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Synthesis and biological evaluation of 4β-acrylamidopodophyllotoxin congeners as DNA damaging agents

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ABSTRACT

A series of new 4β-acrylamidopodophyllotoxin derivatives (**13a–o**) were synthesized by coupling of substituted acrylic acids (**10a–l** and **11m–o**) to the 4β-aminopodophyllotoxin. The synthesized derivatives **13a–o** were evaluated for their cytotoxicity against five human cancer cell lines (breast, oral, colon, lung and ovarian). These podophyllotoxin conjugates have shown promising activity with GI_{50} values ranging from <0.1 to 0.29 μM. Some of the compounds **13j**, **13k** and **13l** that showed significant antiproliferative activity were also evaluated for related cytotoxic effects in MCF-7 cells, and compared to etoposide. These compounds (**13j**, **13k** and **13l**) showed G2/M cell cycle arrest and the apoptotic event was found to be due to both the single-strand DNA breaks as observed by comet assay as well as double-strand breaks as observed by the large accumulation of gamma H2AX foci.

1. Introduction

Podophyllotoxin (**1**), a well-known naturally occurring aryltetralin lignan is extracted from the roots of *Podophyllotoxin peltatum*. The pharmacological properties of podophyllotoxin have been well-recognized for centuries.[1–3] Podophyllotoxin inhibits the assembly of tubulin polymerization, thus preventing the formation of the achromatic spindle and arresting cell division in metaphase.[4] However, the high toxicity and severe gastrointestinal side effect of podophyllotoxin has limited its application as a drug in cancer chemotherapy.[5] The biological activity of podophyllotoxin has led to extensive structural modifications resulting in several more potent and less toxic anticancer agents such as etoposide[6] (**2**) and teniposide (**3**).[7] Etoposide is the most widely used anticancer agent for the treatment of leukemia, testicular cancer and small cell lung cancer. Its clinical efficacy is due to its ability to inhibit the enzyme DNA-topoisomerase II. These compounds **2** and **3** block the catalytic activity of DNA-topoisomerase II by stabilizing a cleavable enzyme–DNA-complex in which the DNA is cleaved and covalently linked to enzyme.[8] This lead has stimulated a renewed interest in the chemical and biochemical studies of podophyllotoxin derived antitumor agents.[9] The replacement of the C-4 sugar moiety of etoposide and teniposide with a non-sugar substituent has improved the therapeutic value of etoposide.[10] 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[7,11–13] like GL-331(**4**) and NPF (**5**), as shown in Figure 1. These are proved to be more potent than etoposide. GL-331 is also an inhibitor of topo-II,[14] and induces apoptotic cell death through independent mechanism which contributes to its cytotoxicity. This compound underwent phase II clinical trials for the treatment of various cancers,[15] however this did not proceed further. A recent study on molecular-area-oriented chemical modifications of podophyllotoxin has revealed certain structural features that are critical for the topo-II inhibition.[16] The comparative molecular field analysis[12,16] model further demonstrated that bulky substituents at C-4 might be favorable for topo-II inhibition. It is observed that, among the C4-N-substituted congeners of podophyllotoxin that have been synthesized and developed, C4β-*N*-amidopodophyllotoxin derivatives have received less attention. In the past few years, a variety of compounds have been prepared and evaluated for their biological activity.[17,18] It is observed from the results that

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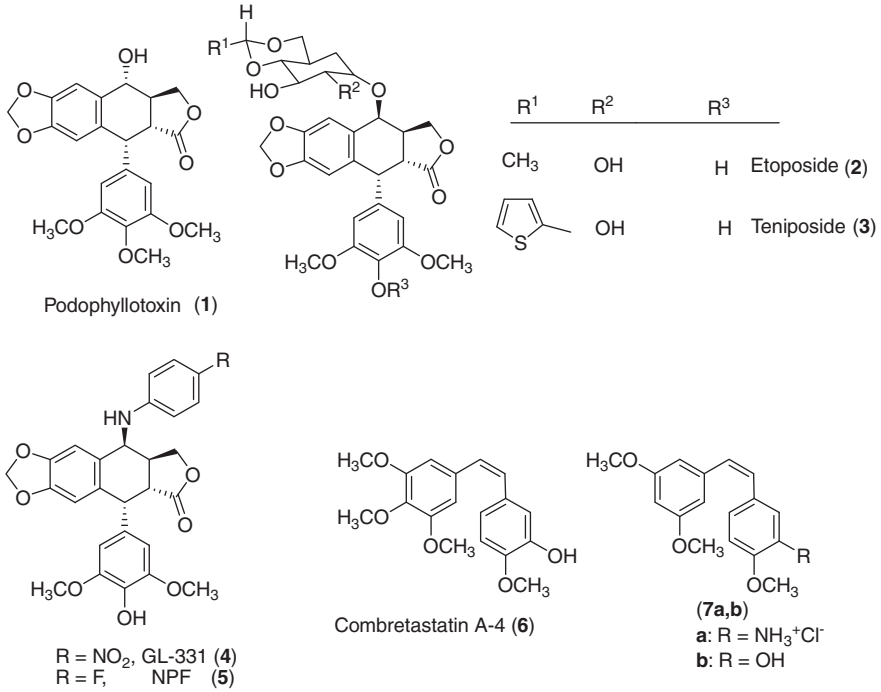


Figure 1.

some of these compounds exhibit enhanced DNA topoisomerase-II inhibition in comparison to etoposide.

The naturally occurring *cis*-stilbene, combretastatin A-4 (**6**) was isolated from the bark of the south African tree *Combretum caffrum*.[19] The combretastatin shows strong cytotoxicity against a variety of cancer cells, including multidrug-resistant cell lines.[20] Several active stilbene based compounds were identified, among them, *cis*-3,4′,5-trimethoxy-3′-aminostilbene (**7a**) and *cis*-3,4′,5-trimethoxy-3′-hydroxystilbene (**7b**) were found to induce HL60 apoptosis at nonomolar concentration.[21]

We have been involved in the development of new synthetic procedures[22] for the podophyllotoxin-based compounds and also in the design and synthesis of new analogues of podophyllotoxin as potential anticancer agents.[23] Based on the available structure–activity relationship (SAR) studies, we decided to explore some new podophyllotoxin analogues further by introducing an amide functionality at 4β-position. In continuation of these efforts, in the present study, we report the synthesis of a series conjugates of 4β-acrylamidopodophyllotoxin congeners, wherein the podophyllotoxin is linked to a stilbene moiety. All these congeners have been evaluated for their anticancer activity against a panel of five human cancer cell lines and promising compounds **13j**, **13k** and **13l** were evaluated for their cell viability, cell cycle analysis, and DNA strand breaks.

2. Chemistry

Acrylic acids (**10b**, **10e**, **10i**, **10m** and **10n**) were synthesized by means of a Perkin reaction, as previously reported.[24] Synthesis of acrylic acids (**10a–l** and **11m–o**) starting from appropriate methoxy substituted phenyl acetic acids with substituted aromatic aldehydes in the presence of Ac_2O and Et_3N and the mixture was refluxed to afford the acids (**10a–l**) and acetates (**10m–o**). As **10m–o** are in acetate form, these upon further treatment of a base provide the required acids (**11m–o**), as shown in Scheme 1.

Synthesis of 4β-acrylamidopodophyllotoxin conjugates (**13a–o**) was carried out from the key intermediate, 4β-aminopodophyllotoxin (**12**).[22e,23a] Further the intermediate 4β-aminopodophyllotoxin (**12**) has been coupled to various substituted acrylic acids (**10a–l** and **11m–o**) in presence of EDCI and HOBt to afford the corresponding 4β-acrylamidopodophyllotoxin derivatives (**13a–o**) as shown in Scheme 2. All the synthesized compounds were characterized by ^{1}H NMR, ^{13}C NMR and mass spectral data.

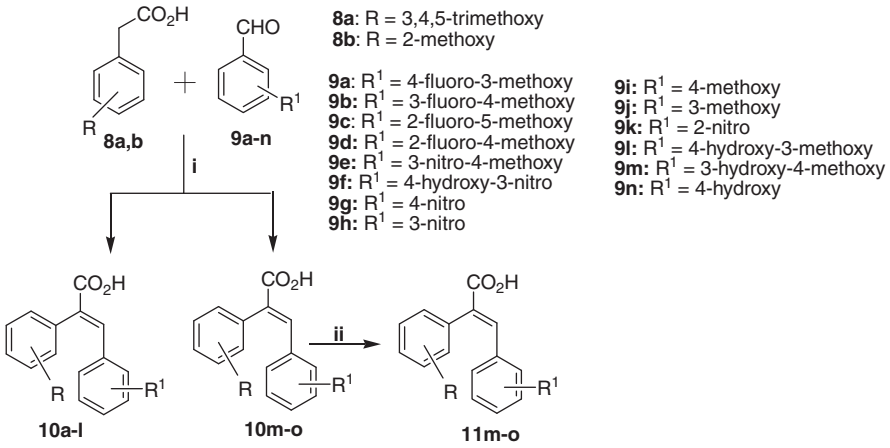
3. Biological evaluation

3.1. Invitro cytotoxicity assay

The synthesized compounds **13a–o** were evaluated for their anticancer activity in selected human cancer cell lines of breast, oral, colon, lung and ovarian by using sulforhodamine B (SRB) method.[25] The compounds that exhibit $GI_{50} \leqslant 10^{-5}$ M are considered to be active on the respective cell lines and the results are illustrated in Table 1. All the compounds (**13a–o**) exhibited significant anticancer activity with GI_{50} values ranging from <0.1 to 2.9 μM, while the positive controls, etoposide and adriamycin demonstrated the GI_{50} in the range of <0.1 to 1.15 μM and 0.13 to 3.08 μM, respectively. The significant anticancer activity showed by the promising compounds **13j**, **13k** and **13l** prompted us to evaluate the cell viability in the MCF-7 cells, with a view to study their detailed biological effects in the cell line. The MCF-7 cells were treated with compounds etoposide (Eto), combretastatin (CA-4), podophyllotoxin (Podo), **10k** (*cis*-stilbene intermediate), **13j**, **13k** and **13l** at 2 and 4 μM for 24 h. The effective cytotoxicity observed after the treatment of these conjugates can be deduced from the MTT assay which is based on mitochondrial function. Podophyllotoxin and **10k** are the starting materials have shown less cytotoxicity. But hybrid molecules (**13j**, **13k**, **13l**) have shown pronounced cytotoxicity and the increase was observed to be synergistic. The data suggests that these conjugates (**13j**, **13k**, **13l**) have almost similar activity to that of etoposide as shown in Figure 2.

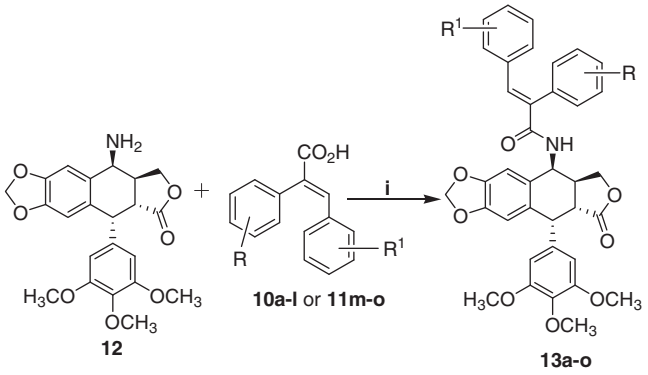
3.2. Effect of podophyllotoxin conjugates on cell cycle

Previous studies[26,27] have reported that etoposide was known to cause G2/M cell cycle arrest and its action on cell cycle was



10a: R = 3,4,5-trimethoxy, R^1 = 4-fluoro-3-methoxy
10b: R = 3,4,5-trimethoxy, R^1 = 3-fluoro-4-methoxy
10c: R = 3,4,5-trimethoxy, R^1 = 2-fluoro-5-methoxy
10d: R = 3,4,5-trimethoxy, R^1 = 2-fluoro-4-methoxy
10e: R = 3,4,5-trimethoxy, R^1 = 3-nitro-4-methoxy
10f: R = 3,4,5-trimethoxy, R^1 = 4-hydroxy-3-nitro
10g: R = 3,4,5-trimethoxy, R^1 = 4-nitro
10h: R = 3,4,5-trimethoxy, R^1 = 3-nitro
10i: R = 3,4,5-trimethoxy, R^1 = 4-methoxy
10j: R = 3,4,5-trimethoxy, R^1 = 3-methoxy
10k: R = 2-methoxy, R^1 = 4-nitro
10l: R = 2-methoxy, R^1 = 2-nitro
10m: R = 3,4,5-trimethoxy, R^1 = 4-O-acetyl-3-methoxy
10n: R = 3,4,5-trimethoxy, R^1 = 3-O-acetyl-4-methoxy
10o: R = 3,4,5-trimethoxy, R^1 = 4-O-acetyl
11m: R = 3,4,5-trimethoxy, R^1 = 4-hydroxy-3-methoxy
11n: R = 3,4,5-trimethoxy, R^1 = 3-hydroxy-4-methoxy
11o: R = 3,4,5-trimethoxy, R^1 = 4-hydroxy

Scheme 1. Reagents and conditions: (i) Ac_2O, Et_3N, reflux, 140 °C; (ii) K_2CO_3, MeOH/H_2O 3/1, rt.



13a: R = 3,4,5-trimethoxy, R^1 = 4-fluoro-3-methoxy
13b: R = 3,4,5-trimethoxy, R^1 = 3-fluoro-4-methoxy
13c: R = 3,4,5-trimethoxy, R^1 = 2-fluoro-5-methoxy
13d: R = 3,4,5-trimethoxy, R^1 = 2-fluoro-4-methoxy
13e: R = 3,4,5-trimethoxy, R^1 = 3-nitro-4-methoxy
13f: R = 3,4,5-trimethoxy, R^1 = 4-hydroxy-3-nitro
13g: R = 3,4,5-trimethoxy, R^1 = 4-nitro
13h: R = 3,4,5-trimethoxy, R^1 = 3-nitro
13i: R = 3,4,5-trimethoxy, R^1 = 4-methoxy
13j: R = 3,4,5-trimethoxy, R^1 = 3-methoxy
13k: R = 2-methoxy, R^1 = 4-nitro
13l: R = 2-methoxy, R^1 = 2-nitro
13m: R = 3,4,5-trimethoxy, R^1 = 4-hydroxy-3-methoxy
13n: R = 3,4,5-trimethoxy, R^1 = 3-hydroxy-4-methoxy
13o: R = 3,4,5-trimethoxy, R^1 = 4-hydroxy

Scheme 2. Reagents and conditions: (i) EDCI, HOBt, CH_2Cl_2, rt, 3 h.

purely cell type dependent. Similar to etoposide, the compounds of the present study possess a podophyllotoxin scaffold and it was considered of interest to study the effect of some of these promising molecules on the cell cycle regulatory mechanism in MCF-7 cells. Therefore, MCF-7 cells were treated by these conjugates at 2 μM concentration for 24 h, and FACS analysis was carried out. These conjugates **13j**, **13k** and **13l** show 36%, 39% and 35% of cells in G2/M phase, respectively, with concomitant decrease of cells in G1 phase. Whereas, the untreated cells, positive control (Etoposide), CA-4 (**6**), podophyllotoxin (Podo), **10k** (*cis*-stilbene intermediate) have shown 26%, 62%, 84%, 86% and 20% of cells in G2/M phase, respectively. Interestingly one of conjugate **10k** (stilbene intermediate) has shown G1/S phase cell cycle arrest and where as podophyllotoxin (Podo) has shown very high G2/M cell cycle arrest. Similar kind of observation was made by Nijmeijer et al., with respect to **10k**.[28] Thus these results from FACS analysis indicate G2/M cell cycle arrest is caused by these podophyllotoxin conjugates in MCF-7 cells as shown in Figure 3.

3.3. Effect of podophyllotoxin conjugates on single-strand DNA breaks

Cancer can be treated with drugs that can induce double-strand DNA breaks (DSBs) that preferentially kills the fast growing tumor

Table 1
Cytotoxic activity (GI_{50} μM) of compounds **13a–o**

Compound	Breast		Oral		Colon	Lung		Ovarian
	Zr-75-1	MCF7	Gurav	DWD	Colo 205	A-549	Hop62	A2780
13a	2.2	–	2.4	–	–	–	2.5	–
13b	–	2.7	–	–	2.4	–	–	–
13c	2.7	–	2.5	–	–	–	–	–
13d	2.5	2.4	0.18	0.19	–	2.1	0.17	2.7
13e	2.2	–	2.6	2.9	2.7	–	2.3	2.5
13f	0.18	2.7	0.19	<0.1	2.0	–	0.19	2.2
13g	2.2	–	2.1	2.4	2.1	–	2.7	2.6
13h	–	2.7	2.1	<0.1	–	2.4	0.17	2.5
13i	2.9	2.9	2.2	2.3	0.17	–	–	2.5
13j	0.18	<0.1	2.1	2.2	–	2.7	0.18	<0.1
13k	2.1	<0.1	2.1	2.3	–	2.7	2.0	2.3
13l	2.2	<0.1	2.2	2.3	–	2.8	2.1	2.2
13m	2.9	–	–	–	–	2.4	2.5	–
13n	–	–	–	–	–	2.9	2.9	–
13o	2.7	–	2.7	2.7	–	2.3	2.6	–
Etoposide	0.2	2.1	0.5	0.6	0.13	3.08	0.8	1.3
ADR	0.11	0.13	<0.1	<0.1	<0.1	<0.1	0.15	<0.1

ADR = adriamycin is one of the control drug.

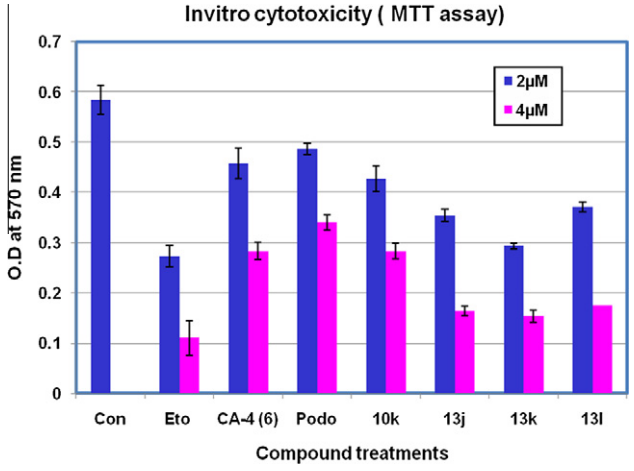


Figure 2. Cell viability assay was observed after the treatment with compounds etoposide (Eto), CA-4 (**6**), Podophyllotoxin (Podo), **10k** (*cis*-stilbene intermediate), **13j**, **13k** and **13l** at 2 and 4 μM concentration by MTT assay for 24 h in 96 well plates at 10,000 cells per well. Etoposide (Eto) was used as standard control. Control indicates the untreated cells.

cells by the inhibition of topoisomerase-II (topo-II). During this event there is a possibility that drugs can induce single-strand breaks (SSBs).[29–31] Further to examine the single-strand breaks comet assay has been performed wherein MCF-7 cells were treated with compounds etoposide (positive control), CA-4 (**6**), podophyllotoxin (Podo), **10k** (*cis*-stilbene intermediate) and **13k** at 2 μM for 24 h followed by agarose gel electrophoresis of slides on which cells were plated. Surprisingly, single-strand DNA breaks have been observed and are visualized as cells having bright head and tail in the cells treated with etoposide, **10k** and **13k** in comparison to the control cells and the percentage of cells with single-strand (ssDNA) breaks was found to be 10%, 20% and 30%, respectively, for as shown in Figure 4.

3.4. Effect of podophyllotoxin conjugates on double-strand DNA breaks

Etoposide is well-known drug that causes double-strand DNA breaks and is a topo-II inhibitor.[26,27,31] It has been well documented that phosphorylation at Ser-139 on H2AX protein (i.e., γ-H2AX) is a direct indicator of double-strand DNA breaks in cells that occur during DNA damage.[32,33] As these podophyllotoxin congeners are closely related molecules to etoposide, it was considered of interest to examine the effect of double-strand DNA breaks. Thus MCF-7 cells were treated with etoposide, CA-4 (**6**), podophyllotoxin (Podo), **10k** (*cis*-stilbene intermediate) and **13k** at 2 μM for 24 h. Interestingly a large number of γ-H2AX foci were observed in etoposide (70%), CA-4 (**6**) (50%), **10k** (30%) and **13k** treated cells when compared to the control (DMSO) cells. Podophyllotoxin treated cells have shown very negligible effect on double-strand DNA breaks. This was strongly supported by the fact that podophyllotoxin cannot act as topo II inhibitor.[34] The percentage of cells with double-strand breaks was observed to be 70% in case of **13k** as shown in Figure 5.

4. Conclusion

These new 4β-acrylamidopodophyllotoxin derivatives (**13a–o**) were synthesized by coupling substituted acrylic acids (**10a–l** and **11m–o**) to the 4β-aminopodophyllotoxin. These compounds (**13a–o**) were evaluated for their cytotoxic activity in five human cancer cell lines. Some of these podophyllotoxin conjugates like **13j**, **13k** and **13l** have shown enhanced cytotoxicity at 2 μM concentration and caused the G2/M cell cycle arrest. Among these compounds, **13k** has slightly better effectiveness in causing cell cycle arrest. Interestingly, the conjugate **13k** causes DNA damage at both single strand (30%) as well as double-strand (70%) and eventually killing the cancerous cells. Where as etoposide has shown 10% of single strand and 70% of double strand specific DNA damage. Thus our hybrid molecules are acting on par with etoposide. These results are supported by previous findings on podophyllotoxin conjugates exhibiting dual inhibitory activities on (topo-I and topo-II).[35] Based on the studies, it seems that the effect of these compounds is more on DNA double-strand breaks compared to single-strand breaks. Thus these compounds can be considered as leads for the effective treatment against cancer.

5. Experimental section

All chemicals and reagents were obtained from Aldrich (Sigma–Aldrich, St. Louis, MO, USA), Lancaster (Alfa Aesar, Johnson Matthey Company, Ward Hill, MA, USA) and were used without further purification. Reactions were monitored by TLC, performed on silica

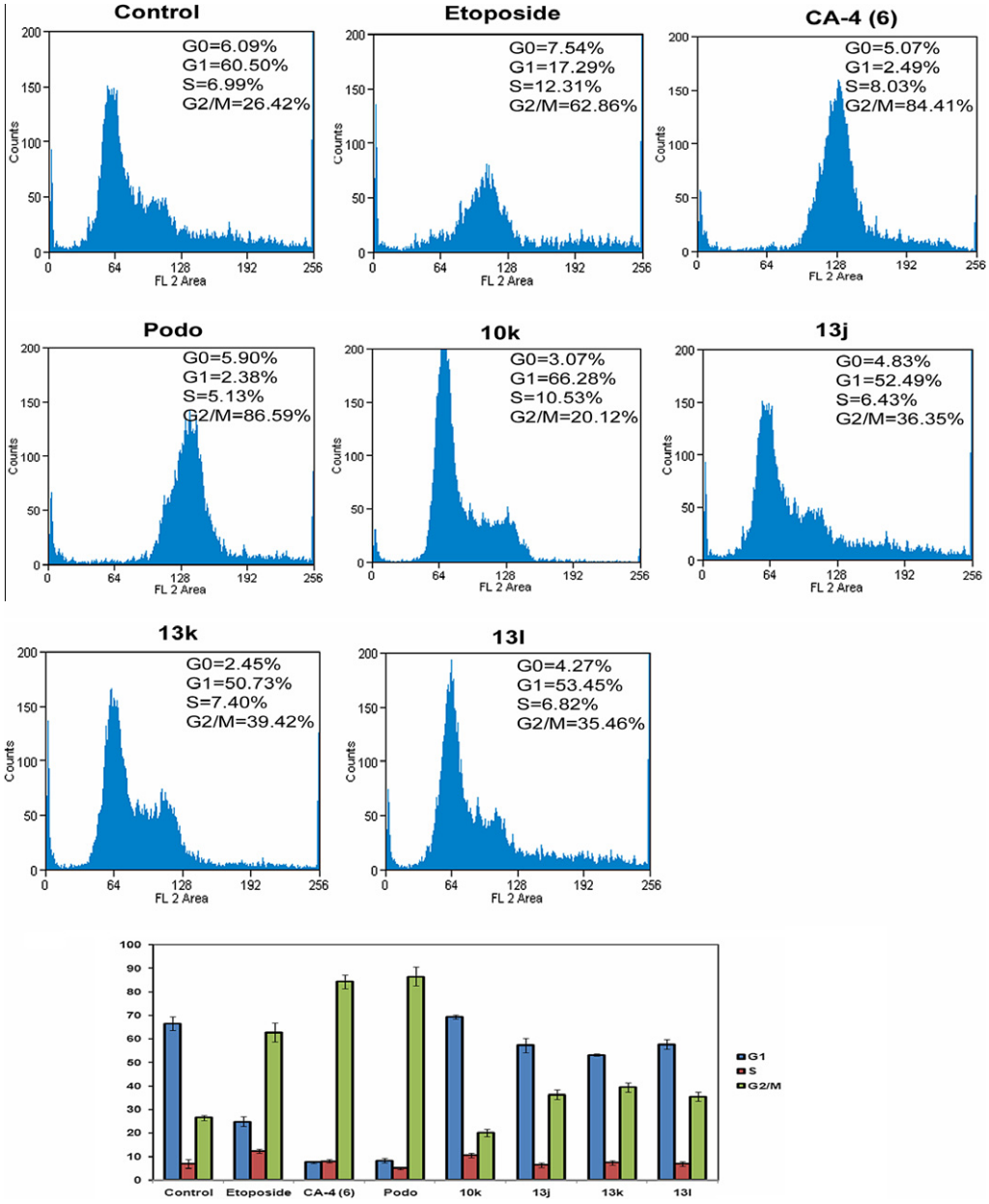


Figure 3. FACS analysis of cell cycle distribution of MCF-7 breast carcinoma cells after treatment with compounds etoposide, CA-4 (6), podophyllotoxin (Podo), **10k** (*cis*-stilbene intermediate), **13j**, **13k** and **13l** at 2 μM concentration for 24 h. Etoposide was used as a standard control.

gel glass plates containing 60 F-254, and visualization on TLC was achieved by UV light or iodine indicator. ^{1}H and ^{13}C NMR spectra were recorded on Gemini Varian-VXR-unity (200, 400 and 500 MHz) or Bruker UXNMR/XWIN-NMR (300 MHz) instruments. Chemical shifts (δ) are reported in ppm downfield from internal TMS standard. ESI spectra were recorded on Micro mass, Quattro LC using ESI^{+} software with capillary voltage 3.98 kV and ESI mode positive ion trap detector. Melting points were determined with an electrothermal melting point apparatus, and are uncorrected.

5.1. (*E*)-3-(4-Fluoro-3-methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)acrylic acid (10a)

The compound 3,4,5-trimethoxyphenyl acetic acid (**8a**) (200 mg, 0.88 mmol) was dissolved in 10 mL of Ac_2O, followed by addition of substituted aldehyde **9a** (136 mg, 0.88 mmol) and Et_3N (1 mL). The reaction mixture was heated at 140 °C for 12 h. After cooling, the reaction mixture was acidified with 35% aq HCl. The reaction mixture was left overnight and the precipitated product was filtered. The precipitate was recrystallized from absolute ethanol afford the pure compound **10a**, 130 mg in 40% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.48 (s, 3H), 3.79 (s, 6H), 3.83 (s, 3H), 6.43 (s, 2H), 6.49 (s, 2H), 6.58 (d, 1H, J = 8.4 Hz), 6.80–6.81 (m, 1H), 6.93 (t, 1H, J = 7.6, 9.3 Hz), 7.79 (s, 1H); MS (ESI): 376 $[M+H]^{+}$.

5.2. (*E*)-3-(3-Fluoro-4-methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)acrylic acid (10b)

This compound **10b** was prepared by the method described for **10a**, employing **8a** (200 mg, 0.88 mmol) and **9b** (136 mg, 0.88 mmol) afford the pure compound **10b**, 143 mg in 44% yield. ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.79 (s, 6H), 3.85 (s, 3H), 3.87 (s, 3H), 6.40 (s, 2H), 6.74–6.85 (m, 3H), 7.73 (s, 1H); MS (ESI): 382 $[M+Na]^{+}$.

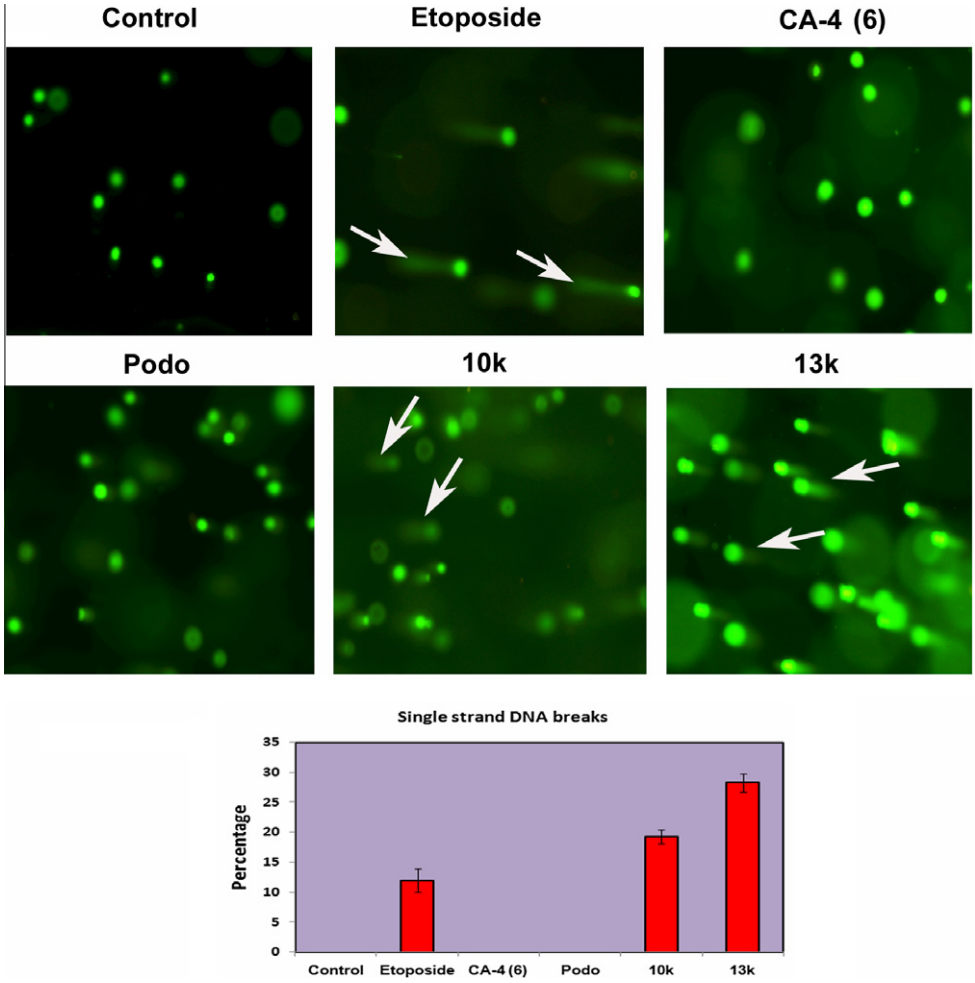


Figure 4. Effect of podophyllotoxin conjugate (**13k**) on the single-strand DNA breaks. The compounds etoposide, CA-4 (**6**), podophyllotoxin (Podo), **10k** (*cis*-stilbene intermediate) and effective conjugate **13k** were added to MCF-7 cells at 2 μM concentration for 24 h. Sybr green I dye was used to stain the DNA in both control as well as compound treated cells. Etoposide has been employed as the positive control. Single-strand DNA breaks were detected due to the bright head and tail formation in compound treated cells. White colored arrows indicate the tail pattern that appears during single-strand DNA breaks. Etoposide, **10k** and **13k** has shown single-strand DNA breaks. Where as podophyllotoxin (Podo) did not as shown in this comet assay.

5.3. (*E*)-3-(2-Fluoro-5-methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)acrylic acid (10c)

This compound **10c** was prepared by the method described for **10a**, employing **8a** (200 mg, 0.88 mmol) and **9c** (136 mg, 0.88 mmol) afford the pure compound **10c**, 120 mg in 37% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.36 (s, 3H), 3.79 (s, 6H), 3.86 (s, 3H), 6.31–6.33 (m, 1H), 6.49 (s, 2H), 6.76–6.80 (m, 1H), 6.96 (t, 1H, J = 8.8, 9.6 Hz), 8.12 (s, 1H); MS (ESI): 385 [M+Na]$^+$.

5.4. (*E*)-3-(2-Fluoro-4-methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)acrylic acid (10d)

This compound **10d** was prepared by the method described for **10a**, employing **8a** (200 mg, 0.88 mmol) and **9d** (136 mg, 0.88 mmol) afford the pure compound **10d**, 145 mg in 45% yield. ^{1}H NMR (500 MHz, DMSO-d_6): δ 3.66 (s, 6H), 3.70 (s, 3H), 3.72 (s, 3H), 6.42 (s, 2H), 6.55–6.58 (m, 1H), 6.70–6.75 (m, 1H), 6.80–6.85 (m, 1H), 7.82 (s, 1H), 12.57 (s, 1H); MS (ESI): 331 [M+H]$^+$.

5.5. (*E*)-3-(4-Methoxy-3-nitrophenyl)-2-(3,4,5-trimethoxyphenyl)acrylic acid (10e)

This compound **10e** was prepared by the method described for **10a**, employing **8a** (200 mg, 0.88 mmol) and **9e** (160 mg, 0.88 mmol) afford the pure compound **10e**, 156 mg in 45% yield. ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.80 (s, 6H), 3.89 (s, 3H), 3.94 (s, 3H), 6.40 (s, 2H), 6.66 (d, 1H, J = 7.3 Hz), 6.84–6.90 (m, 1H), 7.16 (d, 1H, J = 7.3 Hz), 7.77 (s, 1H); MS (ESI): 385 [M+Na]$^+$.

5.6. (*E*)-3-(4-Hydroxy-3-nitrophenyl)-2-(3,4,5-trimethoxyphenyl)acrylic acid (10f)

This compound **10f** was prepared by the method described for **10a**, employing **8a** (200 mg, 0.88 mmol) and **9f** (147 mg, 0.88 mmol) afford the pure compound **10f**, 121 mg in 67% yield. ^{1}H NMR (300 MHz, DMSO-d_6): δ 4.31 (s, 6H), 4.37 (s, 3H), 6.94 (s, 2H), 7.48 (d, 1H, J = 8.8 Hz), 7.73–7.77 (dd, 1H, J = 2.2, 2.0 Hz), 8.20 (s, 1H), 8.33 (br s, 1H); MS (ESI): 382 [M+Na]$^+$.

5.7. (*E*)-3-(4-Nitrophenyl)-2-(3,4,5-trimethoxyphenyl)acrylic acid (10g)

This compound **10g** was prepared by the method described for **10a**, employing **8a** (200 mg, 0.88 mmol) and **9g** (133 mg, 0.88 mmol) afford the pure compound **10g**, 210 mg in 66% yield. ^{1}H NMR (200 MHz, DMSO-d_6): δ 3.72 (s, 6H), 3.81 (s, 3H), 6.37 (s, 2H), 7.25 (d, 2H, J = 8.8 Hz), 7.25 (s, 1H), 8.00 (d, 2H, J = 8.8 Hz); MS (ESI): 322 [M+Na]$^+$.

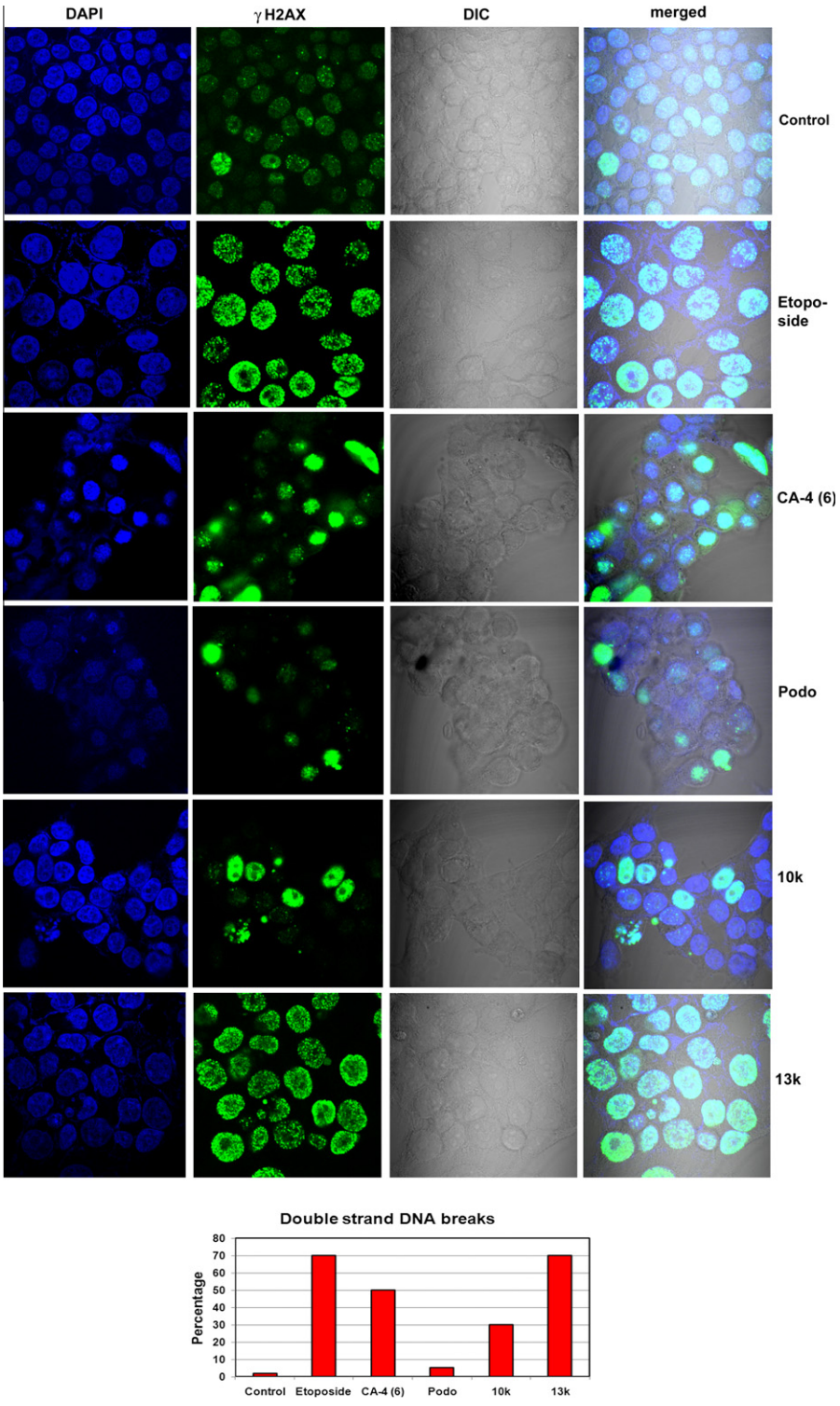


Figure 5. Effect of podophyllotoxin conjugate (**13k**) on the double-strand DNA breaks. The compounds etoposide, CA-4 (**6**), podophyllotoxin (Podo), **10k** (*cis*-stilbene intermediate) and effective conjugate (**13k**) were added to MCF-7 cells at 2 μM concentration for 24 h. γ-H2AX antibody was used to detect serine 139 phosphorylation in cells during double-strand DNA breaks. Etoposide has been employed as the positive control. Podophyllotoxin (Podo) has shown negligible effect on double-strand DNA breaks. Whereas stilbene intermediate (**10k**) has shown considerable double-strand DNA breaks. CA-4 has shown increased foci size as well as effective double-strand DNA breaks.

5.8. (*E*)-3-(3-Nitrophenyl)-2-(3,4,5-trimethoxyphenyl)acrylic acid (10h)

This compound **10h** was prepared by the method described for **10a**, employing **8a** (200 mg, 0.88 mmol) and **9h** (133 mg, 0.88 mmol) afford the pure compound **10h**, 196 mg in 61% yield. ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.65 (s, 6H), 3.69 (s, 3H), 6.48 (s, 2H), 7.51–7.62 (m, 2H), 7.81 (s, 1H), 7.85 (t, 1H, J = 1.7, 1.6 Hz), 8.06–8.09 (m, 1H), 12.85 (br s, 1H); MS (ESI): 391 $[M+H]^+$.

5.9. (*E*)-3-(4-Methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)acrylic acid (10i)

This compound **10i** was prepared by the method described for **10a**, employing **8a** (200 mg, 0.88 mmol) and **9i** (0.12 mL, 0.88 mmol) afford the pure compound **10i**, 176 mg in 57% yield. ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.77 (s, 3H), 3.78 (s, 6H), 3.89 (s, 3H), 6.44 (s, 2H), 6.72 (d, 2H, J = 9.0 Hz), 7.05 (d, 2H, J = 9.0 Hz), 7.85 (s, 1H); MS (ESI): 362 $[M+Na]^+$.

5.10. (*E*)-3-(3-Methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)acrylic acid (10j)

This compound **10j** was prepared by the method described **10a**, employing **8a** (200 mg, 0.88 mmol) and **9j** (0.12 mL, 0.88 mmol) afford the pure compound **10j**, 157 mg in 51% yield. ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.76 (s, 3H), 3.78 (s, 6H), 3.87 (s, 3H), 6.41 (s, 2H), 6.65–6.70 (m, 2H), 6.86 (t, 1H, J = 7.1, 7.3 Hz), 7.04 (d, 1H, J = 8.8 Hz), 7.83 (s, 1H); MS (ESI): 383 $[M+Na]^+$.

5.11. (*E*)-2-(2-Methoxyphenyl)-3-(4-nitrophenyl)acrylic acid (10k)

This compound **10k** was prepared by the method described for **10a**, employing **8b** (200 mg, 1.20 mmol) and **9k** (181 mg, 1.20 mmol) afford the pure compound **10k**, 161 mg in 44% yield. ^{1}H NMR (200 MHz, DMSO-d_6): δ 3.74 (s, 3H), 6.83–6.98 (m, 3H), 7.21 (d, 2H, J = 8.5 Hz), 7.29–7.38 (m, 1H), 7.76 (s, 1H), 7.97 (d, 2H, J = 8.5 Hz); MS (ESI): 604 $[M+H]^+$.

5.12. (*E*)-2-(2-Methoxyphenyl)-3-(2-nitrophenyl)acrylic acid (10l)

This compound **10l** was prepared by the method described for **10a**, employing **8b** (200 mg, 1.20 mmol) and **9k** (181 mg, 1.20 mmol) afford the pure compound **10l**, 152 mg in 42% yield. ^{1}H NMR (500 MHz, DMSO-d_6): δ 3.66 (s, 3H), 6.72–6.95 (m, 4H), 7.18 (t, 1H, J = 7.3, 7.3 Hz), 7.27–7.32 (m, 2H), 7.98 (d, 1H, J = 7.3 Hz), 8.05 (s, 1H); MS (ESI): 322 $[M+Na]^+$.

5.13. (*E*)-3-(4-Acetoxy-3-methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)acrylic acid (10m)

The compound 3,4,5-trimethoxyphenyl acetic acid (**8a**) (200 mg, 0.88 mmol) was dissolved in 4 mL of Ac_2O, followed by addition of aromatic aldehyde **9l** (134 mg, 0.88 mmol) and Et_3N (2 mL). The reaction mixture was heated at 140 °C for 24 h. After cooling, the reaction mixture was acidified with 35% aqueous HCl and kept at room temperature overnight. Then the forming O-acetylated precipitated was collected by filtration, recrystallized from ethanol afford the pure compound **10m**, 170 mg in 50% yield. ^{1}H NMR (200 MHz, DMSO-d_6): δ 1.93 (s, 3H), 3.42 (s, 3H), 3.76 (s, 3H), 3.77 (s, 6H), 6.40–644 (m, 2H); MS (ESI): 403 $[M+H]^+$.

5.14. (*E*)-3-(3-Acetoxy-4-methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)acrylic acid (10n)

This compound **10n** was prepared by the method described for **10m**, employing **8a** (200 mg, 0.88 mmol) and **9m** (134 mg, 0.88 mmol) afford the pure compound **10n**, 175 mg in 52% yield. ^{1}H NMR (200 MHz, DMSO-d_6): δ 1.96 (s, 3H), 3.76 (s, 6H), 3.81 (s, 3H), 3.82 (s, 3H), 6.41 (s, 2H), 6.53–6.66 (m, 3H); MS (ESI): 403 $[M+H]^+$.

5.15. (*E*)-3-(4-Acetoxyphenyl)-2-(3,4,5-trimethoxyphenyl)acrylic acid (10o)

This compound **10o** was prepared by the method described for **10m**, employing **8a** (200 mg, 0.88 mmol) and **9n** (107 mg, 0.88 mmol) afford the pure compound **10o**, 191 mg in 58% yield. ^{1}H NMR (500 MHz, DMSO-d_6): δ 3.35 (s, 3H), 3.70 (s, 6H), 3.74 (s, 3H), 6.49 (s, 2H), 7.06 (d, 2H, J = 8.3 Hz), 7.19 (d, 2H, J = 8.3 Hz), 7.76 (s, 1H); MS (ESI): 373 $[M+H]^+$.

5.16. (*E*)-3-(4-Hydroxy-3-methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)acrylic acid (11m)

A solution of K_2CO_3 (202 mg, 1.47 mmol) in water (0.5 mL) was added to solution of **10m** (200 mg, 0.49 mmol) in methanol (1.5 mL). The reaction mixture was stirred overnight at room temperature and then neutralized with 1 N aqueous HCl. After concentration, the resulting solution was extracted with ethyl acetate. The combined organic layers were washed with water, brine and dried over anhydrous $MgSO_4$, filtered and evaporated to dryness. The crude product was purified by column chromatography with ethyl acetate/hexane (1:1) afford the pure compound **11m**, 147 mg in 82% yield. ^{1}H NMR (200 MHz, DMSO-d_6): δ 3.99 (s, 3H), 4.32 (s, 3H), 4.33 (s, 6H), 6.94–7.00 (m, 3H), 7.23 (s, 2H), 8.18 (s, 1H), 9.35 (br s, 1H); MS (ESI): 604 $[M+H]^+$.

5.17. (*E*)-3-(3-Hydroxy-4-methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)acrylic acid (11n)

This compound **11n** was prepared by the method described for **11m**, employing **10n** (200 mg, 0.49 mmol) and K_2CO_3 (202 mg, 1.47 mmol) afford the pure compound **11n**, 139 mg in 77% yield. ^{1}H NMR (200 MHz, DMSO-d_6): δ 3.75 (s, 6H), 3.80 (s, 3H), 3.81 (s, 3H), 6.40 (s, 2H), 6.52–6.65 (m, 3H), 7.62 (s, 1H); MS (ESI): 383 $[M+Na]^+$.

5.18. (*E*)-3-(4-Hydroxyphenyl)-2-(3,4,5-trimethoxyphenyl)acrylic acid (11o)

This compound **11o** was prepared following the method described for **11m**, employing **10o** (200 mg, 0.54 mmol) and K_2CO_3 (223 mg, 1.62 mmol) afford the pure compound **11o**, 127 mg in 71% yield. ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.70 (s, 6H), 3.78 (s, 6H), 3.74 (s, 3H), 6.49 (s, 2H), 7.04 (d, 2H, J = 8.3 Hz), 7.19 (d, 2H, J = 8.3 Hz), 7.76 (s, 1H); MS (ESI): 345 $[M+H]^+$.

5.19. 4β-(*E*)-3-(4-Fluoro-3-methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)acrylamido podophyllotoxin (13a)

The compound 4β-aminopodophyllotoxin (**12**) (200 mg, 0.48 mmol) was dissolved in 10 mL of dry CH_2Cl_2, followed by addition of acrylic acid **10a** (205 mg, 0.57 mmol), EDCI (108 mg, 0.57 mmol) and HOBt (88 mg, 0.57 mmol). The reaction mixture was stirred at room temperature for 3 h. The reaction mixture was washed with saturated solution of $NaHCO_3$ and extracted with CH_2Cl_2, dried over anhydrous Na_2SO_4. The crude product was purified by column chromatography with ethyl acetate/hexane (4:6) afford the pure compound **13a**, 330 mg in 90% yield. Mp: 144–146 °C, $[\alpha]_D^{25}$ −78.0 (*c* 0.5 in $CHCl_3$), ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.52–2.63 (dd, 1H, J = 5.1, 5.1 Hz), 2.85–3.01 (m, 1H), 3.53 (s, 3H), 3.74 (s, 6H), 375 (s, 6H), 3.77 (s, 3H), 3.82 (s, 3H), 3.88 (t, 1H), 4.41–4.50 (dd, 2H, J = 6.6, 5.8 Hz), 5.23–5.30 (m, 1H), 5.73 (d, 1H, J = 6.6 Hz), 5.95 (d, 2H, J = 3.6 Hz), 6.22 (s, 2H), 6.43 (s, 2H), 6.44 (s, 1H), 6.48–6.54 (dd, 1H, J = 1.4, 1.4 Hz), 6.72–6.76 (m, 2H),

6.87–6.98 (dd, 1H, J = 8.0, 8.0 Hz), 7.70 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 36.7, 40.3, 43.1, 47.8, 55.1, 55.6, 55.7, 59.7, 59.8, 68.5, 101.1, 107.0, 108.0, 109.1, 109.2, 114.3, 115.4, 115.6, 123.1, 123.2, 130.0, 130.8, 131.8, 132.1, 133.5, 135.7, 135.8, 136.2, 137.2, 146.5, 147.1, 149.4, 151.9, 153.1, 167.4, 174.5; MS (ESI): 758 $[M+H]^+$.

5.20. 4β-(*E*)-3-(3-Fluoro-4-methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)acrylamido podophyllotoxin (13b)

This compound **13b** was prepared by the method described for **13a**, employing **12** (200 mg, 0.48 mmol), acrylic acid **10b** (205 mg, 0.57 mmol), EDCI (108 mg, 0.57 mmol) and HOBt (88 mg, 0.57 mmol). The crude product was purified by column chromatography with ethyl acetate/hexane (2:8) afford the pure compound **13b**, 350 mg in 95% yield. Mp: 159–160 °C, $[\alpha]_D^{25}$ +13.9 (*c* 0.5 in $CHCl_3$), ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.54–2.63 (dd, 1H, J = 5.2, 5.2 Hz), 2.87–2.97 (m, 1H), 3.38 (t, 1H), 3.74 (s, 6H), 3.75 (s, 6H), 3.77 (s, 3H), 3.86 (s, 3H), 3.87 (s, 3H), 4.41–4.49 (m, 2H), 5.26 (t, 1H), 5.74 (d, 1H, J = 6.7 Hz), 5.95 (d, 2H, J = 6.0 Hz), 6.22 (s, 2H), 6.40 (s, 2H), 6.44 (s, 1H), 6.62 (d, 1H, J = 12.8 Hz), 6.73 (s, 1H), 6.76–6.87 (m, 2H), 7.66 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 36.7, 40.4, 43.1, 47.7, 55.6, 55.7, 59.7, 60.0, 68.5, 101.1, 106.9, 108.0, 109.1, 109.2, 113.2, 116.2, 116.5, 126.9, 127.8, 130.0, 130.7, 132.0, 133.1, 134.9, 135.7, 136.2, 137.3, 146.5, 147.1, 151.9, 152.2, 153.1, 167.5, 174.5; MS (ESI): 758 $[M+H]^+$.

5.21. 4β-(*E*)-3-(2-Fluoro-5-methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)acrylamido podophyllotoxin (13c)

This compound **13c** was prepared by the method described for **13a**, employing **12** (200 mg, 0.48 mmol), acrylic acid **10c** (205 mg, 0.57 mmol), EDCI (108 mg, 0.57 mmol) and HOBt (88 mg, 0.57 mmol). The crude product was purified by column chromatography with ethyl acetate/hexane (3:7) afford the pure compound **13c**, 350 mg in 95% yield. Mp: 123–126 °C, $[\alpha]_D^{25}$ −12.0 (*c* 0.5 in $CHCl_3$), ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.67–2.74 (dd, 1H, J = 5.2, 5.2 Hz), 2.95–3.08 (m, 1H), 3.40 (s, 3H), 3.74 (s, 6H), 375 (s, 6H), 3.80 (s, 3H), 3.84 (s, 3H), 3.85–3.95 (m, 2H), 4.49–4.55 (dd, 2H, J = 60, 6.0 Hz), 5.32–5.37 (dd, 1H, J = 4.5, 4.5 Hz), 5.96 (d, 2H, J = 9.0 Hz), 5.99 (d, 1H, J = 7.5 Hz), 6.20–6.25 (m, 1H), 6.28 (s, 2H), 6.45 (s, 2H), 6.48 (s, 1H), 6.72–6.77 (m, 1H), 6.78 (s, 1H), 6.93 (t, 1H), 7.94 (s, 1H); MS (ESI): 758 $[M+H]^+$.

5.22. 4β-(*E*)-3-(2-Fluoro-4-methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)acrylamido podophyllotoxin (13d)

This compound **13d** was prepared by the method described for **13a**, employing **12** (200 mg, 0.48 mmol), acrylic acid **10d** (205 mg, 0.57 mmol), EDCI (108 mg, 0.57 mmol) and HOBt (88 mg, 0.57 mmol). The crude product was purified by ethyl acetate/hexane (3:7) afford the pure compound **13d**, 340 mg in 92% yield. Mp: 123–125 °C, $[\alpha]_D^{25}$ +48.9 (*c* 0.5 in $CHCl_3$), ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.61–2.66 (dd, 1H, J = 4.7, 4.7 Hz), 2.94–3.03 (m, 1H), 3.73 (s, 6H), 3.75 (s, 6H), 3.77 (s, 3H), 3.81 (s, 3H), 3.89 (s, 3H), 3.90–3.92 (m, 1H), 4.49–4.55 (m, 2H), 5.30–5.34 (m, 1H), 5.73 (d, 1H, J = 6.2 Hz), 5.96 (d, 2H, J = 11.7 Hz), 6.27 (s, 2H), 6.37–6.41 (m, 1H), 6.42 (s, 2H), 6.49 (s, 1H), 6.56–6.61 (m, 2H), 6.74 (s, 1H), 7.96 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 36.7, 40.2, 40.3, 43.1, 47.8, 55.9, 59.8, 59.9, 68.6, 101.0, 101.2, 101.4, 106.6, 107.0, 108.0, 109.3, 110.3, 114.8, 115.0, 125.9, 130.1, 130.4, 130.7, 132.1, 135.7, 136.2, 137.2, 146.5, 147.1, 151.9, 152.9, 160.8, 162.8, 167.6, 174.5; MS (ESI): 758 $[M+H]^+$.

5.23. 4β-(*E*)-3-(4-Methoxy-3-nitrophenyl)-2-(3,4,5-trimethoxyphenyl)acrylamido podophyllotoxin (13e)

This compound **13e** was prepared by the method described for **13a**, employing **12** (200 mg, 0.48 mmol), acrylic acid **10e** (221 mg, 0.57 mmol), EDCI (108 mg, 0.57 mmol) and HOBt (88 mg, 0.57 mmol). The crude product was purified by column chromatography with ethyl acetate/hexane (2:8) afford the pure compound **13e**, 310 mg in 79% yield. Mp: 140–143 °C, $[\alpha]_D^{25}$ −23.9 (*c* 0.5 in $CHCl_3$), ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.56–2.63 (dd, 1H, J = 5.3, 5.3 Hz), 2.87–3.00 (m, 1H), 3.36 (t, 1H, J = 7.0, 7.0 Hz), 3.73 (s, 6H), 3.75 (s, 6H), 3.76 (s, 3H), 3.94 (s, 3H), 3.95 (s, 3H), 4.39–4.46 (m, 2H), 5.25 (t, 1H, J = 5.3, 6.2 Hz), 5.81 (d, 1H, J = 6.2 Hz), 5.94 (d, 2H, J = 5.3 Hz), 6.20 (s, 2H), 6.38 (s, 2H), 6.71 (s, 1H), 6.91 (d, 1H, J = 7.9 Hz), 7.25 (s, 1H), 7.27 (d, 1H, J = 7.9 Hz), 7.34 (s, 1H), 7.68 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 36.7, 43.1, 46.5, 47.8, 48.5, 55.7, 55.8, 56.7, 59.8, 60.0, 101.2, 106.9, 108.1, 109.2, 109.3, 114.0, 125.8, 127.4, 130.0, 130.4, 131.8, 132.1, 135.7, 136.2, 136.3, 137.6, 138.6, 146.5, 147.1, 151.6, 151.9, 153.3, 167.4, 169.1, 174.4; MS (ESI): 785 $[M+H]^+$.

5.24. 4β-(*E*)-3-(4-Hydroxy-3-nitrophenyl)-2-(3,4,5-trimethoxyphenyl)acrylamido podophyllotoxin (13f)

This compound **13f** was prepared by the method described for **13a**, employing **12** (200 mg, 0.48 mmol), acrylic acid **10f** (214 mg, 0.57 mmol), EDCI (108 mg, 0.57 mmol) and HOBt (88 mg, 0.57 mmol). The crude product was purified by column chromatography with ethyl acetate/hexane (3:7) to afford pure compound **13f**, 330 mg in 88% yield. Mp: 184–187 °C, $[\alpha]_D^{25}$ −84.9 (*c* 0.5 in $CHCl_3$), ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.55–2.61 (dd, 1H, J = 4.5, 4.5 Hz), 2.88–3.02 (m, 1H), 3.74 (s, 6H), 3.76 (s, 6H), 3.77 (s, 3H), 3.88 (s, 3H), 4.38–4.44 (m, 2H), 5.21–5.31 (m, 3H), 5.96 (d, 2H, J = 4.5 Hz), 6.22 (s, 2H), 6.40 (s, 2H), 6.43 (s, 1H), 6.73 (s, 1H), 7.00 (d, 1H, J = 9.0 Hz), 7.28 (d, 1H, J = 9.0 Hz), 7.70 (s, 1H), 7.74 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 36.7, 40.4, 43.1, 47.7, 55.6, 55.8, 59.8, 60.0, 101.1, 106.9, 108.0, 109.1, 109.2, 118.7, 126.1, 126.2, 130.0, 130.4, 132.1, 135.7, 136.3, 136.4, 137.5, 146.5, 147.1, 151.9, 153.2, 167.4, 174.5; MS (ESI): 794 $[M+Na]^+$.

5.25. 4β-(*E*)-3-(4-Nitrophenyl)-2-(3,4,5-trimethoxyphenyl) acrylamido podophyllotoxin (13g)

This compound **13g** was prepared by the method described for **13a**, employing **12** (200 mg, 0.48 mmol), acrylic acid **10g** (204 mg, 0.57 mmol), EDCI (108 mg, 0.57 mmol) and HOBt (88 mg, 0.57 mmol). The crude product was purified by column chromatography with ethyl acetate/hexane (2:8) afford the pure compound **13g**, 350 mg in 95% yield. Mp: 187–190 °C, $[\alpha]_D^{25}$ +71.0 (*c* 0.5 in $CHCl_3$), ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.56–2.68 (dd, 1H, J = 5.1, 5.1 Hz), 2.91–3.10 (m, 1H), 3.73 (s, 6H), 3.75 (s, 6H), 381 (s, 3H), 3.91 (s, 3H), 4.25–4.35 (m, 1H), 4.46–4.57 (m, 2H), 5.35 (t, 1H), 5.82 (d, 1H, J = 7.3 Hz), 5.97 (d, 2H, J = 5.1 Hz), 6.27 (s, 2H), 6.38 (s, 2H), 6.51 (s, 1H), 6.73 (s, 1H), 7.22 (d, 2H, J = 8.8 Hz), 7.86 (s, 1H), 8.07 (d, 2H, J = 8.8 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 36.6, 43.0, 47.7, 55.6, 59.8, 60.0, 68.5, 101.2, 107.0, 109.0, 109.2, 109.3, 123.1, 129.9, 130.5, 131.7, 132.1, 135.7, 136.2, 137.5, 138.0, 139.9, 142.5, 146.2, 146.5, 147.1, 147.5, 151.9, 152.9, 167.5, 174.5; MS (ESI): 777 $[M+Na]^+$.

5.26. 4β-(*E*)-3-(3-Nitrophenyl)-2-(3,4,5-trimethoxyphenyl) acrylamido podophyllotoxin (13h)

This compound **13h** was prepared by the method described for **13a**, employing **12** (200 mg, 0.48 mmol), acrylic acid **10h** (204 mg,

0.57 mmol), EDCI (108 mg, 0.57 mmol) and HOBt (88 mg, 0.57 mmol). The crude product was purified by column chromatography with ethyl acetate/hexane (2:8) afford the pure compound **13h**, 340 mg in 93% yield. Mp: 181–184 °C, $[\alpha]_D^{25}$ +80.5 (*c* 0.5 in $CHCl_3$), ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.54–2.62 (dd, 1H, *J* = 5.0, 5.0 Hz), 2.90–3.03 (m, 1H), 3.73 (s, 6H), 3.74 (s, 6H), 3.77 (s, 3H), 3.88 (s, 3H), 3.89–3.90 (m, 1H), 4.43–4.50 (m, 2H), 5.25–5.30 (m, 1H), 5.82 (d, 1H, *J* = 6.9 Hz), 5.94–5.98 (dd, 2H, *J* = 1.1, 1.1 Hz), 6.22 (s, 2H), 6.38 (s, 2H), 6.45 (s, 1H), 6.73 (s, 1H), 7.40–7.43 (m, 2H), 7.77–7.81 (m, 2H), 8.04–8.09 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 36.7, 40.4, 43.1, 47.7, 55.7, 55.8, 59.8, 60.0, 68.5, 101.2, 107.0, 108.1, 109.2, 109.3, 122.6, 123.6, 129.6, 130.0, 131.7, 132.1, 135.7, 136.1, 136.2, 137.0, 137.7, 138.7, 146.5, 147.2, 147.4, 151.9, 153.2, 167.4, 174.5; MS (ESI): 755 $[M+H]^+$.

5.27. 4β-(*E*)-3-(4-Methoxyphenyl)-2-(3,4,5-trimethoxyphenyl) acrylamido podophyllotoxin (13i)

This compound **13i** was prepared by the method described for **13a**, employing **12** (200 mg, 0.48 mmol), acrylic acid **10i** (196 mg, 0.57 mmol), EDCI (108 mg, 0.57 mmol) and HOBt (88 mg, 0.57 mmol). The crude product was purified by column chromatography with ethyl acetate/hexane (3:7) afford the pure compound **13i**, 320 mg in 94% yield. Mp: 128–130 °C, $[\alpha]_D^{25}$ −26.4 (*c* 0.5 in $CHCl_3$), ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.55–2.66 (dd, 1H, *J* = 4.4, 5.1 Hz), 2.87–3.07 (m, 1H), 3.74 (s, 12H), 3.78 (s, 3H), 380 (s, 3H), 3.91 (s, 3H), 4.25–4.34 (m, 1H), 4.45–4.55 (m, 2H), 5.32 (t, 1H), 5.69 (d, 1H, *J* = 6.6 Hz), 5.96 (d, 2H, *J* = 4.4 Hz), 6.26 (s, 2H), 6.43 (s, 2H), 6.48 (s, 1H), 6.69–6.74 (m, 3H), 6.99 (d, 2H, *J* = 8.8 Hz), 7.78 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 36.7, 40.2, 40.3, 43.1, 47.7, 55.0, 55.6, 59.7, 59.9, 68.6, 101.1, 104.1, 107.0, 108.0, 109.2, 113.5, 127.2, 130.1, 131.2, 132.0, 133.6, 134.2, 135.7, 136.2, 137.0, 146.5, 147.1, 151.9, 153.0, 159.2, 167.8, 174.5; MS (ESI): 740 $[M+H]^+$.

5.28. 4β-(*E*)-3-(3-Methoxyphenyl)-2-(3,4,5-trimethoxyphenyl) acrylamido podophyllotoxin (13j)

This compound **13j** was prepared by the method described for **13a**, employing **12** (200 mg, 0.48 mmol), acrylic acid **10j** (196 mg, 0.57 mmol), EDCI (108 mg, 0.57 mmol) and HOBt (88 mg, 0.57 mmol). The crude product was purified by column chromatography with ethyl acetate/hexane (3:7) afford the pure compound **13j**, 310 mg in 88% yield. Mp: 187–190 °C, $[\alpha]_D^{25}$ −69.9 (*c* 0.5 in $CHCl_3$), ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.51–2.58 (dd, 1H, *J* = 4.8, 4.8 Hz), 2.85–3.01 (m, 1H), 3.71 (s, 3H), 3.72 (s, 6H), 3.75 (s, 6H), 3.76 (s, 3H), 3.86 (s, 3H), 4.40–4.48 (m, 2H), 5.22–5.37 (m, 3H), 5.93 (d, 2H, *J* = 8.0 Hz), 6.20 (s, 2H), 6.36–6.44 (m, 4H), 6.66 (d, 1H, *J* = 8.8 Hz), 6.72 (s, 1H), 6.93–7.02 (m, 2H), 7.70 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 36.8, 40.4, 43.1, 47.8, 55.0, 55.7, 55.8, 59.8, 60.0, 68.6, 101.2, 106.5, 107.0, 108.0, 109.2, 109.3, 113.5, 113.7, 127.3, 130.1, 131.2, 131.3, 132.0, 132.1, 132.2, 133.7, 134.2, 135.7, 136.2, 137.1, 138.5, 146.5, 147.1, 151.9, 153.1, 159.2, 159.9, 167.9, 174.6; MS (ESI): 740 $[M+H]^+$.

5.29. 4β-(*E*)-2-(2-Methoxyphenyl)-3-(4-nitrophenyl)acrylamido podophyllotoxin (13k)

This compound **13k** was prepared by the method described for **13a**, employing **12** (200 mg, 0.48 mmol), acrylic acid **10k** (170 mg, 0.57 mmol), EDCI (108 mg, 0.57 mmol) and HOBt (88 mg, 0.57 mmol). The crude product was purified by column chromatography with ethyl acetate/hexane (2:8) afford the pure compound **13k**, 320 mg in 95% yield. Mp: 181–183 °C, $[\alpha]_D^{25}$ −42.5 (*c* 0.5 in $CHCl_3$), ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.42–2.51 (dd, 1H, *J* = 4.7, 4.7 Hz), 2.86–3.01 (m, 1H), 3.74 (s, 6H), 3.77 (s, 3H), 383 (s, 3H), 4.05–4.14 (m, 2H), 4.24–4.30 (m, 1H), 4.40–4.49 (m, 1H), 5.61 (d, 1H, *J* = 6.7 Hz), 5.95 (s, 2H), 6.19 (s, 2H), 6.42 (s, 1H), 6.68 (s, 1H), 6.92–7.07 (m, 3H), 7.12 (d, 2H, *J* = 8.8 Hz), 7.36–7.45 (m, 1H), 7.83 (s, 1H), 8.02 (d, 2H, *J* = 8.8 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 36.6, 43.1, 47.1, 55.2, 55.6, 59.8, 64.5, 68.3, 101.1, 108.0, 108.9, 109.3, 111.5, 119.2, 120.7, 123.3, 124.0, 130.3, 131.3, 132.0, 132.1, 135.7, 136.2, 137.9, 139.2, 142.4, 146.2, 146.5, 147.1, 151.9, 157.0, 174.6; MS (ESI): 696 $[M+H]^+$.

5.30. 4β-(*E*)-2-(2-Methoxyphenyl)-3-(2-nitrophenyl)acrylamido podophyllotoxin (13l)

This compound **13l** was prepared by the method described **13a**, employing **12** (200 mg, 0.48 mmol), acrylic acid **10l** (170 mg, 0.57 mmol), EDCI (108 mg, 0.57 mmol) and HOBt (88 mg, 0.57 mmol). The crude product was purified by column chromatography with ethyl acetate/hexane (2:8) afford the pure compound **13l**, 320 mg in 95% yield. Mp: 187–190 °C, $[\alpha]_D^{25}$ −59.9 (*c* 0.5 in $CHCl_3$), ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.47–2.54 (dd, 1H, *J* = 5.2, 2.2 Hz), 2.83–3.02 (m, 1H), 3.73 (s, 6H), 3.73 (s, 6H), 3.76 (s, 3H), 3.83 (s, 3H), 4.02 (t, 1H, *J* = 9.8, 9.8 Hz), 4.40–4.50 (m, 2H,) 5.27–5.33 (m, 1H), 5.54 (d, 1H, *J* = 6.7 Hz), 5.94 (d, 2H, *J* = 3.0 Hz), 6.20 (s, 2H), 6.39 (s, 1H), 6.70–6.92 (m, 4H), 7.17–7.33 (m, 5H), 8.03 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 36.6, 40.5, 43.1, 47.1, 55.2, 55.7, 59.8, 68.3, 101.2, 108.0, 108.9, 109.3, 111.0, 120.3, 124.3, 128.9, 129.8, 130.3, 131.2, 131.5, 131.6, 131.8, 132.0, 133.2, 135.7, 136.2, 136.4, 146.5, 147.1, 147.7, 151.9, 157.2, 167.6, 174.6; MS (ESI): 695 $[M+H]^+$.

5.31. 4β-(*E*)-3-(4-Hydroxy-3-methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)acrylamido podophyllotoxin (13m)

This compound **13m** was prepared by the method described for **13a**, employing **12** (200 mg, 0.48 mmol), acrylic acid **11m** (220 mg, 0.57 mmol), EDCI (108 mg, 0.57 mmol) and HOBt (88 mg, 0.57 mmol). The crude product was purified by column chromatography with ethyl acetate/hexane (3:7) afford the pure compound **13m**, 300 mg in 82% yield. Mp: 134–137 °C, $[\alpha]_D^{25}$ −12.9 (*c* 0.5 in $CHCl_3$), ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.55–2.63 (dd, 1H, *J* = 4.5, 4.5 Hz), 2.86–3.00 (m, 1H), 3.53 (s, 3H), 3.73 (s, 6H), 3.75 (s, 9H), 3.81 (s, 3H), 4.23–4.31 (m, 1H), 4.39–4.48 (m, 2H), 5.30 (t, 1H), 5.72 (d, 1H, *J* = 6.7 Hz), 5.95 (d, 2H, *J* = 3.0 Hz), 6.14–6.24 (m, 3H), 6.34–6.47 (m, 4H), 6.70–6.77 (m, 3H), 7.68 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 36.8, 40.4, 43.1, 47.8, 54.6, 55.6, 55.8, 59.8, 60.0, 68.6, 101.1, 102.8, 107.2, 108.0, 109.1, 109.2, 109.4, 112.6, 115.0, 121.1, 124.7, 126.0, 130.1, 131.4, 131.6, 132.1, 132.4, 133.3, 135.2, 135.7, 136.2, 137.0, 146.5, 146.7, 147.1, 147.3, 151.9, 153.2, 167.6, 174.5; MS (ESI): 756 $[M+H]^+$.

5.32. 4β-(*E*)-3-(3-Hydroxy-4-methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)acrylamido podophyllotoxin (13n)

This compound **13n** was prepared by the method described for **13a**, employing **12** (200 mg, 0.48 mmol), acrylic acid **11n** (220 mg, 0.57 mmol), EDCI (108 mg, 0.57 mmol) and HOBt (88 mg, 0.57 mmol). The crude product was purified by column chromatography with ethyl acetate/hexane (3:7) afford the pure compound **13n**, 320 mg in 88% yield. Mp: 145–147 °C, $[\alpha]_D^{25}$ −60.9 (*c* 0.5 in $CHCl_3$), ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.52–2.63 (dd, 1H, *J* = 4.6, 4.6 Hz), 2.85–3.02 (m, 1H), 3.75 (s, 9H), 3.78 (s, 3H), 389 (s, 6H), 4.24–4.33 (dd, 1H, *J* = 4.6, 4.6 Hz), 4.43–4.52 (dd, 2H, *J* = 6.2, 6.2 Hz), 5.31 (t, 1H), 5.67 (d, 1H, *J* = 7.8 Hz), 5.97 (d, 2H, *J* = 4.6 Hz), 6.24 (s, 2H), 6.42 (s, 2H), 6.47 (s, 1H), 6.52–6.70 (m, 3H), 6.75 (s, 1H), 7.68 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 36.7, 40.4, 43.1, 47.7, 55.3, 55.6, 55.7, 59.8, 60.0, 68.6, 101.1, 107.1, 108.0, 109.1, 109.2, 111.3, 116.6, 121.9, 127.5, 130.2, 131.1,

132.0, 133.5, 134.5, 135.7, 136.2, 137.1, 145.7, 146.5, 147.1, 148.0, 151.9, 152.9, 168.0, 174.5; MS (ESI): 778 $[M+Na]^+$.

5.33. 4β-(*E*)-3-(4-Hydroxyphenyl)-2-(3,4,5-trimethoxyphenyl) acrylamido podophyllotoxin (13o)

This compound **13o** was prepared by the method described for **13a**, employing **12** (200 mg, 0.48 mmol), acrylic acid **11o** (188 mg, 0.57 mmol), EDCI (108 mg, 0.57 mmol) and HOBt (88 mg, 0.57 mmol). The crude product was purified by column chromatography with ethyl acetate/hexane (3:7) afford the pure compound **13o**, 330 mg in 94% yield. Mp: 137–140 °C, $[\alpha]_D^{25}$ −46.9 (*c* 0.5 in $CHCl_3$), ^1H NMR (400 MHz, $CDCl_3$): δ 2.52–2.64 (dd, 1H, *J* = 5.1, 5.1 Hz), 2.84–3.03 (m, 1H), 3.71 (s, 6H), 3.74 (s, 6H), 3.77 (s, 3H), 3.85 (s, 3H), 4.23–4.32 (m, 1H), 4.40–4.51 (m, 2H), 5.29 (t, 1H), 5.76 (d, 1H, *J* = 6.6 Hz), 5.96 (d, 2H, *J* = 2.9 Hz), 6.22 (s, 2H), 6.38 (s, 2H), 6.44 (s, 1H), 6.75 (s, 1H), 6.91 (d, 2H, *J* = 8.8 Hz), 7.03 (d, 2H, *J* = 8.8 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 36.7, 40.4, 43.1, 47.7, 55.6, 55.7, 59.8, 60.0, 68.6, 101.2, 107.1, 108.1, 109.2, 109.3, 121.5, 130.1, 130.6, 132.1, 132.8, 133.3, 135.7, 136.2, 136.4, 137.2, 146.5, 147.1, 150.0, 151.9, 152.9, 167.8, 169.0, 174.5; MS (ESI): 764 $[M+K]^+$.

6. Procedure of the SRB-assay

The synthesized compounds (**13a–o**) have been evaluated for their in vitro cytotoxicity in human cancer cell lines. A protocol of 48 h continuous drug exposure has been used and a sulforhodamine B (SRB) protein assay has been used to estimate cell viability or growth. The cell lines were grown in DMEM medium containing 10% fetal bovine serum and 2 mM L-glutamine and were inoculated into 96 well microtiter plates in 90 mL at plating densities depending on the doubling time of individual cell lines. The microtiter plates were incubated at 37 °C, 5% CO_2, 95% air, and 100% relative humidity for 24 h prior to addition of experimental drugs. Aliquots of 10 mL of the drug dilutions were added to the appropriate microtiter wells already containing 90 mL of cells, resulting in the required final drug concentrations. For each compound four concentrations (0.1, 1, 10 and 100 μM) were evaluated and each was done in triplicate wells. Plates were incubated further for 48 h and assay was terminated by the addition of 50 mL of cold trichloroacetic acid (TCA) (final concentration,10% TCA) and incubated for 60 min at 4 °C. The plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (50 mL) at 0.4% (w/v) in 1% acetic acid was added to each of the cells, and plates were incubated for 20 min at room temperature. The residual dye was removed by washing five times with 1% acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on an ELISA plate reader at a wavelength of 540 nm with 690 nm reference wavelengths. Percent growth was calculated on a plate by plate basis for test wells relative to control wells. The above determinations were repeated three times. Percentage growth was expressed as the (ratio of average absorbance of the test well to the average absorbance of the control wells) * 100. Growth inhibition of 50% (GI_{50}) was calculated from [(Ti − Tz)/(C − Tz)] * 100 ¼ 50, which is the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. Where, Tz¼ Optical density at time zero, OD of control¼ C, and OD of test growth in the presence of drug ¼ Ti.

7. Cell culture

The human breast cancer cell line MCF-7 was purchased from American type culture collection was maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen), supplemented with 10% fetal calf serum and 100 U/ml Pencillin and 100 μg/ml streptomycin sulfate (Sigma). The cell line was maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in the incubator.

8. In vitro evaluation of cytotoxicity (MTT assay)

Cell viability was assessed by the MTT assay, a mitochondrial function assay. It is based on the ability of viable cells to reduce the MTT to insoluble formazan crystals by mitochondrial dehydrogenase. In this assay MCF-7 cells were seeded in a 96-well plate at a density of 10,000 cells/well. After overnight incubation cells were treated with compounds etoposide, combretastatin (CA-4), podophyllotoxin (Podo), **10k**, **13j**, **13k** and **13l** at 2 and 4 μM concentration and incubated for 24 h. Then the medium was discarded and replaced with 10 μL MTT dye. Plates were incubated at 37 °C for 2 h. The resulting formazan crystals were solubilized in 100 μL extraction buffer. The optical density (OD) was read at 570 nm with micro plate reader.

9. Cell cycle analysis

5×10^5 MCF-7 cells were seeded in 60 mm dish and were allowed to grow for 24 h, 2 μM concentration of etoposide, CA-4 (**6**), podophyllotoxin (Podo), **10k** and **13j**, **13k**, **13l** compounds were added to the culture media, and the cells were incubated for an additional 24 h. Cells were harvested with Trypsin–EDTA, fixed with ice-cold 70% ethanol at 4 °C for 30 min, washed with PBS and incubated with 1 mg/ml RNAase solution (Sigma) at 37 °C for 30 min. Cells were collected by centrifugation at 2000 rpm for 5 min and further stained with 250 μL of DNA staining solution [10 mg of Propidium Iodide (PI), 0.1 mg of trisodium citrate, and 0.03 mL of Triton X-100 were dissolved in 100 mL of sterile MilliQ water at room temperature for 30 min in the dark]. The DNA contents of 20,000 events were measured by flow cytometer (DAKO CYTOMATION, Beckman Coulter, Brea, CA). Histograms were analyzed using Summit Software.

10. Comet assay (single cell gel electrophoresis)

The MCF-7 cells were seeded in 60 mm dishes and were treated with compounds such as etoposide, CA-4 (**6**), podophyllotoxin (Podo), **10k** and the most effective hybrid (**13k**) at a concentration for 2 μM concentration for 24 h. Here we have employed the protocol of Feridoun karimi Busheri et al., 2010 with slight modification. Briefly, 20,000 cells were embedded in agarose and deposited in microscope slides. The slides were incubated 45 min in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, 1% SDS, 1% Triton X-100) and incubated on ice for 30 min followed by immersion in an alkaline solution (300 mM, 1 mM Na_2EDTA) for 30 min. Electrophoresis was carried out for 20 min at 40 V in 0.5% TBE buffer (pH 8). During electrophoresis the damaged DNA migrates away from the nucleus towards anode. Slides were then stained with a 20 mg/ml Sybr green I solution for 30 min. Images from were taken in fluorescent microscope at 20×.

11. γ-H2AX staining

MCF-7 breast cancer cells were seeded on cover slips and treated with as etoposide, CA-4 (**6**), podophyllotoxin (Podo), **10k** and the most effective hybrid (**13k**) at concentration of 2 μM for 24 h. After treatment, cover slips were fixed with paraformaldehyde solution (4% in 1× PBS) for 20 min at room temperature. Cell permeabilization was achieved by administration of a TritonX-100

solution (0.2% in 1× PBS) for 5 min. Then cover slips were kept in 100% methanol at 4 °C over night. Subsequently, cover slips were blocked with a 1% BSA solution for 60 min and then incubated with anti γ-H2AX (1:100) antibody at room temperature for 2 h. The slides were washed three times each of 5 min with PBST and incubated with a FITC-conjugated anti-rabbit secondary antibody (Jackson Immuno Research Laboratories Inc., Pennsylvania, USA) for one hour. The cover slips were washed three times with PBST solution and mounted with DAPI solution. Finally, cells were observed using confocal microscope (Olympus FV1000). Images taken were processed with the support of the flow view version 1.7c software program.

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