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Research Article

Synthesis and biodistribution studies of two novel radioiodinated areno-annelated estra-1,3,5(10), 16-tetraene-3-ols as promising estrogen receptor radioligands

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Summary

Two novel radioiodinated areno-annelated estra-1,3,5(10),16-tetraenes, [^{125}I]2-iodo-1′-methoxybenzo[4′,3′:16,17]estra-1,3,5(10),16-tetraene-3-ol (**2-[^{125}I]-MEBE**) and [^{125}I]4-iodo-1′-methoxybenzo[4′,3′:16,17]estra-1,3,5(10),16-tetraene-3-ol, (**4-[^{125}I]-MEBE**) were synthesized for evaluation as potential ligands for the estrogen receptor. Radioiodination of 1′-methoxybenzo[4′,3′:16,17]estra-1,3,5(10),16-tetraene-3-ol at the A ring was accomplished by electrophilic aromatic substitution using [^{125}I] sodium iodide and chloramine-T as oxidant. After purification by reverse phase HPLC, the two radioisomers (**2-[^{125}I]-MEBE** and **4-[^{125}I]-MEBE**) were obtained in a radiochemical yield of 42 and 48%, respectively, in a radiochemical purity of greater than 95% and a high specific activity. The effect of the site of radioiodination (C_2 vs C_4) on the biological behaviour of the molecules was evaluated through biodistribution studies in immature female Sprague-Dawley rats. Both **2-[^{125}I]-MEBE** and **4-[^{125}I]-MEBE** are stable *in vivo* and are mainly excreted through the hepatobiliary pathway. Both localize in the uterus and ovaries via a receptor-mediated process, where the **2-[^{125}I]-MEBE** isomer has the higher specific ER binding and uterus selectivity.

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The favourable *in vitro*/*in vivo* stability and biodistribution profiles suggest that these radioligands are good candidates for further exploration of their potential clinical application.

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Key Words: estrogen receptor; estradiol derivatives; radioiodination; breast tumour imaging

Introduction

Breast cancer still remains the leading cause of cancer mortality among women in Western countries regardless of the progress achieved in its early detection and treatment.[1] Breast cancer of about two-thirds of the patients express estrogen receptors (ER) in the primary tumour. Knowledge of the tumour ER status increases the chance of survival of these patients, allowing for a better diagnosis of the illness and for a better determination of proper adjuvant or palliative hormonal therapy. Since one-third of breast cancer patients respond to hormonal therapy, drugs based on ER ligands are valuable tools for the diagnosis and oriented therapy of the disease and the search for novel ligands to specifically target the ER continues to be a very demanding task. Both gamma- and positron-emitting derivatives of estradiol have been developed to visualize and quantify ER densities in breast tumours using single photon emission (SPECT) imaging or positron emission tomography (PET). In the case of radiopharmaceutical development for SPECT using ^{123}I as a tracer, the most studied compounds are the 16α-iodo[2,3] and the 17α-iodovinyl estradiols.[4,5] The presence of radioiodine at either position is well tolerated by the ER and introduction of additional substituents at 11β- or 7α- can enhance receptor binding.[6,7] While most of the emphasis has been focused on the radioimaging potential of these radioligands, the presence of Auger electrons from the decay of $^{123/125}$I also initiated interest in their radiotherapeutic applications. Studies by McLaughlin *et al.*[8] and DeSombre *et al.*[9] have shown that estrogens labelled with Auger emitters can destroy ER-positive cells while sparing ER-negative cells.

As a part of our investigation on new specific ligands[10] for targeted therapy and/or nuclear imaging of ER-rich tissues, we became interested in the synthesis and evaluation of *E*-ring expanded estrane derivatives radiolabelled in the aromatic A ring. In this study, two radioiodinated isomers of 1′-methoxybenzo[4′,3′:16,17]estra-1,3,5(10),16-tetraene-3-ol (**MEBE**) were synthesized and characterized. Biological behaviour of the molecules was studied in immature female rats attempting to evaluate the effect of altering the position of radioiodination.

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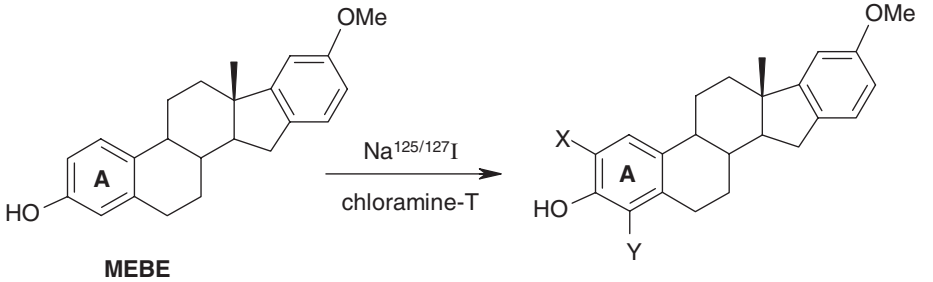
Results and discussion

Iodination of **MEBE**[11] with sodium iodide was accomplished by electrophilic aromatic substitution at the aromatic A ring using chloramine-T as the oxidant and aqueous sodium metabisulphite as quenching agent[12] and gave a mixture of three iodinated compounds (Scheme 1). The reaction products were separated by semi-preparative reverse phase HPLC with UV detection (254 nm), using MeOH/H_2O (85/15) as eluent. The iodinated areno-annelated estra-1,3,5(10),16-tetraene derivatives **2-IMEBE**, **4-IMEBE** and **2, 4-IMEBE** were obtained in yields of 30, 16 and 21%, respectively, and in high chemical purity. Their structures were confirmed by FTIRC/MS (M^+ ions at *m/e* 460, 460 and 586, respectively) and by ^{1}H NMR and ^{13}C NMR spectroscopy. The substitution pattern of the aromatic A ring was elucidated for each compound by ^{1}H NMR. The ^{1}H NMR spectrum of the 2-iodinated isomer gave two singlets at 7.52 and 6.73 ppm, corresponding to the aromatic protons at C1 and C4, respectively. In contrast, the 4-iodinated isomer has two doublets at 7.21 and 6.95 ppm with *ortho* coupling constants of $J = 8.4$ Hz assigned to the aromatic protons at C1 and C4, respectively. The di-iodinated compound shows one singlet at 7.63, confirming the double iodination at C2 and C4.

Radioiodination of **MEBE** was achieved by substituting the non-radioactive NaI used in the synthesis with Na[^{125}I].[11] The radioiodinated derivatives **2-[^{125}I]IMEBE** and **4-[^{125}I]IMEBE** were isolated by analytical reverse phase HPLC with simultaneous UV (254 nm) and radioactivity detection using MeOH/H_2O (85/15) (Figure 1). During HPLC purification eluting **2-[^{125}I]IMEBE** and **4-[^{125}I]IMEBE** could not be detected by the UV monitor, and it was consequently assumed that their specific activities are in the same range as that of the starting Na[^{125}I], 2200 Ci/mmol. **2-[^{125}I]IMEBE** and **4-[^{125}I]IMEBE**



Scheme 1. Synthesis of the iodinated areno-annelated estra-1,3,5(10),16-tetraene derivatives (2-IMEBE, 4-IMEBE) and their radioiodinated analogues (2-[^{125}I]IMEBE and 4-[^{125}I]IMEBE)

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were compared with their corresponding unlabelled analogues, **2-IMEBE** and **4-IMEBE**, by HPLC with simultaneous UV and radioactivity detection (Figure 2) and were shown to be the expected products on the basis of the elution profile. The two isomers were obtained in radiochemical yields of 42

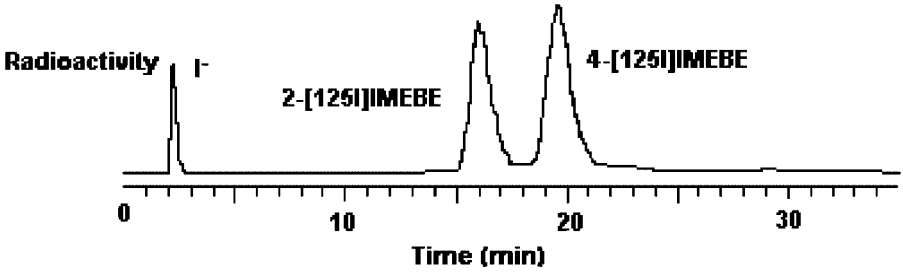

Figure 1. Analytical HPLC chromatogram for the separation of 2-[^{125}I]IMEBE and 4-[^{125}I]IMEBE. Experimental conditions: EC 250/4 Nucleosil C_{18} column, 10 µm Macherey Nagel; flow rate 1.0 ml/min; mobile phase 85% aqueous methanol

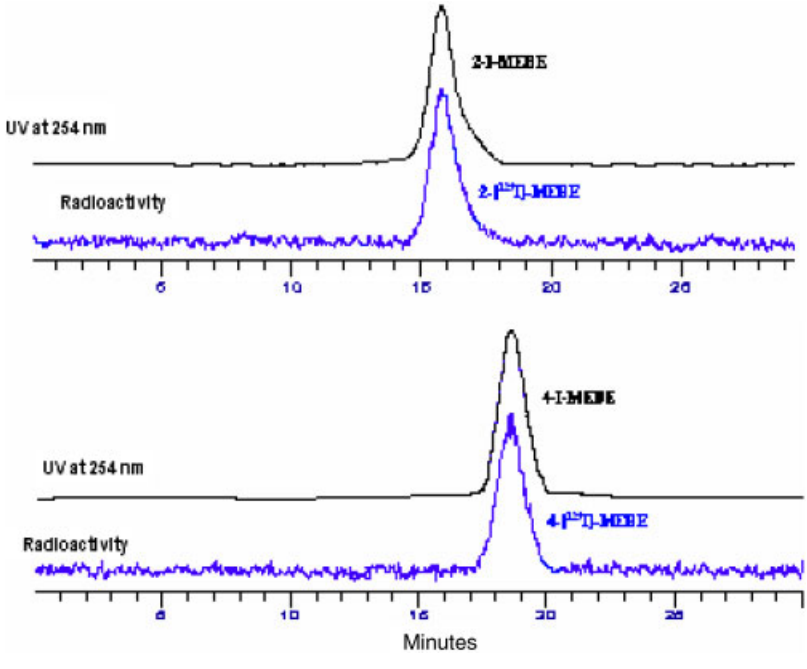

Figure 2. HPLC analysis of 2-[^{125}I]IMEBE and 4-[^{125}I]IMEBE, co-injected with unlabelled 2-IMEBE and 4-IMEBE. Experimental conditions: EC 250/4 Nucleosil C_{18} column, 10 µm Macherey Nagel; flow rate 1.0 ml/min; mobile phase 85% aqueous methanol

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and 48%, respectively, and in radiochemical purity of higher than 95%. Only a trace of radioactive di-iodinated compound **2,4-[^{125}I]IMEBE** was observed in the radioiodination.

The *in vitro* stability of the radioiodinated compounds under physiological conditions is a very important parameter to be taken into account in the evaluation of their potential interest for clinical application since dehalogenation gives free iodide, which may result in undesirable biodistribution of radioactivity causing radiation dose exposure of non-target tissues and limiting the clinical efficacy. Thus, stability of both compounds was studied in the presence of 0.9% NaCl solution and human serum up to 6 days.

Studies have demonstrated that both compounds are radiochemically stable in 0.9% NaCl solution for 2 days at 37°C and for 6 days at 6°C. A series of human serum stability tests were also conducted on each of the two labelled compounds. The serum stabilities of the compounds are shown in the radiochromatograms of Figure 3. Both isomers still show high human serum stability after 3 days at 37°C.

The lipophilicity was assessed using the octanol/PBS distribution coefficients (log *P*) defined as the concentration ratio of the compounds in octanol phase and in aqueous phase at physiological pH. Both radiolabelled isomers present a very lipophilic character as most unconjugated steroids. Nevertheless, they show suitable partition coefficients to minimize non-specific binding ($\log P < 3$). **2-[^{125}I]-MEBE** (1.8) is somewhat less lipophilic than **4-[^{125}I]-MEBE** (2.5) as was already expected on the basis of the observed HPLC elution profile.

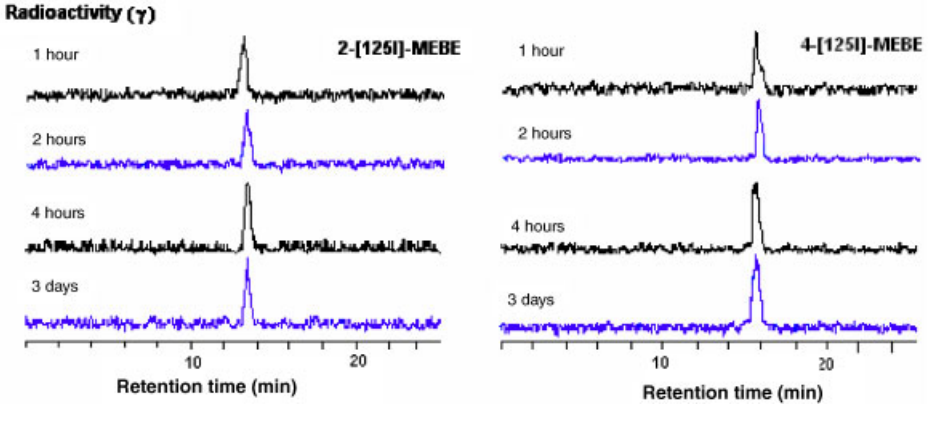

Figure 3. Radioactive HPLC-RP profile of 2-[^{125}I]-MEBE and 4-[^{125}I]-MEBE: Serum stability at 37°C. Experimental conditions: EC 250/4 Nucleosil C_{18} column, 10 µm Macherey Nagel; flow rate 1.0 ml/min; mobile phase 85% aqueous methanol

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The effect of the site of radioiodination (C_2 vs C_4) on the biological behaviour of the radioiodinated **MEBE** was studied through biodistribution studies in immature female Sprague-Dawley rats after intravenous administration of the radiotracer at 1, 2 and 24 h. The biodistribution profiles (graphically presented in Figures 4 and 5) are quite similar for the two radioisomers with rapid blood clearance and high liver uptake that clears into the intestines suggesting the hepatobiliary tract as the main excretory route, which is in agreement with the lipophilicity of the compounds and which has been reported before for this animal model. The main differences are related to a slightly faster blood clearance and higher liver uptake and retention of **4**-[^{125}**I**]**IMEBE**, which can be explained by its higher lipophilicity. **2**-[^{125}**I**]**IMEBE** and **4**-[^{125}**I**]**IMEBE** also show uptake in target tissues, uterus and ovaries. In order to evaluate the receptor-mediated uptake in these ER-rich tissues, a separate group of animals was co-injected with unlabelled estradiol to block receptors (Figure 6). As can be seen, uterus and ovaries uptake were effectively blocked by cold estradiol, resulting in decreased radioactivity in these organs. In particular, the uptake of **2**-[^{125}**I**]-**MEBE** decreased to about one half. The uptake selectivity was assessed through the uterus-to-blood and uterus-to-muscle radioactivity ratios over time (Table 1). High uterus-to-non-targeting ratios were found at 24 h indicating that both compounds are selective. Thus, *in vivo* data demonstrated a specific uptake mechanism with **2**-[^{125}**I**]-**MEBE** exhibiting the higher specific ER binding and uterus selectivity.

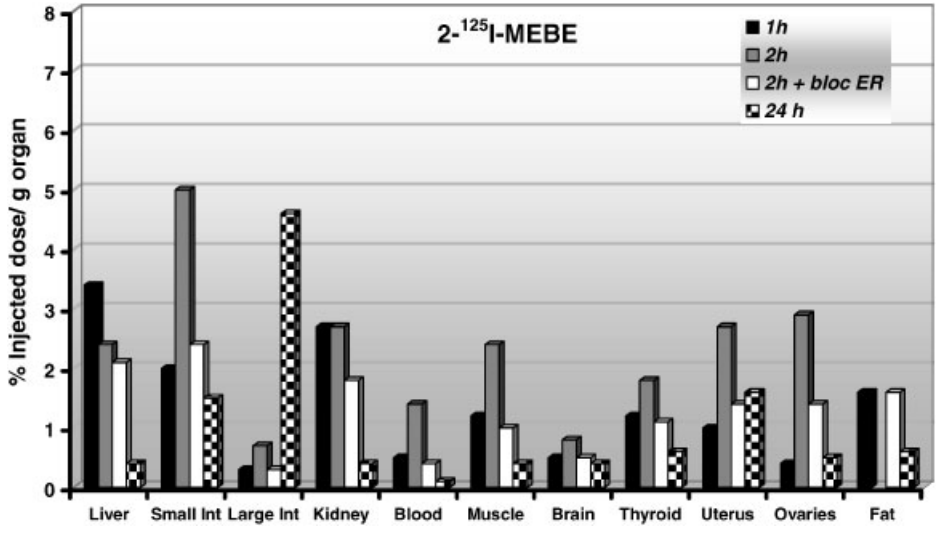

Figure 4. Biodistribution data in most relevant organs, expressed as %ID/g organ for 2-[^{125}I]IMEBE at 1, 2 and 24 h after IV administration in immature Sprague-Dawley female rats ($n = 5$)

DOI: 10.1002/jlcr

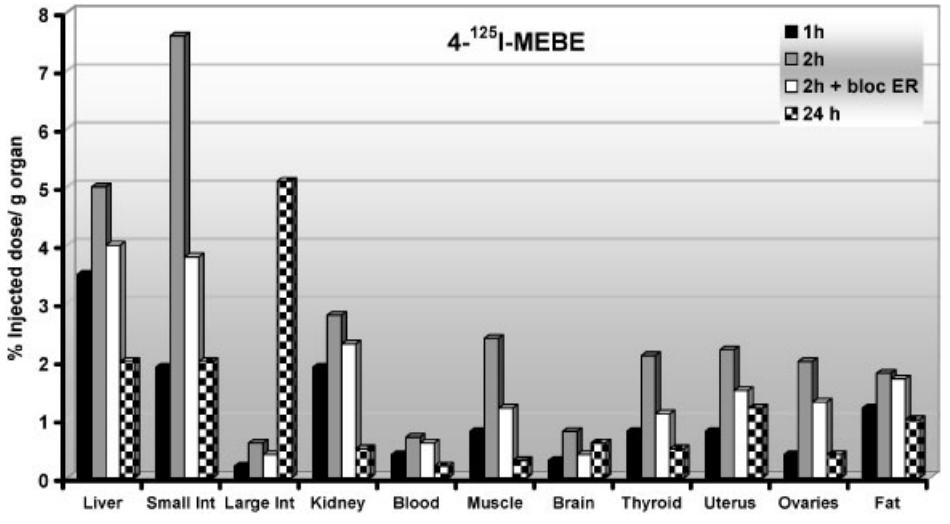

Figure 5. Biodistribution data in most relevant organs, expressed as %ID/g organ for 4-[^{125}I]IMEBE at 1, 2 and 24 h after IV administration in immature Sprague-Dawley female rats ($n = 5$)

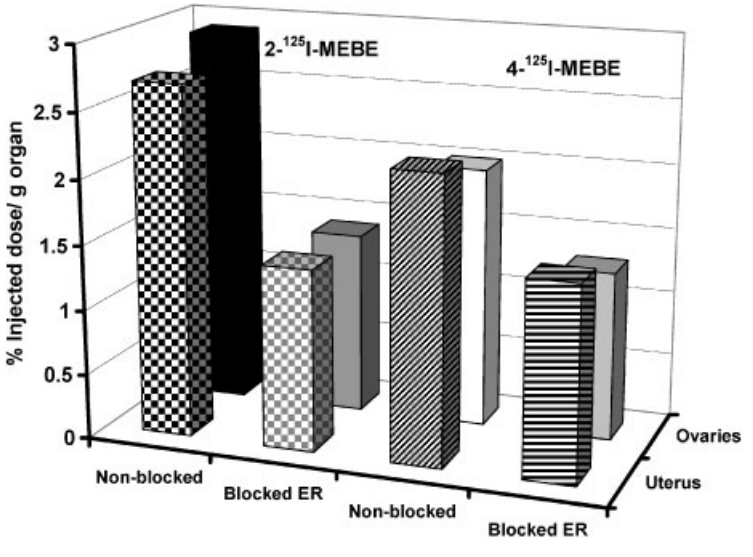

Figure 6. Effect of ER blocking by injection of β-estradiol on the uterus and ovaries uptake of 2-[^{125}I]IMEBE and 4-[^{125}I]IMEBE, at 2 h after administration

The amount of activity excreted in urine is very small, indicating a very low metabolization of the radiolabelled steroids. However, the radioactivity is totally excreted as radiochemical species with the same retention time of free

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Table 1. Uterus-to-blood and uterus-to-muscle radioactivity ratios in immature Sprague-Dawley female rats at 1, 2 and 24 h after IV administration of 2-[^{125}I] IMEBE and 4-[^{125}I]IMEBE

Time after administration (h)	2-[^{125}I]IMEBE		4-[^{125}I]IMEBE	
	Uterus/blood	Uterus/muscle	Uterus/blood	Uterus/muscle
1	2.0	0.8	2.2	1.0
2	2.2	1.1	3.1	0.9
24	16.0	4.0	6.7	4.0

radioiodine as demonstrated by HPLC analysis of urine samples taken at sacrifice time. The *in vivo* stability of the radioiodinated steroids is mainly reflected in the thyroid radioactivity concentration. It can be seen from the low thyroid radioactivity levels that both compounds are relatively stable *in vivo*.

Conclusions

Two novel *E*-ring expanded estrane derivatives, **2-[^{125}I]-MEBE** and **4-[^{125}I]-MEBE**, were prepared in good radiochemical yields by direct electrophilic radioiodination on the aromatic A ring. Both radioisomers were obtained with high radiochemical purity and high specific activity after reversed phase HPLC purification. Additional to the favourable radiochemical features and *in vitro* stability, the biological behaviour in immature animal model supports the potential interest of this kind of radioligands to be further explored as therapeutic agents. The data have encouraged us to proceed with studies of related and less lipophilic compounds bearing a hydroxyl substituent on the *E*-ring (**HIBE**). Hence, the evaluation of **HIBE** radioisomers is currently under investigation.

Experimental

General: All commercial reagents and solvents were of analytical grade and were used without further purification. Na[^{125}I] (carrier free) was obtained from Amersham Biosciences. Proton and carbon nuclear magnetic resonance spectra (^{1}H and ^{13}C) were performed on a Varian Unity 300 MHz spectrometer using $CDCl_3$ as solvent. Mass spectra were obtained with a Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FTICR/MS) Finnigan FT/MS 2001-DT. Purification of the iodinated compounds was performed by semi-preparative reverse phase HPLC on a Perkin Elmer system equipped with UV (SPD-10 AV, Shimadzu) and radioactivity detector (LB 509, Berthold) with an ODS 250/8 Hypersil C_{18} column, 10 µm Macherey Nagel. Purification of the radioiodinated compounds was performed on an EC 250/4 Nucleosil C_{18} column, 10 µm Macherey Nagel.

DOI: 10.1002/jlcr

Chemistry: 1′-Methoxybenzo[4′,3′:16,17]estra-1,3,5(10),16-tetraene-3-ol (**MEBE**) (11 mg, 0.033 mmol)[11] was dissolved in 1,4-dioxane (1 ml) and a NaI solution (200 µl, 0.033 mmol, 5 mg in 0.05 M phosphate buffer, pH = 7.4) was added, followed by a chloramine-T solution (500 µl, 0.034 mmol, 9.5 mg in 0.05 M phosphate buffer, pH = 7.4). The reaction was allowed to proceed for 5 min. at room temperature with constant mixing. Thereafter, it was quenched by the addition of a solution of sodium metabisulphite (500 µl, 0.033 mmol, 6.5 mg in 0.05 M phosphate buffer, pH = 7.4). Water (2 ml) was added and the mixture extracted with ethyl acetate (2 × 10 ml). The combined organic extracts were dried over anhydrous $MgSO_4$ and evaporated to dryness. The residue obtained was dissolved in a mixture of methanol in water (1 ml, 85% (v/v). The dissolved reaction products were separated by semi-preparative HPLC using 85% (v/v) methanol in water. The elution rate was 2 ml/min and the effluent was continuously monitored at $\lambda = 254$ nm. Separate fractions were collected, evaporated and the residues obtained were crystallized from methanol to give 2-iodo-1′-methoxybenzo[4′,3′:16,17]estra-1,3,5(10),16-tetraene-3-ol (**2-IMEBE**) (4,4 mg, 30%), 4-iodo-1′-methoxybenzo [4′,3′:16,17]estra-1,3,5(10),16-tetraene-3-ol (**4-IMEBE**) (2.4 mg, 16%) and 2,4-diiodo-1′-methoxybenzo[4′,3′:16,17] estra-1,3,5(10),16-tetraene-3-ol (**2, 4-IMEBE**) (4 mg, 21%).

(**2-IMEBE**): **^{1}H-NMR** (300 MHz, $CDCl_3$) δ: 0.92 (s, 3H, 18-CH_3); 1.37–2.87 (m, 13H); 3.78 (s, 3H, -OCH_3); 5.04 (s, 1H, -OH); 6.64 (d, 1H, J*meta* = 1.8 Hz, 2′-H); 6.67 (dd, 1H, J*meta* = 1.8 Hz, J*ortho* = 8.1 Hz, 6′-H); 6.73 (s, 1H, 4-H); 7.12 (d, 1H, J*ortho* = 8.1 Hz, 5′-H); 7.52 (s, 1H, 1-H); **^{13}C-NMR** ($CDCl_3$, 75 MHz) δ: 19.20 (C18); 26.48 (C7); 27.55 (C11); 29.20 (C6); 31.13 (C12); 34.82 (C15); 37.43 (C8); 44.02 (C9); 45.61 (C14); 55.41 (-O<u>C</u>H_3); 56.81 (C13); 82.40 (C2); 107.20 (C2′); 110.82 (C6′); 114.93 (C4); 125.48 (C5′); 134.37 (C5); 134.72 (C16); 135.34 (C10); 139.36 (C1); 152.48 (C17); 155.84 (C3); 158.63 (C1′); **HRMS** (EI (+) 10 eV, $T \sim 150$°C) Found: 460.08903, calculated $C_{23}H_{25}O_2I$ (M^+): 460.089379.

(**4-IMEBE**): **^{1}H-NMR** (300 MHz, $CDCl_3$) δ: 0.92 (s, 3H, 18-CH_3); 1.55–2.84 (m, 13H); 3.79 (s, 3H, -OCH_3); 5.36 (s, 1H, -OH); 6.64–6.71 (m, 2H, 2′-H e 6′-H); 6.84 (d, 1H, J*ortho* = 8.7 Hz, 2-H); 7.13 (d, 1H, J*ortho* = 8.1 Hz, 5′-H); The signal for one proton overlaps with the signal for the chloroform. **^{1}H-NMR** (300 MHz, CD_3COCD_3) δ: 0.93 (s, 3H, 18-CH_3); 1.27–2.82 (m, 13H); 3.75 (s, 3H, -OCH_3); 6.65 (d, 1H, J*meta* = 1.8 Hz, 2′-H); 6.76 (dd, 1H, J*meta* = 1.8 Hz, J*ortho* = 8.1 Hz, 6′-H); 6.95 (d, 1H, J*ortho* = 8.7 Hz, 2-H); 7.13 (d, 1H, J*ortho* = 8.1 Hz, 5′-H); 7.21 (d, 1H, J*ortho* = 8.4 Hz, 1-H); **HRMS** (EI (+) 10 eV, T ~ 150°C) Found: 460.08893, calculated $C_{23}H_{25}O_2I$ (M^+): 460.089379.

(**2, 4-IMEBE**): **^{1}H-NMR** (300 MHz, $CDCl_3$) δ: 0.91 (s, 3H, 18-CH_3); 1.23–2.88 (m, 13H); 3.79 (s, 3H, -OCH_3); 5.77 (s, 1H, -OH); 6.64 (d, 1H, J*meta* = 2.4 Hz, 2′-H); 6.69 (dd, 1H, J*meta* = 2.4 Hz, J*ortho* = 6 Hz, 6′-H);

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7.13 (d, 1H, J*ortho* = 7.8 Hz, 5′-H); 7.63 (s, 1H, 1-H); **^{13}C-NMR** ($CDCl_3$, 75 MHz) δ: 19.13 (C18); 26.76 (C7); 28.54 (C11); 31.09 (C6); 34.78 (C12); 36.71 (C15); 37.18 (C8); 44.25 (C9); 45.54 (C14); 55.42 (-O<u>C</u>H$_3$); 56.60 (C13); 78.17 (C2); 92.16 (C4); 107.24 (C2′); 110.90 (C6′); 125.52 (C5′); 134.28 (C5); 135.62 (C16); 136.71 (C10); 140.79 (C1); 151.30 (C17); 155.64 (C3); 158.65 (C1′); **HRMS** (EI (+) 15 eV, $T \sim 150$°C) Found: 585.98564, calculated $C_{23}H_{24}O_2I_2$ (M^+): 585.986027.

Radiochemistry: To a solution of **MEBE** in ethanol (25 µl, 3 µg, 9 µmol) was added [^{125}I]NaI (10 µl, 274 µCi, 10.1 MBq) and chloramine-T (10 µl, 40 µg, 4 mg/ml in water). Then, the mixture was diluted with phosphate buffer (100 µl, 0.01 M, pH = 7.4). The reaction was allowed to proceed for 3 min. with constant mixing at room temperature, before being quenched by the addition of a solution of sodium metabisulphite (50 µl, 40 µg, 0.8 mg/ml in water).

In vitro stability studies: The radioiodinated compounds were studied by radiochemical purity evaluation at several time points after incubation in 0.9% NaCl and human serum at different temperatures in order to detect any radiochemical impurities or free radioiodide. Briefly, the radiolabelled compound (200 µl) was incubated in fresh human serum (400 µl) at room temperature and at 37°C. Aliquots (100 µl) were taken before incubation started (for zero time point analysis), as well as at various time intervals during incubation. The mixture was vortexed and centrifuged (3000 rpm, 15 min, 4°C) and the supernatant solution was analysed by HPLC to check the radiochemical stability.

Lipophilicity: log *P* was determined by the 'shakeflask' method. The radiolabelled compound was added to a mixture of octanol (1 ml) and phosphate buffer (1 ml, 0.01 M, pH = 7.4), previously saturated in each other by stirring the mixture. The mixture was vortexed and centrifuged (3000 rpm, 10 min, rt) to allow for phase separation. Aliquots of both octanol and phosphate buffer were counted for radioactivity in a γ-counter. The log *P* of each isomer was calculated by dividing the counts in the octanol by those in the water.

Biodistribution: Studies were carried out in immature female Sprague-Dawley rats, obtained from IFFA, CREDO, Spain, 19–20 days, injected into the tail vein under light isofluorane anaesthesia with 10 µCi of the radiolabelled steroid in saline (100 µl) containing 1% Tween 20. A separate group of rats was co-injected with unlabelled estradiol (5 µg) in order to block the estrogen receptors. Animals were maintained on normal diet *ad libitum* and were sacrificed by excess anaesthesia at 1, 2 and 24 h post injection with radiotracer and 2 h in the case of co-injected cold estradiol. Tissue samples of the main organs were removed, weighed and the activity measured in a γ-counter (Berthold LB2111, Germany). Results were expressed as per cent of injected

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dose per gram of organ (%ID/g organ) and presented as mean values $\pm$ SD. For blood and muscle, this value was calculated assuming that these organs constitute 6% and 40% of the total weight, respectively.

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