etoposide (**1**) and teniposide (**2**) are semisynthetic derivatives of podophyllotoxin (**3**), a bioactive component of *Podophyllum peltatum* L (Chart 1). Although podophyllotoxin is known as an antimicrotubule agent acting at the colchicine-binding site on tubulin, etoposide and teniposide inhibit DNA topoisomerase II (topo II) by stabilizing the covalent topo II−DNA cleavable complex.[2,3] Etoposide and teniposide are clinically useful against various cancers, including small-cell lung cancer, testicular carcinoma, lymphoma, and Kaposi's sarcoma;[4,5] however, their therapeutic use is often hindered by problems such as acquired drug-resistance and poor water solubility. To obtain better therapeutic agents, extensive synthetic efforts have been devoted to overcome the aforementioned problems.

As highlights, etopophos (**4**), NK 611 (**5**), GL-331 (**6**), and TOP-53 (**7**) were developed. Etopophos (**4**), a water-soluble prodrug of **1**, is readily converted in vivo by endogenous phosphatase to the active drug **1** and exhibits similar pharmacological and pharmacokinetic profiles to those of **1**. The in vivo bioavailability was increased from 0.04% to over 50% through this prodrug approach.[6,7] NK 611 (**5**) is a 2′′-dimethylamino analogue of **1** and, thus, is more water-soluble than **1**. Although **5** was more potent than **1** in topo II inhibition and cytotoxicity assays against a variety of human cancer lines, evidence for cross-resistance between **1** and **5** was found.[8,9] GL-331 (**6**) is a 4β-arylamino **1** analogue developed by our group. As a highly potent topo II inhibitor, GL-311 overcomes multidrug resistance in many, including **1**-resistant, cancer cell lines.[10] TOP-53 (**7**), a 4β-alkylated derivative of **1**, stimulated significant levels of topo II-mediated DNA cleavage when incubated with either wild-type topo II or two **1**-resistant yeast topo II mutants. TOP-53 exhibits high activity against non-small-cell lung cancer, which is usually insensitive to treatment with **1**.[11]

Prior molecular area-oriented structure−activity relationship (SAR) studies[12] and the composite pharmacophore model proposed by MacDonald et al.[13] designated the C_4 molecular area of **1** analogues as a variable region. The comparative molecular field analysis (CoMFA) models generated by us[14,15] further demonstrated that bulky substituents at C_4 might be favorable for topoisomerase II inhibition. These postulates are compatible with the excellent activity profiles of NK 611, GL-331, and TOP-53. In addition, both GL-331 and TOP-53 showed topoisomerase II inhibitory activity, antitumor spectra, and drug-resistance profiles significantly different from those of **1**, which suggested an important role of various C_4 substitutions in the activity profiles of **1** analogues and the feasibility of optimizing this compound class through rational C_4 modification. It was also implied that the C_4 molecular area would

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Chart 1. Structures of Etoposide (**1**), Teniposide (**2**), Podophyllotoxin (**3**), Etopophos (**4**), NK 611 (**5**), GL-331 (**6**), and TOP-53 (**7**)

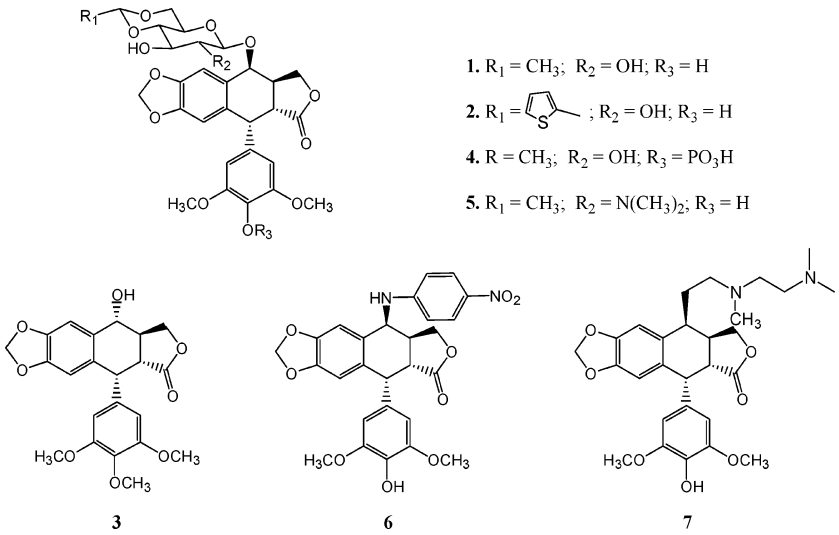
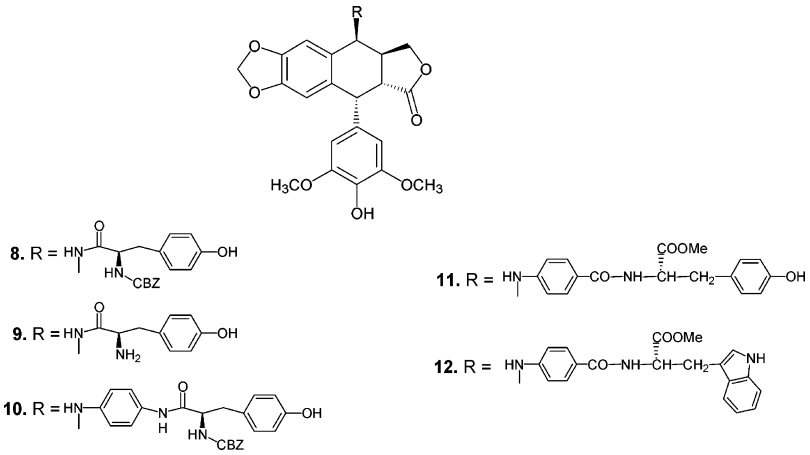


Chart 2. Structures of Compounds **8**−**12**



be appropriate for introducing water-solubility-enhancing moieties.

These critical SAR clues prompted us to perform further chemical exploration on this molecular area to address the acquired drug resistance and poor water solubility problems associated with **1** analogues. Accordingly, we recently designed and synthesized several 4β-*O*-demethyl-epipodophyllotoxin derivatives bearing bulky tails at the C_4 side chain.[16] Five of these compounds (**8**−**12**, Chart 2) showed good activity in either enzymatic or cytotoxic assays. The 4β-[(4″-benzamido)-amino]-4′-*O*-demethyl-epipodophyllotoxin derivatives **11** and **12** exhibited the most favorable activity profiles. This observation indicated that introduction of bulky substituents at the para position of the 4β-anilino moiety would enhance topo II inhibition and maintain superior cell growth inhibition and drug-resistance profiles. In this paper, we report the synthesis and biological evaluation of a series of novel 4β-[(4″-benzamido)-amino]-4′-*O*-demethyl-epipodophyllotoxin derivatives (**13**−**23**) following the previous molecular design. The 4β-[(4″-benzamido)-amino] moiety was included to retain the optimal topo II inhibitory activity and drug-resistance profiles of the 4β-arylamino epipodophyllotoxins. Protected α-amino acids were introduced to modulate the water solubility and, presumably, the bioavailability of this compound class. These amino acids and other bulky tails were introduced at the C_4 position to optimize activity profiles of the target compounds.

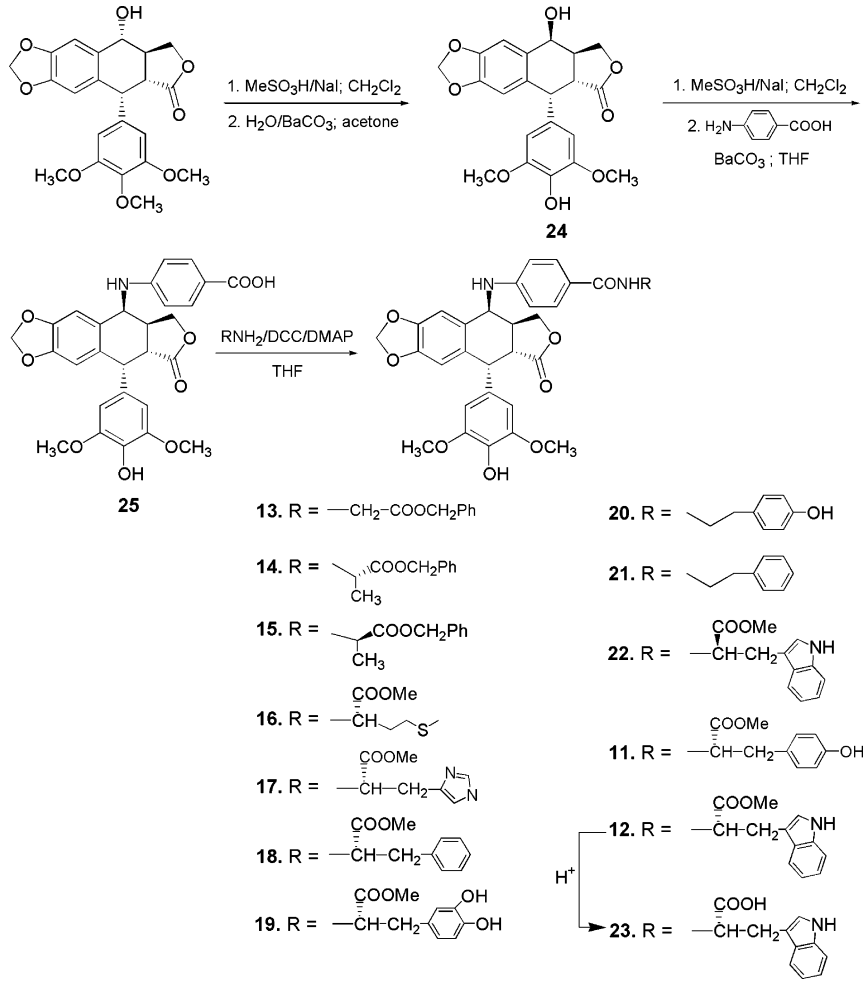
Chemistry

Compounds **11**−**23** were synthesized from podophyllotoxin (**3**) according to previously published methods (Scheme 1).[16] Briefly, 4′-demethyl-epipodophyllotoxin (DMEP, **24**) was prepared stereoselectively from **3** through successive 4′-demethylation, 4-iodination with methanesulfonic acid/sodium iodide, and nucleophilic substitution with water.[17] Nucleophilic displacement of the C_4 hydroxyl in **24** with 4-amino benzoic acid afforded intermediate **25**. Compound **25** was then condensed with the appropriate amines in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino) pyridine (DMAP) to provide compounds **11**−**22**. Acidic hydrolysis (2 N HCl/THF 1:1) of compound **12** gave compound **23**.

Results and Discussion

Compounds **11**−**23** were first evaluated for their inhibition against KB and **1**-resistant KB-7d cells and ability to induce cellular protein-linked DNA breaks

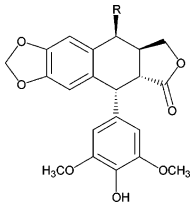
Scheme 1. Synthesis of Compounds **11**−**23**

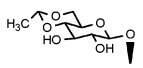
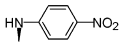
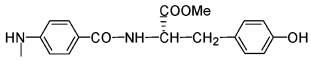
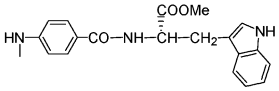
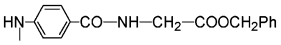
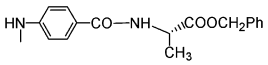
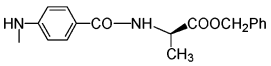
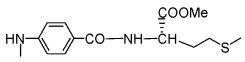
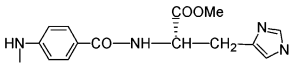
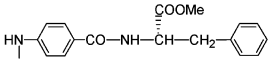
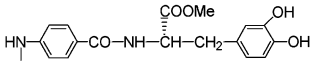
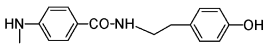
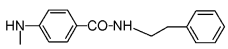
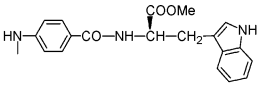
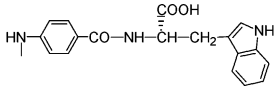


(PLDB) (Table 1). Most compounds effectively inhibited the KB cell growth and, more notably, retained inhibition against **1**-resistant KB-7d cells, thereby sharing the excellent drug-resistance profile of **6**. Nine compounds (**13**−**18** and **20**−**22**) induced more cellular protein-linked DNA breaks in KB cells than **1**, and three compounds (**16**, **20**, and **21**) were even superior to the more potent topo II inhibitor, GL-331 (**6**). The promising activity profiles of compounds **11**−**22** support our postulation that extending the 4β-[(4″-benzamido)-amino] moiety with bulky tails at the para position could favorably affect the activity of epipodophyllotoxins. Consistent with the previous observations,[18,19] the cytotoxicity of these compounds did not correlate with their ability to cause cellular protein-linked DNA breaks. Other possible mechanisms of action, such as potential effects on tubulin polymerization and differences in the drug-induced DNA break patterns, which may confer different effects on cell growth, may contribute to the lack of correlation between these parameters.

The results in Table 1 reveal some important SAR information. The large activity range of compounds **11**−**23** indicated that the substituents on the α carbon of the amino acids markedly affect the activity profiles of this compound class. The impressive ability of compounds **14**, **16**, and **18** to induce intracellular protein-linked DNA breaks suggests that a hydrophobic interaction might exist between the enzyme/DNA and this molecular area of the drugs. Inclusion of moieties containing nitrogen or oxygen atoms in the amino acid side chains (e.g., **11**, **12**, **17**, **19**, and **22**) decreased the induction of protein-linked DNA breakage. As seen with compounds **11**, **18**, and **19**, adding hydroxyl groups on the phenyl ring decreased the activity significantly. Many factors could possibly contribute to this decrease. First, hydrogen bonding between the nitrogen or oxygen atoms and the enzyme/DNA might cause the loss of activity, presumably by preventing the molecules from assuming an optimal conformation. Second, it is also possible that the relatively polar (e.g., hydroxyls in **11** and **19**, imidazole in **17**) and steric (e.g., indolyl in **12** and **22**) moieties might impede an important hydrophobic interaction between the molecules and topo II/DNA. Substitutions on the α carbon of the amino acids also showed a stereo preference. Bulky substitution was more favorable in an α-orientation than a β-orientation (**14** vs **15**; **22** vs **12**). Omission of the methyl ester on the α carbon of the amino acids significantly increased the inhibitory potency against KB cell growth; however, it also led to unfavorable drug-resistance profiles (**20** vs **11**; **21** vs **18**). In addition, although the absence of the methyl ester moiety enhanced the induction of protein-linked DNA breaks, it also reversed the activity rank (**18** was superior to **11**, while **21** was inferior to **20** in cellular protein−DNA complex formation). The unique activity profiles of **20** and **21** implied that their action mode or cellular uptake mechanisms might be different from that of their methyl ester congeners.

Table 1. Induction of Protein-Linked DNA Breaks and Inhibition of Tumor Cells by **11**−**23**



Comp.	R	%PLDB formation [a]	KB ED_{50} [b] (μg/ml)	KB-7d ED_{50} [b] (μg/ml)	Relative Resistance [c] (fold)
1		100 (100)	0.5	> 10	> 20
6		**244 (218)**	**0.2**	**2**	**10**
11		76	1.9	5	2.6
12		58	0.5	2.5	5
13		**178**	**0.5**	**0.3**	**0.6**
14		**210**	**0.5**	**0.25**	**0.5**
15		**133**	**0.1**	**0.25**	**2.5**
16		**295**	**0.8**	**2.4**	**3**
17		**122**	**0.23**	**2.2**	**9.6**
18		**226**	**0.55**	**1.0**	**1.8**
19		33	1	5.5	5.5
20		**368 (275)**	**0.025**	**0.8**	**32**
21		**(227)**	**0.035**	**0.5**	**14**
22		**121**	**0.065**	**0.4**	**6.2**
23		7	4	8	2

[a] % PLDB formation was determined by the SDS/potassium precipitation method (see Experimental Section). Percentage values are levels of protein-linked DNA breaks induced by drug treatment relative to the VP-16 control set arbitrarily at 100%. Values in parentheses reflect effects at a concentration of 5 μg/mL. Other values reflect effects at a concentration of 10 μg/mL. [b] ED_{50} is the concentration of drug that afforded 50% reduction in cell number after a 3-day incubation. [c] Relative resistance (fold) values are the ED_{50} values against KB-7d over those against KB cells.

Table 2. Inhibition of Multiple Human Tumor Cell Lines by Selected Compounds[a]

	ED_{50}[b] (μM)							
compd	KB	1A9	U-87-MG	HCT-8	MCF-7	PC-3	CAKI-1	A549
1	**0.3**	**0.15**	73.6	62.9	62.2	>100	>100	10.6
8	**3.9**	**0.94**	100	77.7	17.4	94.8	>100	15.9
9	18.8	**3.3**	>100	>100	41.7	>100	*c*	54.3
10	**0.49**	**0.0086**	>100	>100	1.9	>100	*c*	0.77
13	**0.86**	**0.12**	16.9	3.5	2.3	>100	>100	0.61
14	**0.90**	**0.05**	8.0	2.4	1.6	3.8	>100	0.70
15	**1.0**	**0.057**	39.9	2.2	2.7	>100	>100	0.47
16	**1.9**	**0.12**	45.2	11.4	9.3	82.7	>100	2.0
17	7.6	**0.69**	>100	66.0	29.8	>100	>100	16.1
18	**1.4**	**0.36**	87.1	3.8	9.6	>100	>100	1.2
22	**0.44**	**0.026**	94.3	3.4	3.6	>100	>100	0.63

[a] Cell lines: KB, nasopharyngeal; 1A9, ovarian; U-87-MG, glioblastoma; HCT-8, ileocecal; MCF-7, breast; PC-3, prostate; CAKI-1, renal; A549, lung. [b] ED_{50} is the concentration of drug that afforded 50% reduction in cell number after a 3-day incubation. [c] Not applicable.

Table 3. Inhibition of Multiple Drug-Resistant Tumor Cell Lines by Selected Compounds[a]

	ED_{50}[b] (μM)					
compd	KB	KB-VIN	KB-7d	KB-CPT	1A9	1A9-PTX10
1	0.3	>100	17.2	7.0	0.15	>5
8	3.9	>100	11.9	9.0	0.94	5.0
9	18.8	>100	30.2	44.8	3.3	>5
10	0.49	>100	0.41	1.3	0.0086	0.92
13	0.86	38.9	2.6	1.3	0.12	0.78
14	0.90	10.4	1.4	1.7	0.05	0.2
15	1.0	3.7	0.67	1.9	0.057	1.2
16	1.9	65.3	5.6	3.3	0.12	2.1
17	7.6	11.1	14.7	17.6	0.69	4.0
18	1.4	4.2	1.7	3.7	0.36	1.7
22	0.44	54.9	3.5	0.28	0.026	0.35

[a] Cell lines: KB, nasopharyngeal; KB-VIN, vincristine-resistant; KB-7d, etoposide-resistant; KB-CPT, camptothecin-resistant; 1A9, ovarian; 1A9-PTX10, paclitaxel-resistant. [b] ED_{50} is the concentration of drug that afforded 50% reduction in cell number after a 3-day incubation.

Hydrolysis of **12** to give the free acid **23** resulted in a dramatic reduction in cellular protein−DNA complex formation and cell growth inhibition. This observation agreed with the previous hypotheses that the C_4 molecular area might interact with the DNA minor groove,[14] and thereby, a potentially negative charge in this area was disfavored.

Ten compounds selected from **8**−**23** were screened against multiple human tumor cell lines to further determine their antitumor spectra and drug-resistance profiles (Tables 2 and 3). Although etoposide (**1**) was only active against KB and 1A9 tumor cells, chemical modifications of **1** produced potent antitumor agents with broader antitumor spectra (Table 2). Like **1**, all compounds showed the highest inhibition against the replication of KB (nasopharyngeal) and 1A9 (ovarian) cell lines and were basically inactive against U-87-MG (glioblastoma) and CAKI-1 (renal) tumor cells. However, most 4β-arylamino derivatives bearing an additional aromatic ring in the C_4 side chains (e.g., **10**, **13**−**15**, **18**, and **22**) exhibited inhibitory activity against HCT-8 (ileocecal), MCF-7 (breast), and A549 (lung) tumor cells (Table 2). Compound **14** showed evident inhibition against PC-3 (prostate) tumor cells. When tested against multiple drug-resistant tumor cells, these compounds retained good inhibition against the etoposide- (KB-7d) and camptothecin- (KB-CPT) resistant KB subclones, as well as the paclitaxel (1A9-PTX10)-resistant 1A9 subclone. However, some compounds (**8**−**10**, **13**, **16**, and **22**) displayed certain levels of cross-resistance with vincristine, an anticancer drug targeting tubulin, and resulted in significant loss of activity against the vincristine-resistant KB subclone (KB-VIN) (Table 3). These results implied that tubulin could be involved in the cell growth inhibitory effects of the tested compounds. The improved antitumor activity and spectra of these newly synthesized compounds demonstrated that C_4 chemical modification was indeed an effective approach to optimize the activity profiles of epipodophyllotoxin derivatives.

Compounds **16** and **20** were selected and evaluated in preliminary metabolism studies using rat serum and rat liver microsomes. Compound **16** was extensively metabolized by both serum and liver enzymes (Figure 1a,b). It was rapidly hydrolyzed in rat serum with only 4% remaining after 1 h incubation. The free acid metabolite (M − 14) was observed, and its amount increased over the incubation time (Figure 1a). Compound **16** was oxidized to give an M + 16 metabolite when incubated with rat liver microsomes. Although the parent compound continued to degrade as time proceeded, the accumulation of the metabolite M + 16 seemed to reach a plateau after 30 min (Figure 1b). The methyl ester moiety and the sulfur atom in **16** are assumed to be the primary metabolic sites by rat serum and liver microsomes, respectively. When incubated with rat serum, compound **20** was relatively stable, and no significant metabolite appeared (Figure 1c). The amount of **20** gradually decreased to 67% when incubated with rat liver microsomes for 1 h, and an oxidized metabolite (M + 16) was detected as the major metabolite. After an initial accretion, the amount of the metabolite increased very slowly over the incubation time (Figure 1d). The phenol moiety in the C_4 side chain might be responsible for the oxidative metabolism pathway.

To summarize, a series of novel 4β-[(4″-benzamido)-amino]-4′-*O*-demethyl-epipodophyllotoxin derivatives was designed based on previous chemical modification and molecular modeling studies. Nine of these compounds (**13**−**18**, **20**−**22**) showed superior preclinical activity profiles to **1**, including ability to cause cellular protein-linked DNA breaks and inhibition against KB and KB-7d cells. Selected compounds from these and related derivatives exhibited high potency against KB and 1A9 tumor cells and excellent drug-resistance profiles superior to **1**. Preliminary metabolism studies on compounds **16** and **20** indicated that **20**, but not **16**, was relatively stable in rat serum and liver microsomes. The

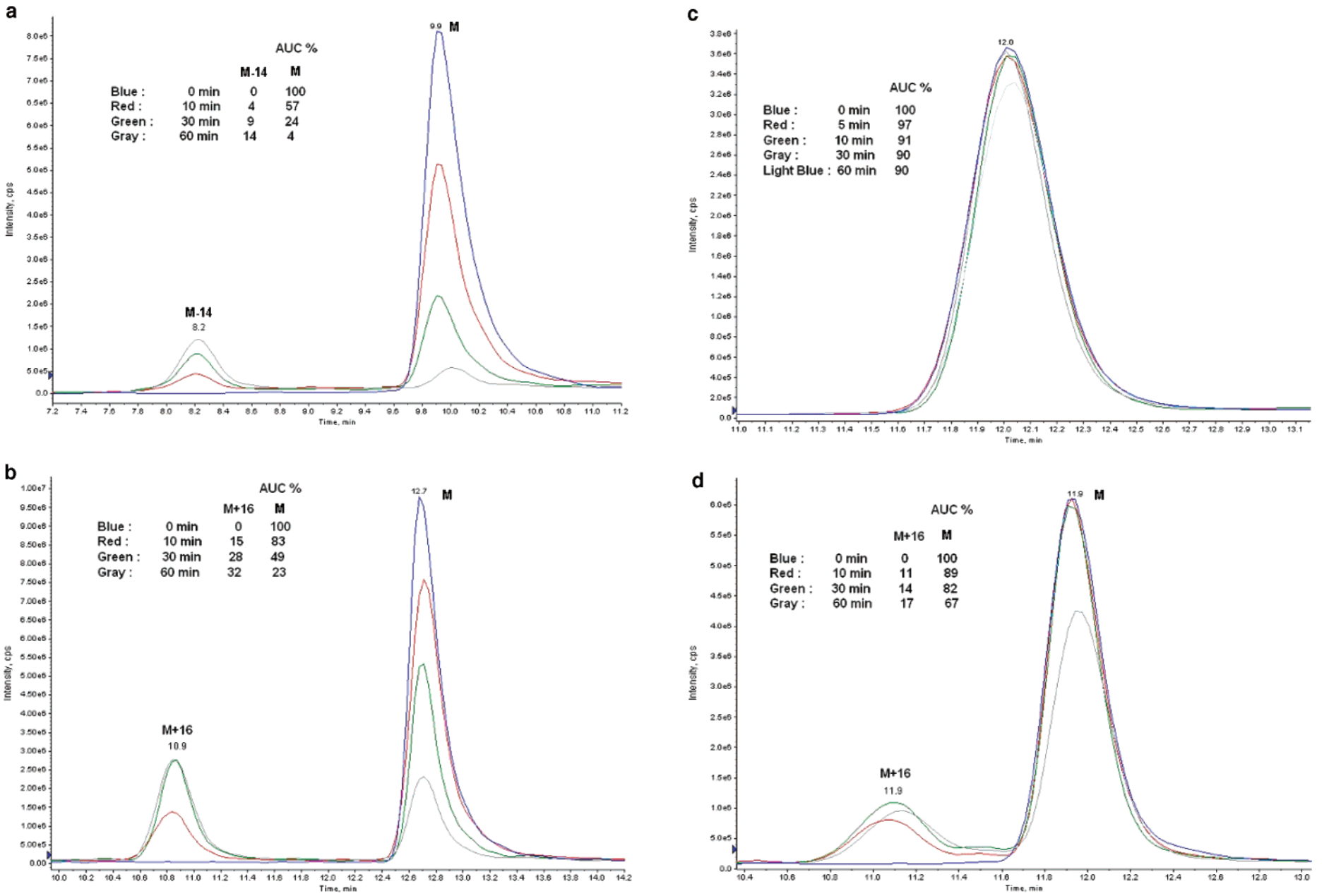


Figure 1. Metabolism of compounds **16** and **20** by rat serum or liver microsomes: (a) metabolism of **16** by rat serum; (b) metabolism of **16** by rat liver microsomes; (c) metabolism of **20** by rat serum; (d) metabolism of **20** by rat liver microsomes. Metabolites at time periods of 0, 10, 30, and 60 min were analyzed (see Experimental Section). The percentage values are AUC (area under the curve) levels at the time periods relative to those of parent compounds at 0 min set arbitrarily at 100%.

attempt to simultaneously optimize activity and modulate water solubility by including protected amino acids in the C_4 side chain was not advantageous due to significant loss of activity upon deprotection (e.g., **23**); however, extending the 4β-[(4″-benzamido)-amino] moiety with bulky tails at the para position did improve the activity profiles of this compound class. Further analogue synthesis following this molecular design is ongoing and will be reported when the studies are completed.

Experimental Section

All melting points were taken on Fisher-Johns and Mel-Temp II melting point instruments 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. Optical rotations were measured with a JASCO DIP-1000 polarimeter. HPLC analyses were carried out on an Agilent 1100 HPLC system with XIC detector. Mass spectra were recorded on a PE-Sciex API-3000 LC/MS/MS instrument equipped with a Turbo IonsSpray ion source. All new target compounds were characterized by melting point, ^{1}H and IR spectral analyses, and elemental analyses.

General Preparation of Compounds 13−23. To a solution of compound **25** (0.1 mmol) in THF (3 mL) was added dicyclohexylcarbodiimide (DCC, 22 mg, 0.11 mmol). After 15 min, an appropriate amine (0.1 mmol) was added to the reaction mixture, and the mixture was stirred at ambient temperature overnight. The suspension was diluted with 10 mL of EtOAc and was filtered. After the solvent was removed under reduced pressure, the crude product was chromatographed on a FlashElute system using a 12M silica cartridge and EtOAc/hexanes as eluant.

4′-*O*-Demethyl-4β-[4″(benzyl glycyl-*N*-carbonyl)-anilino]-4-desoxy-podophyllotoxin (13): yield 65%; mp 156−157 °C; $[\alpha]^{25}_D$ −99.0 (*c* = 0.1, acetone); IR (film) 1758 (lactone), 1728 (ester), 1701 (amide), 1653, 1508, 1458, 1376 (aromatic C=C), 1123 (phenol) cm^{-1}; MS *m/e* 665 $[M - 1]^+$; ^{1}H NMR (acetone-d_6) δ 7.79 (d, *J* = 8.7 Hz, 2 H, 3″,5″-H), 7.38 (m, 5 H, 2‴−6‴-H), 6.85 (s, 1 H, 5-H), 6.80 (d, *J* = 8.7 Hz, 2 H, 2″,6″-H), 6.54 (s, 1 H, 8-H), 6.40 (s, 2 H, 2′,6′-H), 5.98 (dd, *J* = 2.4, 0.9 Hz, 2 H, −OCH$_2$O-), 5.18 (s, 2 H, −CH$_2$−Ph), 5.05 (m, 1 H, 1-H), 4.57 (d, *J* = 4.8 Hz, 1 H, 4-H), 4.41 (t, *J* = 7.8 Hz, 1 H, 11-H), 4.16 (d, *J* = 6.0 Hz, 2 H, −CO−CH$_2$−NH−), 3.87 (t, *J* = 7.8 Hz, 1H, 11-H), 3.70 (s, 6 H, 3′,5′-OCH$_3$), 3.25 (m, 2 H, 2,3-H). Anal. ($C_{37}H_{34}N_2O_{10}$·2H$_2$O) C, H, N.

4′-*O*-Demethyl-4β-[4″(benzyl L-alanyl-*N*-carbonyl)-anilino]-4-desoxy-podophyllotoxin (14): yield 32%; mp 143−145 °C; $[\alpha]^{25}_D$ −80.0 (*c* = 0.05, acetone); IR (film) 1760 (lactone), 1726 (ester), 1700 (amide), 1470, 1455, 1365 (aromatic C=C) cm^{-1}; MS *m/e* 703 $[M + Na]^+$; ^{1}H NMR (acetone-d_6) δ 7.78 (d, *J* = 8.7 Hz, 2 H, 3″,5″-H), 7.38 (m, 5 H, 2‴−6‴-H), 6.84 (s, 1 H, 5-H), 6.78 (d, *J* = 8.7 Hz, 2 H, 2″,6″-H), 6.55 (s, 1 H, 8-H), 6.39 (s, 2 H, 2′,6′-H), 5.98 (d, *J* = 2.4 Hz, 2 H, −OCH$_2$O−), 5.18 (d, *J* = 3.0 Hz, 2 H, −CH$_2$−Ph), 5.05 (m, 1 H, 1-H), 4.90 (m, 1 H, −CO−CH−NH−), 4.56 (d, *J* = 4.8 Hz, 1 H, 4-H), 4.40 (t, *J* = 7.8 Hz, 1 H, 11-H), 3.85 (t, *J* = 7.8 Hz, 1 H, 11-H), 3.70 (s, 6 H, 3′,5′-OCH$_3$), 3.25 (m, 2 H, 2,3-H), 1.47 (d, *J* = 7.2 Hz, 3 H, −CH-*CH*$_3$−). Anal. ($C_{38}H_{36}N_2O_{10}$·2H$_2$O) C, H, N.

4′-*O*-Demethyl-4β-[4″(benzyl D-alanyl-*N*-carbonyl)-anilino]-4-desoxy-podophyllotoxin (15): yield 43%; mp 137−138 °C; $[\alpha]^{25}_D$ −130.0 (*c* = 0.1, acetone); IR (film) 1760 (lactone), 1726 (ester), 1699 (amide), 1470, 1455, 1366 (aromatic C=C), 1123 (phenol) cm^{-1}; MS *m/e* 679 $[M - 1]^+$; ^{1}H NMR (acetone-d_6) δ 7.78 (d, *J* = 8.7 Hz, 2 H, 3″,5″-H), 7.37 (m, 5 H, 2‴−6‴-H), 6.84 (s, 1 H, 5-H), 6.79 (d, *J* = 8.7 Hz, 2 H, 2″,6″-H), 6.55 (s, 1 H, 8-H), 6.39 (s, 2 H, 2′,6′-H), 5.98 (dd, *J* = 2.4, 0.9 Hz, 2 H, −OCH$_2$O−), 5.18 (d, *J* = 2.7 Hz, 2 H, −CH$_2$−Ph), 5.06 (m, 1 H, 1-H), 4.91 (m, 1 H, −CO−CH−NH−), 4.56 (d, *J* = 4.8 Hz, 1 H, 4-H), 4.40 (t, *J* = 7.8 Hz, 1 H, 11-H), 3.85 (t, *J* = 7.8 Hz, 1 H, 11-H), 3.70 (s, 6 H, 3′,5′-OCH$_3$), 3.26 (m, 2 H, 2, 3-H), 1.47 (d, *J* = 7.5 Hz, 3 H, −CH-*CH*$_3$−). Anal. ($C_{38}H_{36}N_2O_{10}$·1·1/2H$_2$O) C, H, N.

4′-*O*-Demethyl-4β-[4″(methyl L-methionyl-*N*-carbonyl)-anilino]-4-desoxy-podophyllotoxin (16): yield 66%; mp 152−157 °C; $[\alpha]^{25}_D$ −170.0 (*c* = 0.05, acetone); IR (film) 1760 (lactone), 1729 (ester), 1702 (amide), 1508, 1465, 1458 (aromatic C=C), 1121 (phenol) cm^{-1}; MS *m/e* 687 $[M + Na]^+$; ^{1}H NMR (acetone-d_6) δ 7.78 (d, *J* = 8.7 Hz, 2 H, 3″,5″-H), 6.83 (s, 1 H, 5-H), 6.79 (d, *J* = 8.7 Hz, 2 H, 2″,6″-H), 6.55 (s, 1 H, 8-H), 6.39 (s, 2 H, 2′,6′-H), 5.98 (dd, *J* = 2.7, 0.9 Hz, 2 H, −OCH$_2$O−), 5.06 (m, 1 H, 1-H), 4.80 (m, 1 H, −CO−CH−NH−), 4.57 (d, *J* = 4.5 Hz, 1 H, 4-H), 4.40 (t, *J* = 7.8 Hz, 1 H, 11-H), 3.87 (t, *J* = 7.8 Hz, 1 H, 11-H), 3.70 (s, 6 H, 3′,5′-OCH$_3$), 3.68 (s, 3 H, COOMe), 3.26 (m, 4 H, 2,3-H, −NH−CH-*CH*$_2$−). Anal. ($C_{34}H_{36}N_2O_{10}S$·1·1/2H$_2$O) C, H, N.

4′-*O*-Demethyl-4β-[4″(methyl L-histidyl-*N*-carbonyl)-anilino]-4-desoxy-podophyllotoxin (17): yield 44%; mp 189−192 °C; $[\alpha]^{25}_D$ −50.0 (*c* = 0.04, acetone); IR (film) 2955, 2911 (carboxylic acid), 1762 (lactone), 1728 (ester), 1699 (amide), 1466, 1458, 1366 (aromatic C=C), 1117 (phenol) cm^{-1}; MS *m/e* 670 $[M]^+$; ^{1}H NMR (acetone-d_6) δ 7.82 (d, *J* = 9.0 Hz, 2 H, 3″,5″-H), 7.77 (d, *J* = 8.7 Hz, 1 H, 2‴-H), 6.85 (s, 1 H, 5-H), 6.82 (d, *J* = 9.0 Hz, 2 H, 2″,6″-H), 6.81 (d, *J* = 9.3 Hz, 1 H, 5‴-H), 6.56 (s, 1 H, 8-H), 6.40 (s, 2 H, 2′,6′-H), 5.99 (dd, *J* = 2.7, 0.6 Hz, 2 H, −OCH$_2$O−), 5.09 (m, 1 H, 1-H), 4.79 (m, 1 H, −CO−CH−N−), 4.59 (d, *J* = 4.2 Hz, 1 H, 4-H), 4.43 (m, 1 H, 11-H), 3.85 (m, 1 H, 11-H), 3.71 (s, 6 H, 3′,5′-OCH$_3$), 3.62 (s, 3 H, COOMe), 3.27−3.02 (m, 4 H, 2,3-H, −NH−CH-*CH*$_2$−). Anal. ($C_{35}H_{34}N_4O_{10}$·2·1/2H$_2$O) C, H, N.

4′-*O*-Demethyl-4β-[4″(methyl l-phenylalanyl-*N*-carbonyl)-anilino]-4-desoxy-podophyllotoxin (18): yield 68%; mp 158−159 °C; $[\alpha]^{25}_D$ −142.0 (*c* = 0.2, acetone); IR (film) 1758 (lactone), 1728 (ester), 1702 (amide) 1508, 1458, 1365 (aromatic C=C) cm^{-1}; MS *m/e* 703 $[M + Na]^+$; ^{1}H NMR (acetone-d_6) δ 7.70 (d, *J* = 8.7 Hz, 2 H, 3″,5″-H), 7.29 (m, 5 H, 2‴−6‴-H), 6.83 (s, 1 H, 5-H), 6.76 (d, *J* = 8.7 Hz, 2 H, 2″,6″-H), 6.54 (s, 1 H, 8-H), 6.39 (s, 2 H, 2′,6′-H), 5.97 (dd, *J* = 2.4, 0.9 Hz, 2 H, −OCH$_2$O−), 5.05 (m, 1 H, 1-H), 4.90 (m, 1 H, −CO−CH−NH−), 4.56 (d, *J* = 4.8 Hz, 1 H, 4-H), 4.40 (t, *J* = 7.8 Hz, 1 H, 11-H), 3.85 (t, *J* = 7.8 Hz, 1 H, 11-H), 3.70 (s, 6 H, 3′,5′-OCH$_3$), 3.69 (s, 3 H, COOMe), 3.30−3.09 (m, 4 H, 2,3-H, −NH−CH-*CH*$_2$−). Anal. ($C_{38}H_{36}N_2O_{10}$·1·1/2H$_2$O) C, H, N.

4′-*O*-Demethyl-4β-[4″(methyl l-3,4-dihydroxy-phenylalanyl-*N*-carbonyl)-anilino]-4-desoxy-podophyllotoxin (19): yield 33%; mp 218 °C (dec); $[\alpha]^{25}_D$ −145.0 (*c* = 0.2, acetone); IR (film) 1773 (lactone), 1729 (ester), 1675 (amide), 1508, 1458, 1380 (aromatic C=C), 1125 (phenol) cm^{-1}; MS *m/e* 711 $[M - 1]^+$; ^{1}H NMR (acetone-d_6) δ 7.71 (d, *J* = 8.7 Hz, 2 H, 3″,5″-H), 7.08 (s, 1 H, 5-H), 6.84 (d, *J* = 8.7 Hz, 2 H, 2″,6″-H), 6,77 (m, 2 H, 2‴,3‴-H), 6.75 (m, 1 H, 6‴-H), 6.54 (s, 1 H, 8-H), 6.40 (s, 2H, 2′,6′-H), 5.98 (dd, *J* = 2.4, 0.9 Hz, 2 H, −OCH$_2$O−), 5.02 (m, 1 H, 1-H), 4.57 (d, *J* = 4.5 Hz, 1 H, 4-H), 4.42 (m, 1 H, 11-H), 3.87 (t, *J* = 7.8 Hz, 1 H, 11-H), 3.70 (s, 6 H, 3′,5′-OCH$_3$), 3.30−2.98 (m, 4 H, 2,3-H, −CO−CH-*CH*$_2$−). Anal. ($C_{38}H_{36}N_2O_{12}$·2H$_2$O) C, H, N.

4′-*O*-Demethyl-4β-[4″(tyramido)-anilino]-4-desoxy-podophyllotoxin (20): yield 82%; mp 173−175 °C; $[\alpha]^{25}_D$ −103.0 (*c* = 0.1, acetone); IR (film) 1767 (lactone), 1695 (amide), 1470, 1448, 1382 (aromatic C=C), 1122 (phenol) cm^{-1}; MS *m/e* 638 $[M]^+$; ^{1}H NMR (acetone-d_6) δ 7.72 (d, *J* = 8.7 Hz, 2 H, 3″,5″-H), 7.07 (d, *J* = 8.7 Hz, 2 H, 2‴,6‴-H), 6.84 (s, 1 H, 5-H), 6.78 (d, *J* = 8.7 Hz, 2 H, 2″,6″-H), 6.76 (d, *J* = 8.7 Hz, 2 H, 3‴,5‴-H), 6.54 (s, 1 H, 8-H), 6.39 (s, 2H, 2′,6′-H), 5.98 (dd, *J* = 2.4, 0.9 Hz, 2 H, −OCH$_2$O−), 5.06 (m, 1 H, 1-H), 4.60 (d, *J* = 4.5 Hz, 1 H, 4-H), 4.40 (t, *J* = 7.8 Hz, 1 H, 11-H), 3.85 (t, *J* = 7.8 Hz, 1 H, 11-H), 3.70 (s, 6 H, 3′,5′-OCH$_3$), 3.50 (m, 2 H, −NH−CH$_2$−), 3.26 (m, 2 H, 2,3-H), 2.79 (t, *J* = 7.8 Hz, 2 H, −CH$_2$−Ph−). Anal. ($C_{36}H_{34}N_2O_9$· 2·1/4H$_2$O) C, H, N.

4′-*O*-Demethyl-4β-[4″-(phenylethylamido)-anilino]-4-desoxy-podophyllotoxin (21): yield 84%; mp 166−167 °C; $[\alpha]^{25}_D$ −96.0 (*c* = 0.1, acetone); IR (film) 1732 (lactone), 1463 (aromatic C=C), 1120 (phenol); MS *m/e* 621 $[M - 1]^+$; ^{1}H NMR ($CDCl_3$) δ 7.50 (d, *J* = 8.4 Hz, 2 H, 3″,5″-H), 7.25−7.12 (m, 5

H, 2‴−6‴-H), 6.67 (s, 1 H, 5-H), 6.44 (d, J = 8.4 Hz, 2 H, 2″,6″-H), 6.41 (s, 1 H, 8-H), 6.23 (s, 2H, 2′,6′-H), 5.84 (dd, J = 11.4, 0.9 Hz, 2 H, −OCH_2O−), 4.65 (d, J = 3.3 Hz, 1 H, 1-H), 4.45 (d, J = 4.2 Hz, 1 H, 4-H), 4.22 (t, J = 7.5 Hz, 1 H, 11-H), 3.78 (t, J = 3.9 Hz, 1 H, 11-H), 3.65 (s, 6 H, 3′,5′-OCH_3), 3.55 (m, 2 H, −NH−CH_2−), 3.08−2.90 (m, 2 H, 2,3-H), 2.82 (t, J = 6.9 Hz, 2 H, −CH_2−Ph). Anal. ($C_{36}H_{34}N_2O_8$·$2H_2O$) C, H, N.

4′-*O*-Demethyl-4*β*-[4″(methyl d-trytophanyl-*N*-carbonyl)-anilino]-4-desoxy-podophyllotoxin (22): yield 84%; mp 172−174 °C; $[\alpha]^{25}_D$ −77.0 (c = 0.1, acetone); IR (film) 1762 (lactone), 1719 (ester), 1701 (amide) 1508, 1465, 1458 (aromatic C═C), 1121 (phenol) cm^{-1}; MS *m/e* 718 $[M - 1]^+$; ^1H NMR (acetone-d_6) δ 7.69 (d, J = 8.7 Hz, 2 H, 3″,5″-H), 7.62 (d, J = 7.8 Hz, 1 H, 4‴-H), 7.38 (d, J = 7.8 Hz, 1 H, 7‴-H), 7.07 (m, 3 H, 2‴,5‴,6‴-H), 6.83 (s, 1 H, 5-H), 6.75 (d, J = 8.7 Hz, 2 H, 2″,6″-H), 6.54 (s, 1 H, 8-H), 6.40 (s, 2 H, 2′,6′-H), 5.98 (dd, J = 2.1, 0.9 Hz, 2 H, −OCH_2O−), 5.05 (m, 1 H, 1-H), 4.95 (m, 1 H, −CO−CH−N−), 4.56 (d, J = 4.8 Hz, 1 H, 4-H), 4.40 (t, J = 7.8 Hz, 1 H, 11-H), 3.85 (t, J = 7.8 Hz, 1 H, 11-H), 3.71 (s, 6 H, 3′,5′-OCH_3), 3.67 (s, 3 H, COOMe), 3.35 (m, 4 H, 2,3-H, −NH−CH-*CH_2*−). Anal. ($C_{40}H_{37}N_3O_{10}$·2·1/$2H_2O$) C, H, N.

Preparation of 4′-*O*-demethyl-4*β*-[4″(L-trytophanyl-*N*-carbonyl)-anilino]-4-desoxy-podophyllotoxin (23). Compound **12** (0.15 mmol) was suspended in 10 mL of 2 N HCl/THF (1:1). The reaction mixture was refluxed at 75 °C for 1 h, then was neutralized to pH 7, extracted with EtOAc, dried over $NaSO_4$, and concentrated under vacuum. The crude product was chromatographed on a FlashElute system using EtOAc/hexanes as eluant to give **23**: yield 82%; mp 260 °C (dec); $[\alpha]^{25}_D$ −196.0 (c = 0.05, acetone); IR (film) 2957, 2925 (carboxylic acid), 1762 (lactone), 1694 (amide), 1463, 1456, 1366 (aromatic C═C), 1122 (phenol) cm^{-1}; MS *m/e* 704 $[M - 1]^+$; ^1H NMR (CD_3OD) δ 7.52 (d, J = 9.0 Hz, 2 H, 3″,5″-H), 7.30 (d, J = 9.0 Hz, 1 H, 1‴-H), 7.25 (d, J = 8.1 Hz, 1 H, 2‴-H), 7.02 (m, 4 H, 4‴−7‴-H), 6.67 (s, 1 H, 5-H), 6.55 (d, J = 9.0 Hz, 2 H, 2″,6″-H), 6.42 (s, 1 H, 8-H), 6.30 (s, 2 H, 2,6-H), 5.94 (d, J = 2.1 Hz, 2 H, −OCH_2O−), 4.90 (m, 1 H, 1-H), 4.49 (d, J = 4.8 Hz, 1 H, 4-H), 4.32 (t, J = 7.8 Hz, 1 H, 11-H), 3.75 (t, J = 7.8 Hz, 1 H, 11-H), 3.66 (s, 6 H, 3′,5′-OCH_3), 3.45−3.06 (m, 4 H, 2,3-H, −NH−CH-*CH_2*−). Anal. $C_{39}H_{35}N_3O_{10}$·2·H_2O.

Cell Growth Inhibition Assay. Cell growth inhibition was assayed using the sulforhodamine B (SRB) protocol developed by Rubinstein et al.[20] 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 cells were cultured at 37 °C in RPMI-1640 supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 2% (w/v) sodium bicarbonate, 10% (v/v) fetal bovine serum, and 100 μg/mL kanamycin in a humidified atmosphere containing 5% CO_2. Drug exposure was for 3 days, and the ED_{50} value, the drug concentration that reduced the cell number by 50%, was interpolated from dose−response data. Each test was performed in triplicate with variation no more than 5%. ED_{50} values determined in independent tests varied no more than 30%.

Cellular Protein−DNA Complex Formation Assay. Stimulation of intracellular protein-associated DNA breaks was measured using a standard assay method.[21] Briefly, KB cells were labeled overnight with 0.5 μCi/mL [methyl-^3H]-thymidine (60 Ci/mol), chased for 2 h, and then treated in duplicate with test compounds at 10 μg/mL. After 1 h, samples were processed, and protein-associated DNA complexes were measured as potassium−SDS precipitable radioactivity.

Metabolism Studies. For serum drug metabolism experiments, compounds (20 μM) were incubated in undiluted rat serum (Pel-Freez biologicals, Rogers, AR) for time periods of 0, 10, 30, and 60 min. Reactions were stopped by adding an equal volume of acetonitrile, and precipitated proteins were pelleted by centrifugation. Samples were then analyzed by LC/MS to identify metabolites. For liver drug metabolism studies, compounds were incubated with 1 mg/mL male Sprague−Dawley rat liver microsomes (In Vitro Technologies, Baltimore, MD) in 25 mM Tris buffer containing 0.125 mg/mL β-nicotinamide adenine dinucleotide phosphate (NADP), 0.5 mg/mL glucose-6-phosphate, and 0.5 units/mL glucose-6-phosphate dehydrogenase. Similarly to the serum assay, the reactions were terminated by adding acetonitrile at different time periods and then analyzed by LC/MS analyses.

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Supporting Information Available: Elemental analysis data for compounds **13**−**23**. This material is available free of charge via the Internet at http://pubs.acs.org.

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