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Synthesis of 4β-carbamoyl epipodophyllotoxins as potential antitumour agents

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ABSTRACT

A series of new 4β-carbamoyl epipodophyllotoxin analogues have been synthesized and evaluated for their anticancer activity against eleven cancer cell lines including Zr-75-1, MCF7, KB, Gurav, DWD, Colo 205, A-549, Hop62, PC3, SiHa and A-2780. Most of the compounds exhibited better growth-inhibition activities against tested cell lines than that of etoposide. Further, compounds **6g** and **6i** are also evaluated for their DNA topoisomerase-II (topo-II) inhibition activity and they exhibited significant inhibition of topo-II catalytic activity comparable to etoposide.

1. Introduction

Podophyllotoxin (Fig. 1) is a naturally occurring aryltetralin lignan isolated from the roots of *Podophyllum peltatum* and *Paeonia emodi*. Podophyllotoxin shows strong cytotoxic activity against various cancer cell lines.[1] It is effective in the treatment of Wilms tumours, various genital tumours, non-Hodgkin's, lymphomas and lung cancer.[2,3] However, the high toxicity and severe gastrointestinal side effect of podophyllotoxin has limited its application as a drug in cancer chemotherapy. The biological activity of podophyllotoxin has led to extensive structural modifications resulting in several more potent and less toxic anticancer agents. The semisynthetic podophyllotoxin derivatives like etoposide and teniposide are currently used in the chemotherapy for a variety of malignancies including germ-cell malignancies, small-cell lung cancer, non-Hodgkin's lymphoma, leukemia, Kaposi's sarcoma, neuroblastoma, and soft tissue sarcoma.[4–6]

Podophyllotoxin inhibits the assembly of tubulin into microtubules through interaction with protein at the colchicine binding site, preventing the formation of the spindle. However, its semisynthetic derivatives showed different mechanisms of action. Etoposide and congeners induce a premitotic blockade in late S stage of the cell cycle because of the inhibition of DNA topoisomerase II (top-II), an enzyme required for the unwinding of DNA during replication.[7,8]

Although etoposide is widely used in therapy, it presents several limitations, such as moderate potency, poor water solubility, development of drug resistance, metabolic inactivation, and toxic effects.[9] Therefore, the structure of etoposide has been extensively modified, thus increasing the information about its structure–activity relationships. The most important modification is that of the substituent in the 4β-position leading to potent inhibitors of topoisomerase II. The replacement of C-4 sugar unit of etoposide with a heteroatom (*O*, *N*, or *S*) linked moieties helped in overcoming the problem of drug resistance to etoposide.[10] Subsequently, a large number of analogues have been reported exhibiting comparable or better activity than etoposide and teniposide.[11,12]

In recent years, we have been involved in the development of new methodologies, as well as synthesis of podophyllotoxin derivatives with improved bioactivities and reduced toxicity.[12,13] From our previous studies as well as the investigations carried out in the literature have shown that N-linked derivatives of podophyllotoxin possess lower bioavailability compared to O-linked congeners. This finding has prompted us to design new carbamate derivatives of podophyllotoxin that have promising anticancer activity. In view of the available information on structure–activity relationship studies and in continuation of our ongoing projects on the design and development of structurally modified podophyllotoxin congeners we describe herein synthesis and structure–activity relationships of a series of 4β-carbamoyl substituted epipodophyllotoxin analogues.

2. Chemistry

The synthetic route for these new derivatives **6a–j** is depicted in Scheme 1. The podophyllotoxin (**1**) was first treated with $BF_3{\cdot}OEt_2$/NaI followed by basic hydrolysis with barium carbonate to afford

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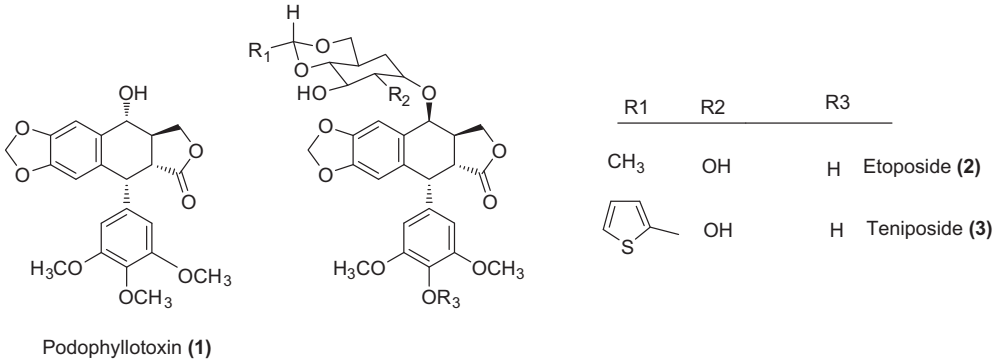


Figure 1.

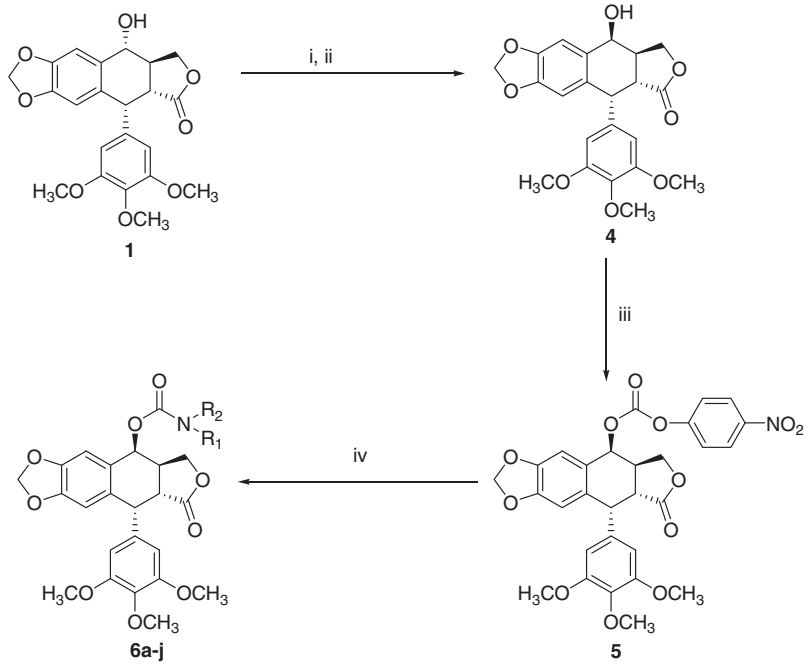
epipodophyllotoxin **4**.[13a] Compound **4** was then converted into its 4β-activated derivative **5** by simple treatment with *p*-nitrophenylchloroformate in pyridine. Introduction of the carbamate chains was performed by reacting **5** with the appropriate amines in the presence of triethylamine to afford compounds **6a–j** in good yields.

3. Biological activity

Compounds **6a–j** were evaluated for their anticancer activity in selected human cancer cell lines, that is, breast (Zr-75-1, MCF7), oral (KB, Gurav, DWD)), colon (Colo-205), lung (A-549, Hop62), prostate (PC-3), cervix (SiHa) and ovarian (A-2780) origin by employing the sulforhodamine B (SRB) assay method.[14] The results are summarized with standard drug etoposide and 5-fluorouracil in Table 1 and reveal that all the new podophyllotoxin congeners are significantly cytotoxic, with the concentration of the drug that produces 50% inhibition of cell growth (GI_{50}) ranging from <0.1 to 2.7 μM.

In the earlier SAR studies on the podophyllotoxin based compounds it has been observed that the O-linked derivatives of podophyllotoxin shows lower anticancer activity in comparison to the N-linked congeners.[11–13,15,16] However, in the present investigation these new class of 4β-carbamoyl podophyllotoxins possess potent anticancer activity, that is comparable to previously reported 4β-N-sustituted podophyllotoxin derivatives.

In the literature, it has been observed that inversion of configuration at 4-position to provide epipodophyllotoxin is essential for DNA topo-II inhibition but there is no clarity about the role of 4′-O-methylation or 4′-O-demethylation. DNA topo-II is the pharmacological target of clinical relevance for the podophyllotoxin lignans, therefore representative compounds **6g** and **6i** have been evaluated for its inhibition. These compounds exhibit comparable in vitro inhibition of topo-II catalytic activity to etoposide and the results are illustrated in Figure 2. The topo-II-mediated relaxation assay performed is in accordance to the previously reported procedure.[17]



Scheme 1. Reagents and conditions: (i) $BF_3{\cdot}OEt_2$/NaI, CH_3CN, rt, 30 min; (ii) H_2O–acetone, $BaCO_3$, rt, 30 min; (iii) 4-nitrochloroformate, pyridine, 0 °C, 45 min; (iv) R_1R_2NH, CH_2Cl_2, rt, 3–6 h.

Table 1
Anticancer activity data of compounds **6a–j**

Compound	GI_{50} (μM)										
	Breast		Oral			Colon	Lung		Prostate	Cervix	Ovarian
	Zr-75-1	MCF7	KB	Gurav	DWD	Colo 205	A-549	Hop62	PC3	SiHa	A-2780
6a	0.19	2.0	2.1	0.17	2.0	2.7	<0.1	0.16	0.17	1.2	0.19
6b	0.15	0.19	0.18	0.16	<0.1	2.7	<0.1	2.1	0.19	0.19	0.14
6c	0.19	2.3	2.4	0.18	2.0	2.5	<0.1	2.5	2.3	2.3	2.0
6d	0.12	0.16	0.18	0.17	<0.1	2.1	<0.1	0.13	0.15	2.1	0.18
6e	<0.1	0.15	0.15	0.14	<0.1	2.2	<0.1	0.12	0.16	2.4	0.15
6f	0.14	0.19	0.17	0.14	<0.1	2.7	<0.1	0.14	0.16	1.9	0.16
6g	0.14	0.16	0.12	0.14	<0.1	2.7	<0.1	0.13	0.16	1.4	0.13
6h	0.16	0.18	0.18	0.19	2.0	0.15	<0.1	2.3	2.1	2.0	0.18
6i	0.13	0.16	0.18	0.12	<0.1	0.12	<0.1	2.1	0.16	1.8	0.11
6j	<0.1	0.11	0.16	0.15	<0.1	2.1	<0.1	0.12	0.15	2.1	0.12
VP-16[a]	0.2	2.1	0.3	0.5	0.6	0.13	3.08	0.8	2.6	3.1	1.3
5FU[b]	2.3	2.8	5.2	3.1	6.1	2.8	2.4	4.1	12	6.3	3.5

[a] VP-16 = Etoposide.
[b] 5FU = 5-Fluorouracil.

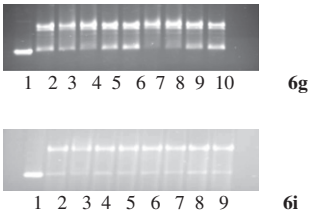


Figure 2. Topoisomerase-II inhibition for compounds **6g** and **6i**. Lane 1: contain supercoiled PBR322 DNA in the absence of topoisomerase-II. Lane 2: CCC DNA related by topoisomerase-II. Lane 3: inhibitory effect of etoposide 10 μM. Lanes 4–10: **6g** and **6i** at concentrations 10, 50, 100, 200, 300, 500 μM, and 1 mM.

4. Conclusion

In summary, new 4β-carbamoyl substituted epipodophyllotoxin analogues with different alkyl and aryl side chains have been prepared. Interestingly these 4β-carbamoyl substituted epipodophyllotoxin congeners exhibit promising anticancer activity. The compounds of this series like etoposide also inhibit DNA topo-II and have the potential for further development.

5. Experimental section

5.1. General methods

The NMR spectra are recorded on Varian Gemini 200 MHz spectrometer, using TMS as an internal reference. IR spectra are recorded on Perkin-Elmer model 683 or 1310 spectrometer with sodium chloride optics. Mass spectra are recorded on CEC-21-100B, Finnigan Mat 1210, or Micromass 7070 spectrometer operating at 70 eV using a direct inlet system. Optical rotations are measured on Jasco Dip 360 digital polarimeter. Melting points are determined on an electrothermal melting point apparatus and are uncorrected. TLC is performed with E. Merck precoated silica gel plates (60F-254) with iodine as a developing agent. Acme, India silica gel (100–200 mesh) is used for column chromatography.

5.1.1. Epipodophyllotoxin (4)

To a solution of podophyllotoxin (**1**) (4.14 g, 10 mmol) in dry CH_3CN (100 mL), NaI (2.98 g, 20 mmol) was added and stirred for 5 minutes. To this stirred suspension, $BF_3{\cdot}OEt_2$ (2.5 mL, 20 mmol) was added drop wise at 0 °C and stirring continued for another 30 min at room temperature. Then a mixture of H_2O/Me_2CO, v/v (1:1) and $BaCO_3$ (2.17 mg, 11 mmol) were added successively. After stirring for 30 min at room temperature, the solvent was evaporated and the residue was treated with 10% $Na_2S_2O_3$ solution and extracted with ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4 and concentrated under vacuum to give the crude product. This was purified by column chromatography (CH_2Cl_2/CH_3COCH_3, 92:8) to afford **4**, 3.52 g in 85% yield. Mp: 148–152 °C, $[\alpha]_D^{25}$: −64.0 (*c* 1.0, $CHCl_3$). ^1H NMR ($CDCl_3$): δ 2.77–2.80 (m, 1H), 3.21 (dd, 1H, *J* = 14.3, 5.2 Hz), 3.73 (s, 6H), 3.76 (s, 3H), 4.33–4.41 (m, 2H), 4.56 (d, 1H, *J* = 5.2 Hz), 4.81 (d, 1H, *J* = 3.0 Hz), 5.96 and 5.99 (ABq, 2H, *J* = 1.5 Hz), 6.22 (s, 2H), 6.53 (s, 1H), 6.84 (s, 1H); IR(KBr) cm^{-1}: 3513, 2921, 2849, 1764, 1589, 1504, 1479; MS (FAB): 414 [M^+].

5.1.2. Epipodophyllotoxin 4-(*p*-nitrophenyl)carbonate (5)

To a solution of *p*-nitrophenylchloroformate (2.5 g, 12.5 mmol) in dry CH_2Cl_2 (10 mL) was added dry pyridine (1.2 mL). Instantaneously a white precipitate was formed. A solution of epipodophyllotoxin (2.07 g, 5 mmol) in dry CH_2Cl_2 (10 mL) was added drop wise under nitrogen atmosphere. The mixture was stirred for 45 min at room temperature and washed with water and dried over Na_2SO_4 and concentrated in vacuo. This residue was purified by column chromatography using ethyl acetate/hexane (1:1) to afford compound **5**, 2.46 g in 85% yield. Mp: 115–120 °C, $[\alpha]_D^{25}$: −122.0 (*c* 1.0, $CHCl_3$). ^1H NMR ($CDCl_3$): δ 3.07–3.16 (m, 1H), 3.29 (dd, 1H, *J* = 14.8, 5.4 Hz), 3.76 (s, 6H), 3.80 (s, 3H), 4.13 (t, 1H, *J* = 4.6 Hz), 4.44 (t, 1H, *J* = 8.5 Hz), 4.71 (d, 1H, *J* = 4.6 Hz), 6.03–6.12 (m, 3H), 6.23 (s, 2H), 6.60 (s, 1H), 7.01 (s, 1H), 7.41 (d, 2H, *J* = 9.3 Hz), 8.32 (d, 2H, *J* = 9.3 Hz); ^{13}C NMR ($CDCl_3$): δ 36.8, 41.2, 43.8, 43.9, 56.3, 60.7, 66.9, 74.2, 101.9, 108.5, 109.6, 110.5, 121.6, 125.4, 126.3, 133.4, 134.3, 145.6, 147.6, 148.5, 149.6, 152.8, 155.2, 173.7; IR(KBr) cm^{-1}: 2911, 2836, 1766, 1720, 1590, 1506, 1485; MS (FAB): 580 [M+1]. Anal. Calcd for $C_{29}H_{25}NO_{12}$: C, 60.10; H, 4.35; N, 2.42. Found: C, 60.05; H, 4.39; N, 2.41.

5.1.3. Epipodophyllotoxin 4-[*N*-(*n*-propyl)]carbamate (6a)

To a stirred solution of epipodophyllotoxin 4-(*p*-nitrophenyl)carbonate (**5**) (290 mg, 0.5 mmol) in dry CH_2Cl_2 (10 mL) was added *n*-propylamine (50 μL, 600 μmol) and triethylamine (85 μL, 600 μmol) at room temperature. The mixture was stirred for 3 h at room temperature and washed with cold saturated $NaHCO_3$ and water. The extract was dried over anhydrous Na_2SO_4 and concentrated in vacuo. This residue was purified by column chromatography using ethyl acetate/cyclohexane (1:1) to afford compound **6a**, 212 mg in 85% yield. Mp: 120–123 °C, $[\alpha]_D^{25}$: −87.0 (*c* 1.0, $CHCl_3$). ^1H NMR ($CDCl_3$): δ 0.94–1.12 (m, 3H), 1.55–1.78 (m,

2H), 2.92–2.99 (m, 1H), 3.13–3.53 (m, 3H), 3.73 (s, 6H), 3.77 (s, 3H), 3.94 (t, 1H, J = 10.2 Hz), 4.36 (t, 1H, J = 7.9 Hz), 4.60 (d, 1H, J = 5.1 Hz), 4.73 (t, 1H, J = 5.8 Hz), 5.98–6.12 (m, 3H), 6.22 (s, 2H), 6.52 (s, 1H), 6.89 (s, 1H); ^{13}C NMR ($CDCl_3$): δ 11.2, 23.1, 38.5, 40.9, 44.1, 44.5, 54.2, 56.8, 60.9, 68.8, 101.5, 108.2, 108.9, 111.0, 132.1, 131.5, 134.2, 136.8, 146.4, 147.8, 148.1, 155.4, 174.8; IR(KBr) cm^{-1}: 3378, 2920, 2851, 1770, 1713, 1590, 1508, 1485; MS (FAB): 500 [M+1]. Anal. Calcd for $C_{26}H_{29}NO_9$: C, 62.52; H, 5.85; N, 2.80. Found: C, 62.47; H, 5.88; N, 2.82.

5.1.4. Epipodophyllotoxin 4-[*N*-(iso-propyl)]carbamate (6b)

This compound was prepared according to the method described for **6a** employing *iso*-propylamine (37 μL, 600 μmol) and epipodophyllotoxin 4-(*p*-nitrophenyl)carbonate (**5**) (290 mg, 0.5 mmol) to afford compound **6b**, 200 mg in 80% yield. Mp: 110–115 °C, $[\alpha]_D^{25}$: −81.0 (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 1.19 (d, 6H, J = 6.8 Hz), 2.91–2.95 (m, 1H), 3.09 (dd, 1H, J = 13.6, 4.5 Hz), 3.73 (s, 6H), 3.77 (s, 3H), 3.79–3.86 (m, 1H), 3.93 (t, 1H, J = 9.0 Hz), 4.37 (t, 1H, J = 9.0 Hz), 4.55 (d, 1H, J = 7.5 Hz), 4.60 (d, 1H, J = 5.2 Hz), 5.96–6.12 (m, 3H), 6.22 (s, 2H), 6.52 (s, 1H), 6.89 (s, 1H); ^{13}C NMR ($CDCl_3$): δ 23.3, 38.6, 41.0, 44.2, 46.5, 54.1, 56.9, 61.0, 68.9, 101.6, 108.5, 108.9, 110.9, 131.6, 132.0, 134.4, 136.7, 146.6, 147.5, 148.3, 155.3, 174.6; IR(KBr) cm^{-1}: 3362, 2927, 2854, 1770, 1720, 1585, 1508, 1485; MS (FAB): 500 [M+1]. Anal. Calcd for $C_{26}H_{29}NO_9$: C, 62.52; H, 5.85; N, 2.80. Found: C, 62.55; H, 5.79; N, 2.81.

5.1.5. Epipodophyllotoxin 4-[*N*-(cyclopropylmethyl)]carbamate (6c)

This compound was prepared according to the method described for **6a** employing cyclopropylmethyl amine (52 μL, 600 μmol) and epipodophyllotoxin 4-(*p*-nitrophenyl)carbonate (**5**) (290 mg, 0.5 mmol) to afford compound **6c**, 204 mg in 80% yield. Mp: 134–137 °C, $[\alpha]_D^{25}$: −85.0 (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 0.21–0.29 (m, 2H), 0.53–0.65 (m, 2H), 0.98–1.13 (m, 1H), 2.92–2.98 (m, 1H), 3.07–3.45 (m, 3H), 3.73 (s, 6H), 3.77 (s, 3H), 3.96 (t, 1H, J = 10.5 Hz), 4.36 (t, 1H, J = 8.3 Hz), 4.6 (d, 1H, J = 4.5 Hz), 4.81 (t, 1H, J = 5.2 Hz), 5.97–6.14 (m, 3H), 6.22 (s, 2H), 6.52 (s, 1H), 6.90 (s, 1H); ^{13}C NMR ($CDCl_3$): δ 3.5, 10.7, 38.5, 41.4, 43.6, 49.1, 54.2, 56.5, 60.9, 68.4, 101.5, 108.2, 108.8, 110.5, 131.6, 132.1, 134.6, 136.8, 146.4, 147.2, 147.7, 155.1, 174.3; IR(KBr) cm^{-1}: 3387, 2927, 2845, 1780, 1723, 1592, 1510, 1487; MS (FAB): 511 [M^+]. Anal. Calcd for $C_{27}H_{29}NO_9$: C, 63.40; H, 5.71; N, 2.74. Found: C, 63.34; H, 5.75; N, 2.79.

5.1.6. Epipodophyllotoxin 4-(*N*,*N*-dimethyl)carbamate (6d)

This compound was prepared according to the method described for **6a** employing *N*,*N*-dimethylamine hydrochloride (49 mg, 0.6 mmol) and epipodophyllotoxin 4-(*p*-nitrophenyl)carbonate (**5**) (290 mg, 0.5 mmol) to afford compound **6d**, 218 mg in 90% yield. Mp: 160–164 °C, $[\alpha]_D^{25}$: −101.0 (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 2.92–3.0 (m, 7H), 3.10 (dd, 1H, J = 14.3, 4.9 Hz), 3.74 (s, 6H), 3.77 (s, 3H), 3.91 (t, 1H, J = 10.3 Hz), 4.36 (t, 1H, J = 8.8 Hz), 4.61 (d, 1H, J = 4.7 Hz), 5.98–6.12 (m, 3H), 6.23 (s, 2H), 6.53 (s, 1H), 6.91 (s, 1H); ^{13}C NMR ($CDCl_3$): δ 37.8, 38.6, 41.6, 44.2, 54.5, 57.1, 61.0, 68.9, 101.8, 108.5, 108.9, 111.0, 131.7, 132.1, 134.5, 137.1, 146.8, 147.5, 147.8, 155.6, 174.9; IR(KBr) cm^{-1}: 2924, 1775, 1720, 1595, 1505, 1483; MS (FAB): 485 [M^+]. Anal. Calcd for $C_{25}H_{27}NO_9$: C, 61.85; H, 5.61; N, 2.89. Found: C, 61.79; H, 5.66; N, 2.91.

5.1.7. Epipodophyllotoxin 4-[*N*-(2-methoxyethyl)]carbamate (6e)

This compound was prepared according to the method described for **6a** employing 2-methoxyethylamine (51 μL, 600 μmol) and epipodophyllotoxin 4-(*p*-nitrophenyl)carbonate (**5**) (290 mg, 0.5 mmol) to afford compound **6e**, 206 mg in 80% yield. Mp: 130–135 °C, $[\alpha]_D^{25}$: −86.0 (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 2.93–2.97 (m, 1H), 3.13 (dd, 1H, J = 14.8, 5.4 Hz), 3.34 (s, 3H), 3.40–3.56 (m, 4H), 3.73 (s, 6H), 3.77 (s, 3H), 3.97 (t, 1H, J = 10.1 Hz), 4.36 (t, 1H, J = 7.8 Hz), 4.61 (d, 1H, J = 4.6 Hz), 5.09 (t, 1H, J = 5.4 Hz), 5.98–6.15 (m, 3H), 6.23 (s, 2H), 6.52 (s, 1H), 6.90 (s, 1H); ^{13}C NMR ($CDCl_3$): δ 38.1, 40.8, 41.0, 43.9, 54.5, 56.9, 59.3, 61.3, 68.9, 70.7, 101.9, 108.6, 108.9, 110.9, 131.8, 132.3 , 134.6, 136.7, 146.9, 147.8, 147.8, 155.7, 174.9; IR(KBr) cm^{-1}: 3376, 2928, 2847, 1778, 1715, 1596, 1505, 1484; MS (FAB): 515 [M^+]. Anal. Calcd for $C_{26}H_{29}NO_{10}$: C, 60.58; H, 5.67; N, 2.72. Found: C, 60.61; H, 5.62; N, 2.76.

5.1.8. Epipodophyllotoxin 4-[*N*-(2-propynyl)]carbamate (6f)

This compound was prepared according to the method described for **6a** employing 2-propynylamine (41 μL, 600 μmol) and epipodophyllotoxin 4-(*p*-nitrophenyl)carbonate (**5**) (290 mg, 0.5 mmol) to afford compound **6f**, 186 mg in 75% yield. Mp: 163–166 °C, $[\alpha]_D^{25}$: −90.0 (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 2.22 (s, 1H), 2.93–2.95 (m, 1H), 3.10 (dd, 1H, J = 14.3, 5.2 Hz), 3.73 (s, 6H), 3.77 (s, 3H), 3.90–4.10 (m, 3H), 4.36 (t, 1H, J = 9.0 Hz), 4.60 (d, 1H, J = 4.5 Hz), 4.99 (t, 1H, J = 5.2 Hz), 5.99–6.13 (m, 3H), 6.22 (s, 2H), 6.53 (s, 1H), 6.90 (s, 1H); ^{13}C NMR ($CDCl_3$): δ 30.1, 38.1, 41.3, 43.5, 54.3, 56.8, 61.1, 69.1, 72.9, 80.3, 101.8, 108.5, 108.8, 110.3, 131.1, 132.2, 134.6, 136.8, 146.8, 147.2, 147.8, 155.8, 175.1; IR(KBr) cm^{-1}: 3392, 2932, 2843, 1776, 1715, 1598, 1507, 1484; MS (FAB): 495 [M^+]. Anal. Calcd for $C_{26}H_{25}NO_9$: C, 63.03; H, 5.09; N, 2.83. Found: C, 62.96; H, 5.13; N, 2.81.

5.1.9. Epipodophyllotoxin 4-(morpholine)carbamate (6g)

This compound was prepared according to the method described for **6a** employing morpholine (52 μL, 600 μmol) and epipodophyllotoxin 4-(*p*-nitrophenyl)carbonate (**5**) (290 mg, 0.5 mmol) to afford compound **6g**, 237 mg in 90% yield. Mp: 155–158 °C, $[\alpha]_D^{25}$: −88.0 (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 2.90–2.96 (m, 1H), 3.06 (dd, 1H, J = 14.3, 5.2 Hz), 3.36–3.71 (m, 8H, morpholine), 3.73 (s, 6H), 3.77 (s, 3H), 3.88 (t, 1H, J = 10.5 Hz), 4.37–4.46 (m, 1H), 4.61 (d, 1H, J = 5.2 Hz), 5.99–6.17 (m, 3H), 6.22 (s, 2H), 6.53 (s, 1H), 6.89 (s, 1H); ^{13}C NMR ($CDCl_3$): δ 38.9, 41.3, 43.5, 49.7, 54.3, 56.8, 60.9, 66.3, 68.4, 101.7, 108.5, 108.5, 110.6, 131.6, 131.7, 134.1, 136.8, 146.7, 147.6, 148.1, 155.9, 175.3; IR(KBr) cm^{-1}: 3357, 2946, 2841, 1775, 1715, 1596, 1505, 1488; MS (FAB): 527 [M^+]. Anal. Calcd for $C_{27}H_{29}NO_{10}$: C, 61.47; H, 5.54; N, 2.66. Found: C, 61.41; H, 5.60; N, 2.63.

5.1.10. Epipodophyllotoxin 4-[*N*-(4-trifluoromethylbenzyl)] carbamate (6h)

This compound was prepared according to the method described for **6a** employing 4-trifluoromethyl benzylamine (86 μL, 600 μmol) and epipodophyllotoxin 4-(*p*-nitrophenyl)carbonate (**5**) (290 mg, 0.5 mmol) to afford compound **6h**, 215 mg in 70% yield. Mp: 180–183 °C, $[\alpha]_D^{25}$: −65.0 (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 2.99–3.06 (m, 1H), 3.18 (dd, 1H, J = 14.4, 5.0 Hz), 3.74 (s, 6H), 3.8 (s, 3H), 4.03 (t, 1H, J = 10.1 Hz), 4.42– 4.54 (m, 3H), 4.63 (d, 1H, J = 4.5 Hz), 6.01–6.21 (m, 3H), 6.26 (s, 2H), 6.55 (s, 1H), 6.93 (s, 1H), 7.39 (d, 2H, J = 7.6 Hz), 7.61 (d, 2H, J = 7.6 Hz); ^{13}C NMR ($CDCl_3$): δ 37.1, 41.5, 43.8, 44.7, 56.3, 60.6, 67.4, 69.1, 101.6, 108.4, 109.6, 110.1, 125.6, 125.6, 132.8, 134.5, 142.1, 147.5, 148.9, 152.7, 156.0, 174.2; IR(KBr) cm^{-1}: 3362, 2924, 1778, 1720, 1590, 1507, 1484; MS (FAB): 615 [M^+]. Anal. Calcd for $C_{31}H_{28}F_3NO_9$: C, 60.49; H, 4.58; N, 2.28. Found: C, 60.54; H, 4.51; N 2.24.

5.1.11. Epipodophyllotoxin 4-[*N*-2-(3-indolyl)ethyl]carbamate (6i)

This compound was prepared according to the method described for **6a** employing tryptylamine hydrochloride (118 mg,

0.6 mmol) and epipodophyllotoxin 4-(*p*-nitrophenyl)carbonate (**5**) (290 mg, 0.5 mmol) to afford compound **6i**, 240 mg in 80% yield. Mp: 170–175 °C, $[\alpha]_D^{25}$: −82.0 (*c* 1.0, $CHCl_3$). ^1H NMR ($CDCl_3$): δ 2.98–3.14 (m, 4H), 3.58–3.62 (m, 2H), 3.73 (s, 6H), 3.77 (s, 3H), 3.85–3.88 (m, 1H), 4.33–4.36 (m, 1H), 4.60 (d, 1H, J = 4.5 Hz), 4.79 (t, 1H, J = 5.4 Hz), 5.98–6.12 (m, 3H), 6.23 (s, 2H), 6.51 (s, 1H), 6.88 (s, 1H), 7.00–7.25 (m, 3H), 7.32–7.62 (m, 2H); ^{13}C NMR ($CDCl_3$): δ 25.8, 38.2, 41.2, 41.4, 43.7, 54.3, 56.8, 60.8, 68.7, 101.8, 108.3, 108.8, 110.5, 111.7, 113.5, 119.5, 120.5, 121.7, 123.5, 127.8, 131.9, 132.3, 134.3, 136.8, 136.8, 146.6, 147.5, 148.1, 155.8, 175.3; IR(KBr) cm^{-1}: 3380, 2923, 2852, 1774, 1715, 1589, 1506, 1483; MS (FAB): 601 [M+1]. Anal. Calcd for $C_{33}H_{32}N_2O_9$: C, 65.99; H, 5.37; N, 4.66. Found: C, 66.05; H, 5.32; N, 4.63.

5.1.12. Epipodophyllotoxin 4-[2-(dimethylamino)ethyl]carba mate (6j)

This compound was prepared according to the method described for **6a** employing N^1,N^1-dimethylethane-1,2-diamine (53 mg, 0.6 mmol) and epipodophyllotoxin 4-(*p*-nitrophenyl)carbonate (**5**) (290 mg, 0.5 mmol) to afford compound **6j**, 227 mg in 86% yield. Mp: 172–174 °C, $[\alpha]_D^{25}$: −105.0 (*c* 1.0, $CHCl_3$). ^1H NMR ($CDCl_3$): δ 2.42–2.92 (m, 9H), 3.10 (dd, 1H, J = 14.3, 4.9 Hz), 3.32–3.38 (m, 2H), 3.75 (s, 6H), 3.79 (s, 3H), 3.91 (t, 1H, J = 10.3 Hz), 4.36 (t, 1H, J = 8.8 Hz), 4.61 (d, 1H, J = 4.7 Hz), 5.01 (t, 1H, J = 5.1 Hz), 5.98–6.12 (m, 3H), 6.23 (s, 2H), 6.53 (s, 1H), 6.92 (s, 1H); ^{13}C NMR ($CDCl_3$): δ 38.5, 38.6, 41.8, 44.5, 47.1, 54.8, 56.9, 60.8, 61.1, 68.8, 101.9, 108.6, 109.2, 111.3, 131.8, 132.5, 134.7, 136.9, 147.0, 147.8, 148.7, 155.5, 175.6; IR(KBr) cm^{-1}: 3378, 2925, 1775, 1720, 1591, 1505, 1483; MS (FAB): 529 [M+1]. Anal. Calcd for $C_{27}H_{32}N_2O_9$: C, 61.35; H, 6.11; N, 5.30. Found: C, 61.28; H, 6.18; N, 5.28.

6. Procedure of the SRB-assay

Tumour cells were grown in tissue culture flasks in growth medium (RPMI-1640 with 2 mM glutamine, pH 7.4, 10% fetal calf serum, 100 μg/ml streptomycin, and 100 units/ml penicillin) at 37 °C in an atmosphere of 5% CO_2 and 95% relative humidity in a CO_2 incubator. The cells at subconfluent stage were harvested from the flask by treatment with trypsin (0.05% trypsin in PBS containing 0.02% EDTA) and suspended in growth medium. Cells with more than 97% viability (trypan blue exclusion) were used for determination of cytotoxicity. An aliquot of 100 μl of cells were transferred to a well of 96-well tissue culture plate. The cells were allowed to grow for 24 h at 37 °C in a CO_2 incubator as stated above. Test materials (100 μl) were then added to the wells and cells were further allowed to grow for another 48 h. Suitable blanks and positive controls were also included. Each test was done in triplicate. The cell growth was stopped by gently layering of 50 μl of 50% trichloroacetic acid. The plates were incubated at 4 °C for an hour to fix the cells attached to the bottom of the wells. Liquids of all the wells were gently pipette out and discarded. The plates were washed five times with distilled water to remove TCA, growth medium, etc and were air-dried. 100 μl of SRB solution (0.4% in 1% acetic acid) was added to each well and the plates were incubated at room temperature for 30 min. The unbound SRB was quickly removed by washing the wells five times with 1% acetic acid. Plates were air dried, tris-buffer (100 μl of 0.01 M, pH 10.4) was added to all the wells and plates were gently stirred for 5 minutes on a mechanical stirrer. The optical density was recorded on ELISA reader at 540 nm. The cell growth at absence of any test material was considered 100% and in turn growth inhibition was calculated. IC_{50} values were determined by regression analysis.

7. DNA Topoisomerase-II inhibition assay

Topoisomerase-II activity was determined using kit (Topogen Inc., USA, Cat No. 2000H). Reaction was assembled in micro centrifuge tube that contains super coiled DNA 250 ng/μl & Topoisomerase-II (4 units) in assay buffer (A 0.1 volume and B 1 volume). In each reaction 2 μl sample was added then volume was made up to 20 μl with water and then incubated at 37 °C. Reaction was terminated by addition of 2 μl of 10% SDS. Each sample tube was treated with proteinase K and extracted once with chloroform/isoamyl alcohol (24:1). Products were resolved by 1% agarose gel electrophoresis in TAE buffer (40 mM tris-acetate, pH 8.0, and 1 mM EDTA) and stained with 0.5 μg/ml ethidium bromide (EtBr).

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