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Novel carbamate derivatives of 4-*β*-amino-4′-*O*-demethyl-4-desoxypodophyllotoxin as inhibitors of topoisomerase II: synthesis and biological evaluation

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A novel series of carbamate derivatives of 4-*β*-amino-4′-*O*-demethyl-4-desoxypodophyllotoxin were synthesized. Their effect on human DNA topoisomerase II and antiproliferative activity was evaluated. Compounds **4a**–**c**, **4g**, **4j** and **4k** are topoisomerase II poisons that induce double-stranded breaks in DNA and exhibit increased cytotoxicity compared to etoposide.

Introduction

Etoposide (VP16) is a semisynthetic glycoside derivative of podophyllotoxin (Fig. 1) and is one of the most extensively used anticancer drugs in the treatment of several types of tumors, including testicular and small cell lung cancer, lymphoma, leukemia and Kaposi's sarcoma.[1–3] Despite its extensive use in cancer chemotherapy, it presents several limitations, such as poor water solubility, the development of drug resistance, metabolic inactivation, myelosuppression and toxicity.[4] Etoposide and the 4′-demethylepipodophyllotoxin (4′-DMEP) derivatives in general (Fig. 1) unlike podophyllotoxin, do not inhibit tubuline polymerisation, but inhibit an ubiquitous and essential enzyme: human DNA topoisomerase II.[5,6] This enzyme controls DNA topology by transient cleavage of the DNA double helix.[7,8] When the concentration of these transient intermediates (called cleavage complexes) increases, DNA strand breaks occur. 4′-DMEP derivatives, such as VP16, kill cells by increasing the levels of topoisomerase II-mediated DNA cleavage and are thus called topoisomerase II poisons.[5,9,10]

In order to overcome the limitations cited above, and to develop more active and more potent analogues, several chemical modifications have been carried out on the 4′-demethylepipodophyllotoxin structure. QSAR studies suggested that the essential structural features for antitopoisomerase II activity are: the 4′-hydroxyl group, the 4-β-stereochemistry and the 4-substitution of 4′-demethylepipodophyllotoxin.[11,12,13] The latter has been extensively modified and the glycoside moiety of etoposide has been replaced with non-sugar groups leading to, in some cases, better activity compared to etoposide.[14,15]

Recently, we reported that some non glycoside 4′-demethylepipodophyllotoxin derivatives bearing a carbamate residue at the 4 position (4′-demethylepipodophyllotoxin-4-aminoalkyl carbamates, Fig. 1) strongly inhibited topoisomerase II and showed antiproliferative effects *in vitro* and antitumor activity *in vivo*.[16]

This prompted us to further investigate carbamate substitution in this position. We report herein the synthesis of a novel series of carbamates bearing variable aliphatic or aromatic side chains obtained by introducing a nitrogen atom in the 4β position (Scheme 1, **4**). The relationship between cytotoxicity and inhibition of topoisomerase II, as well as the DNA binding affinity, were studied. The compounds are more cytotoxic than etoposide and some are good topoisomerase II inhibitors. Finally, structure–activity relationships were established for this new class of compounds.

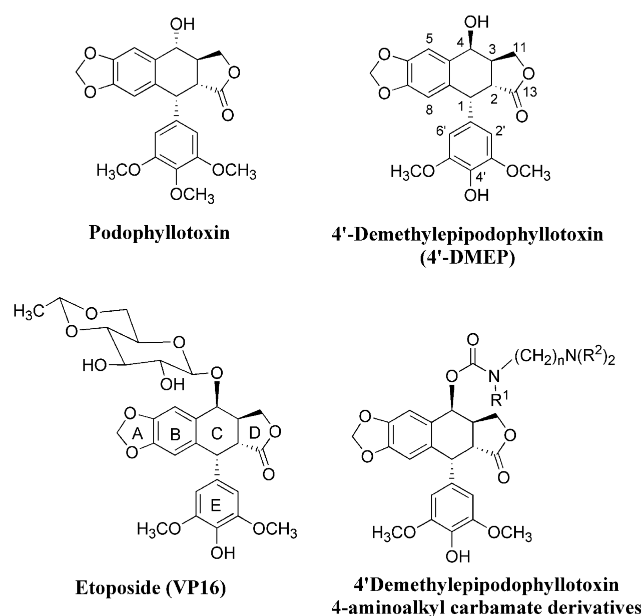

Fig. 1 Chemical structures of podophyllotoxin, 4′-DMEP, VP16 and the 4′-demethylepipodophyllotoxin 4-aminoalkyl carbamates previously synthesized.

Results and discussion

Chemistry

The synthesis of these novel 4-β-amino-4′-*O*-demethyl-4-desoxypodophyllotoxin derivatives (**4a**–**m**) is depicted in Scheme 1. The

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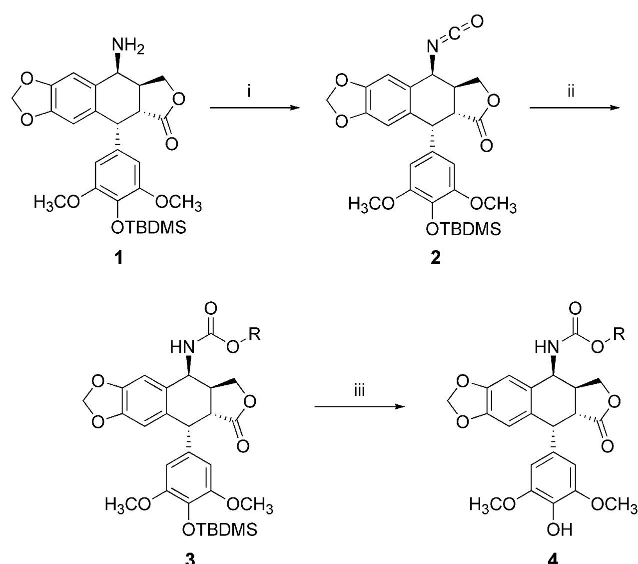


Scheme 1 Synthesis of the 4-β-*N*-carbamates of 4′-demethylepipodophyllotoxin. Reagents: (i) phosgene, dry CH_2Cl_2; (ii) ROH, dry CH_2Cl_2; (iii) Dowex® 50 × 2–200, methanol.

4′-*tert*-butyldimethylsilyloxy-4β-amino-4′-*O*-demethyl-4-desoxypodophyllotoxin (**1**), required as starting material, was prepared as previously described.[14] The 4-β-isocyanate-4′-*tert*-butyldimethylsilyloxy-4′-*O*-demethyl-4-desoxypodophyllotoxin (**2**) was prepared by reacting **1** with phosgene in dry dichloromethane at room temperature. The preparation of 4-β-(alkyloxycarbonyl)amino-4′-*tert*-butyldimethylsilyloxy-4′-*O*-demethyl-4-desoxypodophyllotoxins **3a**–**i**, and 4-β-(aryloxycarbonyl)amino-4′-*tert*-butyldimethylsilyloxy-4′-*O*-demethyl-4-desoxypodophyllotoxins **3j**–**m**, was achieved *in situ* by adding the appropriate alcohol to the reaction mixture of the previous step. It must be noted that, during the course of this reaction, an important desilylation occurred and only a small amount of **3** was isolated, along with the desired deprotected compound **4**. The subsequent complete regenerations of the 4′-hydroxyl group from compounds **3a**–**m** were performed in methanol using a DOWEX® 50 × 2–200 ion exchange resin to provide the desired phenolic compounds **4a**–**m** in good to excellent yields.

Biological evaluation and DNA topoisomerase II inhibition

We investigated drug–DNA interactions by perturbation of ethidium bromide–DNA complexes in agarose gel at two concentrations (50 and 100 μM) of carbamate derivatives **4a**–**m** (data not shown). While under the same conditions, the known intercalating drug daunorubicin shifts the ethidium bromide complexed in supercoiled DNA; the carbamate derivatives had no effect. This result argues for a non-intercalative binding mode such as already known for etoposide.[17,18,19]

Biological results for the novel 4-β-*N*-carbamate derivatives of 4′-demethylpodophyllotoxin are shown in Table 1. All compounds were tested for their ability to poison topoisomerases I and II. Furthermore, their effect on L1210 cell proliferation and on the cell cycle were studied.

All compounds resulted inactive for topoisomerase I inhibition (data not shown).

Topoisomerase II poisoning was evaluated by the amount (by percentage) of linear DNA mediated by the action of topoisomerase II on a supercoiled plasmid in the presence of etoposide or of the 4-β-*N*-carbamates (Fig. 2). Supercoiled DNA was treated with human DNA topoisomerase IIα in the presence of an increasing concentration of the tested compounds. Results obtained with three of the most potent derivatives (**4a**, **4b**, **4c**) are shown in Fig. 2; etoposide was used as a reference. In the presence of increasing concentrations of these compounds, a band corresponding to linear DNA can be clearly observed, attesting that these molecules stabilize the cleavage complex, in which the double helix is cleaved by the enzyme on both strands. Compounds **4a** and **4b** produced much more double-stranded DNA cleavage than **4c** and are almost equivalent to etoposide. This shows that introducing an aliphatic chain on the carbamate group is favourable for topoisomerase inhibition and that increasing the length of this chain leads to a decrease in activity. These compounds presented a four to six fold better cytotoxicity on murine L1210 cell lines than VP16. They also exhibited an important effect on the cell cycle. Most compounds of the series showed good activity, confirming that the introduction of a carbamate group in the 4-position, instead of the glycopyranose group of etoposide, is favourable for antitopoisomerase activity. Compound **4g**, bearing a terminal methoxy group, presented the same biological properties as **4b** while the introduction of a terminal chlorine (**4d**) conferred a decrease in topoisomerase II inhibition activity. Compounds **4e** and **4f**, bearing a terminal double and triple bond, induced a strong effect on cytotoxicity and on the cell cycle, while topoisomerase II activity decreased. The introduction of an heterocycle, as in the case of compounds **4h** or **4i**, which present a pyrane or a maleimide substituent, respectively, conferred an almost complete loss of topoisomerase II poisoning. Furthermore, while compound **4h** presented the same cytotoxicity, **4i** was four times less active. In fact, the degree of topoisomerase II inhibition does not always correlate with cytotoxicity. To examine this point, we investigated the ability of **4e**, **4f**, **4g** and **4h**, to inhibit tubulin polymerization, as the precursor (podophyllotoxin) is a potent antimicrotubule agent. We observed that only compound **4g** presented a weak inhibition activity of tubulin polymerization/depolymerization (40% at 20 μM, compared to an IC_{50} = 3 μM for podophyllotoxin). On the contrary, compounds **4e**, **4f** and **4h** have no effect on tubulin polymerisation. Furthermore, the weak inhibition of topoisomerase II observed with these latter compounds indicates that, in these cases, this enzyme is probably not the only cellular target.

The perturbation of the cell cycle induced by these compounds was also studied on the L1210 cell line. All the tested compounds induced a marked accumulation (>65%) of cells in the G2 + M phases at a concentration between 0.25 and 5 μM.

In order to further investigate structure–activity relationships, we also prepared aromatic carbamates. We observed that a benzyl group (**4j**), a 4-fluorobenzyl group (**4k**) or a 4-fluorophenethyl group (**4l**) maintained good activity (Table 1). In particular,

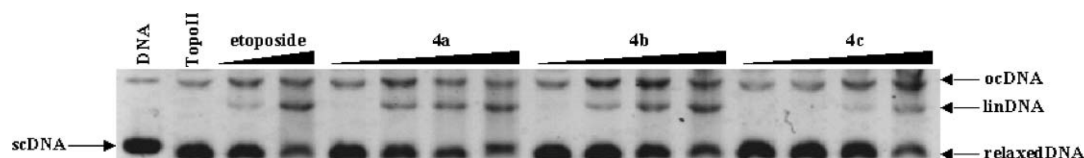
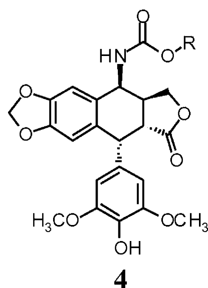


Fig. 2 Stimulation of the topoisomerase II-mediated DNA double stranded cleavage. Native supercoiled pAT (lane DNA) was incubated for 30 min at 30 °C with 6 units of topoisomerase II in the absence (lane TopoII) or in the presence of increasing concentrations of the drugs at the indicated concentrations. Reactions were stopped with SDS and treatment with proteinase K. Samples were analyzed by native agarose gel electrophoresis 1% in Tris–borate–EDTA buffer (TBE 1×) containing ethidium bromide (1 μg mL^{-1}) at room temperature. Gels were washed and photographed under UV light. The positions of supercoiled (form I), open-circular (form II), linear (form III) and relaxed DNA species are indicated. Etoposide was used at 5 and 20 μM; **4a**–**c** at 1, 5, 10 and 20 μM.

Table 1 Biological activities



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Compound	R	Inhibition of topoisomerase II[a] (% linear DNA)	Cytotoxicity	
			$IC_{50}/\mu M$[b]	Cell cycle effect[c]
VP-16	—	50	0.83	76% (2.5 μM)
4a	CH_3	50	0.18	74% (0.5 μM)
4b	CH_2CH_3	44	0.14	71% (1 μM)
4c	$(CH_2)_3CH_3$	40	0.23	72% (2 μM)
4d	$(CH_2)_3Cl$	31	0.15	70% (1 μM)
4e	$CH_2CH{=}CH_2$	25	0.11	72% (1 μM)
4f	$CH_2C{\equiv}CH$	29	0.09	69% (0.25 μM)
4g	$(CH_2)_2OCH_3$	42	0.14	N.T.
4h	CH_2 O	10	0.14	N.T.
4i	$(CH_2)_2$—N O O	15	0.44	N.T.
4j	CH_2	50	0.27	73% (2 μM)
4k	CH_2 F	45	0.36	71% (2 μM)
4l	$(CH_2)_2$ F	37	0.39	70% (5 μM)
4m	Cl CH_2 Cl	4	0.57	78% (5 μM)

[a] Each value reported here is a mean value of at least 3 independent experiments at 20 μM of drug. [b] IC_{50}: concentration of drug required to reduce L1210 cell growth to 50%. [c] % of L1210 cells in the G2M phase at the specified drug concentration. N.T. = not tested.

compound **4j** was comparable to etoposide for topoisomerase II inhibition activity. The fluoro atom in the *para* position (**4k**) does not markedly influence the effect on the enzyme. As in the case of aliphatic chains, the increase in the length of the side chain caused a decrease of topoisomerase II inhibition activity (**4k** *vs.* **4l**), which is completely lost by the introduction of 2,4-dichlorobenzyl group (**4m**). All of these compounds displayed a two-fold lower cytotoxicity than that observed in the aliphatic series, except for compound **4m**, which is four-fold less efficient. This latter is in correlation with the loss of topoisomerase II poisoning activity.

In agreement with the lower cytotoxicity, the perturbation of the cell cycle is induced for these aromatic derivatives at higher concentrations.

Conclusion

All of these results confirm that the replacement of the glycoside moiety of etoposide with a 4-β-*N*-carbamate residue is favorable to topoisomerase II poisoning. As previously reported,[14] the 4-β-amino-4′-*O*-demethyl-4-desoxypodophyllotoxin used as the starting material to prepare this series of 4-β-*N*-carbamates showed no inhibition of topoisomerase II. This lack of activity can be compared to that of 4′-DMEP[13] which is, in contrast, a poison of topoisomerase II. This is in agreement with the observation that the 4-*N*-carbamates of 4′-demethylepipodophyllotoxin here studied showed lower antitopoisomerase II activity than the previously studied 4-*O*-carbamates.[16] Whether there is a structure–activity relationship concerning 4-β-*N* and 4-β-*O* groups is currently under investigation in our laboratories. Concerning the side chain, methyl and benzyl groups gave the best results, strongly suggesting that, in this series, shorter side chains increased the topoisomerase II inhibition. As in our previously published works,[14,16] we have underlined that the spatial organization is fundamental for topoisomerase inhibition and even small changes can compromise this activity.

Materials and methods

Solvents and most of the starting materials were purchased from Acros, Aldrich or Avocado. Melting points were measured on a

Köfler hot stage apparatus and are uncorrected. Mass spectra were obtained with a Nermag–Ribermag R10–10C spectrometer applying either desorption chemical ionization (CI, operating in the positive ion mode using ammonia as the reagent gas) or fast atom bombardment (FAB). Infrared spectra were obtained with a Perkin-Elmer 1710 spectrophotometer using chloroform solvent. Specific rotations were measured with a Perkin Elmer 241 polarimeter. The ^{1}H NMR (300 MHz) spectra were recorded on a Bruker AC 300 spectrometer. Chemical shifts are expressed as parts per million from tetramethylsilane. Splitting patterns have been designated as follows: s (singlet), d (doublet), dd (doublet of doublet), t (triplet), m (multiplet), and br (broad signal). Coupling constants (*J* values) are listed in hertz (Hz). Reactions were monitored by analytical thin-layer chromatography and products were visualized by exposure to UV light. Merck silica gel (230–400 mesh ASTM) was used for column chromatography. Acetone, methanol, and dichloromethane, employed as eluents for column chromatography, were distilled on a rotary evaporator prior to use. All yields reported are unoptimized. Elemental analysis for most of the new substances was performed by CNRS Laboratories (Vernaison, France), and unless noted otherwise, the results obtained are within 0.4% of the theoretical values.

Synthesis of 4-β-isocyanate-4′-*tert*-butyldimethylsilyloxy-4′-*O*-demethyl-4-desoxypodophyllotoxin (2)

To a solution of 4-β-amino-4′-*tert*-butyldimethylsilyloxy-4′-*O*-demethyl-4-desoxypodophyllotoxin (**1**) (108 mg, 0.2 mmol) in dry dichloromethane (10 mL) were added a solution of phosgene in toluene 1.93 N (1.2 mL) and triethylamine (30 µL). The mixture was stirred overnight at room temperature. After concentration under reduced pressure, the product was purified by flash chromatography (CH_2Cl_2 : acetone, 97 : 3) leading to **2**. Yield 90%; R_f 0.8 (CH_2Cl_2 ; acetone, 97 : 3); ^{1}H-NMR ($CDCl_3$): δ (ppm) 6.83 (1H, s, 5-H); 6.54 (1H, s, 8-H); 6.21 (2H, s, 2′,6′-H); 6.00 (2H, d, J = 5.8 Hz, CH_2O_2); 4.93 (1H, d, J = 3.9 Hz, 4-H); 4.61 (1H, d, J = 5.3 Hz, 1-H); 4.46 (1H, t, J = 8.2 Hz, 11a-H); 4.16 (1H, t, J = 7.1 Hz, 11b-H); 3.66 (6H, s, 3′,5′-OCH_3); 3.17–3.05 (2H, m, 2,3-H); 0.97 (9H, s, *t*Bu); 0.1 (6H, s, Me_2); MS (CI) m/z 540 [M + H]$^+$, 557 [M + NH_4]$^+$.

General procedure for the synthesis of compounds 3a–m

1.5 eq. of the appropriate alcohol were added (0.3 mmol) to the reaction mixture obtained from the previous step. The reaction mixture was further stirred at room temperature for the reported times (monitored by TLC).

Evaporation of the solvent *in vacuo* afforded a crude material chromatographed on silica gel (100 g, eluent: various mixtures CH_2Cl_2 : acetone, see R_f) to provide satisfactory pure compounds with the reported yields. In the case of **3a**, **3b**, **3j** and **3m** the products were simultaneously deprotected after reaction into **4a**, **4b**, **4j** and **4m** respectively. Chromatographed compounds **3c**–**i** and **3k**–**l** were directly employed in the subsequent deprotection step without further purification.

4-β-(Butyloxycarbonyl)amino-4′-*tert*-butyldimethylsilyloxy-4′-*O*-demethyl-4-desoxypodophyllotoxin (3c). Yield 71%; R_f = 0.6 (CH_2Cl_2 : acetone, 97 : 3); ^{1}H-NMR ($CDCl_3$) δ: 6.81 (1H, s, 5-H); 6.51 (1H, s, 8-H); 6.23 (2H, s, 2′,6′-H); 5.97 (2H, d, J = 4.6 Hz, CH_2O_2); 5.02–4.93 (1H, m, NH); 4.84–4.78 (1H, m, 4-H); 4.42 (1H, t, J = 4.6 Hz, 11a-H); 4.56 (1H, d, J = 3.8 Hz, 1-H); 4.18–4.08 (2H, m, $CH_2\alpha$); 3.98 (1H, t, J = 7.6 Hz, 11b-H); 3.71 (3H, s, OCH_3); 3.67 (6H, s, 3′,5′-OCH_3); 2.94–2.86 (2H, m, 2,3-H); 1.70–1.58 (2H, m, $CH_2\beta$); 1.42–1.32 (2H, m, $CH_2\gamma$); 1.00 (9H, s, *t*Bu); 0.98 (3H, t, J = 7.2 Hz, CH_3); 0.10 (6H, s, 2Me); MS (CI) m/z 631 [M + NH_4]$^+$.

4-β-(3-Chloropropyloxycarbonyl)amino-4′-*tert*-butyldimethylsilyloxy-4′-*O*-demethyl-4-desoxypodophyllotoxin (3d). Yield 84%; R_f = 0.7 (CH_2Cl_2 : acetone, 97 : 3); ^{1}H-NMR ($CDCl_3$) δ: 6.80 (1H, s, 5-H); 6.52 (1H, s, 8-H); 6.23 (2H, s, 2′,6′-H); 5.97 (2H, d, J = 4.3 Hz, CH_2O_2); 4.98–4.09 (1H, m, NH); 4.83–4.78 (1H, m, 4-H); 4.56 (1H, d, J = 4.5 Hz, 1-H); 4.42 (1H, t, J = 7.3 Hz, 11a-H); 4.32 (2H, t, J = 6.2 Hz, $CH_2\alpha$); 3.94 (1H, t, J = 9.0 Hz, 11b-H); 3.67 (6H, s, 3′,5′-OCH_3); 3.61 (2H, t, J = 6.5 Hz, $CH_2\gamma$); 2.98–2.89 (2H, m, 2,3-H); 2.12–2.00 (2H, m, $CH_2\beta$); 1.00 (9H, s, *t*Bu); 0.10 (6H, s, 2Me); MS (CI) m/z 651 [M + NH_4]$^+$.

4-β-(Allyloxycarbonyl)amino-4′-*tert*-butyldimethylsilyloxy-4′-*O*-demethyl-4-desoxypodophyllotoxin (3e). Yield 80%; R_f = 0.55 (CH_2Cl_2 : acetone, 97 : 3); ^{1}H-NMR ($CDCl_3$) δ: 6.81 (1H, s, 5-H); 6.51 (1H, s, 8-H); 6.20 (2H, s, 2′,6′-H); 5.99 (2H, d, J = 5.1 Hz, CH_2O_2); 5.95–5.86 (1H, m, $CH_2CH{=}CH_2$); 5.34 (2H, d, J = 7.0 Hz, $CH_2CH{=}CH_2$); 5.02–4.94 (1H, m, 4-H); 4.96 (1H, d, J = 4.1 Hz, NH); 4.62–4.50 (3H, m, $CH_2\alpha$, 1-H); 4.40 (1H, t, J = 8.1 Hz, 11a-H); 3.94 (1H, t, J = 9.6 Hz, 11b-H); 3.67 (6H, s, 3′,5′-OCH_3); 2.94–2.86 (2H, m, 2,3-H); 1.00 (9H, s, *t*Bu); 0.10 (6H, s, 2Me); MS (CI) m/z 615 [M + NH_4]$^+$.

4-β-(Propargyloxycarbonyl)amino-4′-*tert*-butyldimethylsilyloxy-4′-*O*-demethyl-4-desoxypodophyllotoxin (3f). Yield 67%; R_f = 0.38 (CH_2Cl_2 : acetone, 97 : 3); ^{1}H-NMR ($CDCl_3$) δ: 6.80 (1H, s, 5-H); 6.52 (1H, s, 8-H); 6.23 (2H, s, 2′,6′-H); 5.97 (2H, d, J = 5.4 Hz, CH_2O_2); 5.04–4.92 (2H, m, 4-H, NH); 4.74–4.62 (3H, m, $CH_2C{\equiv}CH$); 4.56 (1H, d, J = 4.0 Hz, 1-H); 4.41 (1H, t, J = 7.9 Hz, 11a-H); 3.94 (1H, t, J = 9.5 Hz, 11b-H); 3.67 (6H, s, 3′,5′-OCH_3); 2.98–2.85 (2H, m, 2,3-H); 1.00 (9H, s, *t*Bu); 0.10 (6H, s, Me); MS (CI) m/z 596 [M + H]$^+$, 613 [M + NH_4]$^+$.

4-β-(2-Methoxyethoxycarbonyl)amino-4′-*tert*-butyldimethylsilyloxy-4′-*O*-demethyl-4-desoxypodophyllotoxin (3g). Yield 95%; R_f = 0.46 (CH_2Cl_2 : acetone, 80 : 20); ^{1}H-NMR ($CDCl_3$) δ: 6.80 (1H, s, 5-H); 6.51 (1H, s, 8-H); 6.23 (2H, s, 2′,6′-H); 5.97 (2H, d, J = 3.3 Hz, CH_2O_2); 4.98–4.90 (2H, m, NH, 4-H); 4.57 (1H, d, J = 3.8 Hz, 1-H); 4.42 (1H, t, J = 7.2 Hz, 11a-H); 4.30–4.18 (2H, m, $CH_2\alpha$); 3.98 (1H, t, J = 9.4 Hz, 11b-H); 3.67 (6H, s, 3′,5′-OCH_3); 3.60–3.52 (2H, m, $CH_2\beta$); 3.39 (3H, s, OCH_3); 2.97–2.84 (2H, m, 2,3-H); 1.00 (9H, s, *t*Bu); 0.10 (6H, s, Me); MS (CI) m/z 616 [M + H]$^+$, 633 [M + NH_4]$^+$.

4-β-[(Tetrahydropyran-2-yl)methoxycarbonyl]amino-4′-*tert*-butyldimethylsilyloxy-4′-*O*-demethyl-4-desoxypodophyllotoxin (3h). Yield 96%; R_f = 0.34 (CH_2Cl_2 : acetone, 97 : 3); ^{1}H-NMR ($CDCl_3$) δ: 6.79 (1H, s, 5-H); 6.50 (1H, s, 8-H); 6.22 (2H, s, 2′,6′-H); 5.97 (2H, d, J = 4.8 Hz, CH_2O_2); 5.02–4.85 (2H, m, NH, 4-H); 4.56 (1H, d, J = 3.7 Hz, 1-H); 4.39 (1H, t, J = 7.5 Hz, 11a-H); 4.08–3.90 (4H, m, $CH_2\alpha$, CHO, 11b-H); 3.67 (6H, s, 3′,5′-OCH_3); 3.55–3.40 (2H, m, CH_2O); 3.00–2.85 (2H, m, 2,3-H); 1.72–1.45 (6H, m, CH_2); 1.00 (9H, s, *t*Bu); 0.10 (6H, s, Me); MS (CI) m/z 656 [M + H]$^+$, 673 [M + NH_4]$^+$.

4-β-[(2,5-Dioxopyrrolidin-1-yl)ethoxycarbonyl]amino-4′-*tert*-butyldimethylsilyloxy-4′-*O*-demethyl-4-desoxypodophyllotoxin (3i). Yield 65%; R_f = 0.45 (CH_2Cl_2 : acetone, 80 : 20); ^{1}H-NMR ($CDCl_3$) δ: 6.88 (1H, s, 5-H); 6.51 (1H, s, 8-H); 6.23 (2H, s, 2′,6′-H); 5.97 (2H, d, J = 3.8 Hz, CH_2O_2); 4.98–4.90 (1H, m, NH); 4.89–4.82 (1H, m, 4-H); 4.55 (1H, d, J = 3.93 Hz, 1-H); 4.23 (1H, t, J = 5.0 Hz, 11a-H); 4.30–4.20 (2H, m, $CH_2\beta$); 4.04 (1H, t, J = 9.5 Hz, 11b-H); 3.85–3.78 (2H, m, $CH_2\alpha$); 3.67 (6H, s, 3′,5′-OCH_3); 2.98–2.85 (2H, m, 2,3-H); 2.73 (4H, t, J = 8.5 Hz, $2CH_2$); 1.00 (9H, s, *t*Bu); 0.10 (6H, s, Me); MS (CI) m/z 683 [M + H]$^+$, 700 [M + NH_4]$^+$.

4-β-(4-Fluorobenzyloxycarbonyl)amino-4′-*tert*-butyldimethylsilyloxy-4′-*O*-demethyl-4-desoxypodophyllotoxin (3k). Yield 98%; R_f = 0.42 (CH_2Cl_2 : acetone, 97 : 3); ^{1}H-NMR ($CDCl_3$) δ: 7.38–7.30 (2H, m, Ph); 7.08–6.98 (2H, m, Ph); 6.79 (1H, s, 5-H); 6.51 (1H, s, 8-H); 6.22 (2H, s, 2′,6′-H); 5.96 (2H, d, J = 5.3 Hz, CH_2O_2); 5.11 (2H, br s, CH_2Ph); 5.28–5.10 (1H, m, NH); 5.18–5.12 (1H, m, 4-H); 4.57 (1H, d, J = 4.8 Hz, 1-H);

4.42 (1H, t, $J = 7.8$ Hz, 11a-H); 3.98 (2H, t, $J = 9.8$ Hz, 11b-H); 3.67 (6H, s, 3′,5′-OCH_3); 2.98–2.85 (2H, m, 2,3-H); 1.00 (9H, s, *t*Bu); 0.10 (6H, s, Me); MS (CI) *m/z* 666 $[M + H]^+$, 683 $[M + NH_4]^+$.

4-β-(4-Fluorophenethyloxycarbonyl)amino-4′-*tert*-butyldimethylsilyloxy-4′-*O*-demethyl-4-desoxypodophyllotoxin (3l). Yield 92%; $R_f = 0.45$ (CH_2Cl_2 : acetone, 97 : 3); ^{1}H-NMR ($CDCl_3$) δ: 7.20–7.12 (2H, m, Ph); 7.08–6.98 (2H, m, Ph); 6.76 (1H, s, 5-H); 6.51 (1H, s, 8-H); 6.22 (2H, s, 2′,6′-H); 5.97 (2H, d, $J = 4.4$ Hz, CH_2O_2); 5.00–4.90 (1H, m, NH); 4.82–4.77 (1H, m, 4-H); 4.56 (1H, d, $J = 4.5$ Hz, 1-H); 4.31 (1H, t, $J = 6.4$ Hz, 11a-H); 3.09–3.80 (3H, m, 11b-H, $CH_2\alpha$); 3.67 (6H, s, 3′,5′-OCH_3); 2.98–2.80 (4H, m, 2,3-H, $CH_2\beta$); 1.00 (9H, s, *t*Bu); 0.10 (6H, s, Me); MS (CI) *m/z* 680 $[M + H]^+$, 697 $[M + NH_4]^+$.

General procedure for the synthesis of compounds 4a–m

Dowex 50 × 2–200 ion-exchange resin (3 g), previously washed with water, then MeOH, was added to a solution of **3a**–**m** (0.6 mmol) in MeOH (65 mL). The mixture was vigorously stirred for 16 h at room temperature. The resin was removed by filtration and thoroughly washed with MeOH. Evaporation of the filtrate *in vacuo* gave the desired compound in the reported yields. This was also performed on compounds **3a**, **3b**, **3j** and **3m**, which have not been isolated in order to deprotect them completely.

4-β-(Methyloxycarbonyl)amino-4′-*O*-demethyl-4-desoxypodophyllotoxin (4a). Yield 80%; $R_f = 0.45$ (CH_2Cl_2 : acetone, 90 : 10); mp = 150–152 °C (white crystals); $[\alpha]^D_{20} = -109.2$ (c = 0.610, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ: 6.81 (1H, s, 5-H); 6.49 (1H, s, 8-H); 6.28 (2H, s, 2′,6′-H); 5.96 (2H, d, $J = 7.6$ Hz, CH_2O_2); 5.41 (1H, br s, 4′-OH); 4.97 (1H, br d, $J = 3.2$ Hz, 4-NH); 4.95–4.86 (1H, m, 4-H); 4.55 (1H, d, $J = 4.1$ Hz, 1-H); 4.41 (1H, t, $J = 7.8$ Hz, 11a-H); 3.95 (1H, t, $J = 9.6$ Hz, 11b-H); 3.77 (6H, s, 3′,5′-OCH_3); 3.71 (3H, s, OCH_3); 2.94–2.82 (2H, m, 2,3-H); IR ($CHCl_3$): ν 3366 (NH), 3020 (aliphatic C–H), 1777 (C=O lactone), 1587, 1507, 1485 (aromatic C=C); MS (CI) *m/z* 475 $[M + NH_4]^+$.

4-β-(Ethyloxycarbonyl)amino-4′-*O*-demethyl-4-desoxypodophyllotoxin (4b). Yield 99%; $R_f = 0.57$ (CH_2Cl_2 : acetone, 90 : 10); mp = 146–148 °C (white crystals); $[\alpha]^D_{20} = -104.2$ (c = 0.515, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ: 6.82 (1H, s, 5-H); 6.50 (1H, s, 8-H); 6.28 (2H, s, 2′,6′-H); 5.97 (2H, d, $J = 6.2$ Hz, CH_2O_2); 5.41 (1H, br s, 4′-OH); 4.97 (1H, br d, $J = 3.1$ Hz, 4-NH); 4.90–4.80 (1H, m, 4-H); 4.57 (1H, d, $J = 3.9$ Hz, 1-H); 4.42 (1H, t, $J = 7.4$ Hz, 11a-H); 4.21–4.09 (2H, m, CH_2); 3.95 (1H, t, $J = 9.2$ Hz, 11b-H); 3.77 (6H, s, 3′,5′-OCH_3); 2.97–2.85 (2H, m, 2,3-H); 1.26 (3H, t, $J = 7.0$ Hz, CH_3); IR ($CHCl_3$): ν 3324 (NH), 3020 (aliphatic C–H), 1776 (C=O lactone), 1604, 1485 (aromatic C=C); MS (CI) *m/z* 489 $[M + NH_4]^+$.

4-β-(Butyloxycarbonyl)amino-4′-*O*-demethyl-4-desoxypodophyllotoxin (4c). Yield 76%; $R_f = 0.48$ (CH_2Cl_2 : acetone, 90 : 10); mp = 126–128 °C (white crystals); $[\alpha]^D_{20} = -99.5$ ($c = 0.485$, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ: 6.82 (1H, s, 5-H); 6.50 (1H, s, 8-H); 6.29 (2H, s, 2′,6′-H); 5.97 (2H, d, $J = 5.7$ Hz, CH_2O_2); 5.41 (1H, br s, 4′-OH); 4.82 (1H, br d, $J = 6.9$ Hz, 4-NH); 4.90–4.80 (1H, m, 4-H); 4.55 (1H, d, $J = 4.1$ Hz, 1-H); 4.41 (1H, t, $J = 7.8$ Hz, 11a-H); 4.20–4.10 (2H, m, $CH_2\alpha$); 3.95 (1H, t, $J = 9.6$ Hz, 11b-H); 3.77 (6H, s, 3′,5′-OCH_3); 3.71 (3H, s, OCH_3); 2.94–2.82 (2H, m, 2,3-H); 1.65–1.55 (2H, m, $CH_2\beta$); 1.40–1.30 (2H, m, $CH_2\gamma$). IR ($CHCl_3$): ν 3367 (NH), 3038 (aliphatic C–H), 1776 (C=O lactone), 1622, 1505, 1485 (aromatic C=C); MS (CI) *m/z* 517 $[M + NH_4]^+$.

4-β-(3-Chlororopyloxycarbonyl)amino-4′-*O*-demethyl-4-desoxypodophyllotoxin (4d). Yield 58%; $R_f = 0.35$ (CH_2Cl_2 : acetone, 90 : 10); mp = 132–134 °C (white crystals); $[\alpha]^D_{20} = -102.6$ (c = 0.425, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ: 6.82 (1H, s, 5-H); 6.51 (1H, s, 8-H); 6.28 (2H, s, 2′,6′-H); 5.97 (2H, d, $J = 5.7$ Hz, CH_2O_2); 5.41 (1H, br s, 4′-OH); 5.01–4.85 (1H, m, 4-H); 4.86 (1H, br d, $J = 7.0$ Hz, 4-NH); 4.57 (1H, d, $J = 4.2$ Hz, 1-H); 4.42 (1H, t, $J = 7.7$ Hz, 11a-H); 4.27 (2H, t, $J = 6.0$ Hz, $CH_2\alpha$); 3.94 (1H, t, $J = 9.5$ Hz, 11b-H); 3.78 (6H, s, 3′,5′-OCH_3); 3.61 (2H, t, $J = 6.3$ Hz, $CH_2\alpha$); 3.00–2.87 (2H, m, 2,3-H); 2.16–2.04 (2H, m, $CH_2\beta$); IR ($CHCl_3$): ν 3366 (NH), 3020 (aliphatic C–H), 1777 (C=O lactone), 1587, 1507, 1485 (aromatic C=C); MS (CI) *m/z* 537 $[M + NH_4]^+$.

4-β-(Allyloxycarbonyl)amino-4′-*O*-demethyl-4-desoxypodophyllotoxin (4e). Yield 62%; $R_f = 0.60$ (CH_2Cl_2 : acetone, 90 : 10); mp = 130–132 °C (white crystals); $[\alpha]^D_{20} = -113.5$ (c = 0.620, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ: 6.82 (1H, s, 5-H); 6.50 (1H, s, 8-H); 6.28 (2H, s, 2′,6′-H); 5.97 (2H, d, $J = 6.2$ Hz, CH_2O_2); 5.93–5.86 (1H, m, $CH_2CH{=}CH_2$); 5.41 (1H, br s, 4′-OH); 5.28 (2H, d, $J = 8.0$ Hz, $CH_2CH{=}CH_2$); 5.15–4.98 (1H, m, 4-H); 4.91 (1H, br d, $J = 7.1$ Hz, 4-NH); 4.68–4.54 (3H, m, 1-H, $CH_2CH{=}CH_2$); 4.42 (1H, t, $J = 7.0$ Hz, 11a-H); 3.95 (1H, t, $J = 9.8$ Hz, 11b-H); 3.77 (6H, s, 3′,5′-OCH_3); 2.95–2.87 (2H, m, 2,3-H); IR ($CHCl_3$): ν 3358 (NH), 3020 (aliphatic C–H), 1773 (C=O lactone), 1559, 1508, 1458 (aromatic C=C); MS (CI) *m/z* 501 $[M + NH_4]^+$.

4-β-(Propargyloxycarbonyl)amino-4′-*O*-demethyl-4-desoxypodophyllotoxin (4f). Yield 50%; $R_f = 0.55$ (CH_2Cl_2 : acetone, 90 : 10); mp = 140–142 °C (white crystals); $[\alpha]^D_{20} = -109.4$ (c = 0.575, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ: 6.81 (1H, s, 5-H); 6.50 (1H, s, 8-H); 6.28 (2H, s, 2′,6′-H); 5.97 (2H, d, $J = 4.5$ Hz, CH_2O_2); 5.41 (1H, s, 4′-OH); 5.02–4.95 (2H, m, NH, 4-H); 4.57 (1H, d, $J = 4.2$ Hz, 1-H); 4.41 (1H, t, $J = 7.7$ Hz, 11a-H); 4.35–4.20 (3H, m, $CH_2C{\equiv}CH$); 3.96 (1H, t, $J = 9.5$ Hz, 11b-H); 3.77 (6H, s, 3′,5′-OCH_3); 2.98–2.85 (2H, m, 2,3-H); IR ($CHCl_3$): ν 3358 (NH), 3020 (aliphatic C–H), 1773 (C=O lactone), 1559, 1508, 1458 (aromatic C=C); MS (CI) *m/z* 482 $[M + H]^+$, 499 $[M + NH_4]^+$.

4-β-(2-Methoxyethoxycarbonyl)amino-4′-*O*-demethyl-4-desoxypodophyllotoxin (4g). Yield 77%; $R_f = 0.62$ (CH_2Cl_2 : acetone, 90 : 10); mp = 136–138 °C (white crystals); $[\alpha]^D_{20} = -104.8$ (c = 0.425, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ: 6.81 (1H, s, 5-H); 6.51 (1H, s, 8-H); 6.28 (2H, s, 2′,6′-H); 5.97 (2H, d, $J = 6.1$ Hz, CH_2O_2); 5.42 (1H, br s, 4′-OH); 5.15–4.92 (2H, m, NH, 4-H); 4.57 (1H, d, $J = 4.2$ Hz, 1-H); 4.42 (1H, t, $J = 7.9$ Hz, 11a-H); 4.45–4.29 (2H, m, $CH_2\alpha$); 3.96 (1H, t, $J = 9.6$ Hz, 11b-H); 3.77 (6H, s, 3′,5′-OCH_3); 3.65–3.58 (2H, m, $CH_2\beta$); 3.39 (3H, s, OCH_3); 2.97–2.84 (2H, m, 2,3-H); IR ($CHCl_3$): ν 3358 (NH), 3020 (aliphatic C–H), 1773 (C=O lactone), 1559, 1508, 1458 (aromatic C=C); MS (CI) *m/z* 502 $[M + H]^+$, 519 $[M + NH_4]^+$.

4-β-[(Tetrahydropyran-2-yl)methoxycarbonyl]amino-4′-*O*-demethyl-4-desoxypodophyllotoxin (4h). Yield 55%; $R_f = 0.45$ (CH_2Cl_2 : acetone, 90 : 10); mp = 139–141 °C (white crystals); $[\alpha]^D_{20} = -98.1$ (c = 0.615, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ: 6.80 (1H, s, 5-H); 6.49 (1H, s, 8-H); 6.28 (2H, s, 2′,6′-H); 5.97 (2H, d, $J = 4.2$ Hz, CH_2O_2); 5.42 (1H, br s, 4′-OH); 4.98–4.88 (2H, m, NH, 4-H); 4.57 (1H, d, $J = 4.0$ Hz, 1-H); 4.41 (1H, t, $J = 7.2$ Hz, 11a-H); 4.12–3.95 (2H, m, $CH_2\alpha$, CHO, 11b-H); 3.77 (6H, s, 3′,5′-OCH_3); 3.50–3.35 (2H, m, CH_2O); 3.00–2.85 (2H, m, 2,3-H); 1.72–1.50 (6H, m, CH_2); IR ($CHCl_3$): ν 3358 (NH), 3020 (aliphatic C–H), 1773 (C=O lactone), 1559, 1508, 1458 (aromatic C=C); MS (CI) *m/z* 542 $[M + H]^+$, 559 $[M + NH_4]^+$.

4-β-[(2,5-Dioxopyrrolidin-1-yl)ethoxycarbonyl]amino-4′-*O*-demethyl-4-desoxypodophyllotoxin (4i). Yield 57%; $R_f = 0.54$ (CH_2Cl_2 : acetone, 90 : 10); mp = 145–147 °C (white crystals); $[\alpha]^D_{20} = -80.3$ (c = 0.590, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ: 6.89 (1H, s, 5-H); 6.50 (1H, s, 8-H); 6.28 (2H, s, 2′,6′-H); 5.97 (2H, d, $J = 5.1$ Hz, CH_2O_2); 5.42 (1H, br s, 4′-OH); 4.98–4.84 (2H, m, NH, 4-H); 4.56 (1H, d, $J = 4.8$ Hz, 1-H); 4.37 (1H, t, $J = 6.3$ Hz, 11a-H); 4.28–4.17 (2H, m, $CH_2\beta$); 4.06 (1H, t,

$J = 9.1$ Hz, 11b-H); 3.92–3.88 (2H, m, $CH_2\alpha$); 3.77 (6H, s, 3′,5′-OCH_3); 2.93–2.82 (2H, m, 2,3-H); 2.75–2.68 (4H, m, CH_2); IR ($CHCl_3$): ν 3358 (NH), 3020 (aliphatic C–H), 1773 (C=O lactone), 1559, 1508, 1458 (aromatic C=C); MS (CI) m/z 569 $[M + H]^+$, 586 $[M + NH_4]^+$.

4-β-(Benzyloxycarbonyl)amino-4′-*O*-demethyl-4-desoxypodophyllotoxin (4j). Yield 75%; $R_f = 0.48$ (CH_2Cl_2 : acetone, 90 : 10); mp = 128–130 °C (white crystals); $[\alpha]_{20}^{D} = -91.6$ ($c = 0.470$, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ: 7.36 (5H, br s, Ph); 6.81 (1H, s, 5-H); 6.50 (1H, s, 8-H); 6.28 (2H, s, 2′,6′-H); 5.97 (2H, d, J = 6.3 Hz, CH_2O_2); 5.41 (1H, br s, 4′-OH); 5.15 (2H, s, CH_2Ph); 5.05–4.98 (1H, br s, NH); 4.95–4.88 (1H, m, 4-H); 4.56 (1H, d, $J = 4.5$ Hz, 1-H); 4.42 (1H, t, $J = 7.0$ Hz, 11a-H); 3.98 (2H, t, $J = 9.0$ Hz, 11b-H); 3.77 (6H, s, 3′,5′-OCH_3); 2.90–2.82 (2H, m, 2,3-H); IR ($CHCl_3$): ν 3358 (NH), 3020 (aliphatic C–H), 1773 (C=O lactone), 1559, 1508, 1458 (aromatic C=C); MS (CI) m/z 534 $[M + H]^+$, 551 $[M + NH_4]^+$.

4-β-(4-Fluorobenzyloxycarbonyl)amino-4′-*O*-demethyl-4-desoxypodophyllotoxin (4k). Yield 50%; $R_f = 0.60$ (CH_2Cl_2 : acetone, 90 : 10); mp = 140–142 °C (white crystals); $[\alpha]_{20}^{D} = -88.8$ ($c = 0.525$, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ: 7.38–7.30 (2H, m, Ph); 6.80 (1H, s, 5-H); 6.49 (1H, s, 8-H); 6.28 (2H, s, 2′,6′-H); 5.97 (2H, d, $J = 7.7$ Hz, CH_2O_2); 5.41 (1H, br s, 4′-OH); 5.10 (2H, br s, CH_2Ph); 5.04–4.98 (1H, m, NH); 4.95–4.89 (1H, m, 4-H); 4.56 (1H, d, $J = 4.4$ Hz, 1-H); 4.41 (1H, t, J = 7.7 Hz, 11a-H); 4.39 (2H, t, $J = 9.6$ Hz, 11b-H); 3.77 (6H, s, 3′,5′-OCH_3); 2.98–2.85 (2H, m, 2,3-H); IR ($CHCl_3$): ν 3358 (NH), 3020 (aliphatic C–H), 1773 (C=O lactone), 1559, 1508, 1458 (aromatic C=C); MS (CI) m/z 552 $[M + H]^+$, 570 $[M + NH_4]^+$.

4-β-(4-Fluorophenethyloxycarbonyl)amino-4′-*O*-demethyl-4-desoxypodophyllotoxin (4l). Yield 75%; $R_f = 0.58$ (CH_2Cl_2 : acetone, 80 : 20); mp = 127–129 °C (white crystals); $[\alpha]_{20}^{D} = -94.2$ ($c = 0.600$, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ: 7.17 (2H, t, $J = 6.7$ Hz, Ph); 7.00 (2H, t, $J = 8.6$ Hz, Ph); 6.77 (1H, s, 5-H); 6.50 (1H, s, 8-H); 6.28 (2H, s, 2′,6′-H); 5.97 (2H, d, J = 4.8 Hz, CH_2O_2); 5.42 (1H, br s, 4′-OH); 4.98–4.92 (1H, m, NH); 4.85–4.78 (1H, m, 4-H); 4.56 (1H, d, $J = 4.2$ Hz, 1-H); 4.38 (1H, t, $J = 7.8$ Hz, 11a-H); 4.31 (2H, t, $J = 6.6$ Hz, $CH_2\alpha$); 3.87 (1H, t, $J = 9.3$ Hz, 11b-H); 3.77 (6H, s, 3′,5′-OCH_3); 2.98–2.82 (4H, m, 2,3-H, $CH_2\beta$); IR ($CHCl_3$): ν 3358 (NH), 3020 (aliphatic C–H), 1773 (C=O lactone), 1559, 1508, 1458 (aromatic C=C); MS (CI) m/z 566 $[M + H]^+$, 583 $[M + NH_4]^+$.

4-β-(2,4-Dichlorobenzyloxycarbonyl)amino-4′-*O*-demethyl-4-desoxypodophyllotoxin (4m). Yield 40%; $R_f = 0.35$ (CH_2Cl_2 : acetone, 90 : 10); mp = 242–244 °C (white crystals); $[\alpha]_{20}^{D} = -81.9$ ($c = 0.430$, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ: 7.43 (1H, s, Ph); 7.35 (2H, d, $J = 5.9$ Hz, Ph); 6.81 (1H, s, 5-H); 6.50 (1H, s, 8-H); 6.28 (2H, s, 2′,6′-H); 5.97 (2H, d, $J = 6.3$ Hz, CH_2O_2); 5.41 (1H, br s, 4′-OH); 5.21 (2H, d, $J = 6.3$ Hz, CH_2Ph); 5.05–4.93 (2H, m, NH, 4-H); 4.56 (1H, d, $J = 4.0$ Hz, 1-H); 4.42 (1H, t, $J = 7.3$ Hz, 11a-H); 3.96 (2H, t, $J = 9.4$ Hz, 11b-H); 3.77 (6H, s, 3′,5′-OCH_3); 2.90–2.80 (2H, m, 2,3-H); IR ($CHCl_3$): ν 3358 (NH), 3020 (aliphatic C–H), 1773 (C=O lactone), 1559, 1508, 1458 (aromatic C=C); MS (CI) m/z 534 $[M + H]^+$, 551 $[M + NH_4]^+$.

DNA and biochemicals

The plasmid pBS was obtained from Stratagene (France). Purified human topoisomerase I and IIα were purchased from TopoGEN Inc. (USA) and etoposide from Sigma Chemicals. Compounds were dissolved in dimethyl sulfoxide at 5 mM, then diluted to working concentrations in distilled water immediately before use.

Topoisomerase I-mediated DNA cleavage assay

Each reaction mixture (20 µl total volume) contained 0.1 µg of pBS supercoiled DNA, the reaction buffer consisting of 35 mM Tris-HCl, pH 8, 72 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 5 mM spermidine and 0.01% BSA. Drugs were added at different concentrations (from 1 to 20 µM). The reaction was initiated by the addition 2 units topoisomerase I and allowed to proceed at 30 °C for 20 min.

The reaction was stopped by adding 10 × loading buffer (0.25% bromophenol blue, 50% glycerol) and 10% sarkosyl.

Samples were electrophoresed for 3 h at 100 V in 1% agarose gel in Tris–borate–EDTA buffer (TBE). The gel was stained with SYBR Gold 1X (Molecular Probes, USA) in TBE 1x buffer for 20 min and photographed under UV light.

Topoisomerase II-mediated DNA cleavage assay

Supercoiled pBS DNA (0.1 µg) was incubated for 15 min at 30 °C, in a 50 mM Tris–HCl buffer, pH 7.5, containing 1 mM ATP, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA and 30 µg BSA, in the presence of the drug at the desired concentration (5, 10, 20 or 50 µM, total reaction volume 10 µl). 6 units of human DNA topoisomerase IIα were added to the duplex, preincubated as described, and incubated for 30 min at 30 °C. The DNA-topoisomerase II cleavage complexes were dissociated by addition of SDS (GibcoBRL), final concentration 0.5%, and of proteinase K (Sigma) to 500 µg mL^{-1}, followed by incubation for 30 min at 50 °C. DNA samples were then added to the electrophoresis dye mixture (5 µL) and electrophoresed (35 V cm^{-1}) in a 1% agarose gel in TBE 1×, containing ethidium bromide (1 µg mL^{-1}), at room temperature for 2 h. Gels were washed and photographed under UV light.

Cell culture and cytotoxicity assays and cell cycle analysis

Assays were performed as previously described.[20,21]

Tubuline test

Tubulin polymerization inhibition was determined as previously reported.[22]

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