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Synthesis of 4β-*N*-polyaromatic substituted podophyllotoxins: DNA topoisomerase inhibition, anticancer and apoptosis-inducing activities

Ahmed Kamal [a,*], B. Ashwini Kumar [a], Paidakula Suresh [a], Satyam Kumar Agrawal [b], Gousia Chashoo [b], Shashank K. Singh [b], A. K. Saxena [b]

[a] Division of Organic Chemistry, Indian Institute of Chemical Technology, Hyderabad 500 607, India
[b] Pharmacology Division, Indian Institute of Integrative Medicine, Jammu 180 001, India

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ABSTRACT

A new class of 4β-*N*-polyaromatic substituted podophyllotoxin congeners have been synthesized and evaluated for their DNA topoisomerase-II (topo-II) inhibition as well as anticancer potential in some human cancer cell lines. The ease of synthesis and interesting biological activities make the present series of polyaromatic-podophyllotoxin congeners as a promising new structure for the development of new anticancer agents based on podophyllotoxin scaffold.

1. Introduction

Podophyllotoxin (**1**), a well-known naturally occurring aryltetralin lignan, extracted from the roots of *Podophyllotoxin peltatum*, whose medicinal properties particularly cytotoxicity have been well-known for centuries.[1–3] Attempts to use **1** for the treatment of cancer were mostly unsuccessful due to the severe side effects, hence this cyclolignan has been employed as a lead compound for the design and development of anticancer agents. This led to the synthesis of a number of semisynthetic derivatives that are in clinical use for the treatment of a variety of malignancies. The prominent ones are etoposide (**2**), teniposide (**3**), and etopophos (**4**), interestingly these differ substantially in their mechanism of action compared to the parent compound podophyllotoxin. Etoposide and other analogs inhibit DNA topo-II, while podophyllotoxin inhibit the assembly in the microtubulin.[4,5] In view of the development of drug resistance by cancer cells as well as side effects associated with the use of these agents in clinic (which include myelosuppression, eutropenia, and nausea), the search for new effective anticancer drugs with **1** as a lead compound still remains as an intense area of research.

The recent synthetic studies on podophyllotoxin have been focused on the synthesis of the C-4 non-sugar substituted analogs, which exhibit improved topoisomerase II inhibition. The replacement of the C-4 sugar moiety of etoposide and teniposide with a non-sugar substituent has improved the therapeutic value of etoposide.[6] The C-4 non-sugar substituent can be linked through O-, S- or N-linkage. In general, the O-linked (ethers, esters) and S-linked (thioethers) compounds are less active in comparison to the N-linked congeners[7–10] like NPF (**7**) and GL-331 (**8**). Prior molecular area-oriented structure–activity relationship (SAR) studies[11] and the composite pharmacophore model proposed by MacDonald et al.[12] designated the C-4 molecular area of **1** analogs as a variable region. The comparative molecular field analysis (CoMFA) models generated by Lee and co-workers[13,14] further demonstrated that bulky substituents at C-4 might be favorable for DNA topo-II inhibition. These postulates are compatible with the excellent activity profiles of NK 611 (**5**), TOP-53 (**6**), and GL-331.[15] In addition, both GL-331 and TOP-53 showed good topoisomerase II inhibitory and antitumor activity, while the drug-resistance profiles are significantly different from those of **1**. This investigation thus suggest the important role played by various substitutions at C-4 in the activity profile of such analogs and the feasibility of optimizing the activity through rational C-4 modification in this class of compounds.[16]

Polycyclic aromatic hydrocarbons have been considered as one of the most prevalent antitumor agents. Becker and co-workers, explained an exploratory synthetic and biological evaluation of unique polycyclic aromatic compounds such as chrysene (**9**) and 1-pyrene methylamine derivatives (**10**).[17–19] These compounds

* Corresponding author. Tel.: +91 40 27193157; fax: +91 40 27193189.
E-mail address: ahmedkamal@iict.res.in (A. Kamal).

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possess a broad spectrum of chemotherapeutic activity against both murine and human tumors. Interestingly, some of them like crisnatol (**11**) and 502U83 (**12**) were chosen for clinical development, as shown in Figure 1. Based on the information available on the structure–activity relationship studies and in continuation of our ongoing project on the design and development of structurally modified podophyllotoxin analogs,[16,20] we became interested in the synthesis of different polycyclic aromatic hydrocarbons linked to the epipodophyllotoxin ring system.[21] Hence, in the present work we report the synthesis of a series of 4β-*N*-polyaryl substituted podophyllotoxin congeners, and their cytotoxic activities against certain tumor cell lines (502713, HCT-15, HEP-2, IMR-32, A-549, DU-145, and PC-3). Furthermore, DNA topo-II inhibition and cell cycle analysis was evaluated for some representative compounds of this series.

2. Chemistry

These new 4β-polyarylamino podophyllotoxin congeners were synthesized by coupling of various substituted polyarylamine compounds with podophyllotoxin by employing $BF_3{\cdot}OEt_2$/NaI reagent system.[22] The reaction proceeded via the formation of 4β-iodopodophyllotoxin or 4β-iodo-4′-*O*-demethylpodophyllotoxin as intermediates. Since these iodo intermediates are highly reactive and susceptible to nucleophilic attack in presence of moisture, these were reacted in the crude form for the next step to yield the final products. The synthetic strategy developed for the preparation of these new 4β-*N*-polyaryl substituted podophyllotoxin congeners is shown in Scheme 2. Two of the amino substituted precursors, that is, benzophenone amine **16** and its mustard **19** were obtained from 4-fluoro-4′-nitrobenzophenone **15**, which in turn was obtained by the reaction of fluorobenzene **13** and 4-nitrobenzoyl chloride **14** as shown in Scheme 1. All the synthesized compounds were characterized by ^{1}H NMR, ^{13}C NMR, IR, and mass spectral data.

3. Biological activity

Compounds **20a–f** and **21a–e** were evaluated for their anticancer activity in selected human cancer cell lines, that is, colon (502713, HCT-15), liver (HEP-2), neuroblastoma (IMR-32), lung (A-549), and prostate (DU-145, PC-3) origin by employing the sulforhodamine B (SRB) assay method.[23] The results are summarized in Table 1 and compared to etoposide and 5-fluorouracil. Most of these new compounds exhibited significant anticancer activity compared to etoposide and camptothecin as well as 5-fluorouracil.

DNA topo-II is the pharmacological target of clinical relevance for most of the podophyllotoxin lignans;[24] therefore, some of the representative compounds (**20c, 20d**, and **21d**) were evaluated for its inhibition. All these compounds exhibited comparable

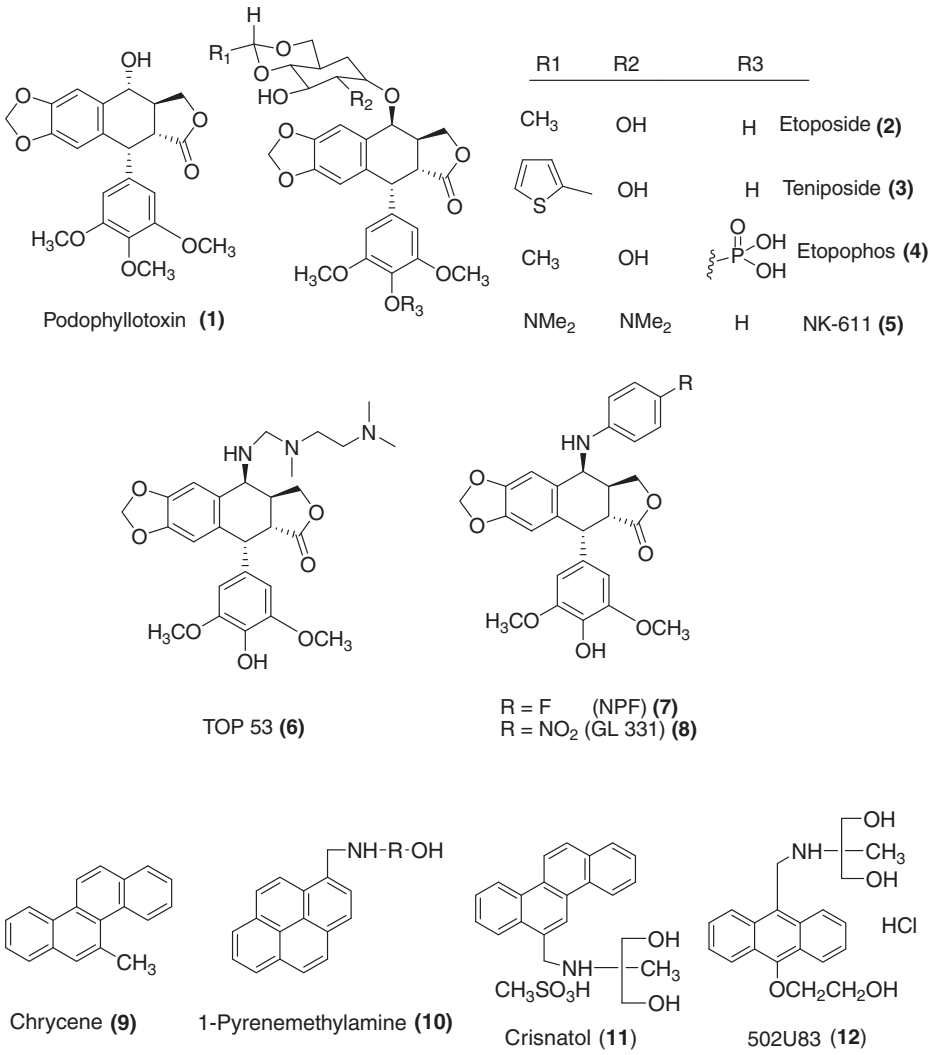
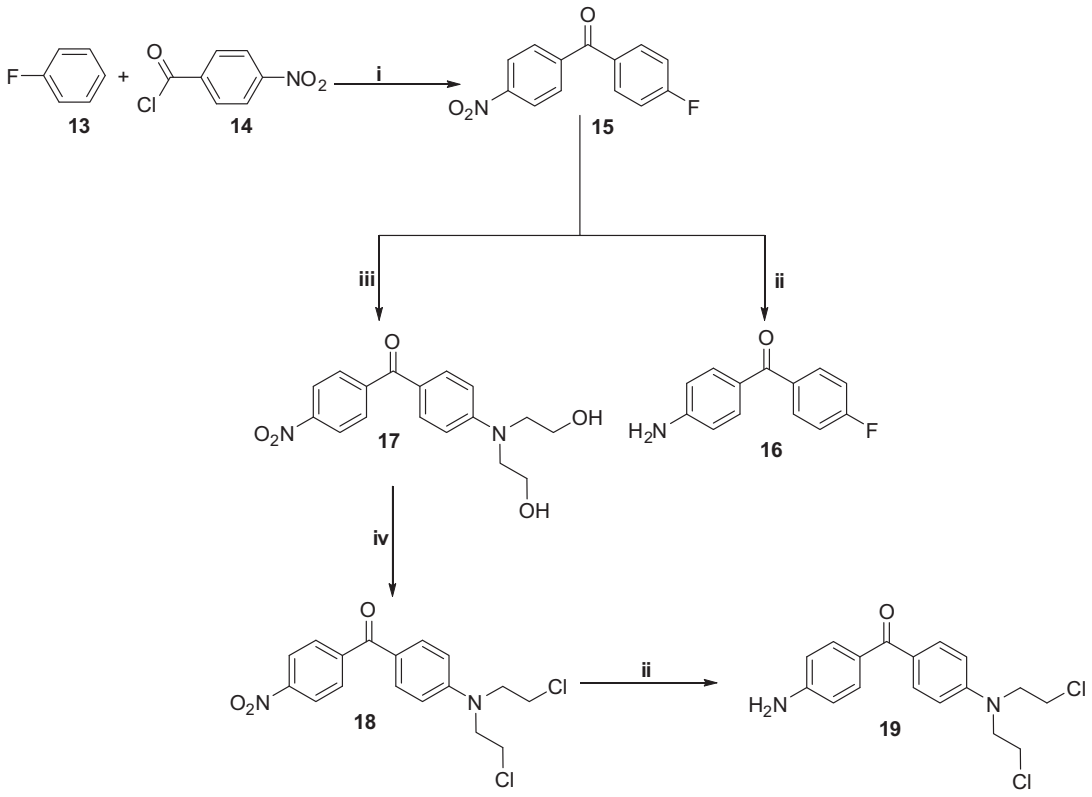


Figure 1.



Scheme 1. Reagents and conditions: (i) $AlCl_3$, CH_2Cl_2, 30 min, rt. (ii) $SnCl_2{\cdot}2H_2O$, MeOH, reflux, 3 h. (iii) Diethanolamine, DMSO, reflux, 5 h. (iv) (a) MsCl, TEA, CH_2Cl_2, rt, 4 h. (b) NaCl, DMF, reflux, 15 min.

inhibition of DNA topo-II catalytic activity comparable to etoposide and the results are illustrated in Figure 2.

Further it was considered of interest to evaluate the DNA fragmentation aspect for **20d**. This compound at 0.5, 1, 5, and 10 μM concentrations induced DNA fragmentation in leukemia (MOLT-4) cells after 24 h incubation as shown in Figure 3. In order to understand the mode of action of **20d**, its cell cycle effects were examined. Thus **20d** was tested at 0.5, 1, 5, and 10 μM concentrations by treatment with leukemia (MOLT-4) cells and compared to camptothecin (5 μM). This study indicated that it blocks the G_1 phase of cell cycle and there was an increase in sub-G_1 cell population indicating apoptosis as seen from Figure 4.

4. Conclusion

In summary, new 4β-*N*-polyaromatic-podophyllotoxin congeners having different polyaryl substitutions including a benzophenone and its mustard moieties were prepared and evaluated against some human cancer cell lines. Almost all the compounds exhibited significant in vitro anticancer activity at micromolar (μM) concentration. These new podophyllotoxins also inhibited DNA topo-II and interestingly the cell cycle studies revealed that these compounds possess apoptosis-inducing activity. The insights obtained from these investigations are considered useful for the development of newer structures based on the podophyllotoxin lignans.

5. Experimental section

5.1. General methods

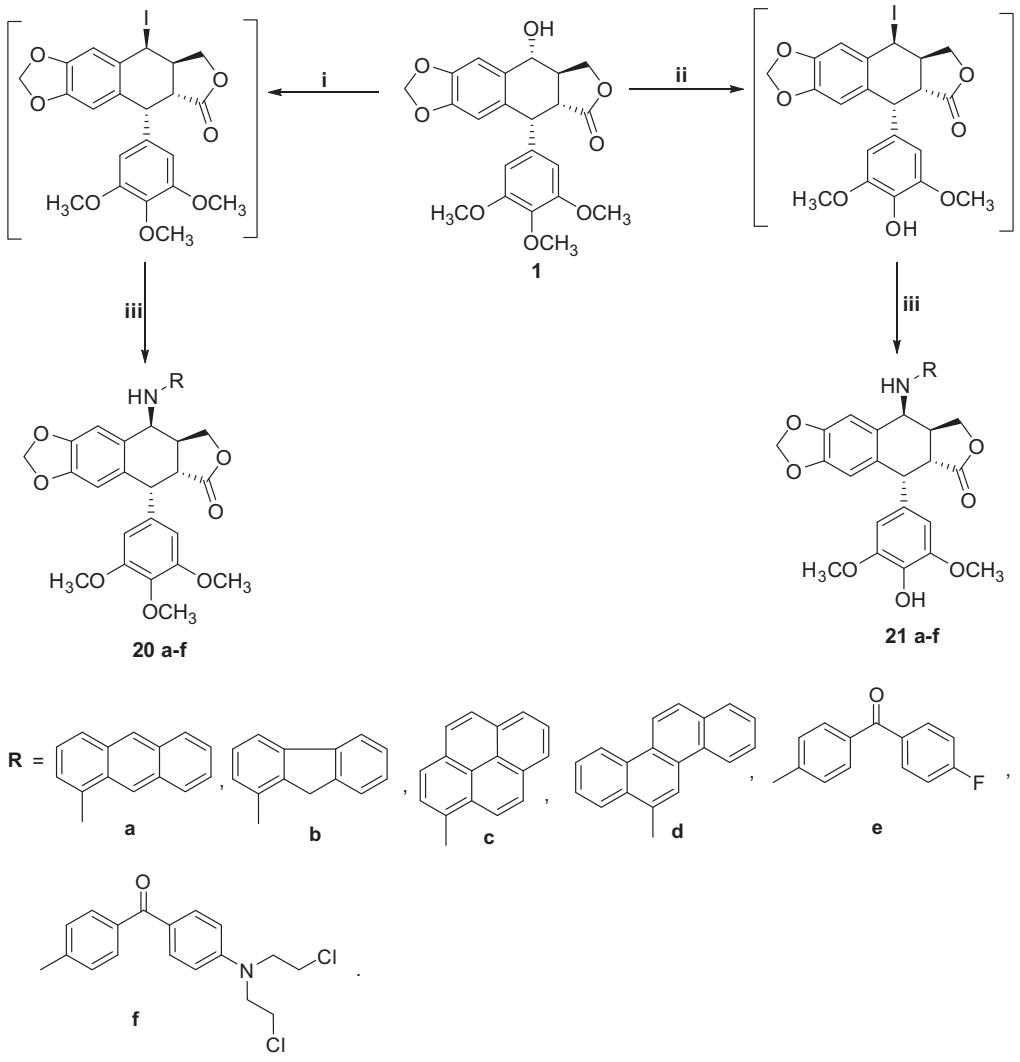
The NMR spectra are recorded on Varian Gemini 200 MHz spectrometer, using TMS as an internal reference. IR spectra are recorded on Perkin–Elmer model 683 or 1310 spectrometer with sodium chloride optics. Mass spectra are recorded on CEC-21-100B, Finnigan Mat 1210, or Micromass 7070 spectrometer operating 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. 4-Fluoro-4′-nitrobenzophenone (15)

To a solution of fluorobenzene (**13**) (2.71 mL, 20 mmol) in dry CH_2Cl_2 was added 4-nitrobenzoyl chloride (**14**) (4.44 g, 24 mmol) and stirred for 5 min. To this stirred solution $AlCl_3$ (5.32 g, 40 mmol) was added and stirred for 30 min. This reaction mixture was poured in ice cold water. This solution was filtered, extracted with ethyl acetate, and dried over anhydrous Na_2SO_4. This solvent was removed under vacuum and the crude product was subjected to column chromatography using ethyl acetate/hexane (2:3) affords **14**, 3.70 g in 75% yield. ^{1}H NMR (200 MHz, DMSO-d_6 + $CDCl_3$): δ 7.26 (t, 2H, J = 8.2 Hz), 7.82–7.98 (m, 4H), 8.38 (d, 2H, J = 8.2 Hz).

5.1.2. 4-Amino-4′-fluorobenzophenone (16)

The compound **15** (735 mg, 3 mmol) dissolved in methanol (40 mL) and added $SnCl_2{\cdot}2H_2O$ (3.38 g, 15 mmol) was refluxed for 3 h. The reaction mixture was cooled and the methanol was evaporated under vacuum, and the residue was carefully adjusted to pH 8 with saturated $NaHCO_3$ solution and then extracted with ethyl acetate (2 × 30 mL). The combined organic phase was washed with brine (15 mL), dried over anhydrous Na_2SO_4, and evaporated under vacuum. This crude product was subjected to column chromatography using ethyl acetate/hexane (1:1) affords **16**, 516 mg in 80%



Scheme 2. Reagents and conditions: (i) $BF_3{\cdot}OEt_2$/NaI, CH_3CN, rt, 30 min. (ii) $BF_3{\cdot}OEt_2$/NaI, CH_2Cl_2, rt, 5 h. (iii) R-NH_2, THF, $BaCO_3$, rt, 8 h.

Table 1
Anticancer activity data of compounds **20a–f** and **21a–e**

Compound	IC_{50} (μM)						
	Colon		Liver	Neuroblastoma	Lung	Prostate	
	502713	HCT-15	HEP-2	IMR-32	A-549	DU-145	PC-3
20a	9.4	6.3	6.7	6.7	8.8	21	7.9
20b	5.5	0.09	3	5.5	7.1	6.6	4.3
20c	0.002	0.0001	—	—	0.0009	—	0.0001
20d	0.1	0.002	0.002	0.003	0.04	2.2	0.1
20e	4.1	6.2	7.1	19	17	—	6.7
20f	0.1	0.9	0.9	6.3	3.08	3.7	2.6
21a	—	8.2	8.6	2.3	8.3	18	—
21b	0.001	0.0001	1	10	1	2.7	0.5
21c	0.002	0.0001	0.0002	0.2	1.7	2.2	—
21d	0.1	0.1	0.01	0.001	0.0003	0.0003	0.0003
21e	1.4	0.08	1.3	1.3	0.2	0.3	6.0
Etoposide	0.1	0.9	0.9	6.3	3.08	3.7	2.6
Camptothecin	nd	0.067	0.7	0.08	0.03	0.02	0.03
5FU[a]	4.6	3.6	0.3	18	2.4	4.8	12

nd = not determined.
[a] 5FU = 5-Fluorouracil is one of the control drug.

yield. ^{1}H NMR (200 MHz, DMSO-d_6 + $CDCl_3$): δ 5.34 (br s, 2H), 6.65 (d, 2H, J = 9.0 Hz), 7.16 (t, 2H, J = 9.0 Hz), 7.58 (d, 2H, J = 9.0 Hz), 7.69–7.78 (m, 2H).

5.1.3. 4-[Di(2-hydroxyethyl)amino]-4′-nitrorobenzophenone (17)

The compound **15** (2.45 g, 10 mmol) dissolved in DMSO (25 mL) and added diethanolamine (3.15 g, 15 mmol) was refluxed for 5 h.

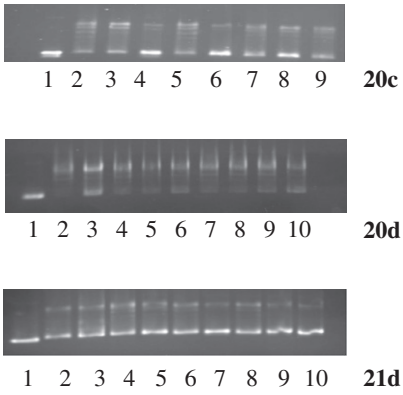


Figure 2. Topoisomerase-II inhibition for compounds **20c**, **20d**, and **21d**. Lane 1: contain supercoiled PBR322 DNA in the absence of topoisomerase-II. Lane 2: CCC DNA related by topoisomerase-II. Lane 3: inhibitory effect of etoposide (VP-16) 10 μM. Lanes 4–10: **20c**, **20d**, and **21d** at concentrations 10, 50, 100, 200, 300, 500 μM, and 1 mM.

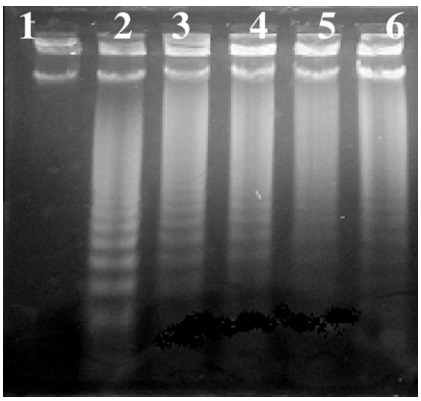


Figure 3. DNA fragmentation assay for compound **20d**. Lane 1: untreated cells. Lane 2: camptothecin (5 μM). Lanes 3–6: **20d** at concentration 0.5, 1, 5, and 10 μM.

The reaction mixture was cooled and extracted with ethyl acetate and ice cold water. The combined organic phase was washed with brine (15 mL), dried over anhydrous Na_2SO_4, and evaporated under vacuum. This crude product was subjected to column chromatography using ethyl acetate/hexane (8:1) affords **17**, 2.64 g in 80% yield. ^{1}H NMR (200 MHz, DMSO-d_6 + $CDCl_3$): δ 3.65–3.88 (m, 4H), 3.90–4.46 (m, 4H), 6.65 (d, 2H, J = 8.2 Hz), 7.65 (d, 2H, J = 8.2 Hz), 7.82 (d, 2H, J = 8.2 Hz), 8.35 (d, 2H, J = 8.2 Hz).

5.1.4. 4-[Di(2-chloroyethyl)amino]-4′-nitrorobenzophenone (18)

The compound **17** (1.65 g, 5 mmol) dissolved in dry CH_2Cl_2 (20 mL) was added triethylamine (2.09 mL, 15 mmol) at 0 °C and stirred for 5 min. To this reaction mixture mesyl chloride (0.77 mL, 10 mmol) was added drop wise and stirred for 4 h at room temperature, dichloromethane was removed under vacuum and extracted with ethyl acetate. The combined organic phase was dried over anhydrous Na_2SO_4 and evaporated under vacuum. This crude product was subjected to next step without any further purification. The above crude compound was dissolved in DMF was added NaCl (1.45 g, 25 mmol) and heated to reflux for 15 min. The reaction mixture was cooled and extracted with ethyl acetate, dried over anhydrous Na_2SO_4, and evaporated under vacuum. This crude product was subjected to column chromatography using ethyl acetate/hexane (9:1) as eluent affords **18**, 1.28 g in 70% yield. ^{1}H NMR (200 MHz, DMSO-d_6 + $CDCl_3$): δ 3.74 (t, 4H, J = 6.7 Hz), 3.91 (t, 4H, J = 6.0 Hz), 6.80 (d, 2H, J = 9.0 Hz), 7.74 (d, 2H, J = 8.2 Hz), 7.87 (d, 2H, J = 8.2 Hz), 8.35 (d, 2H, J = 8.2 Hz).

5.1.5. 4-Amino-4′-[di(2-chloroyethyl)amino]benzophenone (19)

The compound **18** (1.10 g, 3 mmol) dissolved in methanol (30 mL) and added $SnCl_2{\cdot}2H_2O$ (3.38 g, 15 mmol) was refluxed for 1.5 h. The reaction mixture was cooled and the methanol was evaporated under vacuum, and the residue was carefully adjusted to pH 8 with saturated $NaHCO_3$ solution and then extracted with ethyl acetate (2×30 mL). The combined organic phase was washed with brine (15 mL), dried over anhydrous Na_2SO_4, and evaporated under vacuum. This crude product was subjected to

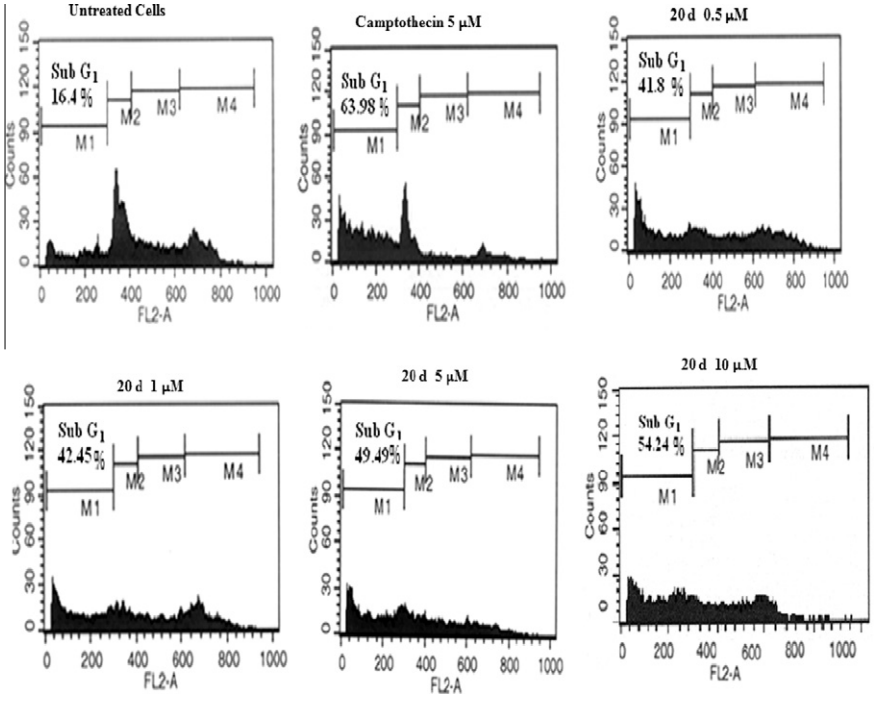


Figure 4. DNA cell cycle analysis for compound **20d**.

column chromatography using ethyl acetate/hexane (9:1) affords **19**, 809 mg in 80% yield. ^{1}H NMR (200 MHz, DMSO-d_6 + $CDCl_3$): δ 3.36 (t, 4H, J = 6.6 Hz), 3.50 (t, 4H, J = 6.5 Hz), 6.27–6.40 (m, 4H), 7.20–7.37 (m, 4H).

5.1.6. 4β-(1″-Anthrylamino)-4-desoxypodophyllotoxin (20a)

To a solution of podophyllotoxin (414 mg, 1 mmol) in dry acetonitrile (10 mL), sodium iodide (298 mg, 2 mmol) was added and stirred for 5 min to this stirred suspension $BF_3{\cdot}OEt_2$ (0.13 mL, 2 mmol) was added dropwise at 0 °C and the stirring was continued for another 0.5 h at room temperature. This solution was then evaporated in vacuo and used for the next reaction without further purification. To the crude product, anhydrous barium carbonate (395 mg, 2 mmol) and 1-anthraceneamine (231 mg, 1.2 mmol) in 10 mL of dry THF under nitrogen was added and stirred for 8 h at room temperature. The reaction mixture was filtered, diluted with ethyl acetate and washed with water, 10% aqueous sodium thiosulphate solution, dried, and purified via column chromatography using ethyl acetate/hexane (2:3) as eluent affords **20a**, 530 mg in 90% yield. Mp: 195–200 °C, $[\alpha]_D^{25}$: −39.0 (c = 1.0, $CHCl_3$). ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.87–3.10 (m, 1H), 3.30–3.43 (dd, 1H, J = 13.6, 4.5 Hz), 3.78 (s, 6H), 3.80 (s, 3H), 3.97 (t, 1H, J = 9.0 Hz), 4.43 (t, 1H, J = 8.0 Hz), 4.67 (d, 2H, J = 4.5 Hz), 4.95 (br s, 1H), 5.97 (d, 2H, J = 3.0 Hz), 6.33 (s, 2H), 6.43 (d, 1H, J = 7.5 Hz), 6.61 (s, 1H), 6.80 (s, 1H), 7.28–7.37 (m, 1H), 7.43–7.78 (m, 3H), 7.94–8.12 (m, 2H), 8.24 (s, 1H), 8.35 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 38.7, 42.3, 43.7, 52.5, 56.4, 60.7, 69.0, 101.5, 108.7, 109.3, 110.0, 118.4, 118.8, 122.9, 125.4, 125.5, 125.8, 126.9, 127.8, 128.2, 130.6, 131.0, 131.8, 132.2, 132.5, 135.2, 142.4, 147.7, 148.4, 152.7, 174.6; IR (KBr) cm^{-1}: 3409, 2903, 2834, 1774, 1586, 1503, 1481; MS (FAB): 589 [M$^+$]. Anal. Calcd for $C_{36}H_{31}NO_7$: C, 73.33; H, 5.30; N, 2.38. Found: C, 73.29; H, 5.28; N, 2.35.

5.1.7. 4β-(1″-Fluorenylamino)-4-desoxypodophyllotoxin (20b)

This compound was prepared by method described for **20a** employing 1-fluorenylamine (220 mg, 1.2 mmol) and podophyllotoxin (414 mg, 1 mmol) affords **20b**, 432 mg in 75% yield. Mp: 209–212 °C, $[\alpha]_D^{25}$: −129.0 (c = 1.0, $CHCl_3$). ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.89–3.07 (m, 1H), 3.20–3.49 (dd, J = 13.6, 4.5 Hz), 3.77 (s, 6H), 3.82 (s, 3H), 3.84 (s, 2H), 3.92 (br s, 1H), 4.07 (t, 1H, J = 9.0 Hz), 4.44 (t, 1H, J = 8.3 Hz), 4.63 (d, 1H, J = 4.5 Hz), 4.76–4.80 (m, 1H), 5.98 (d, 2H, J = 3.0 Hz), 6.34 (s, 2H), 6.55 (s, 1H), 6.58 (dd, 1H, J = 8.3, 2.2 Hz), 6.75–6.79 (m, 1H), 6.81 (s, 1H), 7.10–7.70 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 36.9, 38.6, 42.4, 43.8, 52.4, 56.4, 60.7, 69.0, 101.4, 108.8, 109.3, 109.9, 111.2, 118.2, 118.3, 119.9, 122.9, 125.0, 126.6, 126.8, 127.0, 131.1, 131.8, 133.7, 135.1, 140.7, 142.3, 143.6, 147.8, 148.3, 152.8, 174.5; IR (KBr) cm^{-1}: 3364, 2906, 2834, 1774, 1615, 1585, 1503, 1457; MS (FAB): 577 [M$^+$]. Anal. Calcd for $C_{35}H_{31}NO_7$: C. 72.78; H, 5.41; N, 2.42. Found: C, 72.75; H, 5.39; N, 2.43.

5.1.8. 4β-(1″-Pyrenylamino)-4-desoxypodophyllotoxin (20c)

This compound was prepared by method described for **20a** employing 1-pyrenylamine (265 mg, 1.2 mmol) and podophyllotoxin (414 mg, 1 mmol) affords **20c**, 413 mg, in 67% yield. Mp: 190–193 °C, $[\alpha]_D^{25}$: −122.0 (c = 1.0, $CHCl_3$). ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.97–3.19 (m, 1H), 3.34–3.67 (dd, 1H, J = 14.1, 5.3 Hz), 3.82 (s, 6H), 3.84 (s, 3H), 4.02 (t, 1H, J = 10.1 Hz), 4.50 (t, 1H, J = 8.0 Hz), 4.70 (d, 1H, J = 4.7 Hz), 4.86–4.92 (m, 1H), 5.11–5.23 (m, 1H), 6.01 (s, 2H), 6.37 (s, 2H), 6.62 (s, 1H), 6.83 (s, 1H), 7.12–8.10 (m, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 38.6, 42.2, 43.8, 52.4, 56.3, 60.6, 69.1, 101.4, 108.9, 109.4, 110.1, 110.8, 114.1, 118.3, 122.2, 125.0, 125.9, 125.9, 127.8, 129.12, 131.2, 131.0, 134.2, 135.2, 142.4, 147.8, 148.4, 152.8, 174.7; IR (KBr) cm^{-1}: 3394, 2924, 1770, 1615, 1505, 1483; MS (FAB): 613 [M$^+$]. Anal. Calcd for $C_{38}H_{35}NO_7$: C, 74.38; H, 5.09; N, 2.28. Found: C, 74.21; H, 5.15; N, 2.31.

5.1.9. 4β-(6″-Chrycenylamino)-4-desoxypodophyllotoxin (20d)

This compound was prepared by method described for **20a** employing 6-chrycenylamine (296 mg, 1.2 mmol) and podophyllotoxin (414 mg, 1 mmol) affords **20d**, 456 mg, in 71% yield. Mp: 157–160 °C, $[\alpha]_D^{25}$: −48.0 (c = 1.0, $CHCl_3$). ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.98–3.30 (m, 2H), 3.82 (s, 6H), 3.83 (s, 3H), 4.07 (t, 1H, J = 9.5 Hz), 4.61 (t, 2H, J = 7.1 Hz), 4.72–4.82 (m, 2H), 5.21–5.28 (m, 1H), 6.01 (d, 2H, J = 2.3 Hz), 6.38 (s, 2H), 6.57 (s, 1H), 6.86 (s, 1H), 7.40–9.01 (m, 11H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 38.6, 42.2, 43.7, 52.5, 56.3, 60.6, 69.0, 101.4, 108.6, 109.2, 110.0, 111.3, 111.4, 118.4, 119.1, 122.4, 123.3, 123.5, 126.9, 129.2, 124.4, 129.9, 131.1, 131.4, 131.8, 134.0, 134.2, 135.1, 142.4, 147.8, 148.3, 152.6, 174.5; IR (KBr) cm^{-1}: 3409, 2906, 1774, 1598, 1503, 1483; MS (FAB): 639 [M$^+$]. Anal. Calcd for $C_{40}H_{37}NO_7$: C, 74.10; H, 5.20; N, 2.19. Found: C, 74.21; H, 5.16; N, 2.17.

5.1.10. 4β-[4″-(4″-Fluorobenzoyl)anilino]-4-desoxypodophyllotoxin (20e)

This compound was prepared by method described for **20a** employing 4-amino-4′-fluorobenzophenone (258 mg, 1.2 mmol) and podophyllotoxin (414 mg, 1 mmol) affords **20e**, 458 mg, in 75% yield. Mp: 106–110 °C, $[\alpha]_D^{25}$: −106.0 (c = 1.0, $CHCl_3$). ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.99–3.02 (m, 2H), 3.75 (s, 6H), 3.78 (s, 3H), 4.40–4.50 (m, 2H), 4.58–4.63 (m, 1H), 4.80–4.89 (m, 1H), 5.95 and 5.98 (ABq, 2H, J = 1.5 Hz), 6.25 (s, 2H), 6.55–6.67 (m, 3H), 6.78 (s, 1H), 7.12–7.65 (m, 2H), 7.72–7.89 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 38.7, 42.3, 43.6, 52.5, 56.4, 60.7, 69.0, 101.5, 108.8, 109.3, 110.0, 114.2, 115.6, 117.2, 118.3, 118.9, 131.1, 131.7, 132.5, 135.1, 144.2, 145.2, 146.2, 147.8, 148.4, 152.7, 164.5, 174.6, 194.2; IR (KBr) cm^{-1}: 3348, 2923, 1772, 1641, 1596, 1504, 1481; MS (FAB): 611 [M$^+$]. Anal. Calcd for $C_{35}H_{30}FNO_8$: C, 68.73; H, 4.94; N, 2.29. Found: C, 68.75; H, 4.91; N, 2.27.

5.1.11. 4β-(4″-{4″-[Di(2″-chloroethyl)amino]benzoyl}anilino)-4-desoxypodophyllotoxin (20f)

This compound was prepared by method described for **20a** employing 4-amino-4′-[di(2-chloroethyl)amino]benzophenone (404 mg, 1.2 mmol) and podophyllotoxin (414 mg, 1 mmol) affords **20f**, 476 mg, in 65% yield. Mp: 186–190 °C, $[\alpha]_D^{25}$: −110.0 (c = 1.0, $CHCl_3$). ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.97–3.13 (m, 2H), 3.64–3.91 (m, 17H), 3.99–4.10 (m, 1H), 4.26–4.48 (m, 2H), 4.63–4.70 (m, 1H), 4.81–4.90 (m, 1H), 5.99 (d, 2H, J = 6.8 Hz), 6.33 (s, 2H), 6.55 (s, 1H), 6.57–6.74 (m, 4H), 6.80 (s, 1H), 7.66–7.80 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): 38.6, 42.2, 43.2, 43.7, 52.5, 53.2, 56.3, 60.6, 69.0, 101.5, 108.7, 109.2, 110.0, 111.3, 113.6, 116.9, 118.4, 128.1, 131.1, 131.2, 131.8, 133.4, 135.2, 146.2, 147.3, 147.8, 148.4, 152.7, 153.4, 174.6, 193.2; IR (KBr) cm^{-1}: 3380, 2924, 2854, 1773, 1727, 1596, 1507, 1480; MS (FAB): 732 [M$^+$]. Anal. Calcd for $C_{39}H_{38}Cl_2N_2O_8$: C, 63.85; H, 5.22; N, 3.82. Found: C, 63.81; H, 5.19; N, 3.85.

5.1.12. 4′-*O*-Demethyl-4β-(1″-anthrylamino)-4-desoxypodophyllotoxin (21a)

To a solution of podophyllotoxin (414 mg, 1 mmol) in dry CH_2Cl_2 (10 mL), sodium iodide (298 mg, 2 mmol) was added and stirred for 5 min to this stirred suspension $BF_3{\cdot}OEt_2$ (0.13 mL, 2 mmol) was added dropwise at 0 °C and the stirring was continued for another 8 h at room temperature. Nitrogen was bubbled through the solution to drive of the excess hydrogen iodide. This solution was then evaporated in vacuo and used for the next reaction without further purification. To the above crude product, anhydrous barium carbonate (395 mg, 2 mmol) and 1-anthraceneamine (231 mg, 1.2 mmol) in 10 mL of dry THF under nitrogen was

added and stirred for 8 h at room temperature. The reaction mixture was filtered, diluted with ethyl acetate and washed with water, 10% aqueous sodium thiosulphate solution, dried and purified via column chromatography using ethyl acetate/hexane (1:1) as eluent affords **21a**, 374 mg in 65% yield. Mp: 180–182 °C, $[\alpha]_D^{25}$: −59.0 (c = 1.0, $CHCl_3$). ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.97–3.11 (m, 1H), 3.39–3.68 (dd, 1H, J = 13.6, 4.5 Hz), 3.83 (s, 6H), 3.97 (t, 1H, J = 9.1 Hz), 4.46 (t, 1H, J = 8.3 Hz), 4.73–4.80 (m, 2H), 5.45 (br s, 1H), 5.98 (d, 2H, J = 1.5 Hz), 6.40 (s, 2H), 6.47 (d, 1H, J = 7.5 Hz), 6.63 (s, 1H), 6.82 (s, 1H), 7.35–7.40 (m, 1H), 7.48–7.69 (m, 3H), 7.97–8.12 (m, 2H), 8.24 (s, 1H), 8.35 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 38.7, 42.3, 43.81, 52.5, 56.4, 69.1, 101.6, 108.7, 109.3, 110.0, 118.4, 118.8, 123.0, 125.3, 125.6, 125.8, 126.9, 127.7, 128.1, 130.7, 131.1, 131.8, 132.2, 132.5, 134.4, 142.6, 148.1, 148.4, 152.7, 175.3; IR (KBr) cm^{-1}: 3416, 2924, 2852, 1773, 1576, 1481; MS (FAB): 575 [M$^+$]. Anal. Calcd for $C_{35}H_{29}NO_7$: C, 73.03; H, 5.08; N, 2.43. Found: C, 72.99; H, 5.11; N, 2.41.

5.1.13. 4′-*O*-Demethyl-4β-(1″-fluorenylamino)-4-desoxypodophyllotoxin (21b)

This compound was prepared by method described for **21a** employing 1-fluorenylamine (220 mg, 1.2 mmol) and podophyllotoxin (414 mg, 1 mmol) affords **21b**, 354 mg, in 63% yield. Mp: 250–252 °C, $[\alpha]_D^{25}$: −105.0 (c = 1.0, $CHCl_3$). ^{1}H NMR (200 MHz, $CDCl_3$): δ 3.01–3.30 (m, 2H), 3.74 (s, 6H), 3.79 (s, 2H), 3.88–4.23 (m, 1H), 4.34 (t, 1H, J = 7.8 Hz), 4.52 (d, 1H, J = 5.2 Hz), 4.86–4.98 (m, 1H), 5.96 (s, 2H), 6.28 (s, 2H), 6.5 (s, 1H), 6.67–6.78 (m, 1H), 6.81 (s, 1H), 6.86–6.98 (m, 1H), 7.06–7.58 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 36.9, 38.7, 42.2, 43.6, 52.5, 60.6, 68.9, 101.4, 108.8, 109.2, 109.9, 111.2, 118.3, 118.5, 119.9, 122.9, 125.0, 126.6, 126.8, 127.0, 131.1, 131.9, 133.8, 135.1, 140.8, 142.3, 143.7, 147.8, 148.3, 152.8, 174.4; IR (KBr) cm^{-1}: 3349, 2925, 2854, 1758, 1610, 1515, 1458; MS (FAB): 563 [M$^+$]. Anal. Calcd for $C_{34}H_{29}NO_7$: C, 72.46; H, 5.19; N, 2.49. Found: C, 72.45; H, 5.21; N, 2.45.

5.1.14. 4′-*O*-Demethyl-4β-(1″-pyrenylamino)-4-desoxypodophyllotoxin (21c)

This compound was prepared by method described for **21a** employing 1-pyrenylamine (265 mg, 1.2 mmol) and podophyllotoxin (414 mg, 1 mmol) affords **21c**, 313 mg, in 55% yield. Mp: 148–153 °C, $[\alpha]_D^{25}$: −76.0 (c = 1.0, $CHCl_3$). ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.89–3.13 (m, 1H), 3.29–3.67 (dd, 1H, J = 13.6, 4.5 Hz), 3.83 (s, 6H), 3.98 (t, 1H, J = 10.5 Hz), 4.44 (t, 1H, J = 8.3 Hz), 4.55–4.60 (m, 1H), 4.66 (d, 1H, J = 5.2 Hz), 5.07–5.25 (m, 1H), 5.34 (br s, 1H), 5.97 (s, 2H), 6.35 (s, 2H), 6.59 (s, 1H), 6.8 (s, 1H), 7.18–7.67 (m, 1H), 7.90–8.29 (m, 8H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 38.6, 42.2, 43.8, 52.5, 60.6, 69.1, 101.4, 108.8, 109.4, 110.1, 110.8, 114.2, 118.3, 122.1, 125.1, 125.9, 125.9, 127.7, 131.0, 131.8, 134.1, 135.3, 142.2, 147.8, 148.3, 152.8, 174.6; IR (KBr) cm^{-1}: 3381, 2920, 1775, 1603, 1510, 1483; MS (FAB): 599 [M$^+$]. Anal. Calcd for $C_{37}H_{33}NO_7$: C, 74.11; H, 4.87; N, 2.34. Found: C, 74.03; H, 4.91; N, 2.29.

5.1.15. 4′-*O*-Demethyl-4β-(1″-chrycenylamino)-4-desoxypodophyllotoxin (21d)

This compound was prepared by method described for **21a** employing 6-chrycenylamine (296 mg, 1.2 mmol) and podophyllotoxin (414 mg, 1 mmol) affords **21d**, 327 mg, in 52% yield. Mp: 158–160 °C, $[\alpha]_D^{25}$: −39.0 (c = 1.0, $CHCl_3$). ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.99–3.34 (m, 2H), 3.84 (s, 6H), 4.08 (t, 1H, J = 10.2 Hz), 4.59–4.63 (m, 1H), 4.74–4.89 (m, 2H), 5.21–5.69 (m, 1H), 5.98 (s, 2H), 6.34 (s, 2H), 6.51 (s, 1H), 6.83 (s, 1H), 7.40–9.01 (m, 11H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 38.6, 42.3, 43.7, 52.4, 60.6, 69.0, 101.5, 108.7, 109.3, 110.0, 111.2, 111.3, 118.4, 119.3, 122.3, 123.2, 123.5, 131.1, 131.3, 131.7, 134.1, 134.3, 135.1, 142.3, 147.7, 148.3, 152.7, 174.6; IR (KBr) cm^{-1}: 3394, 2923, 1768, 1615, 1503, 1482; MS (FAB): 625 [M$^+$]. Anal. Calcd for $C_{39}H_{35}NO_7$: C, 74.87; H, 4.99; N, 2.24. Found: C, 74.79; H, 5.03; N, 2.23.

5.1.16. 4′-*O*-Demethyl-4β-[4″-(4″-fluorobenzoyl)anilino]-4-desoxypodophyllotoxin (21e)

This compound was prepared by method described for **21a** employing 4-amino-4′-fluorobenzophenone (258 mg, 1.2 mmol) and podophyllotoxin (414 mg, 1 mmol) affords **21e**, 388 mg in 65% yield. Mp: 162–165 °C, $[\alpha]_D^{25}$: −129.0 (c = 1.0, $CHCl_3$). ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.68–3.02 (m, 2H), 3.79 (s, 6H), 4.36–4.45 (m, 1H), 4.52–4.63 (m, 2H), 4.79–4.88 (m, 1H), 5.35 (br s, 1H), 5.95 and 5.98 (ABq, 2H, J = 1.5 Hz), 6.28 (s, 2H), 6.51 (s, 1H), 6.57 (d, 2H, J = 8.6 Hz), 6.76 (s, 1H), 7.13–7.46 (m, 2H), 7.68–7.79 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 38.7, 42.3, 43.6, 52.5, 60.7, 69.0, 101.4, 108.7, 109.3, 110.0, 114.2, 115.7, 117.2, 118.3, 118.9, 131.0, 131.7, 132.5, 135.1, 144.2, 145.2, 146.4, 147.6, 148.3, 152.7, 164.5, 174.5, 194.2; IR (KBr) cm^{-1}: 3402, 2924, 1775, 1610, 1503, 1481; MS (FAB): 597 [M$^+$]. Anal. Calcd for $C_{34}H_{28}FNO_8$: C, 68.34; H, 4.72; N, 3.18. Found: C, 68.29; H, 4.75; N, 3.16.

5.1.17. 4′-*O*-Demethyl-4β-(4″-{4″-[di(2″-chloroethyl)amino]-benzoyl}anilino)-4-desoxy podophyllotoxin (21f)

This compound was prepared by method described for **21a** employing 4-amino-4′-[di(2-chloroethyl)amino]benzophenone (404 mg, 1.2 mmol) and podophyl-lotoxin (414 mg, 1 mmol) affords **21f**, 366 mg in 51% yield. Mp: 173–175 °C, $[\alpha]_D^{25}$: −124.0 (c = 1.0, $CHCl_3$). ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.69–3.12 (m, 2H), 3.65–3.88 (m, 14H), 3.99 (t, 1H, J = 10.5 Hz), 4.48–4.56 (m, 2H), 4.62 (d, 1H, J = 4.5 Hz), 4.82–4.98 (m, 1H), 5.34 (br s, 1H), 5.98 (d, 2H, J = 6.8 Hz), 6.33 (s, 2H), 6.55 (s, 1H), 6.57–6.74 (m, 4H), 6.8 (s, 1H), 7.66–7.80 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 38.7, 42.3, 43.2, 43.7, 52.5, 53.6, 60.7, 69.0, 101.5, 108.7, 109.2, 109.9, 111.5, 113.6, 117.3, 118.4, 118.8, 127.9, 131.1, 131.2, 131.7, 133.5, 135.1, 144.8, 146,2, 147.3, 147.8, 148.4, 152.7, 153.3, 174.6, 195.1; IR (KBr) cm^{-1}: 3395, 2920, 1772, 1598, 1507, 1481; MS (FAB): 718 [M$^+$]. Anal. Calcd for $C_{38}H_{36}Cl_2N_2O_8$: C, 63.42; H, 5.04; N, 3.89. Found: C, 63.38; H, 4.99; N, 3.91.

6. Procedure of the SRB-assay

Tumor 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. Hundred microliters 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 min on a mechanical stirrer. The optical density was recorded on ELISA reader at 540 nm. The cell growth in 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).

8. DNA gel electrophoresis

DNA fragmentation was determined by electrophoresis of extracted genomic DNA from leukemia cell line (MOLT-4). Briefly, exponentially growing cells (2×10^6 cells/ mL) in 6-well plate were treated with compound **20d** in 0.5, 1, 5, and 10 μM concentrations for 24 h. Cells were harvested, washed with PBS, pellets were dissolved in lysis buffer (10 mM EDTA, 50 mM Tris pH 8.0, 0.5% w/v SDS, and proteinase K (0.5 mg/mL) and incubated at 50 °C for 1 h. Finally the DNA obtained was heated rapidly to 70 °C, supplemented with loading dye and immediately resolved on to 1.5% agarose gel at 50 V for 2–3 h.

9. Flow-cytometric analysis of phase distribution of nuclear DNA

Effect of compound **20d** on DNA content by cell cycle phase distribution was assessed using Molt-4 cells by incubating the cells (1×10^6 mL/well) with **20d** (0.5, 1, 5, and 10 μM) for 24 h. The cells were then washed twice with ice-cold PBS, harvested, fixed with ice cold PBS in 70% ethanol, and stored at −20 °C for 30 min. After fixation, these cells were incubated with RNase A (0.1 mg/mL) at 37 °C for 30 min, stained with propidium iodide (50 μg/mL) for 30 min on ice in dark, and then measured for DNA content using BD-LSR flow cytometer (Becton Dickinson, USA) equipped with electronic doublet discrimination capability using blue (488 nm) excitation from Argon laser. Data were collected in list mode on 10,000 events for FL2-A versus FL2-W.

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