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Synthesis and structure–activity relationships of 16-modified analogs of 2-methoxyestradiol

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Abstract—A series of 16-modified 2-methoxyestradiol analogs were synthesized and evaluated for antiproliferative activity toward HUVEC and MDA-MB-231 cells, and for susceptibility to conjugation. In addition, the estrogenicity of these analogs was accessed by measuring cell proliferation of the estrogen-dependent cell line MCF7 in response to compound treatment. It was observed that antiproliferative activity dropped as the size of the 16 substituent increased. Selected analogs tested in glucuronidation assays had similar rates of clearance to 2-methoxyestradiol, but had enhanced clearance in sulfonate conjugation assays.

1. Introduction

2-Methoxyestradiol (2ME2, **1**, Fig. 1—carbon numbering and ring labels added for clarity), an endogenous metabolite of estradiol (**2**), is produced in vivo by hydroxylation of estradiol at the 2-position and subsequent methylation of the 2-hydroxyestradiol by catechol-*O*-methyltransferase (COMT). 2ME2 has low affinity for the estrogen receptors and its physiological function is unknown. Although this endogenous metabolite was long thought to have no physiological function, several studies during the past decade demonstrated that it has antiproliferative activity in a wide range of cells from varied origins. Furthermore, numerous studies have demonstrated the efficacy of orally administered 2ME2 in animal oncology models.[1] Currently, 2ME2 is undergoing clinical evaluation in oncology in a number of Phase 1 and 2 trials under the name Panzem® NCD.

While the angiogenic, pro-apoptotic and tumor inhibitory effects of 2ME2 and its ability to induce apoptosis are well documented,[2] the mechanism of action at the molecular level is still being investigated. Several targets have been proposed, such as binding to tubulin, effects

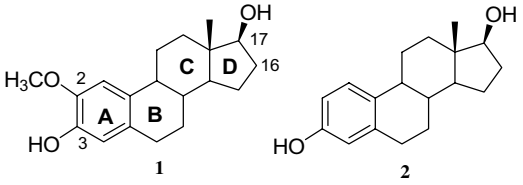

Figure 1.

on microtubule dynamics,[3–5] effects on cell cycle kinases, dysregulating hypoxia-inducible factor-1 (HIF-1),[6] and inhibition of superoxide dismutase (SOD) enzymatic activity.[7] Consistent with the low affinity of 2ME2 for the estrogen receptors α and β, its antiproliferative activity is independent of interaction with the estrogen receptors.[8] Apoptosis induced by treatment with 2ME2 occurs irrespective of p53 status,[9–11] and recent evidence suggests this activity is mediated through activation of the extrinsic pathway following upregulation of Death Receptor 5 (DR5).[12] 2ME2 binds the colchicine binding site of tubulin and destabilizes microtubules in tumor cells in vitro and in vivo. Through its disruption of microtubules, 2ME2 causes reduction in HIF-1α protein levels.[6] HIF-1α is a component of the dimeric transcription factor HIF-1 which regulates over 70 genes involved in cell survival, proliferation, metastasis, and angiogenesis.[13,14]

To elucidate the determinants of 2ME2 activity and to prepare more efficacious analogs, structure–activity

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relationship (SAR) studies have been conducted by several groups. In general, these SARs were evaluated in terms of antiproliferative activity across the NCI 60 tumor cell line screen (GI_{50}), and/or by binding to tubulin and inhibition of tubulin polymerization. Most of these SARs have concentrated on modifying or incorporating new substituents in the A, B, and D rings of 2ME2. In 1995, Cushman reported several 2-modified analogs of estradiol.[15] In this series of compounds, substituents that resembled the 2-methyl ether such as ethoxy and 2′,2′-difluoroethoxy as well as propynyl and propene had similar activities or were more potent than 2ME2. In 1997, Cushman[16] reported that modification of position 6 in some 2-modified analogs of 2ME2 resulted in compounds with similar or improved GI_{50} values compared to 2ME2, when the position 6 substituents were OH, NH_2 or ketone. In 1997, Miller and Macdonald reported that expansion of the A-ring to give tropinones had better binding to the cholchicine site of tubulin and inhibited tubulin polymerization more effectively than 2ME2, with the best analog reported to be approximately 5-fold more potent on tubulin.[17,18] In 2000, Wang[19] reported that expansion of the steroid B-ring to a seven membered ring caused an increase in the GI_{50} value for the NCI 60 tumor cell line screen, indicating these substitutions did not result in better antiproliferative activity. The exception in this series was the 6-acetamide, which retained similar activity to 2ME2. In 2002, Hughes reported cell cycle analysis (via inhibition of DNA synthesis) and ER affinity data for a series of 2-methoxy and 2-ethoxyestradiol analogs.[20] In the same publication, the authors also presented a CoMFA model for ER affinity and inhibition of DNA synthesis. In 2002, Cushman reported a series of 2-modified analogs of 2ME2 that were cytotoxic, and of which some were potent inhibitors of tubulin polymerization.[21] This work was continued in 2004 with additional 2- and 17-modified 2ME2 analogs.[22] Rao has reported on 2ME2 analogs with multiple A, B, and D ring modifications which have in vitro antiproliferative activity.[23] Several members of this series are ~2- to 10-fold more potent inhibitors of MDA-MB-231 and SK-OV-3 cell proliferation than 2ME2 and ~25-fold more potent at inhibition of tubulin polymerization. In summary, a number of groups have demonstrated that 2ME2 can be modified to improve inhibition of tubulin polymerization and antiproliferative activity.

The metabolism of hormonal steroids is well known. In the case of estradiol (E_2), oxidation of the 17-hydroxy functionality to a ketone by 17β-hydroxysteroid dehydrogenase (type 2, 17β-HSD) is a major metabolic route, which yields estrone (E_1) from E_2.[24] Additionally, conjugation of estradiols in the form of glucuronides and sulfonate esters is a major metabolic pathway for these compounds, with >95% of the E_2 analog 17-ethynylestradiol being converted to conjugates at steady state.[25] Recent evidence has revealed that similar transformations occur in humans and rats following oral administration of 2ME2.[26,27] Recently, Newman reported that 3,17-bissulfamate-2-methoxyestradiol had antiproliferative activity and was not a substrate for 17-β-hydroxysteroid dehydrogenase.[28] This suggests that other D-ring modifications may yield analogs with increased metabolic stability.

As 17-oxidation and conjugation are major pathways for metabolism and deactivation of 2ME2, we hypothesized that the addition of steric and/ or electronic bulk at position 16 may prevent or slow oxidation and/or conjugation at position 17 and consequently improve the antiangiogenic and antitumor effects observed with 2ME2 in vivo. To the best of our knowledge, there has been no structure–activity relationships established for 16-alkyl or 16-heteroalkyl substituted 2ME2 analogs. In this paper, we report a novel series of 2ME2 analogs with substitutions at position 16 of the D-ring and their structure–activity relationships. The biological activities assessed in vitro are antiproliferative activity toward HUVEC and MDA-MD-231 cells, level of estrogenic activity as reflected in MCF-7 cells, and glucuronide and sulfate conjugation by human liver enzymes.

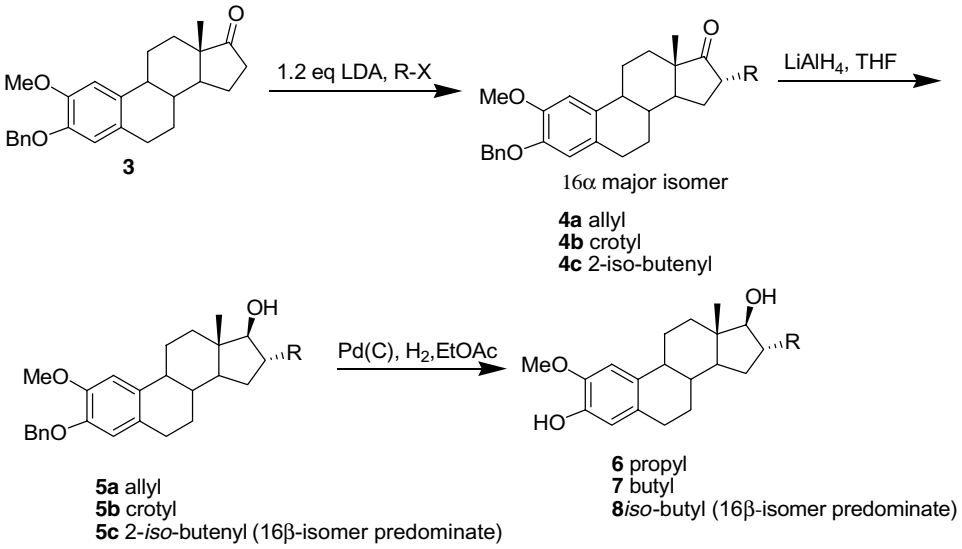
2. Results

2.1. Chemistry

All analogs were prepared using similar reaction conditions reported for estradiol analogs. 2ME2 was first protected as a benzyl ether using K_2CO_3/benzyl bromide in ethanol, then oxidized to 3-benzyl-2-methoxyestrone (**3**) using Swern conditions.[29] All synthetic schemes utilized 3-benzyloxy-2-methoxy estrone (**3**) as a starting material to prepare the 16-substituted analogs.

Compound **3** gave access to multiple 16-substituted analogs using enolate chemistry. It has been reported that direct alkylation of estrone with unactivated alkyl halides gives a mix of products including both α and β isomers as well as disubstituted products.[30–32] Only activated electrophiles, such as allyl halides, are reported to yield monosubstituted products by direct alkylation of estrones. Based on this precedence, and established chemistry for estradiol analogs, several of the 16-substituted analogs that had predominantly the α isomer were prepared as in Scheme 1.[31] Thus, 3-benzyloxy-$2ME_1$ (**3**) was treated with LDA, and alkylated with the appropriate allylic halide to give **4a**, **b**, and **c**. Subsequent low temperature reduction with lithium aluminum hydride gave 17β-alcohols **5a**, **b**, and **c** from the corresponding ketone. Concurrent debenzylation and olefin reduction using H_2 Pd/C gave the final products **6**, **7**, and **8**.

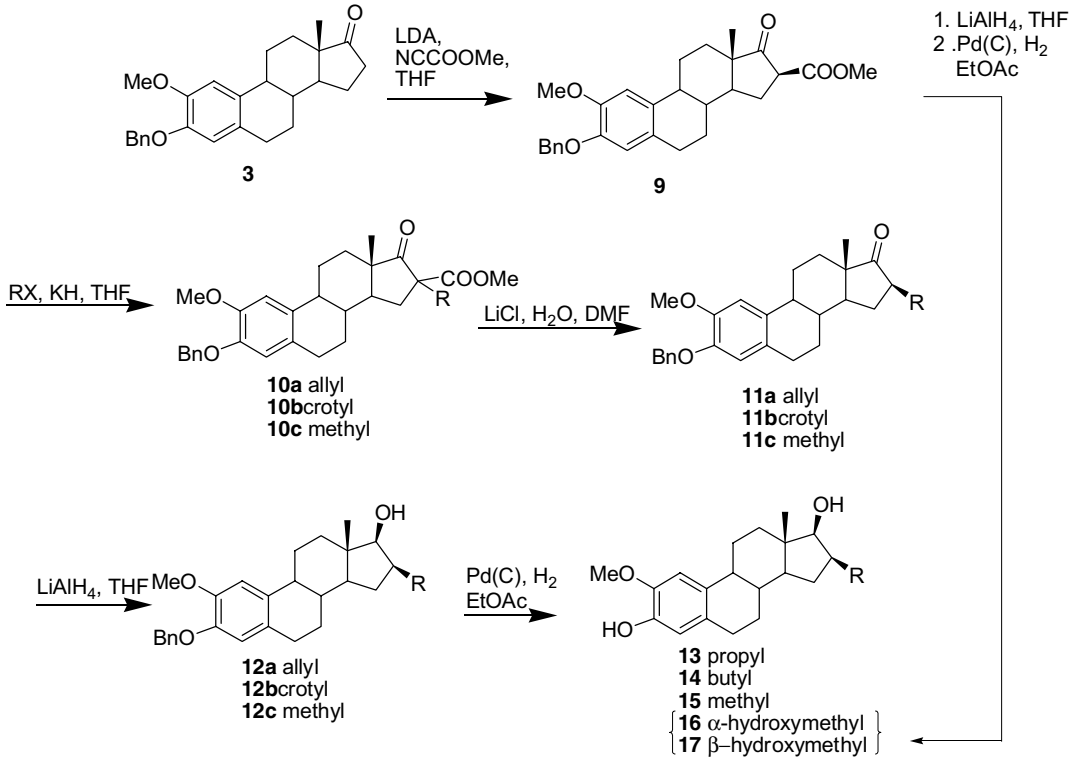
Stereochemical assignments were made by observing the 18-CH_3 group and H-16 ^{1}H NMR signals. It is well established that for 18-CH_3 the 16α-stereoisomer is upfield from the 16β isomer (generally about 0.02–0.05 ppm and the chemical shift is around 0.9 ppm). In the case of H-16, the 16β-stereoisomer is about 0.4–0.5 ppm downfield from the 16α-stereoisomer and these signals are centered around 3.5 ppm.[31] The reason for the preferential formation of the α-stereoisomer in synthesis of **6** and **7** can be explained by the steric shielding of the β-face of position 16 by the C18-axial methyl group, which forces the electrophile to approach from



Scheme 1.

the less hindered alpha face. The exception to this precedence in this series is the case of 16-*iso*-butylene **4c** which could be a result of isomerization.

When the 16β isomer was the desired isomer, Scheme 2 was utilized.[31] Thus, 3-benzyloxy-2-methoxyestrone (**3**) was methoxycarbonylated with LDA and cyanomethyl carboxylate to give **9**, then alkylated with KH and the appropriate alkyl halide giving **10a**, **b**, and **c**. Subsequent decarboxymethylation[33] gave **11a**, **b**, and **c** followed by ketone reduction as above gave protected 16β-alkyl-3-benzyloxy 2ME2 **12a**, **b**, and **c**. Deprotection and olefin reduction by catalytic hydrogenation gave final products **13**, **14**, and **15**. 16-Hydroxymethyl-2ME2 (**16**, **17**) was prepared by reduction of carboxymethyl intermediate **9** with lithium aluminum hydride at a low temperature to reduce the 17-ketone to the β-stereoisomer, followed by warming to ambient temperature to reduce the ester functionality giving **12d**.[34] Benzyl deprotection using catalytic hydrogenation and separation by column chromatography gave products **16** and **17**. It was observed by ^{1}H NMR that the ester **9** was approximately a 3:1 mixture of β:α stereoisomers. Purification by column chromatography isomerized the majority to the more stable



Scheme 2.

β-stereoisomer. However, upon LAH reduction of **9** and subsequent purification by column chromatography, a small amount of the α-stereoisomer was observed and was isolated by column chromatography. The α and β isomers' ^{1}H and ^{13}C NMR data are in agreement with Goto.[35] The 17β,16β-stereoisomer H-16 had a doublet at 3.94 ppm with a coupling constant of 9.8 Hz and the 17β, 16α-stereoisomer had the corresponding doublet at 3.47 ppm with coupling constant of 7.5 Hz.[15]

Two 16α-substituted analogs were prepared using Scheme 3. The use of hydrazones to give 16α-substituted steroids is well established.[36–39] Thus, **3** was converted to hydrazone **18** using *N*,*N*-dimethyl hydrazine, which was alkylated using LDA and the appropriate alkyl halide giving **19a** and **b**. Hydrolysis of the hydrazone with $CuCl_2$ gave ketones **20a** and **b**, which were reduced to the β-alcohols **21a** and **b** at low temperatures, with lithium aluminum hydride. Removal of the benzyl ether was accomplished by catalytic hydrogenation giving **22** and **23**. Additionally, 16α-(dimethylaminomethyl)-2ME2 was prepared as shown in Scheme 4, thus alkylation of **3** with neat *tert*-butoxy-bis(dimethylamino)methane at 155 °C followed by catalytic hydrogenation and ketone reduction yielded **26**.[40]

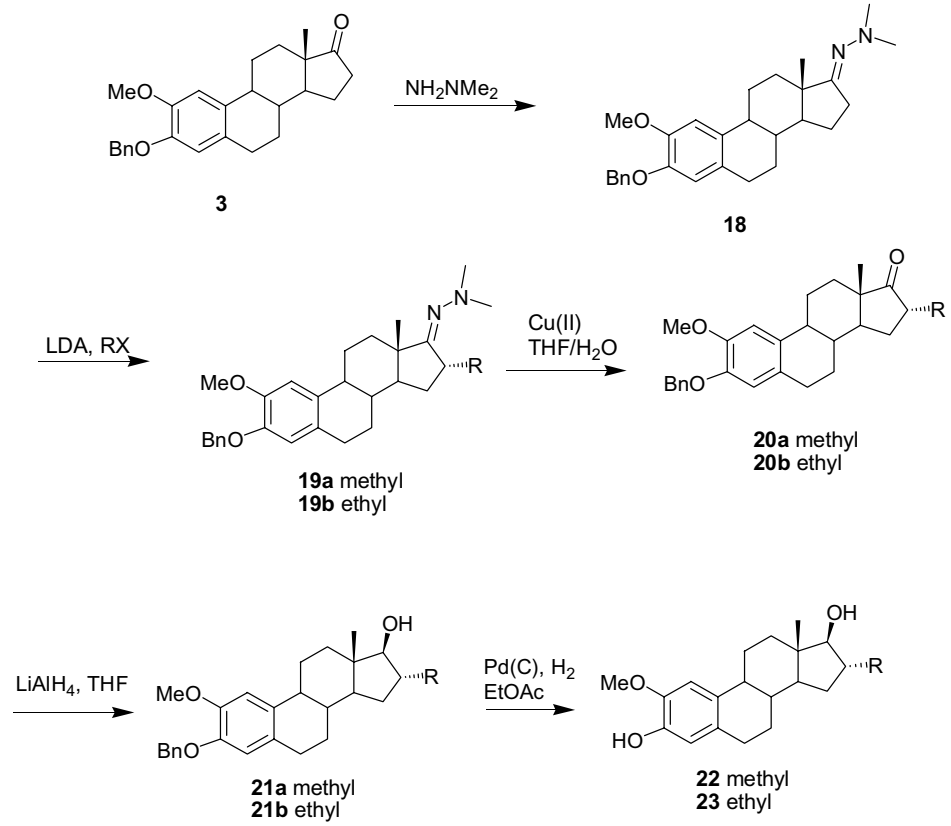
2.2. Biology

Screening of these analogs for antiproliferative activity was performed using two different cell types. Human umbilical vein endothelial cell (HUVEC, early passage p2–p4) was employed as an in vitro surrogate for antiangiogenic activity and MDA-MB-231 (human breast cancer cell line) was used for antitumor activity. The ability to sustain the proliferation of estrogen-dependent MCF-7 breast carcinoma cells in media depleted of other steroidal estrogens was assessed as a measure of estrogenicity. Selected analogs were tested as substrates for glucuronide and sulfate conjugation in an in vitro assay using human liver enzymes.

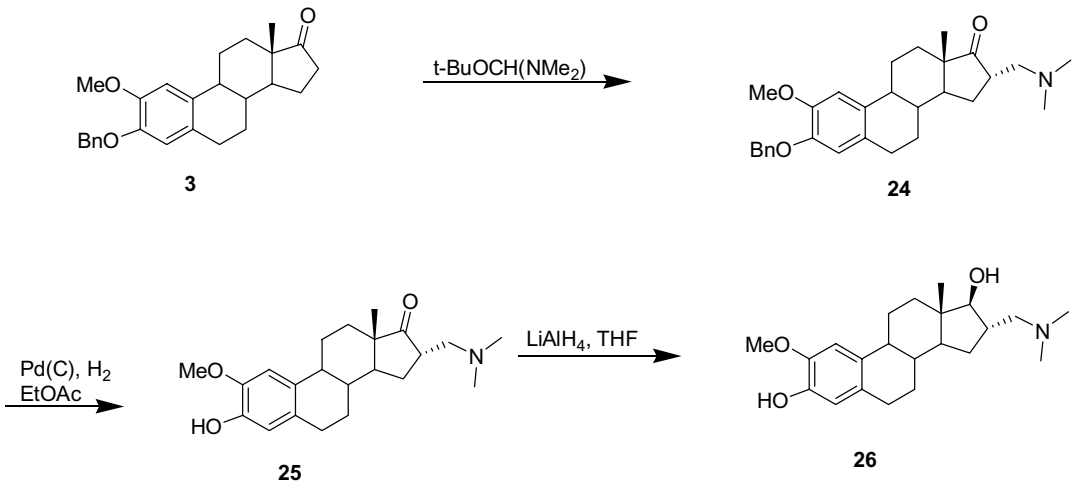
3. Discussion

2ME2 has been shown to have similar antiproliferative activities across a wide variety of tumor and non-tumor cell lines from various species.[1] In the current study, we also found similar IC_{50} values for 2ME2 in human endothelial cells and MDA-MB-231 tumor cells. In contrast, substitutions at the 16-position of the D-ring have different effects on the activity of the resulting analogs and several structure–activity relationships can be determined from the in vitro data in Table 1.

There is a general trend for the 16-substituted analogs that as steric bulk increases, the IC_{50} value for MDA-MB-231 proliferation increases. While the 16-methyl (**15**, **22**) and ethyl (**23**) analogs have approximately the same activity as 2ME2, when propyl (**6**, **13**), butyl (**7**, **14**) or *iso*-butyl (**8**) are incorporated at position 16, there is a 35- to 45-fold drop in the antiproliferative activity assessed with MDA-MB-231 tumor cells.



Scheme 3.



Scheme 4.

Table 1. 16-Substituted 2-methoxyestradiol analogs antiproliferative activity

Compound	Compound (α/β)	MDA-MB-231 IC_{50} (μM)	HUVEC IC_{50} (μM)	MCF7 SI (relative to 2-methoxyestradiol)
1	2-Methoxyestradiol	1.00 ± 0.05	0.84 ± 0.02	1.00
22	16α-Methyl	0.74 ± 0.21	0.35 ± 0.15	2.07 ± 0.43
15	16β-Methyl (1:2)	2.40 ± 0.07	2.26 ± 0.28	1.62 ± 0.43
23	16α-Ethyl	3.59 ± 1.91	1.35 ± 0.57	1.09 ± 0.30
16	16α-Hydroxymethyl	4.26 ± 3.52	2.92 ± 1.40	0.93 ± 0.48
17	16β-Hydroxymethyl	6.09 ± 2.62	6.36 ± 1.99	1.14 ± 0.20
6	16α-Propyl (7.3:1)	35.10 ± 7.68	6.55 ± 1.57	0.83 ± 0.16
13	16β-Propyl (2:1)	45.15 ± 2.75	17.11 ± 1.74	0.93 ± 0.14
14	16β-Butyl (1:2.6)	37.93 ± 1.97	8.17 ± 0.87	0.82 ± 0.18
7	16α-Butyl (2:1)	27.94 ± 0.09	18.17 ± 1.94	0.73 ± 0.11
8	16β-*iso*-Butyl (1:2)	38.33 ± 1.73	7.15 ± 2.04	0.41 ± 0.02
26	16α-Dimethylaminomethyl	22.73 ± 2.10	36.82 ± 0.97	1.43 ± 0.45

Values are averages ±SD of at least two different experiments, in which the effect at each concentration of the analog was assessed by triplicate.

Structure–activity relationships for antiproliferative activity toward endothelial cells (HUVEC) are different, as the results show that larger 16-substituents are better tolerated in these cells. Methyl (**15**, **22**), ethyl (**23**), α-propyl (**6**), β-butyl (**14**) and β-*iso*-butyl (**7**) retain activities within one order of magnitude of the antiproliferative activity of 2ME2. In the case of the larger alkyl substituents, β-propyl (**13**) and α-butyl (**7**), there is an approximate 20-fold drop in activity.

No clear stereochemical structure–activity correlation was observed for the 16-substituted 2ME2 derivatives in the cell types tested. Although this observation was made with the analogs prepared with only a moderate degree of enantiomeric selectivity (up to 7.3:1), there is not a large preference for α or β substitution in either cell line.

Several 16-substituted analogs display disparity between their activities in HUVEC and MDA-MB-231 tumor cells. For example, 16α-propyl (**6**) has a ~5-fold difference in activity between cell types, and similar effects are observed with *iso*-butyl (**8**) and β-butyl (**14**). These analogs are potentially selective for antiproliferative activity against endothelial cells and, in principle, could serve as more specific antiangiogenic agents. However, this would need to be investigated further by testing the antiproliferative activity of these analogs with other tumor cell lines in order to evaluate whether this is a general structure–activity relationship or a specific property directed only toward certain tumor cell lines.

When polar substituents such as hydroxymethyl (**16**, **17**) are incorporated at position 16, there is a drop in antiproliferative activity toward HUVEC ranging from 3- to 8-fold compared to 2ME2. There was little discrimination between the α and β 16-hydroxymethyl moiety. In the case of 16-dimethylaminomethyl (**26**), for which the amine would have a positive charge at the pH of the assay, there is a 43-fold drop in activity compared to 2ME2. On the other hand, if we compare the 16-α-ethyl (**23**) to the 16-α-hydroxymethyl (**16**), which are sterically similar but electronically dissimilar, there is not a large difference in their activities in HUVEC or MDA-MB-231 antiproliferative activity, or in their estrogenic activity for MCF-7 cells. In contrast, while in MDA-MB-231 cells, there is a less than 2-fold difference in activity between congener isomers 16-dimethylaminomethyl (**26**) and 16-*iso*-butyl (**8**), there is a significant difference when these compounds are com-

pared on HUVEC. There are two possible interpretations of these data. First, the positively charged nitrogen of **26** may be detrimental for HUVEC antiproliferative activity. Alternatively, there may be a stereochemical component for positively charged heterosubstituents (e.g. **26**) whereas there is no such component for neutral substituents since a large discrimination between 16-α and β isomers was not observed, as discussed previously.

The 16-methyl substituent (both α and β stereochemistry) is significantly more estrogenic than 2ME2, as is the 16-dimethylaminomethyl substituent. All other 16-substituted 2ME2 analogs had about equal activity to 2ME2 in sustaining proliferation of estrogen-dependent MCF-7 tumor cells. We have recently shown that the estrogenic activity observed in this assay with 2ME2 is related to its metabolism. We found that in the presence of 2ME2 and a metabolic inhibitor, there was a decrease in the proliferation of MCF-7 cells accompanied by an increase in the antiproliferative activity of the 2ME2 (reduced IC_{50})[6]. It is unclear with the series of analogs presented here whether compounds such as **15**, **22**, and **26** are intrinsically estrogenic, or whether their metabolites are responsible for this activity. More detailed studies on metabolism and estrogen-like proliferative mechanism for these compounds are outside the scope of this investigation.

Human clinical PK, preclinical ADME, and in vitro data indicate that in addition to 17-oxidation, conjugation is a major metabolic pathway for 2ME2. To explore the effect of 16-substitutions on this process, 16α-butyl-2ME2 (**7**), 16α-methyl 2ME2 (**22**), 16β-hydroxymethyl-2ME2 (**17**), 16α-ethyl (**22**), and racemic 16-ethyl-2ME2 (**27**) were tested as substrates for UDP-glucuronosyl transferase (UDPGT) activity in human liver microsome preparations compared to 2ME2 as a control. 2ME2 was rapidly conjugated in this in vitro assay and, after 60 min, only 9.4% of parent remained unchanged. Similar results were obtained with all the 16-modified analogs assessed, suggesting that a similar extent of conjugation could be expected in vivo for these analogs. Steroids having larger 16 substitutents (e.g. isopropyl) were not tested since they had a significant reduction in in vitro activity and would not be considered as potential drug candidates. The results are summarized in Table 2.

Sulfonylation is another potential conjugation pathway for 2ME2. Both 2ME2 and racemic 16-ethyl-2ME2 were assessed as substrates for liver cytosolic sulfotransferases. After 60 min of incubation, 85% of 2ME2 remained unchanged. Consistent with these results, recent in vivo data from humans and rats showed that conjugation of 2ME2 occurs mainly via glucuronidation and not sulfonation.[41] Under the same assay conditions, **24** was rapidly conjugated and only 8.3% of parent remained after 60-min incubation. These results indicate that this substitution, which had only very minor effects on the other properties assessed, would probably enhance the metabolic clearance of this analog by virtue of its enhanced rate of sulfonation.

4. Conclusion

A novel series of 2ME2 analogs modified at position 16 in the D-ring have been synthesized, and various structure–activity relationships were determined. These analogs were prepared by incorporating activated alkyl halides via a reactive enolate. In general, the analogs showed good antiproliferative activity toward HUVEC, an assay in which a wide range of substituents were well tolerated at this position. On the other hand, only methyl and ethyl substituents were well tolerated for antiproliferative activity against MDA-MB-231 breast tumor cells. Most of these analogs have approximately the same activity as 2ME2 in sustaining the proliferation of the estrogen-dependant MCF-7 breast tumor cells when assessed at levels below their antiproliferative concentrations.

Analogs that had similar antiangiogenic activity to 2ME2 were similarly good substrates for conjugation by UDPGT. Interestingly, substitution of the 16 position of 2ME2 with an ethyl group unveiled a modulatory role for this position, since this analog has the propensity to form sulfonate esters much more efficiently than 2ME2.

This series of analogs demonstrate that certain modifications can be made at position 16 of 2ME2 without affecting the overall antiproliferative activity, and other modifications result in agents with selective antiproliferative activity for endothelial cells. Future studies for these molecules could include determination of the activity of these analogs over a wide variety of tumor cell types and in animal tumor models. Additionally, other assays such as chorioallantoic membrane, cell migration and matrigel plug assay could be used to explore the role these molecules have in inhibiting specific stages in the angiogenic cascade.

Table 2. Conjugation of 16-substituted 2ME2 analogs

Compound	16-Substitutent	% Parent remaining at $t = 0$ min	Glucuronidation (% remaining at $t = 60$ min)	Sulfonylation (% remaining at $t = 60$ min)
1	H	100	9.4	85
7	α-Butyl	100	0	—
22	α-Methyl	100	12	—
17	β-Methanol	100	17	—
23	α-Ethyl	100	0	—
27	Ethyl (racemic)	100	—	8.3

All assays were performed by BD Biosciences, Woburn, MA. Each assay was performed in duplicate, reported results are the average value from each determination.

5. Experimental

5.1. General

Chemicals and reagents of high purity were obtained from Aldrich, Fluka, or Acros chemical companies and were used without further purification. 2ME2 was purchased from Tetrionics (now SAFC), Madison, WI. Chemical reactions were monitored by thin layer chromatography using Merck precoated silica gel $60F_{254}$ plates. Flash columns packed manually with silica gel (Merck, grade 9385, 230–400 mesh, 40 Å) or Biotage (FLASH 12™ or FLASH 40™) columns were used for flash chromatography. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DPX 300 MHz spectrometer. Chemical shifts are reported in parts per million (δ) relative to tetramethylsilane as internal standard (0.0 δ). IR spectra were recorded on a Perkin-Elmer 783 spectrometer and wave numbers are reported in cm^{-1}. Elemental analysis was performed by Atlantic Micro Lab., Norcross, GA. Both glucuronidation and sulfotransferase assays were preformed by BD Gentest, Woburn, MA.

5.2. 3-(Benzyloxy)-2-methoxyestra-1,3,5(10)-triene-17-one (3)

2ME2 (10.09 g, 33.4 mmol) and potassium carbonate (22 g, 278 mmol) were suspended in anhydrous ethanol and cooled to 0 °C. Benzyl bromide (11.4 mL, 95.8 mmol) was added dropwise and, following the addition, the mixture was brought to reflux for 8 h. The solution was cooled to rt, and the solvent was removed under reduced pressure. The resulting residue was diluted with approximately 200 mL water, and washed with ethyl acetate (3×200 mL). The combined organic phases were washed with water (200 mL), saturated $NaHCO_3$ (200 mL), and saturated NaCl (200 mL). The organic layer was dried with Na_2SO_3, filtered, and solvent was removed under reduced pressure. The product was dried in vacuo with gentle heating with a heat gun to give a yellowish glass (13.54 g, quantitative yield) and was used without further purification. Selected spectral data: ^{1} H NMR (300 MHz, $CDCl_3$) δ 7.29–7.53 (m, 5H), 6.88 (s, 1H), 6.65 (s, 3H), 5.11 (s, 2H), 3.87 (s, 3H), 3.7 (t, J = 8 Hz, 1H), 0.80 (s, 3H). FT-IR (neat, ATR, cm^{-1}) 3341, 2920, 2864, 1605, 1513, 1453, 1254, 1211, 1117, 1022. 3-(benzyloxy)-2-methoxyestra-1,3,5(10)-triene-17β-ol was converted to **3** using the Swern oxidation. Oxalyl chloride (38 mmol, 19 mL, 2M, CH_2Cl_2) was added to anhydrous CH_2Cl_2 (25 mL) and cooled to −46 °C. DMSO (5.40 mL, 76 mmol) was added dropwise and stirred for 2 min. 3-Benzyloxy-2-methoxyestra-1,4,5(10)-triene-3,17β-diol in CH_2Cl_2/DMSO (10 mL/15 mL) and added within 5 min and the resulting mixture was stirred for 1 h. Triethylamine (170 mmol, 23.5 mL) was added dropwise, stirred 5 min and warmed to rt. Water (200 mL) was added and the mixture was washed with CH_2Cl_2 (3×200 mL). The combined organics were washed with water (200 mL), dilute HCl (1% aq, 200 mL), saturated $NaHCO_3$ (200 mL), and saturated NaCl (200 mL). The organics were dried with $MgSO_4$, filtered, and solvent was removed under reduced pressure to give a white solid. The solid was crystallized with hot ethanol to give white crystals (9.94 g, 25.5 mmol, 76% overall yield from 2ME2). Selected spectral data: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.28–7.48 (m, 5H), 6.86 (s, 1H), 6.66 (s, 1H), 3.88 (s, 3H), 0.94 (s, 3H). IR (cm^{-1}, neat) 2920, 1731, 1519, 1202, 1012.

5.3. 3-(Benzyloxy)-16α-(*E*-crotyl)-2-methoxyestra-1,3,5(10)-triene-17-one (4b)

LDA (2M Aldrich, heptane/THF/ethylbenzene, 1.2 mL, 2.4 mmol) was added to anhydrous THF (10 mL) and cooled to −78 °C, and **3** (780 mg, 2.0 mmol) in THF (10 mL) was added dropwise. Following addition, the mixture was warmed to 0 °C and stirred 1 h. The mixture was then cooled to −78 °C and DMPU (1 mL), then *E*-crotyl bromide (205 μL, 2.0 mmol) were added dropwise. The mixture was warmed to rt over 4 h. The reaction was quenched by carefully adding water (100 mL) and washing with ethyl acetate (2×100 mL). The combined organics were washed with water (100 mL) and saturated NaCl (100 mL). The solution was dried with $MgSO_4$, filtered, and solvent was removed under reduced pressure. The crude product was purified using hexane–ethyl acetate (9:1) with a SiO_2 Biotage FLASH apparatus. Obtained 680 mg (1.53 mmol) product and recovered 121 mg (0.31 mmol) of starting material (90% yield based on recovered starting material). Selected spectral data: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.28–7.48 (m, 5H), 6.86 (s, 1H), 6.66 (s, 1H), 5.34–5.59 (m, 2H), 5.13 (s, 2H), 3.88 (s, 3H), 0.87 & 0.97 (s, total 3H, ratio 1:2).

5.4. 16α-(Allyl)-3-(benzyloxy)-2-methoxyestra-1,3,5(10)-triene-17-one (4a)

Using the same general procedure for **4b**, obtained 822 mg **4a** (64%) from 3-benzyloxy-2-methoxyestra-1,3,5(10)-triene-17-one (1.17 g, 3.0 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.50–7.27 (m, 5H), 6.86 (s, 1H), 6.65 (s, 1H), 5.88–5.72 (m, 1H), 5.13 (s, 2H), 5.09–5.02 (m, 2H), 3.88 (s, 3H), 2.89–2.74 (m, 2H), 2.68–2.47 (m, 2H), 2.45–2.13 (m, 3H), 2.13-1.90 (m, 3H), 1.68–1.24 (m, 4H), 0.98 (s) and 0.89 (s, total 3H, ratio 2:1).

5.5. 3-(Benzyloxy)-16α-(*iso*-butenyl) 2-methoxyestra-1,3,5(10)-triene-17-one (4c)

Using the same general procedure for **4b**, obtained 749 mg **4c** (48%) from **3** (1.17 g, 3.0 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.54–7.26 (m, 5H), 6.87 (s, 1H), 6.60 (s, 1H), 5.13 (s, 2H), 4.88–4.69 m, 2H), 3.89 (s, 3H), 2.91–1.24 (m, 17 H), 1.00 (s) and 0.91 (s, total 3H, ratio 1:2.5).

5.6. 3-(Benzyloxy)-16β-(carbomethoxy)-2-methoxyestra-1,3,5(10)-triene-17-one (9)

Compound **3** (1.6113 g, 2.978 mmol) was dissolved in THF (15 mL), cooled to − 78 °C and LDA (1.80 mL, 2M, Aldrich, Heptane/THF/ethylbenzene) was added dropwise and stirred for 1 h. Methyl cyanoformate (237 μL, 3 mmol) in DMPU (1 mL) was added and the

mixture warmed to rt over 18 h. Water (100 mL) was carefully added, and the mixture was washed with ethyl acetate (3 × 100 mL). The combined organics were washed with saturated NaCl (100 mL), dried with Na_2SO_4, filtered and solvent was removed under reduced pressure. Final purification of product using hexane–ethyl acetate (85:15) then switching to hexane–ethyl acetate (75:25) with a SiO_2 flash column yielded 806 mg product (1.8 mmol, 60%). Selected spectral data: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.28–7.48 (m, 5H), 6.85 (s, 1H), 6.66 (s, 1H), 5.13 s (2H), 3.88 (s, 3H), 3.78 (s, 3H), 3.23 (dd, J = 9, 10 Hz, 1H), 1.0 (s, 3H). FT-IR (neat, ATR, cm^{-1}) 2929, 2860, 1750, 1723, 1604, 1508, 1211, 1014.

5.7. 3-(Benzyloxy)-16α/β-hydroxymethyl-2-methoxyestra-1,3,5(10)-triene-17β-ol (12d)

Compound **9** (826 mg, 1.84 mmol) was dissolved in anhydrous THF (120 mL) and cooled to −78 °C, LAH (1M THF, Aldrich, 3.68 mL) was added dropwise, stirred for 1 h at −78 °C then warmed to rt for an additional h. The reaction was quenched by careful addition of ethyl acetate (3 mL) and water (3 mL). Additional water (100 mL) and ethyl acetate (100 mL) were added, and the aqueous layer was washed with ethyl acetate (100 mL). The combined organics were washed with saturated NaCl (100 mL), dried with $NaSO_4$, filtered and solvent was removed under reduced pressure. Product was purified by Biotage FLASH apparatus using a hexanes–ethyl acetate gradient elution (85:15 to 4:6 hexanes–ethyl acetate). Obtained 420 mg of product with 16β stereochemistry. This material was used for the preparation of **17**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.51–7.27 (m, 5H), 6.87 (s, 1H), 6.65 (s, 1H), 5.13 (s, 2H), 3.97 (d, J = 9.8 Hz, 1H), 3.88 (s, 3H),), 3.67 (dd, J = 3.8, 7 Hz, 1H), 2.90–2.65 (m, 2H), 2.60–1.00 (m, 15H), 0.87 (s, 3H). Also obtained 53 mg of product containing mixture of α/β stereochemistry (ratio undetermined). This material was used for the preparation of **16**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.50–7.27 (m, 5H), 6.87 (s, 1H), 6.64 (s, 1H), 5.13 (s, 3H), 3.88 (s, 3H), 3.87–3.79 (m) and 3.69 (app t, J = 8.7, 9.8 Hz, total 1H), 3.55 (d, J = 7.2 Hz, 1H), 2.88–2.66 (m, 2H), 2.46–1.11 (m, 15H), 0.88 (s, 3H).

5.8. 16-(Allyl)-3-(benzyloxy)-16-(carbomethoxy)-2-methoxyestra-1,3,5(10)-triene-17-one (10a)

Compound **9** (0.840 g, 1.87 mmol), potassium hydride (1.5 g, 10.9 mmol, 30% mineral oil dispersion, washed in hexanes), and 18-crown-6 (120 mg, 0.4 mmol) were mixed in THF (40 mL) and refluxed for 1 h. The mixture was cooled to rt, and allyl bromide (537 μL, 6.2 mmol) was added, then refluxed for 18 h. After cooling to rt, the reaction was quenched by carefully adding water (2 mL), stirring, then adding additional water (100 mL). This mixture was washed with ethyl acetate (2 × 100 mL) and the combined organics were washed with saturated NaCl (100 mL). The organics were dried with $MgSO_4$, filtered, and solvent was removed under reduced pressure. Purification using 85:15 hexanes–ethyl acetate using a SiO_2 Biotage FLASH apparatus yielded 697 mg of product (1.42 mol, 76% yield). Selected spectral data: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.28–7.48 (m, 5H), 6.85 (s, 1H), 6.66 (s, 1H), 566–5.79 (m, 1H), 5.15–5.20 (m, 2H), 5.13 (s, 2H), 3.88 (s, 3H), 3.75 (s, 3H), 0.99 (s, 3H).

5.9. 3-(Benzyloxy)-16-(*E*-crotyl)-16-carbomethoxy-2-methoxyestra-1, 3,5(10)-triene-17-one (10b)

Using the same general procedure as in **10a**, obtained **10b** (879 mg, 57%) from **9** (1.0892, 2.43 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ7.50–7.26 (m, 5H), 6.86 (s, 1H), 6.67 (s, 1H), 5.71–5.50 (m, 1H), 5.40–5.25 (m, 1H), 5.13 (s, 2H), 3.88 (s, 3H), 3.75 (s, 3H), 2.87–2.71 (m, 3H), 2.46–2.20 (m, 4H), 2.17–1.93 (m, 3H), 1.80–1.26 (m, 10H), 0.99 (s, 3H).

5.10. 3-(Benzyloxy)-16-(carbomethoxy)-16-methyl-2-methoxyestra-1,3,5(10)-triene-17-one (10c)

Using the same general procedure as in **10a**, obtained **10c** (275 mg, 46%) from **9** (584, 1.3 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.50–7.24 (m, 5H), 6.84 (s, 1H), 6.65 (s, 1H), 5.13 (s, 2H), 3.88 (s, 3H), 3.74 (s, 3H), 2.90–2.74 (m, 2H), 2.58–2.24 (m, 3H), 2.09–1.89 (m, 4H), 1.71–0.84 (m, 9H), 1.03 (s, 3H).

5.11. 16β-(Allyl)-3-(benzyloxy)-2-methoxyestra-1,3,5(10)-triene-17-one (11a)

16-Allyl-16-carbomethoxy-3-benzyloxy-2-methoxyestra-1,3,5(10)-triene-17-one (697 mg, 1.42 mmol), LiCl (1.15 g, 27 mmol), water (485 μL, 27 mmol) were dissolved in DMF (63 mL) and refluxed for 20 h. The reaction was cooled to rt and 1 N HCl (100 mL) was added. The layers were separated and the aqueous phase was washed with ether (2 × 100 mL) the combined organics were washed with water (100 mL), and saturated NaCl (100 mL). The organics were dried with $MgSO_4$, filtered, and solvent was removed under reduced pressure. Purification by 85:15 hexanes–ethyl acetate SiO_2 Biotage FLASH apparatus gave purified product 66% (401 mg, 0.93 mmol). Selected spectral data: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.28–7.48 (m, 5H), 6.85 (s, 1H), 6.65 (s, 1H), 5.69–5.88 (m, 1H), 5.13 (s, 2H), 5.00–5.08 (m, 2H), 5.88 (s, 3H), 0.98 and 0.88 (s, total 3H, ratio 1:1.4). FT-IR (neat, ATR, cm^{-1}) 2925, 2855, 1726, 1514, 1214, 1103.

5.12. 16β-(*E*-Crotyl)-3-benzyl-2-methoxyestra-1,3,5(10)-triene-17-one (11b)

Using the same general procedure as in **11a** obtained **11b** (700 mg, 92%) from **10b** (879 mg, 1.74 mmol). Product was used as is without additional purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.50–7.26 (m, 5H), 6.85 (s, 1H), 6.65 (s, 1H), 5.60–5.34 (m, 2H), 5.13 (s, 2H), 3.88 (s, 3H), 2.86-2.74 (m, 2H), 2.62-1.19 (m, 18H), 0.86 (s, 3H).

5.13. 3-(Benzyloxy)-16β-methyl-2-methoxyestra-1,3,5(10)-triene-17-one (11c)

Using the same general procedure as in **11a**, obtained **11c** (180 mg, 44%) from **10c** (482 mg, 1.0 mmol). Product was purified using Biotage FLASH apparatus using 85:15

Hexanes–ethyl acetate as eluent. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.50–7.26 (m, 5H), 6.86 (s, 1H), 6.66 (s, 1H), 5.13 (s, 2H), 3.88 (s, 3H), 2.86–2.72 (m, 2H), 2.68–1.11 (m, 15H), 0.97 (s) and 0.90 (s, total 3H, ratio 1:2).

5.14. 3-(Benzyloxy)16α-(*N*,*N*-(dimethyl)aminomethyl)-2-methoxyestra-1,3,5(10)-triene-17-one (24)

Compund **3** (1.51 g, 3.87 mmol) was suspended in *tert*-butoxy bis(dimethylamino)methane (1.64 mL, 8.13 mmol) and heated in an oil bath (155 °C) for 1.5 h, during which time the steroid dissolved. The reaction mixture was cooled to rt, and poured into ice water (100 mL) and washed with CH_2Cl_2 (2 × 100 mL). The organics were washed with brine (100 mL), dried with magnesium sulfate, filtered, and solvent was removed under reduced pressure gave product which was used without further purification (1.82 g, quantitative yield). Selected spectral data: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.23–7.47 (m, 5H), 6.87 (s, 1H), 6.64 (s, 1H), 5.12 (s, 2H), 3.88 (s, 3H), 3.07 (s, 6H), 0.91 (s, 3H).

5.15. 16α-(*N*,*N*-(Dimethyl)aminomethyl)-2-methoxyestra-1,3,5(10)-triene-17-one-3-ol (25)

Compound **24** (473 mg, 1.06 mmol) was dissolved in ethyl acetate (25 mL) in a Parr pressure bottle. The bottle was flushed with Ar, and Pd/C (10%) (2.5 g) was added. The bottle was fitted to a Parr hydrogenator, filled and purged with H_2 five times, and then pressurized with H_2 to 50 psi. The reaction mixture was agitated for 48 h, then filtered through celite and solvent was removed under reduced pressure. The crude product was purified using a Biotage FLASH apparatus 99:1:1 $CHCl_3$–CH_3OH: NH_4 OH (30% aq) eluent. Product **25** was obtained (70 mg, 18%). Selected spectral data: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.79 (s, 1H), 6.66 (s, 1H), 3.86 (s, 3H), 3.04–0.91 (m, 14H), 2.28 (s, 6H), 0.87 (s, 3H).

5.16. 16α-(*N*,*N*-(Dimethyl)aminomethyl)-2-methoxyestra-1,3,5(10)-triene-3,17β-diol (26)

Compound **25** (70 mg, 0.2 mmol) was dissolved in anhydrous THF (10 mL) and cooled to −78 °C. LAH (17 mg, 0.45 mmol) was added and the mixture was stirred for 2.5 h. The mixture was quenched by careful addition of ethyl acetate (3 mL) then water (3 mL), the mixture was warmed to rt, then additional ethyl acetate (30 mL) and water (30 mL) were added. The layers were separated, and the aqueous layer was washed with ethyl acetate (30 mL). The combined organic layers were washed with water (30 mL) and saturated NaCl (30 mL), dried with $MgSO_4$, filtered, and solvent was removed under reduced pressure. Product was purified using Biotage FLASH apparatus with 99:1:1 $CHCl_3$–CH_3OH–NH_4OH (30% aq) as eluent. Obtained **26** (18 mg, 5%). Selected spectral data: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.81 (s, 1H), 6.65 (s, 1H), 3.88 (s) and 3.85 (obscured d) (total 4H), 2.28 (s, 6H), 0.87 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 145.1, 143.8, 132.2, 129.7, 115.1, 108.7, 83.1, 77.6, 63.6, 56.4, 49.5, 45.03, 44.98, 44.55, 38.6, 38.1, 34.9, 30.5, 29.3, 28.0, 27.0, 13.2. Anal. Calcd for $C_{22}H_{33}O_3N1/4H_2O$: C, 72.59; H, 9.28; N, 3.85 Found: C, 72.80; H, 9.17; N, 3.66.

5.17. 3-(Benzyloxy)-2-(methoxy)-17-(*N*,*N*-dimethylhydrazone)-estra-1,3,5(10)-triene (18)

Compound (**3**) (4.6733 g, 11.99 mmol) was suspended in absolute ethanol (80 mL) and 1,1-dimethylhydrazine (9.492 g, 158 mmol), then DMF (2 mL) was added. The mixture was refluxed for 20 h, cooled to rt, and poured into water (200 mL) The mixture was washed with ether (2 × 200 mL) and the combined organic layers were washed with water (2 × 200 mL), then saturated NaCl (1 × 200 mL). The combined organics were dried with Na_2SO_4, filtered, and solvent was removed under reduced pressure. Product was purified with Biotage FLASH apparatus using 3:1 hexanes–ethyl acetate (1% triethylamine) as eluent, starting material was recovered (1.9037 g, 4.88 mmol) and **18** was obtained as a clear colorless oil that crystallized in vacuo (1.5229 g, 49% based on recovered starting material). Selected spectral data: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.50–7.16 (m, 5H), 6.86 (s, 1H), 6.65 (s, 1H), 5.13 (s, 2H), 3.89 (s, 3H), 2.86–2.74 (m, 2H), 2.59–1.22 (m, 11 H), 2.50 (s, 6H), 0.93 (s, 3H).

5.18. 3-(Benzyloxy)-16α-(methyl)-17-(*N*,*N*-dimethylhydrazone)estra-1,3,5(10)-triene (19a)

Compound **18** (1.555 g, 3.6 mmol) was dissolved in THF (20 mL) and cooled to 0 °C. *n*-Butyllithium (1.46 M, hexanes, Aldrich, 2.9 mL, 4.32 mmol) was added dropwise and stirred 30 min, methyl iodide (263 μL, 4.22 mmol) was then added and the mixture was warmed to rt over 30 min and stirred an additional 2 h. The reaction was quenched by addition of saturated ammonium chloride (100 mL) and the resulting solution was washed with ether (2 × 100 mL) and the combined organics were washed with saturated NaCl (1 × 100 mL), dried with Na_2SO_4, filtered, and solvent was removed under reduced pressure. The product was purified with a Biotage FLASH apparatus using 6:1 hexanes–ethyl acetate (1% triethylamine) as eluent. Obtained **19a** (715 mg, 45.5%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.51–7.25 (m, 5H), 6.87 (s, 1H), 6.65 (s, 1H), 5.13 (s, 2H), 3.88 (s, 3H), 2.88–2.69 (m, 2H), 2.61–1.21 (m, 13H), 2.44 (s, 6H), 1.32 (d, J = 7.16 Hz, 3H), 0.89 (s, 3H).

5.19. 3-(Benzyloxy)-16α-(ethyl)-17-(*N*,*N*-dimethylhydrazone)estra-1,3,5(10)-triene (19b)

Using the same general procedure as in **19a**, with ethyl iodide (824 mg, 5.28 mmol) obtained **19b** (1.334 g, 66%) from **18** (1.9258 g, 4.4 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.52–7.22 (m, 5H), 6.90 (s, 1H), 6.67 (s, 1H), 5.14 (s, 2H), 3.91 (s, 3H), 2.48 (s, 6H), 2.90–0.86 (m, 19H), 0.93 (s, 3H).

5.20. 3-(Benzyloxy)-16α-(methyl)estra-1,3,5(10)-triene-17-one (20a)

Copper(II) chloride dihydrate (860 mg, 6.4 mmol) was dissolved in water (10 mL) and (**19a**) (715 mg, 1.6 mmol) in THF/H_2 O (35 mL/7 mL) was added. The mixture was stirred for 4 h, and was poured into water (200 mL). The mixture was washed with ether (2 × 200 mL) and the

combined organics were washed with saturated NaCl (1 × 200 mL). The organics were dried over Na_2SO_4, filtered, and solvent was removed under reduced pressure. The product was purified with Biotage FLASH apparatus using 8:1 hexanes–ethyl acetate as eluent. Obtained **20a** (380 mg, 58%), which was used as is for ketone reduction. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.50–7.27 (m, 5H), 6.87 (s, 1H), 6.67 (s,1H), 5.14 (s, 2H), 3.89 (s, 3H), 2.87–2.76 (m, 2H), 2.67–2.54 (m, 1H), 2.46–2.54 (m, 1H), 2.46–2.21 (m, 2H), 2.03–1.90 (m, 2H), 1.80–1.22 (m, 8H), 1.17 (d, J = 7.5 Hz, 3H), 0.98 (s, 3H).

5.21. 3-(Benzyloxy)-16α-(ethyl)estra-1,3,5(10)-triene-17-one (20b)

Using the same general procedure as in **20a**, obtained **20b** (838 mg, 68%) from **19b** (1.334 g, 2.9 mmol), which was used as is for ketone reduction. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.50–7.25 (m, 5H), 6.86 (s, 1H), 6.66 (s, 1H), 5.13 (s, 2H), 3.88 (s, 3H), 2.91–2.66 (m, 2H), 2.49–1.18 (m, 15H), 0.97 (s, 3H).

5.22. 3-(Benzyloxy)-16α-(*E*-crotyl)-2-methoxyestra-1,3,5(10)-triene-17β-ol (5b)

Compound **4b** (680 mg, 1.53 mmol) was dissolved in anhydrous THF (10 mL), and cooled to −78 °C. Lithium aluminum hydride (3.06 mmol, 116 mg) was added and the solution was stirred for 2 h. The reaction was quenched by carefully adding water (2 mL) and warming to rt, then adding additional 50 mL portion of water. The mixture was washed with ethyl acetate (2 × 50 mL) and the combined organics were washed with water (50 mL), saturated NaCl (50 mL), dried with $MgSO_4$, filtered, and solvent was removed under reduced pressure. The mixture was purified with 3:1 hexane–ethyl acetate using a SiO_2 Biotage FLASH apparatus to give 500 mg purified product (**5b**, 1.12 mmol, 73% yield). Selected spectral data: ^{1} H NMR (300 MHz, $CDCl_3$) δ 7.28–7.48 (m, 5H), 6.87 (s, 1H), 6.64 (s, 1H), 5.47–5.56 (m, 2H), 5.12 (s, 2H), 3.88 (s, 3H), 3.8 (d, J = 9 Hz) and 3.33 (d, J = 8 Hz) total 1H, ratio 1:1.7, 0.84 and 0.81 (s, 3H total).

5.23. 3-(Benzyloxy)-16α-(allyl)-2-methoxyestra-1,3,5(10)-triene-17β-ol (5a)

Using the same general procedure for **5b**, obtained **5a** (914 mg, quantitative) from **4a** (822 mg, 1.91 mmol). Product was used as is without additional purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.50–7.25 (m, 5H), 6.86 (s, 1H), 6.64 (s, 1H), 5.89–5.79 (m, 1H), 5.12 (s, 2H), 5.10–4.97 (m, 2H), 3.87 (s, 3H), 3.87–3.76 (m) and 3.39–3.30 (m, total 1H, ratio not determined), 2.87 (m, 2H), 2.43 (m) and 1.03 (m, 15H), 0.83 (s) and 0.82 (s, total 3H, ratio ∼3:1).

5.24. 3-(Benzyloxy)-16β-(isobutenyl)-2-methoxyestra-1,3,5(10)-triene-17β-ol (5c)

Using the same general procedure for **5b**, obtained **5c** (841 mg, quantitative) from **4c** (749 mg, 1.43 mmol). Product was used as is without additional purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.51–7.26 (m, 5H), 6.88 (s, 1H), 6.65 (s, 1H), 5.13 s, 2H), 4.79 (s, 2H), 3.89 (s, 3H), 3.87–3.80 (m) and 3.37–3.28 (m, total 1H, ratio undetermined), 3.88–2.66 (m, 2H), 2.53–1.00 (m, 15H), 0.86 (s) and 0.83 (s, total 3H, ratio 1:2.3).

5.25. 3-(Benzyloxy)-16β-(allyl)-2-methoxyestra-1,3,5(10)-triene-17βol (12a)

Using the same general procedure for **5b**, obtained **12a** (268 mg, 72%) from **11a** (401 mg, 0.93 mmol). Product was purified with Biotage FLASH apparatus using 4:1 hexanes–ethyl acetate as eluent. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.50–7.25 (m, 5H), 6.87 (s, 1H), 6.64 (s, 1H), 5.97–5.79 (m, 1H), 5.12 (s, 2H), 5.12–4.97 (m 2H), 3.88 (s, 3H), 3.82 (d, J = 10.2 Hz) and 3.43 (d, J = 7.5 Hz, total 1 H, ratio ∼2:1), 2.85–2.67 (m, 2H), 2.57–1.03 (m, 15H), 0.84 (s) and 0.82 (s, total 3H, ratio ∼2:1).

5.26. 3-(Benzyloxy)-16β-(*E*-crotyl)-2-methoxyestra-1,3,5(10)-triene-17β-ol (12b)

Using the same general procedure for **5b**, obtained **12b** (300 mg, 42%) from **11b** (700 mg, 1.61 mmol). Product was purified with Biotage FLASH apparatus using 4:1 hexanes–ethyl acetate as eluent. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.51–7.26 (m, 5H), 6.87 (s, 1H), 6.64 (s, 1H), 5.59–5.38 (m, 2H), 5.13 (s, 2H), 3.88 (s, 3H), 3.81 (dd, J = 4, 7 Hz) and 3.33 (d, J = 7 Hz, total 1H, ratio ∼4:1), 2.88–2.66 (m, 2H), 2.48–0.98 (m, 11H), 0.84 (d, J = 3 Hz, 2H), 0.81 (s, 3H).

5.27. 3-(Benzyloxy)-16β-(methyl)-2-methoxyestra-1,3,5(10)-triene-17β-ol (12c)

Using the same general procedure for **5b**, obtained **12c** (158 mg, 88%) from **11c** (180 mg, 0.44 mmol). Product was purified with Biotage FLASH apparatus using 3:1 hexanes–ethyl acetate as eluent. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.50–7.27 (m, 5H), 6.87 s, 1H), 6.64 (s, 1H), 5.13 (s, 2H), 3.88 (s, 3H), 3.73 (d, J = 9.8 Hz) and 3.23 (d, J = 7.5 Hz, total 1H, ratio ∼2:1), 2.86–2.68 (m, 2H), 2.40–1.22 (m, H), 1.16 (d, J = 7 Hz) and 1.05 (d, J = 7.5 Hz, total 3H, ratio ∼2:1), 0.82 (s, and 0.80 (s, total 3H, ratio ∼2:1).

5.28. 3-(Benzyloxy)-16α-(methyl)-2-methoxyestra-1,3,5(10)-triene-17β-ol (21a)

Using the same general procedure for **5b**, obtained **21a** (116 mg, 73%) from **20a** (156 mg, 0.39 mmol). Product was purified with Biotage FLASH apparatus using 3:1 hexanes–ethyl acetate as eluent. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.50–7.27 (m, 5H), 6.88 (s, 1H), 6.65 (s, 1H), 5.13 (s, 2H), 3.88 (s, 3H), 3.23 (d, J = 7.5 Hz, 1H), 2.84–2.66 (m, 2H), 2.37–1.21 (m, 13H), 1.17 (d, J = 6.8 Hz, 3H), 0.83 (s, 3H).

5.29. 3-(Benzyloxy)-16α-(ethyl)-2-methoxyestra-1,3,5(10)-triene-17β-ol (21b)

Using the same general procedure for **5b**, obtained **21b** (873 mg, 43%) from **20b** (838 mg, 4.83 mmol). Product

was purified with Biotage FLASH apparatus using 3:1 hexanes–ethyl acetate as eluent. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.50–7.25 (m, 5H), 6.87 (s, 1H), 6.64 (s, 1H), 5.13 (s, 2H), 3.88 (s, 3H), 3.53–3.23 (m, 1H), 2.87–0.74 (m, 17H), 0.83 (s, 3H).

5.30. 16α-(*n*-Butyl)-2-methoxyestra-1,3,5(10)-triene-3,17β-diol (7)

Compound **5b** (500 mg, 1.12 mmol) was dissolved in ethyl acetate (25 mL) in Parr reaction bottle. The bottle was flushed with argon, and Pd/C (10%, 2.5 g) was added. The bottle was fitted to a Parr hydrogenator, filled and purged with hydrogen five times, pressurized to 50 psi, and agitated for 24 h. The mixture was filtered through a celite pad, the solvent was removed under reduced pressure and purified with a 3:1 hexane ethyl acetate SiO_2 flash column. Crystallization from ethyl acetate hexanes yielded 358 mg product (1.0 mmol, 89%). Selected spectral data: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.81 (s, 1H), 6.66 (s, 1H), 3.87 (s, 3H), 3.76 (d, J = 10 Hz) and 3.29 (d, J = 8 Hz) (total 1H, ratio 1:2), 0.82 and 0.79 (s, 3H). FT-IR (neat, ATR, cm^{-1}) 3245, 2914, 1606, 1523, 1414, 1258, 1028. Anal. Calcd for $C_{20}H_{34}O_3$: C, 77.44; H, 9.56. Found: C, 76.64; H, 9.51.

5.31. 16α-(*n*-Propyl)-2-methoxyestra-1,3,5(10)-triene-3,17β-diol (6)

Using the same general procedure for **7**, obtained **6** (577 mg, 78%) from **5a** (914 mg, 2.12 mmol). Selected spectral data: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.81 (s, 1H), 6.66 (s, 1H), 5.43 (s, 1H), 3.87 (s, 3H), 3.29 (t, J = 7 Hz, 1H), 0.95 (t, J = 7 Hz, 3H), 0.83 and 0.80 (s, total 3H, ratio 7.3:1). ^{13}C NMR (75 MHz, $CDCl_3$) δ 145.0, 143.8, 132.2, 130.0, 115.0, 108.5, 88.6, 56.6, 49.0 and 48.8, 44.7, 44.5, 44.2, 39.0 and 38.7, 38.4, 37.3, 30.5, 29.4, 27.7, 26.9, 22.1 and 22.0, 14.8, 12.3. Anal. Calcd for $C_{22}H_{32}O_3$: C, 76.69; H, 9.37. Found: C, 76.55; H, 9.44.

5.32. 16β-(*iso*-Butyl)-2-methoxyestra-1,3,5(10)-triene-3,17β-diol (8)

Using the same general procedure for **7**, obtained **8** (427 mg, 63%) from **5c** (841 mg, 1.89 mmol). Selected spectral data: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.81 (s, 1H), 6.66 (s, 1H), 5.43 (s, 1H), 3.88 (s, 3H), 3.77 (dd, J = 9, 10 Hz) and 3.26 (t, J = 7 Hz) (total 1 H, ratio 2:1), 0.84 and 0.80 (s, total 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 145.0, 143.9, 132.2, 129.9, 115.0, 108.5, 89.1 and 82.9, 56.5, 49.0 and 48.7, 44.7 and 44.6, 42.2 and 40.8, 39.0 and 38.7, 38.2, 37.8 and 37.3, 32.7, 30.6, 29.4, 27.9 and 27.7, 27.2 and 27.0, 26.9 and 26.7, 24.7 and 23.8, 22.7 and 21.7, 12.8 and 12.3. IR (neat, cm^{-1}, ATR) 3525, 2913, 1506, 1258, 1202, 1026. Anal. Calcd for $C_{22}H_{30}O_3$: C, 76.69; H, 9.37. Found: C, 76.82; H, 9.47.

5.33. 16β-(*n*-Propyl)-2-methoxyestra-1,3,5(10)-triene-3,17β-diol (13)

Using the same general procedure for **7**, obtained **13** (111 mg, 51%) from **12a** (268 mg, 0.665 mmol). Selected spectral data: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.81 (s, 1H), 6.66 (s, 1H), 3.87 (s, 3H), 3.76 (d, J = 10 Hz) and 3.29 (t, J = 7 Hz) (total 1H, ratio 2:1), 0.95 (t, J = 7 Hz, 3H), 0.83 and 0.80 (s, total 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 145.1, 143.8, 132.2, 129.9, 115.1, 108.7, 88.4 and 82.8, 56.5, 48.9 and 48.7, 44.7 and 44.5, 44.4 and 44.0, 40.1, 39.0 and 38.7, 38.4 and 38.1, 34.1, 32.7, 30.4 and 29.4, 27.5 and 27.4, 27.0 and 26.9 and 22.1 and 22.0, 14.7 and 14.67, 12.8 and 12.3. FT-IR (neat, ATR, cm^{-1}) 3411, 2923, 1504,1446, 1267, 1202, 1118, 1024. Anal. Calcd for $C_{22}H_{32}O_31/4H_2O$: C, 75.71; H, 9.39. Found: C, 75.61; H, 9.33.

5.34. 16β-(*n*-Butyl)-2-methoxyestra-1,3,5(10)-triene-3,17β-diol (14)

Using the same general procedure for **7**, obtained **14** (170 mg, 70%) from **12b** (300 mg, 0.67 mmol). Selected spectral data: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.81 (s, 1H), 6.66 (s, 1H), 5.43 (s, 1H), 3.88 (s, 3H), 3.76 (d, J = 10 Hz) 3.29 (d, J = 8 Hz) (total 1H, ratio 2.6:1), 0.83 and 0.80 (s, total 3H). ^{13} C NMR (75 MHz, $CDCl_3$) δ 145.1, 143.8, 132.2, 129.9, 115.1, 108.7, 88.4 and 82.8, 56.5, 48.9 and 48.7, 47.7 and 44.5, 44.4 and 44.2, 40.4 and 38.1, 37.2 and 35.8, 32.8 and 31.6 and 31.3, 31.0 and 30.5, 29.4, 27.9 and 27.7, 27.0 and 26.9, 23.4 and 23.3, 14.54 and 14.50, 12.8 and 12.3. FT-IR (neat, ATR, cm^{-1}) 3221, 2921, 1594, 1504, 1416, 1265, 1200, 1021. Anal. Calcd for $C_{23}H3_4O_3$: C, 77.04; H, 9.56. Found: C, 77.06; H, 9.65.

5.35. 16β-(Methyl)-2-methoxyestra-1,3,5(10)-triene-3,17β-diol (15)

Using the same general procedure for **7**, obtained **15** (65 mg, 52%) from **12c** (157 mg, 0.38 mmol). Selected spectral data: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.82 (s, 1H), 6.66 (s, 1H), 3.88 (s, 3H), 3.73 (d, J = 9.8 Hz) and 3.23 (d, J = 7.5 Hz, total 1 H, ratio 2:1), 2.84–2.74 (m 2H), 2.41–1.22 (m, H), 1.17 (d, J = 6.8 Hz) and 1.05 (d, J = 7.5 Hz, total 3H, ratio 1:2), 0.83 (s) and 0.81 (s, total 3H), ratio 2:1). ^{13} C NMR (75 MHz, $CDCl_3$) δ 145.1, 143.8, 132.2, 129.9, 115.1, 108.7, 90.0 and 82.5, 56.4, 49.0 and 48.5, 44.7 and 44.5, 38.9 and 38.7, 38.2 and 37.2, 34.5, 32.4, 29.4, 27.9 and 27.6, 26.9, 20.8, 16.8, 12.8 and 12.2. FT-IR (neat, ATR, cm^{-1}) 3411, 2923, 1504, 1446, 1267, 1202, 1118, 1024. Anal. Calcd for $C_{20}H_{28}O_31/4H_2O$: C, 74.85; H, 8.95. Found: C, 74.83; H, 8.86.

5.36. 16α-(Hydroxymethyl)-2-methoxyestra-1,3,5(10)-triene-3,17β-diol (16)

Using the same general procedure for **7**, obtained 16α-hydroxymethyl-2-methoxyestra-1,3,5(10)-triene-3,17β-diol (**16**) (14 mg, 33%) after purification using Biotage FLASH apparatus with 95:5 $CHCl_3$–CH_3OH as eluent from **12d**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.78 (s, 1H), 6.62 (s, 1H), 3.84 (s, 3H), 3.75 (dd, J = 6,8 Hz, 1H), 3.61 (dd, J = 8.3, 9.2 Hz, 1H), 3.47 (d, J = 7.5 Hz, 1H), 2.80–2.66 m, 2H), 2.36–1.10 (m, 13H), 0.835 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 145.0, 143.8, 132.0, 129.9, 115.0, 108.6, 86.2, 67.1, 56.4, 49.1, 45.9, 44.63, 44.56, 38.9, 37.0, 29.3, 27.7,

27.1, 26.9, 12.4. Anal. Calcd for $C_{20}H_{28}O_4 1/4H_2O$: C, 71.28; H, 8.53. Found: C, 71.64; H, 8.42.

5.37. 16β-(Hydroxymethyl)-2-methoxyestra-1,3,5(10)-triene-3,17β-diol (17)

Obtained **17** (229 mg, 66%) from **12d** (420 mg, 1.0 mmol) after purification using Biotage Flash apparatus with 95:5 $CHCl_3$–CH_3OH as eluent.^{1}H NMR (300 MHz, $CDCl_3$) δ 6.79 (s, 1H), 6.64 (s, 1H), 3.94 (d, $J = 9.8$ Hz, 1H), 3.84 (s, 3H), 3.83 (d, $J = 9.8$ Hz, 1H), 3.65 (dd, $J = 4.2$, 7.4 Hz, 1H), 2.81–2.72 (m, 2H), 2.56–1.00 (m, 13H), 0.85 (s, 3H).^{13} C NMR (75 MHz, $CDCl_3$) δ 145.0, 143.8, 132.2, 130.0, 115.0, 108.5, 88.6,56.5, 49.0 and 48.8, 44.7, 44.5, 44.2, 39.0 and 38.7, 38.4, 37.3, 30.5, 29.4, 27.7, 26.9, 22.1 and 22.0, 14.8, 12.3. FT-IR (neat, ATR, cm^{-1}) 3283, 3091, 2919, 1602, 1513, 1445, 1204, 1119, 1013. Anal. Calcd for $C_{20}H_{28}O_4$: C, 72.25; H, 8.49. Found: C, 72.24; H, 8.48.

5.38. 16α-(Methyl)-2-methoxyestra-1,3,5(10)-triene-3,17β-diol (22)

Using the same general procedure for **7**, obtained **22** (142 mg, 44%) from **21a** (404 mg, 1.01 mmol). Selected spectral data: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.81 (s, 1H), 6.66 (s, 1H), 3.87 (s, 3H), 3.23 (d, $J = 7$ Hz) (s, 1H), 0.81 (s, 3 H). ^{13} C NMR (75 MHz, $CDCl_3$) δ 145.0, 143.8, 132.2, 130.0, 115.0, 108.5, 90.1, 56.5, 48.6. 44.7, 39.0, 38.8, 37.2, 32.4, 29.4, 27.7, 26.9, 20.8, 12.2. Anal. Calcd for $C_{20}H_{28}O_3 1/4H_2O$: C, 74.85; H, 8.95. Found: C, 74.98; H, 8.65.

5.39. 16α-(Ethyl)-2-methoxyestra-1,3,5(10)-triene-3,17β-diol (23)

Using the same general procedure for **7**, obtained **23** (156 mg, 24%) from **21b** (873 mg, 2.09 mmol). Selected spectral data: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.79 (s, 1H), 6.62 (s, 1H), 3.85 (s, 3H), 3.26 (d, $J = 7.2$ Hz, 1H), 2.80–2.72 (m, 2H), 2.32–0.83 (m, 17H), 0.81 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 145.1, 143.8, 132.2, 129.9, 115.1, 108.7, 88.0, 56.4, 48.7, 45.9, 44.7, 44.5, 39.0, 37.2, 30.0, 29.3, 28.6, 27.7, 26.9, 13.1, 12.3 Anal. Calcd for $C_{21}H_{30}O_3$: C, 76.33; H, 9.15. Found: C, 76.55; H, 9.21.

5.40. 16α/β-(Ethyl)-2-methoxyestra-1,3,5(10)-triene-3,17β-diol (27)

Using the same general procedure as for the preparation of **4b**. Obtain 467 mg (1.1 mmol, 38% yield) of 3-benzyloxy-16α/β-ethyl-2-methoxyestra-1,3,5(10)-triene- 17-one from ethyl iodide (700 mg, 4.4 mmol, 1.5 eq) and 1.1315 g (2.9 mmol) of **4b**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.49–7.30 (m, 5H), 6.86 (s, 1H), 6.66 (s, 1H), 5.13 (s, 2H), 3.88 (s, 3H), 2.86−2.75 (m, 2H), 2.46–1.24 (m, 17H), 0.97 (s) and 0.88 (s) (total 3H, 1:1 isomer ratio). Di substituted product (306 mg, 0.68 mmol) was also isolated.

Racemic 3-benzyloxy-16α/β-ethyl-2-methoxyestra-1,3,5(10)-triene-17-one was reduced with lithium aluminum hydride (1.65 mmol, 63 mg) in THF using the same procedure as in **5b**. Obtained 463 mg (0.9 mmol, 82% yield) of 16α/β-ethyl-2-methoxyestra-1,3,5(10)-triene-17β-ol from 467 mg (1.1 mmol) from the corresponding ketone. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.51–7.24 (m, 5H), 6.87 (s, 1H), 6.65 (s, 1H), 5.13 (s, 2H), 3.88 (s, 3H), 3.81–3.72 (m, isomer) and 3.34–3.27 (m, isomer) (total 1H, 1:1 ratio), 2.87–2.67 (m, 2H), 2.38–0.86 (m, 17 H), 0.83 (s, isomer) and 0.79 (s, isomer) (total 3H, 1:1 ratio).

The benzyl protecting group was removed by dissolving 3-benzyoxy-16α/β-ethyl-2-methoxyestra-1,3,5(10)-triene-17β-ol (463 mg, 0.9 mmol) in THF (20 mL) in a Parr reactor bottle. The mixture was then charged with a catalytic amount of $Pd(OH)_2$ purged five times with H_2 gas, then finally pressurized to 50 psi. The mixture was agitated at rt for 24 h, filtered through Celite. The filtrate was evaporated under reduced pressure and crystallized with chloroform to give 50 mg final product (0.15 mmol, 17% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.81 (s, 1H), 6.66 (s, 1H), 5.44 (br s, 1H), 3.88 (s, 3H), 3.81–3.71 (m, β isomer), 3.34–3.26 (m, α isomer) (total 1 H, 1:1 isomer ratio), 2.85–2.72 (m, 2H) 2.36–0.89 (m, 17 H), 0.83 (s) and 0.79 (s) (total 3H, 1:1 isomer ratio). Anal. Calcd for $C_{21}H_{30}O_3$: C, 76.33; H, 9.15. Found: C, 76.18; H, 9.16.

6. Biology

6.1. Proliferation assays

MDA-MB-231 human breast carcinoma cells were grown in DMEM containing 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Irvine Scientific, Santa Anna, CA). Proliferation was assessed by detection of DNA synthesis by use of the 5-bromo-2′-deoxyuridine (BrdU) cell proliferation colorimetric ELISA kit from Roche (Indianapolis, IN) according to the manufacturer's instructions. For BrdU assays, the cells were seeded at 2000 cells/well in a 96-well plate, allowed to attach overnight then exposed to increasing concentrations of the different compounds for 48 h. Each condition was assessed in triplicate and the experiments were carried out a minimum of two times. Results in Table 1 are means of the data from each independent experiment ±SD.

HUVEC were grown in EGM (Clonetics). HUVEC were seeded at 5000 cells/well in 96-well plates. After being allowed to attach overnight, cells were then treated with increasing concentrations of drug for 48 h at 37 °C. The preparation of the drugs and BrdU proliferation assay were performed as indicated above.

MCF-7, an estrogen-dependent breast carcinoma cell line, was maintained in DMEM/F12 (1:1) containing 10% (v/v) FBS (Hyclone Laboratories, Logan, UT) and 1X antibiotic–antimycotic. MCF-7 cells, which were used between passage 60 and passage 90, were the kind

gift of Dr. Dorraya El Ashry of Georgetown University. For MCF-7 estrogen-dependent proliferation assays, the cells were seeded in complete media at 20–30,000 cells/well in 24-well plates. After allowing the cells to adhere overnight, the seeding density was determined by cell counts. Cells were then washed with PBS (37 °C) and starved by placing them in IMEM–phenol red free media containing 2% charcoal–dextran fetal bovine-stripped serum (Georgetown University) and 1X antibiotic–antimitotic. After 3 days of starvation, cells were treated with or without increasing concentrations of compounds, replacing the media every 2–3 days with the same stripped serum medium with test compounds, and counted after 8 or 10 days of treatment. Proliferation was measured by cell counting using a Coulter Z1 cell counter (Coulter Corporation, Hialeah, FL). Each zcondition was done in triplicate in at least two independent experiments. Results are presented as the stimulation index (SI) relative to 2-methoxyestradiol (defined as 1.00).

6.2. Glucuronidation conjugation assays

Incubation with human liver microsomes was preformed with 50 mM Tris, pH 7.5 containing 10 mM magnesium chloride, 2 mM Uridine-5′-dihydrophosphoglucuronic acid (UDPGA), 25 μg alamethicin/mg liver microsomal protein and 5 μM of each test substance. The sample was worked up by precipitation of protein by acetonitrile containing internal standard (2-(2′,2′,2′-trifluoroethoxy)estradiol) and direct injection of the supernatant onto an LC/MS at 0, 15, 30, and 60 min (details below).

The control incubations were as follows: For samples lacking UDPGA, incubations were performed in 50 mM Tris, pH 7.5, containing 10 mM magnesium chloride, 25 μg alamethicin/mg liver microsomal protein, 0.5 mg/ml human liver microsomes, and 5 μM of each test substance. Sample workup was performed as above at 0 and 60 min. For control samples lacking liver microsomes, incubations were performed in 50 mM Tris, pH 7.5, containing 10 mM magnesium chloride, alamethicin (same amount as in other control sample), 2 mM UDPGA, 0.5 mg/ml BSA and 5 μM of each test substance. Sample workup was performed as above at 0 and 60 min.

A comparator (estradiol) for liver microsomal-mediated loss of parent was tested at 1, 10, 30 and 60 min time points similar to that for the test substances. The substrate concentration was 5 μM.

Analytical method parameters for HPLC/MS: analysis of samples used C_{18} Waters Symmetry column 2.1 mm × 50 mm with water and methanol as mobile phase. The flow rate was 2.0 mL/min and the flow gradient initiated with 50% water, 50% methanol. Over 5 min, gradient changed to 100% methanol which was held for 2 min. The gradient was changed back to initial conditions over 1.5 min and was held for another 2 min. The mass spectrometer was a Micromass Quattro LC operated in the negative ion mode using ESI.

6.3. Sulfonyl transferase assays

Incubations with human liver cytosol were performed in 50 mM Tris buffer, pH 7.5 containing 0.1 mM 3′-phospho-adenosyl-5′-phosphosulfate (PAPS) and 5 μM of each test substance. During sample workup, the protein was precipitated by acetonitrile containing an internal standard (2-(2′,2′,2′-trifluoroethoxy)estradiol) and the supernatant was directly injected into an LC/MS system at 0, 15, 30, and 60 min.

In the control samples lacking PAPS, incubations were performed in 50 mM Tris buffer, pH 7.5, containing human liver cytosol and 5 μM of each test substance. Samples were worked up as above at 0 and 60 min.

For control samples lacking human liver cytosol, incubations were performed in 50 mM Tris buffer, pH 7.5, with 0.1 mM PAPS and 5 μ M of each test substance. Sample workup was performed as described above at 0 and 60 min.

A positive control used 7-hydroxycoumarin for human liver cytosolic-mediated loss of parent compound mediated by sulfonyl transferase. The substrate concentration was 5 μM.

Analytical method parameters for HPLC/MS: analysis of samples used a C_{18} Waters Symmetry column 2.1 × 150 mm with water and methanol as mobile phase. The flow rate was 0.2 ml/min, 20 μL injection volume, and the flow gradient initiated with 50% water/50% methanol, over 5 min gradient changed to 100% methanol, was held for 2 min, then returned to initial conditions over 2 min. The mass spectrometer was a Micromass Quattro LC and was operated in the negative ion mode using ESI. Selected ions were as follows: 2-(2′,2′,2′-trifluorethoxy)estradiol was *m*/*z* 330, for 2-methoxyestradiol *m*/*z* 286, and 16-ethyl-2-methoxyestradiol *m*/*z* 314.

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