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Synthesis of unique 17β-estradiol homo-dimers, estrogen receptors binding affinity evaluation and cytocidal activity on breast, intestinal and skin cancer cell lines

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ABSTRACT

A rapid and efficient synthesis of a series of C_2-symmetric 17β-estradiol homo-dimers is described. The new molecules are linked at position 17α of the steroid nucleus with either an alkyl chain or a polyethylene glycol chain. They are made from estrone in only five chemical steps with an overall yield exceeding 30%. The biological activity of these compounds was evaluated *in vitro* on estrogen dependent and independent (ER^+ and ER^-) human breast tumor cell lines: MCF-7 and MDA-MB-231. Some of the dimers present selective cytotoxic activity against the ER^+ cell line. However, they are not very cytotoxic when compared to the antiestrogen tamoxifen. Unfortunately, they show only weak affinity for the estrogen receptor alpha (ERα) and no affinity for the estrogen receptor beta (ERβ). The new compounds were also tested on human intestinal (HT-29) cancer and on murine skin cancer (B16-F10) cell lines for further biological assessment. Interestingly, the dimers were found to be cytotoxic to the murine skin cancer cell line but were inactive towards the intestinal cancer cell line.

1. Introduction

Symmetry is very frequently found in nature. It is often considered the mark of beauty when one thinks of the human body or any living and non-living component of the world. The simplest and most common of all symmetries is bilateral symmetry [1]. A literature survey related to molecular bilateral symmetry of natural products was recently performed in order to assess a possible role of symmetry in nature [2]. Not surprisingly, the design of C_2-symmetric ligands (bivalent ligands) as bioactive molecules have attracted considerable attention over the years because of their promising therapeutic value in treating a number of diseases. Symmetry plays a crucial role in a variety of biological processes. For instance, many protein receptors, upon activation, dimerize to its active form and subsequently produce its biological actions. Hence, there is a renewal of curiosity for the synthesis of dimeric molecules (or bivalent ligands) capable, not only of interacting with specific biologic receptors, but also of inducing greater biological responses than the corresponding monomeric counterpart. This is a vast and diverse theme of research that was recently reviewed in the literature [3].

Some attempts have been made to design estrogenic bivalent ligands (BL) in order to interfere with the process of estrogen receptor dimerization [4,5]. Fig. 1 illustrates the binding interactions of a bivalent ligand competing with 17β-estradiol

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(E_2) for the estrogen receptor (ER). There are several possible interactions of a BL and/or E_2 with the ER. The native hormone, following binding, results in the dimerization of the receptor (**A**) while BL, with a suitable chain length, could allow ER dimerization (**B**) and subsequent biological actions [6]. On the other hand, BL with an inappropriate chain length could forbid dimerization and biological actions through the formation of several dead-end complexes, for example C_1 and C_2. Estrogens are known to regulate many physiological processes in mammals such as reproduction, cardiovascular health, bone integrity and behaviour [6]. Hence, the development of estrogenic BL is a subject of utmost importance as it could lead to the discovery of original ER antagonists.

In an earlier study the dimers were made of two hexestrol molecules of the erythro (or threo) configuration with either a carbon linker or a polyethylene glycol linker. The dimers **1** and **2** were readily synthesised from appropriate hexestrol derivatives in good yields. Some of these non-steroidal bivalent ligands possess antiestrogenic activity [4]. The bivalent ligands bind to the ER with a relative affinity of 1–7%. In some cases, as for dimer **1** ($n = 8$), a biphasic interaction with the ER was observed indicating possible structure-specific negative site-site interaction [4].

We have synthesized a series of six non-steroidal homo- and heterobifunctional-estrogenic dimers **3** designed for the treatment of breast cancer. They are made of two triphenylethylene moieties linked by an aliphatic chain. It was observed that the cytotoxicity of the dimers increased with the number of hydroxyl groups present on the aromatic rings. The symmetrical triphenylethylene dimer bearing six hydroxyl functions **3** ($R = R' = OH$), possesses a cytotoxic activity similar to that of tamoxifen (see compound **4**) [5]. However, this type of molecule did not present any selectivity towards ER^+ (MCF-7) human breast cancer cells [5].

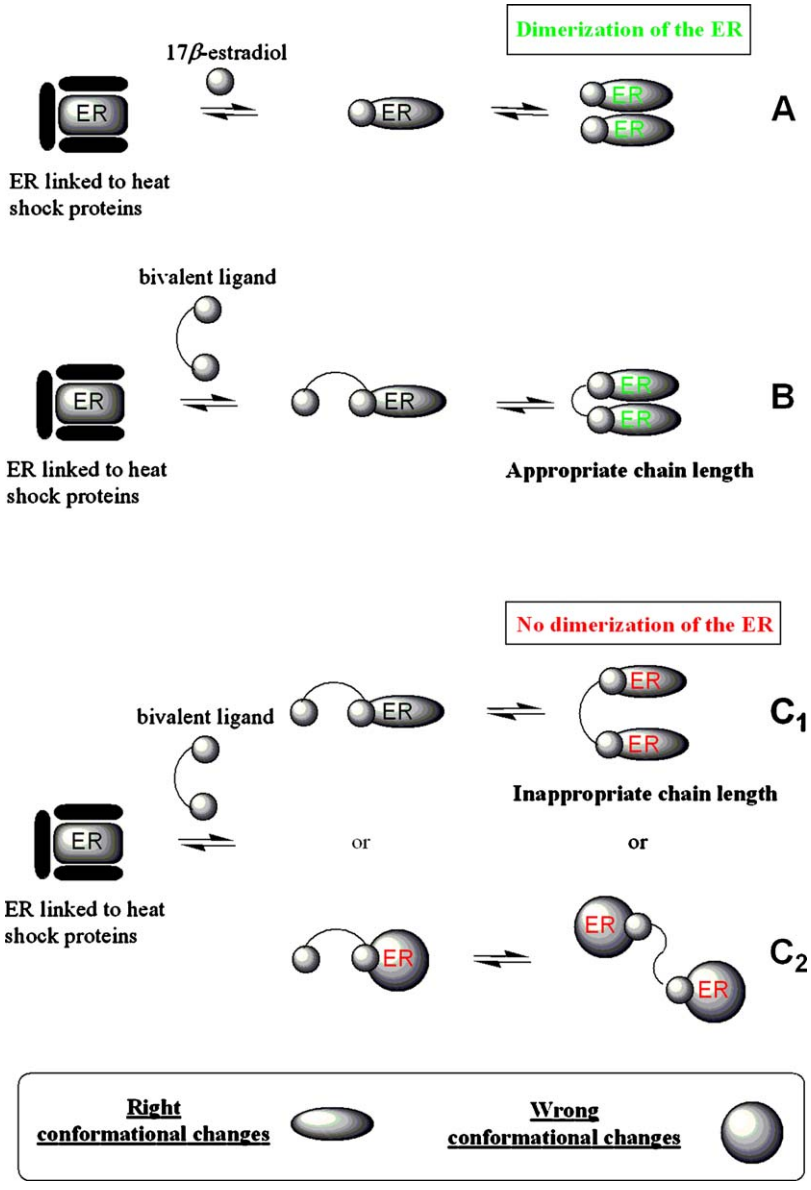


Fig. 1 – Models of the binding interactions of a bivalent ligand competing with 17β-estradiol for the estrogen receptor (ER) [3,4]. Natural conformational changes (⬭) lead to the dimerization of the ER (green). Inappropriate chain length or altered conformational changes (●) of the ER forbid the ER dimerization (red).

Recently, we have synthesized a series of estrone dimers **5** linked at position 16 of the steroid nucleus [7]. They are made from estrone in six chemical steps with an overall yield exceeding 45%. These dimers were linked via two ester groups with an alkyl chain or a polyethylene glycol (PEG) chain using carbodiimide chemistry performed on a suitable acid. It was shown that the diastereoisomeric mixture of estrone dimers **5** were non-toxic towards ER^+ (MCF-7) and ER^- (MDA-MB-231) human breast cancer cell lines [7]. With these results in hand, there was no need to pursue the stereoselective synthesis of this kind of dimers.

In order to obtain dimers with a stronger linkage than the ester bonds found in the previously described molecules, we planned the synthesis of 17β-estradiol dimers that are linked together via ether bonds [8]. The ether connection should increase the stability of the dimeric molecules while improving its solubility. We believed that the use of the natural steroid nucleus, 17β-estradiol, in the design of such dimeric molecules could increase the chance of interactions with the ER. Therefore, by increasing its interaction with the ER, one would theoretically enhance its biological activity as compared to the non-steroidal dimers. It is also believed that these types of molecules synthesized from the natural female sex hormone, 17β-estradiol, would present essentially no toxicity towards healthy or cancerous cells but could possess unforeseen biological properties. This manuscript gives the detailed description of the synthesis of several members of this unique family of C_2-symmetric 17β-estradiol dimers (**6**) [8]. It also reports the *in vitro* cytotoxic activity of the dimers on two neoplastic human breast cancer cell lines: MCF-7 and MDA-MB-231 (ER^+ and ER^-). The new compounds were also tested on skin (B16-F10) and intestine (HT-29) cancer cell lines for further biological appraisal. Moreover, the dimers were assessed for their ERα and ERβ binding affinities.

2. Experimental

2.1. Chemistry

Anhydrous reactions were performed under an inert atmosphere, the set-up assembled and cooled under dry nitrogen. Unless otherwise noted, starting material, reactant and solvents were obtained commercially and were used as such or purified and dried by standard means [9]. Estrone was purchased from Steraloids Inc., Wilton, NH, USA and the polyethylene glycols were purchased from Sigma–Aldrich Canada Ltd., Oakville, Ont., Canada. Organic solutions were dried over magnesium sulfate ($MgSO_4$), evaporated on a rotatory evaporator and the residue put under reduced pressure. All reactions were monitored by UV fluorescence or staining with iodine. Commercial TLC plates were Sigma T 6145 (polyester silica gel 60 Å, 0.25 mm). Preparative TLC was performed on 1 mm silica gel 60 Å, 20 × 20 plates (Whatman, 4861 840). Flash column chromatography was performed according to the method of Still et al. [10] on Merck grade 60 silica gel, 230–400 mesh. All solvents used in chromatography had been distilled.

The infrared spectra were taken on a Nicolet Impact 420 FT-IR. Mass spectral assays were obtained using a VG Micromass 7070 HS instrument using ionization energy of 70 eV (University of Sherbrooke).

Nuclear magnetic resonance (NMR) spectra were recorded either on a Bruker AMX-II-500 equipped with a reversed or QNP probe (Pharmacor Inc.) or (when indicated) on a Varian 200 MHz NMR apparatus. Samples were dissolved in deuterochloroform ($CDCl_3$), deuteroacetone (acetone-d_6) or deuterodimethylsulfoxide (DMSO-d_6) for data acquisition using tetramethylsilane or chloroform as internal standard (TMS, δ 0.0 ppm for ^1H NMR and $CDCl_3$ δ 77.0 ppm for ^{13}C NMR). Chemical shifts (δ) are expressed in parts per million (ppm), the coupling constants (J) are expressed in hertz (Hz). Multiplicities are described by the following abbreviations: s for singlet, d for doublet, dd for doublet of doublets, t for triplet, q for quartet, m for multiplet, #m for several multiplets and br s for broad singlet.

2.1.1. *General procedure for the reparation of the dibromo-PEGs chains* **8** *(**a–e**)*

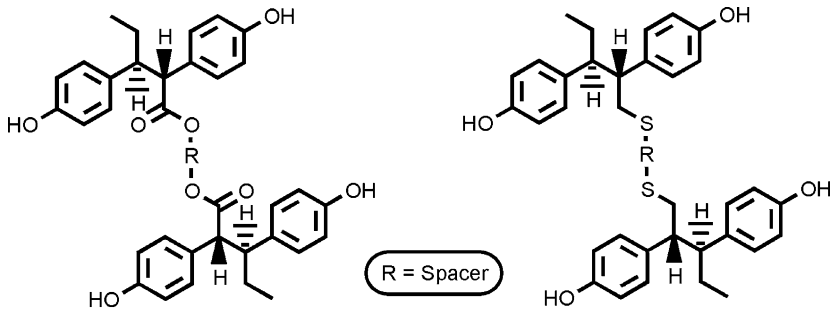
To a solution of an appropriate ethylene glycol (6.66 mmol) in diethyl ether, triethylamine (2.32 mL, 16.65 mmol) was added. The resulting mixture was cooled to 0 °C before slowly adding methanesulfonyl chloride (1.03 mL, 13.32 mmol). The mixture was stirred for 1 h at 0 °C, then was warmed up to room temperature 22 °C and stirred for 2 h. Afterwards, the ether was evaporated and 12 mL dry acetone added, the solution stirred and the triethylamine hydrochloride precipitate was filtered rapidly through a short celite pad. The filtrate containing the dimesylated PEG was treated with lithium bromide (2.31 g, 26.64 mmol) and the mixture was heated to reflux for 20 h. The mixture was filtered on a silica gel (3 cm) and celite (1 cm) pad using hexanes as the eluant. The resulting filtrate was dried, filtered and evaporated to an oil. The dibromo-PEGs **8** (**a–e**) were obtained with yields varying from 50 to 83%.

*2.1.1.1. Spectral data for 1,5-dibromo-3-oxapentane (**8a**).* Fifty percent yield. IR (NaCl, ν_{max}, cm^{-1}): 1279 and 1117 (C–O). ^1H NMR ($CDCl_3$, δ ppm): 3.83 (4H, t, J = 6.3 Hz, 2× CH_2O), 3.47 (4H, t, J = 6.3 Hz, 2× CH_2Br). ^{13}C NMR ($CDCl_3$, δ ppm): 71.0 (CH_2O), 30.0 (CH_2Br).

*2.1.1.2. Spectral data for 1,8-dibromo-3,6-dioxaoctane (**8b**).* Sixty-nine percent yield. IR (NaCl, ν_{max}, cm^{-1}): 1277 and 1121 (C–O). ^1H NMR ($CDCl_3$, δ ppm): 3.80 (4H, t, J = 6.2 Hz, $BrCH_2CH_2O$), 3.67 (4H, s, 2× CH_2O), 3.46 (4H, t, J = 6.4 Hz, 2× CH_2Br). ^{13}C NMR ($CDCl_3$, δ ppm): 71.2 ($BrCH_2CH_2O$), 70.4 (CH_2O), 30.3 (CH_2Br).

*2.1.1.3. Spectral data for 1,11-dibromo-3,6,9-trioxadecane (**8c**).* Seventy-one percent yield. IR (NaCl, ν_{max}, cm^{-1}): 1277 and 1116 (C–O). ^1H NMR ($CDCl_3$, δ ppm): 3.80 (4H, t, J = 6.3 Hz, 2× $BrCH_2CH_2O$), 3.66 (8H, s, 4× CH_2O), 3.46 (4H, t, J = 6.3 Hz, 2× CH_2Br). ^{13}C NMR ($CDCl_3$, δ ppm): 71.2 ($BrCH_2CH_2O$), 70.6 and 70.5 (CH_2O), 30.3 (CH_2Br).

*2.1.1.4. Spectral data for 1,14-dibromo-3,6,9,12-tetraoxatetradecane (**8d**).* Fifty five percent yield. IR (NaCl, ν_{max}, cm^{-1}): 1277 and 1115 (C–O). ^1H NMR ($CDCl_3$, δ ppm): 3.79 (4H, t, J = 6.4 Hz, 2× $BrCH_2CH_2O$), 3.65 (12H, s, 6× CH_2O), 3.45 (4H, t, J = 6.3 Hz, 2× CH_2Br). ^{13}C NMR ($CDCl_3$, δ

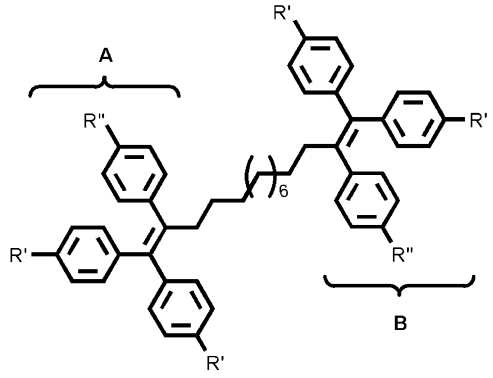


1, Hexestrol dimers

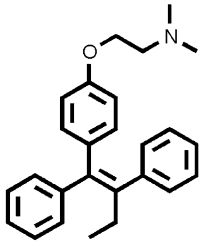
R = -$(CH_2)_n$- (n = 2, 3, 4, 8) or
R = -$CH_2(CH_2OCH_2)_n$-CH_2- (n = 1, 2, 3, 4)

2, Hexestrol dimers

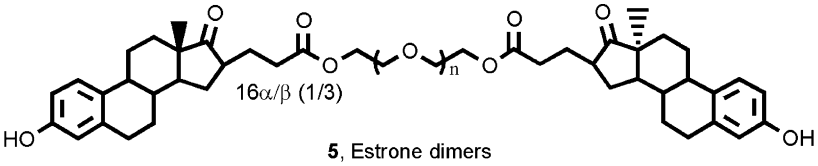
R = -$(CH_2)_n$- (n = 2, 3, 4, 8)



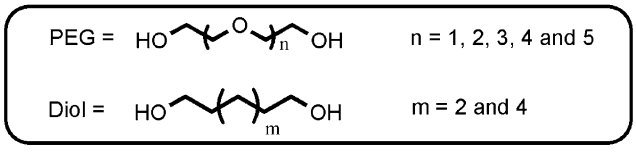
3, Triphenylethylene dimers
R' and R'' = H or OH
Homobifonctionnel (A = B)
Heterobifonctionnel (A ≠ B)

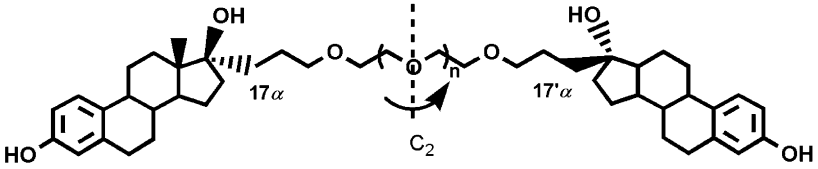


4, Tamoxifen



5, Estrone dimers





6, 17β-Estradiol dimers

ppm): 71.1 ($BrCH_2CH_2O$), 70.6 and 70.53 and 70.48 (CH_2O), 30.3 (CH_2Br).

2.1.1.5. *Spectral data for 1,17-dibromo-3,6,9,12,15-pentaoxaheptadecane (**8e**)*. Eighty-three percent yield. IR (NaCl, ν_{max}, cm^{-1}): 1277 and 1113 (C–O). ^1H NMR ($CDCl_3$, δ ppm): 3.78 (4H, t, J = 6.4 Hz, 2× $BrCH_2CH_2O$), 3.64 (16H, m, 8× CH_2O), 3.45 (4H, t, J = 6.3 Hz, 2× CH_2Br). ^{13}C NMR ($CDCl_3$, δ ppm): 71.1 ($BrCH_2CH_2O$), 70.6 and 70.52 and 70.47 (CH_2O), 30.2 (CH_2Br).

2.1.2. *Synthesis of 17β-estradiol homo dimers **6** (**a–f**)*

2.1.2.1. *Synthesis of 3-benzyloxy-1,3,5(10)-estratrien-17-one (**10**)*. A solution of estrone **9** (1.00 g, 3.70 mmol) in dichloromethane (10 mL), was treated with benzylbromide (0.53 mL, 4.44 mmol), tetrabutylammonium hydrogen sulfate

(100 mg), and a solution of sodium hydroxide (10% w/v, 5 mL). The reaction mixture was stirred vigorously at reflux for 24 h. Then, the mixture was diluted with diethyl ether (30 mL) and water (30 mL) and washed with water (4 mL × 75 mL). The organic phase was dried with magnesium sulfate, filtered and evaporated to yield a solid compound. The residue was triturated with hexanes to give a white solid in 99% yield which was homogeneous by TLC. It was used without further purification at the next step. mp: 128–129 °C. IR (KBr, ν_{max}, cm^{-1}): 1731 (C=O), 1614 (C=C), 1230 and 1008 (C–O). ^1H NMR ($CDCl_3$, δ ppm): 7.41 (2H, d, J = 7.6 Hz, a-CH), 7.36 (2H, t, J = 7.5 Hz, b-CH), 7.29 (1H, t, J = 7.2 Hz, c-CH), 7.18 (1H, d, J = 8.6 Hz, 1-CH), 6.78 (1H, dd, J = 2.5 Hz and J = 8.5 Hz, 2-CH), 6.71 (1H, d, J = 2.1 Hz, 4-CH), 5.01 (2H, s, CH_2Ph), 2.88 (2H, m, 6-CH_2), 2.50–1.39 (13H, #m, 3× CH and 5× CH_2), 0.89 (3H, s, 18-CH_3). ^{13}C NMR ($CDCl_3$, δ ppm): 220.5 (17-C), 156.8 (3-C), 137.7 (CCH_2O), 137.2 (5-C), 132.2 (10-C), 128.4 (b-C), 127.7 (c-C), 127.3 (a-C), 126.2 (1-C), 114.8 (4-C), 112.3 (2-C), 69.8 (CH_2Ph), 50.3, 47.9, 43.9, 38.3, 35.8, 31.5, 29.6, 26.5, 25.8, 21.5, 13.8 (C-18). MS (*m/e*): 360 (M^+), 269 (M^+ - C_7H_7). Exact mass: calculated for $C_{25}H_{28}O_2$ = 360.2089; found = 360.2095. NB: a-CH, b-CH and c-CH are *ortho*, *meta* and *para* protons on the benzyl protecting group. Similarly, a-C, b-C and c-C are the corresponding carbons on the benzyl group.

*2.1.2.2. Synthesis of 3-O-benzyl-17α-(prop-2′-enyl)-1,3,5(10)-estratrien-17β-ol (**11**).* The benzylated estrone **10** (1.50 g, 4.17 mmol) was dissolved in dry diethyl ether (50 mL). Then, a solution of allylmagnesium bromide in diethyl ether (53 mL, 41.70 mmol), prepared form allyl bromide and magnesium, was added until the starting material was completely consumed as indicated by TLC. The resulting mixture was stirred for a further 30 min. Afterwards, the mixture was diluted with diethyl ether (30 mL), washed with a hydrochloric acid solution (75 mL, 4% aquous) and with water (4 mL × 75 mL). The organic phase was dried with magnesium sulfate, filtered and evaporated to a viscous oil. Flash chromatography with a mixture of hexanes and acetone (4:1) gave 1.60 g (95%) of the title compound **11**. IR (KBr, ν_{max}, cm^{-1}): 3558–3470 (O–H), 1601 (C=C), 1222 and 1025 (C–O). ^1H NMR ($CDCl_3$, δ ppm): 7.39 (2H, d, J = 7.5 Hz, a-CH), 7.34 (2H, t, J = 7.5 Hz, b-CH), 7.28 (1H, t, J = 7.4 Hz, c-CH), 7,17 (1H, d, J = 8.5 Hz, 1-CH), 6.75 (1H, dd, J = 8.5 Hz and J = 2.5 Hz, 2-CH), 6.69 (1H, d, J = 2.1 Hz, 4-CH), 6.00 (1H, m, CH_2CH=CH_2), 5.18 (2H, dd, J = 11.0 Hz and J = 18.1 Hz, CH_2CH=CH_2), 4.98 (2H, s, CH_2Ph), 2.84 (2H, m, 6-CH_2), 2.50–1.22 (16 H, #m, 1× OH, 3× CH, 6× CH_2), 0.90 (3H, s, 18-CH_3). ^{13}C NMR ($CDCl_3$, δ ppm): 156.7 (C-3), 137.9 (CCH_2O), 137.3 (C-5), 134.9 (C-2′), 132.9 (C-10), 128.5 (C-b), 127.7 (C-c), 127.4 (C-a), 126.2 (C-1), 119.0 (C-3′), 114.8 (C-4), 112.2 (C-2), 82.4 (C-17), 69.9 (CH_2Ph), 49.6, 46.5, 43.8, 41.8, 39.5, 34.9, 31.8, 29.8, 27.5, 26.3, 23.4, 14.3 (C-18). MS (*m/e*): 402 (M^+), 361 (M^+–C_3H_5). Exact mass: calculated for $C_{28}H_{34}O_2$ = 402.2559; found = 402.2566.

*2.1.2.3. Synthesis of 3-O-benzyl-17α-(3′-hydroxypropyl)-1,3,5(10)-estratrien-17β-ol (**7**).* A stirred solution of alkene **11** (2.20 g, 5.47 mmol) dissolved in anhydrous THF (22 mL) was treated with a 1 M solution BH_3-THF (21.89 mL, 21.89 mmol) at 0 °C under nitrogen atmosphere. The reaction mixture was stirred for 3 h after which time 1 mL water was added to destroy the excess reagent. Then, the mixture was treated with a 30% hydrogen peroxyde solution (2.47 mL, 21.89 mmol) and with an aqueous 3% NaOH solution (29 mL, 21.89 mmol) at 22 °C. The mixture was stirred for 60 min, diluted with ethyl acetate (40 mL), and the resulting organic phase was washed with a saturated ammonium chloride solution (50 mL) and with water (4 mL × 50 mL). The organic phase was dried, filtered and evaporated to an oil. Flash chromatography with a mixture of hexanes and acetone (3:2) gave the desired material **7** in 75% yield. IR (KBr, ν_{max}, cm^{-1}): 3360 (O–H), 1600 (C=C), 1254 and 1017 (C–O). ^1H NMR ($CDCl_3$, δ ppm): 7.41 (2H, d, J = 7.0 Hz, a-CH), 7.36 (2H, t, J = 7.2 Hz, b-CH), 7.30 (1H, d, J = 6.7 Hz, c-CH), 7.18 (1H, d, J = 8.5 Hz, 1-CH), 6.76 (1H, dd, J = 7.9 Hz and J = 2.0 Hz, 2-CH), 6.71 (1H, sl, 4-CH), 5,00 (2H, s, CH_2Ph), 3.67 (2H, m, 3′-CH_2OH), 2.86 (2H, m, 6-CH_2), 2.59 (2H, sl, 2× OH), 2.31–1.20 (17 H, #m, 3× CH and 7× CH_2), 0.90 (3H, s, 18-CH_3). ^{13}C NMR ($CDCl_3$, δ ppm): 156.7 (C-3), 138.0 (CCH_2O), 137.3 (C-5), 132.9 (C-10), 128.5 (C-b), 127.8 (C-c), 127.4 (C-a), 126.2 (C-1), 114.8 (C-4), 112.2 (C-2), 83.2 (C-17), 69.9 (CH_2Ph), 63.3 (C-3′), 49.5, 46.7, 43.8, 39.6, 34.4, 33.4, 31.5, 29.8, 27.5, 26.9 (C-2′), 26.3, 23.4, 14.3 (C-18). MS (*m/e*): 420 (M^+), 402 (M^+–H_2O), 362 (M^+–C_3H_6O). Exact mass: calculated for $C_{28}H_{36}O_3$ = 420.2664; found = 420.2656.

*2.1.2.4. General procedure for the synthesis of dimers **6** (**a–e**) (R = Bn).* A solution of diol **7** (200 mg, 0.48 mmol) dissolved in 3 mL of a mixture of THF:DMF (7:3) was treated with NaH (48 mg, 1.19 mmol). The mixture was stirred for 30 min at 22 °C. Then, the appropriate dibromo-PEG **8** (**a–e**) (0.24 mmol) was added and the resulting solution was stirred for 24 h at 22 °C. Afterwards, the mixture was diluted with 25 mL ethyl acetate and the organic phase was washed with 30 mL saturated NH_4Cl solution as well as with water (4 mL × 30 mL). The organic phase was dried with ahnydrous magnesium sulfate, filtered and evaporated to give the crude product. The residue was adsorbed on silica gel and purified by flash chromatography with hexanes and acetone (3:2) as the eluant to give the protected dimer **6** (**a–e**) (R = Bn) with yield ranging from 40% to 52%.

*2.1.2.5. Spectral data for dimer **6a** (R = Bn).* Forty percent yield. IR (NaCl, ν_{max}, cm^{-1}): 3443 (O–H), 1598 (C=C), 1248 and 1103 (C–O). ^1H NMR ($CDCl_3$, δ ppm): 7.43 (4H, d, J = 7.5 Hz, 2× a-CH), 7.38 (4H, t, J = 7.4 Hz, 2× b-CH), 7.32 (2H, t, J = 7.1 Hz, 2× c-CH), 7.20 (2H, d, J = 8.7 Hz, 2× 1-CH), 6.77 (2H, dd, J = 8.7 Hz and J = 2.4 Hz, 2× 2-CH), 6.73 (2H, s, 2× 4-CH), 5.03 (4H, s, 2× CH_2Ph), 3.68–3.52 (12H, 3m, 6× CH_2O), 2.84 (4H, m, 2× 6-CH_2), 2.40–1,20 (34H, #m, 14× CH_2, 6× CH), 0.92 (6H, s, 2× 18-CH_3). ^{13}C NMR ($CDCl_3$, δ ppm): 156.7 (C-3), 138.0 (CCH_2O), 137.3 (C-5), 133.0 (C-10), 128.5 (C-b), 127.8 (C-c), 127.4 (C-a), 126.2 (C-1), 114.8 (C-4), 112.2 (C-2), 82.9 (C-17), 71.9, 70.5, 70.0, 69.9 (CH_2Ph), 49.5, 46.7, 43.8, 39.6, 34.3, 33.4, 31.6, 29.8, 27.5, 26.3, 24.0, 23.4, 14.4 (C-18). MS (*m/e*): 892 (M^+–H_2O), 874 (M^+–2 H_2O), 801 (M^+–H_2O,–C_7H_7). Exact mass: calculated for $C_{60}H_{76}O_6$ (M^+–H_2O) = 892.5642; found = 892.5638.

*2.1.2.6. Spectral data for dimer **6b** (R = Bn).* Fifty-two percent yield. IR (NaCl, ν_{max}, cm^{-1}): 3418 (O–H), 1614 (C=C), 1230 and 1092 (C–O). ^1H NMR ($CDCl_3$, δ ppm): 7.43 (4H, d, J = 7.6 Hz, 2× a-CH), 7.38 (4H, t, J = 7.7 Hz, 2× b-CH), 7.32 (2H, t, J = 7.1 Hz, 2× c-CH), 7.20 (2H, d, J = 8.6 Hz, 2× 1-CH), 6.78 (2H, dd, J = 8.6 Hz and

2.8 Hz, 2× 2-CH), 6.72 (2H, s, 2× 4-CH), 5.03 (4H, s, 2× CH_2Ph), 3.80–3.50 (16H, s and #m, 8× CH_2O), 2.84 (4H, m, 2× 6-CH_2), 2.66 (2H, sl, 2× OH), 2.40–1.20 (34H, #m, 14× CH_2, 6× CH), 0.92 (6H, s, 2× 18-CH_3). ^{13}C NMR ($CDCl_3$, δ ppm): 156.7 (C-3), 138.0 (CCH_2O), 137.3 (C-5), 133.0 (C-10), 128.5 (C-b), 127.8 (C-c), 127.4 (C-a), 126.3 (C-1), 114.8 (C-4), 112.2 (C-2), 83.0 (C-17), 72.0, 70.6, 70.5, 70.0, 69.9 (CH_2Ph), 49.5, 46.8, 43.8, 39.6, 34.3, 33.4, 31.7, 29.8, 27.5, 26.3, 24.0, 23.4, 14.4 (C-18). MS (*m/e*): 937 (M^+–H_2O), 919 (M^+–2 H_2O), 828 (M^+–H_2O,–C_7H_7). Exact mass: calculated for $C_{62}H_{80}O_7$ (M–H_2O) = 936.5904; found = 936.5887.

*2.1.2.7. Spectral data for dimer **6c** (R = Bn).* Forty-three percent yield. IR (NaCl, ν_{max}, cm^{-1}): 3426 (O–H), 1607 (C=C), 1234 and 1025 (C–O). ^1H NMR (C0DCl_3, δ ppm): 7.42 (4H, d, *J* = 7.2 Hz, 2× a-CH), 7.38 (4H, t, *J* = 7.6 Hz, 2× b-CH), 7.32 (2H, t, *J* = 7.2 Hz, 2× c-CH), 7.20 (2H, d, *J* = 8.6 Hz, 2× 1-CH), 6.77 (2H, dd, *J* = 8.6 Hz and 2.5 Hz, 2× 2-CH), 6.72 (2H, s, 2× 4-CH), 5.03 (4H, s, 2× CH_2Ph), 3.75–3.40 (20H, s, #m, 10× CH_2O), 2,84 (4H, m, 2× 6-CH_2), 2.48 (2H, sl, 2× OH), 2.35–1.20 (34H, #m, 14× CH_2, 6× CH), 0.91 (6H, s, 2× 18-CH_3). ^{13}C NMR ($CDCl_3$, δ ppm): 156.7 (C-3), 138.0 (CCH_2O), 137.3 (C-5), 133.0 (C-10), 128.5 (C-b), 127.8 (C-c), 127.4 (C-a), 126.2 (C-1), 114.8 (C-4), 112.2 (C-2), 83.0 (C-17), 72.0, 70.6, 70.5, 70.0, 69.9 (CH_2Ph), 49.5, 46.7, 43.8, 39.6, 34.4, 33.5, 31.6, 29.8, 27.5, 26.3, 24.0, 23.4, 14.4 (C-18). MS (*m/e*): 980 (M^+–H_2O), 962 (M^+–2 H_2O), 889 (M^+–H_2O,–C_7H_7). Exact mass: calculated for $C_{64}H_{84}O_8$ (M–H_2O) = 980.6166; found = 980.6187.

*2.1.2.8. Spectral data for dimer **6d** (R = Bn).* Forty percent yield. IR (NaCl, ν_{max}, cm^{-1}): 3448 (O–H), 1608 (C=C), 1235 and 1104 (C–O). ^1H NMR ($CDCl_3$, δ ppm): 7.42 (4H, d, *J* = 7.5 Hz, 2× a-CH), 7.37 (4H, t, *J* = 7.4 Hz, 2× b-CH), 7.31 (2H, t, *J* = 7.2 Hz, 2× c-CH), 7.20 (2H, d, *J* = 8.6 Hz, 2× 1-CH), 6.77 (2H, dd, *J* = 8.7 Hz and *J* = 2.4 Hz, 2× 2-CH), 6.71 (2H, d, *J* = 2.3 Hz, 2× 4-CH), 5.03 (4H, s, 2× CH_2Ph), 3.70–3.45 (24H, 2s and #m, 12× CH_2O), 2.83 (4H, m, 2× 6-CH_2), 2.42 (2H, sl, 2× OH), 2.35–1.20 (34H, #m, 14× CH_2, 6× CH), 0.91 (6H, s, 2× 18-CH_3). ^{13}C NMR ($CDCl_3$, δ ppm): 156.7 (C-3), 138.0 (CCH_2O), 137.3 (C-5), 133.0 (C-10), 128.5 (C-b), 127.8 (C-c), 127.4 (C-a), 126.2 (C-1), 114.8 (C-4), 112.2 (C-2), 82.9 (C-17), 72.0, 70.5, 70.0, 69.9 (CH_2Ph), 49.5, 46.7, 43.8, 39.6, 34.4, 33.5, 31.6, 29.8, 27.5, 26.3, 24.0, 23.4, 14.4 (C-18). MS: above the detection limit of the mass spectrometer used.

*2.1.2.9. Spectral data for dimer **6e** (R = Bn).* Forty percent yield. IR (NaCl, ν_{max}, cm^{-1}): 3458 (O–H), 1603 (C=C), 1252 and 1025 (C–O). ^1H NMR-($CDCl_3$, δ ppm): 7.42 (4H, d, *J* = 7.3 Hz, 2× a-CH), 7.38 (4H, t, *J* = 7.4 Hz, 2× b-CH), 7.31 (2H, t, *J* = 7.3 Hz, 2× c-CH), 7.20 (2H, d, *J* = 8.7 Hz, 2× 1-CH), 6.77 (2H, dd, *J* = 8.7 Hz and *J* = 2.4 Hz, 2× 2-CH), 6.72 (2H, d, *J* = 2.3 Hz, 2× 4-CH), 5.03 (4H, s, 2× CH_2Ph), 3.70–3.45 (28H, 2s, m and t, 14× CH_2O), 2.84 (4H, m, 2× 6-CH_2), 2.40–1.20 (36H, #m, 14× CH_2, 6× CH, 2× OH), 0.91 (6H, s, 2× 18-CH_3). ^{13}C NMR ($CDCl_3$, δ ppm): 157.7 (C-3), 139.0 (CCH_2O), 138.3 (C-5), 134.0 (C-10), 129.5 (C-b), 128.7 (C-c), 128.4 (C-a), 127.2 (C-1), 115.7 (C-4), 113.2 (C-2), 83.9 (C-17), 73.0, 71.5, 71.0, 70.9 (CH_2Ph), 50.5, 47.7, 44.8, 40.6, 35.4, 34.5, 32.6, 30.8, 28.5, 27.3, 25.0, 24.4, 14.4 (C-18). MS: above the detection limit of the mass spectrometer used.

*2.1.2.10. General procedure for the synthesis of dimers **6** (**a**–**e**) (R = H).* Palladium on carbon (10%, 10 mg) was added to a solution of protected dimer **6** (**a**–**e**) (R = Bn) (0.07 mmol) dissolved in dry THF (2.5 mL). Then, hydrogen was bubbled into the reaction mixture for 3 min and the hydrogenolysis was performed under 1 atmosphere of hydrogen for 24 h at 22 °C. Afterwards, the catalyst was filtered off and the filtrate evaporated to give the pure 17β-estradiol dimers **6** (**a**–**e**) (R = H) quantitatively.

*2.1.2.11. Spectral data for dimer **6a** (R = H).* IR (NaCl, ν_{max}, cm^{-1}): 3372 (O–H), 1609 (C=C), 1240 and 1093 (C–O). ^1H NMR ($CDCl_3$, CD_3OD, δ ppm): 7.06 (2H, d, *J* = 8.4 Hz, 2× 1-CH), 6.58 (2H, dd, *J* = 8.6 Hz and *J* = 1.6 Hz, 2× 2-CH), 6.52 (2H, s, 2× 4-CH), 3.65–3.40 (12H, #m, 6× CH_2O), 3.03 (4H, sl, 3-OH and 17-OH), 2.76 (4H, m, 2× 6-CH_2), 2.30–1.15 (34H, #m, 14× CH_2, 6× CH), 0.85 (6H, s, 2× 18-CH_3). ^{13}C NMR ($CDCl_3$, CD_3OD, δ ppm): 154.1 (C-3), 137.9 (C-5), 131.8 (C-10), 126.1 (C-1), 115.1 (C-4), 112.6 (C-2), 82.9 (C-17), 72.0, 70.3, 69.9, 49.5, 46.7, 43.7, 39.6, 33.8, 33.2, 31.5, 29.6, 27.4, 26.3, 23.8, 23.3, 14.4 (C-18). MS (*m/e*): 712 (M^+–H_2O), 694 (M^+–2 H_2O). Exact mass: calculated for $C_{46}H_{64}O_6$ (M^+–H_2O) = 712.4703; found = 712.4710.

*2.1.2.12. Spectral data for dimer **6b** (R = H).* IR (NaCl, ν_{max}, cm^{-1}): 3346 (O–H), 1608 (C=C), 1240 and 1092 (C–O). ^1H NMR (acetone-d_6, CD_3OD, δ ppm): 7.07 (2H, d, *J* = 8.4 Hz, 2× 1-CH), 6.56 (2H, dd, *J* = 8.6 Hz and *J* = 2.5 Hz, 2× 2-CH), 6.50 (2H, d, *J* = 1.5 Hz, 2× 4-CH), 3.65–3.40 (16H, s and #m, 8× CH_2O), 3.28 (4H, sl, 3-OH and 17-OH), 2.78 (4H, m, 2× 6-CH_2), 2.35–1.20 (34H, #m, 14× CH_2, 6× CH), 0.91 (6H, s, 2× 18-CH_3). ^{13}C NMR (acetone-d_6, CD_3OD, δ ppm): 155.9 (C-3), 138.5 (C-5), 132.1 (C-10), 127.0 (C-1), 115.9 (C-4), 113.5 (C-2), 83.1 (C-17), 72.7, 71.4, 71.3, 70.9, 50.5, 47.7, 44.8, 40.9, 34.7, 34.2, 32.6, 29.6 (hidden by acetone), 28.5, 27.4, 25.1, 24.2, 15.1 (C-18). MS (*m/e*): 756 (M^+–H_2O), 738 (M^+–2 H_2O). Exact mass: calculated for $C_{48}H_{68}O_7$ (M^+–H_2O) = 756.4965; found = 756.4976.

*2.1.2.13. Spectral data for dimer **6c** (R = H).* IR (NaCl, ν_{max}, cm^{-1}): 3385 (O–H), 1610 (C=C), 1287 and 1036 (C–O). ^1H NMR ($CDCl_3$, δ ppm): 7.05 (2H, d, *J* = 8.5 Hz, 2× 1-CH), 6.59 (2H, d, *J* = 8.2 Hz, 2× 2-CH), 6,51 (2H, d, *J* = 1,4 Hz, 2× 4-CH), 3.65–3.42 (20H, s and #m, 10× CH_2O), 3.06 (4H, br s, 3-OH and 17-OH), 2.76 (4H, m, 2× 6-CH_2), 2.30–1.15 (34H, #m, 14× CH_2, 6× CH), 0.84 (6H, s, 2× 18-CH_3). ^{13}C NMR ($CDCl_3$, δ ppm): 155.1 (C-3), 138.9 (C-5), 132.7 (C-10), 127.1 (C-1), 116.2 (C-4), 113.6 (C-2), 84.0 (C-17), 73.0, 71.3, 70.8, 50.4, 47.7, 44.6, 40.6, 34.8, 34.2, 32.5, 30.6, 28.4, 27.2, 24.8, 24.3, 15.4 (C-18). MS (*m/e*): 800 (M^+–H_2O), 782 (M^+–2 H_2O). Exact mass: calculated for $C_{50}H_{72}O_8$ (M^+–H_2O) = 800.5227; found = 800.5231.

*2.1.2.14. Spectral data for dimer **6d** (R = H).* IR (NaCl, ν_{max}, cm^{-1}): 3383 (O–H), 1611 (C=C), 1248 and 1100 (C–O). ^1H NMR ($CDCl_3$, δ ppm): 7.08 (2H, d, *J* = 8.6 Hz, 2× 1-CH), 6.61 (2H, dd, *J* = 8.6 Hz and *J* = 2.5 Hz, 2× 2-CH), 6.54 (2H, d, *J* = 1.4 Hz, 2× 4-CH), 3.68–3.48 (24H, 2m, 12× CH_2O), 2.76 (4H, m, 2× 6-CH_2), 2.37–1.10 (38H, #m, 14× CH_2, 6× CH, 3-OH, 17-OH), 0.87 (6H, s, 2× 18-CH_3). ^{13}C NMR ($CDCl_3$, δ ppm): 154.0 (C-3), 137.9 (C-5), 132.0 (C-10), 126.2 (C-1), 115.3 (C-4), 112.8 (C-2), 83.2 (C-17), 72.1, 70,5, 70.0, 49.4, 46.7, 43.6, 39.6, 34.2, 33.5, 31.5, 29.7, 27.4, 26.3, 23.9, 23.3, 14,4 (C-18). MS (*m/e*): 844 (M^+–H_2O), 826 (M^+–2 H_2O). Exact mass: calculated for $C_{52}H_{76}O_9$ (M^+–H_2O) = 844.5489; found = 844.5515.

2.1.2.15. *Spectral data for dimer* **6e** *(R = H)*. IR (NaCl, ν_{max}, cm^{-1}): 3428 (O–H), 1613 (C=C), 1286 and 1103 (C–O). ^1H NMR ($CDCl_3$, δ ppm): 7.08 (2H, d, J = 8.63 Hz, 2× 1-CH), 6.73 (2H, sl, 3-OH), 6.62 (2H, d, J = 8.1 Hz, 2× 2-CH), 6.54 (2H, s, 2× 4-CH), 3.68–3.48 (28H, 2s and 2m, 14× CH_2O), 2.76 (4H, m, 2× 6-CH_2), 2.37–1.10 (36H, #m, 14× CH_2, 6× CH, 17-OH), 0.87 (6H, s, 2× 18-CH_3). ^{13}C NMR ($CDCl_3$, δ ppm): 154.0 (C-3), 137.9 (C-5), 132.0 (C-10), 126.2 (C-1), 115.3 (C-4), 112.8 (C-2), 83.2 (C-17), 72.1, 70.47, 70.44, 70.0, 49.4, 46.7, 43.6, 39.6, 34.2, 33.6, 31.5, 29.7, 27.4, 26.3, 23.9, 23.3, 14.4 (C-18). MS (*m/e*): 888 (M^+–H_2O), 870 (M^+–2 H_2O). Exact mass: calculated for $C_{54}H_{80}O_{10}$ (M^+–H_2O) = 888.5751; found = 888.5731.

2.1.2.16. *Synthesis of 3-O-benzyl-17α-(10-bromo-4-oxadecyl)-1,3,5(10)-estratrien-17β-ol (13)*. A solution of the diol **7** (230 mg, 0.55 mmol) in a mixture of THF and DMF (3 mL, 7:3) was treated with NaH (55 mg, 1.37 mmol). The reaction mixture was stirred for 30 min at 22 °C. Afterwards, two equivalent of 1,6-dibromohexane (666 mg, 2.73 mmol) was added and the resulting solution stirred for 24 h at 22 °C. Then, the reaction mixture was diluted with 25 ml ethyl acetate and the organic phase washed with 30 mL saturated NH_4Cl solution and with water (4 mL × 30 mL). The organic phase was dried with magnesium sulfate, filtered and evaporated to an oil. The residue was adsorbed on silica gel and purified by flash chromatography with hexanes and acetone (3:2) as the eluant to give bromide **13** with 66% yield. IR (NaCl, ν_{max}, cm^{-1}): 3418 (O–H), 1609 (C=C), 1230 and 1022 (C-O). ^1H NMR ($CDCl_3$, δ ppm): 7.42 (2H, d, J = 7.5 Hz, a-CH), 7.38 (2H, t, J = 7.5 Hz, b-CH), 7.31 (1H, t, J = 7.3 Hz, c-CH), 7.20 (1H, d, J = 8.7 Hz, 1-CH), 6.77 (1H, dd, J = 1.9 Hz and J = 8.4 Hz, 2-CH), 6.72 (1H, s, 4-CH), 5.03 (2H, s, CH_2Ph), 3.42 (6H, m, 2× CH_2O and CH_2Br), 2,83 (2H, m, 6-CH_2), 2.40–1.20 (26H, #m, 11× CH_2, 3× CH, 1× OH), 0.91 (6H, s, 18-CH_3). ^{13}C NMR ($CDCl_3$, δ ppm): 156.7 (C-3), 138.0 (CCH_2O), 137.3 (C-5), 133.0 (C-10), 128.5 (C-b), 127.8 (C-c), 127.4 (C-a), 126.2 (C-1), 114.8 (C-4), 112.2 (C-2), 82.9 (C-17), 71.6, 70.8, 69.9 (CH_2Ph), 49.5, 46.7, 43.9, 39.6, 34.7, 33.8 (2 carbons), 32.7, 31.7, 29.8, 29.4, 27.9, 27.5, 26.3, 25.4, 24.2, 23.4, 14.4 (C-18). MS (*m/e*): 584 (M^+), 566 (M^+–H_2O), 475 (M^+–H_2O,–C_7H_7). Exact mass: calculated for $C_{34}H_{47}BrO_3$ = 582.2708; found = 582.2704.

2.1.2.17. *Synthesis of dimer* **6f** *(R = Bn)*. NaH (56 mg, 1.41 mmol) was added to a solution of diol **7** (237 mg, 0.56 mmol) in 3 mL of a mixture of THF:DMF (7:3). The mixture was stirred for 30 min at 22 °C. Then, 1,6-dibromohexane (69 mg, 0.28 mmol) was added and the resulting solution was stirred for 24 h at 22 °C. Afterwards, the mixture was diluted with 25 mL ethyl acetate and the organic phase was washed with 30 mL saturated NH_4Cl solution as well as with water (4 mL × 30 mL). The organic phase was dried with ahnydrous magnesium sulfate, filtered and evaporated to give the crude product. The residue was adsorbed on silica gel and purified by flash chromatography with hexanes and acetone (3:2) as the eluant to give the protected dimer **6f** (R = Bn) with 47% yield. IR (NaCl, ν_{max}, cm^{-1}): 3417 (O–H), 1604 (C=C), 1230 and 1025 (C–O). ^1H NMR ($CDCl_3$, δ ppm): 7.42 (4H, d, J = 7.5 Hz, 2× a-CH), 7.37 (4H, t, J = 7.3 Hz, 2× b-CH), 7.31 (2H, t, J = 7.1 Hz, 2× c-CH), 7.20 (2H, d, J = 8.6 Hz, 2× 1-CH), 6.77 (2H, d, J = 8.5 Hz, 2× 2-CH), 6.71 (2H, s, 2× 4-CH), 5.03 (4H, s, 2× CH_2Ph), 3.45 (8H, m, 2× CH_2OCH_2), 2.83 (4H, m, 2× 6-CH_2), 2.40–1,10 (44H, #m, 18× CH_2, 6× CH, 2× OH), 0.91 (6H, s, 2× 18-CH_3). ^{13}C NMR ($CDCl_3$, δ ppm): 156.7 (C-3), 138.0 (CCH_2O), 137.3 (C-5), 133.0 (C-10), 128.5 (C-b), 127.8 (C-c), 127.4 (C-a), 126.3 (C-1), 114.8 (C-4), 112.2 (C-2), 82.9 (C-17), 71.5, 70.9, 69.9 (CH_2Ph), 49.5, 46.7, 43.8, 39.6, 34.6, 33.8, 31.7, 29.8, 29.5, 27.5, 26.3, 26.0, 24.1, 23.4, 14.4 (C-18). MS (*m/e*): 905 (M^+–H_2O), 887 (M^+–2 H_2O). Exact mass: calculated for $C_{62}H_{80}O_5$ (M–H_2O) = 904.6005; found = 904.6024.

2.1.2.18. *Synthesis of dimer* **6f** *(R = H)*. Palladium on carbon (10%, 10 mg) was added to a solution of protected dimer **6f** (R = Bn) (50 mg, 0.05 mmol) dissolved in dry THF (1.5 mL). Then, hydrogen was bubbled into the reaction mixture for 3 min and the hydrogenolysis was performed under 1 atmosphere of hydrogen for 24 h at 22 °C. Afterwards, the catalyst was filtered off and the filtrate evaporated to give the pure 17β-estradiol dimer **6f** (R = H) quantitatively. IR (NaCl, ν_{max}, cm^{-1}): 3333 (O–H), 1610 (C=C), 1248 and 1100 (C–O). ^1H NMR (acetone-d_6, CD_3OD, δ ppm): 7.08 (2H, d, J = 8.3 Hz, 2× 1-CH), 6.56 (2H, dd, J = 8.3 Hz and J = 1.7 Hz, 2× 2-CH), 6.50 (2H, d, J = 1.5 Hz, 2× 4-CH), 3.43–3.38 (8H, m, 2× CH_2OCH_2), 3.30–3.10 (2H, sl, 3-OH and 17-OH), 2.78 (4H, m, 2× 6-CH_2), 2.40–1.10 (42H, #m, 18× CH_2, 6× CH), 0.91 (6H, s, 2× 18-CH_3). ^{13}C NMR (acetone-d_6, CD_3OD, δ ppm): 155.9 (C-3), 138.5 (C-5), 132.2 (C-10), 127.0 (C-1), 115.9 (C-4), 113.5 (C-2), 83.0 (C-17), 72.3, 71.2, 50.5, 47.7, 44.8, 40.9, 34.8, 34.3, 32.6, 30.6, 28.5, 27.4, 26.9, 25.1, 24.2, 15,1 (C-18). 1 carbon hidden by acetone-d_6. MS (*m/e*): 724 (M^+–H_2O), 706 (M^+–2 H_2O). Exact mass: calculated for $C_{48}H_{68}O_5$ (M–H_2O) = 724.5066; found = 724.5071.

2.2. *Biological activity*

2.2.1. *In vitro cytotoxic activity*

The cytotoxicity of the dimers was evaluated on MCF-7, MDA-MB-231, B16-F10 and HT-29 tumor cell lines using the Sulforhodamine B (SRB) colorimetric assay [11,12]. Tumor cell lines were inoculated into 96-well tissue culture plates in 100 μL containing 2 × 103 cells and incubated at 37 °C in a 5% CO_2 atmosphere. After 24 h, freshly solubilized drugs in DMSO were diluted in fresh culture medium. Aliquots of 100 μL containing escalating concentration of drugs (0.1–200 μM) were added to the appropriate microtiter wells already containing 100 μL of culture medium. The cells were incubated 72 h. The supernatant was removed, and the cells were washed with sterile phosphate-buffered saline (PBS, pH 7.4). Assays were stopped by addition of cold trichloracetic acid (TCA) to the wells (final concentration, 10%) followed by their incubation for 60 min at 4 °C. The supernatant was discarded, and the plates were washed five times with tap water and air-dried. Sulforhodamine B solution (50 μL) at 0.1% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 15 min at room temperature. After staining, unbound dye was removed by washing five times with 1% acetic acid, and the plates were air-dried. Bound stain was solubilized with 10 mM Trizma base, and the absorbance was read using a (micro)Quant Universal Microplate Spectrophotometer (Biotek, Winooski, VT) at 585 nm. The results were compared with those of a control reference plate fixed on the treatment day, and the growth inhibition percentage was calculated for each drug contact period.

2.2.2. *Estrogen receptor binding affinity*

The estrogen receptor alpha and beta (ERα and ERβ) affinity assay was performed using recombinant hERα and hERβ (Calbiochem/EMD BioSciences, Darmstadt, Germany) and the HitHunter™ Enzyme Fragment Complementary (EFC) Estrogen Receptor Assay kit (Discoverx Corporation, Fremont, CA) according to manufacturer's protocol [13]. HitHunter™ EFC technology is based on a genetically engineered β-galactosidase enzyme that consists of two fragments termed enzyme acceptor (EA) and enzyme donor (ED). Briefly, estrogen analogs were diluted in DMSO at concentrations ranging from 0.10 to 72.3 nM (10 μL/well) and added to respective wells containing 50 μL of ES Receptor + ED in a 96-well black plate. The plate was then incubated with gentle rocking for 90 min at room temperature to compete for the estrogen receptor binding against labelled enzyme donor-estrogen steroid hormone conjugate (ED-ES conjugate), a small peptide fragment of β-galactosidase (β-gal). Then, 20 μL of EA, an inactive β-gal protein fragment, and 20 μL of Fluorescent substrate was added to each well and unbound ED-ES was free to complement with EA, to form an active enzyme, which subsequently hydrolyse the fluorescent substrate for EFC detection by a microplate reader after an incubation of 1 h at room temperature. The amount of free ED conjugate in the assay is proportional to the concentration of estrogen analogs bound to the estrogen receptor [13]. A standard curve of 17β-estradiol was run in parallel. All assays were done in triplicates.

3. Results and discussion

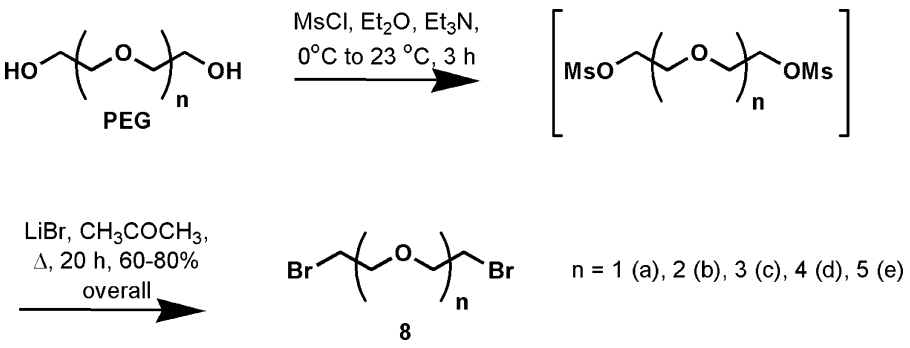
3.1. *Synthesis of 17β-estradiol homo-dimers*

As shown in Scheme 1, the di-, tri- tetra-, penta- and hexaethylene glycols were readily transformed into the dibrominated PEGs in two chemical steps with high yields. Initially, the PEGs were treated with methanesulfonyl chloride and triethylamine in ether to give the dimesylate intermediate which was not isolated. The precipitated triethylamine hydrochloride salt was filtered off and the residue evaporated to an oil. Afterwards, the dimesylate was treated with lithium bromide in acetone at reflux for 20 h to give the desired dibromides **8** with an average overall yield of 70%.

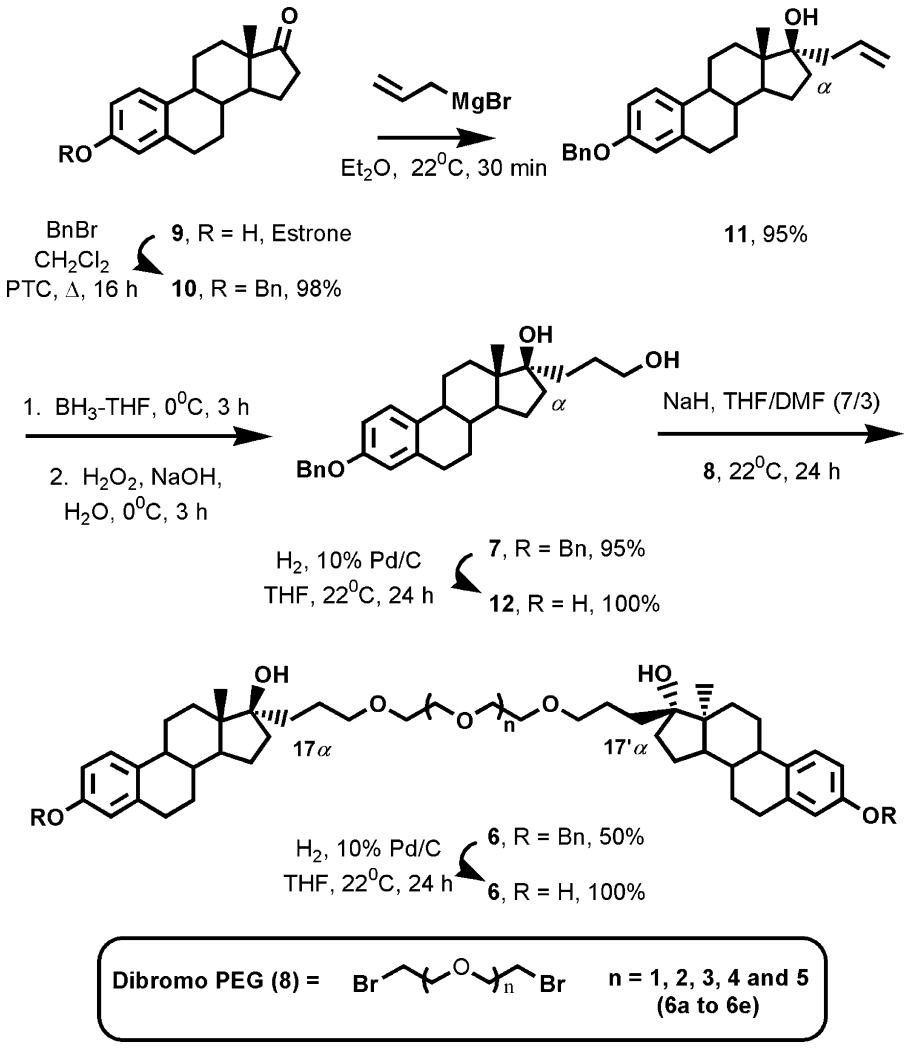
As shown in Scheme 2, five 17β-estradiol dimers were obtained using a straightforward reaction sequence. Initially, estrone (**9**) was protected as a benzyl ether using phase transfer catalysis (PTC) methodology. Thus, estrone was treated with benzyl bromide and tetrabutylammonium hydrogen sulfate in dichloromethane in the presence of a 10% aqueous sodium hydroxide solution [14]. The yield of the protection reaction is 98%. The derivative **10** was transformed into the 17β-(prop-2′-enyl) estradiol **11** upon treatment with freshly prepared allylmagnesium bromide in dry diethylether [15]. Derivative **11** was obtained with 95% yield. The hydroboration-oxydation sequence performed on compound **11** gave the diol **7** in 72% yield [15]. Hydrogenolysis of this intermediate with 10% Pd/C in tetrahydrofuran gave quantitatively the triol **12** [14]. Selective *O*-alkylation of diol **7** was performed upon treatment with sodium hydride for 30 min in a mixture of dry tetrahydrofuran and dimethylformamide to which the appropriate dihalogenated PEG chain **3** (or α,ω-dibromoalkane, see Scheme 3) was added at room temperature for 20 h. The dimers were obtained with an average yield of 50%. The final dimeric molecules were obtained quantitatively by hydrogenolysis of the benzyl ether with 10% Pd/C in tetrahydrofuran. The dimers were obtained with an average overall yield varying from 27% to 36%. All new compounds synthesized were characterized by their respective IR, ^{1}H NMR, ^{13}C NMR and mass spectra.

The ^{1}H NMR and ^{13}C NMR spectra are showing, in the case of dimer **6a** (R = Bn), the presence of only three –CH_2O– signals at 70.0, 70.5, 71.9 ppm, respectively. This clearly indicates that we have in our hands a dimer. The symmetry of the molecule greatly simplifies the NMR spectra, as all the protons and carbons that are equivalent give a single signal. Moreover, mass spectrometry further validates the dimeric structure. All the dimers synthesised in this manuscript present the same spectral characteristic caused by the symmetry of the molecules.

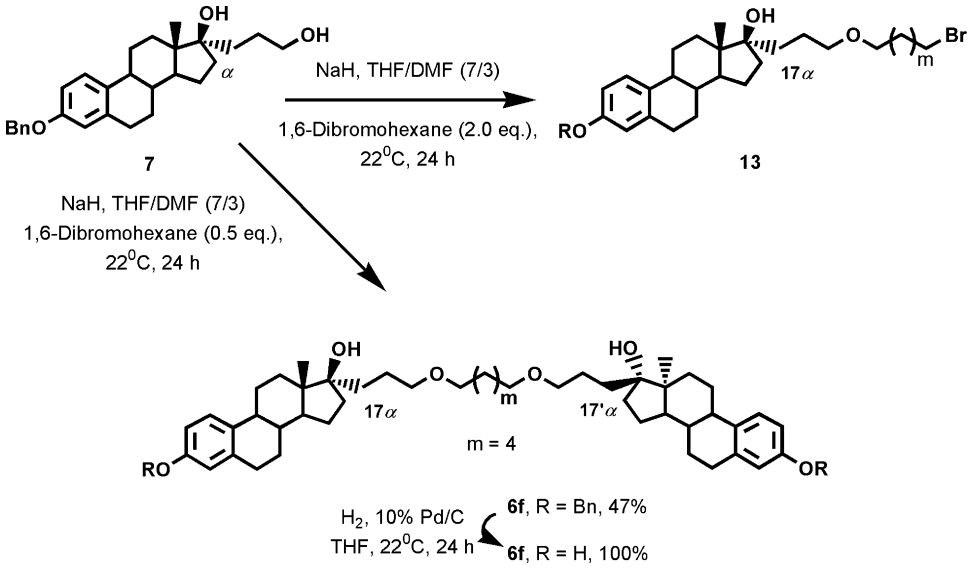
Scheme 3 shows the synthesis of dimer **6f** from alkylation of diol **7** with 0.5 equivalent of 1,6-dibromohexane. The dimer **6f** was obtained with 47% yield. Interestingly, the same alkylation of diol **7** with 2.0 equivalent of 1,6-dibromohexane yielded the monoalkylated derivative **13** in 66% yield. In an attempt to improve the overall yield of formation of the dimers **6f**, derivative **13** was treated with the sodium salt of diol **7** under similar reaction conditions as previously used. Unfortunately, the stepwise formation of the dimer was not successful. Derivative **13** could, however, be a useful synthetic intermediate for the synthesis of other biologically active steroids.



Scheme 1 – Synthesis of dihalogenated PEG chains.



Scheme 2 – Synthesis of 17β-estradiol dimers 6 (a–e).



Scheme 3 – Synthesis of 17β-estradiol dimer 6f and derivative 13.

Table 1 – Inhibitory concentration of drug on ER+ and ER− breast cancer cell lines, on intestine (HT-29) and on skin (B16-F10) cancer cell lines, chain length and C log P

Compounds (R = H)	MCF-7 (ER^+) IC_{50} (μM)[a]	MDA-MB-231 (ER^-) IC_{50} (μM)[a]	HT-29 IC_{50} (μM)[a]	B16-F10 IC_{50} (μM)[a]	Chain length *n* or *m*	C log P[b]
17β-E_2	32	>100	85	29	–	3.78
Tamoxifen	11	19	11	7	–	6.82
12	71	>100	70	57	–	3.37
6a	>200	>200	>200	4.7	*n* = 1	8.22
6b	62	>100	70	18	*n* = 2	8.28
6c	63	>100	83	30	*n* = 3	8.35
6d	>100	>100	>100	5.1	*n* = 4	8.41
6e	>100	>100	>100	20	*n* = 5	8.47
6f	>100	>100	>100	13	*m* = 4	9.32

[a] Inhibitory concentration as obtained by the SRB assay. Experiments were performed in octuplicate, errors are within ±10%.
[b] Calculated log *P* as obtained with ChemDraw Ultra 6.0.

3.2. *In vitro cytotoxic activity*

The cytotoxicity of the dimers was evaluated on several tumor cell lines using the Sulforhodamine B colorimetric assay [11,12]. The cytotoxicity of the compounds was tested along with controls (17β-estradiol and tamoxifen) on both estrogen-receptor positive (ER^+, MCF-7) and estrogen-receptor negative (ER^-, MDA-MB-231) human mammary carcinomas [16]. They were also tested on human intestinal cancer (HT-29) and on murine skin cancer (B16-F10) for additional comparison of activities.

As shown by the SRB assays, the dimers are not very toxic towards human breast cancer cell lines (Table 1). The reference products, 17β-estradiol and tamoxifen are more toxic than the dimers. 17β-Estradiol possesses a IC_{50} of 32 μM on ER^+ MCF-7 cell line but show no apparent activity on ER^- MDA-MB-231 cell line with a IC_{50} of >100 μM. In contrast, the second reference compound, tamoxifen shows cytotoxicity on both ER^+ and ER^- breast cancer cells lines with IC_{50} of 11 and 19 μM, respectively. Interestingly, there is, as it was observed for 17β-estradiol, a selective activity of dimers **6b** (IC_{50} = 62 μM) and **6c** (IC_{50} = 63 μM) against the ER^+ cell line. The lower homologue **6a** and the higher homologues **6d** and **6e** present no cytotoxicity showing an IC_{50} of more than 100 μM. The total length of the chain linking compounds **6b** and **6c** is 16 and 19 atoms long. The shorter linkage seen in derivative **6a** (13 atoms long) as well as the longer linkages seen in compounds **6d** and **6e** (22 and 25 atoms long) appear to be inadequate for selective biological activity on ER^+ cancer cells. The calculated log *P* of the dimers is relatively constant throughout the sequence varying from 8.22 to 8.47 (Table 1). The dimer **6f** bearing a carbon atom linking chain is not toxic and possesses a C log *P* of 9.32 and is therefore less soluble than the PEG dimeric analogs. On the other hand, the triol **12** shows some toxicity against the ER^+ (MCF-7) breast cancer cells with a IC_{50} of 71 μM.

In an attempt to verify if the dimers are active towards other types of cell lines, they were tested on human intestinal cancer (HT-29) and on murine skin cancer (B16-F10) cell lines. The dimers were essentially inactive towards intestinal HT-29 cancer cell line with IC_{50} ranging from 71 to >200 μM. Surprisingly, all the dimers showed cytotoxic activities towards the murine skin cancer (B16-F10) cells with IC_{50} ranging from 4.7 to 30 μM. To the best of our knowledge, this is the first example of selective anticancer activity of 17β-estradiol dimers towards skin cancer. This unique property, if observed on human skin cancers, could in the future provide alternate treatment for skin cancer that is known to afflict numerous people world wide [17]. It is noteworthy that the murine melanoma cells B16-F10 are commonly used in the literature as the standard model to test the efficacy of anticancer agents in *in vitro* as well as *in vivo*, which can be eventually applied in the treatment of human melanomas [18,19].

3.3. *Estrogen receptor binding affinity*

The estrogen receptor alpha and beta (ERα and ERβ) affinity assay was performed using the HitHunter™ EFC Estrogen Fluorescence assay kit (Discoverx, Fremont, CA) according to manufacturer's instructions [13]. The estrogen receptor binding studies showed strong affinities for the reference derivatives with IC_{50} of 0.84 and 0.66 nM, respectively for 17β-estradiol and 4-HO-tamoxifen on the ERα and IC_{50} of 1.70 and 1.23 nM, respectively for 17β-estradiol and 4-HO-tamoxifen on the ERβ. 4-HO-Tamoxifen was used as a reference compound as it is the bioactive metabolite of tamoxifen *in vivo*. Although the affinities of the dimers are stronger for the ERα, they remain below physiologic concentration with IC_{50} in the μM range. The dimers present no affinity for the ERβ. It is likely that the introduction of the 17β PEG tether chain greatly reduces the ability of the estrogenic portion to bind to the ER, contrary to our initial hypothesis. Despite the low affinity for the ERα, dimers **6a** and **6b** are still mildly cytotoxic on ER^+ breast cancer cell lines. Thus, the cytotoxic activities of dimers **6b** and **6c** cannot be attributed to their interactions with the ER. These results demonstrated that the biological action of the dimers is due to an alternate mechanism that remains to be elucidated.

4. Conclusion

In summary, this manuscript presents a facile synthesis of 17β-estradiol homo-dimers. They are made form estrone in five chemical steps with an overall yield exceeding 30%. The

key steps for the synthesis of these compounds is the selective O-alkylation of diol **7** with the dibrominated PEG chains **8**. The dimers are generally less cytotoxic towards breast cancer cells as compared to the cognate hormone 17β-estradiol and the antiestrogen tamoxifen. Interestingly, dimers **6b** and **6c** show specific toxicity towards MCF-7, a hormone-dependent breast cancer cell line. The dimers present low affinity for the ERα and no affinity for the ERβ. These new dimeric molecules show significant cytotoxicity towards the murine skin cancer cell line; a property that could be exploited for the selective topical treatment of human skin cancers. Further investigation will be necessary to assess the complete biological potential of these new C_2-symmetric 17β-estradiol dimers particularly on skin cancers.

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REFERENCES

[1] Hargittai I, Hargittai M. Symmetry through the eyes of a chemist. New-York: VCH Publishers; 1987.
[2] Voloshchuk T, Farina NS, Wauchope OR, Kiprowska M, Haberfield P, Greer A. Molecular bilateral symmetry of natural products; Prediction of selectivity of dimeric molecules by density functional theory and semiempirical calculations. J Nat Prod 2004;67:1141–6.
[3] Bérubé G. Natural and synthetic biologically active dimeric molecules: anticancer agents, anti-HIV agents, steroid derivatives and opioid antagonists. Curr Med Chem 2006;13:131–54.
[4] Bergmann KE, Wooge CH, Carlson KE, Katzenellenbogen BS, Katzenellenbogen JA. Bivalent ligands as probes of estrogen receptor action. J Steroid Biochem Mol Biol 1994;49:139–52.
[5] Groleau S, Nault J, Lepage M, Couture M, Dallaire N, Bérubé G, Gaudreault RC. Synthesis and preliminary *in vitro* cytotoxic activity of new triphenylethylene dimers. Bioorg Chem 1999;27:383–94.
[6] Hewitt SC, Deroo BJ, Korach KS. A new mediator for an old hormone. Science 2005;307:1572–3.
[7] Rabouin D. Synthèse et activité biologique in vitro de dimères de l'estrone et de l'estradiol; Nouvelles molécules antiestrogéniques. Université du Québec à Trois-Rivières, Trois-Rivières, Québec, 2001 (Thesis).
[8] Rabouin D, Perron V, N'Zemba B, Gaudreault RC, Bérubé G. A facile synthesis of C_2-symmetric 17β-estradiol dimers. Bioorg Med Chem Lett 2003;13:557–60.
[9] Perrin DD, Armarego WLF. Purification of laboratory chemicals. 3rd ed. Oxford: Pergamon Press; 1988.
[10] Still WC, Kahn M, Mitra A. Rapid chromatographic techniques for preparative separation with moderate resolution. J Org Chem 1978;43:2923–5.
[11] Boyd MR, Paull KD. Some practical considerations and applications of the National Cancer Institute *in vitro* anticancer drug discovery screen. Drug Dev Res 1995;34:91–109.
[12] Martin A, Clynes M. Comparison of 5 microplate colorimetric assays for *in vitro* cytotoxicity testing and cell proliferation assays. Cytotechnology 1993;11:49–58.
[13] Eglen RM. Enzyme fragment complementation: a flexible high throughput screening assay technology. Assay Drug Dev Technol 2002;1:97–104. The HitHunter™ EFC Estrogen Receptor Fluorescence assay kit and protocol, were obtained from Discoverx, Fremont, California, USA (http://www.discoverx.com/pf.php?p=25).
[14] Greene TW, Wuts PGM. Protective groups in organic synthesis. 3rd ed. New-York: John Wiley & Sons; 2000.
[15] Dionne P, Ngatcha BT, Poirier D. D-ring allyl derivatives of 17β- and 17α-estradiols: chemical synthesis and ^{13}C NMR data. Steroid 1997;62:674–81.
[16] Horwitz KB, Zava DT, Thilagar AK, Jensen EM, McGuire WL. Steroid receptor analyses of nine human breast cancer cell lines. Cancer Res 1978;38:2434–7.
[17] Parkin DM, Pisani P, Ferlay J. Global cancer statistics. CA Cancer J Clin 1999;49:33–64.
[18] Bae J-S, Jang K-H, Yim H, Jin H-K. Polysaccharides isolated from *Phellinus gilvus* inhibit melanoma growth in mice. Cancer Lett 2005;218:43–52.
[19] Cheng Z, Mahmood A, Li H, Davison A, Jones AG. [^{99m}TcOAADT]-$(CH_2)_2$-NEt_2: a potential small-molecule single-photon emission computed tomography probe for imaging metastatic melanoma. Cancer Res 2005;65:4979–86.