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Design, synthesis and biological evaluation of estradiol–chlorambucil hybrids as anticancer agents

Atul Gupta, Pijus Saha, Caroline Descôteaux, Valérie Leblanc, Éric Asselin, Gervais Bérubé *

Département de Chimie-Biologie, Groupe de Recherche en Oncologie et Endocrinologie Moléculaires, Université du Québec à Trois-Rivières, C.P. 500, Trois-Rivières, Québec, Canada G9A 5H7

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ABSTRACT

A series of estradiol–chlorambucil hybrids was synthesized as anticancer drugs for site-directed chemotherapy of breast cancer. The novel compounds were synthesized in good yields through efficient modifications of estrone at position 16α of the steroid nucleus. The newly synthesized compounds were evaluated for their anticancer efficacy in different hormone-dependent and hormone-independent breast cancer cell lines. The novel hybrids showed significant in vitro anticancer activity when compared to chlorambucil. Structure–activity relationship (SAR) reveals the influence of the length of the spacer chain between carrier and drug molecule.

Breast cancer, a leading pandemic, affects women of all ages.[1] It is the most frequent site of cancer in women. The successful treatment of this disease is too often limited by the fact that essentially all breast cancers become resistant to chemotherapy and endocrine therapy.[2] Throughout a women lifetime, increased exposure to the estradiol (**1**, Fig. 1) is an important factor for the development of breast, uterine, and ovarian cancers. At menopausal and post-menopausal conditions, despite low level of circulating estrogens, the tissular concentration of estrogens in tissues such as breast, is significantly high due to its local production. The possible therapies generally used for estrogen-dependent cancers such as breast cancer are: (a) use of aromatase inhibitors[3] or estrogen sulfatase inhibitors[4] and (b) use of pure estrogen antagonists such as fulvestrant[5] or selective estrogen receptor modulators (SERMs) such as tamoxifen.[6] Furthermore, the use of estrogen–cytotoxic conjugates linking platinum-based drugs[7] and various alkylating agents including chlorambucil (**2**) is the subject of intense research.[8]

Chlorambucil (**2**) is an alkylating agent of the nitrogen mustard group and is used as cytostatic drug in cancer therapy.[8] In general, alkylating agents are both mutagenic and genotoxic.[9] The alkylating agents form adducts with DNA. However, they also form adducts with RNA and protein which are likely to contribute to the overall cytotoxicity.[9]

The main side effects of chlorambucil (**2**) are bone marrow suppression, anemia, and weak immune system.[10] Therefore, there is a need to design new chemotherapeutic agents able to target not only breast cancer, but also to display increased efficacy and overall decreased systemic toxicity.

In medicinal chemistry, prodrug approach provides a method to reduce the gap between physiological and pharmacological doses of a drug through its slow release in the body.[11] Furthermore, if a drug molecule is converted into a prodrug using a molecule having affinity with the receptor for which the drug molecule have been designed, can also improve the efficacy of the drug molecule. Therefore, site-directed prodrug approach could be an alternative of choice for the treatment of various diseases such as hormone-dependent cancers which possibly could lead to increased activity with reduced adverse side effects.

In our relentless effort to design new anticancer drugs for site-directed chemotherapy, we have reported a unique class of estradiol–Pt(II) hybrid derivatives which showed good in vitro[12–17] and in vivo[18] potential for the treatment of hormone-dependent cancers, in particular breast cancer, without any apparent side effects. Their interactions with DNA and RNA was also studied using various spectroscopic methods.[19–21]

In our quest to develop alternative anticancer agents for site-directed chemotherapy of hormone-dependent cancers, we have designed a series of estradiol–chlorambucil hybrids of type **4**. In the present approach, chlorambucil (**2**), a known nitrogen mustard drug was linked to the estradiol framework of type (**3**) at position 16α (Fig. 1). This position albeit close to the estrogenic binding site apparently does not affect its binding mode. From our previous studies, we observed that the use of compound **3** as a carrier moiety seemed, in many cases, to be even superior over estradiol **1** in

* Corresponding author. Tel.: +1 819 376 5011x3353.
E-mail address: Gervais.Berube@uqtr.ca (G. Bérubé).

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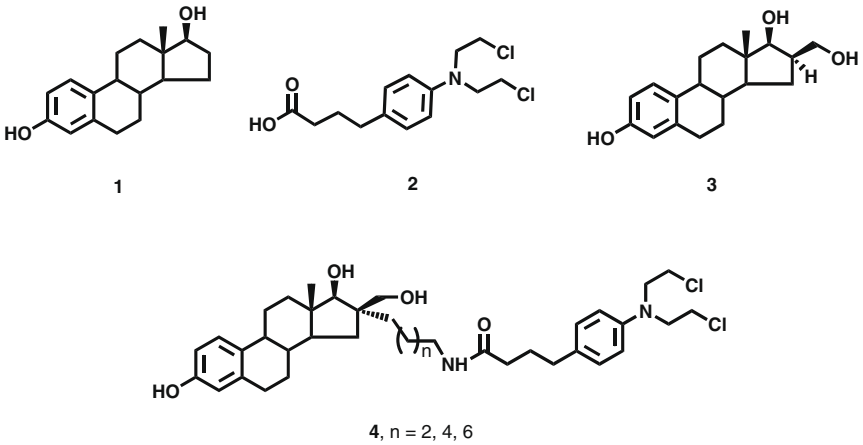
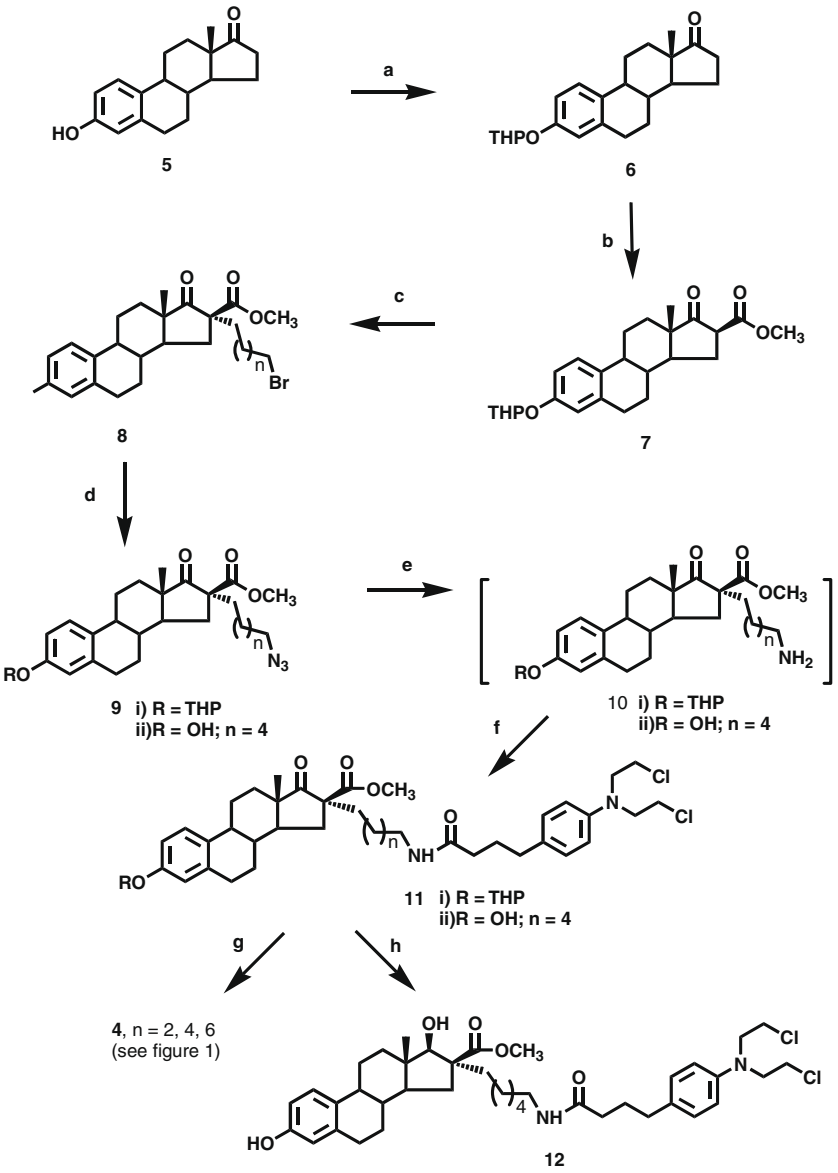


Figure 1. Structures of estradiol (**1**), chlorambucil (**2**), and estradiol derivatives **3** and **4**.



Scheme 1. Reagent and conditions: (a) dihydropyran, pyridinium-*p*-toluenesulfonate, dichloromethane (DCM), 22 °C; (b) dimethyl carbonate, KH, dry tetrahydrofuran (THF), reflux; (c) dibromoalkane, 10% aq NaOH solution, benzyltriethylammonium chloride ($Bn(Et)_3N^+Cl^-$), DCM, reflux; (d) sodium azide (NaN_3), methanol, reflux, 20 h; (e) 10% Pd/C, H_2 gas, 22 °C; (f) chlorambucil, HOBt, DCC, Et_3N, dry dimethylformamide (DMF), 22 °C; (g) (i) $LiBH_4$ excess, dry diethyl ether (Et_2O), 22 °C; (ii) PPTs, EtOH, reflux, 3 h; (h) see condition g with 1 equiv $LiBH_4$.

term of receptor binding affinity. This study presents the design, synthesis and biological activities of these new estradiol–chlorambucil hybrid molecules (**4**).

The synthesis of the target compounds of type **4** was initiated from estrone (**5**) (Scheme 1). As previously reported, estrone (**5**) was protected using dihydropyran in dichloromethane at room temperature (22 °C) yielding 3-tetrahydropyranyloxy estrone (**6**) in 100% yield.[12] Compound **6** was activated at position 16 through the formation of the β-ketoester (**7**). Compound **7** was obtained from the reaction of **6** with dimethyl carbonate in presence of potassium hydride (KH) in dry THF at reflux in 90% yield. Alkylation of compound **7** with an appropriate dibromoalkane, in biphasic medium using 10% aqueous solution of sodium hydroxide and dichloromethane, in presence of benzyltriethylammonium chloride ($Bn(Et)_3N^+Cl^-$, a phase transfer catalyst) at reflux gave compound **8** in 50–85% yield.[12] Nucleophilic substitution reaction of compound **8** with sodium azide (NaN_3) in methanol at reflux gave compound **9** in 75–85% yield. Catalytic reduction of compound **9** by hydrogen gas in presence of 10% Pd/C gave compound **10** quantitatively. Condensation of compound **10** with chlorambucil in presence of 1-hydroxybenzotriazole (HOBt), dicyclohexylcarbodiimide (DCC) and triethylamine at room temperature (22 °C) yielded compound **11** in 55–70% yield. Borohydride reduction of compound **11** with excess of lithium borohydride ($LiBH_4$) in dry diethyl ether (Et_2O) at room temperature (22 °C) and subsequent deprotection of the tetrahydropyranyl ether group by refluxing with ethanol in the presence of pyridinium-*p*-toluenesulfonate (PPTs) gave the targeted compounds **4 (a–c)** in good yields. It is noteworthy that reduction with a limited amount of lithium borohydride under similar reaction conditions yielded, after deprotection, the estradiol–chlorambucil conjugate, compound **12**, on which the carboxymethyl group at position 16β of estradiol skeleton is retained. The compound **12** was also obtained as side product during the synthesis of **4b**. The estradiol–chlorambucil hybrids **4 (a–c)** (Table 1) were fully characterized by the use of infrared (IR), nuclear magnetic resonance spectroscopy (NMR) and their respective high resolution mass analysis.[22]

In addition, for complete characterization, deprotected compounds **9ii**, **10ii** and **11ii** were synthesized. It was observed that the coupling reaction after deprotection (between **10ii** and chlorambucil) gave an hybrid that was more distinct on thin layer chromatography and easier to purify than the coupling of **10i** with chlorambucil but the yield in both cases was almost the same. However, the reduction of the hybrids with $LiBH_4$ was easier before deprotection of the hydroxy function at position 3 of the steroid nucleus.

The newly synthesized compounds were evaluated for their in vitro cytotoxic activity using estrogen receptor positive (MCF-7) and negative (MDA-MB-436, MDA-MB-468, MDA-MB-231) breast cancer cell lines. Chlorambucil (**2**) was used as positive control. MTT assays showed that the newly synthesized estradiol–chlorambucil hybrids of type **4** (Table 1) possess moderate to significant cytotoxic activity.[23,24]

Biological activity results of the compounds showed cytotoxic activity at higher doses than that of chlorambucil except for compound **4a**, which showed anticancer activity at lower dose (IC_{50} = 40 μM) as compared with chlorambucil (**2**) (IC_{50} >160 μM) on MDA-MB-436 cell line (Fig. 2). In general, compounds inhibited effectively cellular proliferation of estrogen receptor negative cancer cell lines as compared with estrogen receptor positive cancer cell lines (Figs. 2–4). However for the ER^- MDA-MB-231 breast cancer cells, the various derivatives were completely inactive while chlorambucil itself showed an IC_{50} of about 130 μM (data not shown).

The observed biological activity of the hybrids in hormone-dependent cancer cell line could be due to the potential estrogenic effect of the estradiol (**1**) carrier molecule which may trigger counteracting effect over the cytotoxicity of the hybrids leading to overall moderate activities in ER^+ cancer cell line (MCF-7).

A complete structure–activity relationship (SAR) was attempted to establish in terms of length of spacer between the carrier

Table 1
Compound numbers and descriptors

Compound No.	Compound descriptors
2	Chlorambucil
4a	$n = 2$
4b	$n = 4$
4c	$n = 6$
12	$n = 4$

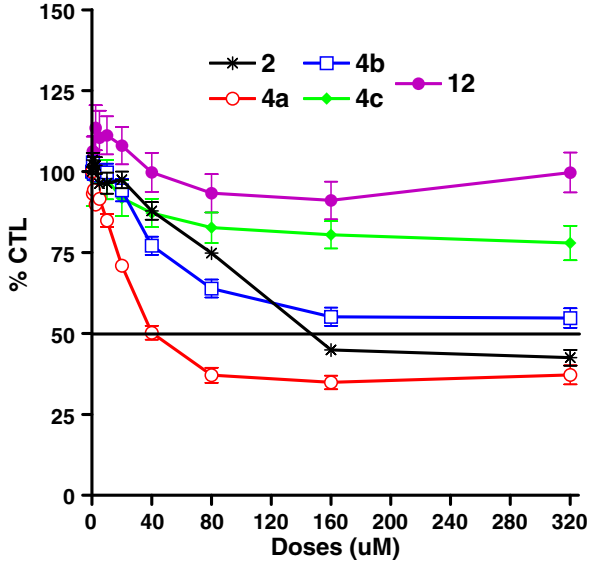


Figure 2. Percent of cell survival in MDA-MB-436 cell line at different concentrations of synthesized compounds after 72 h.

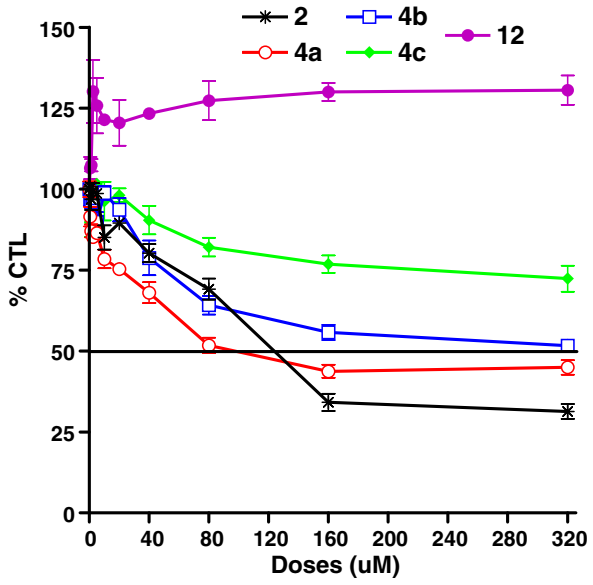


Figure 3. Percent of cell survival in MDA-MB-468 cell line at different concentrations of synthesized compounds after 72 h.

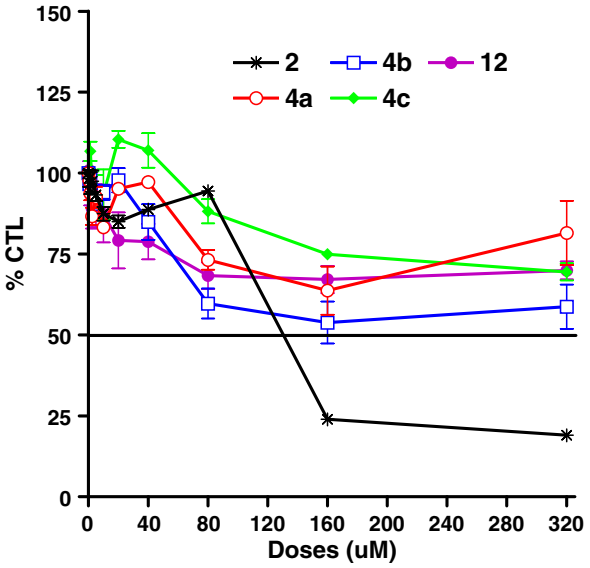


Figure 4. Percent of cell survival in MCF-7 cell line at different concentrations of synthesized compounds after 72 h.

estradiol framework of type (**3**) and cytotoxic molecule chlorambucil (**2**). Cytotoxic activity of these compounds in hormone-independent cancer cell lines (MDA-MB-436 and MDA-MB-486) showed the effect of chain length. In fact, there is a gradual decrease in activity of the hybrids with an increase in spacer length. Compound **4a**, with a four carbon atoms spacer, was found to be most active in hormone-independent cell lines. However, such structure–activity relationship could not be established for these compounds in hormone-dependent cell line (MCF-7). In MCF-7 cell line, the hybrid with six carbon atoms spacer was found to be most active followed by compound with four carbon atoms spacer among this series of hybrids. It is noteworthy that derivative **12**, bearing a carboxymethyl function at position 16β, is essentially inactive in all the breast cancer cells tested. This further indicates that the hydroxymethyl function at position 16β is beneficial for biological activity of the hybrids **4**.

In conclusions, the new estradiol–chlorambucil hybrids **4** showed moderate to significant cytotoxic activity in hormone-dependent (MCF-7) and hormone-independent (MDA-MB-436 and MDA-MB-486) breast cancer cell lines. The novel hybrids were synthesized in good yields through modification of estrone (**5**) at position 16 using simple and efficient chemistry. The MTT cytotoxicity studies of the compounds indicated that these novel hybrid molecules of type **4** have chemotherapeutic potential which could be used as cytotoxic agent in treatment of cancer. The true capacity of the molecules to treat hormone-dependent cancer will be more clearly expressed in vivo. Further evaluation of these compounds would be useful for the development of new anticancer drugs.

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22. *Typical procedure for the synthesis of estradiol–chlorambucil conjugate* **4***:* The synthesis of derivatives 3-tetrahydropyranyloxy-1,3,5(10)-estratrien-17-one (**6**) and 3-tetrahydropyranyloxy-16α,β-methoxycarbonyl-1,3,5(10)-estratrien-17-one (**7**) was already described in an earlier report.[16]
Synthesis of 3-tetrahydropyranyloxy-16β-(methoxycarbonyl)-16α-(6-bromohexyl)-1,3,5(10)-estratrien-17-one (**8**): Compound **7** (2.01 g, 4.87 mmol) was dissolved in 58 mL of dichloromethane, to it 1,6-dibromohexane (29.22 mmol), triethylbenzyl-ammonium chloride (525 mg) and 30 mL of 10% w/v NaOH solution were added. The reaction mixture was refluxed for 24 h with vigorous stirring, diluted with ether, washed with saturated NH_4Cl solution and then with water (4×). The organic phase was dried over $MgSO_4$, filtered, evaporated and the residue was purified by column chromatography using acetone-hexane (1:9) as the eluent to give **8** in 50% yield. Spectral data for **8** ($n = 4$): IR (ν_{max}, cm^{-1}): 1751 (C=O, ester), 1724 (C=O, ketone), 1611 (C=C); ^1H NMR (200 MHz, $CDCl_3$, δ ppm): 7.18 (d, $J = 8.60$ Hz, 1H, ArH), 6.88–6.79 (m, 2H, ArH), 5.39 (m, 1H, OCHO), 3.91–3.77 and 3.69–3.55 (two m, 2H, OCH_2) 3.72 (s, 3H, OCH_3), 3.38 (t, $J = 6.64$ Hz, 2H, CH_2Br), 2.89 (m, 2H, CH_2), 2.44–1.22 (several m, 27H, 3 × CH and 12 × CH_2), 0.92 (s, 3H, CH_3); ^{13}C NMR (50 MHz, $CDCl_3$, δ ppm): 214.3, 172.1, 155.3, 137.8, 132.9, 126.4, 116.7, 114.3, 96.6, 62.2, 60.4, 52.9, 49.8, 46.2, 44.3, 38.1, 35.6, 34.1, 32.9, 32.3, 30.8, 30.6, 29.8, 29.1, 28.1, 26.7, 25.9, 25.5, 18.9, 14.3; ESI+ HRMS: $(M+H)^+$ and $(M+Na)^+$ calculated for $C_{31}H_{43}BrO_5$: 575.2367 and 597.2186; found = 575.2369 and 597.2184, respectively.
Synthesis of 3-tetrahydropyranyloxy-16β-(methoxycarbonyl)-16α-(6-azidohexyl)-1,3,5(10)-estratrien-17-one (**9i**): Compound **8** (1.5 g, 2.61 mmol) was dissolved in methanol (20 mL) and sodium azide (NaN_3, 5 equiv) was added to this solution. The solution was heated to reflux for 20 h with stirring. The solvent was evaporated and diethyl ether was added to the residue. The ethereal solution was washed with water, dried over magnesium sulfate, filtered and concentrated to the oily product. The crude oily product was purified by flash chromatography using acetone–hexane (1:9) as the eluent which yielded compound **9i** in 85% yield. Spectral data for **9i** ($n = 4$): IR (ν_{max}, cm^{-1}): 2094 (N_3), 1752 (C=O, ester), 1725 (C=O, ketone), 1609 (C=C); ^1H NMR (200 MHz, $CDCl_3$, δ ppm): 7.18 (d, $J = 8.60$ Hz, 1H, ArH), 6.88–6.79 (m, 2H, ArH), 5.38 (m, 1H, OCHO), 3.97–3.85 and 3.69–3.55 (two m, 2H, OCH_2), 3.72 (s, 3H, OCH_3), 3.25 (t, $J = 6.64$ Hz, 2H, CH_2N_3), 2.88 (m, 2H, CH_2), 2.44–1.22 (several m, 27H, 3 × CH and 12 × CH_2), 0.92 (s, 3H, CH_3); ESI+ HRMS: $(M+Na)^+$ calculated for $C_{31}H_{43}N_3O_5$: 560.31010; found = 560.30988. A portion of this product was deprotected (reflux with ethanol in presence of pyridinium-*p*-toluenesulfonate (PPTs) for 3 h) for full characterization: *3-hydroxy-16β-(methoxycarbonyl)-16α-(6-azidohexyl)-1,3,5(10)-estratrien-17-one* (**9ii,** 3-OH): IR (ν_{max}, cm^{-1}): 3435 (OH), 2093 (N_3), 1750 (C=O, ester), 1722 (C=O, ketone), 1611 (C=C); ^1H NMR

(200 MHz, $CDCl_3$, δ ppm): 7.15 (d, J = 8.60 Hz, 1H, ArH), 6.70–6.58 (m, 2H, ArH), 5.00 (br s, 1H, OH), 3.72 (s, 3H, OCH_3), 3.24 (t, J = 6.64 Hz, 2H, CH_2N_3), 2.88 (m, 2H, CH_2), 2.44–1.10 (several m, 21H, 3 × CH and 9 × CH_2), 0.92 (s, 3H, CH_3); ^{13}C NMR (50 MHz, $CDCl_3$, δ ppm): 214.9, 172.1, 153.9, 138.2, 132.0, 126.7, 115.5, 113.1, 60.4, 52.9, 51.6, 49.8, 46.2, 44.2, 38.1, 35.7, 32.3, 30.8, 29.6, 29.5, 29.0, 26.7, 26.0, 25.5, 14.3; ESI+ HRMS: $(M+Na)^+$ calculated for $C_{26}H_{35}N_3O_4$: 476.2520; found = 476.2519.

Synthesis of 3-tetrahydropyranyloxy-16β-(methoxycarbonyl)-16α-(6-aminohexyl)-1,3,5(10)-estratrien-17-one (**10i**): To the solution of compound **9i** (0.856 g, 1.67 mmol) in tetrahydrofuran (THF, 15 mL), 10% Pd/C (0.150 g) was added. Hydrogen gas was bubbled to this solution for 10 min and the solution stirred for 24 h. The reaction mixture was filtered through a pad of Celite™ that washed with excess diethyl ether to recover the product. The solvent was evaporated and the crude product was used as such in the next reaction (99% yield). Spectral data for **10i** (n = 4): IR (ν_{max}, cm^{-1}): 3374 (NH), 1754 (C=O, ester), 1725 (C=O, ketone), 1612 (C=C); ^1H NMR (200 MHz, $CDCl_3$, δ ppm): 7.18 (d, J = 8.60 Hz, 1H, ArH), 6.88–6.81 (m, 2H, ArH), 5.39 (m, 1H, OCHO), 3.96–3.85 and 3.62–3.56 (two m, 2H, OCH_2), 3.73 (s, 3H, OCH_3), 3.00 (m, 2H, CH_2NH_2), 2.89 (m, 2H, CH_2), 2.65 (m, 2H, CH_2NH_2), 2.37–1.25 (several m, 27H, 3 × CH, 12 × CH_2), 0.93 (s, 3H, CH_3).

For full characterization the deprotected derivative (**10ii**) was prepared from **9i** using PPTs as described above: *3-hydroxy-16β-(methoxycarbonyl)-16α-(6-aminohexyl)-1,3,5(10)-estratrien-17-one* (**10ii,** 3-OH): ^1H NMR (200 MHz, $CDCl_3$, δ ppm): 7.08 (d, J = 8.60 Hz, 1H, ArH), 6.62–6.56 (m, 2H, ArH), 3.71 (s, 3H, OCH_3), 3.60 (m, 2H, CH_2NH_2), 2.85 (m, 2H, CH_2), 2.71 (m, 3H, OH and CH_2NH_2), 2.43–1.17 (several m, 21H, 3 × CH, 9 × CH_2), 0.91 (s, 3H, CH_3); ^{13}C NMR (50 MHz, $CDCl_3$, δ ppm): 214.2, 171.9, 154.5, 137.7, 131.0, 126.3, 115.5, 113.1, 60.1, 52.6, 49.5, 46.0, 44.0, 41.8, 38.0, 35.5, 33.0, 32.1, 30.6, 29.5, 29.4, 26.5, 25.7, 25.6, 25.3, 14.1; ESI+ HRMS: $(M+H)^+$ and $(M+Na)^+$ calculated for $C_{26}H_{37}NO_4$: 428.2795 and 450.2615; found = 428.2795 and 450.2611, respectively.

Synthesis of 3-tetrahydropyranyloxy-16β-methoxycarbonyl-16α-(4,4-([bis-(2-chloroethyl)-amino]-phenyl)-butanoylaminobutyl)-1,3,5(10)-estratrien-17-one (**11i**): Chlorambucil (0.192 g, 0.63 mmol), 1-hydroxybenzotriazole (HOBt, 0.085 g, 0.63 mmol) and dicyclohexylcarbodiimide (DCC, 0.130 g, 0.63 mmol) were dissolved in dry dimethylformamide (DMF, 1.5 mL) and stirred at room temperature (22 °C) for 15–20 min until a white precipitate was separated out. To this mixture, compound **10i** (0.322 g, 0.63 mmol), dissolved in dry DMF (1.5 mL), and 2–4 drops of triethylamine were added under constant stirring for 20 h. The excess solvent was evaporated under rotary evaporator. The residue was dissolved in ether and extracted with water. The organic layer was dried over magnesium sulfate and concentrated to oily residue. The crude product was purified by flash chromatography using acetone-hexane (3:7) as the eluent which gave pure compound **11i** with 55% yield. Spectral data for **11i** (n = 4): IR (ν_{max}, cm^{-1}): 3315 (NH), 1752 (C=O, ester), 1725 (C=O, ketone); ^1H NMR (200 MHz, $CDCl_3$, δ ppm): 7.14–7.04 (m, 3H, ArH), 6.65–6.57 (m, 4H, ArH), 5.45 (br s, 1H, NH), 5.38 (m, 1H, OCHO), 3.95–3.85 and 3.69–3.55 hidden (two m, 2H, OCH_2), 3.71 (s, 3H, OCH_3), 3.66–3.49 (m, 8H, $N(CH_2CH_2Cl)_2$, 3.20 (m, 2H, CH_2N), 2.80 (m, 2H, CH_2), 2.54 (t, J = 7.20 Hz, 2H, CH_2CONH), 2.41–1.10 (several m, 31H, 3 × CH, 14 × CH_2), 0.92 (s, 3H, CH_3); ESI+ HRMS: $(M+H)^+$ calculated for $C_{45}H_{62}Cl_2N_2O_6$: 797.40630; found = 797.40651.

For full characterization deprotected derivative (**11ii**) was synthesized from the coupling reaction between **10ii** and chlorambucil using the same procedure as for the preparation of **11i**: *3-hydroxy-16β-methoxycarbonyl-16α-(4,4-([bis-(2-chloroethyl)-amino]-phenyl)-butanoylaminobutyl)-1,3,5(10)-estratrien-17-one* (**11ii,** 3-OH): IR (ν_{max}, cm^{-1}): 3296 (OH), 1752 (C=O, ester), 1723 (C=O, ketone); ^1H NMR (200 MHz, $CDCl_3$, δ ppm): ^1H NMR (200 MHz, $CDCl_3$, δ ppm): 7.14–7.04 (m, 3H, ArH), 6.68–6.61 (m, 4H, ArH), 5.46 (br s, 1H, NH), 3.71(s, 3H, –OCH_3), 3.70–3.61 (m, 9H, $N(CH_2CH_2Cl)_2$, OH hidden) 3.22 (q, J = 6.2 Hz, 2H, CH_2N), 2.82 (m, 2H, CH_2), 2.55 (t, J = 7.40 Hz, 2H, CH_2CONH), 2.43–1.09 (several m, 25H, 3 × CH, 11 × CH_2), 0.92 (s, 3H, CH_3). ^{13}C NMR (50 MHz, $CDCl_3$, δ ppm): 214.2, 172.9, 171.9, 154.0, 144.2, 137.8, 131.5, 130.8, 129.7, 126.4, 115.4, 113.0, 112.3, 60.1, 53.6, 52.6, 49.5, 49.3, 46.0, 44.0, 40.5, 39.5, 37.9, 36.0, 35.4, 34.1, 33.8, 32.1, 30.7, 29.6, 29.4, 27.4, 26.6, 25.8, 25.6, 25.3, 24.9, 14.1; ESI+ HRMS: $(M+H)^+$ and $(M+Na)^+$ calculated for $C_{40}H_{54}Cl_2N_2O_5$: 713.3483 and 7735.3302; found = 713.3484 and 735.3293, respectively.

Synthesis of 3-hydroxy-16β-hydroxymethyl-16α-(4,4-([bis-(2-chloroethyl)-amino]-phenyl)-butanoylaminohexyl)-1,3,5(10)-estratrien-17-ol **4b** (**4**, n = 4): In a round bottomed flask, compound **11i** (0.45 g, 0.56 mmol) was dissolved in dry diethyl ether (10 mL), to this solution an excess of lithium borohydride (6 equiv $LiBH_4$) was added in portions at 0 °C. The reaction mixture was stirred at room temperature (22 °C) for 24 h. After completion of the reaction, the reaction mixture was neutralized with saturated ammonium chloride solution and extracted with ether. The organic layer was separated and dried over magnesium sulfate. The ether layer was concentrated on rotatory evaporator and the crude residue was then refluxed with pyridinium-*p*-toluenesulfonate in ethanol for 3 h; neutralized with saturated ammonium chloride solution, diluted with diethyl ether, then washed with saturated ammonium chloride solution 2× and water 4×, respectively. Thereafter organic phase was dried over $MgSO_4$, evaporated and purified by column chromatography on silica gel using acetone–hexane (4:6) as the eluent which gave pure compound with 44% yield. Spectral data for **4b**: IR (ν_{max}, cm^{-1}): 3306 (OH); ^1H NMR (200 MHz, $CDCl_3$, δ ppm): 7.14–7.04 (m, 3H, ArH), 6.65–6.57 (m, 4H, ArH), 6.10 (br s, 1H, OH), 5.58 (t, J = 5.6 Hz, 1H, NH), 3.82–3.44 (m, 13H, $N(CH_2CH_2Cl)_2$, CH, CH_2OH, OH), 3.22 (q, J = 6.2 Hz, 2H, CH_2N), 2.79 (m, 2H, CH_2), 2.55 (t, J = 7.40 Hz, 2H, CH_2CONH), 2.30–1.03 (several m, 25H, 3 × CH, 11 × CH_2), 0.86 (s, 3H, CH_3). ^{13}C NMR (50 MHz, $CDCl_3$, δ ppm): 173.4, 154.2, 144.6, 138.2, 132.3, 130.9, 129.9, 126.5, 115.6, 113.1, 112.4, 90.8, 67.2, 53.8, 47.9, 47.0, 45.1, 44.1, 40.8, 39.7, 39.5, 38.2, 36.3, 34.3, 33.4, 30.1, 29.8, 27.7, 27.0, 26.5, 24.8, 12.2. ESI+ HRMS: $(M+H)^+$ and $(M+Na)^+$ calculated for $C_{39}H_{56}Cl_2N_2O_4$: 687.36899 and 709.35093; found = 687.36964 and 709.35232, respectively.

Synthesis of 3-hydroxy-16β-methoxycarbonyl-16α-(4,4-([bis-(2-chloroethyl)-amino]-phenyl)-butanoylaminohexyl)-1,3,5(10)-estratrien-17-ol (**12**): This derivative was synthesized using the same procedure as above for derivative **4b**, in this case, only 1 equiv of $LiBH_4$. Spectral data: IR (ν_{max}, cm^{-1}): 3325 (OH), 1730 (C=O, ester), 1704 (C=O, ketone); NMR (200 MHz, $CDCl_3$, δ ppm): 7.11–7.04 (m, 3H, ArH), 6.64–6.59 (m, 4H, ArH), 5.80 (br s, 1H, OH), 5.48 (m, 1H, NH), 4.65 (d, J = 7.4 Hz, 1H, *CH*OH), 3.71 (s, 3H, OCH_3), 3.69–3.40 (m, 9H, $N(CH_2CH_2Cl)_2$, OH), 3.22 (q, J = 6.3 Hz, 2H, CH_2N), 2.80 (m, 2H, CH_2), 2.54 (t, J = 7.40 Hz, 2H, CH_2CONH), 1.94–1.03 (several m, 25H, 3 × CH, 11 × CH_2), 0.72 (s, 3H, CH_3). ^{13}C NMR (50 MHz, $CDCl_3$, δ ppm): 178.9, 173.2, 154.1, 144.6, 138.1, 132.3, 130.9, 129.9, 126.7, 115.5, 113.1, 112.4, 90.0, 66.9, 53.8, 52.3, 52.0, 46.6, 45.3, 44.2, 42.4, 40.8, 39.7, 38.4, 37.2, 36.6, 36.3, 34.3, 29.8, 27.7, 27.5, 26.9, 26.4, 25.6, 11.9. ESI+ HRMS: $(M+H)^+$ and $(M+Na)^+$ calculated for $C_{40}H_{56}Cl_2N_2O_5$: 715.36390 and 737.34585; found = 715.36324 and 737.34571, respectively.

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