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Antitumor agents. Part 235: Novel 4′-ester etoposide analogues as potent DNA topoisomerase II inhibitors with improved therapeutic potential ☆

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Abstract—Eight 4′-ester epipodophyllotoxin derivatives (**9**–**16**) were designed and synthesized with the aim to overcome drug-resistance and improve water-solubility simultaneously. These compounds were superior to etoposide (**1**) in causing cellular protein-linked DNA breaks and inhibiting KB and **1**-resistant KB-7d cell replication. Compounds **9** and **10** showed significant inhibitory activity against DNA topoisomerase II in vitro. Compound **10** also exhibited an in vitro DNA cleavage pattern similar to that of GL-331 (**5**). A hypothetical model on the action mode of **1**-analogues is proposed based on the results.

1. Introduction

Etoposide (**1**) and teniposide (**2**) are semisynthetic derivatives of the bioactive natural product podophyllotoxin (**3**). Although **3** is known as an antimitotic agent preventing the polymerization of tubulin, **1** and **2** inhibit the catalytic activity of DNA topoisomerase II (topo II).[2,3] Compounds **1** and **2** are currently used in front-line cancer chemotherapy against various cancers, including small-cell lung cancer, testicular carcinoma, lymphoma, and Kaposi's sarcoma.[4,5] However, their therapeutic uses are often hindered by problems such as poor water-solubility and acquired drug-resistance.[6] Etopophos (**4**), a water-soluble prodrug of **1**, was developed and approved for intravenous use in 1996.[7] As a phosphate, **4** improved the poor water-solubility of **1**. It was efficiently converted to **1** in vivo by endogenous phosphatase, thereby increasing the bioavailability of **1** from 0.04% to over 50%. However, **4** shared the drug-resistance profile with **1**, therefore was ineffective against **1**-resistant cancers.[8,9]

To address the afore-mentioned problems and develop better therapeutic agents, we have concentrated on chemical modification of podophyllotoxin.[6,10] Our earlier approaches focused mainly on the synthesis of unique 4β-amino and -anilino **1**-analogues, which led to the development of GL-331(**5**).[11] GL-331 was more potent than **1** as a topo II inhibitor, and more notably, overcame multidrug-resistance in many cancer cells, including **1**-resistant cancer cell lines.[10,11] GL-331 is currently in Phase II clinical evaluation against several forms of cancer, especially **1**-resistant malignancies.[12] As part of a continuing search for potential anticancer **1**-analogues, we recently discovered that three 4′-ester derivatives (**6**–**8**) (Chart 1) of GL-331 inhibited KB and **1**-resistant KB-7d tumor cell replication and exhibited in vitro topo II inhibitory activity.[13] These results, especially the in vitro topo II inhibition, challenged the long-standing structure–activity relationships (SAR) premise that a free 4′-phenol group is essential for **1**-related topo II inhibitors,[14,15] and in addition, suggested that the 4′-position might tolerate chemical modifications such as esterification. Because 4β-arylamino substitution is regarded as a structural feature critical for improved activity profiles,[16–18] we hypothesize that introduction of solubility-enhancing moieties at the 4′-position of 4β-arylamino analogues will result in both potent topo II inhibition and superior drug-resistance profiles. Based on this hypothesis, we designed a series of novel 4β-arylamino analogues with the 4′-hydroxyl group esterified by α-amino acids to simultaneously circumvent the

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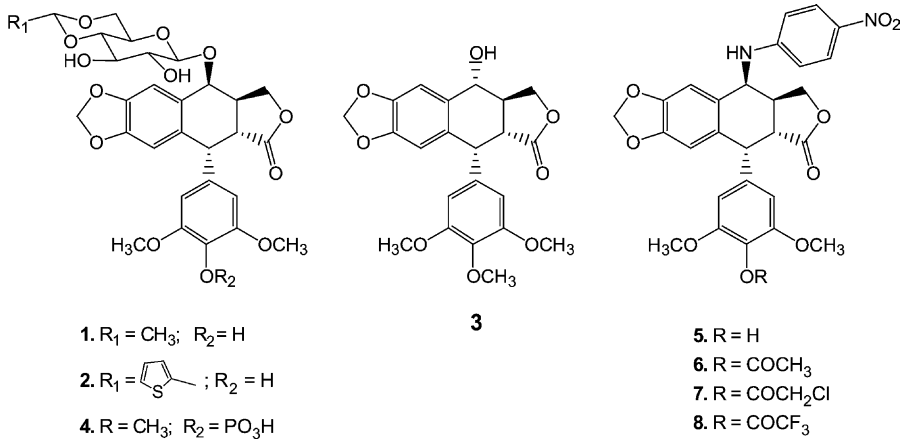


Chart 1. Structures of etoposide (**1**), teniposide (**2**), podophyllotoxin (**3**), etopophos (**4**), GL-331 (**5**), and 4′-esters of GL-331 (**6**–**8**).

drug-resistance and poor water-solubility problems associated with **1**. We report herein the synthesis and biological evaluation of eight novel 4′-ester 4β-arylamino analogues.

2. Results

2.1. Chemistry

Compounds **9**, **13**, and **16** were prepared from condensation of the appropriate 4β-arylamino **1**-analogues with *N*,*N*-dimethylglycine. Compounds **10** and **14** were obtained by treating **9** and **13**, respectively, with hydrogen chloride. Compounds **11**, **12**, and **15** were synthesized through successive condensation of 4′-*O*-demethyl-4β-(4″-fluoroanilino)-4-desoxy-podophyllotoxin (NPF, **17**) with *t*-Boc-protected α-amino acids and deprotection with *p*-toluenesulfonyl acid in dichloromethane and tetrahydrofuran (Scheme 1).

2.2. Biological evaluation

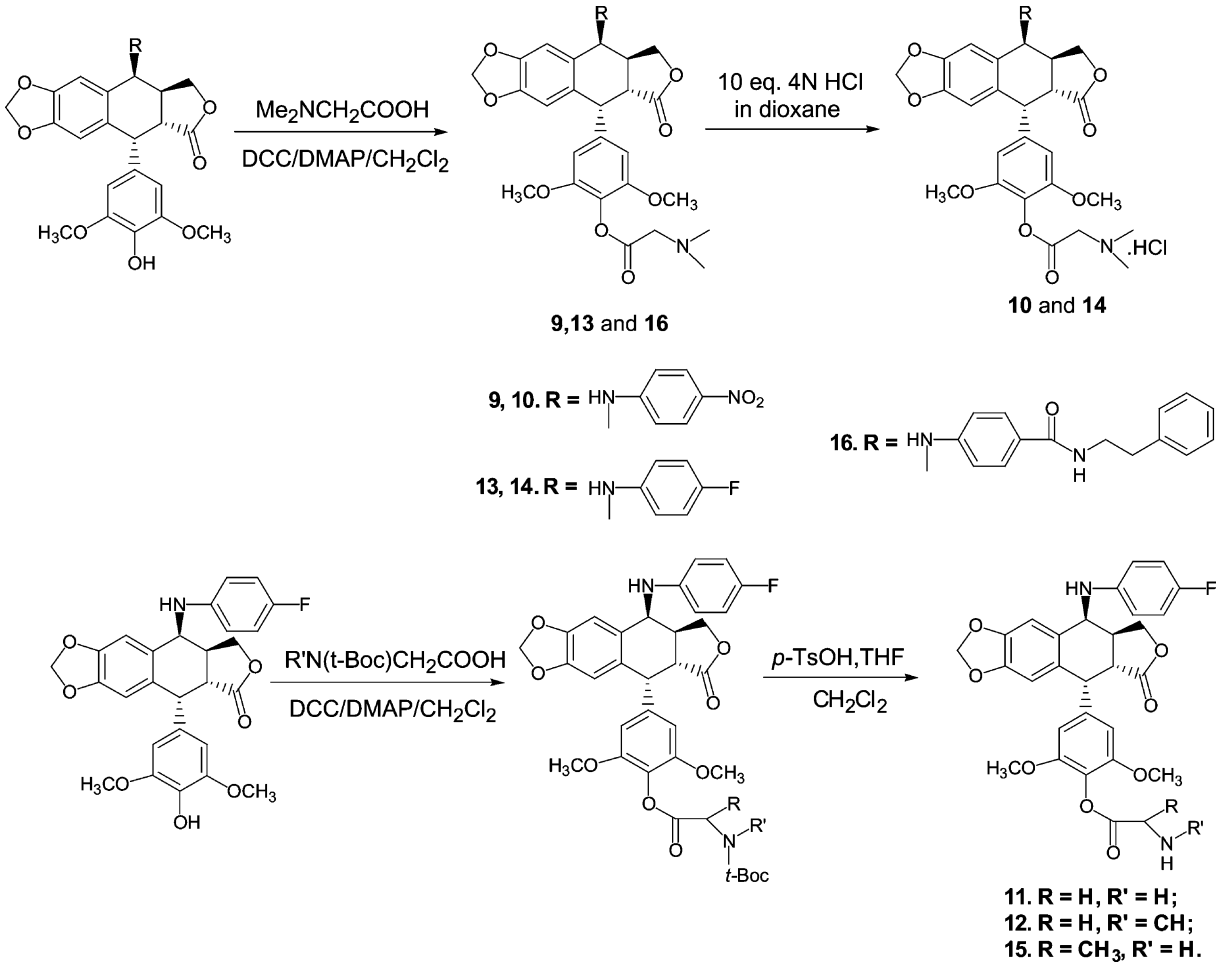
Compounds **9**–**16** were evaluated for their ability to induce cellular protein-linked DNA breaks (PLDB) and inhibit KB and **1**-resistant KB-7d cell replication (Table 1). For the purpose of comparison, their 4′-phenol congeners and etoposide (**1**) were tested in parallel. Compounds **9**–**16** showed inhibition of KB cell replication comparable or superior to that of **1**. Remarkably, these compounds exhibited significant inhibitory activity against the **1**-resistant KB-7d cells (with relative resistance of 5- to 29-fold), and thus retained the superior drug-resistance profile of the parental 4β-arylamino derivatives (with relative resistance of 6- to 21-fold).

Compared with **1**, both the prototype 4β-arylamino analogues (**5**, **17**, and **18**) and their 4′-ester congeners (**9**–**16**) caused more cellular protein-linked DNA breaks. However, this activity was generally decreased by the esterification of the 4′-phenol (**5** vs **9** and **10**; **17** vs **11**–**15**; **18** vs **16**) and was further decreased by the formation of hydrochloride salts with the terminal amines (**9** vs **10**; **13** vs **14**). The effects of 4′-esterification on PLDB induction varied with changing 4β-substitution. For example, while 4′-(*N*,*N*-dimethyl-glycyl) esterification had little effect on the PLDB induction activity of compound **5** (vs **9**), it markedly decreased the level of topo II-mediated DNA breaks in **17** (vs **13**) or **18** (vs **16**). The ability to induce topo II-mediated DNA breaks was decreased by methylation of the nitrogen or the α-carbon of the 4′-amino acid side chain as shown by the following relative activity rankings: 4′-glycyl (**11**) > 4′-sarcrosyl (**12**) > 4′-*N*,*N*′-dimethyl-glycyl (**13**) > 4′-L-alanyl (**15**). These SAR results implied an unfavorable steric effect of 4′-substitution on PLDB induction. Consistent with previous observations,[19,20] the cytotoxicity of compounds **9**–**16** did not correlate with their ability to cause cellular protein-linked DNA breaks.

Among compounds **9**–**16**, **9** showed the best activity profile, which was comparable to that of GL-331 (**5**), the epipodophyllotoxin derivative currently in Phase II clinical trials. Furthermore, with the presence of a tertiary amine, **9** can readily form water-soluble salts with inorganic or organic acids and thereby achieve the goal of simultaneous optimization of water-solubility and activity.

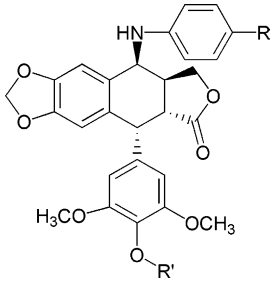
Both compounds **9** and **10** were potent topo II catalytic inhibitors and showed in vitro topo II inhibition comparable to the parent compound **5**. When the most active ester **9** was incubated at 37 °C for 1 h in the Tris–HCl buffer used for the enzymatic assay, no significant amount of hydrolytic metabolite **5** was detected using either TLC or LC-MS. Compound **9** was also incubated with topo II under the experimental conditions used for in vitro catalytic activity, and subsequently analyzed using LC-MS. The ester remained as the major component and no visible hydrolysis (metabolite **5**) was detected after incubation. These results indicated that compound **9** was an enzyme inhibitor in vitro and the intact ester was the active form responsible for in vitro activity.

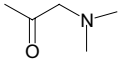
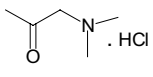
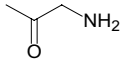
The patterns of DNA breaks induced by compounds **1**, **5**, and **10** were analyzed using an in vitro DNA cleavage



Scheme 1. Preparation of **9**–**16**.

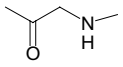
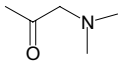
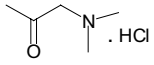
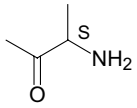
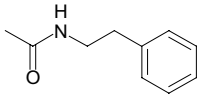
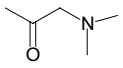
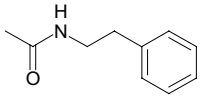
Table 1. Biological evaluation of **9**–**16**



Compound	R	R′	% PLDB formation[a]	ED_{50} (μg/mL)[b]		Relative resistance (fold)[c]
				KB	KB-7d	
1	—	—	100	0.5	>10	>20
5	NO_2	H	228	0.33	2	6
9	NO_2		219	0.4	8	20
10	NO_2		156	2	10	5
11	F		199	0.35	10	29

(*continued on next page*)

Table 1 (*continued*)

Compound	R	R′	% PLDB formation[a]	ED_{50} (μg/mL)[b]		Relative resistance (fold)[c]
				KB	KB-7d	
12	F		172	1	9	9
13	F		165	0.4	8.0	20
14	F		102	0.9	10	11
15	F		116	0.5	4	8
16			161	0.2	2	10
17[d]	F	H	213	0.24	5	21
18[e]		H	227	0.035	0.5	14

[a] PLDB formation %, percentage of cellular protein-linked DNA breaks formed relative to etoposide. All compounds were tested at 5 μg/mL.
[b] ED_{50} = concentration of drug that afforded 50% reduction in cell number after a 3-days incubation.
[c] Relative resistance = ED_{50} (KB-7d) divided by ED_{50} (KB).
[d] From Ref. 9.
[e] From Ref. 1.

assay. As shown in Figure 1, compounds **5** and **10** exhibited similar DNA cleavage patterns, which were distinct from that of **1**. Compared with **10**, compound **5** had a greater effect on in vitro topo II-mediated DNA cleavage, which is consistent with the relative abilities to induce cellular PLDB (Table 1).

3. Discussion

A free 4′-hydroxyl has been considered to be essential for topo II inhibition; however, results presented herein and elsewhere[13,21] suggest that esterification might be tolerable at this position. Solubility-enhancing moieties such as α-amino acids were introduced via esterification at the 4′-position of 4β-arylamino epipodophyllotoxins, which are **1**-analogues with superior activity profiles. This chemical manipulation led to the discovery of a series of novel 4′-esters (**9**–**16**) with improved activity profiles and water-solubility as compared to **1**. The promising results have validated the design of these novel 4′-esters and pioneered a new approach to obtain **1**-analogues with improved therapeutic potential.

While the chemical and enzymatic instability of esters makes it tempting to conclude that our novel ester derivatives might simply act as prodrugs, the results of in vitro enzymatic assays suggest otherwise and implicate a molecular interaction between the enzyme and the intact esters. These results conflicted with the previous SAR premise that a 4′-hydroxyl is required for topo II inhibition and prompted us to speculate on the roles of different molecular areas in **1**-analogues on drug–DNA–enzyme interaction.

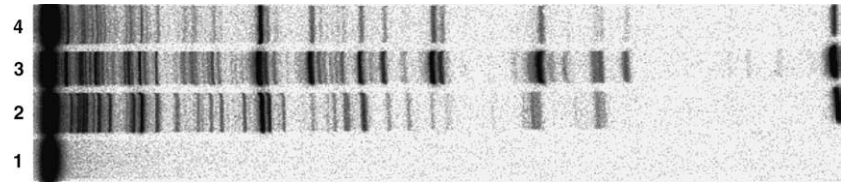


Figure 1. In vitro topo II-mediated PLDBs induced by **1**, **5**, and **10**. Lane 1 is a control lane that contains labeled DNA and topo II, lanes 2–4 also contain DNA and topo II plus compound **1** (lane 2), compound **5** (lane 3), or compound **10** (lane 4) at 25 μM.

Inhibition of topo II catalytic activity by **1**-analogues has been widely appreciated in cancer chemotherapy. These compounds trigger programmed cell death by inhibiting the ability of topo II to ligate cleaved DNA and consequently increasing the cellular level of topo II-associated DNA breaks. However, it is still unclear how these drugs interact with the enzyme–DNA complex to eventually stabilize the cleavable complex. Previous modeling studies[22,23] suggested that the pendant phenyl E ring of **1**-analogues might interact with the DNA minor groove. Indeed, we observed that steric substitution on the *para*-hydroxyl of E ring was detrimental to the ability to induce cellular PLDB, presumably by interfering with the drug–DNA interaction. Consistent with the previous report,[24] the cleavage patterns induced by **1** and **5** in this study were clearly different. The distinct cleavage patterns are probably attributed to the structural differences between the two compounds, which implies that the 4β-substitution plays a critical role in mediating drug–enzyme interactions. The unique drug-resistance profiles of 4β-arylamino analogues might also endorse such a role. In contrast, 4′-esterification did not appear to affect the site specificity of **1**-analogues, because **10** exhibited a DNA cleavage pattern identical to that of **5** and a drug-resistance profile similar to that of **5**. These results suggested a lack of crucial molecular interaction between the enzyme and the 4′-position of **1**-analogues. However, compound **10** did cause a decreased level of topo II-mediated cellular DNA breaks as compared to **5**, which might result from a less favored interaction with DNA.

Our current results shed more light on the action mode of **1**-analogues, and motivated us to propose a new hypothetical model (Fig. 2). In this composite model, the pendent E ring and the variable 4β-substitution are defined as the DNA interacting domain (**A**) and enzyme interacting domain (**B**), respectively. In a drug–DNA–topo II complex, we propose that the pendant E ring (**A**) of the **1**-analogue interacts with DNA and 'anchors' the molecule into a position that will allow the variable 4β-substitution (**B**) to extend to the active site of the enzyme. Subsequent drug–topo II interaction will likely trigger conformational and topological alterations on DNA and/or topo II, stabilize the cleavable complex, and eventually, cause cell death. Thus, molecular area **A** lacks important interaction with the enzyme, and therefore, does not affect the DNA cleavage specificity and drug-resistance profile. Instead, its interaction with DNA affects the level of DNA cleavage by **1**-analogues. In contrast, interaction between molecular area **B** and the enzyme is critical and dictates the DNA cleavage specificity and drug-resistance profile of **1**-analogues. This simplified model designates DNA and topo II interaction to disparate molecular areas. Its aim is to emphasize the different roles of various molecular areas in the drug–DNA–enzyme interactions. However, it is highly possible that promiscuous interactions exist. Further understanding of how both the E ring and 4β-substitution affect the action mode of **1**-analogues should facilitate the rational design of this important class of antitumor compounds.

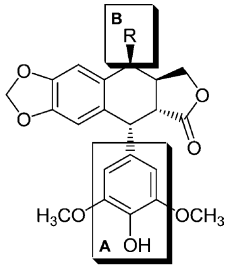


Figure 2. Hypothetical model on the action mode of **1**-analogues.

4. Experimental

4.1. General methods

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, as well as elemental analyses.

4.2. General preparation of 9, 13, and 16

To a solution of the appropriate 4β-arylamino **1**-analogues (0.2 mmol) and *N*,*N*-dimethylglycine (32 mg, 0.3 mmol) in dichloromethane (10 mL) were added *N*,*N*-dicyclohexylcarbodiimide (DCC, 62 mg, 0.3 mmol) and 4-(dimethylamino)pyridine (DMAP, 24 mg, 0.2 mmol). The reaction mixture was stirred under nitrogen at room temperature for 24 h. Then, the suspension was filtered, concentrated, and purified with a FlashElute system using EtOAc/hexanes/Et_3N as eluant.

4.2.1. 4′-*O*-Demethyl-4′-(*N*,*N*-dimethyl-glycyl)-4β-(4″-nitroanilino)-4-desoxy-podophyllotoxin (9). Ninety percent yield; mp 179–180 °C; $[\alpha]_D^{25}$ −252.0 (*c* 0.05, acetone); MS *m*/*e*: 605 $[M-1]^+$; ^{1}H NMR ($CDCl_3$) δ 8.11 (d, $J = 9.3$ Hz, 2H, 3″, 5″-H), 6.74 (s, 1H, 5-H), 6.56 (d, $J = 9.0$ Hz, 2H, 2″, 6″-H), 6.53 (s, 1H, 8-H), 6.32 (s, 2H, 2′, 6′-H), 5.95 (dd, $J = 4.8$, 1.5 Hz, 2H, –OCH_2O–), 4.79 (m, 1H, 1-H), 4.68 (d, $J = 4.8$ Hz, 1H, 4-H), 4.38 (m, 1H, 11-H), 3.79 (m, 1H, 11-H), 3.67 (s, 6H, 3′, 5′ –OCH_3), 3.45 (s, 2H, –CO–CH_2–), 2.41 (s, 6H, –$N(CH_3)_2$).

4.2.2. 4′-*O*-Demethyl-4′-(*N*,*N*-dimethyl-glycyl)-4β-(4″-fluoroanilino)-4-desoxy-podophyllotoxin (3). Eighty-four percent yield; mp 127–129 °C; $[\alpha]_D^{25}$ −101.0 (*c* 0.1, acetone); MS *m*/*e*: 577 $[M-1]^+$; ^1H NMR (acetone-d_6) δ 6.94 (dd, $J = 8.7$, 8.7 Hz, 2H, 3″, 5″-H), 6.82 (s, 1H, 5-H), 6.75 (dd, $J = 9.0$, 4.5 Hz, 2H, 2″, 6″-H), 6.55 (s, 1H, 8-H), 6.47 (s, 2H, 2′, 6′-H), 5.97 (dd, $J = 6.9$, 1.0 Hz, 2H, –OCH_2O–), 4.91 (m, 1H, 1-H), 4.64 (d, $J = 4.8$ Hz, 1H, 4-H), 4.42 (t, $J = 7.2$ Hz, 1H, 11-H), 3.92 (t, $J = 7.2$ Hz, 1H, 11-H), 3.68 (s, 6H, 3′, 5′-OCH_3), 3.39 (s, 2H, –CO–CH_2–), 3.32 (m, 1H, 2-H), 3.18 (m, 1H, 3-H), 2.35 (s, 6H, –$N(CH_3)_2$).

4.2.3. 4′-*O*-Demethyl-4′-(*N*,*N*-dimethyl-glycyl)-4β-[4″-(phenylethylamido)-anilino]-4-desoxy-podophyllotoxin (16). Forty-four percent yield; mp 150–151 °C; $[\alpha]_D^{30}$ −100.0 (*c* 0.1, acetone); MS *m*/*e*: 706 $[M-1]^+$; ^1H NMR (acetone-d_6) δ 7.75 (d, $J = 9.0$ Hz, 2H, 3″, 5″-H), 7.27–7.19 (m, 5H, 2‴–6‴H), 6.86 (s, 1H, 5-H), 6.78 (d, $J = 9.0$ Hz, 2H, 2, 6″-H), 6.56 (s, 1H, 8-H), 6.47 (s, 2H, 2′, 6′-H), 5.98 (dd, $J = 4.5$, 0.9 Hz, 2H, –OCH_2O–), 5.03 (m, 1H, 1-H), 4.65 (d, $J = 4.8$ Hz, 1H, 4-H), 4.40 (t, $J = 7.5$ Hz, 1H, 11-H), 3.88 (t, $J = 5.4$ Hz, 1H, 11-H), 3.69 (s, 6H, 3′, 5′-OCH_3), 3.56 (m, 2H, –NH–CH_2–), 3.39 (s, 2H, –CO–CH_2–), 3.32–3.15 (m, 2H, 2, 3-H), 2.89 (t, $J = 7.2$ Hz, 2H, –CH_2–Ph), 2.35 (s, 6H, –$N(CH_3)_2$).

4.3. General preparation of 10 and 14

Compound **9** or **13** (0.05 mmol) was dissolved in acetone (1 mL), and 125 μL 4.0 N hydrogen chloride in dioxane was added. After being concentrated under vacuum, the residue was washed with dichloromethane to give **10** and **14**, respectively.

4.3.1. 4′-*O*-Demethyl-4′-(*N*,*N*-dimethyl-glycyl)-4β-(4″-nitroanilino)-4-desoxy-podophyllotoxin hydrochloride (10). Ninety-two percent yield; mp 201–202 °C; $[\alpha]_D^{25}$ −118.0 (*c* 0.2, acetone); ^1H NMR (CD_3OD) δ 8.11 (d, $J = 9.3$ Hz, 2H, 3″, 5″-H), 6.77 (s, 1H, 5-H), 6.72 (d, $J = 9.3$ Hz, 2H, 2″, 6″-H), 6.54 (s, 1H, 8-H), 6.46 (s, 2H, 2′, 6′-H), 5.94 (d, $J = 4.5$ Hz, 2H, –OCH_2O–), 4.89 (m, 1H, 1-H), 4.68 (d, $J = 4.8$ Hz, 1H, 4-H), 4.36 (m, 1H, 11-H), 3.74 (m, 1H, 11-H), 3.71 (s, 2H, –CO–CH_2–), 3.66 (s, 6H, 3′, 5′-OCH_3), 3.29 (m, 2H, 2, 3-H), 2.92 (s, 6H, –$N(CH_3)_2$).

4.3.2. 4′-*O*-Demethyl-4′-(*N*,*N*-dimethyl-glycyl)-4β-(4″-fluoroanilino)-4-desoxy-podophyllotoxin hydrochloride (14). Ninety-seven percent yield; mp 162–163 °C; $[\alpha]_D^{25}$ −117.0 (*c* 0.05, acetone); ^1H NMR (CD_3OD) 6.83 (dd, $J = 8.7$, 8.7 Hz, 2H, 3″, 5″-H), 6.68 (s, 1H, 5-H), 6.58 (dd, $J = 9.0$, 4.5 Hz, 2H, 2″, 6″-H), 6.44 (s, 1H, 8-H), 6.40 (s, 2H, 2′, 6′-H), 5.86 (d, $J = 7.8$ Hz, 2H, –OCH_2O–), 4.72 (m, 1H, 1-H), 4.63 (d, $J = 4.8$ Hz, 1H, 4-H), 4.35 (t, $J = 7.2$ Hz, 1H, 11-H), 3.88 (t, $J = 7.2$ Hz, 1H, 11-H), 3.65 (s, 6H, 3′, 5′-OCH_3), 3.59 (s, 2H, –CO–CH_2–), 3.30 (m, 2H, 2, 3-H), 2.98 (s, 6H, –$N(CH_3)_2$).

4.4. General preparation of 11, 12, and 15

To a solution of NPF (0.2 mmol) and the appropriate *t*-BOC protected amino acid (0.22 mmol) in CH_2Cl_2 (10 mL) were added DCC (62 mg, 0.3 mmol) and DMAP (24 mg, 0.2 mmol). The reaction mixture was stirred under nitrogen at room temperature for 24 h. Then, the suspension was filtered, concentrated, and purified to provide N-*t*-BOC protected esters, which were treated with *p*-TsOH in CH_2Cl_2 and THF for 1 h at room temperature. The reaction mixtures were diluted with CH_2Cl_2, washed with 5% $NaHCO_3$ solution, and dried over Na_2SO_4. A FlashElute system with EtOAc/hexanes/Et_3N as eluant was used to purify **11**, **12**, and **15**.

4.4.1. 4′-*O*-Demethyl-4′-glycyl-4β-(4″-fluoroanilino)-4-desoxy-podophyllotoxin (11). Twenty-five percent yield (from NPF); mp 133–135 °C; $[\alpha]_D^{25}$ −69.0 (*c* 0.1, acetone); MS *m*/*e*: 549 $[M-1]^+$; ^1H NMR (CD_3OD) δ 6.89 (dd, $J = 8.7$, 8.7 Hz, 2H, 3″, 5″-H), 6.75 (s, 1H, 5-H), 6.63 (dd, $J = 9.0$, 4.5 Hz, 2H, 2″, 6″-H), 6.51 (s, 1H, 8-H), 6.44 (s, 2H, 2′, 6′-H), 5.92 (d, $J = 4.5$ Hz, 2H, –OCH_2O–), 4.67 (m, 1H, 1-H), 4.53 (d, $J = 4.5$ Hz, 1H, 4-H), 4.41 (m, 1H, 11-H), 3.90 (m, 1H, 11-H), 3.69 (s, 6H, 3′, 5′-OCH_3), 3.30 (s, 2H, –CO–CH_2–), 3.28 (m, 1H, 2-H), 3.08 (m, 1H, 3-H).

4.4.2. 4′-*O*-Demethyl-4′-sarcrosyl-4β-(4″-fluoroanilino)-4-desoxy-podophyllotoxin (12). Thirty-eight percent yield (from NPF); mp 138–139 °C; $[\alpha]_D^{25}$ −104.0 (*c* 0.2, acetone); MS *m*/*e*: 563 $[M-1]^+$; ^1H NMR (CD_3OD) δ 6.90 (dd, $J = 8.7$, 8.7 Hz, 2H, 3″, 5″-H), 6.73 (s, 1H, 5-H), 6.63 (dd, $J = 8.7$, 4.5 Hz, 2H, 2″, 6″-H), 6.48 (s, 1H, 8-H), 6.35 (s, 2H, 2′, 6′-H), 5.92 (dd, $J = 4.5$, 1.5 Hz, 2H, –OCH_2O–), 4.75 (d, $J = 4.2$ Hz, 1H, 1-H), 4.56 (d, $J = 4.8$ Hz, 1H, 4-H), 4.39 (t, $J = 7.2$ Hz, 1H, 11-H), 3.90 (t, $J = 7.2$ Hz, 1H, 11-H), 3.70 (s, 6H, 3′, 5′-OCH_3), 3.31 (s, 2H, –CO–CH_2–), 3.16 (m, 1H, 2-H), 3.09 (m, 1H, 3-H), 2.43 (s, 3H, –NH(CH_3)).

4.4.3. 4′-*O*-Demethyl-4′-L-alanyl-4β-(4″-fluoroanilino)-4-desoxy-podophyllotoxin (15). Thirty-one percent yield (from NPF); mp 135–137 °C; $[\alpha]_D^{30}$ −119.0 (*c* 0.2, acetone); MS *m*/*e*: 563 $[M-1]^+$; ^1H NMR ($CDCl_3$) δ 6.92 (dd, $J = 8.7$, 8.7 Hz, 2H, 3″, 5″-H), 6.73 (s, 1H, 5-H), 6.47 (dd, $J = 8.7$, 4.5 Hz, 2H, 2″, 6″-H), 6.46 (s, 1H, 8-H), 6.34 (s, 2H, 2′, 6′-H), 5.96 (dd, $J = 4.5$, 1.8 Hz, 2H, –OCH_2O–), 4.60 (d, $J = 4.2$ Hz, 1H, 1-H), 4.57 (d, $J = 4.8$ Hz, 1H, 4-H), 4.30 (t, $J = 7.2$ Hz, 1H, 11-H), 3.92 (t, $J = 7.2$ Hz, 1H, 11-H), 3.69 (s, 6H, 3′, 5′-OCH_3), 3.62 (m, 1H, –CO–CH–), 3.16 (m, 1H, 2-H), 2.91 (m, 1H, 3-H), 1.55 (d, $J = 6.9$ Hz, 3H, –CH(CH_3)).

5. Biology

5.1. Cell growth inhibition assay

Cell growth inhibition was assayed using the sulforhodamine B (SRB) protocol developed by Rubinstein

et al.[25] Drug exposure was for 3 days, and the ED_{50} value was interpolated from dose–response data.

5.2. K^+-SDS precipitation assay for cellular protein–DNA complex formation

Stimulation of intracellular protein-associated DNA breaks was measured based on a standard method,[26] and the procedure was fully described in a previous publication.[19] All compounds were tested in duplicate at 5 μg/mL.

5.3. In vitro DNA topoisomerase II assay

This assay was carried out according to the procedure described previously.[27] Assays were performed with a drug concentration of 50 μg/mL.

5.4. Mapping of drug-induced topoisomerase II cleavage sites

A singly-labeled 564 base pair DNA substrate was prepared from plasmid pBR322. The plasmid was digested with *Eco*RI and dephosphorylated with alkaline phosphatase followed by phosphorylation with [γ-^{32}] ATP by T4 polynucleotide kinase. After a second digestion with *Sph*I, the fragment of interest was isolated by electrophoresis on a 5% nondenaturing polyacrylamide gel.

Sites of drug-induced DNA cleavage generated by human topoisomerase IIα were determined by incubating topoisomerase IIα (2 units) with ~20 ng labeled DNA in 20 μL of topoisomerase II cleavage buffer in the presence of 25 μM drug. Reactions were initiated by the addition of enzyme and were incubated at 37 °C for 10 min. Cleavage complexes were trapped by the addition of SDS (1% final concentration) and digested with proteinase K. Formamide loading buffer was added and samples were electrophoresed on an 8% polyacrylamide/7 M urea sequencing gel. Cleavage products were visualized using a Molecular Dynamics PhosphorImager.

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