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Synthesis and biological evaluation of estradiol linked pyrrolo[2,1-*c*]-[1,4]benzodiazepine (PBD) conjugates as potential anticancer agents

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ABSTRACT

A series of new estradiol linked pyrrolo[2,1-*c*][1,4]benzodiazepine (E_2-PBD) conjugates (**3a**–**f**, **4a–f** and **5a**–**f**) with different linker architectures including a triazole moiety have been designed and synthesized. All the 18 compounds have been evaluated for their anticancer activity and it is observed that some of the compounds particularly **4c**–**e** and **5c**,**d** exhibited significant anticancer activity. The detailed biological aspects relating to the cell cycle effects and tubulin depolymerization activity have been examined with a view to understand the mechanism of action of these conjugates. Among all these conjugates, one of the compound **5c** could be considered as the most effective compound particularly against MCF-7 breast cancer cell line.

1. Introduction

Pyrrolo[2,1-*c*][1,4]benzodiazepines (PBDs) are a family of naturally occurring antitumour antibiotics originated from *Streptomyces* species,[1] that exert their cytotoxic activity by binding covalently between C11-position of the PBD and C-2 amino group of the guanine residues with in the minor groove of DNA.[2,3] Recently a series of C2-fluorinated PBDs have been synthesized and evaluated for anticancer activity against a number of cancer cell lines,[4] in which fluorine substitution plays an important role for their biological activity. In the past few years, several hybrid compounds, in which known anticancer agents tethered to PBD moiety have been designed, synthesized and evaluated for their biological activity. C2-Fluorinated PBDs significantly increase the kinetic reactivity during covalent adduct formation and exhibited considerable biological activity.[5–9] Moreover, a number of piperazine and 1,2,3-triazole molecules have been synthesized as useful chemotherapeutic agents for various diseases over the past few years.[10,11] Michejda and co-workers[12] reported some promising anticancer class of compounds such as symmetrical bifunctional agents with remarkable selectivity against colon cancers that possess a piperazine moiety in its linker spacer. Furthermore, piperazine containing PBDs have also shown significant anticancer activity.[13] Many 1,2,3-triazole derivatives are known to exhibit interesting biological activities such as antibiotic,[14] tuberculosis,[15] and anticancer activity.[16]

The naturally occurring PBDs such as anthramycin, chicamycin, mazethramycin, porothramycin A, sibiromycin, tomaymycin, oxotomaymycin, prothracarcin and DC-81 (**2**), are known as potent anticancer agents, however they have been excluded from clinical studies due to problems relating to side effects.[17] Hence, there has been considerable interest in the design and synthesis of conjugate agents with active moieties of known anticancer agents to enhance the selectivity as well as anticancer activity. Drug targeting is likely to improve the selectivity of PBDs and this will allow transporting the required drug preferably to those sites that need treatment. When this objectives to be met, not only the efficacy of the treatment can be improved, but also the toxic side effects can be minimized to a great extent.

The estrogen receptor is a key biological target that has fascinated considerable interest over the years and it is expressed by several type of cancers like breast,[18] uterus,[19] and ovarian.[20] The biological affinity between estradiol and its cognate receptor can theoretically be used to direct a cytotoxic agent to the target cells. As a result, over the years various estrogen anticancer hybrids have been investigated for the treatment of hormone-dependent cancers.[21–28]

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In continuation of our efforts in search of new effective anticancer agents, we have designed and synthesized a series of E_2-PBD conjugates. In the present approach, DC-81 (**2**), a known anticancer agent that has been linked to the estradiol (**1**) through the stable alkane spacers or a piperazine or 1,2,3-triazole moieties. The present study describes the design, synthesis and biological activities of these new E_2-PBD conjugates (**3a–f**, **4a–f** and **5a–f**). Representative estradiol (E_2) (**1**), DC-81 (**2**) and their conjugates (**3a–f**, **4a–f** and **5a–f**) are illustrated in Figure 1.

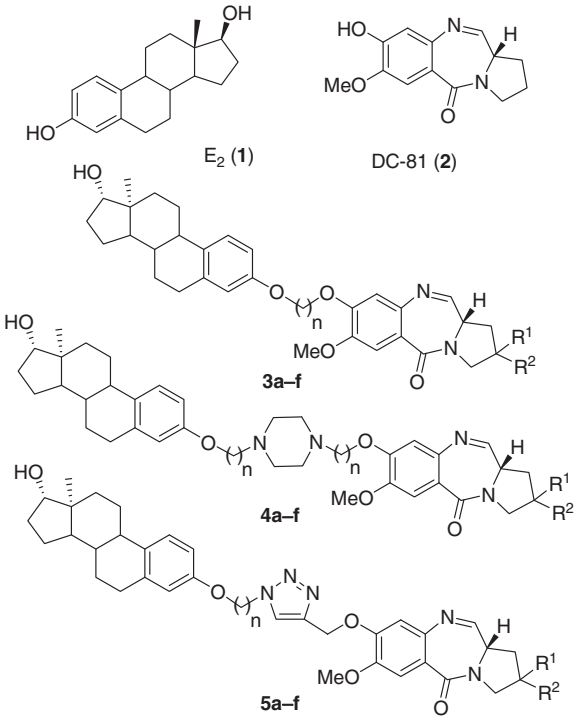


Figure 1. Structures of estradiol (E_2) (**1**), DC-81 (**2**), and E_2-PBD conjugates (**3a–f**, **4a–f** and **5a–f**).

2. Results and discussion

2.1. Chemistry

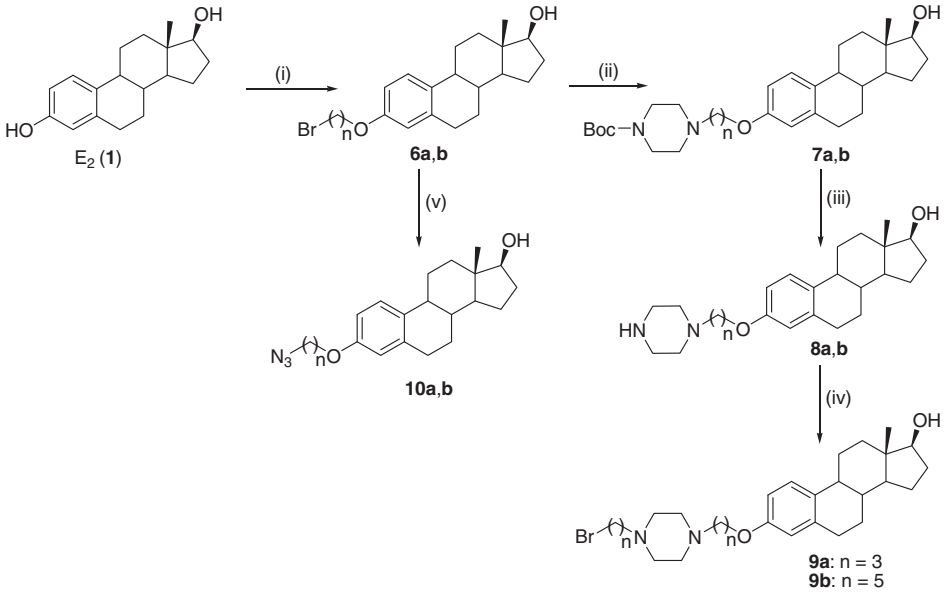
The synthesis of the estradiol derivatives **6a,b**, **9a,b**, and **10a,b** was carried out as outlined in Scheme 1. The etherification of 17β-estradiol (**1**) by employing dibromoalkanes with K_2CO_3 gave compounds **6a,b** and these compounds were treated with *N*-Boc piperazine to obtain compounds **7a,b** which upon deprotection with trifluoroacetic acid provided the intermediates **8a,b**. Further, these compounds on monoalkylation by using dibromoalkanes in the presence of K_2CO_3 afford compounds **9a,b**. Synthesis of the azide precursors was carried out by the reaction of halogen derivatives **6a,b** with sodium azide to attain the azides **10a,b** as illustrated in Scheme 1.

Compounds **11a–c** were obtained by employing our previously reported methods.[29–32] The etherification of **11a–c** with propargyl bromide in the presence of K_2CO_3 afforded the terminal alkyne intermediates **12a–c** in good yields. The synthesis of C8-linked E_2-PBD conjugates (**3a–f** and **4a–f**) was carried out from **11a–c** by treating them with estradiol precursors (**6a,b** and **9a,b**) using K_2CO_3 to provide the corresponding nitro thioacetals (**13a–f** and **14a–f**) as shown in Scheme 2. Terminal alkynes (**12a–c)** were reacted with azides (**10a–c),** that underwent the 'click' reaction in the presence of Cu(I) catalyst and sodium ascorbate to produce the 1,2,3-triazole-containing E_2-PBD nitro thioacetals (**15a–f)** as shown in Scheme 3. Further, reduction of nitro compounds (**13a–f**, **14a–f** and **15a–f)** by using $SnCl_2{\cdot}2H_2O$ in methanol followed by deprotection and cyclisation using $HgCl_2/CaCO_3$ afford the target E_2-PBD conjugates (**3a–f**, **4a–f** and **5a–f**) as shown in Schemes 2 and 3.

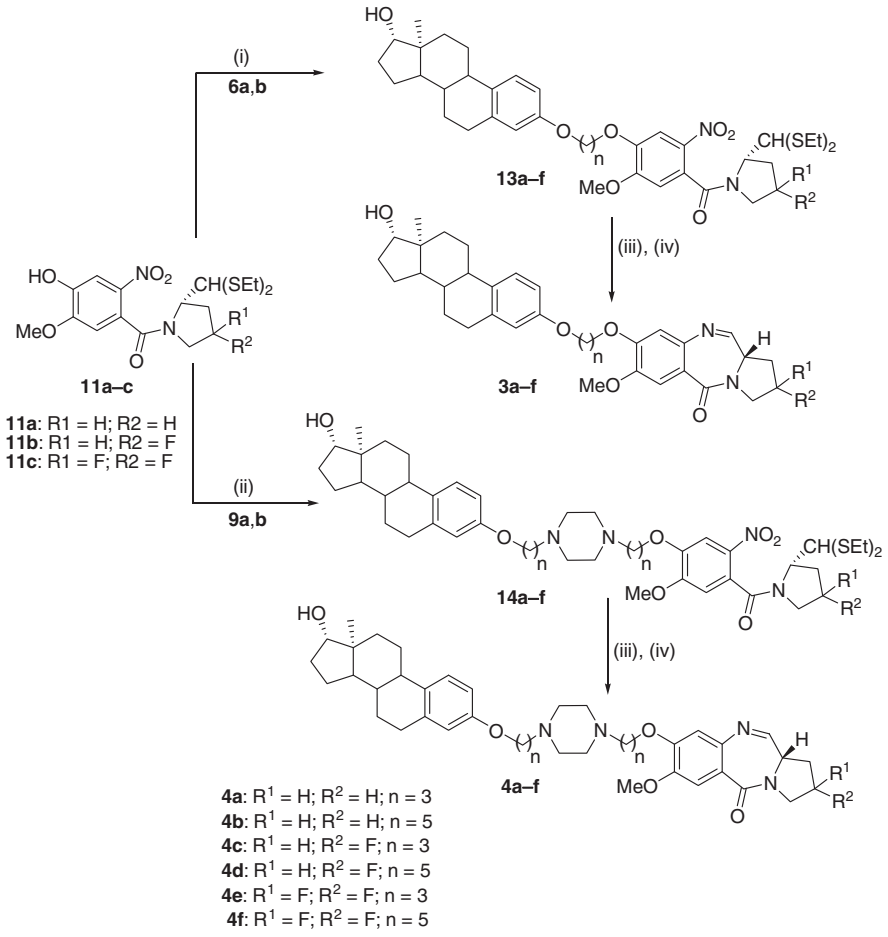
2.2. Evaluation of biological activity

2.2.1. Anticancer activity

The anticancer activity of these conjugates (**3a–f**, **4a–f** and **5a–f**) was carried out in selected human cancer cell lines of breast, cervix, lung, colon, oral, ovarian and prostate by using Sulforhodamine B (SRB) method. The compounds exhibiting $GI_{50} \leqslant 10^{-5}$ M are considered to be active on the respective cell lines. All these conjugates have shown good anticancer activity with GI_{50} values



Scheme 1. Reagents and conditions: (i) dibromoalkanes, K_2CO_3, DMF, rt, 8–10 h, 88–90%; (ii) *N*-Boc piperazine, K_2CO_3, dry CH_3CN, reflux, 24 h, 78–82%; (iii) TFA, dry CH_2Cl_2, rt, 3 h, 95%; (iv) dibromoalkanes, K_2CO_3, dry CH_3CN, reflux, 24 h, 75–78%; (v) NaN_3, DMF,100 °C, 12 h, 90–92%.



Scheme 2. Reagents and conditions: (i) K_2CO_3, dry CH_3COCH_3, 60 °C, 10–12 h, 78–90%; (ii) K_2CO_3, dry CH_3CN, 80 °C, 30–48 h, 70–85%; (iii) $SnCl_2{\cdot}2H_2O$, CH_3OH, reflux, 4–6 h; (iv) $HgCl_2$, $CaCO_3$, CH_3CN–H_2O, (4:1), 10–12 h, 60–70% and 46–55%.

ranging from <0.1 to 2.81 μM, whereas the positive controls adriamycin and DC-81 (**2**) demonstrated the GI_{50} in the range of 0.10–7.25 μM and 0.10–2.37 μM, respectively, as shown in Table 1. Interestingly all the conjugates have shown significant anticancer activity against hormone dependent (estrogen receptor positive) MCF-7 cell line with GI_{50} values ranging from <0.1 to 0.17 μM, C_2-monofluoro-PBD estradiol conjugates have shown slightly better anticancer activity compared to the E_2-PBD conjugates particularly in Zr-75-1, A-549, A-2780, HOP-62, GURAV, and PC-3 cell lines. Further, it is observed that the incorporation of the piperazine as well as triazole moieties in the alkane linker spacer between the estradiol and PBD subunits has slightly enhanced the anticancer activity. Whereas PBD conjugates with propyloxy spacers exhibited slightly more activity than the pentyloxy spacers. Among all the conjugates prepared, compounds **4c–e** and **5c,d** exhibited significant anticancer activity.

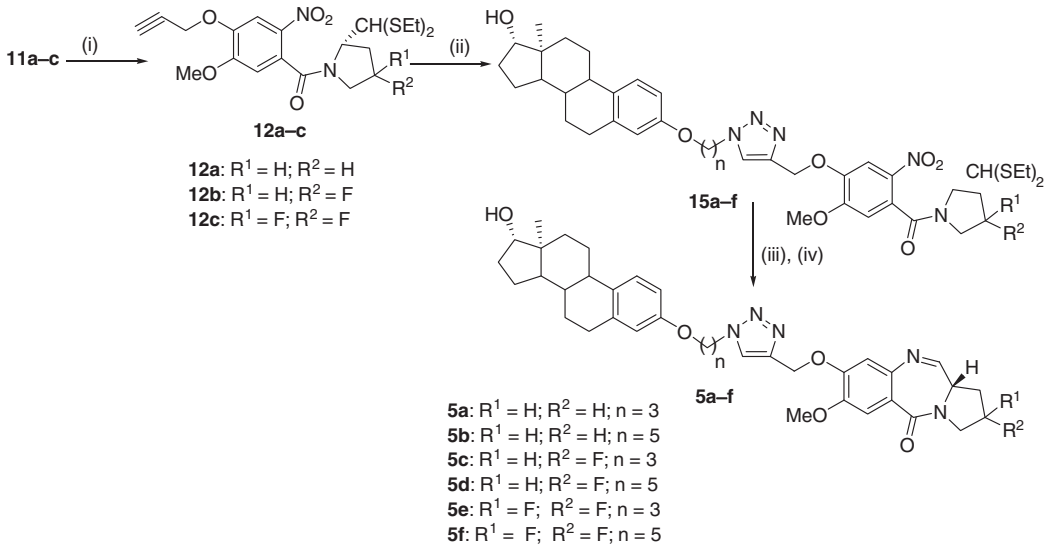
MTT assay was also carried out to identify the cytotoxic effect of some of these E_2-PBD conjugates (**4a**, **4c**, **5c**, **5d** and **5f**) in MCF-7 cells at 4 μM. Further, the cytotoxicity of compounds E_2 (**1**), and a positive control DC-81 (**2**) was also examined to substantiate the cytotoxic effect of these E_2-PBD conjugates and it is interesting to observe that these conjugates have shown higher cytotoxicity than both the controls (**1** and **2**) as shown in Figure 2.

2.2.2. Effect of PBD conjugates on cell cycle

To investigate the mechanism underlying the antiproliferative effect of these E_2-PBD conjugates, we analyzed the cell cycle distribution by treating MCF-7 cells at 4 μM concentration for 24 h by flow cytometry (FACS analysis). The majority of control cells exposed with DMSO showed 14.28% of cells in G2/M phase. E_2-PBD conjugates (**4a**, **4c**, **5c**, **5d** and **5f**) treated cells showed 24.01%, 24.33%, 36.64%, 32.40% and 21.80% of cells in G2/M phase. The left conjugate partner E_2 (**1**) showed 23.80% of cells in G2/M phase, whereas the right side conjugate partner, DC-81 (**2**) has shown more percent of cells in G1 phase and no effect in the G2/M phase as shown in Table 2.

2.2.3. Effect of PBD conjugates on tubulin polymerization

Generally, the inhibition of tubulin polymerization is associated with an arrest in cell cycle progression at the G2/M phase transition by interrupting mitotic spindle formation[33] and chromosome segregation.[34] From previous studies, it is known that molecules that alter the microtubule polymerization, cause mitotic arrest which ultimately leads to apoptosis. Therefore, these E_2-PBD conjugates (**5c** and **5d**) were evaluated for the inhibitory effects on tubulin polymerization at 4 μM for 24 h by taking Nocodazole as the standard. While E_2 (**1**) and DC-81 (**2**), were also used as positive controls. After 24 h **5c** and **5d** cells were fixed and stained with cy3-conjugated β-tubulin antibody and DAPI was used for in situ observation of microtubule network and nuclei fluorescence microscopy. It was observed that the disruption of microtubule network (β-tubulin) was effected significantly in conjugates **5c** than **5d** as shown in Figure 3.



Scheme 3. Reagents and conditions: (i) propargyl bromide, K_2CO_3, dry CH_3COCH_3, 60 °C, 10–12 h, 92–95%; (ii) **10a,b**; $CuSO_4{\cdot}5H_2O$ (1 mol %), $Na_2S_2O_4$, (5 mol %), *t*-BuOH/H_2O (1:1), rt, 8–10 h, 80–90%; (iii) $SnCl_2{\cdot}2H_2O$, CH_3OH, reflux, 6 h; (iv) $HgCl_2$, $CaCO_3$, CH_3CN–H_2O (4:1), 12 h, 55–65%.

Table 1
GI_{50} values[a] (in μM) for E_2-PBD conjugates (**3a**–**f**, **4a**–**f** and **5a**–**f**) in selected human cancer cell lines

Compound	Zr-75-1[c]	A-549[b]	A-2780[d]	HOP-62[b]	KB[e]	SiHa[h]	Gurav[b]	MCF7[c]	Colo205[f]	DWD[e]	PC3[g]
3a	1.19	2.10	1.19	2.23	2.11	2.12	2.05	0.17	0.16	0.17	2.08
3b	1.19	2.20	1.19	2.24	2.13	2.18	2.04	0.17	0.19	0.18	2.19
3c	0.14	0.18	0.17	0.18	2.16	2.12	0.19	0.14	0.16	1.70	0.19
3d	0.17	0.18	0.19	0.19	2.17	2.31	0.18	0.15	0.17	1.90	0.19
3e	0.18	2.81	2.10	2.11	0.15	2.81	2.18	0.17	0.10	2.04	2.40
3f	0.19	2.81	2.12	2.11	0.14	2.81	2.19	0.17	0.20	2.17	2.42
4a	0.12	0.12	1.30	1.14	0.19	0.13	0.14	0.11	0.13	0.12	0.14
4b	0.17	0.16	1.70	1.17	0.19	0.17	0.19	0.14	1.16	0.17	0.19
4c	<0.1	<0.1	<0.1	0.11	0.12	0.15	0.11	<0.1	0.13	0.10	<0.1
4d	<0.1	0.11	0.11	0.13	0.14	0.16	0.13	0.13	0.14	0.15	0.17
4e	0.12	0.12	0.11	0.15	0.17	0.16	0.13	<0.1	0.14	0.10	0.13
4f	0.12	0.13	0.16	0.17	0.81	0.19	0.15	0.14	0.17	1.32	0.19
5a	0.19	0.18	0.17	1.18	0.31	1.14	0.17	0.12	0.17	0.16	0.19
5b	0.11	0.19	0.17	1.69	0.80	1.17	0.18	0.14	0.17	0.17	0.19
5c	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.11	<0.1	<0.1
5d	<0.1	<0.1	<0.1	0.11	<0.1	0.11	0.11	<0.1	0.12	0.12	<0.1
5e	0.17	0.17	1.27	0.18	0.19	1.12	0.17	0.11	0.14	0.17	0.14
5f	0.18	0.17	1.80	0.19	0.19	1.21	0.19	0.11	0.16	0.18	0.17
ADR[i]	1.79	7.25	0.16	0.14	0.17	0.17	0.17	0.17	0.14	0.10	1.81
DC-81 (**2**)	2.37	0.16	0.14	0.15	0.17	0.17	0.16	0.17	0.11	1.49	0.20

[a] 50% growth inhibition and the values are mean of three determinations.
[b] Lung cancer.
[c] Breast cancer.
[d] Ovarian cancer.
[e] Oral cancer.
[f] Colon cancer.
[g] Prostate cancer.
[h] Cervix cancer.
[i] Adriamycin.

2.2.4. Effect of PBD conjugates on the expression of Cdk1

Further, to understand the molecular events involved in G2/M phase arrest, the effect on the expression level of CDKs particularly Cdk1,[35,36] was also investigated in MCF-7 cells that were treated with these conjugates. Accordingly, Western blot analysis was carried out and it was observed that there was a reduction of Cdk1 level as shown in Figure 4. The reduction of Cdk1 protein level is more in case of **5c** compared to compounds E_2 (**1**) and DC-81 (**2**).

2.2.5. Effect of PBD conjugates on apoptosis

From previous studies, it was established that PBD conjugates that alter the microtubule polymerization, cause mitotic arrest which ultimately leads to apoptosis. Hence, it was considered of interest to investigate the apoptosis mediated by these conjugates employing Tunel assay wherein E_2 (**1**) and DC-81 (**2**) are used as positive controls. Results of the Tunel assay reveals that the Apoalert DNA fragmentation is more prominent in case of **5c** and **5d** compared to the controls as shown in Figure 5a.

Further, we have focused our attention on the molecules that are involved in apoptosis. Studies of Basu and co-workers have reported Bax as an important signaling molecule involved in apoptosis and its activity was dependent on p53 status.[37] Moreover, caspases-3 and -7 are also known to cause cleavage of substrates and manifest the apoptotic process.[38] Hence it was considered of

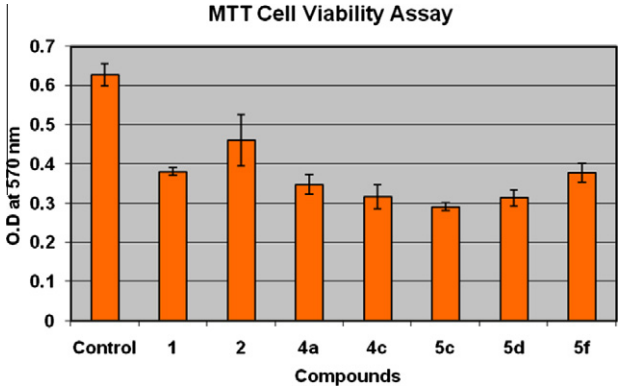


Figure 2. Effect of E_2-PBD conjugates (**4a**, **4c**, **5c**, **5d** and **5f**) on cell viability (in vitro cytotoxicity). MCF-7 cells were treated with 4 μM concentration of PBD conjugates as indicated for 24 h in 96-well plates seeded with 10,000 cells per well. O.D readings were taken at 570 nm wave length to measure the percentage of cell viability after treatment with the respective compounds. E_2 (**1**) and DC-81 (**2**) were used as the positive controls. Control: Control cells treated with DMSO.

Table 2
Cell cycle distribution of MCF-7 cell line at 4 μM concentration of compounds E_2 (**1**), DC-81 (**2**) and E_2-PBD conjugates (**4a**, **4c**, **5c**, **5d** and **5f**)

Compound	G0	G1	S	G2/M
Control (C)	1.03 ± 0.05	61.9.0 ± 1.00	22.10 ± 1.00	14.28 ± 0.72
E_2 (**1**)	0.83 ± 0.13	50.00 ± 1.05	25.26 ± 1.40	23.80 ± 2.60
DC-81 (**2**)	8.50 ± 0.50	73.66 ± 1.52	13.00 ± 1.00	04.83 ± 0.28
4a	2.84 ± 0.19	47.90 ± 0.79	25.20 ± 0.61	24.01 ± 0.23
4c	0.87 ± 0.06	50.53 ± 1.03	24.01 ± 0.53	24.33 ± 0.67
5c	2.00 ± 0.05	30.76 ± 1.78	30.59 ± 1.10	36.64 ± 1.57
5d	0.82 ± 0.09	42.50 ± 1.06	25.80 ± 0.58	32.40 ± 1.69
5f	0.74 ± 0.02	55.00 ± 1.60	22.30 ± 0.77	21.80 ± 1.68

interest to investigate the levels of Bax and procaspase-7. It was observed from the results that there was up regulation of Bax as well as cleavage of procaspase-7 indicating apoptosis in case of **5c** and **5d** as shown in Figure 5b.

2.2.6. Effect of PBD conjugates on tumor suppressor proteins (p53, p21) and modulators of chromation (HDACs)

Moreover, activation of tumor suppressor genes such as p53 and p21 are considered an important in the regulation of apoptotic pathway induced by various stimuli.[39,40] In order to understand the effect of these E_2-PBD conjugates on p53 and p21 dependent apoptotic pathway, MCF-7 cells were treated with conjugates **5c** and **5d** at 4 μM concentration for 24 h and Western blot analysis was carried out. In these studies up regulation of p53 and p21 protein levels is observed particularly in **5c** apart from **5d** as shown in Figure 6a and b.

Recent studies have revealed that HDAC inhibitors,[41] cause up regulation of p21 protein level in estrogen positive breast cancer cells such as MCF-7.[42–45] Moreover, HDAC inhibitors have the potential to inhibit the inducible as well as constitutive NF-κB activation to cause an apoptotic response. Since an increase was observed in the protein levels specific to antitumor genes such as p53, p21 we became further interested to examine the effect of these compounds for the inhibition Histone deacetylases (HDAC) being the main modulators of chromatin. Thus the estrogen positive MCF-7 cells were treated with compounds for 24 h and Western blot analysis was carried out. It was observed from these results that there is down regulation of HDAC-1, 3 and NF-kB (p65) proteins for **5c** treated cells thus suggesting the inhibitory role of this compound as shown in Figure 7.

3. Conclusions

In conclusion, new classes of E_2-PBD conjugates were synthesized and these exhibit promising anticancer activity against several cancer cell lines particularly for the estrogen positive breast cancer (MCF-7) cells. Further, from the MTT proliferation assay it was observed that the conjugates **5c** and **5d** are more effective as antiproliferative agents than the naturally occurring DC-81 (**2**) in MCF-7 cells at 4 μM concentration. Flow cytometric data of these conjugates showed increased cells in G2/M phase, which is suggestive of G2/M cell cycle arrest. Moreover, compound **5c** showed the disruption of microtubules as well as fragmentation of the nuclei and interestingly, this compound was also identified as an effective CDK1 inhibitor. Further, it was observed from the results of the detailed biological assays that the up regulation of p53, p21, BAX and concomitant down regulation of procaspase-7, NF-kB and HDACs when treated with compounds **5c** and **5d**, it was more pronounced in case of **5c**. These studies suggest that compound **5c** has a potential to the taken up for detailed preclinical investigations, particularly in the treatment of breast cancer.

4. Experimental section

4.1. Chemistry

All chemicals were purchased from Aldrich (Sigma–Aldrich, St. Louis, MO, USA), Lancaster (Alfa Aesar, Johnson Matthey Company, Ward Hill, MA, USA) or Spectrochem Pvt. Ltd (Mumbai, India) and were used without further purification. Reactions were monitored by TLC, performed on silica gel glass plates containing 60 GF-254, and visualizat ion on TLC was achieved by UV light or iodine indicator. Column chromatography was performed with Merck 60–120 mesh silica gel. ^{1}H NMR and ^{13}C NMR spectra were recorded on Gemini Varian-VXR-unity (200 MHz) or Bruker UXNMR/XWIN-NMR (300 MHz) instruments. Chemical shifts are reported in parts per million (δ in ppm) relative to the peak for tetramethylsilane (TMS) as an internal standard, coupling constants are reported in hertz (Hz). Elemental analysis was ±0.4% of the theoretical values. 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 Electro thermal melting point apparatus, and are uncorrected.

4.2. General procedures

4.2.1. 3-(3-Bromopropyloxy)-17-hydroxy-1,3,5-estratrien (6a)

To a solution of compound 17β estradiol **1** (E_2, 272 mg, 1 mmol) in dry DMF (1 mL), anhydrous K_2CO_3 (414 mg, 3 mmol) and 1,3-dibromopropane (0.3 mL, 3 mmol) were added and the reaction was stirred at rt over 8 h. After completion of the reaction, ice-cold water (15 mL), added to the reaction mixture. The solution was extracted into ethyl acetate (3 × 30 mL), and the combined organic layers were washed with brine (2 × 30 mL), dried over anhydrous Na_2SO_4, and solvent was evaporated under reduced pressure to get the crude product. This was further purified by column chromatography using ethyl acetate–hexane (15%) to afford the compound **6a** as a white solid. Yield 346 mg, 88%; mp 76–78 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.17 (d, 1H, J = 8.3 Hz, Ar*H*), 6.68 (dd, 1H, J = 2.3, 8.3 Hz, Ar*H*), 6.61 (s, 1H, Ar*H*), 5.32 (br s, 1H, E_2 –O*H*), 4.06 (t, 2H, J = 6.2 Hz, –OC*H*2–, 3.71 (t, 1H, J = 8.3 Hz, E_2 –C*H*–OH), 3.58 (t, 2H, J = 6.8 Hz, –*CH*2Br), 2.80–2.86 (m, 2H, E_2 –C*H* 2–),

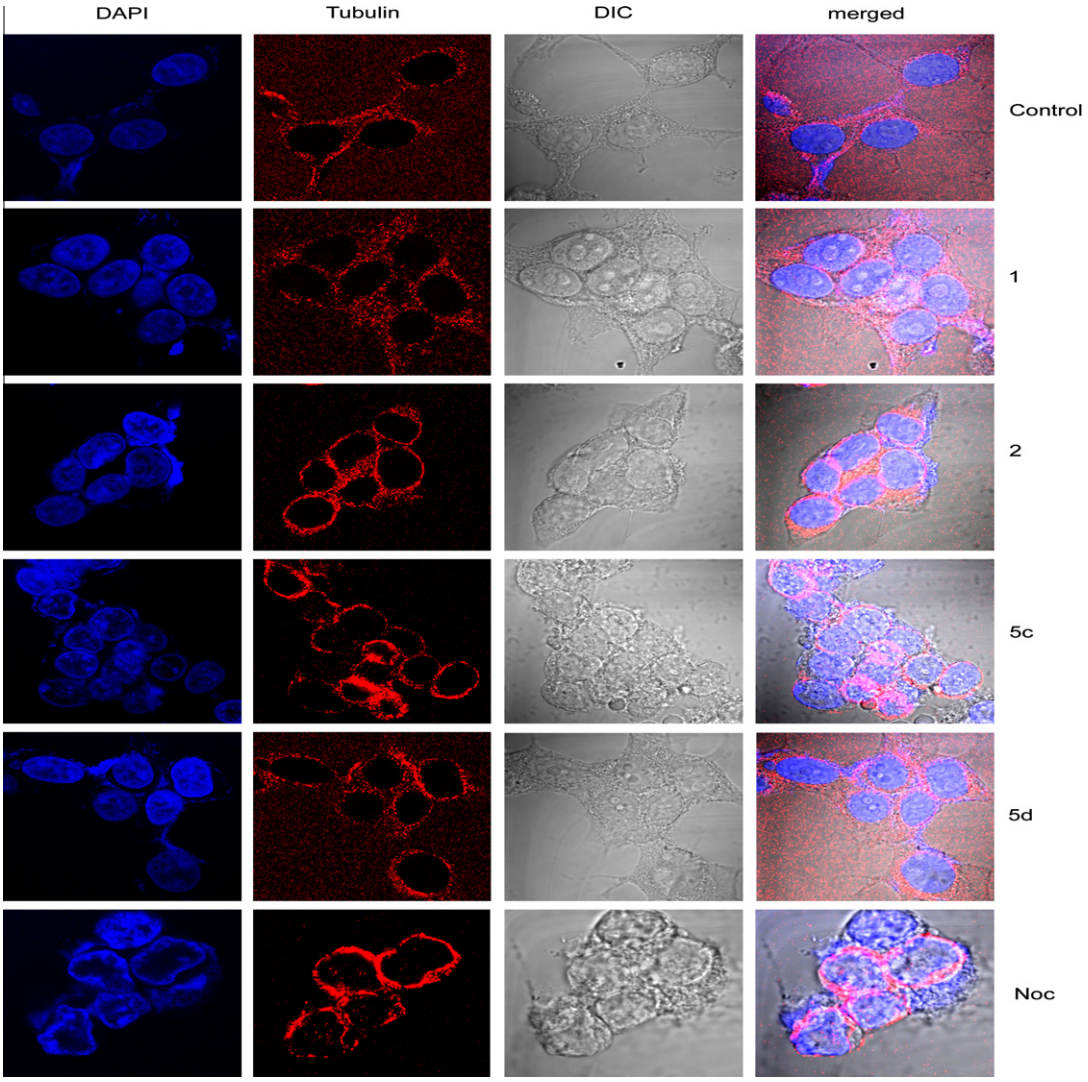


Figure 3. Effect of PBD conjugates on tubulin polymerization. MCF-7 cells were exposed to E_2 (**1**), DC-81 (**2**) and **5c** and **5d** at 4 μM concentration for 24 h. β-Tubulin antibody was used in this immunofluorescence studies. Nocadozole was used as a positive control.

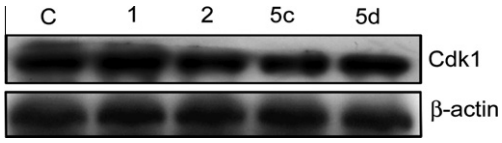


Figure 4. Effect of PBD conjugates on Cdk1 level. MCF-7 cells were treated with E_2 (**1**), DC-81 (**2**), **5c**, and **5d** at 4 μM concentration for 24 h. The cell lysates were collected and expression level of Cdk1 were determined by Western blot analysis. β-Actin was used as a loading control.

2.24–2.36 (m, 5H, E_2 3× –C*H*–, –C*H*2–), 1.12–2.00 (m, 10H, E_2 5× –C*H*2–), 0.77 (s, 3H, E_2 –CH3); MS (ESI): *m/z* 394 $(M+1)^+$.

4.2.2. 3-(5-Bromo pentyloxy)-17-hydroxy-1,3,5-estratrien (6b)

The compound **6b** was prepared following the method described for the preparation of the compound **6a**, employing **1** (E_2, 272 mg, 1 mmol) and 1,5-dibromopentane (0.3 mL, 3 mmol), and the crude product was purified by column chromatography using ethyl acetate–hexane (17%) to afford the compound **6b** as a white solid. Yield 380 mg, 90%; mp 82–84 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.19 (d, 1H, *J* = 9.3 Hz, Ar*H*), 6.69 (dd, 1H, *J* = 2.1, 9.3 Hz, Ar*H*), 6.61 (s, 1H, Ar*H*), 5.33 (br s, 1H, E_2 –O*H*), 3.94 (t, 2H, *J* = 6.2 Hz, –OCH_2–), 3.73 (t, 1H, *J* = 8.2 Hz, E_2 –C*H*–OH), 3.43 (t, 2H, *J* = 7.2 Hz, –OCH_2–), 2.80–2.90 (m, 2H, E_2 –C*H*2–), 2.06–2.35 (m, 3H, E_2 3× –C*H*–), 1.84–1.99 (m, 4H, 2× –C*H*2–), 1.75–1.83 (m, 2H, –C*H*2–), 1.15–1.74 (m, 10H, E_2 5× –C*H*2–), 0.78 (s, 3H, E_2 –CH3); MS (ESI): *m/z* 422 $(M+1)^+$.

4.2.3. 3-{3-Piperazine *tert*-butyl-1-carboxylate)-propyloxy}-17-hydroxy-1,3,5-estra trien (7a)

To a solution of compound **6a** (393 mg, 1 mmol) in dry acetonitrile (20 mL), anhydrous K_2CO_3 (552 mg, 4 mmol) and *tert*-butyl-1-piperazinecarboxylate (374 mg, 2 mmol) were added and the mixture was refluxed for 24 h. After completion of the reaction, potassium carbonate was removed by suction filtration and the solvent was evaporated under reduced pressure to get the crude product. This was further purified by column chromatography using ethyl acetate–hexane (60%) to afford the compound **7a** as a white solid. Yield 388 mg, 78%; mp 118–120 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.18 (d, 1H, *J* = 8.2 Hz, Ar*H*), 6.69 (dd, 1H, *J* = 2.2, 8.2 Hz, Ar*H*), 6.63 (s, 1H, Ar*H*), 5.34 (br s, 1H, E_2 –O*H*), 3.97 (t, 2H, *J* = 6.6 Hz, –OCH_2–), 3.73 (t, 1H, *J* = 8.1 Hz, E_2 –C*H*–OH), 3.38–3.45 (m, 8H, –N(–CH_2–CH_2–)$_2$N–), 2.76–2.90 (m, 2H, E_2 –C*H*2–), 2.56 (t, 2H, *J* = 8.0 Hz, –CH_2N–), 1.80–2.37 (m, 5H, E_2 3× –C*H*–, –CH_2–), 1.12–1.78 (m, 19H, *tert*-butyl 3× –CH_3, E_2 5× –C*H*2–), 0.78 (s, 3H, E_2 –CH3); MS (ESI): *m/z* 499 $(M+H)^+$.

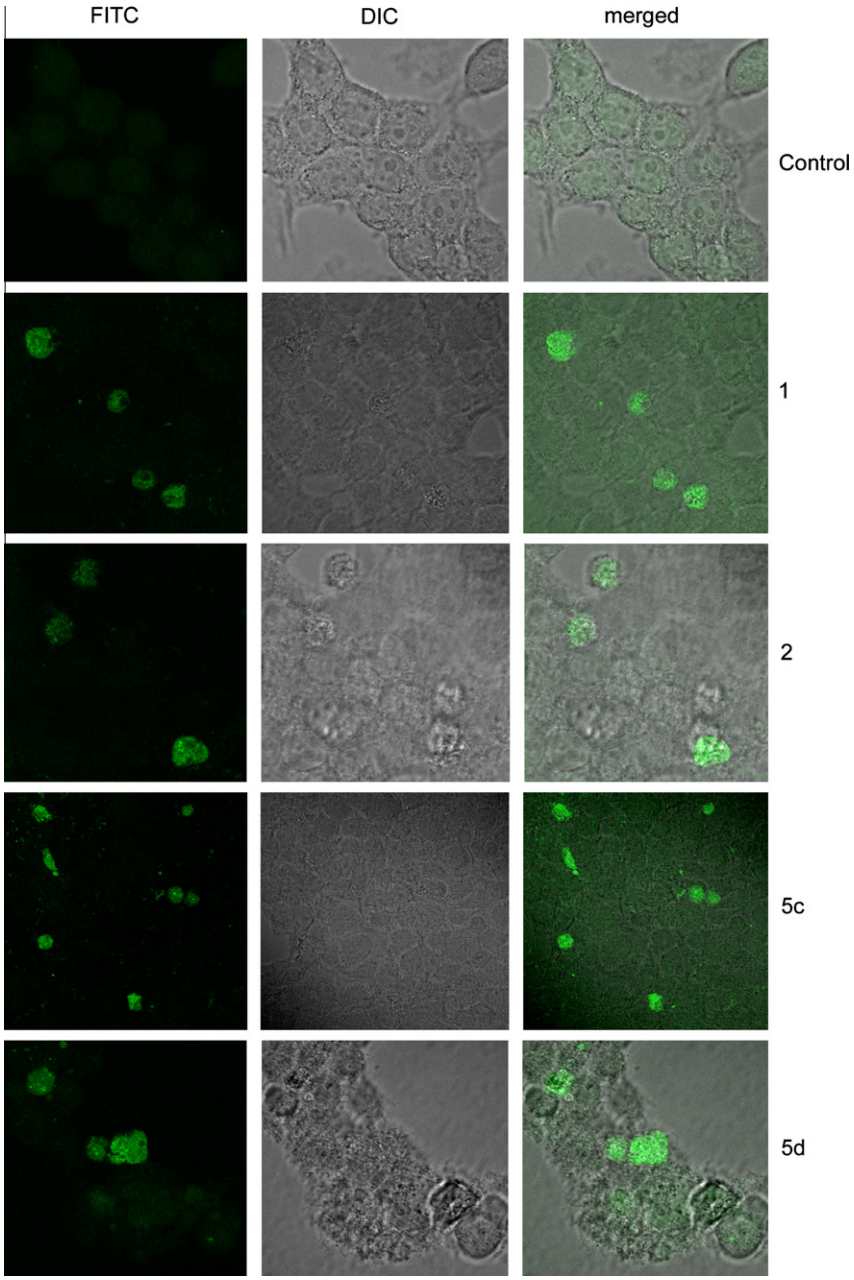


Figure 5a. Effect of PBD conjugates on apoptosis. MCF-7 cells were treated with E_2 (**1**), DC-81 (**2**), **5c** and **5d** at 4 μM concentration for 24 h. The cells were subjected to Tunel staining, green colour stained cells represents Tunel positive cells. Here lack of staining in control (untreated) cells, represents that the cells are actively proliferating, without apoptotic cell death.

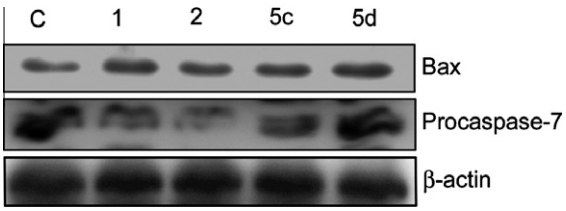


Figure 5b. Effect of PBD conjugates on the expression of Bax and Procaspase-7 proteins. MCF-7 cells were treated with E_2 (**1**), DC-81 (**2**), **5c**, and **5d** at 4 μM concentration for 24 h. The cell lysates were collected and expression levels of Bax and procaspase-7 proteins were determined by Western blot analysis. β-Actin was used as a loading control.

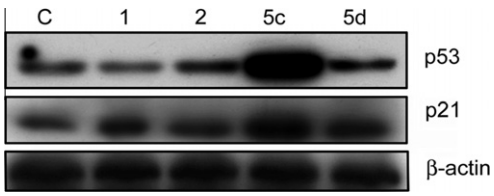


Figure 6a. Effect of PBD conjugates on the expression of p53 and p21 protein levels. MCF-7 cells were treated with E_2 (**1**), DC-81 (**2**), **5c** and **5d** at 4 μM concentration for 24 h. The cell lysates have been collected and the levels of p53, p21 were determined by Western blot analysis using specific antibodies. β-Actin was used as loading control.

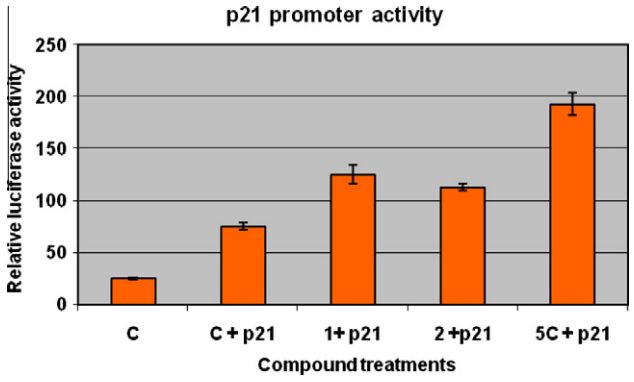


Figure 6b. Effect of PBD conjugates on p21 promoter (−208/+8) activity. MCF-7 cells were transfected with 1.5 μg of p21 promoter fused with luciferase reporter gene and after 24 h, the cells were subjected to compound treatments E_2 (**1**), DC-81 (**2**), and **5c** at 4 μM concentration for 24 h. The cell lysates have been collected and observed for luciferase activity which is the indicator for p21 promoter activity after compound treatment. Here C: Control (untreated).

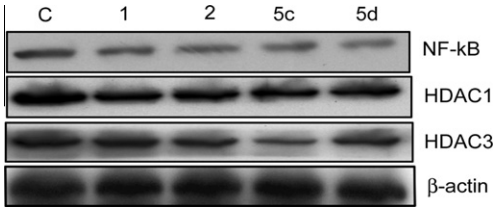


Figure 7. Effect of PBD conjugates on expression of the NF-kB (p65), Histone deacetylases proteins. MCF-7 cells were treated with E_2 (**1**), DC-81 (**2**), **5c** and **5d** at 4 μM concentration for 24 h. Western blot analysis was carried out with antibodies against p65 and HDAC1, 3.

4.2.4. 3-{5-Piperazine *tert*-butyl-1-carboxylate)-pentyloxy}-17-hydroxy-1,3,5-estra trien (7b)

The compound **7b** was prepared following the method described for the preparation of the compound **7a**, employing **6b** (421 mg, 1 mmol) and *tert*-butyl-1-piperazinecarboxylate (374 mg, 2 mmol), and the crude product was purified by column chromatography using ethyl acetate–hexane (64%) to afford the compound **7b** as a white solid. Yield 431 mg, 82%; mp 148–150 °C; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.19 (d, 1H, *J* = 8.3 Hz, Ar*H*), 6.70 (dd, 1H, *J* = 2.3, 8.3 Hz, Ar*H*), 6.61 (s, 1H, Ar*H*), 5.33 (br s, 1H, E_2 –O*H*), 3.98 (t, 2H, *J* = 7.1 Hz, –OCH_2–), 3.72 (t, 1H, *J* = 8.3 Hz, E_2 –C*H*–OH), 3.39–3.46 (m, 8H, –N(–CH_2–CH_2–)$_2$N–), 2.77–2.90 (m, 2H, E_2 –C*H*2–), 2.59 (t, 2H, *J* = 8.1 Hz, –CH_2N–), 2.22–2.51 (m, 3H, E_2 3× –C*H*–), 1.79–2.20 (m, 6H, 3× –C*H*2–), 1.14–1.76 (m, 19H, *tert*-butyl 3 –CH_3, E_2 5× –C*H*2–), 0.78 (s, 3H, E_2 –CH3); MS (ESI): *m/z* 527 $(M+H)^+$.

4.2.5. 3-(3-Piperazinopropyloxy)-17-hydroxy-1,3,5-estratrien (8a)

The compound **7a** (498 mg, 1 mmol) in dry dichloromethane (25 mL), at 0 °C, and trifluoroacetic acid (0.8 mL, 10 mmol) were added slowly to this reaction mixture and the mixture was stirred at room temperature for 2 h. After completion of the reaction, the reaction mixture was concentrated and the resulting compound was suspended in $NaHCO_3$ solution (30 mL), the solution was extracted into dichloromethane (3 × 40 mL), and the combined organic layer were washed with brine (2 × 30 mL), dried over anhydrous Na_2SO_4, and solvent was evaporated under reduced pressure to get the product. Without further purification this compound was used next step, to afford the compound **8a** as a light yellow oil. Yield 380 mg, 95%.

4.2.6. 3-(5-Piperazinopentyloxy)-17-hydroxy-1,3,5-estratrien (8b)

The compound **8b** was prepared following the method described for the preparation of the compound **8a**, employing **7b** (526 mg, 1 mmol) and tri fluoroaceticacid (0.8 mL, 10 mmol), Without further purification this compound was used next step, to afford the compound **8b** as a light yellow oil. Yield 401 mg, 95%.

4.2.7. 3-{3-(4-(3-Bromopropyl)-piperazin-1-yl)-propyloxy}-17-hydroxy-1,3,5-estra trien (9a)

To a solution of compound **8a** (398 mg, 1 mmol) in dry acetonitrile (20 mL), anhydrous K_2CO_3 (553 mg, 4 mmol) and 1,3-dibromopropane (0.3 mL, 3 mmol) were added and the mixture was refluxed for 24 h. After completion of the reaction, potassium carbonate was removed by filtration and the solvent was evaporated under reduced pressure to get the crude product. This was further purified by column chromatography using ethyl acetate–hexane (62%) to afford the compound **9a** as a light yellow oil. Yield 388 mg, 75%; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.19 (d, 1H, *J* = 8.2 Hz, Ar*H*), 6.71 (dd, 1H, *J* = 2.2, 8.2 Hz, Ar*H*), 6.61 (s, 1H, Ar*H*), 5.34 (br s, 1H, E_2 –O*H*), 3.94–4.12 (m, 2H, –OCH_2–), 3.72 (t, 1H, *J* = 8.1 Hz, E_2 –C*H*–OH), 3.56 (t, 2H, *J* = 5.6 Hz, –C*H*2Br), 3.36–3.44 (m, 8H, –N(–CH_2–CH_2–)$_2$N–), 2.77–2.89 (m, 2H, E_2 –C*H* 2–), 2.40–2.66 (m, 4H, 2× –CH_2N–), 1.73–2.36 (m, 7H, 2× –C*H* 2–, E_2 3× –C*H*–), 1.13–1.71 (m, 10H, E_2 5× –C*H* 2–), 0.77 (s, 3H, E_2 –CH3); MS (ESI): *m/z* 520 $(M+1)^+$.

4.2.8. 3-{5-(4-(5-Bromopentyl)-piperazin-1-yl)-pentyloxy}-17-hydroxy-1,3,5-estra trien (9b)

The compound **9b** was prepared following the method described for the preparation of the compound **9a**, employing compound **8b** (426 mg, 1 mmol) and 1,5-dibromopentane (0.3 mL, 3 mmol), and the crude product was purified by column chromatography using ethyl acetate–hexane (64%) to afford the compound **9b** as a light yellow oil. Yield 447 mg, 78%; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.19 (d, 1H, *J* = 8.3 Hz, Ar*H*), 6.71 (dd, 1H, *J* = 2.3, 8.3 Hz, Ar*H*), 6.60 (s, 1H, Ar*H*), 5.34 (br s, 1H, E_2 –O*H*), 3.90–4.00 (t, 2H, 2× –OCH_2–), 3.73 (t, 1H, *J* = 8.3 Hz, E_2 –C*H*–OH), 3.57 (t, 2H, *J* = 5.9 Hz, –C*H*2Br), 3.40–3.47 (m, 8H, –N(–CH_2–CH_2–)$_2$N–), 2.76–2.90 (m, 2H, E_2 –C*H* 2–), 2.45–2.66 (m, 4H, 2× –CH_2N–), 2.38–2.41 (m, 3H, E_2 3× –C*H*–), 1.80–2.30 (m, 12H, 6× –C*H*2 –), 1.12–1.78 (m, 10H, E_2 5× –C*H* 2–), 0.78 (s, 3H, E_2 –CH3); MS (ESI); *m/z* 576 $(M+1)^+$.

4.2.9. 3-(3-Azidopropyloxy)-17-hydroxy-1,3,5-estratrien (10a)

Azidation reaction, compound **6a** (393 mg, 1 mmol) in dry DMF (3 mL), and NaN_3 (265 mg, 4 mmol) was added and the mixture was heated at 60–70 °C for 6–8 h, in this transformation bromo substituted with azide, Later this reaction mixture was poured into ice-cold water and extracted with ethyl acetate (3 × 40 mL), the organic layer was washed with brine (2 × 20 mL), and dried over anhydrous Na_2SO_4, solvent was evaporated under reduced pressure to get the crude product. This was further purified by column chromatography using ethyl acetate–hexane (18%) to afford the compound **10a** as a white solid. Yield 324 mg, 90%; mp 60–62 °C; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.19 (d, 1H, *J* = 8.3 Hz, Ar*H*), 6.69 (dd, 1H, *J* = 2.3, 8.3 Hz, Ar*H*), 6.63 (s, 1H, Ar*H*), 5.34 (br s, 1H, E_2 –O*H*), 4.00 (t, 2H, *J* = 5.4 Hz, –OCH2–), 3.72 (t, 1H, *J* = 8.5 Hz, E_2 –C*H*–OH), 3.41 (t, 2H, *J* = 6.7 Hz, –C*H*2N_3), 2.77–2.89 (m, 2H, E_2 –C*H* 2–), 2.25–2.38 (m, 5H, –C*H* 2–, E_2 3× –C*H*–), 1.14–2.10 (m, 10H, E_2 5× –C*H*2–), 0.78 (s, 3H, E_2 –CH3); MS (ESI): *m/z* 356 $(M+H)^+$.

4.2.10. 3-(5-Azidopentyloxy)-17-hydroxy-1,3,5-estratrien (10b)

The compound **10b** was prepared following the method described for the preparation of the compound **10a**, employing **6b** (421 mg, 1 mmol) and NaN_3 (265 mg, 4 mmol), and the crude

product was purified by column chromatography using ethyl acetate–hexane (20%) to afford the compound **10b** as a white solid. Yield 352 mg, 92%; mp 69–71 °C; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.18 (d, 1H, J = 9.2 Hz, Ar*H*), 6.70 (dd, 1H, J = 3.0, 7.9 Hz, Ar*H*), 6.62 (s, 1H, Ar*H*), 5.32 (br s, 1H, E_2 –O*H*), 3.96 (t, 2H, J = 7.1 Hz, –OCH_2–), 3.71 (t, 1H, J = 8.2 Hz, E_2 –C*H*–OH), 3.42 (t, 2H, J = 7.2 Hz, –CH_2N_3), 2.79–2.90 (m, 2H, E_2 –C*H*2–), 2.06–2.36 (m, 3H, E_2 3× –C*H*–), 1.83–1.96 (m, 4H, 2× –C*H*2–), 1.74–1.80 (m, 2H, –C*H*2–), 1.14–1.72 (m, 10H, E_2 5× –C*H* 2–), 0.78 (s, 3H, E_2 –CH3); MS (ESI): *m/z* 384 $(M+H)^+$.

4.2.11. (2*S*)-*N*-[5-Methoxy-2-nitrobenzoyl-4-(2-propynyloxy]-pyrrolidine-2-carboxaldehyde diethylthioacetal (12a)

To a solution of (2*S*)-*N*-[4-hydroxy-5-methoxy-2-nitrobenzoyl] pyrrolidine-2-carbox aldehyde diethylthioacetal **11a** (400 mg, 1 mmol) in dry acetone (10 mL), anhydrous K_2CO_3 (552 mg, 4 mmol) and the propargyl bromide (302 mg, 2 mmol) were added to the reaction mixture. The reaction mixture was heated to reflux for 4–6 h. After completion of the reaction as indicated by TLC, potassium carbonate was removed by suction filtration and the solvent was removed under vacuum. The crude product thus obtained was purified by column chromatography using ethyl acetate–hexane (35%) to afford the pure compound **12a** as a light yellow solid. Yield 395 mg, 90%; mp 84–86 °C; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.88 (s, 1H, Ar*H*), 6.87 (s, 1H, Ar*H*), 4.88 (d, 2H, J = 3.9 Hz, –OCH_2–), 4.86 (d, 1H, J = 2.3 Hz, –C*H*S_2), 4.68–4.76 (m, 1H, pro –NC*H*–), 3.97 (s, 3H, –OCH_3), 3.18–3.35 (m, 2H, pro –NCH_2–), 2.67–2.89 (m, 4H, 2× –SCH_2–), 2.50–2.62 (m, 1H, ≡C*H*), 1.93–2.32 (m, 4H, pro 2× –CH_2–), 1.20–1.41 (m, 6H, –CH_{32}); MS (ESI): *m/z* 461 $(M+Na)^+$.

4.2.12. (2*S*,4*R*)-*N*-[5-Methoxy-2-nitrobenzoyl-4-(2-propynyl-oxy]-4-fluoropyrrolidine-2-carboxaldehyde diethylthioacetal (12b)

This compound was prepared according to the method described for compound **12a**, employing compound (2*S*,4*R*)-*N*-[4-hydroxy-5-methoxy-2-nitrobenzoyl]-4-fluoropyrroli dine-2-carboxaldehyde diethylthioacetal **11b** (418 mg, 1 mmol) and propargyl bromide (305 mg, 2 mmol). The crude product was purified by column chromatography using ethyl acetate–hexane (38%) to afford the compound **12b** as a light yellow solid. Yield 374 mg, 82%; mp 93–95 °C; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.86 (s, 1H, Ar*H*), 6.92 (s, 1H, Ar*H*), 4.86 (d, 2H, J = 3.3 Hz, –OCH_2–),4.72–4.83 (m, 1H, pro –NC*H*–), 4.62 (d, 2H, J = 2.2 Hz, –C*H*S_2), 3.99 (s, 3H, –OCH_3), 3.95–3.97 (m, 1H, pro –C*H*F–), 3.42–3.70 (m, 2H, pro –NCH_2–), 2.68–2.90 (m, 4H, 2× –SCH_2–), 2.43–2.66 (m, 3H, ≡C*H*, pro –CH_2–), 1.20–1.39 (m, 6H, –CH_{32}); MS (ESI): *m/z* 479 $(M+Na)^+$.

4.2.13. (2*S*)-*N*-[5-Methoxy-2-nitrobenzoyl-4-(2-propynyloxy]-4,4difluoropyrro-lidine-2-carboxaldehyde diethylthioacetal (12c)

This compound was prepared according to the method described for compound **12a**, employing compound (2*S*)-*N*-[4-hydroxy-5-methoxy-2-nitrobenzoyl]-4,4-difluoropyrroli dine-2-carboxaldehyde diethylthioacetal **11c** (435 mg, 1 mmol) and propargyl bromide (305 mg, 2 mmol). The crude product was purified by column chromatography using ethyl acetate–hexane (40%) to afford the compound **12c** as a light yellow solid. Yield 388 mg, 85%; mp 97–99 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.88 (s, 1H, Ar*H*), 6.82 (s, 1H, Ar*H*), 4.87–4.95 (m, 1H, pro –NC*H*–), 4.84 (d, 2H, J = 2.3 Hz, –OCH_2–), 4.78 (d, 1H, J = 3.0 Hz, –C*H*S_2), 3.97 (s, 3H, –OCH_3), 3.42–3.80 (m, 2H, pro –NCH_2–), 2.70–2.92 (m, 4H, 2× –SCH_2–), 2.40–2.69 (m, 3H, ≡C*H*, pro –CH_2–), 1.31–1.42 (m, 6H, –CH_{32}); MS (ESI): *m/z* 475 $(M+H)^+$.

4.2.14. (2*S*)-*N*-[4-{3-(17-Hydroxy-1,3,5-estratrien-3-yl)-oxy}-propyloxy)-5-methoxy-2-nitrobenzoyl]-pyrrolidine-2-carboxaldehyde diethylthioacetal (13a)

To a solution of (2*S*)-*N*-[4-hydroxy-5-methoxy-2-nitrobenzoyl]-pyrrolidine-2-carboxaldehyde diethylthioacetal **11a** (400 mg, 1 mmol) in acetone (20 mL) anhydrous K_2CO_3 (552 mg, 4 mmol) and the compound **6a** (393 mg, 1 mmol) were added. The reaction mixture was heated to reflux for 10 h. After completion of the reaction as indicated by TLC, potassium carbonate was removed by suction filtration and the solvent was removed under vacuum. The crude product was purified by column chromatography using ethyl acetate–hexane (60%) to afford the pure compound **13a** as a light yellow solid. Yield 613 mg, 86%; mp 90–92 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.72 (s, 1H, Ar*H*), 7.20 (d, 1H, J = 9.4 Hz, Ar*H*), 6.81 (s, 1H, Ar*H*), 6.72 (dd, 1H, J = 3.1, 9.4 Hz, Ar*H*), 6.65 (s, 1H, Ar*H*), 5.35 (br s, 1H, E_2 –O*H*), 4.88 (d, 1H, J = 3.9 Hz, –C*H*S_2), 4.66–4.76 (m, 1H, pro –NC*H*–), 4.29 (t, 2H, J = 6.2 Hz, –OCH_2–), 4.15 (t, 2H, J = 5.5 Hz, –OCH_2–), 3.93 (s, 3H, –OCH_3), 3.72 (t, 1H, J = 8.6 Hz, E_2 –CH–OH), 3.18–3.29 (m, 2H, pro –NCH_2–), 2.69–2.90 (m, 6H, 2× –SCH_2–, E_2 –CH_2–), 2.14–2.41 (m, 3H, E_2 3× –CH–), 1.53–2.10 (m, 6H, pro 2× –CH2–, –CH_2–), 1.22–1.48 (m, 16H, E_2 5× –CH_2–, –CH_{32}), 0.78 (s, 3H, E_2 –CH_3); MS (ESI): *m/z* 713 $(M+H)^+$.

4.2.15. (2*S*)-*N*-[4-{3-(17-Hydroxy-1,3,5-estratrien-3-yl)-oxy}-pentyloxy)-5-methoxy-2-nitrobenzoyl]-pyrrolidine-2-carboxaldehyde diethylthioacetal (13b)

This compound was prepared according to the method described for compound **13a**, employing compound **11a** (400 mg, 1 mmol) and compound **6b** (423 mg, 1 mmol). The crude product was purified by column chromatography using ethyl acetate–hexane (65%) to afford the compound **13b** as a light yellow solid. Yield 667 mg, 90%; mp 86–88 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.67 (s, 1H, Ar*H*), 7.19 (d, 1H, J = 8.3 Hz, Ar*H*), 6.82 (s, 1H, Ar*H*), 6.69 (dd, 1H, J = 2.9, 8.3 Hz, Ar*H*), 6.63 (s, 1H, Ar*H*), 5.34 (br s, 1H, E_2 –O*H*), 4.88 (d, 1H, J = 3.8 Hz, –C*H*S_2), 4.68–4.76 (m, 1H, pro –NC*H*–), 4.12 (t, 2H, J = 5.3 Hz, –OCH_2–), 3.97 (t, 2H, J = 6.8 Hz, –OCH_2–), 3.94 (s, 3H, –OCH_3), 3.73 (t, 1H, J = 8.3 Hz, E_2 –CH–OH), 3.19–3.33 (m, 2H, pro –NCH_2–), 2.64–2.89 (m, 6H, 2× –SCH_2–, E_2 –CH_2–), 2.03–2.40 (m, 7H, E_2 3× –CH–, 2× –CH_2–), 1.78–2.01 (m, 6H, pro 2× –CH_2–, –CH_2–), 1.25–1.76 (m, 16H, E_2 5× –CH_2–, –CH_{32}), 0.78 (s, 3H, E_2 –CH_3); MS (ESI): *m/z* 741 $(M+H)^+$.

4.2.16. (2*S*,4*R*)-*N*-[4-{3-(17-Hydroxy-1,3,5-estratrien-3-yl)-oxy}-propyloxy)-5-methoxy-2-nitrobenzoyl]-4-fluoropyrrolidine-2-carboxaldehyde diethylthioacetal (13c)

This compound was prepared according to the method described for compound **13a**, employing compound (2*S*,4*R*)-*N*-[4-hydroxy-5-methoxy-2-nitrobenzoyl]-4-fluoropyrrolidine-2-carboxaldehyde diethylthioacetal **11b** (418 mg, 1 mmol) and compound **6a** (393 mg, 1 mmol). The crude product was purified by column chromatography using ethyl acetate–hexane (65%) to afford the compound **13c** as a light yellow solid. Yield 576 mg, 79%; mp 100–102 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.71 (s, 1H, Ar*H*), 7.20 (d, 1H, J = 9.2 Hz, Ar*H*), 6.79 (s, 1H, Ar*H*), 6.70 (dd, 1H, J = 3.1, 9.2 Hz, Ar*H*), 6.64 (s, 1H, Ar*H*), 5.35 (br s, 1H, E_2 –O*H*), 4.84 (d, 1H, J = 3.7 Hz, –C*H*S_2), 4.67–4.77 (m, 1H, pro –NC*H*–), 4.27 (t, 2H, J = 6.3 Hz, –OCH_2–), 4.13 (t, 2H, J = 5.5 Hz, –OCH_2–), 3.95 (s, 3H, –OCH_3), 3.69–3.77 (m, 1H, E_2 –CH–OH), 3.17–3.29 (m, 3H, pro –CHF–, pro –NCH_2–), 2.67–2.91 (m, 6H, 2× –SCH_2–, E_2 –CH_2–), 2.16–2.39 (m, 3H, E_2 3× –CH–), 1.81–2.11 (m, 4H, pro –CH_2–, –CH_2–), 1.19–1.70 (m, 16H, E_2 5× –CH_2–, –CH_{32}), 0.78 (s, 3H, E_2 –CH_3); MS (ESI): *m/z* 731 $(M+H)^+$.

4.2.17. (2*S*,4*R*)-*N*-[4-{3-(17-Hydroxy-1,3,5-estratrien-3-yl)-oxy)-pentyloxy)-5-methoxy-2-nitrobenzoyl]-4-fluoropyrrolidine-2-carboxaldehyde diethylthioacetal (13d)

This compound was prepared according to the method described for compound **13a**, employing compound **11b** (418 mg, 1 mmol) and compound **6b** (423 mg, 1 mmol). The crude product was purified by column chromatography using ethyl acetate–hexane (66%) to afford the compound **13d** as a light yellow solid. Yield 622 mg, 82%; mp 99–101 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.66 (s, 1H, Ar*H*), 7.20 (d, 1H, *J* = 9.1 Hz, Ar*H*), 6.89 (s, 1H, Ar*H*), 6.70 (dd, 1H, *J* = 3.2, 9.1 Hz, Ar*H*), 6.63 (s, 1H, Ar*H*), 5.34 (br s, 1H, E_2 –O*H*), 4.76–4.84 (m, 1H, pro –NC*H*–), 4.61 (d, 1H, *J* = 6.8 Hz, –C*HS*$_2$), 4.08–4.46 (m, 2H, –OCH_2–), 3.97 (s, 3H, –OCH_3), 3.92–3.96 (m, 2H, –OCH_2–), 3.73 (t, 1H, *J* = 8.3, E_2 –CH–OH), 3.44–3.66 (m, 1H, pro –C*H*F–), 2.69–2.89 (m, 6H, 2× –SCH_2–, pro –NCH_2–), 2.47–2.66 (m, 2H, E_2 –CH_2–), 2.05–2.36 (m, 5H, E_2 3× –CH–, pro –CH_2–), 1.79–2.01 (m, 6H, 3× –CH_2–), 1.14–1.72 (m, 16H, E_2 5× –CH_2–, –CH_{32}), 0.77 (s, 3H, E_2 –CH_3); MS (ESI): *m/z* 759 (M+H)$^+$.

4.2.18. (2*S*)-*N*-[4-{3-(17-Hydroxy-1,3,5-estratrien-3-yl)-oxy}-propyloxy)-5-methoxy-2-nitrobenzoyl]-4,4-difluoro-pyrrolidine-2-carboxaldehyde diethylthioacetal (13e)

This compound was prepared according to the method described for compound **13a**, employing compound (2*S*)-*N*-[4-hydroxy-5-methoxy-2-nitrobenzoyl]-4,4-difluoropyrrolidine-2-carboxaldehyde diethylthioacetal **11c** (436 mg, 1 mmol) and compound **6a** (393 mg, 1 mmol). The crude product was purified by column chromatography using ethyl acetate–hexane (66%) to afford the compound **13e** as a light yellow solid. Yield 584 mg, 78%; mp 104–106 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.73 (s, 1H, Ar*H*), 7.20 (d, 1H, *J* = 8.3 Hz, Ar*H*), 6.77 (s, 1H, Ar*H*), 6.71 (dd, 1H, *J* = 2.6, 8.3, Hz, Ar*H*), 6.65 (s, 1H, Ar*H*), 5.32 (br s, 1H, E_2 –O*H*), 4.91–4.95 (m, 1H, pro –NC*H*–), 4.83 (d, 1H, *J* = 3.8 Hz, –C*HS*$_2$), 4.30 (t, 2H, *J* = 6.0 Hz, –OCH_2–), 4.15 (t, 2H, *J* = 6.0 Hz, –OCH_2–), 3.93 (s, 3H, –OCH_3), 3.66–3.80 (m, 2H, pro –NCH_2–), 3.64 (t, 1H, *J* = 13.0 Hz, E_2 –CH–OH), 2.62–2.92 (m, 6H, 2× –SCH_2–, E_2 –CH_2–), 2.08–2.38 (m, 3H, E_2 3× –CH–), 1.81–2.03 (m, 4H, pro –CH_2–, –CH_2–), 1.15–1.74 (m, 16H, E_2 5×–CH_2–, –CH_{32}), 0.77 (s, 3H, E_2 –CH_3); MS (ESI): *m/z* 749 (M+H)$^+$.

4.2.19. (2*S*)-*N*-[4-{3-(17-Hydroxy-1,3,5-estratrien-3-yl)-oxy}-pentyloxy)-5-methoxy-2-nitrobenzoyl]-4,4-difluoropyrrolidine-2-carboxaldehyde diethylthioacetal (13f)

This compound was prepared according to the method described for compound **13a**, employing compound **11c** (436 mg, 1 mmol) and compound **6b** (423 mg, 1 mmol). The crude product was purified by column chromatography using ethyl acetate–hexane (70%) to afford the compound **13f** as a light yellow solid. Yield 687 mg, 86%; mp 91–93 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.63 (s, 1H, Ar*H*), 7.11 (d, 1H, *J* = 8.2 Hz, Ar*H*), 6.72 (s, 1H, Ar*H*), 6.61 (dd, 1H, *J* = 3.0, 8.2 Hz, Ar*H*), 5.32 (br s, 1H, E_2 –O*H*), 4.86–4.91 (m, 1H, pro –NC*H*–), 4.78 (d, 1H, *J* = 3.8 Hz, –C*HS*$_2$), 4.11 (t, 2H, *J* = 6.2 Hz, –OCH_2–), 3.94–3.96 (m, 2H, –OCH_2–), 3.93 (s, 3H, –OCH_3), 3.65–3.86 (m, 2H, pro –NCH_2–), 3.61 (t, 1H, *J* = 13.4 Hz, E_2 –CH–OH), 2.53–2.93 (m, 6H, 2× –SCH_2–, E_2 –CH_2–), 2.13–2.25 (m, 3H, E_2 3× –CH–), 1.75–2.10 (m, 8H, pro –CH_2–, 3× –CH_2–), 1.16–1.71 (m, 16H, E_2 5× –CH_2–, –CH_{32}), 0.77 (s, 3H, E_2 –CH_3); MS (ESI): *m/z* 799 (M+ Na)$^+$.

4.2.20. (2*S*)-*N*-[4-{3-(17-Hydroxy-1,3,5-estratrien-3-yl)oxypropyl}-piperazino)-propyloxy)-5-methoxy-2-nitrobenzoyl]-pyrrolidine-2-carboxaldehyde diethylthioacetal (14a)

To a solution of compound **11a** (400 mg, 1 mmol) in acetonitrile (20 mL) anhydrous K_2CO_3 (552 mg, 4 mmol) and the compound **9a** (520 mg, 1 mmol) were added. The reaction mixture was heated to reflux for 30 h. After completion of the reaction as indicated by TLC, potassium carbonate was removed by suction filtration and the solvent was removed under vacuum. The crude product was purified by column chromatography using ethyl acetate–hexane (80%) to afford the pure compound **14a** as a light yellow solid. Yield 672 mg, 80%; mp 94–96 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.70 (s, 1H, Ar*H*), 7.20 (d, 1H, *J* = 8.8 Hz, Ar*H*), 6.83 (s, 1H, Ar*H*), 6.67 (dd, 1H, *J* = 2.2, 8.8 Hz, Ar*H*), 6.61 (s, 1H, Ar*H*), 5.34 (br s, 1H, E_2 –O*H*), 4.88 (d, 1H, *J* = 3.7 Hz, –C*HS*$_2$), 4.67–4.76 (m, 1H, pro – NC*H*–), 4.16 (t, 2H, *J* = 6.6, Hz, –OCH_2–), 3.99 (t, 2H, *J* = 5.9, –OCH_2–), 3.94 (s, 3H, –OCH_3), 3.73 (t, 1H *J* = 8.8, E_2 –CH–OH), 3.21–3.40 (m, 2H, pro –NCH_2–) 2.80–3.00 (m, 12H, –N(–CH_2–CH_2–)$_2$N–, 2× –NCH_2–), 2.53–2.78 (m, 6H, 2× –SCH_2–, E_2 –CH_2–), 1.75–2.41 (m, 11H, E_2 3× –CH–, pro 2× –CH2–, 2× –CH_2–), 1.15–1.69 (m, 16H, E_2 5× –CH_2–, –CH_{32}), 0.78 (s, 3H, E_2 –CH_3); MS (ESI): *m/z* 840 (M+H)$^+$.

4.2.21. (2*S*)-*N*-[4-{3-(17-Hydroxy-1,3,5-estratrien-3-yl)-oxy-pentyl}-piperazino)-pentyloxy)-5-methoxy-2-nitrobenzoyl]-pyrrolidine-2-carboxaldehyde diethylthioacetal (14b)

This compound was prepared according to the method described for compound **14a**, employing compound **11a** (400 mg, 1 mmol) and compound **9b** (576 mg, 1 mmol). The crude product was purified by column chromatography using ethyl acetate–hexane (85%) to afford the compound **14b** as a light yellow solid. Yield 736 mg, 85%; mp 97–99 °C; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.67 (s, 1H, Ar*H*), 7.19 (d, 1H, *J* = 8.8 Hz, Ar*H*), 6.82 (s, 1H, Ar*H*), 6.69 (dd, 1H, *J* = 2.3, 8.8, Hz, Ar*H*), 6.61 (s, 1H, Ar*H*), 5.35 (br s, 1H, E_2 –O*H*), 4.88 (d, 1H, *J* = 2.9 Hz, –C*HS*$_2$), 4.69–4.73 (m, 1H, pro –NC*H*–), 4.09–4.14 (m, 4H, 2× –OCH_2–), 3.95 (s, 3H, –OCH_3), 3.68–3.74 (m, 1H, E_2 –C*H*–OH), 3.21–3.32 (m, 2H, pro –NCH_2–), 2.68–2.89 (m, 12H, –N(–CH_2–CH_2–)$_2$N–, –NCH_2–), 2.38–2.60 (m, 2H, E_2 –CH_2), 2.06–2.36 (m, 7H, 2× –SCH_2, E_2 3× –CH–), 2.06–2.23 (m, 4H, pro 2× –CH_2), 1.88–2.02 (m, 6H, 3× –CH_2), 1.75–1.85 (m, 6H, 3× –CH_2), 1.31–1.70 (m, 16H, E_2 5× –CH_2–, –CH_{32}), 0.78 (s, 3H, E_2 –CH_3); MS (ESI): *m/z* 896 (M+H)$^+$.

4.2.22. (2*S*,4*R*)-*N*-[4-{3-(17-Hydroxy-1,3,5-estratrien-3-yl)-oxypropyl}-piperazino)-propyloxy)-5-methoxy-2-nitrobenzoyl]-4-fluoropyrrolidine-2-carboxaldehyde diethyl thioacetal (14c)

This compound was prepared according to the method described for compound **14a**, employing compound **11b** (418 mg, 1 mmol) and compound **9a** (520 mg, 1 mmol). The crude product was purified by column chromatography using ethyl acetate–hexane (90%) to afford the compound **14c** as a light yellow solid. Yield 643 mg, 75%; mp 89–91 °C; mp 114–116 °C; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.66 (s, 1H, Ar*H*), 7.22 (d, 1H, *J* = 8.4 Hz, Ar*H*), 6.86 (s, 1H, Ar*H*), 6.76 (dd, 1H, *J* = 2.2, 8.4 Hz, Ar*H*), 6.62 (s, 1H, Ar*H*), 5.33 (br s, 1H, E_2 –O*H*), 4.71–4.77 (m, 1H, pro –NC*H*–), 4.54 (d, 1H, *J* = 6.7 Hz, –C*HS*$_2$), 4.26 (t, 2H, *J* = 6.0, Hz, –OCH_2–), 4.00 (t, 2H, *J* = 6.6, Hz, –OCH_2–), 3.97 (s, 3H, –OCH_3), 3.84–3.88 (m, 2H *J* = 5.7 Hz, E_2 –C*H*–OH, pro –C*H*F–), 3.40–3.64 (m, 2H, pro –NCH_2–), 2.70–3.21 (m, 12H, –N(–CH_2–CH_2–)$_2$N–, 2× –NCH_2–), 2.68–2.89 (m, 6H, 2× –SCH_2–, E_2 –CH_2–), 2.42–2.66 (m, 9H, E_2 3× –CH–, 2× –CH2–, pro –CH_2–), 1.14–1.78 (m, 16H, E_2 5× –CH_2–, –CH_{32}), 0.78 (s, 3H, E_2 –CH_3); MS (ESI): *m/z* 857 (M+H)$^+$.

4.2.23. (2*S*,4*R*)-*N*-[4-{3-(17-Hydroxy-1,3,5-estratrien-3-yl)-oxypentyl}-piperazino)-pentyloxy)-5-methoxy-2-nitrobenzoyl]-4-fluoropyrrolidine-2-carboxaldehyde diethyl thioacetal (14d)

This compound was prepared according to the method described for compound **14a**, employing compound **11b** (418 mg, 1 mmol) and compound **9b** (576 mg, 1 mmol). The crude product was purified by column chromatography using ethyl acetate–hexane (90%) to afford the compound **14d** as a yellow solid. Yield 618 mg, 70%; mp 100–102 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.68 (s, 1H,

Ar*H*), 7.20 (d, 1H, *J* = 8.2 Hz, Ar*H*), 6.84 (s, 1H, Ar*H*), 6.73 (dd, 1H, *J* = 2.3, 8.2 Hz, Ar*H*), 6.61 (s, 1H, Ar*H*), 5.34 (br s, 1H, E_2 –O*H*), 4.86 (d, 1H, *J* = 6.7 Hz, –C*H*S_2), 4.70–4.72 (m, 1H, pro –NC*H*–), 4.08–4.15 (m, 4H, 2× –OCH_2–), 3.96 (s, 3H, –OCH_3), 3.68–3.73 (m, 2H, pro –NCH_2–), 3.20–3.31 (m, 2H, E_2 –C*H*–OH, pro –C*H*F–), 2.70–2.92 (m, 12H, –N(–CH_2–CH_2–)$_2$N–, 2× –NCH_2–), 2.38–2.60 (m, 2H, E_2 –CH_2–), 2.06–2.36 (m, 7H, 2× –SCH_2, E_2 3× –CH–), 1.88–2.23 (m, 10H, 4× –CH_2, pro –CH_2), 1.76–1.84 (m, 4H, 2× –CH_2), 1.16–1.71 (m, 16H, E_2 5× –CH$_2$–, –CH_{32}), 0.77 (s, 3H, E_2 –CH_3); MS (ESI): *m/z* 913 $(M+H)^+$.

4.2.24. (2*S*)-*N*-[4-{3-(17-Hydroxy-1,3,5-estratrien-3-yl)-oxypropyl}-piperazino)-propyloxy)-5-methoxy-2-nitrobenzoyl]-4,4-difluoropyrrolidine-2-carboxaldehyde diethyl thioacetal (14e)

This compound was prepared according to the method described for compound **14a**, employing compound **11c** (436 mg, 1 mmol) and compound **9a** (520 mg, 1 mmol). The crude product was purified by column chromatography using ethyl acetate–hexane (92%) to afford the compound **14e** as a light yellow solid. Yield 683 mg, 78%; mp 90–92 °C; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.72 (s, 1H, Ar*H*), 7.19 (d, 1H, *J* = 8.8 Hz, Ar*H*), 6.83 (s, 1H, Ar*H*), 6.69 (dd, 1H, *J* = 2.2, 8.8 Hz, Ar*H*), 6.61 (s, 1H, Ar*H*), 5.32 (br s, 1H, E_2 –O*H*), 4.86 (d, 1H, *J* = 4.0 Hz, –C*H*S_2), 4.66–4.77 (m, 1H, pro –NC*H*–), 4.17 (t, 2H, *J* = 6.6, Hz, –OCH_2–), 3.98 (t, 2H, *J* = 5.9, Hz, –OCH_2–), 3.93 (s, 3H, –OCH_3), 3.72 (t, 1H *J* = 8.8, E_2 –CH–OH), 3.21–3.40 (m, 2H, pro –NCH_2–) 2.82–3.10 (m, 12H, –N(–CH_2–CH_2–)$_2$N–, 2× –NCH_2–), 2.54–2.79 (m, 6H, 2× –SCH_2–, E_2 –CH_2–), 1.60–2.51 (m, 9H, E_2 3× –CH–, pro –CH$_2$–, 2× –CH2–), 1.12–1.55 (m, 16H, E_2 5× –CH$_2$–, –CH_{32}), 0.78 (s, 3H, E_2 –CH_3); MS (ESI): *m/z* 876 $(M+H)^+$.

4.2.25. (2*S*)-*N*-[4-{3-(17-Hydroxy-1,3,5-estratrien-3-yl)-oxypentyl}-piperazino)-pentyloxy)-5-methoxy-2-nitrobenzoyl]-4,4-difluoropyrrolidine-2-carboxaldehyde diethyl thioacetal (14f)

This compound was prepared according to the method described for compound **14a**, employing compound **11c** (436 mg, 1 mmol) and compound **9b** (576 mg, 1 mmol). The crude product was purified by column chromatography using ethyl acetate–hexane (95%) to afford the compound **14f** as a yellow solid. Yield 659 mg, 73%; mp 102–103 °C; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.69 (s, 1H, Ar*H*), 7.19 (d, 1H, *J* = 8.7 Hz, Ar*H*), 6.81 (s, 1H, Ar*H*), 6.70 (dd, 1H, *J* = 3.1, 8.7 Hz, Ar*H*), 6.62 (s, 1H, Ar*H*), 5.34 (br s, 1H, E_2 –O*H*), 4.87 (d, 1H, *J* = 3.9 Hz, –C*H*S_2), 4.70–4.74 (m, 1H, pro –NC*H*–), 4.08–4.15 (m, 4H, 2× –OCH_2–), 3.97 (s, 3H, –OCH_3), 3.70–3.77 (m, 3H, pro –NCH_2–, E_2 –CH–OH), 2.77–3.50 (m, 12H, –N(–CH_2–CH_2–)$_2$N–, 2× –NCH_2–), 2.40–2.72 (m, 6H, 2× –SCH_2–, E_2 –CH_2–), 2.37–2.61 (m, 3H, E_2 3× –CH–), 1.89–2.00 (m, 10H, 4× –CH_2– pro –CH$_2$–), 1.76–1.86 (m, 4H, 2× –CH_2), 1.32–1.72 (m, 16H, E_2 5× –CH$_2$–, –CH_{32}), 0.78 (s, 3H, E_2 –CH_3); MS (ESI): *m/z* 932 $(M+H)^+$.

4.2.26. (2*S*)-*N*-[4-{3-(17-Hydroxy-1,3,5-estratrien-3-yl}oxypropyl)-1*H*-1,2,3-triazol-4-yl]-methoxy-5-methoxy-2-nitrobenzoyl]-pyrrolidine-2-carboxaldehyde diethylthio acetal (15a)

To a solution of nitro diethylthioacetal **12a**, (439 mg 1 mmol) and compound **10a** (356 mg, 1 mmol) were dissolved in *t*-BuOH, H_2O 1:1 ratio (20 mL). Freshly prepared sodium ascorbate solution (19.01 mg, 5 mol %) was added followed by $CuSO_4{\cdot}5H_2O$ (1 mol %) the heterogeneous mixture was stirred at room temperature for 12 h, after completion of reaction, 2 mL of 3% ammonia solution was added to the reaction mixture for quenching of excess $CuSO_4{\cdot}5H_2O$ and stirred for further 10 min, *t*-BuOH was removed under reduced pressure, the aqueous layer was diluted with ethyl acetate (30 mL) stirred for another 10 min and then filtered through a Celite bed. The combined organic layer were washed with brine (2 × 30 mL), dried over anhydrous Na_2SO_4, and solvent was evaporated under reduced pressure to get the crude product. This was further purified by column chromatography using ethyl acetate–hexane (65%) to afford the compound **15a** as a yellow solid. Yield 718 mg, 88%; mp 103–105 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.90 (s, 1H, triazole –*H*), 7.69 (s, 1H, Ar*H*), 7.21 (d, 1H, *J* = 9.1 Hz, Ar*H*), 6.84 (s, 1H, Ar*H*), 6.62 (dd, 1H, *J* = 3.0, 9.1 Hz, Ar*H*), 6.61 (s, 1H, Ar*H*), 5.34 (br s, 1H, E_2 –O*H*), 5.31 (s, 2H, –OCH_2–), 4.88 (d, 1H, *J* = 3.77 Hz, –C*H*S_2), 4.68–4.74 (m, 1H, pro –NC*H*–), 4.49 (t, 2H, *J* = 7.5 Hz, –OCH_2–), 3.93 (s, 3H, –OCH_3), 3.74 (t, 1H, *J* = 8.3 Hz, E_2 –CH–OH), 3.40 (t, 2H, *J* = 5.7 Hz, –CH_2N–), 3.18–3.33 (m, 2H, pro –CH_2N–), 2.66–2.90 (m, 2H, E_2 –CH_2–), 2.24–2.48 (m, 4H, 2× –SCH_2–), 2.02–2.22 (m, 3H, E_2 3× –CH–), 1.70–2.00 (m, 6H, pro 2× –CH$_2$–, –CH$_2$–), 1.12–1.68 (m, 16H, E_2 5× –CH$_2$–, –CH_{32}), 0.77 (s, 3H, E_2 –CH_3); MS (ESI): *m/z* 817 $(M+Na)^+$.

4.2.27. (2*S*)-*N*-[4-{3-(17-Hydroxy-1,3,5-estratrien-3-yl}-oxypentyl)-1*H*-1,2,3-triazol-4-yl]-methoxy-5-methoxy-2-nitrobenzoyl]-pyrrolidine-2-carboxaldehyde diethylthio acetal (15b)

This compound was prepared according to the method described for compound **15a**, employing compound **12a** (439 mg, 1 mmol) and compound **10b** (384 mg, 1 mmol). The crude product was purified by column chromatography using ethyl acetate–hexane (70%) to afford the compound **15b** as a yellow solid. Yield 739 mg, 90%; mp 110–112 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.91 (s, 1H, 1H, triazole –*H*), 7.68 (s, 1H, Ar*H*), 7.19 (d, 1H, *J* = 8.5 Hz, Ar*H*), 6.85 (s, 1H, Ar*H*), 6.68 (dd, 1H, *J* = 2.3, 8.5 Hz, Ar*H*), 6.61 (s, 1H, Ar*H*), 5.34 (br s, 1H, E_2 –O*H*), 5.30 (s, 2H, –OCH_2–), 4.87 (d, 1H, *J* = 3.8 Hz, –C*H*S_2), 4.67–4.74 (m, 1H, pro –NC*H*–), 4.40 (t, 2H, *J* = 7.2 Hz, –OCH_2–), 3.94 (s, 3H, –OCH_3), 3.73 (t, 1H, *J* = 8.7 Hz, E_2 –CH–OH), 3.39 (t, 2H, *J* = 6.9 Hz, –CH_2N–), 3.20–3.30 (m, 2H, pro –CH_2N–), 2.68–2.90 (m, 2H, E_2 –CH_2–), 2.23–2.43 (m, 4H, 2× –SCH_2–), 2.05–2.21 (m, 3H, E_2 3× –CH–), 1.72–2.20 (m, 10H, pro 2× –CH$_2$–, 3× –CH$_2$–), 1.13–1.68 (m, 16H, E_2 5× –CH$_2$–, –CH_{32}), 0.77 (s, 3H, E_2 –CH_3); MS (ESI): *m/z* 822 $(M^{+\cdot})$.

4.2.28. (2*S*,4*R*)-*N*-[4-{3-(17-Hydroxy-1,3,5-estratrien-3-yl}-oxypropyl)-1*H*-1,2,3-triazol-4-yl]-methoxy-5-methoxy-2-nitrobenzoyl]-4-fluoropyrrolidine-2-carboxaldehyde diethylthioacetal (15c)

This compound was prepared according to the method described for compound **15a**, employing compound **12b** (457 mg, 1 mmol) and compound **10a** (356 mg, 1 mmol). The crude product was purified by column chromatography using ethyl acetate–hexane (75%) to afford the compound **15c** as a yellow solid. Yield 663 mg, 82%; mp 98–100 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.90 (s, 1H, triazole –*H*), 7.68 (s, 1H, Ar*H*), 7.20 (d, 1H, *J* = 8.6 Hz), 6.89 (s, 1H, Ar*H*), 6.67 (dd, 1H, *J* = 3.1, 8.6 Hz, Ar*H*), 6.61 (s, 1H, Ar*H*), 5.36 (br s, 1H, E_2 –O*H*), 5.32 (s, 2H, –OCH_2–), 4.87 (d, 1H, *J* = 3.8 Hz, –C*H*S_2), 4.55–4.66 (m, 1H, pro –NC*H*–), 4.58 (t, 2H, *J* = 7.0 Hz, –OCH_2–), 3.97 (s, 3H, –OCH_3), 3.89–3.95 (m, 2H, –CH_2N–), 3.45–3.79 (m, 2H, E_2 –CH–OH, pro –CHF–), 3.20–3.30 (m, 2H, pro –CH_2N–), 2.75–2.99 (m, 6H, E_2 –CH_2–, 2× –SCH_2–), 2.38–2.52 (m, 3H, E_2 –CH–), 1.82–2.30 (m, 4H, pro –CH$_2$–, –CH$_2$–), 1.14–1.73 (m, 16H, E_2 5× –CH$_2$–, –CH_{32}), 0.77 (s, 3H, E_2 –CH_3); MS (ESI): *m/z* 835 $(M+Na)^+$.

4.2.29. (2*S*,4*R*)-*N*-[4-{3-(17-Hydroxy-1,3,5-estratrien-3-yl}-oxypentyl)-1*H*-1,2,3-triazol-4-yl]-methoxy-5-methoxy-2-nitrobenzoyl]-4-fluoropyrrolidine-2-carboxaldehyde diethylthioacetal (15d)

This compound was prepared according to the method described for compound **15a**, employing compound **12b** (457 mg, 1 mmol) and compound **10b** (384 mg, 1 mmol). The crude product

was purified by column chromatography using ethyl acetate–hexane (83%) to afford the compound **15d** as a yellow solid. Yield 733 mg, 85%; mp 104–106 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.90 (s, 1H, triazole –*H*), 7.69 (s, 1H, Ar*H*), 7.19 (d, 1H, *J* = 8.3 Hz, Ar*H*), 6.88 (s, 1H, Ar*H*), 6.68 (dd, 1H, *J* = 3.1, 8.3 Hz, Ar*H*), 6.63 (s, 1H, Ar*H*), 5.35 (s, 1H, E_2 –O*H*), 5.30 (s, 2H, –OCH_2–), 4.88 (d, 1H, *J* = 3.0 Hz, –C*H*S_2), 4.59–4.65 (m, 1H, pro –NC*H*–), 4.57 (t, 2H, *J* = 7.0 Hz, –OCH_2–), 3.98 (s, 3H, –OCH_3), 3.87–3.96 (m, 2H, –CH_2N–), 3.44–3.80 (m, 2H, E_2 –C*H*–OH, pro –C*H*F–), 3.19–3.29 (m, 2H, pro –CH_2N–), 2.74–2.98 (m, 6H, E_2 –CH_2–, 2× –SCH_2–), 2.38–2.52 (m, 3H, E_2 3× –C*H*–), 1.78–2.35 (m, 8H, pro –CH_2–, 3× –CH_2–), 1.13–1.71 (m, 16H, E_2 5× –CH_2–, –CH_{32}), 0.78 (s, 3H, E_2 –CH_3); MS (ESI): *m/z* 862 (M+Na)$^+$.

4.2.30. (2*S*)-*N*-[4-{3-(17-Hydroxy-1,3,5-estratrien-3-yl}-oxy-propyl)-1*H*-1,2,3-triazol-4-yl]-methoxy-5-methoxy-2-nitro-benzoyl]-4,4-difluoropyrrolidine-2-carboxaldehyde diethyl-thioacetal (15e)

This compound was prepared according to the method described for compound **15a**, employing compound **12c** (475 mg, 1 mmol) and compound **10a** (356 mg, 1 mmol). The crude product was purified by column chromatography using ethyl acetate–hexane (85%) to afford the compound **15e** as a yellow solid. Yield 682 mg, 80%; mp 109–111 °C; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.87 (s, 1H, triazole –*H*), 7.67 (s, 1H, Ar*H*), 7.19 (d, 1H, *J* = 8.6 Hz, Ar*H*), 6.88 (s, 1H, Ar*H*), 6.67 (dd, 1H, *J* = 3.1, 8.6, Hz, Ar*H*), 6.61 (s, 1H, Ar*H*), 5.36 (br s, 1H, E_2 –O*H*), 5.32 (s, 2H, –OCH_2–), 4.86 (d, 1H, *J* = 3.8 Hz, –C*H*S_2), 4.54–4.65 (m, 1H, pro –NC*H*–), 4.49 (t, 2H, *J* = 7.0 Hz, –OCH_2–), 3.98 (s, 3H, –OCH_3), 3.88–3.95 (m, 2H, –CH_2N–), 3.67–3.79 (m, 1H, E_2 –C*H*–OH), 3.19–3.29 (m, 2H, pro –CH_2N–), 2.74–2.99 (m, 6H, E_2 –CH_2–, 2× –SCH_2–), 2.36–2.51 (m, 3H, E_2 3× –C*H*–), 1.77–2.10 (m, 4H, pro –CH_2–, –CH_2–), 1.15–1.72 (m, 16H, E_2 5× –CH_2–, –CH_{32}), 0.79 (s, 3H, E_2 –CH_3); MS (ESI): *m/z* 853 (M+Na)$^+$.

4.2.31. (2*S*)-*N*-[4-{3-(17-Hydroxy-1,3,5-estratrien-3-yl}oxy-pentyl)-1*H*-1,2,3-triazol-4-yl]-methoxy-5-methoxy-2-nitro-benzoyl]-4,4-difluoropyrrolidine-2-carboxaldehyde diethyl-thioacetal (15f)

This compound was prepared according to the method described for compound **15a**, employing compound **12c** (475 mg, 1 mmol) and compound **10b** (384 mg, 1 mmol). The crude product was purified by column chromatography using ethyl acetate–hexane (85%) to afford the compound **15f** as a yellow solid. Yield 712 mg, 83%; mp 119–121 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.88 (s, 1H, triazole –*H*), 7.61 (s, 1H, Ar*H*), 7.11 (d, 1H, *J* = 8.7 Hz, Ar*H*), 6.74 (s, 1H, Ar*H*), 6.59 (dd, 1H, *J* = 8.7, 2.4 Hz, Ar*H*), 6.51 (s, 1H, Ar*H*), 5.33 (br s, 1H, E_2 –O*H*), 5.30 (s, 2H, –OCH_2–), 4.84–4.89 (m, 1H, pro –NC*H*–), 4.77 (d, 1H, *J* = 2.4 Hz, –C*H*S_2), 4.40 (t, 2H, *J* = 7.2 Hz, –OCH_2–), 3.93 (s, 3H, –OCH_3), 3.89 (t, 2H, *J* = 6.0 Hz, –CH_2N–), 3.69–3.74 (m, 1H, E_2 –C*H*–OH), 3.21–3.32 (m, 2H, pro –CH_2N–), 2.47–2.94 (m, 6H, E_2 –CH_2–, 2× –SCH_2–), 2.02 –2.34 (m, 5H, E_2 3× –C*H*–, pro –CH_2–), 1.76–1.95 (m, 6H, 3× –CH_2–), 1.12–1.70 (m, 16H, E_2 5× –CH_2–, –CH_{32}), 0.77 (s, 3H, E_2 –CH_3); MS (ESI): *m/z* 859 (M+H)$^+$.

4.2.32. Synthesis of 7-methoxy-8-{3-(17-hydroxy-1,3,5-estratrien-3-yl)-oxy}-propyloxy)-(11a*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (3a)

To a solution of compound **13a** (600 mg, 0.84 mmol) in methanol (20 mL), $SnCl_2{\cdot}2H_2O$ (5 mmol) was added and refluxed for 2 h. The methanol was evaporated in vacuum and the aqueous layer was carefully adjusted to pH 7 with 10% $NaHCO_3$ solution and then extracted with ethyl acetate (3 × 30 mL). The combined organic phase was dried over anhydrous Na_2SO_4 and evaporated under vacuum to afford the amino diethylthio acetal, which due to potential stability problems preceded for the next step. A solution of amino diethylthioacetal (0.82 mmol), $HgCl_2$ (2.10 mmol) and $CaCO_3$ (2.10 mmol) in CH_3CN–H_2O (4:1) was stirred slowly at room temperature until TLC indicated complete loss of starting material (12 h). The reaction mixture was diluted with ethyl acetate (25 mL) and filtered through a Celite bed. The clear light yellow organic supernatant was washed with saturated 5% $NaHCO_3$ (20 mL) and brine (20 mL), and the combined organic phase was dried over anhydrous Na_2SO_4. The organic layer was evaporated in vacuum and purified by column chromatography using MeOH/$CHCl_3$ (1.2%) to give the final product **3a** as a white solid. Yield 318 mg, 68%; mp 88–90 °C; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.66 (d, 1H, *J* = 4.40 Hz, imine –*H*), 7.51 (s, 1H, Ar*H*), 7.19 (d, 1H, *J* = 8.1 Hz, Ar*H*), 6.83 (s, 1H, Ar*H*), 6.70 (dd, 1H, *J* = 2.2, 8.1 Hz, Ar*H*), 6.63 (s, 1H, Ar*H*), 5.32 (br s, 1H, E_2 –O*H*), 4.26 (t, 2H, *J* = 4.1 Hz, –OCH_2–), 4.14 (t, 2H, *J* = 5.1 Hz, –OCH_2–), 3.93 (s, 3H, –OCH_3), 3.53–3.86 (m, 3H, pro –NCH_2–, –NC*H*–), 2.96–3.00 (m, 1H, E_2 –C*H*–OH), 2.80–2.86 (m, 2H, E_2 –CH_2–), 2.28–2.33 (m, 3H, E_2 3× –C*H*–), 2.10–2.20 (m, 4H, pro 2× –CH_2–), 1.81–1.98 (m, 2H, –CH_2–), 1.16–1.70 (m, 10H, E_2 5× –CH_2–), 0.77 (s, 3H, E_2 –CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 164.5, 162.3, 156.4, 150.6, 147.6, 140.4, 137.8, 132.6, 126.2, 120.0, 114.3, 111.9, 111.4, 110.4, 81.6, 65.4, 64.0, 56.0, 53.6, 49.8, 46.6, 43.8, 43.1, 38.7, 36.6, 30.6, 29.6, 28.9, 27.1, 26.2, 24.0, 23.0, 22.5, 10.9; IR (KBr) (ν_{max}/cm^{-1}): 3356 (br), 2932, 2872, 1604, 1501, 1433, 1254, 1056, 755; MS (ESI): *m/z* 559 (M+H)$^+$; Anal. Calcd for $C_{34}H_{42}N_2O_5$: C, 73.69; H, 7.78; N, 5.13. Found: C, 73.62; H, 7.72; N, 5.08.

4.2.33. 7-Methoxy-8-{3-(17-Hydroxy-1,3,5-estratrien-3-yl)-oxy}-pentyloxy)-(11a*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (3b)

The compound **3b** was prepared according to the method described for the compound **3a**, employing the compound **13b** (650 mg, 0.88 mmol). The crude product was purified by column chromatography using MeOH/$CHCl_3$ (1.4%) to afford the compound **3b** as a white solid. Yield 356 mg, 70%; mp 92–94 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.69 (d, 1H, *J* = 3.90 Hz, imine –*H*), 7.51 (s, 1H, Ar*H*), 7.19 (d, 1H, *J* = 7.8 Hz, Ar*H*), 6.85 (s, 1H, Ar*H*), 6.69 (dd, 1H, *J* = 1.95,7.81 Hz, Ar*H*), 6.62 (s, 1H, Ar*H*), 5.30 (br s, 1H, E_2 –O*H*), 4.13 (t, 2H, *J* = 6.8 Hz, –OCH_2–), 4.06 (t, 2H, *J* = 6.9 Hz, –OCH_2–), 3.94 (s, 3H, –OCH_3), 3.73–3.84 (m, 2H, E_2 –C*H*–OH, pro –NC*H*–), 3.55–3.61 (m, 2H, pro –NCH_2–), 2.80–2.86 (m, 2H, E_2 –CH_2–), 2.29–2.34 (m, 3H, E_2 3× –C*H*–), 2.01–2.22 (m, 4H, pro –CH_2–), 1.64–1.73 (m, 6H, 3× –CH_2–), 1.16–1.62 (m, 10H, E_2 5× –CH_2–), 0.77 (s, 3H, E_2 –CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 164.3, 162.3, 156.3, 150.4, 147.7, 140.3, 137.4, 132.2, 125.7, 120.0, 113.9, 112.0, 111.5, 110.3, 80.9, 67.1, 66.8, 55.8, 53.1, 49.6, 46.6, 43.5, 42.8, 38.4, 36.3, 33.3, 31.4, 30.6, 29.9, 29.3, 28.8, 26.8, 25.9, 22.6, 22.1, 10.7; IR (KBr) (ν_{max}/cm^{-1}): 3348 (br), 2927, 2869, 1604, 1502, 1433, 1254, 1058, 755; MS (ESI): *m/z* 587 (M+H)$^+$; Anal. Calcd for $C_{36}H_{46}N_2O_5$: C, 73.67; H, 7.91; N, 4.72. Found: C, 73.61; H, 7.86; N, 4.66.

4.2.34. 7-Methoxy-8-{3-(17-Hydroxy-1,3,5-estratrien-3yl)-oxy}-propyloxy)-(2*R*,11a*S*)-2-fluoro-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (3c)

The compound **3c** was prepared according to the method described for the compound **3a**, employing the compound **13c** (550 mg, 0.76 mmol). The crude product was purified by column chromatography using MeOH/$CHCl_3$ (1.6%) to afford the compound **3c** as a white solid. Yield 285 mg, 66%; mp 113–115 °C; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.77 (d, 1H, *J* = 3.69 Hz, imine –*H*), 7.49 (s,

1H, Ar*H*), 7.19 (d, 1H, *J* = 7.7 Hz, Ar*H*), 6.84 (s, 1H, Ar*H*), 6.70 (dd, 1H, *J* = 2.2, 7.7 Hz, Ar*H*), 6.63 (s, 1H, Ar*H*), 5.34 (br s, 1H, E_2 –O*H*), 4.24 (t, 2H, *J* = 6.3 Hz, –OCH_2–), 4.11 (t, 2H, *J* = 6.3 Hz, –OCH_2–), 3.94 (s, 3H, –OCH_3), 3.82–3.87 (m, 2H, pro –C*H*F–, E_2 –CH–OH), 3.71–3.76 (m, 3H, – pro NC*H*–, –NCH_2–), 3.37–3.43 (m, 2H, E_2 –CH_2–), 2.80–2.86 (m, 3H, E_2 3× –CH–), 2.25–2.39 (m, 2H, pro –CH_2–), 1.84–2.20 (m, 2H, –CH_2–), 1.15–1.69 (m, 10H, E_2 5× –CH_2–), 0.77 (s, 3H, E_2 –CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 164.4, 162.4, 156.4, 150.9, 147.4, 140.2, 137.8, 132.6, 126.1, 120.1, 114.4, 111.8, 111.4, 110.5, 90.2, 81.6, 65.5, 64.0, 56.0, 53.2, 49.8, 46.6, 43.7, 43.1, 38.6, 36.5, 30.3, 29.6, 28.9, 27.1, 26.1, 24.0, 23.0, 22.9, 11.0; IR (KBr) (ν_{max}/cm^{-1}): 3354 (br), 2928, 2870, 1606, 1501, 1433, 1253, 1058, 755; MS (ESI): *m/z* 577 (M+H)$^+$; Anal. Calcd for $C_{34}H_{41}FN_2O_5$: C, 70.78; H, 7.27; N, 4.81. Found: C, 70.71; H, 7.22; N, 4.74.

4.2.35. 7-Methoxy-8-{3-(17-hydroxy-1,3,5-estratrien-3-yl)-oxy}-pentyloxy)-(2*R*,11a*S*)-2-fluoro-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (3d)

The compound **3d** was prepared according to the method described for the compound **3a**, employing the compound **13d** (600 mg, 0.80 mmol). The crude product was purified by column chromatography using MeOH/$CHCl_3$ (1.7%) to afford the compound **3d** as a white solid. Yield 329 mg, 69%; mp 128–130 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.85 (d, 1H, *J* = 3.69 Hz, imine –*H*), 7.51 (s, 1H, Ar*H*), 7.19 (d, 1H, *J* = 6.2 Hz, Ar*H*), 6.83 (s, 1H, Ar*H*), 6.70 (dd, 1H, *J* = 2.2, 6.2 Hz, Ar*H*), 6.63 (s, 1H, Ar*H*), 5.31 (br s, 1H, E_2 –O*H*), 4.13 (t, 2H, *J* = 6.2 Hz, –OCH_2–), 4.06 (t, 2H, *J* = 6.4 Hz, –OCH_2–), 3.94 (s, 3H, –OCH_3), 3.84–3.87 (m, 2H, pro –C*H*F–, E_2 –CH–OH), 3.66–3.75 (m, 3H, pro –NC*H*–, –NCH_2–), 3.35–3.40 (m, 2H, E_2 –CH_2–), 2.81–2.87 (m, 3H, E_2 3× –CH–), 2.29–2.35 (m, 2H, pro –CH_2–), 1.85–2.20 (m, 6H, 3× –CH_2–), 1.15–1.65 (m, 10H, E_2 5× –CH_2–), 0.78 (s, 3H, E_2 –CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 164.6, 162.1, 156.7, 151.0, 147.7, 140.5, 137.7, 132.4, 126.1, 121.7, 114.3, 111.8, 111.3, 110.4, 90.5, 81.7, 68.7, 67.7, 56.0, 52.7, 49.8, 46.6, 43.8, 43.1, 38.7, 36.5, 32.2, 30.4, 29.6, 28.9, 28.5. 27.1, 26.2, 23.0, 22.9, 22.4, 11.0; IR (KBr) (ν_{max}/cm^{-1}): 3387 (br), 2928, 2866, 1607, 1502, 1430, 1254, 1057, 785; MS (ESI): *m/z* 605 (M+H)$^+$; Anal. Calcd for $C_{36}H_{45}FN_2O_5$: C, 71.49; H, 7.51; N, 4.64. Found: C, 71.45; H, 7.46; N, 4.59.

4.2.36. 7-Methoxy-8-{3-(17-hydroxy-1,3,5-estratrien-3-yl)-oxy}-propyloxy)-(11a*S*)-2,2-difluoro-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (3e)

The compound **3e** was prepared according to the method described for the compound **3a**, employing the compound **13e** (550 mg, 0.74 mmol). The crude product was purified by column chromatography using MeOH/$CHCl_3$ (2%) to afford the compound **3e** as a white solid. Yield 263 mg, 60%; mp 115–117 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.79 (d, 1H, *J* = 3.66 Hz, imine –*H*), 7.47 (s, 1H, Ar*H*), 7.19 (d, 1H, *J* = 8.8 Hz, Ar*H*), 6.86 (s, 1H, Ar*H*), 6.71 (dd, 1H, *J* = 2.2, 8.8 Hz, Ar*H*), 6.64 (s, 1H, Ar*H*), 5.36 (s, 1H, –E_2 O*H*), 4.25 (t, 2H, *J* = 7.3 Hz, –OCH_2–), 4.14 (t, 2H, *J* = 7.3 Hz, –OCH_2–), 3.93 (s, 3H, –OCH_3), 3.82–3.87 (m, 1H, pro –NC*H*–), 3.71–3.74 (m, 1H, E_2 –CH–OH), 3.36–3.41 (m, 2H, pro –NCH_2–), 2.79–2.87 (m, 2H, E_2 –CH_2–), 2.26–2.40 (m, 3H, E_2 3× –CH–), 1.83–2.19 (m, 4H, pro –CH_2–, –CH_2–), 1.16–1.70 (m, 10H, E_2 5× –CH_2–), 0.76 (s, 3H, E_2 –CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 163.8, 160.4, 156.5, 151.3, 146.4, 140.3, 138.0, 132.8, 126.3, 120.6, 114.4, 111.9, 111.5, 110.6, 96.7, 81.8, 65.6, 64.0, 56.1, 53.1, 49.9, 43.9, 43.2, 38.8, 37.2, 36.6, 30.5, 29.7, 29.0, 27.2, 26.2, 23.0, 22.5, 11.0; IR (KBr) (ν_{max}/cm^{-1}): 3584 (br), 2930, 2870, 1609, 1501, 1435, 1256, 1055, 757; MS (ESI): *m/z* 595 (M+H)$^+$; Anal. Calcd for $C_{34}H_{40}F_2N_2O_5$: C, 68.95; H, 7.21; N, 4.91. Found: C, 68.88; H, 7.15; N, 4.82.

4.2.37. 7-Methoxy-8-{3-(17-hydroxy-1,3,5-estratrien-3-yl)-oxy}-pentyloxy)-(11a*S*)-2,2-difluoro-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (3f)

The compound **3f** was prepared according to the method described for the compound **3a**, employing the compound **13f** (650 mg, 0.84 mmol). The crude product was purified by column chromatography using MeOH/$CHCl_3$ (2.2%) to afford the compound **3f** as a white solid. Yield 323 mg, 62%; mp 121–123 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.79 (d, 1H, *J* = 3.6 Hz, imine –*H*), 7.48 (s, 1H, Ar*H*), 7.19 (d, 1H, *J* = 9.0 Hz, Ar*H*), 6.82 (s, 1H, Ar*H*), 6.69 (dd, 1H, *J* = 2.7, 9.0 Hz, Ar*H*), 6.62 (s, 1H, Ar*H*), 5.34 (br s, 1H, E_2 –O*H*), 4.13 (t, 2H, *J* = 6.3 Hz, –OCH_2–), 4.02 (t, 2H, *J* = 6.4 Hz, –OCH_2–), 3.94 (s, 3H, –OCH_3), 3.83–3.86 (m, 1H, pro –NC*H*–), 3.71–3.74 (m, 1H, E_2 –CH–OH), 3.35–3.40 (m, 2H, pro –NCH_2–), 2.80–2.86 (m, 2H, E_2–CH_2–), 2.28–2.33 (m, 3H, E_2 3× –CH–), 2.10–2.20 (m, 6H, pro –CH_2–, 2× –CH_2–), 1.81–1.98 (m, 2H, –CH_2–), 1.16–1.71 (m, 10H, E_2 5× –CH_2–), 0.77 (s, 3H, E_2 –CH_3); IR (KBr) (ν_{max}/cm^{-1}): 3360 (br), 2938, 2869, 1606, 1501, 1432, 1252, 1133, 1058, 756; MS (ESI): *m/z* 623 (M+H)$^+$; Anal. Calcd for $C_{36}H_{44}F_2N_2O_5$: C, 70.23; H, 7.12; N, 4.50. Found: C, 70.16; H, 7.06; N, 4.43.

4.2.38. 7-Methoxy-8-{3-(17-hydroxy-1,3,5-estratrien-3-yl)-oxypropyl}-piperazino)-propyloxy)-(11a*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (4a)

The compound **4a** was prepared according to the method described for the compound **3a**, employing the compound **14a** (650 mg, 0.78 mmol). The crude product was purified by column chromatography using MeOH/$CHCl_3$ (3.1%) to afford the compound **4a** as a white solid. Yield 266 mg, 50%; mp 82–84 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.67 (d, 1H, *J* = 4.5 Hz, imine –*H*), 7.52 (s, 1H, Ar*H*), 7.19 (d, 1H, *J* = 8.3 Hz, Ar*H*), 6.83 (s, 1H, Ar*H*), 6.67 (dd, 1H, *J* = 2.2, 8.3 Hz, Ar*H*), 6.60 (s, 1H, Ar*H*), 5.29 (br s, 1H, E_2 –O*H*), 4.15 (t, 2H, *J* = 6.0 Hz, –OCH_2–), 4.00 (t, 2H, *J* = 5.3 Hz, –OCH_2–), 3.94 (s, 3H, –OCH_3), 3.78–3.87(m, 2H, pro –NC*H*–, E_2 –CH–OH), 3.69–3.77 (m, 2H, pro –NCH_2–), 3.54–3.63 (m, 4H, 2× –NCH_2–), 2.70–2.98 (m, 8H, –N(–CH_2–CH_2–)$_2$N–), 2.28–2.36 (m, 5H, E_2 –CH_2–, E_2 3× –CH–), 1.82–2.26 (m, 8H, pro 2× –CH_2–, 2× –CH_2–), 1.16–1.73 (m, 10H, E_2 5× –CH_2–), 0.77 (s, 3H, E_2 –CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 164.4, 162.7, 156.6, 149.9, 146.86, 140.6, 138.0, 133.1, 125.1, 120.0, 114.4, 111.8, 111.2, 110.2, 81.5, 65.6, 64.9, 56.1, 53.2, 50.1, 49.1, 48.9, 48.1, 47.9, 46.5, 43.6, 43.1, 38.6, 36.6, 31.2, 30.6, 29.6, 28.9, 27.1, 26.2, 24.0, 23.0, 22.5, 10.9; IR (KBr) (ν_{max}/cm^{-1}): 3381 (br), 2945, 2870, 1603, 1500, 1434, 1253, 1024, 755; MS (ESI): *m/z* 685 (M+H)$^+$; Anal. Calcd for $C_{41}H_{56}N_4O_5$: C, 71.81; H, 8.32; N, 8.29. Found: C, 71.76; H, 8.25; N, 8.22.

4.2.39. 7-Methoxy-8-{3-(17-hydroxy-1,3,5-estratrien-3-yl)-oxypentyl}-piperazino)-pentyloxy)-(11a*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (4b)

The compound **4b** was prepared according to the method described for the compound **3a**, employing the compound **14b** (700 mg, 0.78 mmol). The crude product was purified by column chromatography using MeOH/$CHCl_3$ (3.2%) to afford the compound **4b** as a white solid. Yield 317 mg, 55%; mp 88–90 °C; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.70 (d, 1H, *J* = 3.9 Hz, imine –*H*), 7.50 (s, 1H, Ar*H*), 7.20 (d, 1H, *J* = 7.2 Hz, Ar*H*), 6.84 (s, 1H, Ar*H*), 6.66 (d, 1H, *J* = 7.7 Hz, Ar*H*), 6.61 (s, 1H, Ar*H*), 5.28 (br s, 1H, E_2 –O*H*), 3.99–4.13 (m, 4H, 2× –OCH_2–), 3.93 (s, 3H, –OCH_3), 3.78–3.88(m, 2H, pro –NC*H*–, E_2 –CH–OH), 3.63–3.75 (m, 2H, pro –NCH_2–), 3.54–3.63 (m, 4H, 2× –NCH_2–), 2.70–2.98 (m, 8H, –N(–CH_2–CH_2–)$_2$N–), 2.26–2.38 (m, 5H, E_2 –CH_2–, E_2 3× –CH–), 1.82–2.26 (m, 12H, pro 2× –CH_2–, 4×–CH_2–), 1.71–1.80 (m, 4H, 2× –CH_2–), 1.16–1.69 (m, 10H, E_2 5× –CH_2–), 0.77 (s, 3H, E_2 –CH_3); IR (KBr) (ν_{max}/cm^{-1}): 3378 (br), 2951, 2861, 1605, 1530, 1428, 1264, 1031, 754;

MS (ESI): *m/z* 742 $(M+H)^+$; Anal. Calcd for $C_{45}H_{64}N_4O_5$: C, 72.93; H, 8.64; N, 7.57. Found: C, 72.88; H, 8.58; N, 8.51.

4.2.40. 7-Methoxy-8-{3-(17-hydroxy-1,3,5-estratrien-3-yl)-oxypropyl}-piperazino)-propyloxy)-(2*R*,11a*S*)-2-fluoro-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodia zepin-5-one (4c)

The compound **4c** was prepared according to the method described for the compound **3a**, employing the compound **14c** (600 mg, 0.70 mmol). The crude product was purified by column chromatography using MeOH/$CHCl_3$ (3.4%) to afford the compound **4c** as a white solid. Yield 235 mg, 48%;mp 84–86 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.84 (d, 1H, *J* = 4.3 Hz, imine –*H*), 7.51 (s, 1H, Ar*H*), 7.19 (d, 1H, *J* = 8.7 Hz, Ar*H*), 6.86 (s, 1H, Ar*H*), 6.69 (dd, 1H, *J* = 2.4, 8.7 Hz, Ar*H*), 6.62 (s, 1H, Ar*H*), 5.31 (br s, 1H, E_2 –O*H*), 4.04–4.27 (m, 4H, 2× –OCH_2–), 3.95 (s, 3H, –OCH_3), 3.85–3.92 (m, 3H, E2–CH–OH, pro –CHF–, pro –NC*H*–), 3.62–3.79 (m, 4H, 2× –NCH_2–), 3.36–3.41 (m, 2H, pro –NCH_2–), 2.80–2.88 (m, 8H, –N(–CH_2–CH_2–)$_2$N–), 2.55–2.67 (m, 5H, E_2 –CH_2–, E_2 3× –CH–), 1.83–2.27 (m, 6H, pro –CH_2–, 2× –CH_2–), 1.16–1.72 (m, 10H, E_2 5× –CH_2–), 0.77 (s, 3H, E_2 –CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 164.5, 162.5, 156.6, 149.8, 146.6, 140.1, 138.3, 133.2, 125.1, 120.0, 114.6, 111.6, 111.1, 110.6, 92.3, 81.6, 65.7, 65.2, 56.0, 53.3, 50.2, 49.2, 48.7, 48.0, 46.9, 46.4, 43.6, 43.1, 38.6, 36.6, 30.6, 29.4, 28.9, 27.0, 26.2, 24.1, 23.1, 22.5, 11.0; IR (KBr) (ν_{max}/cm^{-1}): 3382 (br), 2929, 2863, 1605, 1501, 1432, 1215, 1056, 755; MS (ESI): *m/z* 703 $(M+H)^+$; Anal. Calcd for $C_{41}H_{55}FN_4O_5$: C, 70.46; H, 7.89; N, 7.91. Found: C, 70.39; H, 7.83; N, 7.87.

4.2.41. 7-Methoxy-8-{3-(17-hydroxy-1,3,5-estratrien-3-yl)-oxypentyl}-piperazino)-pentyloxy)-(2*R*,11a*S*)-2-fluoro-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodia zepin-5-one (4d)

The compound **4d** was prepared according to the method described for the compound **3a**, employing the compound **14d** (550 mg, 0.60 mmol). The crude product was purified by column chromatography using MeOH/$CHCl_3$ (3.5%) to afford the compound **4d** as a white solid. Yield 208 mg, 46%; mp 99–101 °C; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.79 (d, 1H, *J* = 4.1 Hz, imine –*H*), 7.50 (s, 1H, Ar*H*), 7.20 (d, 1H, *J* = 7.8 Hz, Ar*H*), 6.84 (s, 1H, Ar*H*), 6.67 (d, 1H, *J* = 7.8 Hz, Ar*H*), 6.61 (s, 1H, Ar*H*), 5.30 (br s, 1H, E_2 –O*H*), 3.99–4.13 (m, 4H, 2× –OCH_2–) 3.94 (s, 3H, –OCH_3), 3.80–3.90 (m, 3H, E_2 –CH–OH, pro –CHF–, pro –NC*H*–), 3.63 –3.75 (m, 4H, 2× –NCH_2–), 3.46–3.59 (m, 2H, pro –NCH_2–), 2.78–2.86 (m, 8H, –N(–CH_2–CH_2–)$_2$N–), 2.44–2.58 (m, 5H, E_2 –CH_2–, E_2 3× –CH–), 1.82–2.26 (m, 14H, pro –CH_2–, 6× –CH_2–), 1.16–1.71 (m, 10H, E_2 5× –CH_2–), 0.77 (s, 3H, E_2 –CH_3); IR (KBr) (ν_{max}/cm^{-1}): 3356 (br), 2929, 2864, 1604, 1507, 1433, 1264, 1065, 788; MS (ESI): *m/z* 760 $(M+H)^+$; Anal. Calcd for $C_{45}H_{63}FN_4O_5$: C, 71.52; H, 8.49; N, 7.61. Found: C, 71.46; H, 8.41; N, 7.54.

4.2.42. 7-Methoxy-8-{3-(17-hydroxy-1,3,5-estratrien-3-yl)-oxypropyl}-piperazino)-propyloxy)-(11a*S*)-2,2-difluoro-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodia zepin-5-one (4e)

The compound **4e** was prepared according to the method described for the compound **3a**, employing the compound **14e** (650 mg, 0.74 mmol). The crude product was purified by column chromatography using MeOH/$CHCl_3$ (3.6%) to afford the compound **4e** as a white solid. Yield 266 mg, 50%; mp 105–107 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.79 (d, 1H, *J* = 3.7 Hz, imine –*H*), 7.47 (s, 1H, Ar*H*), 7.19 (d, 1H, *J* = 8.8 Hz, Ar*H*), 6.86 (s, 1H, Ar*H*), 6.71 (dd, 1H, *J* = 2.0, 8.8 Hz, Ar*H*), 6.64 (s, 1H, Ar*H*), 5.31 (br s, 1H, E_2 –O*H*), 4.00–4.35 (m, 4H, 2× –OCH_2–), 3.94 (s, 3H, –OCH_3), 3.83–3.86 (m, 2H, E_2 –CH–OH, pro –NC*H*–), 3.70–3.75 (m, 4H, 2× –NCH_2–), 3.37–3.40 (m, 2H, pro –NCH_2–), 2.80–2.87 (m, 8H, –N(–CH_2–CH_2–)$_2$N–), 2.25–2.40 (m, 5H, E_2 –CH_2–, E_2 3× –CH–), 1.83–2.27 (m, 6H, pro –CH_2–, 2× –CH_2–), 1.14–1.70 (m, 10H, E_2 5× –CH_2–), 0.77 (s, 3H, E_2 –CH_3); IR (KBr) (ν_{max}/cm^{-1}): 3412 (br), 3020, 2870, 1612, 1517, 1425, 1215, 1049, 758; MS (ESI): *m/z* 721 $(M+H)^+$; Anal. Calcd for $C_{41}H_{54}F_2N_4O_5$: C, 68.42; H, 7.55; N, 7.63. Found: C, 68.38; H, 7.48; N, 7.57.

4.2.43. 7-Methoxy-8-{3-(17-hydroxy-1,3,5-estratrien-3-yl)-oxypentyl}-piperazino)-pentyloxy)-(11a*S*)-2,2-difluoro-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodia zepin-5-one (4f)

The compound **4f** was prepared according to the method described for the compound **3a**, employing the compound **14f** (600 mg, 0.64 mmol). The crude product was purified by column chromatography using MeOH/$CHCl_3$ (3.8%) to afford the compound **4f** as a white solid. Yield 248 mg, 50%; mp 111–113 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.78 (d, 1H, *J* = 3.9 Hz, imine –*H*), 7.48 (s, 1H, Ar*H*), 7.20 (d, 1H, *J* = 7.7 Hz, Ar*H*), 6.84 (s, 1H, Ar*H*), 6.70 (dd, 1H, *J* = 3.1, 7.7 Hz, Ar*H*), 6.62 (s, 1H, Ar*H*), 5.30 (br s, 1H, E_2 –O*H*), 3.99–4.32 (m, 4H, 2× –OCH_2–) 3.94 (s, 3H, –OCH_3), 3.80–3.90 (m, 2H, E_2 –CH–OH, pro –NC*H*–), 3.68–3.73 (m, 4H, 2× –NCH_2–), 3.47–3.49 (m, 2H, pro –NCH_2–), 2.79–2.86 (m, 8H, –N(–CH_2–CH_2–)$_2$N–), 2.34–2.48 (m, 5H, E_2 –CH_2–, E_2 3× –CH–), 1.82–2.26 (m, 14H, pro –CH_2–, 6× –CH_2–), 1.15–1.75 (m, 10H, E_2 5× –CH_2–), 0.79 (s, 3H, E_2 –CH_3); IR (KBr) (ν_{max}/cm^{-1}): 3360 (br), 2932, 2863, 1607, 1509, 1432, 1258, 1054, 771; MS (ESI): *m/z* 777 $(M+H)^+$; Anal. Calcd for $C_{45}H_{62}F_2N_4O_5$: C, 69.62; H, 8.34; N, 7.23. Found: C, 69.58; H, 8.31; N, 7.18.

4.2.44. 7-Methoxy-8-{3-(17-hydroxy-1,3,5-estratrien-3-yl}-oxypropyl)-1*H*-1,2,3-triazole-4-yl-methoxy}-(11a*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (5a)

The compound **5a** was prepared according to the method described for the compound **3a**, employing the compound **15a** (650 mg, 0.88 mmol). The crude product was purified by column chromatography using MeOH/$CHCl_3$ (2.6%) to afford the compound **5a** as a white solid. Yield 318 mg, 61%; mp 98–100 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.79 (d, 1H, *J* = 3.4 Hz, imine –*H*), 7.65 (s, 1H, triazole –*H*), 7.52 (s, 1H, Ar*H*), 7.19 (d, 1H, *J* = 9.1 Hz, Ar*H*), 6.98 (s, 1H, Ar*H*), 6.69 (dd, 1H, *J* = 3.0, 9.1 Hz, Ar*H*), 6.63 (s, 1H, Ar*H*), 5.32 (br s, 1H, E_2 –O*H*), 5.30 (s, 2H, –OCH_2–), 4.58 (t, 2H, *J* = 6.8 Hz, –OCH_2–), 3.95 (t, 2H, *J* = 5.3 Hz, –CH_2N–), 3.92 (s, 3H, –OCH_3), 3.71–3.86 (m, 2H, E_2 –CH–OH, pro –NC*H*–), 3.53–3.62(m, 2H, pro –NCH_2–), 2.80–2.87 (m, 2H, E_2 –CH_2–), 2.27–2.40 (m, 3H, E_2 3× –CH–), 1.70–2.12 (m, 6H, pro 2× –CH_2–, –CH_2–), 1.16–1.66 (m, 10H, E_2 5× –CH_2–), 0.77 (s, 3H, E_2 –CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 164.5, 162.7, 156.7, 156.0, 147.7, 144.4, 138.0, 137.6, 133.0, 126.3, 123.7, 119.7, 114.2, 111.7, 111.2, 110.5, 81.6, 67.8, 63.7, 56.0, 49.8, 49.3, 47.1, 43.7, 43.1, 38.6, 36.5, 30.6, 30.3, 29.7, 28.9, 27.0, 26.1, 24.0, 23.0, 22.9, 10.9; IR (KBr) (ν_{max}/cm^{-1}): 3332 (br), 2931, 2872, 1605, 1503, 1434, 1254, 1054, 754; MS (ESI): *m/z* 640 $(M+H)^+$; Anal. Calcd for $C_{37}H_{45}N_5O_5$: C, 69.46; H, 7.09; N, 10.95. Found: C, 69.41; H, 7.02; N, 10.89.

4.2.45. 7-Methoxy-8-{3-(17-hydroxy-1,3,5-estratrien-3-yl}-oxypentyl)-1*H*-1,2,3-triazole-4-ylmethoxy}-(11a*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (5b)

The compound **5b** was prepared according to the method described for the compound **3a**, employing the compound **15b** (700 mg, 0.85 mmol). The crude product was purified by column chromatography using MeOH/$CHCl_3$ (2.8%) to afford the compound **5b** as a white solid. Yield 367 mg, 65%; mp 116–118 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.80 (d, 1H, *J* = 3.5 Hz, imine –*H*), 7.66 (s, 1H, triazole –*H*), 7.53 (s, 1H, Ar*H*), 7.20 (d, 1H, *J* = 8.7 Hz, Ar*H*), 6.99 (s, 1H, Ar*H*), 6.66 (dd, 1H, *J* = 2.5, 8.7 Hz, Ar*H*), 6.61 (s, 1H, Ar*H*), 5.32 (br s, 1H, E_2 –O*H*), 5.29 (s, 2H, –OCH_2–), 4.56 (t, 2H,

J = 6.2 Hz, –OCH_2–), 3.94 (s, 3H, –OCH_3), 3.84–3.92 (m, 2H, –CH_2N–), 3.71–3.79 (m, 2H, E_2 –CH–OH, pro –NCH–), 3.55–3.61 (m, 2H, –NCH_2–), 2.80–2.86 (m, 2H, E_2 –CH_2–), 2.27–2.40 (m, 3H, E_2 3× –CH–), 2.00–2.21 (m, 4H, pro 2× –CH_2–), 1.72–1.90 (m, 6H, 3× –CH_2–), 1.16–1.68 (m, 10H, E_2 5× –CH_2–), 0.77 (s, 3H, E_2 –CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 164.5, 162.7, 155.8, 151.8, 145.0, 144.2, 137.9, 136.8, 133.1, 126.2, 122.5, 119.3, 114.4, 111.9, 111.0, 110.2, 81.5, 68.1, 61.7, 55.9, 49.8, 49.3, 47.1, 43.7, 43.0, 38.5, 36.7, 31.3, 30.2, 29.6, 28.2, 27.0, 26.1, 24.1, 22.9, 22.4, 10.9; IR (KBr) (ν_{max}/cm^{-1}): 3353 (br), 2925, 2868, 1604, 1503, 1433, 1256, 1055, 755; MS (ESI): m/z 668 (M+H)$^+$; Anal. Calcd for $C_{39}H_{49}N_5O_5$: C, 70.34; H, 7.40; N, 10.49. Found: C, 70.28; H, 7.35; N, 10.42.

4.2.46. 7-Methoxy-8-{3-(17-hydroxy-1,3,5-estratrien-3-yl}-oxypropyl)-1*H*-1,2,3-triazole-4-ylmethoxy}-(2*R*,11a*S*)-2-fluoro-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]be nzodiazepin-5-one (5c)

The compound **5c** was prepared according to the method described for the compound **3a**, employing the compound **15c** (600 mg, 0.74 mmol). The crude product was purified by column chromatography using MeOH/$CHCl_3$ (3%) to afford the compound **5c** as a white solid. Yield 267 mg, 55%; mp 84–86 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.83 (d, 1H, J = 4.53 Hz, imine –H), 7.68 (s, 1H, triazole –H), 7.54 (s, 1H, ArH), 7.20 (d, 1H, J = 9.06 Hz, ArH), 7.01 (s, 1H, ArH), 6.68 (d, 1H, J = 9.06 Hz, ArH), 6.61 (s, 1H, ArH), 5.32 (br s, 1H, E_2 –OH), 5.29 (s, 2H, –OCH_2–), 4.39 (t, 2H, J = 6.38 Hz, –OCH_2–), 3.94 (s, 3H, –OCH_3), 3.81–3.86 (m, 2H, –CH_2N–), 3.71–3.80 (m, 3H, E_2 –CH–OH, pro –CHF–, –NCH–), 3.38–3.44 (m, 2H, pro –NCH_2–), 2.81–2.87 (m, 2H, E_2 –CH_2–), 2.24–2.38 (m, 3H, E_2 3× –CH–), 1.86–2.20 (m, 4H, pro –CH_2–, –CH_2–), 1.16–1.69 (m, 10H, E_2 5× –CH_2–), 0.78 (s, 3H, E_2 –CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 164.3, 162.5, 158.5, 156.0, 147.2, 143.4, 138.0, 136.7, 133.1, 126.3, 123.5, 119.5, 114.2, 111.8, 111.1, 110.1, 85.3, 81.6, 63.7, 62.3, 56.0, 49.8, 49.2, 47.2, 43.7, 43.1, 38.6, 36.5, 30.3, 29.5, 28.3, 27.1, 25.4, 24.1, 23.0, 22.9, 10.9; IR (KBr) (ν_{max}/cm^{-1}): 3386 (br), 2930, 2870, 1607, 1501, 1429, 1253, 1053, 754; MS (ESI): m/z 658 (M+H)$^+$; Anal. Calcd for $C_{37}H_{44}FN_5O_5$: C, 67.64; H, 6.64; N, 10.44. Found: C, 67.59; H, 6.58; N, 10.37.

4.2.47. 7-Methoxy-8-{3-(17-hydroxy-1,3,5-estratrien-3-yl}-oxypentyl)-1*H*-1,2,3-triazole-4-ylmethoxy}-(2*R*,11a*S*)-2-fluoro-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]be nzodiazepin-5-one (5d)

The compound **5d** was prepared according to the method described for the compound **3a**, employing the compound **15d** (700 mg, 0.83 mmol). The crude product was purified by column chromatography using MeOH/$CHCl_3$ (3.1%) to afford the compound **5d** as a white solid. Yield 352 mg, 62%; mp 118–120 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): 7.85 (d, 1H, J = 3.9 Hz, imine –H), 7.65 (s, 1H, triazole –H), 7.52 (s, 1H, ArH), 7.19 (d, 1H, J = 8.8 Hz, ArH), 7.01 (s, 1H, ArH), 6.67 (dd, 1H, J = 8.7 Hz, ArH), 6.60 (s, 1H, ArH), 5.35 (br s, 1H, E_2 –OH), 5.32 (s, 2H, –OCH_2–), 4.37 (t, 2H, J = 6.4 Hz, –OCH_2–), 3.94 (s, 3H, –OCH_3), 3.86–3.92 (m, 2H, –CH_2N–), 3.71–3.75 (m, 1H, pro –CHF–), 3.62–3.68 (m, 2H, E_2 –CH–OH, pro –NCH–), 3.36–3.42 (m, 2H, pro –NCH_2–), 2.81–2.86 (m, 2H, E_2 –CH_2–), 2.29–2.56 (m, 3H, E_2 3× –CH–), 2.09–2.22 (m, 4H, 2× –CH_2–), 1.76–2.03 (m, 4H, pro –CH_2–, –CH_2–), 1.15–1.72 (m, 10H, E_2 5× –CH_2–), 0.78 (s, 3H, E_2 –CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 164.3, 162.7, 158.6, 156.4, 147.8, 143.6, 137.8, 136.6, 132.6, 126.2, 123.3, 119.6, 114.3, 111.8, 110.2, 108.2, 84.7, 81.6, 67.1, 62.2, 56.0, 50.2, 49.8, 47.1, 43.8, 43.1, 38.7, 36.4, 31.4, 30.5, 29.8, 28.5, 27.1, 26.2, 25.4, 23.2, 23.0, 22.5, 11.0; IR (KBr) (ν_{max}/cm^{-1}): 3408 (br), 2927, 2866, 1609, 1503, 1430, 1253, 1054, 787; MS (ESI): m/z 686 (M+H)$^+$; Anal. Calcd for $C_{39}H_{48}FN_5O_5$: C, 68.70; H, 7.45; N, 10.31. Found: C, 68.62; H, 7.37; N, 10.24.

4.2.48. 7-Methoxy-8-{3-(17-hydroxy-1,3,5-estratrien-3-yl}-oxypropyl)-1*H*-1,2,3-triazole-4-ylmethoxy}-(11a*S*)-2,2-difluoro-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]be nzodiazepin-5-one (5e)

The compound **5e** was prepared according to the method described for the compound **3a**, employing the compound **15e** (650 mg, 0.78 mmol). The crude product was purified by column chromatography using MeOH/$CHCl_3$ (3.4%) to afford the compound **5e** as a white solid. Yield 315 mg, 60%; mp 113–115 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.80 (d, 1H, J = 3.9 Hz, imine –H), 7.66, (s, 1H, triazole –H), 7.51 (s, 1H, ArH), 7.20 (d, 1H, J = 7.7 Hz, ArH), 7.00 (s, 1H, ArH), 6.67 (dd, 1H, J = 2.8, 7.7 Hz, ArH), 6.61 (s, 1H, ArH), 5.32 (br s, 1H, E_2 –OH), 5.30 (s, 2H, –OCH_2–), 4.37 (t, 2H, J = 7.5 Hz, –OCH_2–), 3.94 (s, 3H, –OCH_3), 3.80–3.91 (m, 2H, –CH_2N–), 3.70–3.77 (m, 2H, E_2 –CH–OH, pro –NCH–), 3.52–3.62 (m, 2H, pro –NCH_2–), 2.22–2.87 (m, 2H, E_2 –CH_2 –), 2.29–2.36 (m, 3H, E_2 3× –CH–), 1.91–2.17 (m, 4H, pro –CH_2–, –CH_2–), 1.16–1.74 (m, 10H, E_2 5× –CH_2–), 0.77 (s, 3H, E_2 –CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 164.4, 162.5, 156.7, 156.1, 147.6, 144.1, 138.1, 136.5, 133.3, 126.4, 123.5, 119.6, 114.3, 111.9, 111.2, 110.4, 92.5, 81.8, 63.8, 62.3, 56.1, 49.9, 49.2, 47.2, 43.8, 43.2, 38.7, 36.6, 30.5, 29.4, 28.7, 27.1, 26.2, 24.1, 23.0, 22.4, 11.1; IR (KBr) (ν_{max}/cm^{-1}): 3381 (br), 3020, 2936, 1607, 1500, 1431, 1216, 1050, 756; MS (ESI): m/z 676 (M+H)$^+$; Anal. Calcd for $C_{37}H_{43}F_2N_5O_5$: C, 65.72; H, 6.49; N, 10.38. Found: C, 65.65; H, 6.42; N, 10.31.

4.2.49. 7-Methoxy-8-{3-(17-hydroxy-1,3,5-estratrien-3-yl}-oxypentyl)-1*H*-1,2,3-triazole-4-ylmethoxy}-(11a*S*)-2,2-difluoro-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (5f)

The compound **5f** was prepared according to the method described for the compound **3a**, employing the compound **15f** (650 mg, 0.75 mmol). The crude product was purified by column chromatography using MeOH/$CHCl_3$ (3.5%) to afford the compound **5f** as a white solid. Yield 310 mg, 59%; mp 116–118 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.81 (d, 1H, J = 3.9 Hz, imine –H), 7.69 (s, 1H, triazole –H), 7.53 (s, 1H, ArH), 7.19 (d, 1H, J = 8.3 Hz, ArH), 6.99 (s, 1H, ArH), 6.67 (dd, 1H, J = 3.3, 8.3 Hz, ArH), 6.60 (s, 1H, ArH), 5.31 (br s, 1H, E_2 –OH), 5.29 (s, 2H, –OCH_2–), 4.38 (t, 2H, J = 6.8 Hz, –OCH_2–), 3.94 (s, 3H, –OCH_3), 3.79–3.91 (m, 2H, –CH_2N–), 3.71–3.76 (m, 2H, E_2 –CH–OH, pro –NCH–), 3.53–3.62 (m, 2H, pro –NCH_2–), 2.79–2.86 (m, 2H, E_2 –CH_2–), 2.28–2.35 (m, 3H, E_2 3× –CH–), 2.08–2.25 (m, 4H, 2× –CH_2–), 1.75–2.01 (m, 4H, pro –CH_2–, –CH_2–), 1.17–1.60 (m, 10H, E_2 5× –CH_2–), 0.77 (s, 3H, E_2 –CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 164.5, 162.8, 158.6, 156.5, 147.0, 143.2, 137.9, 136.7, 132.6, 126.2, 123.3, 119.2, 114.3, 111.8, 111.0, 110.2, 90.5, 81.7, 67.1, 62.3, 56.1, 50.3, 49.1, 47.1, 43.8, 43.1, 38.7, 36.6, 30.4, 29.7, 28.5, 27.1, 26.2, 25.6, 25.2, 24.2, 23.0, 22.9, 11.0; IR (KBr) (ν_{max}/cm^{-1}): 3387 (br), 2981, 2876, 1609, 1531, 1464, 1252, 1051, 777; MS (ESI): m/z 704 (M+H)$^+$; Anal. Calcd for $C_{39}H_{47}F_2N_5O_5$: C, 66.71; H, 6.63; N, 9.76. Found: C, 66.64; H, 6.58; N, 9.71.

4.3. Cell culture

MCF-7 (breast carcinoma cells) were incubated by using DMEM media, supplemented with 10% fetal calf serum, 100 μg/mL pencillin-G and 100 μg/mL streptomycin sulfate. The cell line was maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in the incubator.

4.4. MTT cell viability assay

Cell viability was assessed by the MTT based 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 E_2 (**1**), DC-81 (**2**), **4a**, **4c**, **5c**, **5d** and **5f** at 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 (O.D.) was read at 570 nm with micro plate reader.

4.5. Cell cycle analysis

5×10^5 MCF-7 cells were seeded in 60 mm dish and were allowed to grow for 24 h, 4 μM concentration of E_2 (**1**), DC-81 (**2**), **4a**, **4c**, **5c**, **5d** and **5f** 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.

4.6. Immunofluorescence microscopy studies for tubulin polymerization

MCF-7 cells were seeded on glass cover slips, incubated for 24 h in the presence or absence of test compounds E_2 (**1**), DC-81 (**2**), **5c** and **5d** at 4 μM concentration. Following the termination of incubation, cells were fixed with 4% paraformaldehyde, 0.02% glutaraldehyde in PBS and permeabilized by dipping the cells in 100% methanol (−20 °C). Later, cover slips were blocked with 1% BSA in phosphate buffered saline for 1 h followed by incubation with a primary antitubulin (mouse monoclonal) antibody followed by Cy3 conjugated secondary mouse anti-IgG antibody. At the end of experiments, cells were washed and fixed Photographs were taken using the confocal microscope (Olympus), equipped with Cy3 settings and the pictures were analyzed for the integrity of microtubule network. Here Nocodazole (Noc) (4 μM) was used as positive control for analyzing microtubule integrity. β-Tubulin antibody was purchased from Abcam Company.

4.7. Protein extraction and Western blot analysis

5×10^5 MCF-7 cells were seeded in 60 mm dish and were allowed to grow for 24 h, 4 μM concentration of E_2 (**1**), DC-81 (**2**), **5c** and **5d** compounds were added to the culture media, and the cells were incubated for an additional 24 h. Total cell lysates from cultured MCF-7 cells were obtained by lysing the cells in ice-cold RIPA buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) and containing 100 μg/mL PMSF, 5 μg/mL Aprotinin, 5 μg/mL leupeptin, 5 μg/mL pepstatin and 100 μg/mL NaF. After centrifugation at 12,000 rpm for 10 min, the protein in supernatant was quantified by Bradford method (BIO-RAD) using Multimode varioskan instrument (Thermo-Fischer Scientifics). Fifty micrograms of protein per lane was applied in 12% SDS–polyacrylamide gel. After electrophoresis, the protein was transferred to polyvinylidine difluoride (PVDF) membrane (Amersham Biosciences). The membrane was blocked at room temperature for 2 h in 1× TBS + 0.1% Tween20 (TBST) containing 5% blocking powder (Santacruz). The membrane was washed with TBST for 5 min, and primary antibody was added and incubated at 4 °C overnight. Mouse monoclonal antibodies p21, Cdk1 and Rabbit polyclonal HDAC-1 and HDAC-3 were purchased from Millipore company Ltd. Mouse monoclonal antibodies such as NF-kB (p65), procaspase-7, Rabbit polyclonal antibodies such as β-actin were purchased from Imgenex company. Mouse monoclonal p53 and Rabbit polyclonal Bax antibodies were purchased from santacruz Biotech Company. Membranes were washed with TBST three times for 15 min and the blots were visualized with chemiluminescence reagent (Thermo Fischer Scientifics Ltd). The X-ray films were developed with developer and fixed with fixer solution purchased from Kodak Company.

4.8. Transfection studies

MCF-7 cells were transfected with 1.5 μg of p21 (−208/+8) promoter fused with luciferase reporter gene in one experiment pertaining to observe p21 promoter activity in 6 well plate and transfected into MCF-7 cells using lipofectamine 2000 (invitrogen) according to manufacturer's recommendations. After transfections cells were allowed to grow for 24 h. Then they were subjected with compound treatment E_2 (**1**), DC-81 (**2**), **5c** and **5d** for 24 h at 4 μM concentration. Then the cells were harvested and lysates were subjected to luciferase assay, which is a luminescence based assay.

4.9. Tunel assay

Tunel assay (Terminal Transferase dUTP Nick End Labeling) was conducted by using the Apoalert DNA fragmentation Assay kit (Clone tech). Apoptosis induced nuclear DNA fragmentation was determined using this assay. This assay was conducted according to the manufacturer's recommendations and is based on the principle of terminal deoxy nucleotidyl transferase (TdT)-mediated dUTP nick-end-labeling. TdT catalyzes incorporation of fluorescein-dUTP at the free 3′-hydroxyl ends of fragmented DNA. Flouroscein-labeled DNA can be detected via confocal microscope.

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