

Effect of Fluorination on the Pharmacological Profile of 11 β Isomers of Fulvestrant in Breast Carcinoma Cells

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We describe the synthesis of an 11 β isomer **3** of the steroidal antiestrogen fulvestrant **2**. Partial fluorination of the 11 β side chain in **3** leads to **4**, which still shows strong antiproliferative activity on MCF-7 cells. However, unlike **2** and **3**, compound **4** fails to down-regulate estrogen receptor α (ER α). This result suggests that ER α down-regulation is not a sine qua non condition for the antitumor activity of steroidal antiestrogens.

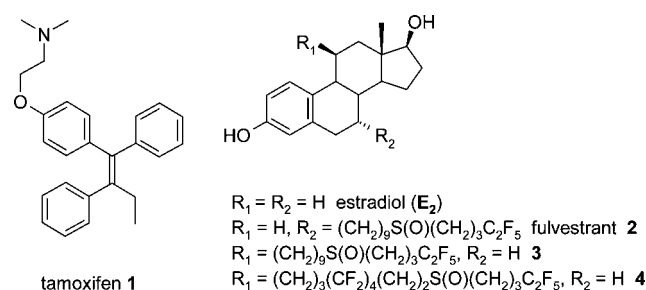
Introduction

The triphenylethylene-based antiestrogen tamoxifen (**1**) remains the gold standard for the treatment of estrogen receptor α (ER α)-positive breast cancer in premenopausal patients, in the adjuvant setting or as the first-line therapy of metastatic disease. Yet the untoward effects associated with the pharmacological profile of this drug (namely, its partial agonism) and the frequent occurrence of drug resistance have prompted the search for antiestrogens devoid of estrogenicity (i.e., “pure antiestrogens”). Several studies have shown that estradiol (E₂) derivatives bearing a functionalized side chain in position 7 α satisfy this criterion. Among these compounds, fulvestrant (ICI 182,780, Chart 1) (**2**) was selected for clinical trials because of its high in vivo antitumor activity, notably in animal models of tamoxifen-resistant breast cancer,¹ and is currently in clinical use.

Structural studies based on X-ray crystallography data of the ligand binding domain (LBD) of the estrogen receptor bound to a steroidal antagonist (ICI 164,384 complexed with the LBD of ER β) revealed that the terminal portion of its 7 α side chain penetrates into a cleft located near the ligand binding pocket, thereby preventing the helix-12 repositioning associated with estrogenic stimulation.² Thus, the conformational change imposed by fulvestrant impedes the binding of coactivators harboring a LxxLL consensus motif (L = leucine, x = any amino acid). Notably, this step is required for the cascade of molecular events that leads to estrogenic responses. Hence, the absence of such coregulator recruitment after a treatment with fulvestrant partly explains its strong antiestrogenicity. The complementary ability of fulvestrant to desensitize breast tumor cells to estrogenic stimulation by inducing a rapid proteasomal degradation of ER α ³ has been reported to be another factor of major importance.^{4,5}

When grafted onto E₂, 7 α /11 β alkyl side chains interact similarly with the surface of ER α protein, provided that they are not too large and/or too rigid to allow for the insertion and/or the rotation of the steroid nucleus within the ligand binding

Chart 1. Estrogens and Antiestrogens



pocket.^{6,7} The possibility that such a property also holds for the 7 α side chain of fulvestrant led us to synthesize an 11 β isomer **3** with the hope of producing a strong antiestrogen with a different endocrine/metabolic profile. Furthermore, our finding that perfluorination of a long 11 β alkyl side chain may modify ligand interactions with the binding pocket⁸ prompted us to synthesize a partially fluorinated derivative **4** of this 11 β fulvestrant isomer. The present paper, which compares the biological properties of these two compounds (**3** and **4**) with those of fulvestrant (**2**), provides new insight in the mechanism of action of steroidal antiestrogens.

Synthesis

Synthesis of the 11 β Isomer **3 of Fulvestrant.** Our synthesis of the 11 β isomer of fulvestrant **3**⁹ started with the conversion of known 11 β -allylestradiol dibenzyl ether **5**¹⁰ to aldehyde **6** by a classical hydroboration^{10b}–Swern oxidation sequence (Scheme 1). Elongation of the alkyl side chain was effected following Wittig's protocol in the presence of protected phosphonium salt **10**,¹¹ leading to olefin **7**. Removal of *tert*-butyldimethylsilyl group by fluoride anion followed by activation of the resulting alcohol function to a mesylate enabled the completion of the side chain by nucleophilic substitution with the sodium salt of 4,4,5,5,5-pentafluoropentylthiol.¹² After selective oxidation of the resulting thioether to sulfoxide **8**,¹³ simultaneous benzyl group deprotection and olefin reduction were planned by catalytic hydrogenation. The use of a “10 mol % Pd/C–catalytic Raney nickel” mixture resulted in partial C3-debenzylation and olefin hydrogenation to deliver advanced intermediate **9** in 71% yield. Alternatively, stoichiometric

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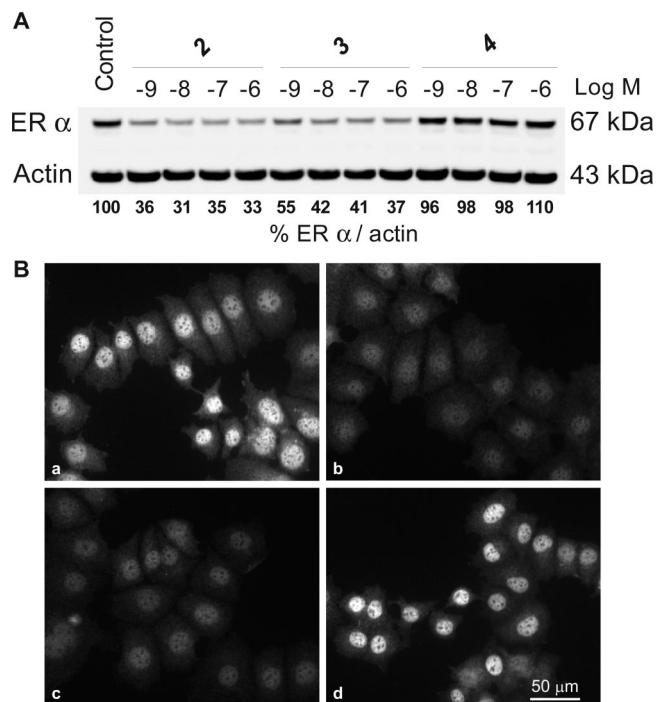


Figure 1. Effect of 2–4 on ER level. (A) Western blot analysis. MCF-7 cells were incubated with increasing amounts of each compound for 24 h. Control untreated cells were maintained in culture in parallel. Equal amounts of solubilized proteins were then subjected to Western blotting. Data refer to an experiment performed twice. (B) Immunofluorescence microscopy. The cells were treated for 24 h with 2, 3, or 4, all at 100 nM. ERα was demonstrated by immunofluorescence staining with HC-20 antiserum, as detailed in Experimental Section: (a) untreated cell culture (control); (b) 2; (c) 3; (d) 4; Texas Red labeling.

copy (Figure 1B) after 24 h of cell exposure to these compounds, was not observed with the perfluorinated derivative 4.

Interestingly, 4 was able to suppress ERα down-regulation induced by E₂ (Figure 2). It also antagonized the receptor elimination caused by fulvestrant and its 11β isomer (Figure S4, Supporting Information), indicating that the antiproliferative activity of the latter compounds was not strictly related to their capacity to influence the receptor turnover rate.

Discussion

Fulvestrant, as well as some other steroidal antiestrogens (i.e., ICI 164,384, RU 58668), is totally devoid of estrogenicity (“pure antiestrogen”). This has been attributed to the fact that these antagonists enhance ERα proteasomal degradation in estrogen responsive cells.^{3,17,18} Such considerations have given rise to the concept of selective estrogen receptor down-regulators (SERDs),¹⁹ defining a pharmacological group of estrogen antagonists distinct from selective estrogen receptor modulators (SERMs) (“partial antiestrogens”) which stabilize ERα.^{3,20} Yet our study reveals that fluorination of the alkyl side chain in an 11β isomer 3 of fulvestrant (i.e., 4), while not affecting antiestrogenicity, suppresses the capacity to down-regulate ERα in breast carcinoma cells.

The response of estrogen target cells to hormonal stimulation is largely controlled by ligand-induced modulation of ERα level.²¹ Previous work²² has unraveled molecular events underlying ERα degradation induced by E₂ or fulvestrant. Binding of these ligands promotes the association of ERα with Uba3/APP-BP1, the activating enzyme complex for NEDD8. This leads to the neddylation and activation of a cullin-based ubiquitin

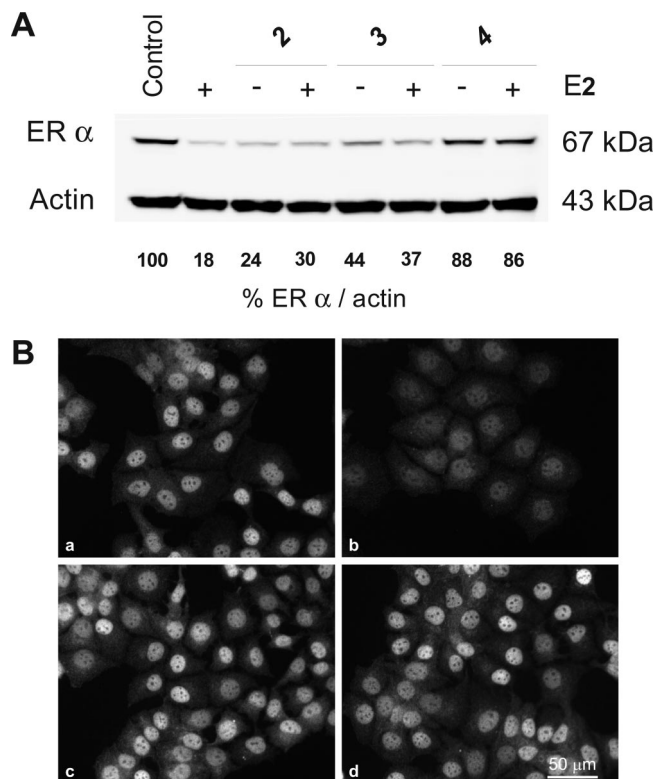


Figure 2. Ability of 2–4 to suppress E₂-induced down-regulation of ERα. (A) Western blot analysis. MCF-7 cells were incubated with each compound at 1 μM, alone or in the presence of 10 nM E₂ (a hormone concentration known to induce a demonstrable down-regulation of ERα) for 24 h. Equal amounts of solubilized proteins were then subjected to Western blotting. Data refer to an experiment performed twice. (B) Immunofluorescence microscopy. The cells were treated for 24 h with E₂ at 1 nM and/or 4 at 1 μM (a concentration selected to produce a clear-cut effect on E₂-induced down-regulation of ERα). ERα was demonstrated by immunofluorescence staining with HC-20 antiserum, as detailed in Experimental Section: (a) untreated cell culture (control); (b) E₂; (c) 4; (d) E₂ and 4 in combination; Texas Red labeling.

ligase (E3). E3, along with an ubiquitin-conjugating enzyme (E2), catalyzes the ubiquitination of ERα. Finally, ubiquitinated ERα is degraded in the proteasome compartment. According to a recent study,²³ two lysines (K302 and K303) located in the hinge region of ERα play a pivotal role in the above-described process, since they seem to be targets for polyubiquitination. In the context of the current study, it is reasonable to assume that the perfluorinated side chain of 4 somehow interferes with ubiquitin–proteasome-mediated breakdown of ERα by preventing E3 neddylation or ERα ubiquitination. Yet it is noted that this occurs without a loss of antiestrogenicity. As far as the antagonist profile of 4 is concerned, it still remains to be determined whether it is a pure antiestrogen despite the fact that it has lost the ability to provoke ERα down-regulation. Although the issue is beyond the scope of the present study, it is of importance, since the properties of 4 suggest that pure antiestrogenicity is not necessarily associated with ERα down-regulation.

As shown by the X-ray crystallographic analysis of the LBD of an estrogen receptor bound to a steroidal antiestrogen (ICI 164,384 complexed with the LBD of ERβ), the side chain of the ligand protrudes from the binding cavity, preventing helix 12 stabilization over the latter and the subsequent recruitment of LxxLL motif-containing coactivators.² The finding that the 11β isomer of fulvestrant (3) antagonizes coactivator binding with a similar efficiency is not surprising in view of the

equivalence of the 7 α and 11 β substitutions in terms of interactions with ER α .^{6,7} The side chain of these two isomers probably protrudes from the LBD in a similar way, resulting in interactions with a same region of the receptor surface. Partial fluorination of this side chain, which limits its flexibility, would logically decrease such an interaction. Could this property be related to the absence of effect of **4** on ER α turnover? Analysis of various 11 β perfluorinated estrogens and antiestrogens may be informative in this respect. Whatever the answer would be, our data suggest that an interaction with a given part of the receptor surface is not required to abrogate the recruitment of coactivators harboring an LxxLL motif. Repulsion of such coregulators from the receptor area seems to be a more likely explanation: the functionalized terminal part of the side chain may shield the receptor against interacting motifs of coactivators rather than obstruct their binding sites. In fact, this concept is in agreement with previous reports suggesting the existence of a "passive" antagonism in addition to an "active" antagonism based on a direct blockade of the recruitment site.²⁴ Studies devoted to the effect of flexible and nonflexible side chains at 11 β are warranted to test this hypothesis.

Experimental Section

General. Usual workup refers to a quench with saturated NH₄Cl solution, extraction with CH₂Cl₂, washing of the organic layers with water, drying over MgSO₄, and concentration under reduced pressure. Additional data may be found in the Supporting Information.

Synthesis of the 11 β Analogue of Fulvestrant. 3-(3,17 β -Bisbenzyloxyestra-1,3,5(10)-trien-11 β -yl)propan-1-ol **6.** To a solution of oxalyl chloride (296 mg, 2.33 mmol, 1.1 equiv) in dichloromethane (5 mL) was added dimethyl sulfoxide (363 mg, 4.66 mmol, 2.2 equiv) at -60 °C, followed after 5 min by a solution of 3-(3,17 β -bisbenzyloxyestra-1,3,5(10)-trien-11 β -yl)propan-1-ol^{10b} (1.08 g, 2.12 mmol) in dichloromethane (5 mL). After 15 min, triethylamine (1.07 g, 10.6 mmol, 5 equiv) was added dropwise. After the mixture was warmed to room temperature and the usual workup, column chromatography on silica gel (CH₂Cl₂) afforded aldehyde **6** (938 mg, 1.85 mmol, 87%) as a white foam.

[9-(3,17 β -Bisbenzyloxyestra-1,3,5(10)-trien-11 β -yl)non-6-enyloxy]-tert-butyldimethylsilane **7.** A solution of *n*-butyllithium (2.5 M in hexanes, 570 μ L, 1.43 mmol, 1.9 equiv) was slowly added to a suspension of the phosphonium salt **10** (817 mg, 1.5 mmol, 2 equiv) in THF (3 mL) at -78 °C. After the mixture was stirred for 15 min, a solution of aldehyde **6** (380 mg, 0.75 mmol) in THF (3 mL) was added. After 30 min of being stirred, the mixture was warmed to room temperature for an additional 2 h. After usual workup, the silylated product **7** (499 mg, 94% yield) was obtained by flash chromatography on silica gel (pentane/CH₂Cl₂, 3/2) as a glass.

3,17 β -Bisbenzyloxy-11 β -[9-(4,4,5,5,5-pentafluoropentane-1-sulfinyl)non-3-enyl]estra-1,3,5(10)-triene **8.** To a solution of steroid **7** (480 mg, 0.68 mmol) in dry THF (3 mL) was added solid tetrabutylammonium fluoride (530 mg, 2.04 mmol, 3 equiv) at 0 °C. The reaction was stopped after completion (TLC) by pouring it into water (2 mL) and CH₂Cl₂ (10 mL). After usual workup, column chromatography (CH₂Cl₂/MeOH, 99.5/0.5) afforded the free alcohol 9-(3,17 β -bisbenzyloxyestra-1,3,5(10)-trien-11 β -yl)non-6-en-1-ol (344 mg, 86%) as a glass. Triethylamine (86 mg, 0.86 mmol, 1.5 equiv) and mesyl chloride (78 mg, 0.68 mmol, 1.2 equiv) were added at 0 °C to a solution of the preceding alcohol (340 mg, 0.57 mmol) in CH₂Cl₂ (4 mL). After usual workup, the crude mixture was purified by column chromatography (CH₂Cl₂) to afford methanesulfonic acid 9-(3,17 β -bisbenzyloxyestra-1,3,5(10)-trien-11 β -yl)non-6-enyl ester (346 mg, 91%) as a white solid. To a solution of the preceding mesylate (55 mg, 0.082 mmol) in distilled THF (0.5 mL) was first added 4,4,5,5,5-pentafluoropentylthiol (32 mg, 0.164 mmol, 2 equiv) and then sodium hydride (80%, 4.5 mg,

0.147 mmol, 1.8 equiv). After usual workup, the crude mixture was purified by column chromatography on silica gel (pentane/CH₂Cl₂, 2/3) to yield 3,17 β -bisbenzyloxy-11 β -[9-(4,4,5,5,5-pentafluoropentylsulfanyl)non-3-enyl]estra-1,3,5(10)-triene (**61** mg, 97%) as a glass. Hydrogen peroxide (35% solution, 31 μ L, 0.32 mmol, 2 equiv) was slowly poured at 0 °C into a solution of the preceding thioether (120 mg, 0.16 mmol) in hexafluoroisopropanol (0.6 mL). After 5 min of stirring, sodium sulfite (25 mg, 0.2 mmol) was added and the mixture was stirred for 30 min. Usual workup afforded, without further purification, the desired sulfoxide **8** in quantitative yield (121 mg).

3-Hydroxy-11 β -[9-(4,4,5,5,5-pentafluoropentane-1-sulfinyl)non-3-enyl]-17 β -benzyloxyestra-1,3,5(10)-triene **9.** Palladium(II) hydride (100% mass) was added to a solution of sulfoxide **8** (65 mg, 0.083 mmol) in ethyl acetate (2 mL). After 24 h of being stirred under hydrogen, the crude mixture was filtered through a pad of Celite and purified by preparative TLC (CH₂Cl₂/MeOH, 95/5) to afford the monodebenzylated product **9** as a glass. Yield 68%.

3,17 β -Dihydroxy-11 β -[9-(4,4,5,5,5-pentafluoropentane-1-sulfinyl)nonyl]estra-1,3,5(10)-triene **3.** Steroid **9** (35 mg, 0.045 mmol) was dissolved in CH₂Cl₂ (1.25 mL), and the solution was cooled to -78 °C. A solution of BCl₃ in CH₂Cl₂ (0.5 M, 0.6 mL, 0.3 mmol, 6.6 equiv) was then slowly added. After 10 min, the reaction was quenched with methanol (1 mL). The final compound was obtained after usual workup by purification on preparative TLC (CH₂Cl₂/MeOH:90/10) as a white solid. Yield 56%.

Synthesis of the Fluorinated 11 β Analogue of Fulvestrant 4. Acetic Acid 3,3,4,4,5,5,6,6-Octafluoro-1,6-diidoheptyl Ester **11.** To a solution of 1,4-diiodoperfluorobutane (4.85 g, 10.7 mmol) in 1,2-dichloroethane (15 mL) was added freshly distilled vinyl acetate (0.4 mL, 4.28 mmol, 0.4 equiv) and 1 M triethylborane (0.2 mL, 0.2 mmol, 1.8 mol%). This addition was repeated two times after a 30 min delay. The solvent was removed under vacuum after 1 h, and the crude mixture was purified by silica gel chromatography (CH₂Cl₂/pentane, 40/60). Monoadduct **11** (2.82 g, 58%) was isolated as a colorless oil (highly unstable when exposed to light).

Acetic Acid 9-[3,17 β -Bisbenzyloxyestra-1,3,5(10)-trien-11 β -yl]-3,3,4,4,5,5,6,6-octafluorononyl Ester **14.** To a solution of 11 β -allylestrane **5** (680 mg, 1.38 mmol) in 1,2-dichloroethane (2.15 mL) were simultaneously added adduct **11** (4 $\times$ 185 mg = 745 mg, 1.38 mmol, 1 equiv) and triethylborane (1 M solution in hexane, $\sim$ 4 $\times$ 0.10 mL = 0.38 mL, 28% mol) every 30 min and in four equal portions. The mixture was stirred 5 min at room temperature, and triphenyltin hydride ($\sim$ 2 $\times$ 0.6 g = 1.21 g, 3.45 mmol, 2.5 equiv) and triethylborane (0.28 mL, 2 $\times$ 10% mol) were introduced in two equal portions. Reaction was allowed to proceed overnight. After usual workup, column chromatography on silica gel (pure pentane to CH₂Cl₂/pentane, 30/70) afforded addition product **14** (354 mg, 33%) as a glass. Acetic acid 7-[1,11 β -ethano-3,17 β -bisbenzyloxyestra-1,3,5(10)-trien-1'-yl]-3,3,4,4,5,5,6,6-octafluorononyl ester **13** was also isolated (15%).

3,17 β -Bisbenzyloxy-11 β -[4,4,5,5,6,6,7,7-octafluoro-9-(4,4,5,5,5-pentafluoropentane-1-sulfinyl)nonyl]estra-1,3,5(10)-triene **16.** The preparation of this compound closely follows that of its nonfluorinated analogue **8** (vide supra). LiAlH₄ (10 mg, 0.262 mmol, 1.5 equiv) was added to a solution of acetate **14** (135 mg, 0.173 mmol) in THF (4.5 mL). After completion of the reaction, water (10 μ L), a 15% NaOH solution (10 μ L), and finally water (30 μ L) were added. The precipitate was removed by filtration and the filtrate was evaporated under reduced pressure to obtain 9-(3,17 β -bisbenzyloxyestra-1,3,5(10)-trien-11 β -yl)-3,3,4,4,5,5,6,6-octafluoronon-1-ol (132 mg, 98%) as a glass. To a solution of the preceding alcohol (43 mg, 0.058 mmol) in CH₂Cl₂ (1 mL) were added triethylamine (17 mg, 0.168 mmol, 2.9 equiv) and methanesulfonyl chloride (16 mg, 0.140 mmol, 2.4 equiv). After usual workup, the crude mixture was purified by column chromatography (CH₂Cl₂) to afford methanesulfonic acid 9-[3,17 β -bisbenzyloxyestra-1,3,5(10)-trien-11 β -yl]-3,3,4,4,5,5,6,6-octafluorononyl ester (45 mg, 95%) as a glass. To a solution of the preceding mesylate (85 mg, 0.10 mmol) and 4,4,5,5,5-pentafluoropentylthiol (100 mg,

0.51 mg, 5 equiv) in THF was added NaH (80%, 12 mg, 0.4 mmol, 4 equiv) at 0 °C. The reaction was allowed to proceed overnight. After usual workup, column chromatography (CH₂Cl₂/pentane, 75:25) afforded 3,17 β -bisbenzyloxy-11 β -[4,4,5,5,6,6,7,7-octafluoro-9-(4,4,5,5,5-pentafluoropentylsulfanyl)nonyl]estra-1,3,5(10)-triene (88 mg, 92%) as a glass. Oxidation of this sulfide, as described above for the preparation of **8**, afforded the title compound yield 100%.

3,17 β -Dihydroxy-11 β -[4,4,5,5,6,6,7,7-octafluoro-9-(4,4,5,5,5-pentafluoropentane-1-sulfinyl)nonyl]estra-1,3,5(10)-triene 4. Brief treatment of dibenzylether **16** by boron trichloride in CH₂Cl₂ as described above for the preparation of **3** gave the title compound **4**. Yield 35%.

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Supporting Information Available: Biological procedures, biological properties of **2–4**, and spectral and analytical data for **3–16** and all intermediates. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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