Journal of Steroid Biochemistry and Molecular Biology 219 (2022) 106064

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Regioselective synthesis, physicochemical properties and anticancer activity of 2-aminomethylated estrone derivatives

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ARTICLE INFO

This paper is dedicated to the memory of Prof. Gyula Schneider.

Keywords:
Modified Mannich reaction
ortho-Quinone methide
aza-Michael-addition
Regioselectivity
Physicochemical characterization
Medchem-driven selection

ABSTRACT

The unique estrogen receptor (ER)-independent antiproliferative and apoptotic activity of 2-methoxyestradiol (2ME2) is well known, however, its use has been limited because of its poor oral bioavailability. In this study, novel 2-aminomethylated estrone (E) and estradiol (E2) derivatives structurally related to 2ME2 were synthesized, and their physicochemical properties as well as their *in vitro* cytotoxic effects were investigated in the hope of finding more selective antiproliferative agents with improved pharmacokinetic profile. The target compounds were synthesized from 2-dimethylaminomethylated E obtained regioselectively by a three-component Mannich reaction. Quaternization with methyl iodide followed by reacting the ammonium salt with various dialkyl and alicyclic secondary amines afforded the desired products in good yields. The reactions proceeded *via* a 1,4-nucleophilic addition of the applied secondary amines to the *ortho*-quinone methide (*o*-QM) intermediates, generated *in situ* from the salt by base-promoted β-elimination. The compound library has been enlarged with structurally similar E2 analogues obtained by stereoselective reduction and with some 17β-benzylamino derivatives prepared by reductive amination. The potential values of the novel E and E2 derivatives were characterised by means of three different approaches. At the first step compounds were virtually screened using physicochemical parameters. Physicochemical characterization was completed by kinetic solubility and *in vitro* intestinal-specific permeability measurement. Antiproliferative effects were additionally determined on a panel of malignant and non-cancerous cell lines. The evaluation of the pharmacological profile of the novel E and E2 derivatives was completed with the calculation of lipophilic efficacy (LiPE).

1. Introduction

One of the most important tasks of medicinal chemistry is to create compound libraries that contain a large number of molecules with high structural diversity [1,2]. Multicomponent Mannich-type aminoalkylation reactions in which a product can be formed by the simultaneous reaction of three reactants (an active hydrogen-containing agent, an aldehyde and an amine reagent) serve as an effective means of achieving this goal [3–6]. Their great advantage is that the order of addition of the components is arbitrary and their structure can be varied independently, vastly increasing the number of molecules that may be obtained in a short amount of time with minimal effort. Because formaldehyde is most commonly used as the aldehyde component, the applicability of miscellaneous types of substrates with an activating functional group (*e.g.* carbonyl compounds, phenols, naphthols, terminal alkynes, different heterocycles) and the variability of amine reagents generally ensures the structural diversity of the aminomethylated products (Mannich bases) [5,6]. The importance of Mannich bases stems from their many practical applications, especially in the field of medicinal chemistry and drug design. In addition to their therapeutic potential in many kinds of diseases, such as cancer (Fig. 1), improvements in the physicochemical and pharmacokinetic properties are also

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https://doi.org/10.1016/j.jsbmb.2022.106064
Received 20 December 2021; Received in revised form 21 January 2022; Accepted 23 January 2022
Available online 25 January 2022

expected due to the presence of the polar aminomethyl moiety [5].

Although different reaction mechanisms have been suggested for the three-component modified Mannich reaction of an electron-rich aromatic compound (phenol or naphthol), an amine and an aldehyde [7–9], the theory of the primary formation of an *ortho*-quinone methide (*o*-QM) from the phenolic compound and the aldehyde partner appears to be the most accepted. 1,4-Conjugate addition of the amine to this very short-lived reactive intermediate followed by rearomatization leads to the aminoalkylated aromatic product. It is important to note that the *o*-QMs formed from naphthols are more stable due to their partially retained aromatic character than those generated from phenols [8]. Nevertheless, several recent reviews [8,10] and articles [11] have addressed the properties, applicability, and biological activity of *o*-QMs derived from naphthols and phenols.

The phenolic A-ring of natural steroids estrone (E) and estradiol (E2) seems suitable for carrying out the Mannich reaction, however, aminomethylation can occur on both carbon atoms (C2 and C4) adjacent to the C—OH group. Therefore, achieving high regioselectivity toward the C2-substituted product may be challenging despite the fact that for steric reasons, the availability of the C2 position appears to be more favourable. However, the preference for the C2 substitution in Mannich reactions is contradicted by the results of Lande and coworkers, who studied the regioselectivity of the aminomethylation of bicyclic phenols [12]. It was found that in the Mannich reaction of phenol condensed with a six-membered ring, which structurally mimics the A- and B-rings of estrogens, the incorporation of the aminomethyl group occurs mainly at the carbon between the phenolic C—OH and the ring junction. This would correspond to position C4 of the sterane skeleton.

Nevertheless, 2-substituted E- or E2-based derivatives are of great interest, since several compounds of this type have been demonstrated to have less or no ability to bind to the estrogen receptor (ER) and thus no hormonal activity [13]. Perhaps the best known and most studied member of this family of compounds is 2-methoxyestradiol (2ME2, Fig. 1), which has an ER-independent mechanism of action to inhibit the proliferation of human cancer cells of diverse origins. Although it lacks hormonal effects, its oral bioavailability is low due to its poor solubility and intestinal absorption as well as rapid metabolism in the body [14]. Its unfavourable pharmacokinetic profile has prompted the production of several modified analogues [15]. Among others, 2-aminomethylated E2 derivatives were also prepared by regioselective 2-formylation of E and subsequent reductive amination with different primary amines [16]. These compounds and their analogues modified by Wittig reaction at C17 position, which are formally considered to be Mannich bases, showed significant antiproliferative activity on various human cancer cell lines (Fig. 1).

Based on the literature background and our research on the synthesis of cytotoxic A-ring modified E derivatives [17–19], the aim of the present study was to develop an efficient method for the highly regioselective aminomethylation of E and E2 at C2 position and to characterize *in silico* and *in vitro* physicochemical properties (solubility, permeability) and cytotoxic activity of our novel derivatives in comparison with 2ME2 as reference compound. According to the results of cytotoxicity studies performed on cancerous (HeLa, A2780, MDA-MB-231) and non-cancerous (NIH/3T3) cell lines, we further aimed to identify the structure – activity/selectivity relationships of the new derivatives and to select them taking into account the potential promiscuous property classified by lipophilic efficiency index (LiPE).

2. Results and discussion

2.1. Synthetic studies

For the introduction of an aminomethyl group onto C2 position of the estrane skeleton, first, a one-step three-component Mannich-type method was carried out using E, dimethylamine (40 % aqueous solution) and formaldehyde (35 % aqueous solution) as reactants (Scheme 1). The amine and the aldehyde were used in excess, and the mixture was stirred in refluxing EtOH under regular thin-layer chromatography (TLC) control. To our delight, complete conversion was achieved within 2 h, and the transformation proved to be highly regioselective toward the formation of the 2-substituted product (**1a**), which was obtained in 85 % yield after chromatographic purification. Trace amounts of the 4-substituted isomer (**1'a**) were thus removed successfully during purification. When the same reaction was performed in a closed vessel at 100

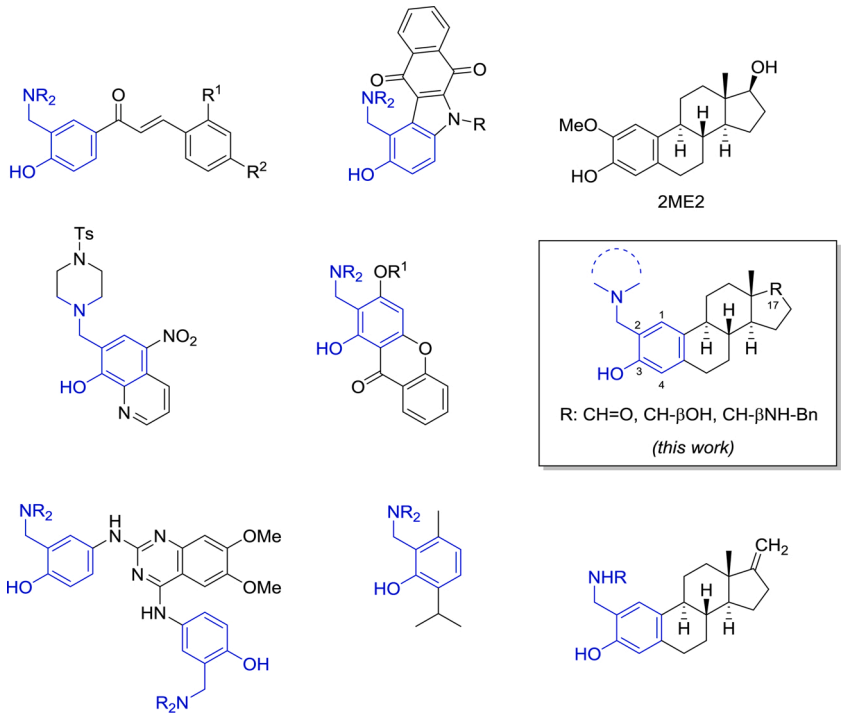
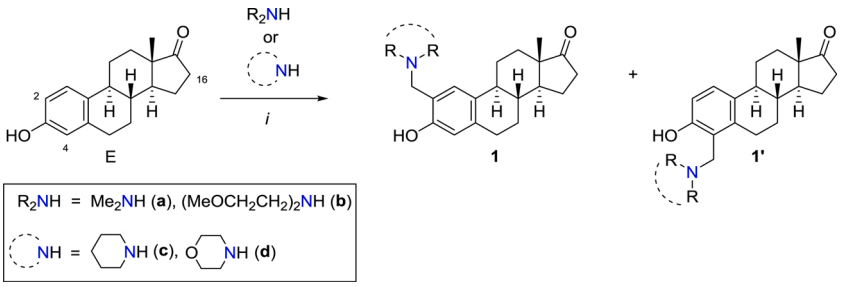


Fig. 1. Design of 2-aminomethylated E and E2 derivatives based on the structures of 2ME2 [14] and some cytototoxic phenolic Mannich bases [5,6,16].

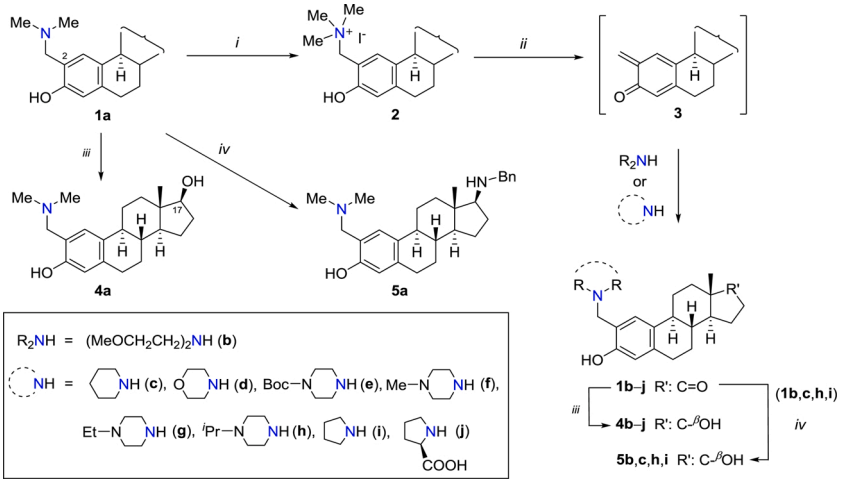


Scheme 1. Modified Mannich aminomethylation of E using formaldehyde and different dialkyl and cyclic secondary amines. *Reaction and conditions*: (*i*) CH_2O (aq. 35 %), EtOH, reflux, 2 h (for **1a**) and 24 h (in all other cases) or 100 °C (MW), 10 min (for **1a**) and 45 min (in all other cases).

°C under microwave (MW) irradiation, the reaction time was reduced to 10 min. Although C16 is also active due to the proximity of the adjacent carbonyl group for the Mannich reaction [20], aminomethylation did not occur at this position despite the use of reagent excess. Encouraged by the initial result, other secondary amines (*bis*(2-methoxyethyl)amine, piperidine and morpholine) were also tested (Scheme 1), but in addition to a significant decrease in the reaction rate (24 h, reflux or 45 min, MW), the desired products proved to be mixtures of the corresponding C2 (**1b**–**d**) and C4 regioisomers (**1′b**–**d**), to which the latter contributes to a much greater extent than in the reaction with dimethylamine. Nevertheless, in all cases, the results supported the formation of the C2-substituted derivative (**1**) as the major product, contrary to previous findings for bicyclic phenols [12], although the ratio of **1b** and **1'b** was already close to 1:1 when *bis*(2-methoxyethyl)amine was used.

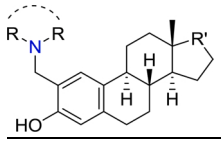
Since the C2 and C4 products (**1** and **1′**) obtained by the reactions of amines other than dimethylamine were difficult to separate by column chromatography, an alternative route for the preparation of 2-aminomethylated derivatives of E had to be developed instead of the direct Mannich-type synthesis. For this purpose, **1a** was used as the starting material, which could be efficiently synthesized regioselectively even in larger quantities. Next, **1a** was subjected to salt formation with methyl iodide (MeI) in diethyl ether (Et_2O) / acetonitrile (MeCN) = 4:1 to obtain tetraalkylammonium iodide (**2**) in a nearly quantitative yield by stirring at room temperature (r.t.) for 24 h (Scheme 2). Since compound **2** proved to be light-sensitive [21], the reaction was performed in dark. The use of a solvent mixture was of great importance: while MeCN aided in the dissolution of the starting material (**1a**), diethyl ether promoted the precipitation of the salt (**2**) formed. After dilution of the reaction mixture with ether and subsequent filtration, the quaternary ammonium iodide **2** was converted to Mannich bases (**1b**–**j**) with different dialkyl and alicyclic secondary amines (Scheme 2). The transformations were carried out in MeCN under reflux in the presence of a base, which induced the *in situ* formation of an *o*-QM intermediate (**3**) by β-elimination [11]. 1,4-*aza*-Michael addition of the amines applied resulted in the desired 2-aminomethylated products in good yields (Table 1). It is important to note that organic bases, such as 1,8-diazabicyclo(5.4.0) undec-7-ene (DBU) or tetramethylethylenediamine (TMEDA) effectively catalysed the reaction to occur towards the formation of 2-aminomethylated E derivatives (**1b**–**j**), while in the presence of KOH or NaOH a considerable amount of by-product, presumably 2-hydroxymethyl-E was formed beside the desired products. Further advantage of this protocol that there was no need to protect the COOH group of proline during the synthesis of **1j** contrarily to the three-component Mannich-type reaction [21]. Additional novel compounds with higher polarity than **1** were also synthesized either by stereoselective reduction of **1a**–**j** to afford 2-aminomethylated E2 derivatives (**4a**–**j**) or by reductive amination of the C17-carbonyl group in **1a**–**c**, **1h** and **1i** with benzylamine ($BnNH_2$) in the presence of sodium cyanoborohydride to provide some 17β-benzylamino derivatives (**5a**–**c**, **5h, 5i**) (Scheme 2, Table 1). It is worth mentioning that the regioselective functionalization of E2 and its 17β-benzylamino analogue (easily accessible from E by reductive amination) at C2 position with different aminomethyl groups is also possible by the described method, but the early-stage derivatization of E and subsequent conversions of C17 is more practical due to easier handling of the less polar E-based Mannich bases.

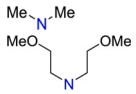
The structures of all synthesized compounds were confirmed by ^1H and ^{13}C NMR spectroscopy as well as by MS spectrometry (Supplementary Material). The chemical shifts, splitting, and integrals of the signals on the ^1H NMR spectra of the products clearly supported the structures. For all derivatives, the substitution at C2 position is evidenced by a singlet of two non-coupled protons (1-H and 4-H) in the aromatic range. For the C4 regioisomer, two doublet signals (1-H and 2-



Scheme 2. Synthesis of 2-aminomethylated E and E2 derivatives. *Reagents and conditions*: (*i*) MeI, MeCN/Et_2O (1:4), r.t., 24 h (in dark); (*ii*) DBU, MeCN, reflux, 1 h; (*iii*) $NaBH_4$, EtOH, r.t., 2 h; (*iv*) $BnNH_2$, $NaBH_3CN$, AcOH (pH 6), MeOH, r.t., 16–24 h.

Table 1
Yields of E-derived Mannich bases.



R' C=O		Yield [a] (1) (%)	R' C–$^{\beta}$OH	Yield [a] (4) (%)	R' C–$^{\beta}$NH-Bn	Yield [a] (5) (%)
	**1a**	85 [b]	**4a**	89	**5a**	64
	1b	75	**4b**	88	**5b**	56
	**1c**	89	**4c**	87	**5c**	66
	**1d**	79	**4d**	78	–	–
	**1e** [c]	82	**4e**	91	–	–
	**1f**	63	**4f**	83	–	–
	**1g**	62	**4g**	81	–	–
	**1h**	71	**4h**	72	**5h**	71
	**1i**	85	**4i**	88	**5i**	61
	**1j**	76	**4j**	86	–	–
	**1k**	70 [d]	**4k**	70	–	–

[a] After chromatographic purification.
[b] Yield obtained from E by direct Mannich reaction (Scheme 1).
[c] Boc = *tert*-butyloxycarbonyl.
[d] Yield calculated from **1a** after 3 steps (salt formation, preparation of **1e** and deprotection with trifluoroacetic acid (TFA) in dichloromethane (DCM) for 24 h at r.t.

H) would be observed in the same range. In case of the 17β-benzylamino derivatives (**5**), the signals between 7.2 and 7.4 ppm are indicative for the incorporation of the aromatic ring, while the diastereotopic CH_2 protons belonging to the Bn group give a complex signal at around 3.7 ppm. Proton peaks characteristic of the various aminomethyl substituents incorporated at C2 can also be identified in the spectra. The ^{13}C NMR spectra, which were recorded using J-MOD pulse sequence, were also in agreement with the proposed structures.

2.2. Characterization of in silico and in vitro physicochemical properties

Following the classical medicinal chemistry approach, physicochemical characterization of E (**1**), E2 (**4**) and 17β-benzylamino derivatives (**5**) was the first step to select possible candidates with lower attrition risks for drug adverse and toxicological effects. Regarding the rules of thumb for clinical candidates (*e.g.* Lipinski's rule of five: Ro5) [22,23] the most relevant physicochemical parameters (molecular weight: Mw, pK_a, log$P$/$D_{7.4}$, topological polar surface area: TPSA) of synthesized compounds were predicted, kinetic solubility (in phosphate buffer saline, pH 7.4) and intestinal-specific PAMPA (pH 6.5 (donor) – pH 7.4 (acceptor)) permeability were also experimentally determined. Reviewing the *in silico* data summarized in Table 2, Ro5 violations were identified only for 17β-benzylamino derivatives. The lipophilicity (log$P$ > 5) for **5a**, **5c**, and **5i**, the molecular weight (Mw > 500) for **5b**, and both parameters for **5h** exceeded the thresholds determined by Ro5. In terms of lipophilicity, it is worth highlighting the E derivatives **4c** and **4e**, which have relatively higher predicted log$P$ values (4.15 and 4.21, respectively) and the log$D_{7.4}$ values of the two proline derivatives (**1j**, **4j**: 0.33, 0.64) are lower (explained by the presence of ionized COOH group at pH 7.4) than the optimal lipophilicity range for oral absorption.

The acid-base character of the test compounds was also characterized by estimating the pK_a of the strongest functional groups. With the exception of proline derivatives (where the p$K_{a,acid}$ values of COOH group are 2.3), the acidic character of the compounds was determined by the aromatic OH function (p$K_{a,acid}$ values between 10.1 and 10.6). Regarding the basic character, a much more diverse picture emerges from the review of p$K_{a,base}$ values (Table 2), which is also closely related to the synthetic strategy of the new E and E2 derivatives. In the case of 2-aminomethylated E and E2, the *N*-centered mono- and dibasic groups, while in the case of the 17β-benzylamino derivatives, an additional *N*-centered basic group determines the basic character of the compounds. It is important to note that the reference compound (2ME2) is a moderately lipophilic monoprotic acid as opposed to the novel amphoteric (in some case amphiphilic) E and E2 Mannich base derivatives.

In correlation with the steroid structure, the TPSA (topological polar surface area) values of the test compounds are relatively low and in the narrow range. This range of polarity is suitable for penetration through cell membranes as well as the blood-brain barrier. Compounds in this polarity range tend to have good permeability across cell membranes (TPSA < 140 Å^2) [24] in general and across blood-brain barrier (TPSA < 90 Å^2) [25] as well. In accordance with ADME screening in the early stage of drug discovery, we investigated the kinetic solubility the intestinal-specific PAMPA permeability relevant to oral absorption of the new E and E2 derivatives and of 2ME2 as a reference compound.

Considering the heterogeneity of the experimental physicochemical data, both kinetic solubility and permeability data were evaluated according to a three-level classification system (see footnote of Table 2). Based on the class categories obtained in the experimental study and the *in silico* parameters, we were able to identify primary and secondary candidates. Compounds that did not identify Ro5 violation and were in the good (medium grey) category in both *in vitro* assays were evaluated as primary candidates. For secondary candidates, moderate (light grey) classification was sufficient based on experimental results. It can be seen from Table 2 that a total of five compounds were selected for E derivatives (primary candidates: **1f**, **1g**, secondary candidates: **1a**, **1c**, **1i**) and three compounds for E2 derivatives (primary candidate: **4i**, secondary candidates: **4c**, **4g**). 17β-benzylamino derivatives were excluded due to Ro5 violation. It is also important to note that the reference compound (2ME2) would also did not meet the established criteria system due to its poor kinetic solubility (54 μM). Summarizing the results of physicochemical pre-screening, *N*-substituted cyclic base piperidine (**c**), *N*-ethylated piperazine (**g**) and pyrrolidine (**i**) derivatives satisfy the general medicinal chemistry rules.

2.3. Antiproliferative assay

In vitro antiproliferative capacity of twenty-seven newly synthesised, 2-aminomethylated E analogues (**1a**–**5i**) was also evaluated on a panel of human adherent cancer cell lines. The compounds were tested against cervical (HeLa), ovarian (A2780) and breast (MDA-MB-231) carcinoma cell lines. Additionally, their tumour selectivity was also determined by using non-cancerous mouse embryo fibroblast (NIH/3T3) cells.

2-Methoxyestradiol (2ME2) as positive control in our study is known for its highly effective antiproliferative activity on several cancer cell lines from different tissue origins [27]. In our panel for antiproliferation screen, 2ME2 similarly displayed low IC_{50} values (0.70–2.5 μM) on the utilized gynecological cancer cell lines (Supplementary Material, Table S1), in addition, it was the most effective against A2780 cells and the less effective against MDA-MB-231 breast carcinoma cells. However, 2ME2 inhibited the proliferation of non-cancerous fibroblast cells at the

Table 2
In silico and experimental physicochemical parameters of E-derived Mannich bases.

Compound[h]	Predicted parameters[i]						Experimental data		
	Mw	$pK_{a,acid}$[b]	$pK_{a,base}$[b]	log*P*	log$D_{7.4}$	TPSA Å^2	Kin. Sol.[d] µM	PAMPA P_e[e] /·10^{-6} cm/s	MR[g] %
R': C=O									
1a	327	10.2	8.7	3.28	2.14	40.5	341±10	18.4±1.9	79.2±0.3
1b	416	10.1	7.0	2.64	2.52	59.0	103±3	13.9±0.4	88.4±0.7
1c	368	10.2	8.9	3.77	2.32	40.5	148±7	23.3±1.8	33.1±3.1
1d	370	10.1	6.5	2.80	2.76	49.8	26±2	10.3±1.4	66.9±2.5
1e	469	10.1	6.2	3.89	3.88	70.1	18±2	23.5±6.6	51.5±7.2
1f	383	10.1	7.6	2.60	2.19	43.8	405±8	22.2±4.2	86.2±1.1
1g	397	10.2	7.8	3.00	2.48	43.8	300±12	20.9±4.2	87.5±1.7
1h	411	10.2	8.0	3.29	2.64	43.8	271±24	12.3±1.0	86.4±0.4
1i	354	10.5	8.3	3.43	1.21	40.5	244±7	17.5±6.4	79.7±3.7
1j	398	2.3[c]	8.9	2.86	0.33	77.8	92±15	6.7±0.4	70.4±3.2
1k	369	10.2	9.1	2.75	1.18	52.6	452±16	12.1±0.9	75.2±1.6
C–$^{\beta}$OH									
4a	329	10.2	8.7	3.54	2.39	43.7	235±5	11.4±1.1	84.9±0.7
4b	418	10.2	7.0	2.96	2.84	62.2	190±2	ND[f]	-
4c	370	10.2	8.9	4.15	2.69	43.7	125±2	21.0±1.4	67.8±1.6
4d	371	10.2	6.5	3.07	3.03	52.9	54±1	29.4±3.3	29.4±3.3
4e	471	10.2	6.2	4.21	4.20	73.2	48±2	ND[f]	-
4f	385	10.2	7.6	2.92	2.55	46.9	287±8	12.1±2.6	78.8±3.5
4g	399	10.2	7.8	3.31	2.78	46.9	306±7	18.1±4.7	86.7±3.3
4h	413	10.2	8.0	3.57	2.92	46.9	120±17	14.8±2.0	76.9±1.2
4i	356	10.6	8.3	3.75	1.51	43.7	328±13	25.7±4.5	83.3±1.5
4j	400	2.3[c]	8.9	3.17	0.64	81.0	459±18	ND[f]	-
4k	371	10.2	9.1	3.05	1.48	55.7	348±6	12.2±1.9	81.8±4.3
C–$^{\beta}$NH-Bn									
5a	419	10.2	9.5	5.20[a]	2.03	35.5	471±12	12.5±3.4	81.5±0.6
5b	507[a]	10.2	9.5	4.58	2.39	54.0	104±3	23.4±1.2	71.8±2.1
5c	459	10.2	9.5	5.96[a]	2.53	35.5	235±5	12.8±2.4	88.3±0.9
5h	502[a]	10.2	9.5	5.34[a]	2.63	38.7	106±4	25.1±2.5	69.9±4.8
5i	445	10.5	9.5	5.60[a]	1.71	35.5	504±4	10.6±1.1	37.8±3.9
2ME2	302	10.3	-	3.36	3.36	49.7	54±2	46.3±1.8	21.1±3.9

[a]Lipinski's Ro5 violation for molecular weight (Mw > 500) or (log*P* > 5), [b]pK_a values calculated for the strongest acidic or basic moiety, [c]decreased predicted pK_a due to the proline COOH group, [d]Classification system for kinetic solubility, kin. sol. (µM): good (medium grey ≥ 300), moderate (300 > light grey ≥ 100), poor (dark grey < 100), [e]Classification system for PAMPA permeability, P_e (10^{-6} cm/s): good (medium grey ≥ 20), moderate (20 > light grey ≥ 15), poor (dark grey < 15), [f]ND: not determined (the compound cannot be detected in the acceptor side), [g]increased lipid partition (MR% > 80), indicating a strong interaction between the active substance and the PAMPA artificial lipid membrane. [h]primary candidates (medium grey): no Ro5 violation and belongs to the good class based on experimental data, secondary candidates (light grey): no Ro5 violation and belongs to at least moderate class based on experimental data. [i]Calculated parameters using ACD/Labs Percepta software package [26].

same concentration range. Consequently, 2ME2 might not be considered as a tumour-selective agent in our experimental circumstances.

Similar to 2ME2, amongst the newly synthesized derivatives, 17β-benzylamino derivatives (**5a**–**i**) also exerted high antiproliferative effectiveness on the investigated cancer cell lines (IC_{50} = 1.07–5.09 µM), however the most susceptible cell line cannot be determined due to the different effectivity order of each analogue in this group. The IC_{50} values of these compounds on non-cancerous fibroblast cells were between 1.15 and 5.32 µM, which demonstrated their non-selective antiproliferative effect towards cancer cells. Considering the promiscuous properties of the **5a**–**i** derivatives and their associated increased lipophilicity, hereinafter, the antiproliferative activity of the test compounds was characterized by their lipophilic efficiency (LiPE = pIC_{50}-log*P*) [23,28] in order to highlight their selectivity and to reduce potential adverse effects (Table 3). Therefore, to compare the antiproliferative effects, we used pIC_{50} values (p$IC_{50,2ME2}$ = 5.60–6.15, p$IC_{50,5a-i}$ = 5.27–5.94) and paired them with LiPE to use a three-class system to select potentially selective and drug-like derivatives of novel E and E2 compounds. The individual effect of data pairs was divided into a classification system as follows: medium grey (elevated effect and low promiscuity risk): pIC_{50} ≥ 5 and LiPE ≥ 1.5 or pIC_{50} ≥ 4.5 and LiPE ≥ 2.0, light grey (moderate effect and moderate promiscuity risk): 5 > pIC_{50} ≥ 4.5 and 2.0 > LiPE ≥ 1.0, dark grey (elevated effect and high promiscuity risk): pIC_{50} ≥ 5 but LiPE < 1.0.

The E2 derivatives (**4a–k**) displayed more diverse antiproliferative effect against the selected gynecological cancer cell lines. Since four analogues from this group (**4a**, **4b**, **4i** and **4e**) possess pIC_{50} values higher than 5.0, HeLa cells can be considered as the most susceptible cell line. Only **4e** was able to inhibit ovarian cancer cell (A2780) proliferation with a pIC_{50} value higher than 5.0. Three compounds (**4a**, **4b** and **4h**) exerted moderate or weak antiproliferative activity against this cell line. The novel E2 derivatives, except **4b**, were not able to inhibit substantially the proliferation of hormone-insensitive breast cancer cell line, MDA-MB-231. On the other hand, **4b** demonstrated good or moderate antiproliferative activity against all investigated cancer cell lines. Moreover, **4b** inhibited the proliferation of non-cancerous fibroblast cells at 1.6–3.2 times higher concentration compared to its IC_{50} values on cancerous gynecological cell lines. Among the remaining 2-aminomethylated E2 compounds, **4a** exerted the most selective antiproliferative effect, because it possesses 15 times higher IC_{50} value on NIH/3T3 cells compared to that on HeLa cells. **4i** and **4g** can be considered as tumour-specific compounds, because they were able to suppress HeLa cell proliferation only. Regarding the antiproliferative effects (pIC_{50}) and the LiPE values of our classification system (Table 3), the primary hits (medium grey) were **4a** and **4i**, and **4b** and **4g** as the secondary hits (light grey) showed good selectivity on cancerous cell lines. Compound **4e** can be also considered as a potential lead molecule, although the LiPE values are lower than 1.0 (due to increased log*P*: 4.21), but it also showed an increased antiproliferative effect selectively (p$IC_{50,HeLa/A2780}$ > 5.0 and p$IC_{50,NIH/3T3}$ = 4.73).

The newly synthesized E derivatives (**1a–k**) displayed lower antiproliferative activities against the investigated cancer cells compared to the compounds of the other two scaffolds. **1a**, **1b** and **1c** demonstrated moderate antiproliferative activity (pIC_{50} = 4.70–4.98) on cervical HeLa cells. Five compounds (**1a**, **1b**, **1c**, **1e** and **1i**) exerted moderate or weak antiproliferative activity (pIC_{50} = 4.49–4.81) against ovarian cancer cell

Table 3
In vitro antiproliferative activity (pIC_{50}) and lipophilic efficiency (LiPE) of E-derived Mannich bases.

Compd.	**pIC_{50}** values; M / **LiPE** = pIC_{50} - log*P* growth inhibition at 30 μM; % ± SEM			
	HeLa	**A2780**	**MDA-MB-231**	**NIH/3T3**
R': C=O				
1a	**4.79 / 1.51**	**4.68 / 1.40**	–[a]	**4.51[b] / 1.23**
	77.5 ± 2.8	76.1 ± 0.8	16.1 ± 2.0	49.9 ± 1.0
1b	**4.98 / 2.34**	**4.49 / 1.85**	**4.75 / 2.11**	**4.46 / 1.82**
	60.8 ± 2.1	51.7 ± 2.4	54.1 ± 1.3	47.8 ± 2.0
1c	**4.70 / 0.93**	**4.74 / 0.97**	**–**	**4.63 / 0.86**
	62.7 ± 1.4	61.7 ± 1.3	39.2 ± 1.8	53.1± 1.7
1d	–	–	–	–
	42.9 ± 2.4	47.3 ± 3.1	n. m.	n. d.
1e	**–**	**4.81 / 0.92**	**–**	**4.19 / 0.30**
	44.2± 1.0	78.8 ± 1.3	40.0 ± 2.2	33.8 ± 2.4
1f	–	–	–	–
	36.6 ± 1.5	21.8 ± 2.2	n. m.	n. d.
1g	–	–	–	–
	39.3 ± 3.4	43.9 ± 3.2	n. m.	n. d.
1h	–	–	–	–
	35.2 ± 2.1	45.4 ± 0.4	n. m.	n. d.
1i	–	**4.66 / 1.23**	–	**4.29 / 0.86**
	36.9 ± 2.0	71.1 ± 0.8	n. m.	44.9 ± 0.7
1j	–	–	–	–
	20.1 ± 2.2	7.3 ± 2.6	20.0 ± 3.2	n. d.
1k	–	–	–	–
	48.1 ± 1.9	11.7 ± 3.3	20.8 ± 2.6	n. d.
C–$^{\beta}$OH				
4a	**5.34 / 1.80**	**4.56 / 1.02**	–	**4.15 / 0.61**
	90.7 ± 1.1	64.8 ± 1.6	45.3 ± 2.9	38.4 ± 1.6
4b	**5.13 / 2.17**	**4.86 / 1.90**	**4.85 / 1.89**	**4.62 / 1.66**
	94.8 ± 1.0	99.0 ± 1.1	95.2 ± 0.7	56.6 ± 2.8
4c	–	–	–	–
	41.8 ± 2.8	32.3 ± 3.1	36.5 ± 2.4	34.0 ± 2.1
4d	–	–	–	–
	41.2 ± 2.2	22.1 ± 2.6	10.6 ± 4.6	n. d.
4e	**5.17 / 0.96**	**5.12 / 0.91**	**–**	**4.73 / 0.52**
	73.1 ± 0.7	74.2 ± 0.6	38.4 ± 2.4	58.7 ± 0.2
4f	–	–	–	–
	45.7 ± 2.4	13.3 ± 2.8	9.7 ± 2.1	n. d.
4g	**4.68 / 1.37**	**–**	**–**	**4.21 / 0.90**
	59.3 ± 0.5	26.4 ± 2.2	21.4 ± 2.0	43.0 ± 0.7
4h	**4.46 / 0.89**	**4.42 / 0.85**	**–**	**4.17 / 0.60**
	53.3 ± 2.0	53.1 ± 2.8	13.8 ± 2.9	42.9 ± 0.3
4i	**5.09 / 1.34**	–	–	**4.51 / 0.76**
	73.5 ± 0.3	42.6 ± 1.7	21.0 ± 3.2	50.2 ± 0.8
4j	–	–	–	–
	5.8 ± 1.3	2.2 ± 2.0	13.8 ± 3.0	n. d.
4k	–	–	–	–
	37.7 ± 3.1	10.2 ± 2.2	22.1 ± 1.9	n. d.
C–$^{\beta}$NH-Bn				
5a	**5.92 / 0.72**	**5.89 / 0.69**	**5.82 / 0.62**	**5.82 / 0.62**
	96.8 ± 1.3	100.6 ± 0.4	98.5 ± 1.2	93.6 ± 1.4
5b	**5.51 / 0.93**	**5.66 / 1.08**	**5.66 / 1.08**	**5.94 / 1.36**
	90.6 ± 0.3	96.5 ± 0.3	103.8 ± 1.1	101.2 ± 0.90
5c	**5.74 / -0.22**	**5.45 / -0.51**	**5.49 / -0.47**	**5.53 / -0.43**
	93.8 ± 2.1	99.5 ± 0.5	96.7 ± 1.3	91.5 ± 0.8
5h	**5.43 / 0.09**	**5.60 / 0.26**	**5.94 / 0.60**	**5.93 / 0.59**
	90.9 ± 0.2	95.9 ± 0.4	104.3 ± 0.6	100.4 ± 1.3
5i	**5.29 / -0.31**	**5.47 / -0.13**	**5.35 / -0.25**	**5.27 / -0.33**
	99.8 ± 0.2	99.2 ± 0.2	97.0 ± 1.1	97.3 ± 2.1
2ME2	**5.94 / 2.58**	**6.15 / 2.79**	**5.87 / 2.51**	**5.60 / 2.24**
	92.6 ± 1.1	80.1 ± 1.8	99.9 ± 0.6	63.6 ± 1.0

[a] pIC_{50} values have been calculated if the growth inhibition value of the compound at 30 μM concentration is higher than 50 %. [b] pIC_{50} values on NIH/3T3 cell line have been calculated if the compound at 30 μM concentration possesses a growth inhibition value higher than 50 % on any investigated cancer cell line. [c] n.m.- no measurable antiproliferative effect. [d] n.d. - not determined.
Classification system for antiproliferative effect: medium grey (elevated effect and low promiscuity risk): $pIC_{50} \geq 5$ and LiPE ≥ 1.5 or $pIC_{50} \geq 4.5$ and LiPE ≥ 2.0, light

grey (moderate effect and moderate promiscuity risk): 5 > pIC_{50} ≥ 4.5 and 2.0 > LiPE ≥ 1.0, dark grey (elevated effect and high promiscuity risk): pIC_{50} ≥ 5 but LiPE < 1.0.

line (A2780). Similar to the E2 derivative **4b**, *bis*(2-methoxyethyl)amino derivative **1b** (modified at C2 position) was able to inhibit MDA-MB-231 cell proliferation to a large extent. The result indicates that regardless of the chemical environment of the C17 position, the presence of the *bis*(2-methoxyethyl)amino group at the C2 position may be responsible for this selective antiproliferative effect. It can be supported by the experience that among the E and E2 derivatives, no other substituent at position C2 provided similar antiproliferative pattern. In this group of E derivatives, **1i** and **1e** can be considered as tumour specific compounds due to their selective inhibition on proliferation of ovarian cancer cells. Moreover, **1e** displayed the highest selectivity toward cancer cells, as a difference four times higher between the IC_{50} values calculated for A2780 and fibroblast cells can be observed.

Regarding the potential structure – antiproliferative activity relations for C2-substituent of E and E2 compounds, a *N*-containing branched-chain acyclic derivatives (**1a** ↔ **4a** and **1b** ↔ **4b**) might be considered to possess more favourable antiproliferative characteristics against cervical and ovarian cancer cell lines than a corresponding *N*-containing cyclic analogues (**1d** ↔ **4d**, **1j** ↔ **4j**, **1k** ↔ **4k**). Another prominent QSAR pattern can be identified for piperazine derivatives of E2, where the antiproliferative effect on HeLa cells increases with increasing lipophilicity of non-substituted (**4k**) and *N*-aliphatic (**4e**–**h**) analogues (log*P*: 2.92, 3.05, 3.31, 3.57, 4.21 and pIC_{50}: -, -, 4.68, 4.46, 5.17, respectively). This observation supports the consideration of LiPE for compound selection in our medicinal chemistry project as well. Furthermore, C2-pyrrolidine derivatives **1i** and **4i** represented cancer-specific antiproliferative activity with moderate or good pIC_{50} values against A2780 and HeLa cells, respectively. Overall, based on the pIC_{50} - LiPE classification system, among E derivatives, **1b** was identified as a primary and **1i** was selected as a secondary hit.

Finally, to collect the primary and secondary candidates of *in silico / in vitro* physicochemical screening and the primary and secondary hits of the antiproliferative assay panel, the most advantageous compound is the E2 derivative **4i**, but **1i** and **4g** may also be preferred as a secondary candidate for *in vivo* pharmacology studies. Although, focusing on the results of antiproliferative studies, **4a** and **4b** can be also served as *in vivo* preclinical candidates due to their HeLa-selective activity.

3. Conclusions

In summary, regioselective synthesis of novel A-ring-modified derivatives in the estrone series was efficiently accomplished. Phenolic Mannich bases substituted with various tertiary aminomethyl groups at C2 position were obtained by *aza*-Michael addition of different secondary amines to the *o*-QM intermediate generated *in situ* from quaternized 2-dimethylaminomethyl-E. Additional 17β-hydroxy and 17β-benzylamino analogues were also prepared to compare physicochemical properties and pharmacological activities.

Two independent approaches were used to characterize the compounds, considering the rules of thumb for drug-likeness related to pharmacokinetic behaviour and the risk of promiscuity in the evaluation of the antiproliferative effect. Based on the rigorous classification system, 17β-benzylamino derivatives (**5**) were excluded due to their increased molecular weight and lipophilicity. The result of the primary *in silico* screen is supported by the fact that the **5** derivatives, although they have an enhanced antiproliferative activity, however, their effect is not selective. Promiscuous behaviour of this scaffold (**5**) was also supported by their low LiPE values (< 1). The selectivity-LiPE relationship was also identified for the piperazine derivatives formed at C2 position. In this closed chemical space, the positive correlation between lipophilicity and antiproliferative effect was confirmed by our observations. Overall, only one estrone (E: **1i**) and two estradiol (E2: **4i**, **4g**) derivatives complied with our rules on *in silico* drug-likeness, on *in vitro* drug absorption and on selective antiproliferative activity. The result is consistent with the general finding of antiproliferative screen, which showed that E2 derivatives exhibited more enhanced effect than E-derived compounds. However, the role of the substituent at C2 position in the antiproliferative effect was also observed, given that the pyrrolidine derivatives of E and E2 satisfy the two-stage classification system. It is also important to highlight that the reference compound, 2ME2 has a substantial antiproliferative effect pIC_{50} (> 5.6) with optimal LiPE (> 2.2) values. However, similar to **5** derivatives, its effect is not selective, and it has poor aqueous solubility. In contrast, **4g**, **4i** and **1i** derivatives may be advantageous clinical candidates in the treatment of cervical and ovarian carcinoma due to their more favourable physicochemical and selective antiproliferative properties.

4. Experimental

4.1. Materials and methods

Chemicals, reagents, and solvents were purchased from commercial suppliers (Sigma-Aldrich and Alfa Aesar) and used without further purification. Reactions under MW irradiation were carried out with a CEM Corporation Focused Microwave System, Model Discover SP. Melting points (Mp) were determined on an SRS Optimelt digital apparatus and are uncorrected. The transformations were monitored by TLC using 0.25 mm thick Kieselgel-G plates (Si 254F, Merck). Compound spots were detected by spraying with 5 % phosphomolybdic acid in 50 % aqueous phosphoric acid. Flash chromatographic purifications were carried out on silica gel 60, 40–63 μm (Merck). All eluent and solvent system compositions are given in volume percent (v/v%). NMR spectra were recorded with a Bruker DRX 500 instrument at room temperature in $CDCl_3$ or DMSO-d_6 using residual solvent signals as an internal reference. Chemical shifts are reported in ppm (δ scale), and coupling constants (J) are given in Hz. Multiplicities of the ^1H signals are indicated as a singlet (s), a doublet (d), doublet of doublets (dd), a triplet (t) or a multiplet (m). ^{13}C NMR spectra are ^1H-decoupled and the J-MOD pulse sequence was used for multiplicity editing. In this spin-echo type experiment, the signal intensity is modulated by the different coupling constants J of carbons depending on the number of attached protons. Both protonated and unprotonated carbons can be detected (CH_3 and CH carbons appear as positive signals, while CH_2 and C carbons as negative signals). Elemental analysis data were obtained with a Perkin Elmer CHN analyzer model 2400 and FT-IR spectra were recorded on a FT/IR-4700 spectrometer (Jasco) using ATR. Automated flow injection analyses were performed by using an HPLC/MSD system. The system comprised an Agilent 1100 micro vacuum degasser, a quaternary pump, a micro-well plate autoinjector and a 1946A MSD equipped with an electrospray ion (ESI) source operated in positive ion mode. The ESI parameters were: nebulizing gas N_2, at 35 psi; drying gas N2, at 350 °C and 12 L/min; capillary voltage (VCap) 3000 V; and fragmentor voltage 70 V. The MSD was operated in scan mode with the mass range m/z 60–620. Samples (0.2 μL) were injected with an automated needle wash directly into the solvent flow (0.3 mL/min) of MeCN/H_2O = 70:30 supplemented with 0.1 % formic acid. The system was controlled by Agilent LC/MSD Chemstation software.

4.2. Chemistry

4.2.1. Synthesis of 2-[(dimethylamino)methyl]-estrone (1a) by Mannich reaction

Estrone (E, 270 mg, 1.00 mmol), dimethylamine (40 % aq., 0.60 mL, 6.0 equiv.) and formalin (35 % aq., 0.60 mL, 7.5 equiv.) were dissolved in abs. EtOH (5.0 mL). The mixture was irradiated with MW in a closed vessel at 100 ° C for 10 min. After completion, the pale yellow

homogeneous mixture was poured onto ice-cold water and the precipitate formed was filtered off, washed with cold water and dried. The crude product was purified by column chromatography using EtOAc / CH_2Cl_2 = 10:90 as eluent to afford **1a** as a white solid (278 mg, 85 %). Scale-up synthesis was performed with the same reactant ratios by refluxing in EtOH for 2 h. Mp 169−171 °C; Anal. calcd for $C_{21}H_{29}NO_2$ C, 77.02; H, 8.93; Found: C, 77.10; H, 8.82; ^{1}H NMR (DMSO-d_6, 500 MHz): δ_H 0.82 (s, 3H, 18-H_3), 1.25–1.61 (overlapping m, 6 H), 1.70–1.80 (m, 1 H), 1.86–2.00 (overlapping m, 2 H) 2.00–2.10 (m, 1 H), 2.10–2.15 (m, 1 H), 2.26 (s, 6H, 2 × *N*-CH_3), 2.28–2.31 (m, 1 H), 2.39–2.47 (m, 1 H), 2.69–2.81 (m, 2H, 6-H_2), 3.44–3.55 (m, 2H, *N*-CH_2), 6.41 (s, 1H, 4-H), 6.94 (s, 1H, 1-H), 10.43 (bs, 1H, 3-OH); ^{13}C NMR (DMSO-d_6, 125 MHz): δ_C 13.5 (C-18), 21.1 (CH_2), 25.7 (CH_2), 26.1 (CH_2), 28.8 (CH_2), 31.4 (CH_2), 35.4 (CH_2), 37.9 (CH), 43.4 (CH), 44.3 (2C, 2 × *N*-CH_3), 47.3 (C-13), 49.6 (CH), 60.8 (*N*-CH_2), 115.0 (C-4), 120.0 (C-2), 125.6 (C-1), 129.7 (C-10), 135.9 (C-5), 154.8 (C-3), 219.7 (C-17); ESI-MS 328 [M + H]$^+$.

4.2.2. General procedure for the synthesis of E-derived Mannich bases (1b–j)

Compound **1a** (327 mg, 1.00 mmol) and MeI (0.6 mL, 10 equiv.) were dissolved in Et_2O/ MeCN = 4:1 and the mixture was stirred in dark at room temperature for 24 h. After TLC control using MeOH / EtOAc = 30:70 as eluent, the suspension was diluted with diethyl ether and the quaternary ammonium iodide salt (**2**) was filtered off and dried. The salt was dissolved in MeCN (20 mL), then secondary amine (2 mmol) and DBU (0.30 mL, 2 equiv.) were added. The mixture was kept at reflux temperature for 1 h until complete conversion (TLC monitoring). The pale yellow homogeneous mixture was poured onto ice-cold water and the precipitate formed was filtered off, washed with cold water and dried. The crude product was purified by column chromatography.

*4.2.2.1. 2-((bis(2-Methoxyethyl)amino)methyl)-estrone (**1b**).* According to the general procedure, *bis*(2-methoxyethyl)amine (0.3 mL) was used. The crude product was purified with EtOAc / hexane = 40:60 to afford **1b** (312 mg, 75 %) as a colourless oil. Anal. calcd for $C_{25}H_{37}NO_4$ C, 72.26; H, 8.97; Found: C, 72.14; H, 9.02; ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 0.90 (s, 3H, 18-H_3), 1.35–1.67 (overlapping m, 6 H), 1.91–2.25 (overlapping m, 5 H) 2.32–2.37 (m, 1 H), 2.49 (dd, 1H, J = 19.0 Hz, J = 8.7 Hz), 2.78–2.87 (overlapping m, 6H, 6-H_2, 2 × *N*-CH_2), 3.32 (s, 6H, 2 × OMe), 3.52 (t, 4H, J = 5.6 Hz, 2 × *O*-CH_2), 3.76–3.88 (m, 2H, *N*-CH_2), 6.56 (s, 1H, 4-H), 6.87 (s, 1H, 1-H), 10.25 (bs, 1H, 3-OH); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 14.0 (C-18), 21.7 (CH_2), 26.1 (CH_2), 26.7 (CH_2), 29.4 (CH_2), 31.7 (CH_2), 36.0 (CH_2), 38.5 (CH), 44.0 (CH), 48.1 (C-13), 50.6 (CH), 53.3 (2C, 2 × *N*-CH_2), 58.4 (*N*-CH_2), 58.9 (2C, 2 × OMe), 70.4 (2C, 2 × *O*-CH_2), 116.3 (C-4), 119.9 (C-2), 125.7 (C-1), 130.4 (C-10), 137.1 (C-5), 155.7 (C-3), 221.1 (C-17); ESI-MS 416 [M + H]$^+$.

*4.2.2.2. 2-[(Piperidin-1-yl)methyl]-estrone (**1c**).* According to the general procedure, piperidine (0.20 mL) was used. The crude product was purified with EtOAc / CH_2Cl_2 = 20:80 to afford **1c** (327 mg, 89 %) as a white solid. Mp 195−197 °C; Anal. calcd. for $C_{24}H_{33}NO_2$ C, 78.43; H, 9.05; Found: C, 78.32; H, 9.01; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 0.91 (s, 3H, 18-H_3), 1.36–1.68 (overlapping m, 12H, 6H of ring), 1.91–2.03 (m, 2 H), 2.01–2.07 (m, 1 H), 2.07–2.17 (m, 1 H), 2.18–2.24 (m, 1 H), 2.32–2.72 (m, 6H, 4H of ring *N*-CH_2), 2.80–2.91 (m, 2H, 6-H_2), 3.54–3.68 (m, 2H, *N*-CH_2), 6.56 (s, 1H, 4-H), 6.86 (s, 1H, 1-H); ^{13}C NMR (126 MHz, $CDCl_3$): δ_C 14.1 (C-18), 21.7 (CH_2), 24.2 (ring CH_2), 26.0 (2C, 2 × ring CH_2), 26.2 (CH_2), 26.8 (CH_2), 29.4 (CH_2), 31.8 (CH_2), 36.0 (CH_2), 38.7 (CH), 44.2 (CH), 48.2 (C-13), 50.7 (CH), 54.1 (2C, 2 × ring CH_2), 62.4 (*N*-CH_2), 116.0 (C-4), 119.4 (C-2), 125.4 (C-1), 130.3 (C-10), 136.9 (C-5), 156.1 (C-3), 220.9 (C-17); ESI-MS 368 [M + H]$^+$.

*4.2.2.3. 2-[(Morpholino)methyl]-estrone (**1d**).* According to the general procedure, morpholine (0.17 mL) was used. The crude product was purified with EtOAc / CH_2Cl_2 = 20:80 to afford **1d** (292 mg, 79 %) as a white solid. Mp 210−212 °C; Anal. calcd. for $C_{23}H_{31}NO_3$ C, 74.76; H, 8.46; Found: C, 74.63; H, 8.38; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 0.90 (s, 3H, 18-H_3), 1.35–1.68 (overlapping m, 6 H), 1.90–2.25 (m, 5 H), 2.33–2.38 (m, 1 H), 2.46–2.53 (m, 1 H), 2.63 (bs, 4H, ring *N*-CH_2), 2.80–2.90 (m, 2H, 6-H_2), 3.60–3.87 (overlapping m, 6H, 2H of *N*-CH_2 and 4H of ring *O*-CH_2), 6.61 (s, 1H, 4-H), 6.92 (s, 1H, 1-H), 10.23 (bs, 1H, 3-OH); ^{13}C NMR (126 MHz, $CDCl_3$): δ_C 14.0 (C-18), 21.7 (CH_2), 26.2 (CH_2), 26.8 (CH_2), 29.4 (CH_2), 31.8 (CH_2), 36.0 (CH_2), 38.6 (CH), 44.1 (CH), 48.1 (C-13), 50.7 (CH), 53.2 (2C, 2 × *N*-CH_2), 62.1 (*N*-CH_2), 67.0 (2C, 2 × *O*-CH_2), 116.1 (C-4), 118.4 (C-2), 125.8 (C-1), 130.8 (C-10), 137.5 (C-5), 155.5 (C-3), 220.8 (C-17); ESI-MS 370 [M + H]$^+$.

*4.2.2.4. 2-[((4-tert-Butoxycarbonyl)piperazin-1-yl)methyl]-estrone (**1e**).* According to the general procedure, 1-*tert*-butoxycarbonyl-piperazine (373 mg) was used. The crude product was purified with EtOAc / CH_2Cl_2 = 10:90 to afford **1e** (384 mg, 82 %) as a white solid. Mp 190−192 °C (decomposes); Anal. calcd. for $C_{28}H_{40}N_2O_4$ C, 71.76; H, 8.60; Found: C, 71.69; H, 8.48; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 0.91 (s, 3H, 18-H_3), 1.40–1.44 (m, 1 H), 1.45 (s, 9H, Boc *tert*-Bu), 1.46–1.68 (overlapping m, 5 H), 1.90–2.26 (overlapping m, 5 H), 2.33–2.38 (m, 1 H), 2.46–2.53 (m, 1 H), 2.63 (bs, 4H, ring CH_2), 2.82–2.90 (m, 2H, 6-H_2), 3.55 (bs, 4H, ring CH_2), 3.68–3.85 (m, 2H, *N*-CH_2), 6.65 (s, 1H, 4-H), 6.92 (s, 1H, 1-H), 10.20 (bs, 1H, 3-OH); ^{13}C NMR (126 MHz, $CDCl_3$): δ_C 14.0 (C-18), 21.7 (CH_2), 26.2 (CH_2), 26.8 (CH_2), 28.6 (3C, 3 × Boc methyls), 29.4 (CH_2), 31.8 (CH_2), 36.0 (CH_2), 38.6 (CH), 44.1 (CH), 48.1 (C-13), 50.7 (CH), 52.6 (broad, 4C, 4 × ring CH_2), 61.8 (*N*-CH_2), 80.1 (Boc *tert*-Bu), 116.2 (C-4), 118.6 (C-2), 125.8 (C-1), 130.8 (C-10), 137.5 (C-5), 154.7 (Boc carbonyl), 155.5 (C-3), 220.8 (C-17); ESI-MS 469 [M + H]$^+$.

*4.2.2.5. 2-[(4-Methylpiperazin-1-yl)methyl]-estrone (**1f**).* According to the general procedure, 1-methyl-piperazine (0.22 mL) was used. The crude product was purified with acetone to afford **1f** (203 mg, 53 %) as a white solid. Mp 165−167 °C; Anal. calcd. for $C_{24}H_{34}N_2O_2$ C, 75.35; H, 8.96; Found: C, 75.32; H, 8.98; ^{1}H NMR (500 MHz, DMSO-d_6): δ_H 0.82 (s, 3H, 18-H_3), 1.26–1.50 (m, 6 H), 1.50–1.58 (m, 1 H), 1.72–1.78 (m, 1 H), 1.87–1.99 (m, 2 H), 2.01–2.09 (m, 1 H), 2.10–2.18 (overlapping m, 4H, 3H of *N*-CH_3), 2.24–2.38 (m, 6 H), 2.38–2.47 (m, 3 H), 2.69–2.80 (m, 2H, 6-H_2), 3.51–3.62 (m, 2H, *N*-CH_2), 6.42 (s, 1H, 4-H), 6.94 (s, 1H, 1-H); ^{13}C NMR (126 MHz, DMSO-d_6): δ_C 13.5 (C-18), 21.0 (CH_2), 25.5 (CH_2), 26.0 (CH_2), 28.6 (CH_2), 31.3 (CH_2), 35.3 (CH_2), 37.9 (CH), 43.3 (CH), 45.5 (*N*-CH_3), 47.2 (C-13), 49.6 (CH), 51.9 (2C, 2 × *N*-CH_2), 54.5 (2C, 2 × *N*-CH_2), 59.1 (*N*-CH_2), 115.0 (C-4), 119.3 (C-2), 125.7 (C-1), 129.7 (C-10), 135.9 (C-5), 154.5 (C-3), 219.4 (C-17). ESI-MS 383 [M + H]$^+$.

*4.2.2.6. 2-[(4-Ethylpiperazin-1-yl)methyl]-estrone (**1g**).* According to the general procedure, 1-ethyl-piperazine (0.26 mL) was used. The crude product was purified with EtOAc to afford **1g** (245 mg, 62 %) as a white solid. Mp 166−168 °C; Anal. calcd. for $C_{25}H_{36}N_2O_2$ C, 75.72; H, 9.15; Found: C, 75.76; H, 9.07; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 0.90 (s, 3H, 18-H_3), 1.12 (t, J = 7.2 Hz, 3H, ethyl CH_3), 1.34–1.67 (m, 6 H), 1.92–2.23 (m, 5 H), 2.34–3.06 (overlapping m, 14H, bs 8H of ring CH_2 2H of 6-H_2, 2H of ethyl CH_2 and two 1 H m), 3.60–3.74 (m, 2H, *N*-CH_2), 6.56 (s, 1H, 4-H), 6.88 (s, 1H, 1-H); ^{13}C NMR (126 MHz, $CDCl_3$): δ_C 11.8 (ethyl CH_3), 14.0 (18-H_3), 21.7 (CH_2), 26.2 (CH_2), 26.7 (CH_2), 29.4 (CH_2), 31.7 (CH_2), 36.0 (CH_2), 38.6 (CH), 44.1 (CH), 48.1 (C-13), 50.6 (CH), 52.27 (2C, 2 × ring CH_2), 52.31 (ethyl CH_2), 52.6 (2C, 2 × ring CH_2), 61.5 (*N*-CH_2), 116.0 (C-4), 118.7 (C-2), 125.7 (C-1), 130.6 (C-10), 137.3 (C-5), 155.6 (C-3), 221.0 (C-17). ESI-MS 397 [M + H]$^+$.

*4.2.2.7. 2-[(4-Isopropylpiperazin-1-yl)methyl]-estrone (**1h**).* According to the general procedure, 1-isopropyl-piperazine (0.29 mL) was used. The crude product was purified with EtOAc to afford **1h** (291 mg, 71 %) as a white solid. Mp 160−163 °C; Anal. calcd. for $C_{26}H_{38}N_2O_2$ C, 76.06;

H, 9.33; Found: C, 75.99; H, 9.23; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 0.91 (s, 3H, 18-H_3), 1.08 (d, J = 6.5 Hz, 6H, 2 × isopropyl CH_3), 1.35–1.67 (overlapping m, 6 H), 1.89–2.25 (overlapping m, 5 H), 2.33–2.38 (m, 1 H), 2.45–2.53 (m, 1 H), 2.64 (bs-like m, 8H, ring CH_2), 2.72–2.77 (m, 1H, isopropyl CH), 2.81–2.89 (m, 2H, 6-H_2), 3.61–3.73 (m, 2H, *N*-CH_2), 6.56 (s, 1H, 4-H), 6.88 (s, 1H, 1-H), 10.55 (bs, 1H, 3-OH); ^{13}C NMR (126 MHz, $CDCl_3$): δ_C 14.0 (C-18), 18.7 (d, J = 2.0 Hz, 2C, 2 × isopropyl CH_3), 21.7 (CH_2), 26.2 (CH_2), 26.8 (CH_2), 29.4 (CH_2), 31.8 (CH_2), 36.0 (CH_2), 38.6 (CH), 44.2 (CH), 48.7 (2C, 2 × ring CH_2), 50.7 (CH), 53.1 (2C, 2 × ring CH_2), 54.5 (isopropyl CH), 61.7 (*N*-CH_2), 116.0 (C-4), 119.0 (C-2), 125.6 (C-1), 130.5 (C-10), 137.1 (C-5), 155.7 (C-3), 220.8 (C-17); ESI-MS 411 $[M + H]^+$.

*4.2.2.8. 2-[(Pyrrolidin-1-yl)methyl]-estrone (**1i**).* According to the general procedure, pyrrolidine (0.17 mL) was used. The crude product was purified with EtOAc / CH_2Cl_2 = 20:80 to afford **1i** (300 mg, 85 %) as a white solid. Mp 161−163 °C; Anal. calcd. for $C_{23}H_{31}NO_2$ C, 78.15; H, 8.84; Found: C, 78.01; H, 8.75; ^{1}H NMR (500 MHz, DMSO-d_6): δ_H 0.83 (s, 3H, 18-H_3), 1.26–1.62 (m, 6 H), 1.68–1.79 (m, 5H, 4H of ring CH_2), 1.87–2.00 (m, 2 H), 2.00–2.09 (m, 1 H), 2.10–2.17 (m, 1 H), 2.26–2.34 (m, 1 H), 2.43 (dd, J = 18.9, 8.5 Hz, 1 H), 2.49–2.54 (m, 4H, overlapping with solvent, ring CH_2), 2.68–2.82 (m, 2H, 6-H_2), 3.62–3.73 (m, 2H, *N*-CH_2), 6.41 (s, 1H, 4-H), 6.94 (s, 1H, 1-H); ^{13}C NMR (126 MHz, DMSO-d_6): δ_C 13.5 (C-18), 21.1 (CH_2), 23.2 (2C, 2 × ring CH_2), 25.6 (CH_2), 26.1 (CH_2), 28.8 (CH_2), 31.4 (CH_2), 35.4 (CH_2), 37.9 (CH), 43.4 (CH), 47.3 (C-13), 49.6 (CH), 53.0 (2C, 2 × *N*-CH_2), 56.7 (*N*-CH_2), 115.0 (C-4), 120.5 (C-2), 125.3 (C-1), 129.6 (C-10), 135.8 (C-5), 154.7 (C-3), 219.7 (C-17); ESI-MS 354 $[M + H]^+$.

*4.2.2.9. 2-[((L)-Prolin-1-yl)methyl]-estrone (**1j**).* According to the general procedure, (*L*)-proline (231 mg) was used, and the pH was adjusted to 7 before work-up. The crude product was purified with MeOH / EtOAc = 30:70 to afford **1 j** (302 mg, 76 %) as a slightly pinkish solid. Mp >210 °C (decomposes); Anal. calcd. for $C_{24}H_{31}NO_4$ C, 72.52; H, 7.86; Found: C, 72.41; H, 7.87; ^{1}H NMR (500 MHz, DMSO-d_6): δ_H 0.83 (s, 3H, 18-H_3), 1.28–1.58 (m, 6 H), 1.63–1.71 (m, 1 H), 1.73–1.78 (m, 1 H), 1.80–1.87 (m, 1 H), 1.93–1.99 (m, 3 H), 2.00–2.09 (m, 1 H), 2.11–2.21 (m, 2 H), 2.28–2.36 (m, 1 H), 2.43 (dd, J = 18.9, 8.5 Hz, 1 H), 2.73–2.78 (m, 2H, 6-H_2), 3.15 (t, J = 3.5 Hz, 1H, ring CH), 3.50 (dd, J = 9.1, 5.3 Hz, 2 H), 3.79 (d, J = 13.0 Hz, 1H, one of *N*-CH_2), 4.15 (d, J = 13.0 Hz, 1H, the other *N*-CH_2), 6.57 (s, 1H, 4-H), 7.14 (s, 1H, 1-H); ^{13}C NMR (126 MHz, DMSO-d_6): δ_C 13.4 (C-18), 21.0 (CH_2), 22.9 (ring CH_2), 25.5 (CH_2), 25.9 (CH_2), 28.4 (ring CH_2), 28.7 (CH_2), 31.2 (CH_2), 35.3 (CH_2), 37.8 (CH), 43.2 (CH), 47.2 (C-13), 49.5 (CH), 52.7 (*N*-CH_2), 54.0 (CH_2), 66.8 (ring CH), 115.1 (C-4), 117.4 (C-2), 127.5 (C-1), 129.9 (C-10), 137.6 (C-5), 154.1 (C-3), 171.4 (COOH), 219.4 (C-17); ESI-MS 398 $[M + H]^+$.

*4.2.3. Synthesis of 2-[(piperazin-1-yl)methyl)-estrone (**1k**)*

2-[((4-*tert*-Butoxycarbonyl)piperazin-1-yl)methyl]-estrone (**1e**, 468 mg, 1.00 mmol) was dissolved in 10 mL of anhydrous CH_2Cl_2 and 1 mL of trifluoroacetic acid was added dropwise to the mixture. The solution was then stirred at room temperature for 24 h. After completion, the colorless mixture was poured onto water, the pH was adjusted to 8 with 1 N KOH solution and the precipitate formed was extracted with EtOAc (3 × 25 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4 and reduced *in vacuo*. The crude product was purified by column chromatography using EtOAc / MeOH / triethylamine = 85:10:5 as eluent to afford **1k** as an off-white solid (313 mg, 85 %). Mp >200 °C (decomposes); Anal. calcd. for $C_{23}H_{32}N_2O_2$ C, 74.96; H, 8.75; Found: C, 74.84; H, 8.65; ^{1}H NMR (500 MHz, DMSO-d_6): δ_H 0.82 (s, 3H, 18-H_3), 1.25–1.61 (m, 6 H), 1.71–1.79 (m, 1 H), 1.86–1.99 (m, 2 H), 2.05 (dt, J = 18.5, 8.8 Hz, 1 H), 2.09–2.17 (m, 1 H), 2.25–2.48 (overlapping m, 6H, 4H of ring CH_2), 2.69 (t, J = 4.9 Hz, 4H, ring CH_2), 2.72–2.77 (m, 2H, 6-H_2), 3.49–3.59 (m, 2H, *N*-CH_2), 6.41 (s, 1H, 4-H), 6.93 (s, 1H, 1-H); ^{13}C NMR (126 MHz, DMSO-d_6): δ_C 13.5 (C-18), 21.1 (CH_2), 25.6 (CH_2), 26.1 (CH_2), 28.7 (CH_2), 31.3 (CH_2), 35.3 (CH_2), 37.9 (CH), 43.4 (CH), 45.6 (2C, 2 × ring CH_2), 47.3 (C-13), 49.6 (CH), 53.4 (2C, 2 × ring CH_2), 60.1 (*N*-CH_2), 115.1 (C-4), 119.2 (C-2), 125.8 (C-1), 129.7 (C-10), 135.9 (C-5), 154.7 (C-3), 219.6 (C-17); ESI-MS 369 $[M + H]^+$.

*4.2.4. General procedure for the synthesis of E2-derived Mannich bases (**4a**−**k**)*

E-based Mannich base (**1a**−**k**, 0.50 mmol) was dissolved in EtOH (10 mL) and $NaBH_4$ (45 mg, 1.20 mmol) was added. The solution was stirred at room temperature for 2 h, then poured into water and neutralized with diluted HCl to decompose the excess of the reagent. After adjusting the pH to 8 with 1 N KOH solution, the resulting precipitate was filtered, washed with water, dried, and purified by flash chromatography.

*4.2.4.1. 2-[(Dimethylamino)methyl]-estradiol (**4a**).* According to the general procedure, compound **1a** (164 mg) was used. The crude product was purified with EtOAc / DCM = 20:80 to afford **4a** (150 mg, 91 %) as a white solid. Mp 148−150 °C; Anal. calcd. for $C_{21}H_{31}NO_2$ C, 76.55; H, 9.48; Found: C, 76.45; H, 9.39; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 0.78 (s, 3H, 18-H_3), 1.14–1.54 (overlapping m, 7 H), 1.65–1.73 (m, 1 H), 1.82–1.88 (m, 1 H), 1.91–1.96 (m, 1 H), 2.07–2.19 (m, 2 H), 2.25–2.30 (m, 1 H), 2.32 (s, 6H, 2 × *N*-CH_3), 2.75–2.87 (m, 2H, 6-H_2), 3.53–3.67 (m, 2H, *N*-CH_2), 3.72 (t, J = 8.4 Hz, 1H, 17-H), 6.56 (s, 1H, 4-H), 6.87 (s, 1H, 1-H); ^{13}C NMR (126 MHz, $CDCl_3$): δ_C 11.1 (C-18), 23.2 (CH_2), 26.4 (CH_2), 27.3 (CH_2), 29.4 (CH_2), 30.7 (CH_2), 36.8 (CH_2), 38.9 (CH), 43.3 (C-13), 43.9 (CH), 44.5 (2C, 2 × *N*-CH_3), 50.1 (CH), 62.9 (*N*-CH_2), 82.0 (C-17), 115.9 (C-4), 119.3 (C-2), 125.2 (C-1), 130.9 (C-10), 137.3 (C-5), 155.6 (C-3); ESI-MS 330 $[M + H]^+$.

*4.2.4.2. 2-((bis(2-Methoxyethyl)amino)methyl)-estradiol (**4b**).* According to the general procedure, compound **1b** (208 mg) was used. The crude product was purified with EtOAc / DCM = 20:80 → 50:50 to afford **4b** (184 mg, 88 %) as an opalescent oil. Anal. calcd for $C_{25}H_{39}NO_4$ C, 71.91; H, 9.41 Found: C, 72.02; H, 9.38; ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 0.78 (s, 3H, 18-H_3), 1.14–1.21 (m, 1 H), 1.23–1.54 (overlapping m, 6 H), 1.65–1.73 (m, 1 H), 1.82–1.87 (m, 1 H), 1.91–1.96 (m, 1 H), 2.06–2.18 (overlapping m, 2 H), 2.27 (dt, J = 13.2, 3.7 Hz, 1 H), 2.76–2.89 (overlapping m, 6H, 2 × *N*-CH_2 and 2H of 6-H_2), 3.33 (s, 6H, 2 × *O*-CH_3), 3.55 (t, 4H, J = 5.5 Hz, 2 × *O*-CH_2), 3.72 (t, 1H, J = 8.5 Hz, 17-H), 3.78–3.91 (m, 2H, *N*-CH_2), 6.57 (s, 1H, 4-H), 6.89 (s, 1H, 1-H), 10.19 (bs, 1H, 3-OH); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 11.2 (C-18), 23.3 (CH_2), 26.6 (CH_2), 27.5 (CH_2), 29.5 (CH_2), 30.8 (CH_2), 37.0 (CH_2), 39.1 (CH), 43.4 (C-13), 44.1 (CH), 50.3 (CH), 53.4 (2C, 2 × *N*-CH_2), 58.7 (*N*-CH_2), 58.9 (2C, 2 × OMe), 70.6 (2C, 2 × *O*-CH_2), 82.1 (C-17), 116.2 (C-4), 119.9 (C-2), 125.6 (C-1), 131.0 (C-10), 137.3 (C-5), 155.7 (C-3); ESI-MS 418 $[M + H]^+$, 440 $[M + Na]^+$.

*4.2.4.3. 2-[(Piperidin-1-yl)methyl]-estradiol (**4c**).* According to the general procedure, compound **1c** (184 mg) was used. The crude product was purified with EtOAc / DCM = 20:80 to afford **4c** (161 mg, 87 %) as a white solid. Mp 92−94 °C; Anal. calcd. for $C_{24}H_{35}NO_2$ C, 78.00; H, 9.55; Found: C, 77.91; H, 9.50; ^{1}H NMR (500 MHz, DMSO-d_6): δ_H 0.66 (s, 3H, 18-H_3), 1.06–1.46 (overlapping m, 9H, 2H of ring), 1.48–1.54 (t-like m, 4H, ring CH_2), 1.54–1.60 (m, 1 H), 1.73–1.78 (m, 1 H), 1.80–1.92 (m, 2 H), 2.01–2.08 (m, 1 H), 2.19–2.25 (m, 1 H), 2.39 (bs like m, 4H, ring CH_2), 2.62–2.75 (m, 2H, 6-H_2), 3.48–3.59 (m, 3H, *N*-CH_2 and 17-H), 4.47 (bs, 1H, 17-OH), 6.37 (s, 1H, 4-H), 6.90 (s, 1H, 1-H); ^{13}C NMR (126 MHz, DMSO-d_6): δ_C 11.2 (C-18), 22.7 (ring CH_2), 23.6 (CH_2), 25.5 (2C, 2 × ring CH_2), 26.1 (CH_2), 26.9 (CH_2), 28.8 (CH_2), 29.9 (CH_2), 36.6 (CH_2), 38.6 (CH), 42.8 (C-13), 43.5 (CH), 49.5 (CH), 53.2 (2C, 2 × ring CH_2), 60.4 (*N*-CH_2), 80.0 (C-17), 115.0 (C-4), 119.3 (C-2), 125.5 (C-1), 130.2 (C-10), 135.9 (C-5), 154.8 (C-3); ESI-MS 370 $[M + H]^+$.

*4.2.4.4. 2-[(Morpholino)methyl]-estradiol (**4d**).* According to the general procedure, compound **1d** (185 mg) was used. The crude product was purified with EtOAc / DCM = 20:80 to afford **4d** (145 mg, 78 %) as a white solid. Mp 169−171 °C; Anal. calcd. for $C_{23}H_{33}NO_3$ C, 74.36; H, 8.95; Found: C, 74.28; H, 8.92; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 0.78 (s, 3H, 18-H_3), 1.14–1.22 (m, 1 H), 1.23–1.52 (m, 6 H), 1.65–1.73 (m, 1 H), 1.82–1.89 (m, 1 H), 1.91–1.96 (dt, J = 12.7, 3.3 Hz, 1 H), 2.06–2.18 (m, 2 H), 2.27 (dt, J = 13.1, 3.6 Hz, 1 H), 2.61 (bs, 4H, ring CH_2), 2.75–2.87 (m, 2H, 6-H_2), 3.64–3.82 (overlapping m, 7H, 1H of 17-H, 2H of *N*-CH_2, and 4H of ring CH_2), 6.58 (s, 1H, 4-H), 6.91 (s, 1H, 1-H); ^{13}C NMR (126 MHz, $CDCl_3$): δ_C 11.2 (C-18), 23.3 (CH_2), 26.6 (CH_2), 27.5 (CH_2), 29.5 (CH_2), 30.8 (CH_2), 37.0 (CH_2), 39.1 (CH), 43.4 (C-13), 44.1 (CH), 50.3 (CH), 53.2 (2C, 2 × ring CH_2), 62.2 (*N*-CH_2), 67.0 (2C, 2 × *O*-CH_2), 82.1 (C-17), 116.1 (C-4), 118.2 (C-2), 125.8 (C-1), 131.4 (C-10), 137.7 (C-5), 155.3 (C-3); ESI-MS 372 $[M + H]^+$.

*4.2.4.5. 2-[((4-tert-Butoxycarbonyl)piperazin-1-yl)methyl]-estradiol (**4e**).* According to the general procedure, compound **1e** (234 mg) was used. The crude product was purified with EtOAc / DCM = 10:90 to afford **4e** (214 mg, 91 %) as a white solid. Mp 181−183 °C; Anal. calcd. for $C_{28}H_{42}N_2O_4$ C, 71.46; H, 9.00; Found: C, 71.40; H, 9.02; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 0.78 (s, 3H, 18-H_3), 1.14–1.21 (m, 1 H), 1.24–1.52 (overlapping m, 15H, 9H s of Boc *tert*-Bu), 1.65–1.73 (m, 1 H), 1.83–1.89 (m, 1 H), 1.91–1.96 (m, 1 H), 2.07–2.17 (m, 2 H), 2.24–2.30 (m, 1 H), 2.50 (bs, 4H, ring CH_2), 2.75–2.87 (m, 2H, 6-H_2), 3.47 (bs, 4H, ring CH_2), 3.60–3.75 (m, 3H, 2H of *N*-CH_2 and 17-H), 6.56 (s, 1H, 4-H), 6.88 (s, 1H, 1-H), 10.24 (bs, 1H, 3-OH); ^{13}C NMR (126 MHz, $CDCl_3$): δ_C 11.1 (C-18), 23.1 (CH_2), 26.4 (CH_2), 27.3 (CH_2), 28.4 (3C, 3 × Boc methyl), 29.4 (CH_2), 30.7 (CH_2), 36.8 (CH_2), 38.9 (CH), 43.3 (C-13), 43.9 (CH), 50.1 (CH), 52.4 (broad, 4C, 4 × ring CH_2), 61.7 (*N*-CH_2), 80.0 (Boc *tert*-Bu), 81.9 (C-17), 116.0 (C-4), 118.2 (C-2), 125.7 (C-1), 131.3 (C-10), 137.6 (C-5), 154.6 (Boc carbonyl), 155.1 (C-3); ESI-MS 471 $[M + H]^+$.

*4.2.4.6. 2-[(4-Methylpiperazin-1-yl)methyl]-estradiol (**4f**).* According to the general procedure, compound **1f** (191 mg) was used. The crude product was purified with acetone to afford **4f** (160 mg, 83 %) as a white solid. Mp 166−167 °C; Anal. calcd. for $C_{24}H_{36}N_2O_2$ C, 74.96; H, 9.44; Found: C, 74.82; H, 9.33; ^{1}H NMR (500 MHz, DMSO-d_6): δ_H 0.66 (s, 3H, 18-H_3), 1.03–1.42 (m, 7 H), 1.53–1.60 (m, 1 H), 1.73–1.79 (m, 1 H), 1.80–1.90 (m, 2 H), 2.01–2.08 (m, 1 H), 2.16 (s, 3H, *N*-CH_3), 2.19–2-25 (m, 1 H), 2.26–2.54 (overlapping m, 8H, ring CH_2), 2.62–2.75 (m, 2H, 6-H_2), 3.48–3.60 (m, 3H, *N*-CH_2 and 17-H), 4.45 (bs, 1H, 17-OH), 6.39 (s, 1H, 4-H), 6.93 (s, 1H, 1-H); ^{13}C NMR (126 MHz, DMSO-d_6): δ_C 11.2 (C-18), 22.7 (CH_2), 26.1 (CH_2), 26.9 (CH_2), 28.8 (CH_2), 29.9 (CH_2), 36.6 (CH_2), 38.6 (CH), 42.8 (C-13), 43.5 (CH), 45.5 (*N*-CH_3), 49.5 (CH), 52.0 (2C, 2 × ring CH_2), 54.6 (2C, 2 × ring CH_2), 59.2 (*N*-CH_2), 80.0 (C-17), 115.1 (C-4), 119.2 (C-2), 125.8 (C-1), 130.3 (C-10), 136.0 (C-5), 154.4 (C-3); ESI-MS 385 $[M + H]^+$.

*4.2.4.7. 2-[(4-Ethylpiperazin-1-yl)methyl]-estradiol (**4g**).* According to the general procedure, compound **1g** (198 mg) was used. The crude product was purified with acetone to afford **4g** (161 mg, 81 %) as a white solid. Mp 179−181 °C; Anal. calcd. for $C_{25}H_{38}N_2O_2$ C, 75.33; H, 9.61; Found: C, 75.30; H, 9.63; ^{1}H NMR (500 MHz, DMSO-d_6): δ_H 0.66 (s, 3H, 18-H_3), 0.98 (t, J = 7.1 Hz, 3H, ethyl CH_3), 1.04–1.43 (m, 7 H), 1.53–1.60 (m, 1 H), 1.73–1.79 (m, 1 H), 1.80–1.91 (m, 2 H), 2.01–2.08 (m, 1 H), 2.19–2.25 (m, 1 H), 2.31 (q, J = 7.2 Hz, 2H, ethyl CH_2), 2.43 (bs, 8H, ring CH_2), 2.62–2.75 (m, 2H, 6-H_2), 3.47–3.60 (m, 3H, *N*-CH_2 and 17-H), 4.45 (bs, 1H, 17-OH), 6.38 (s, 1H, 4-H), 6.93 (s, 1H, 1-H), 10.23 (bs, 1H, 3-OH); ^{13}C NMR (126 MHz, DMSO-d_6): δ_C 11.2 (C-18), 11.9 (ethyl CH_3), 22.7 (CH_2), 26.1 (CH_2), 26.9 (CH_2), 28.8 (CH_2), 29.9 (CH_2), 36.6 (CH_2), 38.6 (CH), 42.8 (C-13), 43.5 (CH), 49.5 (CH), 51.4 (ethyl CH_2), 52.1 (2C, 2 × ring CH_2), 52.2 (2C, 2 × ring CH_2), 59.3 (*N*-CH_2), 80.0 (C-17), 115.0 (C-4), 119.2 (C-2), 125.7 (C-1), 130.2 (C-10), 136.0 (C-5), 154.4 (C-3); ESI-MS 399 $[M + H]^+$.

*4.2.4.8. 2-[(4-Isopropylpiperazin-1-yl)methyl]-estradiol (**4h**).* According to the general procedure, compound **1h** (205 mg) was used. The crude product was purified with acetone to afford **4h** (142 mg, 69 %) as a white solid. Mp 183−185 °C; Anal. calcd. for $C_{26}H_{40}N_2O_2$ C, 75.68; H, 9.77; Found: C, 75.66; H, 9.80; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 0.78 (s, 3H, 18-H_3), 1.05 (d, J = 6.5 Hz, 6H, 2 × isopropyl CH_3), 1.14–1.21 (m, 1 H), 1.22–1.53 (m, 6 H), 1.65–1.73 (m, 1 H), 1.83–1.89 (m, 1 H), 1.91–1.97 (m, 1 H), 2.07–2.19 (m, 2 H), 2.25–2.32 (m, 1 H), 2.59 (bs, 8H, ring CH_2), 2.69 (p, J = 6.5 Hz, 1H, isopropyl CH), 2.75–2.87 (m, 2H, 6-H_2), 3.58–3.77 (overlapping m, 3H, *N*-CH_2 and 17-H), 6.54 (s, 1H, 4-H), 6.89 (s, 1H, 1-H); ^{13}C NMR (126 MHz, $CDCl_3$): δ_C 11.1 (C-18), 18.5 (d, J = 2.0 Hz, 2C, 2 × isopropyl CH_3), 23.2 (CH_2), 26.4 (CH_2), 27.3 (CH_2), 29.4 (CH_2), 30.7 (CH_2), 36.8 (CH_2), 38.9 (CH), 43.3 (C-13), 43.9 (CH), 48.5 (2C, 2 × ring CH_2), 50.1 (CH), 52.9 (2C, 2 × ring CH_2), 54.4 (*i*-Pr CH), 61.5 (*N*-CH_2), 81.9 (C-17), 115.8 (C-4), 118.6 (C-2), 125.5 (C-1), 131.0 (C-10), 137.3 (C-5), 155.4 (C-3); ESI-MS 413 $[M + H]^+$.

*4.2.4.9. 2-[(Pyrrolidin-1-yl)methyl]-estradiol (**4i**).* According to the general procedure, compound **1i** (177 mg) was used. The crude product was purified with EtOAc / DCM = 20:80 to afford **4i** (156 mg, 88 %) as a white solid. Mp >90 °C (decomposes); Anal. calcd. for $C_{23}H_{33}NO_2$ C, 77.70; H, 9.36; Found: C, 77.59; H, 9.28; ^{1}H NMR (500 MHz, DMSO-d_6): δ_H 0.66 (s, 3H, 18-H_3), 1.04–1.42 (m, 7 H), 1.53–1.59 (m, 1 H), 1.70–1.79 (overlapping m, 5H, 4H ring CH_2), 1.80–1.92 (m, 2 H), 2.02–2.08 (m, 1 H), 2.19–2.25 (m, 1 H), 2.46–2.50 (m, overlapping with solvent, 4H, ring CH_2), 2.62–2.72 (m, 2H, 6-H_2), 3.51 (t, J = 8.5 Hz, 1H, 17-H), 3.60–3.71 (m, 2H, *N*-CH_2), 4.49 (bs, 1H, 17-OH), 6.38 (s, 1H, 4-H), 6.93 (s, 1H, 1-H); ^{13}C NMR (126 MHz, DMSO-d_6): δ_C 11.3 (C-18), 22.8 (CH_2), 23.2 (2C, 2 × ring CH_2), 26.2 (CH_2), 27.0 (CH_2), 28.9 (CH_2), 29.9 (CH_2), 36.6 (CH_2), 38.7 (CH), 42.8 (C-13), 43.5 (CH), 49.5 (CH), 53.0 (2C, 2 × ring CH_2), 56.8 (*N*-CH_2), 80.1 (C-17), 114.9 (C-4), 120.5 (C-2), 125.2 (C-1), 130.0 (C-10), 135.7 (C-5), 154.6 (C-3); ESI-MS 356 $[M + H]^+$.

*4.2.4.10. 2-[((L)-Prolin-1-yl)methyl]-estradiol (**4j**).* According to the general procedure, compound **1j** (199 mg) was used, and the pH was adjusted to 7 before work-up. The crude product was purified with MeOH / EtOAc = 30:70 to afford **4j** (172 mg, 86 %) as a white solid. Mp >190 °C (decomposes); Anal. calcd. for $C_{24}H_{33}NO_4$ C, 72.15; H, 8.33; Found: C, 72.01; H, 8.23; ^{1}H NMR (500 MHz, DMSO-d_6): δ_H 0.65 (s, 3H, 18-H_3), 1.04–1.42 (m, 7 H), 1.53–1.61 (m, 1 H), 1.63–1.71 (m, 1 H), 1.73–1.79 (m, 1 H), 1.80–1.91 (m, 3 H), 1.91–1.99 (m, 1 H), 2.02–2.09 (m, 1 H), 2.11–2.20 (m, 1 H), 2.22–2.27 (m, 1 H), 2.65–2.73 (m, 2H, 6-H_2), 2.75–2.83 (m, 1 H), 3.16–3.23 (m, 1 H), 3.48–3.58 (overlapping m, 2H, 1H of 17-H), 3.84 (d, J = 12.9 Hz, 1H of *N*-CH_2), 4.17 (d, J = 12.9 Hz, 1H of *N*-CH_2), 4.51 (s, 1H, 17-OH), 6.58 (s, 1H, 4-H), 7.15 (s, 1H, 1-H); ^{13}C NMR (126 MHz, DMSO-d_6): δ_C 11.2 (C-18), 22.7 (CH_2), 23.0 (ring CH_2), 26.1 (CH_2), 26.8 (CH_2), 28.3 (ring CH_2), 28.9 (CH_2), 29.9 (CH_2), 36.5 (CH_2), 38.5 (CH), 42.8 (C-13), 43.4 (CH), 49.5 (CH), 52.8 (ring CH_2), 53.8 (ring CH_2), 67.0 (ring CH), 80.0 (C-17), 115.2 (C-4), 116.8 (C-2), 127.9 (C-1), 130.5 (C-10), 137.9 (C-5), 154.1 (C-3), 171.1 (COOH); ESI-MS 400 $[M + H]^+$.

*4.2.4.11. 2-[(Piperazin-1-yl)methyl)-estradiol (**4k**).* According to the general procedure, compound **1k** (184 mg) was used. The crude product was purified with EtOAc / MeOH / triethylamine = 85:10:5 to afford **4k** (130 mg, 70 %) as a white solid. Mp 187–189 °C (decomposes); Anal. calcd. for $C_{23}H_{34}N_2O_2$ C, 74.55; H, 9.25; Found C, 74.48; H, 9.19; ^{1}H NMR (500 MHz, DMSO-d_6): δ_H 0.66 (s, 3H, 18-H_3), 1.05–1.43 (m, 7 H), 1.53–1.60 (m, 1 H), 1.73–1.79 (m, 1 H), 1.80–1.92 (m, 2 H), 2.00–2.10 (m, 2 H), 2.19–2.26 (m, 1 H), 2.35 (bs-like m, 4H, ring CH_2), 2.65–2.75 (m, 5H, 2H of 6-H_2), 3.49–3.57 (overlapping m, 3H, 17-H and *N*-CH_2), 4.47 (bs, 1H, 17-OH), 6.38 (s, 1H, 4-H), 6.92 (s, 1H, 1-H), 10.40 (bs, 1H, 3-OH); ^{13}C NMR (126 MHz, DMSO-d_6): δ_C 11.2 (C-18), 22.7 (CH_2), 26.1 (CH_2), 26.9 (CH_2), 28.8 (CH_2), 29.9 (CH_2), 36.6 (CH_2), 38.6 (CH), 42.8

(C-13), 43.5 (CH), 45.6 (2C, 2 × ring CH_2), 49.5 (CH), 53.4 (2C, 2 × ring CH_2), 60.2 (*N*-CH_2), 80.0 (C-17), 115.0 (C-4), 119.1 (C-2), 125.7 (C-1), 130.2 (C-10), 135.9 (C-5), 154.6 (C-3); ESI-MS 371 $[M + H]^+$.

4.2.5. General procedure for the synthesis of 17β-benzylamino Mannich bases (5a–c, 5h, 5i) by reductive amination

To a stirred solution of E-based Mannich base (**1a–c, 1 h, 1i**, 0.50 mmol) in MeOH (10 mL), benzylamine (0.22 mL, 4 equiv.) and $NaCNBH_3$ (47 mg, 1.75 mmol) was added, and the pH was adjusted to 6 by the addition of glacial acetic acid. The solution was stirred at room temperature for 16−24 h, then poured into $NaHCO_3$ solution and extracted with EtOAc (2 × 10 mL). The combined organic layers were washed with water, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The resulting crude product was purified by flash chromatography.

*4.2.5.1. 17β-(Benzylamino)-2-[(dimethylamino)methyl]estra-1,3,5(10)-trien-3-ol (**5a**).* According to the general procedure, compound **1a** (164 mg) was used. The crude product was purified with EtOAc to afford **5a** (134 mg, 64 %) as an off-white solid. Mp 168−170 °C; Anal. calcd. for $C_{28}H_{38}N_2O$ C, 80.34; H, 9.15; Found: C, 80.22; H, 9.10; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 0.79 (s, 3H, 18-H_3), 1.16–1.55 (m, 7 H), 1.67–1.74 (m, 1 H), 1.82–1.87 (m, 1 H), 1.99–2.12 (m, 2 H), 2.11–2.18 (m, 1 H), 2.21–2.28 (m, 1 H), 2.31 (s, 6H, 2 *N*-CH_3), 2.67 (t, $J = 8.6$ Hz, 1H, 17-H), 2.77–2.86 (m, 2H, 6-H_2), 3.55 (d, $J = 13.7$ Hz, 1H, Bn-CH_2), 3.63 (d, $J = 13.7$ Hz, 1H, Bn-CH_2) 3.81–3.90 (m, 2H, *N*-CH_2), 6.55 (s, 1H, 4-H), 6.86 (s, 1H, 1-H), 7.23–7.28 (t-like m, 1H, 4'-H), 7.31–7.37 (m, 4H, 2'-,3'-,5'- and 6'-H); ^{13}C NMR (126 MHz, $CDCl_3$): δ_C 12.1 (C-18), 23.7 (CH_2), 26.7 (CH_2), 27.6 (CH_2), 29.56 (CH_2), 29.65 (CH_2), 38.2 (CH_2), 39.0 (CH), 43.3 (C-13), 44.1 (CH), 44.7 (2C, *N*-CH_3), 52.4 (CH), 52.8 (Bn CH_2), 63.1 (*N*-CH_2), 68.3 (C-17), 115.9 (C-4), 119.4 (C-2), 125.2 (C-1), 127.0 (C-4'), 128.2 (2C, 2 × Bn ring), 128.5 (2C, 2 × Bn ring), 131.0 (C-10), 137.3 (C-5), 140.9 (C-1'), 155.7 (C-3); ESI-MS 419 $[M + H]^+$.

*4.2.5.2. 17β-(Benzylamino)-2-((bis(2-methoxyethyl)amino)methyl)estra-1,3,5(10)-trien-3-ole (**5b**).* According to the general procedure, compound **1b** (208 mg) was used. The crude product was purified with EtOAc / DCM = 20:80 → 50:50 to afford **5b** (142 mg, 56 %) as a colourless oil that slowly crystallized. Mp 97−98 °C; Anal. calcd. for $C_{32}H_{46}N_2O_3$ C, 75.85; H, 9.15; Found: C, 75.72; H, 9.22; ^{1}H NMR (500 MHz, DMSO-d_6): δ_H 0.71 (s, 3H, 18-H_3), 1.07–1.38 (m, 7 H), 1.56–1.64 (m, 1 H), 1.72–1.78 (m, 1 H), 1.86–1.99 (m, 2 H), 2.01–2.09 (m, 1 H), 2.18–2.24 (m, 1 H), 2.52 (t-like m overlapping with solvent, 1H, 17-H), 2.61–2.75 (overlapping m, 6H, 4H, 2 × *N*-CH_2 and 2H of 6-H_2), 3.21 (s, 6H, 2 × OMe), 3.42 (t, $J = 5.7$ Hz, 4H, 2 × *O*-CH_2), 3.63–3.79 (m, 4H, *N*-CH_2 and Bn CH_2), 6.37 (s, 1H, 4-H), 6.93 (s, 1H, 1-H), 7.21 (t-like m, 1H, 4'-H), 7.26–7.37 (m, 4H, 2'-,3'-,5'- and 6'-H); ^{13}C NMR (126 MHz, DMSO-d_6): δ_C 11.9 (C-18), 23.1 (CH_2), 26.2 (CH_2), 27.1 (CH_2), 28.8 (CH_2), 28.9 (CH_2), 37.6 (CH_2), 38.6 (CH), 42.8 (C-13), 43.5 (CH), 51.7 (CH), 51.9 (Bn CH_2), 52.4 (2C, 2 × *N*-CH_2), 56.4 (*N*-CH_2), 58.0 (2C, 2 × OMe), 67.8 (C-17), 69.6 (2C, 2 × *O*-CH_2), 115.1 (C-4), 120.2 (C-2), 125.7 (C-1), 126.3 (C-4'), 127.8 (2C, 2 × Bn ring), 128.0 (2C, 2 × Bn ring), 130.2 (C-10), 135.9 (C-5), 141.6 (C-1'), 154.7 (C-3); ESI-MS 507 $[M + H]^+$, 529 $[M + Na]^+$.

*4.2.5.3. 17β-(Benzylamino)-2-[(piperidin-1-yl)methyl]estra-1,3,5(10)-trien-3-ol (**5c**).* According to the general procedure, compound **1c** (184 mg) was used. The crude product was purified with EtOAc / DCM = 50:50 to afford **5c** (151 mg, 66 %) as a white solid. Mp 160−162 °C; Anal. calcd. for $C_{31}H_{42}N_2O$ C, 81.17; H, 9.23; Found: C, 81.07; H, 9.21; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 0.79 (s, 3H, 18-H_3), 1.15–1.55 (m, 9H, 2H of ring), 1.63 (p, $J = 5.6$ Hz, 4H, 2 × ring CH_2), 1.67–1.74 (m, 1 H), 1.83–1.88 (m, 1 H), 1.98–2.12 (m, 2 H), 2.11–2.18 (m, 1 H), 2.21–2.27 (m, 1 H), 2.50 (bs, 4H, 2 × ring CH_2), 2.67 (t, $J = 8.5$ Hz, 1H, 17-H), 2.73–2.88 (m, 2H, 6-H_2), 3.55–3.68 (m, 2H, Bn CH_2), 3.80–3.89 (m, 2H, *N*-CH_2), 6.54 (s, 1H, 4-H), 6.86 (s, 1H, 1-H), 7.24 (t, $J = 7.0$ Hz, 1H, 4'-H), 7.29–7.38 (m, 4H, 2'-,3'-,5'- and 6'-H); ^{13}C NMR (126 MHz, $CDCl_3$): δ_C 12.1 (C-18), 23.7 (CH_2), 24.3 (*N*-CH_2), 26.1 (2C, 2 × ring CH_2), 26.8 (CH_2), 27.7 (CH_2), 29.6 (CH_2), 29.9 (CH_2), 38.4 (CH_2), 39.1 (CH), 43.4 (C-13), 44.2 (CH), 52.6 (CH), 53.0 (CH_2), 54.1 (2C, 2 × ring CH_2), 62.5 (*N*-CH_2), 68.7 (C-17), 115.9 (C-4), 119.2 (C-2), 125.4 (C-1), 126.9 (C-4'), 128.1 (2C, 2 × Bn ring), 128.4 (2C, 2 × Bn ring), 131.1 (C-10), 137.2 (C-5), 141.5 (C-1'), 155.8 (C-3); ESI-MS 459 $[M + H]^+$.

*4.2.5.4. 17β-(Benzylamino)-2-[(4-isopropylpiperazin-1-yl)methyl]estra-1,3,5(10)-trien-3-ol (**5h**).* According to the general procedure, compound **1 h** (205 mg) was used. The crude product was purified with acetone to afford **5 h** (178 mg, 71 %) as a white solid. Mp 118−120 °C; Anal. calcd. for $C_{33}H_{47}N_3O$ C, 78.99; H, 9.44; Found: C, 78.86; H, 9.34; ^{1}H NMR (500 MHz, DMSO-d_6): δ_H 0.71 (s, 3H, 18-H_3), 0.95 (d, $J = 6.5$ Hz, 6H, 2 × isopropyl CH_3), 1.06–1.38 (m, 7 H), 1.55–1.64 (m, 1 H), 1.73–1.78 (m, 1 H), 1.87–1.98 (m, 2 H), 2.01–2.08 (m, 1 H), 2.18–2.23 (m, 1 H), 2.44 (bs, 8H, ring CH_2), 2.52 (t overlapping with solvent, $J = 8.5$ Hz, 1H, 17-H), 2.61 (p, $J = 6.5$ Hz, 1H, isopropyl CH), 2.65–2.75 (m, 2H, 6-H_2), 3.50–3.60 (m, 2H, Bn CH_2), 3.68–3.79 (m, 2H, *N*-CH_2), 6.37 (s, 1H, 4-H), 6.91 (s, 1H, 1-H), 7.21 (t-like m, 1H, 4'-H), 7.26–7.33 (m, 2H, 3'- and 5'-H), 7.33–7.37 (m, 2H, 2'- and 6'-H); ^{13}C NMR (126 MHz, DMSO-d_6): δ_C 11.9 (C-18), 18.2 (2C, 2 × isopropyl CH_3), 23.1 (CH_2), 26.2 (CH_2), 27.1 (CH_2), 28.8 (CH_2), 28.9 (CH_2), 37.6 (CH_2), 38.5 (CH), 42.8 (C-13), 43.5 (CH), 47.9 (2C, 2 × ring CH_2), 51.7 (CH), 51.9 (Bn CH_2), 52.5 (2C, 2 × ring CH_2), 53.5 (isopropyl CH), 59.4 (*N*-CH_2), 67.8 (C-17), 115.0 (C-4), 119.1 (C-2), 125.7 (C-1), 126.3 (C-4'), 127.8 (2C, 2 × Bn ring), 128.0 (2C, 2 × Bn ring), 130.2 (C-10), 136.0 (C-5), 141.6 (C-1'), 154.5 (C-3); ESI-MS 502 $[M + H]^+$.

*4.2.5.5. 17β-(Benzylamino)-2-[(pyrrolidin-1-yl)methyl]estra-1,3,5(10)-trien-3-ol (**5i**).* According to the general procedure, compound **1i** (177 mg) was used. The crude product was purified with EtOAc / DCM = 50:50 to afford **5i** (135 mg, 61 %) as a white solid. Mp 191−193 °C; Anal. calcd. for $C_{30}H_{40}N_2O$ C, 81.03; H, 9.07; Found: C, 81.00; H, 8.99; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 0.83 (s, 3H, 18-H_3), 1.16–1.54 (m, 7 H), 1.67–1.74 (m, 1 H), 1.80–1.87 (overlapping m, 5H, 4H of ring CH_2), 1.99–2.10 (m, 2 H), 2.10–2.18 (m, 1 H), 2.21–2.28 (m, 1 H), 2.62 (bs, 4H, ring CH_2), 2.67 (t, $J = 8.8$ Hz, 1H, 17-H), 2.77–2.86 (m, 2H, 6-H_2), 3.72 (d, $J = 13.7$ Hz, 1H, Bn CH_2), 3.80–3.87 (overlapping m, 2H, 1H of Bn CH_2, 1H of *N*-CH_2), 3.93 (d, $J = 13.4$ Hz, 1H, *N*-CH_2), 6.54 (s, 1H, 4-H), 6.87 (s, 1H, 1-H), 7.27 (t-like m, 1H, 4'-H), 7.31–7.36 (m, 2H, 2'- and 6'-H), 7.39 (m, 2H, 3'- and 5'-H); ^{13}C NMR (126 MHz, $CDCl_3$): δ_C 12.1 (C-18), 23.7 (CH_2), 23.9 (2C, 2 × ring CH_2), 26.8 (CH_2), 27.7 (CH_2), 29.6 (CH_2), 38.4 (CH_2), 39.1 (CH), 43.4 (C-13), 44.2 (CH), 52.6 (CH), 53.0 (Bn CH_2) 53.7 (2C, 2 × ring CH_2), 59.2 (*N*-CH_2), 68.7 (C-17), 115.9 (C-4), 120.0 (C-2), 124.8 (C-1), 126.9 (C-4') 128.1 (2C, 2 × Bn ring), 128.4 (2C, 2 × Bn ring), 131.0 (C-10), 137.2 (C-5), 141.5 (C-1'), 155.8 (C-3); ESI-MS 445 $[M + H]^+$.

4.3. Cell culture maintenance

The antiproliferative properties of the newly synthesized compounds (**1a-5i**) were determined on a panel of human adherent gynecological cancer cell lines. The HeLa (cervical adenocarcinoma), A2780 (ovarian adenocarcinoma) and MDA-MB-231 (hormone-insensitive breast adenocarcinoma) cells were cultured in minimum essential media (MEM). The cancer selectivity of compounds was tested on the non-cancerous mouse embryo fibroblast cell line NIH/3T3. All cell lines were purchased from European Collection of Cell Cultures (ECCAC, Salisbury, UK). 500 mL MEM was supplemented with 10 % fetal bovine serum (FBS), 5 mL 100X antibiotic-antimycotic mixture and 5 mL 100X non-essential amino acid solution. All media and supplements were obtained from Thermo Fisher Scientific, Waltham, MA USA. The cultured cells were incubated at 37 °C in humidified 5 % CO_2. Cell

maintenance was executed by sub-culturing in 75 cm^2 cell culture flasks. Rapidly growing cells were used for cell viability assay.

4.4. MTT assay for cell viability

The MTT assay was employed to determine the extent of inhibition of cell proliferation by the test compounds. Exponentially growing HeLa, A2780, MDA-MB-231 and NIH/3T3 cells were seeded into 96-well plates (5×10^3 cells per well in 100 µL of media) and incubated for 24 h to allow for cell attachment. First, cells were treated with 10 and 30 µmol concentrations of test compounds formulated in 100 µL of media and incubated for 72 h in a screening experiment. The cells in the control group were treated only with media. After 72 h of incubation, 20 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT-reagent, 5 mg / mL in PBS; Duchefa Biochemie BV, Haarlem, The Netherlands) was added and incubated for 4 h at 37 C in humidified 5 % CO_2. The medium was then aspirated and 100 µL of DMSO added to dissolve formazan crystals produced by the mitochondrial enzymes of intact cells, followed by 30 min of mechanical shaking. Absorbance measurements were subsequently taken using a microplate UV-VIS reader (SPECTROstar Nano, BMG Labtech GmbH, Offenburg, Germany) at 545 nm, followed by calculation for percentage cell viability [29]. 2-Methoxyestradiol (Merck KGaA, Darmstadt, Germany) was used as positive control. For determination of IC_{50} values cells were treated with six different concentrations of compounds in the range of 0.1–30 µmol. The IC_{50} values (the concentration at which the extent of cell proliferation was half that of the untreated control) were calculated using sigmoidal dose response curves fitted to the determined data by means of GraphPad Prism 5.01 (GraphPad Software, San Diego, CA, USA). All *in vitro* experiments were carried out twice with five parallels. Stock solutions of the tested substances (10 mM) were prepared in DMSO. The highest DMSO content of the medium (0.3 %) did not have any substantial effect on cell proliferation.

4.5. In vitro physicochemical test methods

Kinetic solubility was tested with 5 % DMSO as cosolvent in phosphate buffered saline (PBS, 0.01 M phosphate buffer pH 7.4, 0.138 M NaCl, 0.0027 M KCl) at room temperature for 2 h using 96-well MultiScreen HTS-PCF Filter Plates (Merck Millipore). Target concentration was 500 µM for every investigated compounds. After the incubation period all solutions were filtered by MultiScreen HTS Vacuum Manifold (Merck Millipore). Concentration of filtrates were determined by spectrophotometric detection ($AUC_{250\text{-}500nm}$) using Multiskan FC Microplate Photometer (Thermo Scientific).

The intestinal specific PAMPA investigation was based on a 'in house developed' method. Positive and negative control compounds (carbamazepine: $28.7 \pm 1.5 \cdot 10^{-6}$ cm/s and indomethacine and $2.5 \pm 0.2 \cdot 10^{-6}$ cm/s, respectively) were used in every individual PAMPA investigation. Briefly, a 96-well acceptor plate and a 96-well filter plate were assembled into a sandwich. The hydrophobic filter material of the 96 well filter plate was coated by the mixture of phosphatidylcholine / cholesterol = 2:1 (5 µL, 4 % (w/v) in *n*-dodecan). Subsequently, the acceptor wells at the bottom of the sandwich were filled with 300 µL of 10 mM PBS solution adjusted to pH 7.4. The donor wells at the top of the sandwich were hydrated with 150 µL of test compound solution. The test compound solutions were prepared in 100 times dilution. Firstly, a 10 mM stock solution was prepared from the corresponding compound in DMSO, than it was diluted with PBS (pH 6.5) to get 5 % mixture followed by filtration through a MultiScreen Solubility filter plate. The resulting sandwich was then incubated at 37 °C for 4 h. After the incubation, PAMPA sandwich plates were separated and compound concentrations in starting, donor and acceptor solutions were measured similar to kinetic solubility method. The effective permeability and membrane retention of drugs were calculated using the following equation [30]:

$$P_e = \frac{-2.303}{A \times (t - \tau_{ss})} \bullet \left(\frac{1}{1 + r_v}\right) \bullet \lg\left[- r_v + \left(\frac{1 + r_v}{1 - MR}\right) \times \frac{C_D(t)}{C_D(0)} \right]$$

where P_e is the effective permeability coefficient (cm/s), A is the filter area (0.3 cm^2), V_D and V_A are the volumes in the donor (0.15 cm^3) and acceptor phase (0.3 cm^3), t is the incubation time (s), τ_{SS} is the time to reach steady-state (s), $C_D(t)$ is the concentration of the compound in the donor phase at time point t (mol/cm^3), $C_D(0)$ is the concentration of the compound in the donor phase at time point zero (mol/cm^3), r_v is the aqueous compartment volume ratio (V_D/V_A), MR is the membrane retention factor, defined as:

$$MR = 1 - \frac{C_D(t)}{C_D(0)} - \frac{V_A C_A(t)}{V_D C_D(0)}$$

Author statement

Conceptualization, supervision É.F., R.M and Gy.T.B..; Chemical synthesis and optimization experiments B.M; Investigation, data curation, P.L. and G.M.; Pharmacological and physicochemical tests N. I. K.; Writing, original draft preparation B.M., É.F., Gy.T.B.; Resources I.Z.; Writing, review and editing R.M. and I.Z. All authors have read and agreed to the published version of the manuscript.

Acknowledgements

This work was supported by National Research, Development and Innovation Office-NKFIA (Hungary) through project GINOP-2.3.2-15-2016-00038. The publication was funded by The University of Szeged Open Access Fund (FundRef, Grant No. 5602).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.jsbmb.2022.106064.

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