ACS Medicinal Chemistry Letters

Supporting Information

Crucial Role of 3-Bromoethyl in Removing the Estrogenic Activity of 17β-HSD1 Inhibitor 16β-(*m*-Carbamoylbenzyl)-estradiol

René Maltais, Diana Ayan and Donald Poirier*

Laboratory of Medicinal Chemistry, Endocrinology and Genomic Unit, CHUQ (CHUL) - Research Center and Laval University, Quebec (Quebec) G1V 4G2, Canada

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1. Experimental procedures for the synthesis and characterization (IR, ^{1}H NMR, ^{13}C NMR, MS) of representative compound 5

1.1. General

Chemical reagents were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). The usual solvents were obtained from Fisher Scientific (Montréal, QC, Canada) and were used as received. Anhydrous dichloromethane (DCM), dimethylformamide (DMF) and tetrahydrofuran (THF) were obtained from Sigma-Aldrich. Thin-layer chromatography (TLC) and flash-column chromatography were performed on 0.20-mm silica gel 60 F254 plates (E. Merck; Darmstadt, Germany) and with 230-400 mesh ASTM silica gel 60 (Silicyle, Québec, QC, Canada), respectively. Infrared spectra (IR) were recorded on a Horizon MB 3000 ABB FTIR spectrometer (Québec, QC, Canada) and the significant band reported in cm^{-1}. Nuclear magnetic resonance (NMR) spectra were recorded at 400 MHz for ^{1}H and 100.6 MHz for ^{13}C on a Bruker Avance 400 digital spectrometer (Billerica, MA, USA). The chemical shifts (δ) were expressed in ppm and referenced to chloroform (7.26 and 77.0 ppm), acetone (2.06 and 29.24 ppm) or methanol (3.31 ppm and 49.0), respectively for ^{1}H and ^{13}C NMR. Low-resolution mass spectra (LRMS) were recorded on a PE Sciex API-150ex apparatus (Foster City, CA, USA) equipped with a turbo ion-spray source.

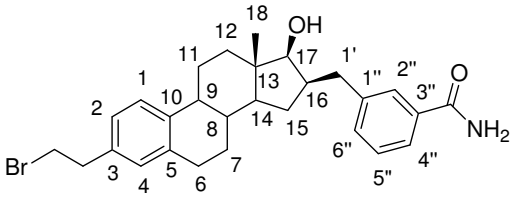
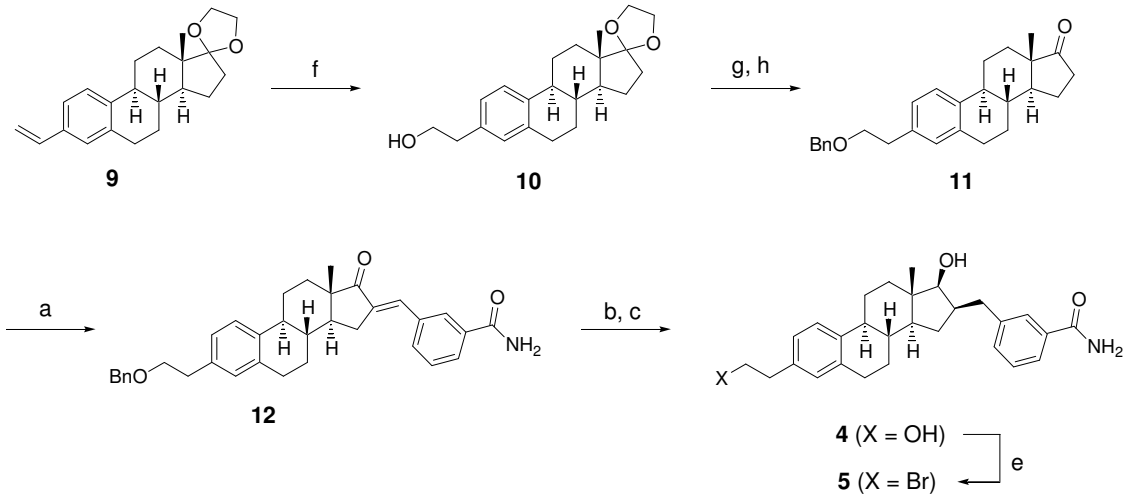


Figure 1. Carbon numbering used for the assignment of representative ^{1}H NMR signals.



Scheme 1. Reagents and conditions: (a) *1)* BH_3-$(CH_3)_2S$, THF, -78°C-rt; *2)* $NaHCO_3$, H_2O_2, rt, 3 h; (b) NaH, benzylbromide, DMF, 0°C-rt, 18 h; (c) 10% HCl : acetone (1:1), rt, 5 h; (d) 3-carboxamide-benzaldehyde, KOH, EtOH, reflux, 0.5 h; (e) $NaBH_4$, MeOH, DCM, rt, 1 h; (f) H_2, 10% Pd/C, MeOH, rt, 36 h; (g) PPh_3, CBr_4, DCM, 0°C, 1.7 h.

*1.2. Synthesis of 3-(2-hydroxyethyl) estra-1(10), 2, 4-trien-17-dioxolane (**10**)*

To a solution of BH_3-dimethylsulfur (2.0 M in THF, 10.4 mL) in anhydrous THF (70 mL) at -78°C was added dropwise 3-vinyl-estra-1(10),2,4-trien-17-dioxolane (**9**) (2.25 g, 6.93 mmol) in anhydrous THF (5 mL) under an atmosphere of argon. The resulting solution was stirred at ambient temperature for 16 h. The solution was then cooled to 0°C and aqueous 1 M $NaHCO_3$ (27.7 mL) was added, immediately followed by addition of 30% H_2O_2 (11.7 mL). The solution was vigorously stirred for 3 h at room temperature and then diluted with EtOAc (50 mL). The resulting solution was poured into water (200 mL), and extracted with EtOAc (5 x 75 mL). The organic layers were combined, washed with brine, dried with $MgSO_4$ and evaporated under reduced pressure. The crude compound was purified by flash chromatography (EtOAc/Hexanes: 4:6) to give 1.18 g (50% yield) of compound **10**. IR (KBr): 3410 (OH). ^{1}H NMR (Acetone-d_6): 0.88 (s, 18-CH_3), 1.27-2.38 (unassigned CH and CH_2), 2.73 (t, J = 7.1 Hz, C$\underline{H_2}$CH_2OH), 2.82 (m, 6-CH_2), 3.62 (broad t, OH), 3.71 (m, CH_2C$\underline{H_2}$OH), 3.88 (m, 2 x CH_2 of dioxolane), 6.92 (s, 4-CH), 6.97 (d, J = 8.0 Hz, 2-CH), 7.19 (d, J = 7.9 Hz, 1-CH). ^{13}C NMR (Acetone-d_6): 13.9, 22.1, 25.9, 27.0, 29.3, 30.7, 33.9, 39.0, 39.1, 44.1, 45.9, 49.3, 63.1, 64.3, 64.9, 118.8, 125.1, 126.2, 129.4, 136.1, 136.5, 137.7. LRMS for $C_{22}H_{31}O_3$ $[M+H]^+$ 343.4 m/z.

*1.3. Synthesis of 3-[2-(benzyloxy)ethyl]estra-1(10),2,4-trien-17-one (**11**)*

To a solution of compound **10** (1.1 g, 3.2 mmol) in anhydrous DMF (50 mL), was added NaH (60% in oil) (168 mg, 4.2 mmol) at 0°C under an atmosphere of argon. The solution was stirred for 1 h at 0°C and benzyl bromide (898 mg, 627 µL, 5.3 mmol) was added in one portion. The solution was allowed to return to room temperature and stirred overnight, poured into water (300 mL) and extracted with EtOAc (3 x 75 mL). The organic layers were combined, washed with brine, dried with $MgSO_4$ and evaporated under reduced pressure. The crude compound was then treated with a solution of aqueous HCl (10%) in acetone (1:1) (50 mL) and stirred for 5 h at room temperature. The resulting solution was neutralized using an aqueous $NaHCO_3$ (10%) solution and extracted with EtOAc (2 x 75 mL). The organic layers were combined, washed with brine, dried with $MgSO_4$ and evaporated under reduced pressure. The crude compound was purified by flash chromatography (EtOAc/Hexanes: 1:9) to give 1.04 g (84% yield, 2 steps) of compound **11**. IR (KBr): 1736 (C=O, ketone). ^{1}H NMR ($CDCl_3$): 0.91 (s, 18-CH_3), 1.30-2.40 (unassigned CH and CH_2), 2.51 (dd, J_1 = 8.5 Hz, J_2 = 18.9 Hz, 16β-CH), 2.88 (m, 6-CH_2 and C$\underline{H_2}$CH_2O), 3.68 (t, J = 7.3 Hz, CH_2C$\underline{H_2}$O), 4.54 (s, OC$\underline{H_2}$Ph), 6.97 (s, 4-CH), 7.02 (d, J = 8.0 Hz, 2-CH), 7.22 (d, J = 7.9 Hz, 1-CH), 7.23-7.38 (m, 5H, $OCH_2$$\underline{Ph}$). ^{13}C NMR ($CDCl_3$): 13.8, 21.6, 25.7, 26.5, 29.4, 31.6, 35.8, 35.9, 38.2, 44.3, 48.0, 50.5, 71.3, 72.9, 125.3, 126.3, 127.5, 127.6 (2x), 128.3 (2x), 129.6, 136.3, 136.4, 137.6, 138.4, 221.2. LRMS for $C_{27}H_{33}O_2$ $[M+H]^+$ 389.4 m/z.

*1.4. Synthesis of 3-{(E)-[(16E)-3-[2-(benzyloxy)ethyl]-17-oxoestra-1(10),2,4-trien-16-ylidene] methyl}benzamide (**12**)*

To a solution of compound **11** (400 mg, 1.03 mmol) in EtOH (25 mL) was added 3-formyl-benzamide (344 mg, 2.05 mmol) and aqueous KOH (10%) solution (4.5 mL). The solution was heated at reflux for 30 min. The resulting solution was diluted with water (200 mL), neutralized with aqueous HCl 10%, and extracted with EtOAc (3 x 50 mL). The organic layers

were combined, washed with brine, dried with $MgSO_4$ and evaporated under reduced pressure to give 400 mg (75 % yield) of compound **12**. IR (KBr): 3356 and 3202 (NH_2), 1713 (C=O, conjugated ketone), 1666 (C=O, amide), 1628 (conjugated double bond). ^{1}H NMR ($CDCl_3$): 1.00 (s, 18-CH_3), 1.35-2.65 (unassigned CH and CH_2), 2.89 (t, J = 7.3 Hz, C$\underline{H_2}$CH_2O), 2.93 (m, 6-CH_2), 3.00 (ddd, J_1 = 1.5 Hz, J_2 = 6.5 Hz, J_3 = 15.9 Hz, 1H of 15-CH_2), 3.69 (t, J = 7.3 Hz, CH_2C$\underline{H_2}$O), 4.54 (s, OC$\underline{H_2}$Ph), 5.82 and 6.17 (2 broad s, NH_2), 6.99 (s, 4-CH), 7.03 (d, J = 8.0 Hz, 2-CH), 7.23 (d, J = 8.0 Hz, 1-CH), 7.25-7.38 (m, 5H of OC$H_2\underline{Ph}$), 7.49 (s, 1'-CH), 7.52 (t, J = 7.8 Hz, 5"-CH), 7.71 (d, J = 7.8 Hz, 6"-CH), 7.77 (d, J = 7.8 Hz, 4"-CH), 8.04 (s, 2"-CH). ^{13}C NMR ($CDCl_3$): 14.5, 25.7, 26.9, 29.1, 29.3, 31.7, 35.8, 37.8, 44.3, 47.9, 48.6, 71.2, 72.9, 125.3, 126.4, 127.5, 127.6, 127.7 (2x), 128.4 (2x), 129.0, 129.2, 129.6, 131.9, 133.5, 133.8, 136.2, 136.3, 136.4, 137.4, 137.5, 138.4, 168.9, 209.4. LRMS for $C_{35}H_{37}NO_3Na$ $[M+Na]^+$ 542.4 m/z.

*1.5. Synthesis of 3-{[(17β)-17-hydroxy-3-(2-hydroxyethyl)estra-1(10),2,4-trien-16-yl]methyl} benzamide (**4**)*

To a solution of compound **12** (390 mg, 0.75 mmol) in a mixture of MeOH and DCM (4:1) was added $NaBH_4$ (85 mg, 2.23 mmol). The solution was stirred at room temperature for 1 h. The resulting solution was concentrated under vacuo, diluted with DCM (30 mL), washed with water, dried with $MgSO_4$ and evaporated under reduced pressure to give 375 mg of the crude 17β-alcohol. To this alcohol intermediate dissolved in EtOH (100 mL) and under an atmosphere of argon at room temperature was added 10% Pd on charcoal (80 mg). The reaction vessel was flushed three times with H_2 and the mixture was stirred for 36 h, then filtered on celite and evaporated under reduced pressure. The crude compound was purified by flash chromatography (EtOAc/Hexanes: 4:6) to give 255 mg (69% yield, 2 steps) of compound **4**. IR (KBr): 3364 (OH and NH_2), 1659 (C=O, amide). ^{1}H NMR (CD_3OD): 0.90 (s, 18-CH_3), 1.10-1.16 (m, 14α-CH and 15β-CH), 1.20-1.55 (m, 7α-CH, 12α-CH, 8β-CH and 11β-CH) 1.68 (t, J = 7.0 Hz, 15α-CH), 1.79-1.84 (m, 7β-CH), 2.01-2.05 (m, 12β-CH), 2.21 (td, J_1 = 3.7 Hz, J_2 = 12.7 Hz, 9α-CH), 2.32-2.37 (m, 11α-CH), 2.47 (q, J = 12.3 Hz, 1H of 1'-CH_2), 2.52 (m broad, 16α-CH), 3.74 (t, J = 7.2 Hz, C$\underline{H_2}$CH_2OH), 2.79 (m, 6-CH_2), 3.17 (dd, J_1 = 2.7 Hz, J_2 = 12.5 Hz, 1H of 1'-CH_2), 3.71 (t, J = 7.2 Hz, CH_2C$\underline{H_2}$OH), 3.84 (d, J = 9.4 Hz, 17α-CH), 6.90 (s, 4-CH), 6.96 (d, J = 8.0 Hz, 2-CH), 7.19 (d, J = 8.0 Hz, 1-CH), 7.40 (m, 5"-CH and 6"-CH), 7.70 (d, J = 7.0 Hz, 4"-CH), 7.76 (s, 2"-CH). ^{13}C NMR (CD_3OD): 13.3 (C18), 27.4 (C11), 28.6 (C7), 30.5 (C6), 33.0 (C15), 38.9 (C1'), 39.0 (C12), 39.7 (C8), 39.8 (C$\underline{H_2}$CH_2OH), 43.3 (C16), 45.4 (C13), 45.7 (C9), 50.0 (C14), 64.4 (CH_2OH), 83.0 (C17), 126.0 (C4"), 126.2 (C1), 127.2 (C2), 129.1 (C2"), 129.4 (C5"), 130.4 (C4), 133.5 (C6"), 134.8 (C3"), 137.2 (C3), 137.5 (C5), 139.3 (C10), 144.3 (C1"), 172.7 ($CONH_2$). LRMS for $C_{28}H_{36}NO_3$ $[M+H]^+$ 434.4.

*1.6. Synthesis of 3-{[(16β,17β)-3-(2-bromoethyl)-17-hydroxyestra-1(10),2,4-trien-16-yl]methyl} benzamide (**5**)*

To a solution of compound **4** (175 mg, 0.40 mmol) in DCM (15 mL) was added at 0°C triphenylphosphine (200 mg, 0.76 mmol) and carbon tetrabromide (252 mg, 0.76 mmol). The solution was stirred at 0°C for 40 min and a second portion of triphenylphosphine (100 mg, 0.38 mmol) and carbon tetrabromide (126 mg, 0.38 mmol) were added and the solution stirred for 1 h at 0°C. The resulting mixture was poured into water (150 mL), extracted with DCM (50 mL), and the organic phase dried with $MgSO_4$ and evaporated under reduced pressure. The crude

compound was purified by flash chromatography (DCM/MeOH: 97:3) to give 168 mg (73% yield) of compound **5**. IR (KBr): 3364 and 3194 (OH and NH_2), 1636 (C=O, amide). ^{1}H NMR (CD_3OD): 0.91 (s, 18-CH_3), 1.12-1.18 (m, 14α-CH and 15β-CH), 1.22-1.56 (m, 7α-CH, 12α-CH, 8β-CH and 11β-CH), 1.68 (t, J = 7.0 Hz, 15α-CH), 1.82-1.86 (m, 7β-CH), 2.02-2.06 (m, 12β-CH), 2.22 (td, J_1 = 3.6 Hz, J_2 = 12.9 Hz, 9α-CH), 2.34-2.38 (m, 11α-CH), 2.44 (d, J = 12.3 Hz, 1H of 1'-CH_2), 2.52 (m broad, 16α-CH), 2.82 (m, 6-CH_2), 3.06 (t, J = 7.3 Hz, $C\underline{H}_2CH_2Br$), 3.17 (dd, J_1 = 2.7 Hz, J_2 = 12.5 Hz, 1H of 1'-CH_2), 3.55 (t, J = 7.2 Hz, $CH_2C\underline{H}_2Br$), 3.84 (d, J = 9.4 Hz, 17α-CH), 6.91 (s, 4-CH), 6.97 (d, J = 8.0 Hz, 2-CH), 7.22 (d, J = 8.0 Hz, 1-CH), 7.40 (m, 5"-CH and 6"-CH), 7.69 (d, J = 7.0 Hz, 4"-CH), 7.75 (s, 2"-CH). ^{13}C NMR (CD_3OD): 13.3 (C18), 27.3 (C11), 28.5 (C7), 30.5 (C15), 33.0 (C6), 33.9 (CH_2Br), 38.8 (C1'), 38.9 (C12), 39.6 (C8), 40.1 ($\underline{C}H_2CH_2Br$), 43.3 (C16), 45.4 (C13), 45.7 (C9), 50.0 (C14), 83.0 (C17), 126.0 (C4"), 126.4 (C1), 127.0 (C2), 129.1 (C2"), 129.4 (C5"), 130.1 (C4), 133.5 (C6"), 134.8 (C3"), 137.4 (C3), 137.8 (C5), 140.0 (C10), 144.3 (C1"), 172.7 ($CONH_2$). LRMS for $C_{28}H_{35}NO_2$ $[M+H]^+$ 496.0 and 498.1; HPLC purity of 98.5% (Retention time = 14.6 min).

2. Chromatogram (HPLC purity) of compound 5

HPLC apparatus (Waters Associates, Milford, MA, USA) using a Luna phenyl-hexyl column (75 x 4.6 mm id, 3 µm, Serial No: 338048-2, 60 Å) and MeOH/H_2O: 70/30 as solvent.

Empower software

Report Produits RM

Reported by User: System Project Name: Purete_UV

SAMPLE INFORMATION

Sample Name:	RM-560-147 Dil	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	11/25/2009 12:20:57 PM
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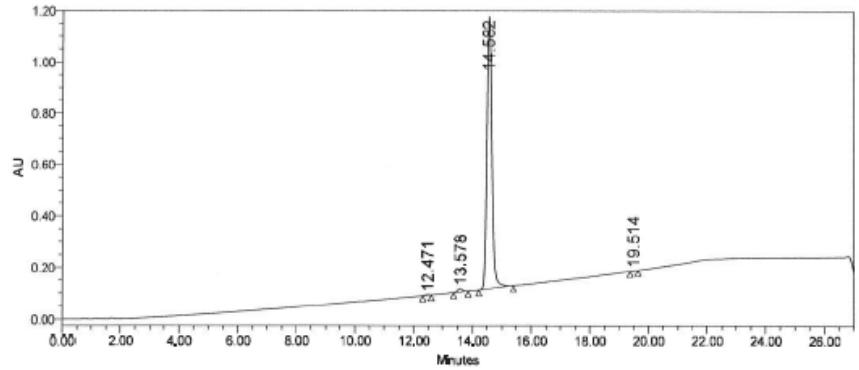
Injection #:	1	Date Processed:	11/25/2009 1:04:40 PM
Injection Volume:	5.00 ul	Processing Method:	Produits RM_532_48
Run Time:	30.0 Minutes	Channel Name:	Wvln Ch2
Sample Set Name:	RB20091125_1	Proc. Chnl. Descr.:	PDA 205.0 nm

MeOH : H2O 70 : 30



Channel W2996 ; Processed Channel: PDA 205.0 nm; Result Id: 1673; Processing Method: Produits RM_532_48

Processed Channel Descr.: PDA 205.0 nm

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2	PDA 205.0 nm	13.578	143051	1.16	13036
3	PDA 205.0 nm	14.582	12142256	98.50	1059054
4	PDA 205.0 nm	19.514	18436	0.15	2278

3. Description of biological assays: inhibition of 17β-HSD1 and estrogenic activity

3.1. Cell culture

The ER-positive breast cancer cell lines T-47D and MCF-7 were obtained from the American Type Culture Collection (ATCC) and maintained in 175 cm^2 culture flasks at 37°C in a humidified atmosphere at 5% CO_2. T-47D cells were grown in RPMI medium supplemented with 10% (v/v) fetal bovine serum (FBS), L-glutamine (2 nM), penicillin (100 IU/mL), streptomycin (100 µg/mL) and estradiol (1 nM). The MCF-7 cells were propagated in Dubelcco's Modified Eagle's Medium nutrient mixture F-12 Ham (DMEM-F12) medium supplemented with 5% FBS, glutamine (2 nM), penicillin (100 IU/mL), streptomycin (100 µg/mL) and estradiol (1 nM).

3.2. Inhibition of 17β-HSD1 in T-47D cells

T-47D cells were seeded in a 24-well plate (3000 cells/well) in 990 µL of medium supplemented with insulin (50 ng/mL) and 5% dextran-coated charcoal treated FBS, which was used rather than untreated 10% FBS, to remove the remaining steroid hormones. Inhibitor stock solutions were previously prepared in ethanol and diluted with culture medium to achieve appropriate concentrations prior to use. After 24 h of incubation, 5 µL of the diluted solution were added to the cells to obtain a final concentration of 0.01 or 0.1 µM for all inhibitors. For the most active inhibitors, concentrations ranging from 0.01 to 10 µM were tested to determine the IC_{50} value. The final concentration of ethanol in the well was adjusted to 0.1%. Additionally, 5 µL of a solution of [^{14}C]-estrone (American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) was added to obtain a final concentration of 60 nM. Cells were incubated for 24 h and each inhibitor was assessed in triplicate. After incubation, the culture medium was removed and labeled steroids (E1 and E2) were extracted with 1 mL of diethyl ether. The organic phases were evaporated to dryness with nitrogen. Residues were dissolved in dichloromethane and dropped on silica gel thin layer chromatography plates (EMD Chemicals Inc., Gibbstown, NJ, USA) and eluted with toluene/acetone (4:1) as solvent system. Substrate [^{14}C]-E1 and metabolite [^{14}C]-E2 were identified by comparison with reference steroids (E1 and E2) and quantified using the Storm 860 system (Molecular Dynamics, Sunnyvale, CA, USA). The percentage of transformation and the percentage of inhibition were calculated as follow: % transformation = 100 x [^{14}C]-E2 / ([^{14}C]-E1 + [^{14}C]-E2) and % of inhibition = 100 x (% transformation without inhibitor -% transformation with inhibitor) / % transformation without inhibitor.

3.3. Estrogenic activity in MCF-7 (ER^+) cells

MCF-7 cells were seeded with medium supplemented with insulin (50 ng/mL) and 5% dextran-coated charcoal treated FBS, which was used rather than untreated 10% FBS, to remove the remaining steroid hormones. Aliquots (100 µL) of the cell suspension were seeded in 96-well plates (3000 cells/well). After 48 h, the medium was changed for a new one containing an appropriate concentration of inhibitors to be tested and was replaced every 2 days. Cells grew either in absence or presence of the inhibitors for 7 days. Quantification of cell growth was determined by using CellTiter 96®Aqueous Solution Cell Proliferation Assay (Promega, Nepean, ON, Canada) according to the manufacturer's instructions. The cell growth in absence of a tested inhibitor (control) was fixed at 100%. Each inhibitor was assessed in triplicate.