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Original article

Synthesis and biological evaluation of salpichrolide analogs as antiestrogenic agents

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ABSTRACT

The antiestrogenic activity of three natural salpichrolides A, G and B (**1**, **3** and **4**) and of five synthetic analogs containing an aromatic D ring and a simplified side chain (**5**–**9**), was evaluated on MCF-7 cells. The 2,3-ene-1-keto steroids **8** and **9** were obtained from 3β-acetoxy-17(13→18)-abeo-5αH-pregna-13,15,17-trien-20-one, the key step for these syntheses being a Wharton carbonyl rearrangement of a 1,2-epoxy-3-keto steroid to the allylic alcohol using hydrazine hydrate. The antiestrogenic activity was evaluated by performing dose–response experiments in ER(+) MCF-7 breast cancer cells. Dose-dependent proliferation was quantified via [^{3}H]-thymidine incorporation after 3 days treatment. Salpichrolides A, G and B and analogs **5**, **8** and **9** were active as antiestrogens with compound **9** being the most active of the synthetic analogs. Compounds **5** and **9** were also evaluated against the ER(−) cell line MDA-MB-231 and shown to be inactive.

1. Introduction

Breast cancer is the most frequent cancer among women with an estimated 1.67 million new cases in 2012 (25.2% of all malignancies), being the most common cause of cancer related deaths in women (over 500,000 in 2012) in both developed and developing regions [1]. Over 70% of breast tumors express estrogen receptor alpha (ERα) and most of them respond to antiestrogen therapies, at least at the beginning of the treatment [2]. In those cases, the endocrine therapy aims to inhibit estrogen signaling resulting in inhibition of cell proliferation or induction of cell death [3]. For this purpose the available antiestrogen agents are either Selective Estrogen Receptor Modulators (SERMs) or Selective Estrogen Receptor Down-regulators (SERDs). The latter (fulvestrant is the best known) bind to ERα and induce its proteasomal degradation [3]. However, most of these ER-positive breast tumors become hormone resistant and patients relapse within 5 years [2], giving rise to a need for novel antiestrogens with new potential properties. The withanolides are C-28 steroidal lactones and lactols isolated from several genera of the Solanaceae family [4], that exhibit a variety of biological activities including potential anticancer activity on breast cancer cells [5–8]. An evaluation of a series of withanolides against a panel of human breast cancer cell lines containing or lacking the estrogen receptor (ERα(+) and ERα(−) respectively), showed that while most of the compounds assayed exhibited antiproliferative activity on all cell lines, two withanolides were selective against hormone dependent ERα(+) cell lines [9]. A distinctive characteristic of the latter compounds isolated from the plant *Salpichroa origanifolia*, was a modified steroid nucleus with a six membered aromatic D ring [10], with salpichrolide A (**1**) being the most active (Fig. 1). The closely related salpichrolide D (**2**) with a five membered D ring was equally active against ERα(+) and ERα(−) cell lines, suggesting that the selectivity might be associated to the presence of the aromatic D ring. The authors proposed that the mode of action of the selective withanolides could involve the inhibition of the ER-dependent pathway, required for proliferation of the hormone-dependent ERα(+) breast cancer cell lines.

Those results prompted us to evaluate the antiestrogenic activity of salpichrolide A (**1**) and two structurally related compounds also isolated from *S. origanifolia* (**3**, **4**), by assaying their capacity to block the response to estradiol in ERα(+) breast cancer cells. We also speculated that if the modified steroid nucleus with an aromatic D ring was relevant to the observed selectivity, synthetic analogs with a simple side chain as **5**–**9** (Fig. 2) might retain this

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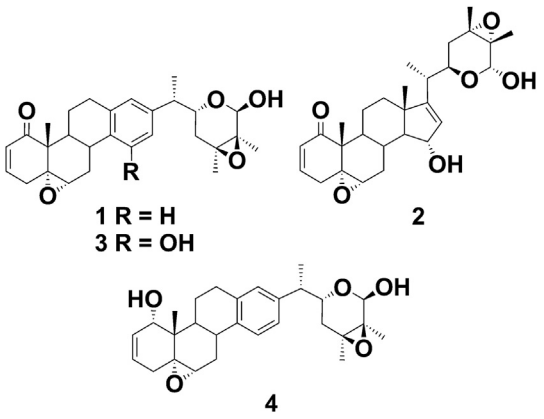


Fig. 1. Structures of the major natural salpichrolides.

property while being more amenable from a synthetic standpoint, to be used as leads for further development.

2. Results and discussion

2.1. Chemistry

The synthetic analogs were selected so as to contain typical substituents present in rings A and B of the salpichrolides and related withanolides, i.e. the 5,6-epoxide (compounds **6** and **7**) or an oxygenated function at position 1 (compounds **8** and **9**). Compound **5** contains a 3β-hydroxyl, a common substituent present in many natural steroids including several withanolides. In all cases the simplified side chain was kept invariant. The synthesis of compounds **5**–**7** has been reported previously by us [11]. Analogs **8** and **9** were obtained from compound **10** as depicted in Scheme 1. Initial attempts to carry out this sequence with the ketone at C-20 were unsuccessful, thus compound **10** was reduced with $NaBH_4$ and the resulting 20-hydroxysteroid was protected as the *tert* butyldimethylsilyl ether to give **11** as a mixture of epimers at C-20. The transformation of the A ring into the enone intermediate **15** was accomplished by deacetylation and oxidation to the 3-ketosteroid **13**, followed by dehydrogenation with 2-iodoxybenzoic acid (IBX) in DMSO at 90 °C to give the Δ^1 steroid **15**. As the 20-silyl ether is cleaved by IBX, it was necessary to change the *tert*-butyldimethylsilyl ether group at position 20 (compound **13**) for an acetate group (compound **14**) prior to the dehydrogenation step. The enone **15** was epoxidized with hydrogen peroxide 30% in MeOH–KOH to give the α,β-epoxy ketone **16**. The key step of the synthesis was a Wharton rearrangement of the epoxyketone **16** to give the allylic alcohol **17**, upon reaction with hydrazine hydrate in EtOH [12,13]. The ^1H NMR spectrum of compound **17** exhibited two olefinic protons at δ 5.87 (H-2 and H-3), and a doublet at δ 3.84 ($J = 4.5$ Hz) corresponding to H-1. These data were consistent with the presence of a 1α-hydroxy group in ring A [13,14]. Compound **17** was treated with 5% KOH in MeOH to give the 20-hydroxy intermediate **18**, regioselective oxidation with MnO_2–Na_2CO_3 gave compound **8** (9% yield from **10**) while oxidation with PCC gave compound **9** (10% yield from **10**).

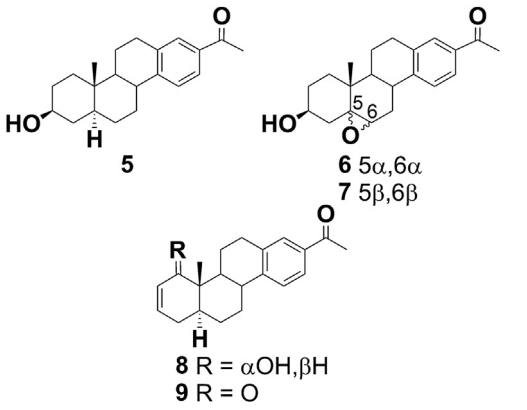


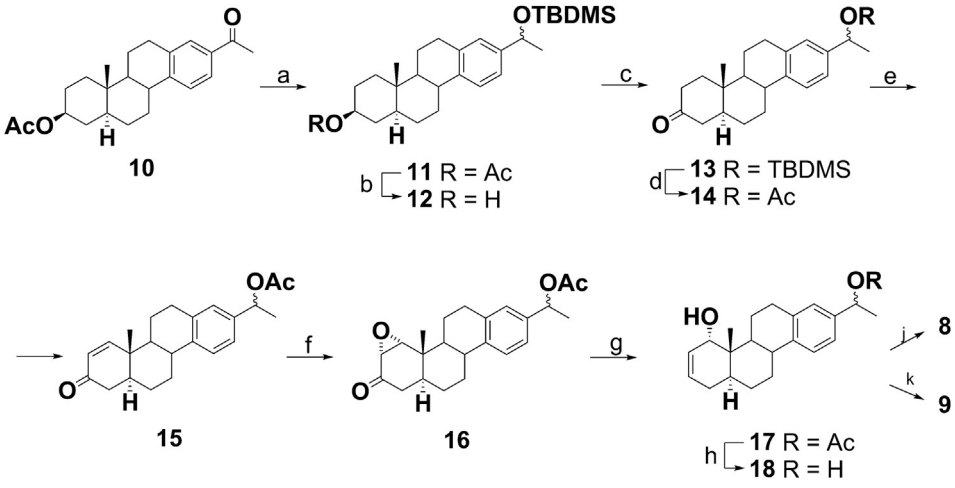
Fig. 2. Structures of the synthetic analogs.

2.2. Antiestrogenic activity

The antiestrogenic activity was assessed by incubating human breast cancer MCF-7 cells in the simultaneous presence of a stimulatory (but not saturating) concentration of estradiol and increasing concentrations of the compound to be tested. Besides salpichrolide A (**1**), two other natural salpichrolides with an aromatic D ring were selected for evaluation, salpichrolide G (**3**) with a phenolic group at position 15 that exhibited cytotoxicity to both ER(+) and ER(−) cell lines [9] and salpichrolide B (**4**) that is a minor component of *S. origanifolia* but can be easily obtained from **1** [14]. As shown in Fig. 3, all three natural salpichrolides significantly reversed the proliferative action of estradiol with an IC_{50} of 1.0×10^{-7} M, 1.4×10^{-7} M and 1.8×10^{-8} M for salpichrolides A (**1**) (panel a), G (**3**) (panel b) and B (**4**) (panel c) respectively. The effect of these compounds was compared to the classical antiestrogen and SERD fulvestrant (ICI 182,780). Fulvestrant at a 10 nM concentration significantly reversed the estrogen effect (IC_{50} 1.9×10^{-9} M) and inhibited cell proliferation below control values (Fig. 3 panel d). The latter effect may be due to the ability of fulvestrant of inducing apoptosis in MCF-7 cells [3]. None of the tested compounds was able to inhibit cell proliferation below control values, suggesting that their mechanism of action is different from the SERD fulvestrant.

The results obtained with the synthetic analogs are shown in Fig. 4. Compound **9** (IC_{50} 5.0×10^{-8} M), with an A ring that maintains the functionality of salpichrolide A (**1**), was the most effective of the synthetic compounds, with a significant antiestrogenic effect at a concentration of 100 nM (Fig. 4c). Compound **8**, that has the same functionality in ring A as compound **4**, showed antiestrogenic activity only at a concentration of 1 μM (Fig. 4b). Compound **6**, containing the 5α,6α-epoxide functionality present in most of the natural salpichrolides, lacks antiestrogenic activity (data not shown), while compound **7** with a 5β,6β-epoxide showed only incipient antiestrogenic activity at a concentration of 1 μM (data not shown). It is noteworthy that compound **5**, a very simple analog, that has neither the side chain nor any of the functionalities present in rings A and B of the salpichrolides, completely reversed estradiol effect at a concentration of 100 nM, with an IC_{50} of 3.3×10^{-7} M (Fig. 4a) while analogs of **5** with a normal steroid framework as pregnenolone, do not significantly bind to ERα [15]. This suggests that the aromatic D ring is a key structural motif for the observed antiestrogenic activity. However, the lack of activity of compounds **6** and **7** indicates that as expected, other structural factors are implied in the interaction with the ER.

In order to better assess if the synthetic analogs exerted their effect through the ER and thus maintained the selectivity for ER(+) cells, the breast cancer ERα(−) MDA-MB-231 cell line was incubated in the same conditions with the most active compounds **5** and **9**. As shown in Fig. 5, neither compound **5** nor compound **9** exerted any action on these cells, supporting the implication of the ERα in their action. This result also suggests that the action of these compounds on MCF-7 cells is not likely to be a toxic one.



Scheme 1. Reagents and conditions: a) i. $NaBH_4$, MeOH, CH_2Cl_2, 0 °C, 40 min; ii. TBDMSCl, DMF, imidazole, 50 °C, 2 h; b) 5% KOH (aq), MeOH-THF, 25 °C, 1 h; c) PCC, CH_2Cl_2, $BaCO_3$, MS (3 Å), 25 °C, 30 min; d) i. Bu_4NF, THF, 25 °C, 6 h; ii. Ac_2O, pyridine, DMAP, 25 °C, 1 h; e) IBX (3 eq), DMSO, 90 °C, 2 h; f) 30% H_2O_2, MeOH–CH_2Cl_2, 10% KOH/MeOH, 25 °C, 50 min; g) i. $NH_2NH_2.H_2O$, EtOH, H_2O, reflux, 2 h; ii. 10% HCl, 25 °C, 10 min; h) 5% KOH (aq), MeOH-THF, 25 °C, 1 h; j) MnO_2, Na_2CO_3, CH_2Cl_2, 25 °C, 24 h; k) PCC, CH_2Cl_2, $BaCO_3$, MS(3 Å), 25 °C, 1 h.

2.3. Molecular modeling

A comparison of the structures of compound **9** and estradiol raises the question of whether compound **9** would bind to the ER in the "normal" way or in an inverted orientation with the D ring occupying the position of the aromatic A ring of estradiol in the ligand binding pocket (LBP). In a preliminary approach to answer this question we used a combination of docking and molecular dynamics to study the ligand binding mode of **9**. Starting from the crystal structure of the ERα/estradiol complex (pdb:1qku) [16] and

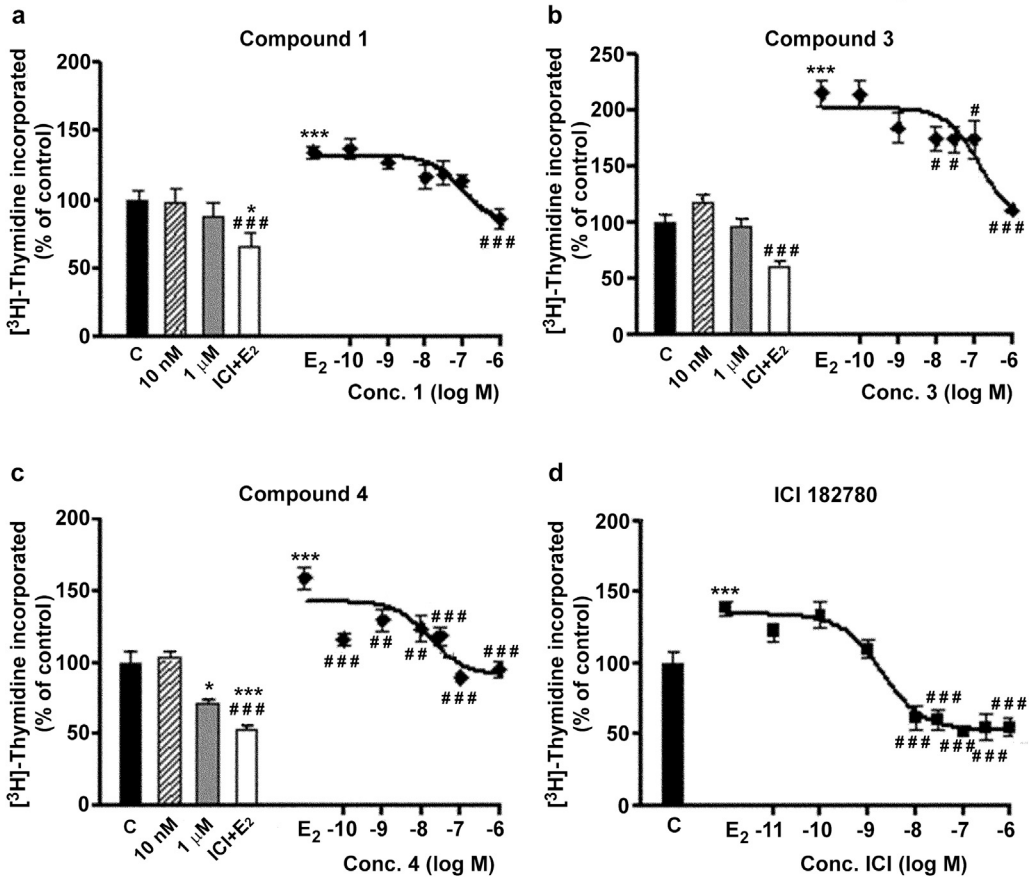


Fig. 3. Antiestrogenic action of the natural salpichrolides **1**, **3** and **4** and of fulvestrant (ICI 182,780). MCF-7 cells was incubated, as described in the Experimental section, in the presence of 0.1 nM 17β-estradiol (E_2) and increasing concentrations of the natural compounds (panels a–c) or ICI 182,780 (panel d). Controls: C: cells incubated only with vehicle (DMSO) in the absence of any treatment; 10 nM and 1 μM: cells treated only with these concentrations of the tested compound; ICI + E_2: cells treated with ICI 182,780 at a concentration of 1 μM in the presence of 0.1 nM E_2. *$p < 0.05$, ***$p < 0.001$ with respect to the control in the absence of any treatment; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ with respect to the cells incubated in the presence of estradiol alone, as analyzed by ANOVA followed by Dunnett's Multiple Comparison Test.

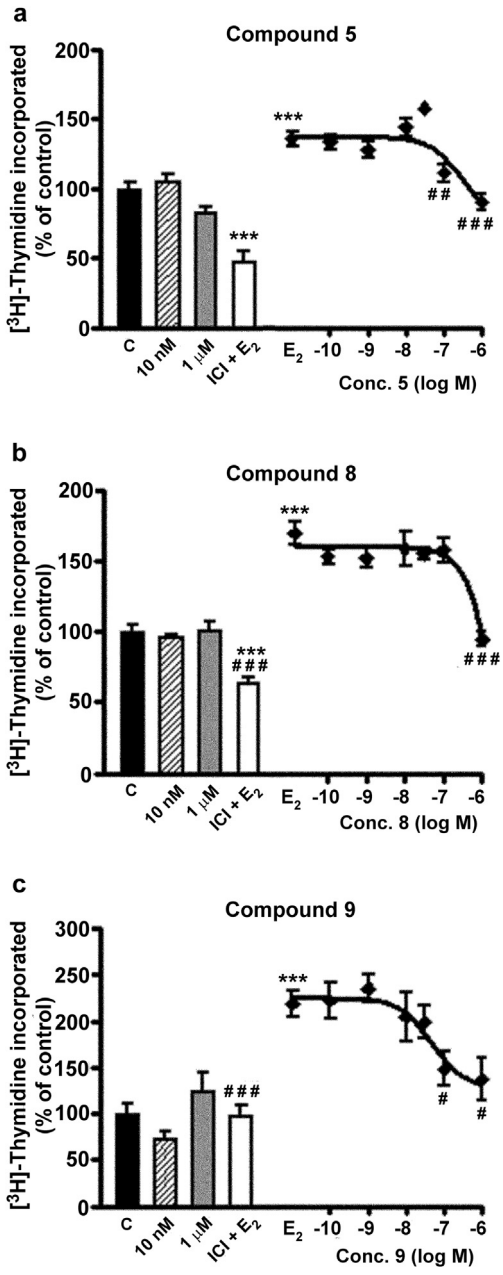


Fig. 4. Antiestrogenic action of the synthetic analogs **5**, **8** and **9**. MCF-7 cells were incubated, as described in the Experimental section, in the presence of 0.1 nM 17β-estradiol (E_2) and increasing concentrations of compounds **5**, **8** or **9** (panels a–c). Controls: C: cells incubated only with vehicle (DMSO) in the absence of any treatment; 10 nM and 1 μM: cells treated only with these concentrations of the tested compound; ICI + E_2: cells treated with ICI 182,780 at a concentration of 1 μM in the presence of 0.1 nM E_2. $^{***}p < 0.001$ with respect to the control in the absence of any treatment; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ with respect to the cells incubated in the presence of estradiol alone, as analyzed by ANOVA followed by Dunnett's Multiple Comparison Test.

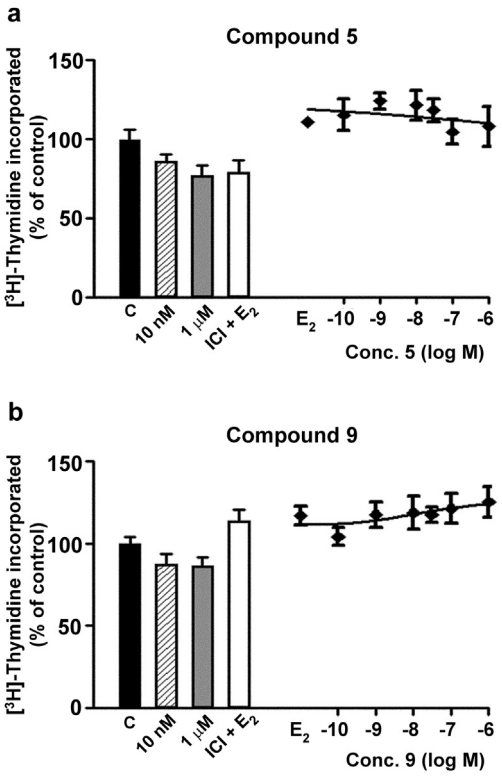


Fig. 5. Action of the synthetic analogs **5** and **9** on the ERα(−) MDA-MB-231 cell line. Cells were incubated, as described in the Experimental section, in the presence of 0.1 nM 17β-estradiol (E_2) and increasing concentrations of compounds **5** or **9** (panels a and b). Controls: C: cells incubated only with vehicle (DMSO) in the absence of any treatment; 10 nM and 1 μM: cells treated only with these concentrations of the tested compound; ICI + E_2: cells treated with ICI 182,780 at a concentration of 1 μM in the presence of 0.1 nM E_2. No significant differences were found as analyzed by ANOVA followed by Dunnett's Multiple Comparison Test.

the HF/6-31G** optimized geometry of **9**, we docked the steroid molecule through 400 runs of genetic algorithm with the Autodock 4.2 program [17]. Using an RMSD cluster tolerance of 1.5 Å, we observed that **9** can acquire three different poses inside the ERα LBP (Fig. 6). Pose A was by far the most frequent (frequency 85%) and corresponded to the inverse oriented binding mode, with the aromatic ring D pointing towards Arg394 and Glu353 residues and ring A positioned close to His524 (Fig. 6a). Pose B (frequency 7.5%) exhibited a double inversion, where in addition to the change in orientation observed in pose A the steroid nucleus was rotated 180° (Fig. 6b). The third orientation (pose C, frequency 6.5%) corresponded to the normal binding mode of the steroid, i.e. the steroid nucleus oriented as that of estradiol in the ERα crystal structure (Fig. 6c). These results point to pose A as the most frequent and energetically favorable orientation for binding of **9** to the ER.

To evaluate the stability of the inverted ligand orientation within the receptor and allow for conformational changes to take place both on the protein and the ligand, we took the structure of the ERα-**9** complex shown in Fig. 6a as a starting point and performed a 20 ns molecular dynamics (MD) simulation with the AMBER 12 software package [18]. The simulation exhibited small and constant RMSD values, with the steroid remaining almost unchanged in its original position (Fig. 7a). As shown in Fig. 7b, the C-20 carbonyl interacted with the Arg394/Glu353 pair, establishing a polar network similar to that observed in the crystal structure of the ERα-estradiol complex (Fig. 7c) [16]. At the other end of the LBP and at variance with the ERα–estradiol complex, the ligand molecule did not show any interactions with His524. The MD simulation results thus shows that the inverse orientation of compound **9** in the ER LBP, may attain a stable binding mode; the lack of interaction with His524 could be responsible for impeding the ER to acquire its active conformation.

3. Conclusions

The selective antiproliferative activity exhibited by two natural salpichrolides with an aromatic D ring, prompted us to evaluate

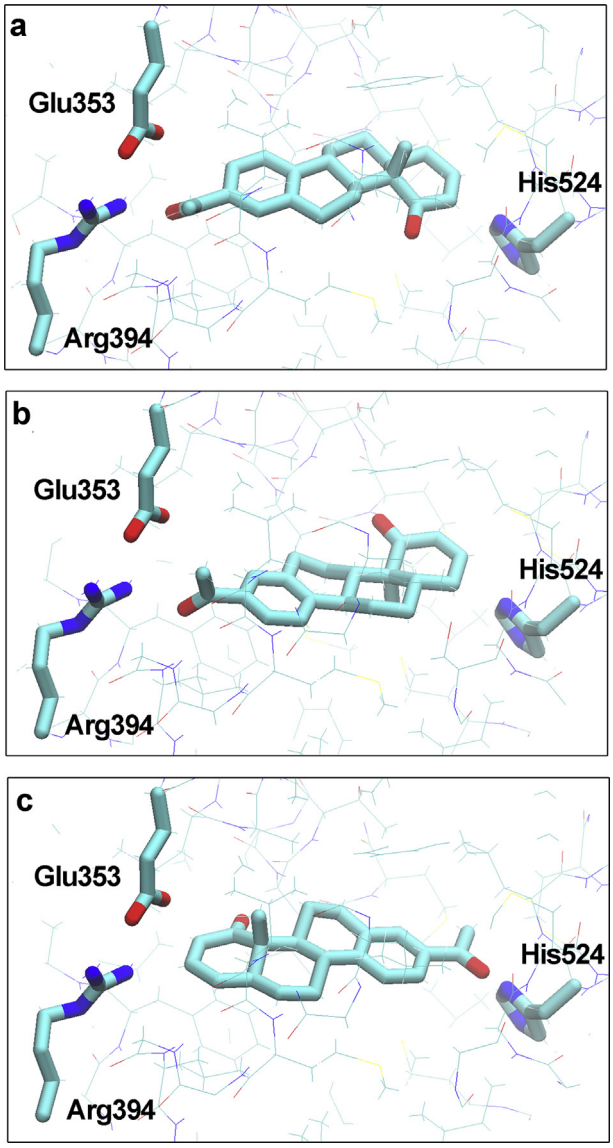


Fig. 6. Docking of compound **9** into the ERα ligand binding pocket (Autodock 4.2). a) Pose A had frequency = 85%, average energy = −5.6 kcal/mol. b) Pose B had frequency = 7.5%, average energy = −5.4 kcal/mol. c) Pose C had frequency = 6.5% and average energy = −5.2 kcal/mol.

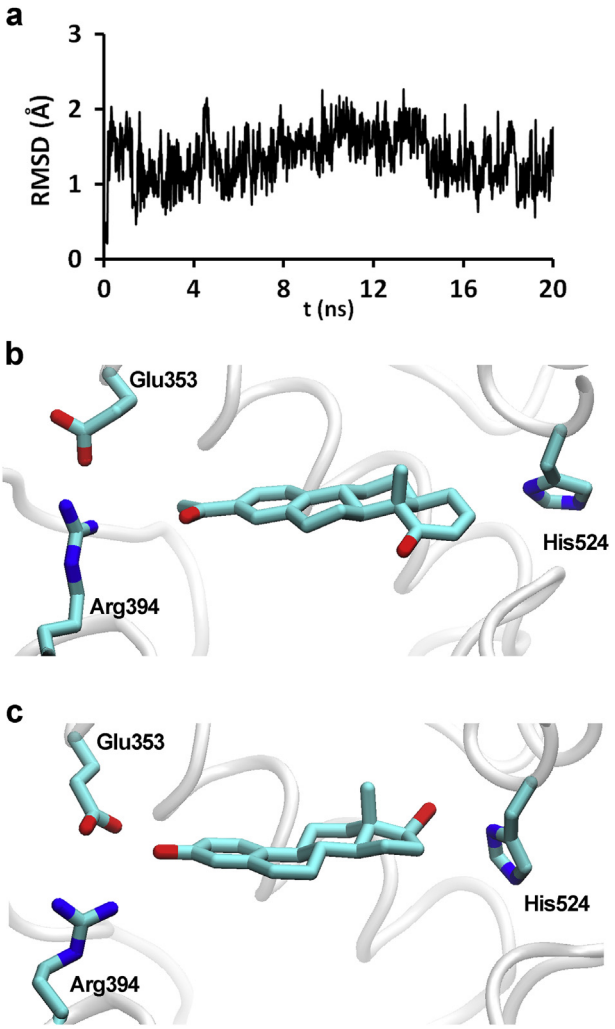


Fig. 7. Molecular dynamics simulation (Amber 12) of ERα-**9** complex corresponding to the most frequent orientation of the ligand in the docking experiment. a) Root-mean squared deviation (RMSD) from the initial structure, measured over the heavy atoms of the steroid. b) Structure of the ERα-**9** complex after 20 ns showing the molecule of compound **9** and the amino acid side chains involved in ligand binding. c) Crystal structure of the ERα-estradiol complex (pdb:1qku) [16] showing the estradiol molecule and the amino acid side chains involved in ligand binding.

the antiestrogenic activity of salpichrolides **1**, **3** and **4** and then that of more simple structures based on this modified steroid nucleus. This led to compound **9** with a simple acetyl residue instead of the complex side chain present in the salpichrolides, that exhibited an antiestrogenic activity similar to that of salpichrolide A (**1**) while maintaining the lack of antiproliferative activity on ERα(−) cells. Most important, our results showed that the lactol side chain of the salpichrolides is not a requirement for the observed activity and that analogs with a simple side chain as **9**, readily available from common steroidal precursors, may be just as efficacious retaining both the antiestrogenic activity and the selectivity for ER(+) cells. Although further studies will be needed to fully assess the potential properties of these compounds, the above findings show that their modified steroid nucleus is a convenient scaffold for the development of new antiestrogens. In addition, these molecules may prove useful as tools for gaining further insight on estrogen receptor-mediated signaling networks.

4. Experimental

4.1. General

Mps were taken on a Fisher-Johns apparatus and are uncorrected. IR spectra were recorded in thin films using KBr disks on a Nicolet Magna 550 FT-IR spectrophotometer, values are given in cm^{-1}. NMR spectra were recorded on Bruker AC-200 (^{1}H at 200.13 MHz, ^{13}C at 50.32 MHz) or Avance II 500 (^{1}H at 500.13 MHz, ^{13}C at 125.77 MHz) spectrometers. Chemical shifts are given in ppm downfield from TMS as internal standard, *J* values are given in Hz. Multiplicity determinations and 2D spectra (COSY, NOESY, HSQC and HMBC) were obtained using standard Bruker software. Exact mass spectra were obtained using a Bruker micrOTOF-Q II mass spectrometer, equipped with an ESI source operating in positive mode. Flash column chromatography was carried out on silica gel S 0.040–0.063 mm. Thin layer chromatography (tlc) analysis was performed on silica gel 60 F254 (0.2 mm thick). The homogeneity of all compounds was confirmed by tlc. Solvents were evaporated at reduced pressure and *ca.* 40–50 °C. Compounds **1** and **3** were isolated from fresh leaves of *S. origanifolia* [19]. Compound **4** was

obtained by reduction of **1** [20]. Compounds **5**–**7** and **10** were obtained from pregnenolone acetate following the procedures described previously by us [11].

4.2. Chemical synthesis

4.2.1. (20R,S)-3β-Acetoxy-20-(t-butyldimethylsilyloxy)-17(13→18)-abeo-5αH-pregna-13,15,17-triene (**11**)

Sodium borohydride (0.286 g, 7.56 mmol) was added to a solution of compound **10** (1.34 g, 3.78 mmol) in dichloromethane (33 mL) and methanol (33 mL) at 0 °C. The reaction mixture was stirred for 40 min at room temperature, acidified (pH 6) with 1 M HCl and concentrated to a third of its volume. Water was added to the residue and then extracted with dichloromethane. The organic layer was washed with water, dried with sodium sulfate and the solvent evaporated under vacuum to give the 20-hydroxy intermediate as a mixture of epimers at C-20.

Imidazole (1.48 g, 21.7 mmol) and *t*-butyldimethylsilyl chloride (2.20 g, 14.6 mmol) were added successively to a solution of the residue obtained above in anhydrous DMF (20 mL) and the solution was stirred for 2 h at 50 °C under a nitrogen atmosphere. The reaction mixture was allowed to cool to room temperature and then extracted with diethyl ether. The organic layer was washed successively with brine and water and dried with sodium sulfate. Evaporation of the solvent followed by flash chromatography (hexane–ethyl acetate 100:0→90:10) gave the 20-silyl ether **11** as a mixture of epimers at C-20 (1:1 determined by NMR) (1.67 g, 94% from **10**); ^{1}H NMR (500.13 MHz, $CDCl_3$) δ_H: 7.24 (0.5H, d, J = 8.1 Hz, H-15 epimer 1); 7.23 (0.5H, d, J = 8.1 Hz, H-15 epimer 2); 7.11 (0.5H, dd, J = 8.2 and 1.6 Hz, H-16 epimer 1); 7.09 (0.5H, dd, J = 8.2 and 1.6 Hz, H-16 epimer 2); 7.03 (0.5H, br s, H-18 epimer 1); 7.00 (0.5H, br s, H-18 epimer 2); 4.82 (1H, q, J = 6.3 Hz, H-20); 4.74 (1H, m, H-3); 2.82 (2H, m, H-12); 2.70 (1H, td, J = 11.7 and 8.6 Hz, H-8); 2.48 (1H, m, H-7α); 2.05 (3H, s, 3-acetate); 1.95 (1H, m, H-11α); 1.91 (1H, m, H-1β); 1.88 (1H, m, H-2α); 1.69 (1H, m, H-4α); 1.58 (1H, m, H-2β); 1.48 (2H, m, H-6); 1.41 (2H, m, H-4β and H-11β); 1.40 (3H, d, J = 6.3 Hz, H-21); 1.28 (1H, m, H-7β); 1.27 (1H, m, H-5); 1.11 (2H, m, H-9 and H-1α); 0.91 (9H, s, $(CH_3)_3$C–Si); 0.87 (1H, s, H-19); 0.06 (6H, s, $(CH_3)_2$–Si); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ_C: 170.7 (3-acetate); 143.9 (C-17); 139.3 (C-14); 136.34 and 136.29 (C-13); 125.72 and 125.70 (C-15); 125.41 and 125.36 (C-18); 122.60 and 122.51 (C-16); 73.6 (C-3); 70.49 and 70.42 (C-20); 51.0 (C-9); 44.0 (C-5); 37.6 (C-8); 36.4 (C-1); 35.8 (C-10); 33.8 (C-4); 31.95 and 31.91 (C-7); 30.92 and 30.89 (C-12); 28.8 (C-6); 27.3 (C-2); 27.2 (C-21); 25.9 (($\mathbf{C}H_3)_3$C–Si); 22.8 (C-11); 21.5 (3-acetate); 18.3 ($(CH_3)_3\mathbf{C}$–Si); 11.6 (C-19); −4.75 and −4.78 (($CH_3)_2$–Si).

4.2.2. (20R,S)-20-(t-Butyldimethylsilyloxy)-17(13→18)-abeo-5αH-pregna-13,15,17-trien-3β-ol (**12**)

To a solution of compound **11** (1.67 g, 3.55 mmol) in tetrahydrofuran (87 mL) and methanol (87 mL) was added a 5% aqueous solution of KOH (20.2 mL). After stirring for 1 h at room temperature, the reaction mixture was neutralized with 1 M HCl and concentrated to a third of its volume. Water was added to the residue and then extracted with dichloromethane. The organic layer was dried with sodium sulfate and the solvent evaporated under vacuum. The resulting solid was purified by flash chromatography (hexane–ethyl acetate 70:30) to give **12** as a mixture of epimers at C-20 (1.48 g, 97%); IR (KBr): 3350.3; 2927.9; 2856.9; 1471.3; 1254.8; 1094.3; 1044.8; 834.3; 775.9 cm^{-1}; ^{1}H NMR (500.13 MHz, $CDCl_3$) δ_H: 7.25 (0.5H, d, J = 8.0 Hz, H-15 epimer 1); 7.24 (0.5H, d, J = 8.1 Hz, H-15 epimer 2); 7.11 (0.5H, dd, J = 8.1 and 1.6 Hz, H-16 epimer 1); 7.09 (0.5H, dd, J = 8.1 and 1.6 Hz, H-16 epimer 2); 7.03 (0.5H, d, J = 1.1 Hz, H-18 epimer 1); 7.00 (0.5H, d, J = 1.1 Hz, H-18 epimer 2); 4.82 (1H, q, J = 6.4 Hz, H-20); 3.65 (1H, m, H-3); 2.82 (2H, m, H-12); 2.71 (1H, td, J = 11.3 and 4.0 Hz, H-8); 2.48 (1H, dq, J = 12.8 and 3.7 Hz, H-7β); 1.96 (1H, m, H-11α); 1.89 (1H, m, H-1β); 1.88 (1H, m, H-2α); 1.65 (1H, m, H-4α); 1.49 (2H, m, H-2β); 1.48 (2H, m, H-6); 1.42 (1H, m, H-11β); 1.40 (3H, d, J = 6.2 Hz, H-21); 1.35 (1H, m, H-4β); 1.28 (1H, m, H-7α); 1.21 (1H, m, H-5); 1.10 (1H, td, J = 11.4 and 2.5 Hz, H-9); 1.08 (1H, m, H-1α); 0.91 (9H, s, $(CH_3)_3$C–Si); 0.86 (1H, s, H-19); 0.06 (6H, s, $(CH_3)_2$–Si); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ_C: 143.9 (C-17); 139.4 (C-14); 136.40 and 136.35 (C-13); 125.75 and 125.73 (C-15); 125.41 and 125.35 (C-18); 122.59 and 122.51 (C-16); 71.2 (C-3); 70.50 and 70.43 (C-20); 51.1 (C-9); 44.2 (C-5); 37.9 (C-2); 37.6 (C-8); 36.7 (C-1); 35.8 (C-10); 32.06 and 32.02 (C-7); 31.4 (C-4); 30.98 and 30.95 (C-12); 29.0 (C-6); 27.2 (C-21); 25.9 (($\mathbf{C}H_3)_3$C–Si); 22.8 (C-11); 18.3 ($(CH_3)_3\mathbf{C}$–Si); 11.7 (C-19); −4.75 and −4.78 (($CH_3)_2$–Si); HRMS-ESI: calculated for $C_{27}H_{44}NaO_2Si$: 451.3003, found 451.2987.

4.2.3. (20R,S)-20-Acetoxy-17(13→18)-abeo-5αH-pregna-13,15,17-trien-3-one (**14**)

A suspension of pyridinium chlorochromate (2.90 g, 13.4 mmol), barium carbonate (1.78 g, 9.02 mmol) and 3 Å molecular sieves (2.37 g) in anhydrous dichloromethane (40 mL) was stirred for 5 min under a nitrogen atmosphere. A solution of **12** (1.45 g, 3.38 mmol) in anhydrous dichloromethane (25 mL) was added and stirring continued at room temperature for 45 min. The reaction mixture was diluted with ether and percolated through silica gel with ether–dichloromethane (1:1). Evaporation of the solvent followed by flash chromatography (hexane–ethyl acetate 95:5) gave the 3-ketosteroid **13** as a mixture of epimers at C-20 (1.21 g, 80%); IR (KBr): 2925.7; 2855.4; 2360.2; 2341.7; 1706.8; 1254.8; 1097.7; 774.7; 672.0 cm^{-1}; ^{1}H NMR (200.13 MHz, $CDCl_3$) δ_H: 7.25 (1H, m, H-15); 7.10 (1H, m, H-16); 7.04 (1H, m, H-18); 4.83 (1H, m, H-20); 1.39 (3H, d, J = 6.0 Hz, H-21); 1.06 (3H, s, H-19); 0.91 (9H, s, $(CH_3)_3$C–Si); 0.06 (6H, s, $(CH_3)_2$–Si); HRMS-ESI: calculated for $C_{27}H_{42}NaO_2Si$: 449.2846, found 449.2851.

Compound **13** (1.10 g, 2.58 mmol) was added to a solution of Bu_4NF in THF 1 M (5.17 mL, 5.17 mmol) and the solution was stirred for 6 h at 25 °C. The solvent was evaporated under vacuum and the resulting solid was purified by flash chromatography (hexane–ethyl acetate 60:40). The residue (740 mg) was treated with acetic anhydride (5.20 mL), pyridine (2.20 mL) and DMAP (0.1 mg) for 1 h at 25 °C. The solvent was evaporated under vacuum and the resulting solid was purified by flash chromatography (hexane–ethyl acetate 80:20) to give **14** as a mixture of epimers at C-20 (798 mg, 88% from **13**); IR (KBr): 2935.6; 2860.2; 1734.5; 1715.4; 1241.0; 669.2 cm^{-1}; ^{1}H NMR (500.13 MHz, $CDCl_3$) δ_H: 7.29 (1H, d, J = 8.2 Hz, H-15); 7.14 (1H, dd, J = 8.1 and 1.6 Hz, H-16); 7.06 (1H, bs, H-18); 5.83 (1H, q, J = 6.6 Hz, H-20); 2.85 (2H, m, H-12); 2.77 (1H, td, J = 11.8 and 3.8 Hz, H-8); 2.50 (1H, dq, J = 13.0 and 3.4 Hz, H-7β); 2.45 (1H, dd, J = 14.4 and 2.3 Hz, H-2α); 2.36 (1H, m, H-2β); 2.32 (1H, t, J = 14.0 Hz, H-4α); 2.17 (1H, m, H-1α); 2.16 (2H, m, H-4β); 2.06 (3H, s, 20-acetate); 1.99 (1H, m, H-11α); 1.62 (1H, m, H-5); 1.54 (2H, m, H-6); 1.52 (3H, d, J = 6.6 Hz, H-21); 1.48 (1H, m, H-11β); 1.44 (1H, m, H-1β); 1.30 (1H, m, H-7α); 1.17 (1H, td, J = 11.8 and 3.8 Hz, H-9); 1.05 (1H, s, H-19); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ_C: 211.63 (C-3); 170.37 (20-acetate); 140.33 (C-17); 138.80 and138.78 (C-14); 136.84 and 136.83 (C-13); 126.6 and 126.5 (C-18), 126.36 and 126.34 (C-15); 123.66 and 123.55 (C-16); 72.16 and 72.10 (C-20); 50.63 and 50.61 (C-9); 45.84 (C-5); 44.40 (C-4); 38.08 (C-1); 37.92 (C-2); 37.62 (C-8); 35.96 (C-10); 31.71 (C-7); 30.79 and 30.77 (C-12); 29.12 (C-6); 22.92 (C-11); 22.03 and 21.99 (C-21); 21.39 (20-acetate); 10.87 (C-19); HRMS-ESI: calculated for $C_{23}H_{30}NaO_3$: 377.2087, found. 377.2081.

4.2.4. (20R,S)-20-Acetoxy-17(13→18)-abeo-5αH-pregna-1,13,15,17-tetraen-3-one (**15**)

A solution of **14** (790 mg, 2.23 mmol) in DMSO (14.1 mL) was treated with IBX (1.87 g, 6.68 mmol) at 90 °C, and stirring was

continued for 2 h. The reaction mixture was diluted with diethyl ether and washed successively with 5% aqueous sodium bicarbonate and brine. The organic layer was dried with sodium sulfate and the solvent evaporated under vacuum. The resulting solid was purified by flash chromatography (hexane–ethyl acetate 90:10→80:20) to give compound **15** as a mixture of epimers at C-20 (393 mg, 50%); IR (KBr): 2932.9; 2864.2; 1733.3; 1678.3; 1443.9; 1371.0; 1243,1; 1065.7; 946.2 cm^{-1}; ^1H NMR (500.13 MHz, $CDCl_3$) δ_H: 7.29 (1H, d, $J = 8.2$ Hz, H-15); 7.23 (1H, d, $J = 10.2$ Hz, H-2); 7.15 (1H, dd, $J = 8.2$ and 1.7 Hz, H-16); 7.07 (1H, d, $J = 1.7$ Hz, H-18); 5.92 (1H, dd, $J = 10.2$ and 0.9 Hz, H-1); 5.83 (1H, q, $J = 6.6$ Hz, H-20); 2.89 (2H, m, H-12); 2.80 (1H, td, $J = 11.7$ and 4.0 Hz, H-8); 2.54 (1H, dq, $J = 13.0$ and 3.7 Hz, H-7β); 2.42 (1H, dd, $J = 17.6$ and 14.0 Hz, H-4β); 2.30 (1H, ddd, $J = 17.6$, 4.1 and 0.9 Hz, H-4α); 2.17 (1H, m, H-11α); 2.06 (3H, s, 20-acetate); 2.02 (1H, m, H-5); 1.63 (2H, m, H-6); 1.55 (1H, m, H-11β); 1.52 (3H, d, $J = 6.6$ Hz, H-21); 1.39 (1H, td, $J = 11.3$ and 2.2 Hz, H-9); 1.34 (1H, m, H-7α); 1.05 (1H, s, H-19); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ_C: 199.85 (C-3); 170.3 (20-acetate); 157.8 (C-2); 139.97 and 139.01 (C-14); 139.0(C-17); 136.57 and 136.56 (C-13); 127.8 (C-1); 126.57 and 126.52 (C-18); 126.07 and 126.06 (C-15); 123.69 and 123.60 (C-16); 72.08 and 72.02 (C-20); 46.43 and 46.41 (C-9); 43.7 (C-5); 40.7 (C-4); 39.0 (C-10); 37.93 and 37.92 (C-8); 31.1 (C-7); 30.57 and 30.55 (C-12); 27.7 (C-6); 23.0 (C-11); 22.01 and 21.98 (C-21); 21.4 (20-acetate); 12.5 (C-19); HRMS-ESI: calculated for $C_{23}H_{28}NaO_3$: 375.1931, found 375.1916.

4.2.5. (20R,S)-20-Acetoxy-1α,2α-epoxy-17(13→18)-abeo-5αH-pregna-13,15,17-trien-3-one (**16**)

To a solution of **15** (300 mg, 0.85 mmol) in CH_2Cl_2 (3.2 mL) and MeOH (12.5 mL), 30% H_2O_2 (0.63 mL) and a solution of 10% KOH in MeOH (0.085 mL) were added successively at 25 °C and stirring was continued for 50 min. The reaction mixture was diluted with water, neutralized with 1 M HCl and concentrated to a third of its volume. The residue was extracted with dichloromethane and the solvent evaporated under vacuum. The resulting solid was purified by flash chromatography (hexane–ethyl acetate 70:30) to give **16** as a mixture of epimers at C-20 (248 mg, 79%); IR (KBr): 2924.4; 2853.5; 1733.4; 1715.6; 1243.2; 1063.3; 1024.5; 743.0 cm^{-1}; ^1H NMR (500.13 MHz, $CDCl_3$) δ_H: 7.28 (1H, d, $J = 8.1$ Hz, H-15); 7.15 (1H, dd, $J = 8.1$ and 1.7 Hz, H-16); 7.08 (1H, bs, H-18); 5.83 (1H, m, H-20); 3.65 (1H, d, $J = 4.2$ Hz, H-1); 3.30 (1H, d, $J = 4.2$ Hz, H-2); 2.91 (2H, m, H-12); 2.73 (1H, td, $J = 11.6$ and 3.8 Hz, H-8); 2.48 (1H, dq, $J = 12.8$ and 3.2 Hz, H-7β); 2.33 (1H, dd, $J = 19.2$ and 5.5 Hz, H-4α); 2.18 (1H, m, H-11α); 2.16 (1H, m, H-5); 2.06 (3H, s, 20-acetate); 1.99 (1H, dd, $J = 19.3$ and 12.7 Hz, H-4β); 1.69 (1H, td, $J = 11.7$ and 2.1 Hz, H-9); 1.59 (1H, m, H-6α); 1.55 (1H, m, H-11β); 1.52 (3H, d, $J = 6.6$ Hz, H-21); 1.42 (1H, qd, $J = 13.0$ and 3.2 Hz, H-6β); 1.30 (1H, qd, $J = 12.4$ and 3.4 Hz, H-7α); 0.93 (1H, s, H-19); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ_C: 205.5 (C-3); 170.3 (20-acetate); 139.7 (C-14); 138.99 and 138.95 (C-17); 136.49 and 136.47 (C-13); 126.59 and 126.48 (C-18); 126.17 and 126.15 (C-15); 123.70 and 123.56 (C-16); 72.08 and 72.01 (C-20); 60.8 (C-1); 55.9 (C-2); 45.4 (C-9); 39.8 (C-4); 37.80 and 37.79 (C-8); 36.9 (C-10); 33.34 (C-5); 31.0 (C-7); 30.36 and 30.35 (C-12); 27.3 (C-6); 23.1 (C-11); 22.0(C-21); 21.3 (20-acetate); 10.5 (C-19); HRMS-ESI: calculated for $C_{23}H_{28}NaO_4$: 391.1880, found 391.1889.

4.2.6. (20R,S)-20-Acetoxy-17(13→18)-abeo-5αH-pregna-2,13,15,17-tetraen-1α-ol (**17**)

To a suspension of **16** (148 mg; 0.40 mmol) in ethanol (6.3 mL), hydrazine hydrate (0.76 mL, 15.69 mmol) and water (0.25 mL, 13.89 mmol) were added at 25 °C. The mixture was heated under reflux for 2 h allowed to cool to room temperature and 10% HCl added. After 10 min the mixture was extracted with dichloromethane, the organic layer was dried with sodium sulfate and the solvent evaporated under vacuum. The resulting solid was purified by flash chromatography (hexane–ethyl acetate 70:30) to give compound **17** as a mixture of epimers at C-20 (72.0 mg, 51%); (KBr): 3389.0, 2918.4, 1735.4, 1443.0, 1371.2, 1243.1, 1022.4, 740.3 cm^{-1}; ^1H NMR (500.13 MHz, $CDCl_3$) δ_H: 7.30 (1H, d, $J = 8.1$ Hz, H-15); 7.13 (1H, dd, $J = 8.1$ and 1.7 Hz, H-16); 7.06 (1H, br s, H-18); 5.87 (2H, m, H-2 and H-3); 5.83 (1H, m, H-20); 3.84 (1H, d, $J = 4.5$ Hz, H-1); 2.86 (2H, m, H-12); 2.70 (1H, td, $J = 11.7$ and 2.8 Hz, H-8); 2.43 (1H, dq, $J = 12.8$ and 3.2 Hz, H-7β); 2.06 (3H, s, 20-acetate); 2.00 (1H, m, H-4α); 1.98 (1H, m, H-11α); 1.81 (1H, t, $J = 11.5$ and 2.4 Hz, H-9); 1.75 (1H, m, H-4β); 1.71 (1H, m, H-6α); 1.65 (1H, m, H-5); 1.52 (3H, d, $J = 6.6$ Hz, H-21); 1.48 (1H, m, H-6β); 1.41 (1H, m, H-11β); 1.26 (1H, m, H-7α); 0.75 (1H, s, H-19); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ_C: 170.40 (20-acetate); 140.94 (C-17); 138.50 and 138.47 (C-14); 137.31 and 137.29 (C-13); 130.6 (C-2); 127.8 (C-3); 126.70 and 126.59 (C-18); 126.55 and 126.54 (C-15); 123.51 and 123.37 (C-16); 72.24 and 72.18 (C-20); 69.6 (C-1); 43.34 and 43.32 (C-9); 39.4 (C-10); 37.67 and 37.66 (C-8); 34.3 (C-5); 31.86 and 31.85 (C-7); 30.74 and 30.73 (C-12); 30.4 (C-4); 28.9 (C-6); 22.11 and 22.09 (C-11); 22.01 and 21.98 (C-21); 21.4 (20-acetate); 10.8 (C-19); HRMS-ESI: calculated for $C_{23}H_{30}NaO_3$: 377.2087, found 377.2087.

4.2.7. 1α-Hydroxy-17(13→18)-abeo-5αH-pregna-2,13,15,17-tetraen-20-one (**8**)

To a solution of compound **17** (69.5 mg, 0.196 mmol) in tetrahydrofuran (30 mL) and methanol (30 mL) a 5% aqueous solution of KOH (1.25 mL) was added. After stirring for 1 h at 25 °C, the reaction mixture was neutralized with 1 M HCl and concentrated to a third of its volume. Water was added to the residue and then extracted with dichloromethane. The organic layer was dried with sodium sulfate and the solvent evaporated under vacuum.

To a solution of the solid obtained above in CH_2Cl_2 (15 mL), Na_2CO_3 (100 mg) and MnO_2 (280 mg; 3.22 mmol) were added. The reaction mixture was stirred for 24 h at 25 °C. The suspension was percolated through celite and the solvent evaporated under vacuum. The resulting solid was purified by flash chromatography (hexane–ethyl acetate 90:10→80:20) to give **8** (43 mg, 71% from **17**) as an amorphous solid; IR (KBr): 3462.9; 3023.3; 2920.9; 1768.2; 1680.5; 1604.1; 1444.2; 1259.3; 736.6 cm^{-1}; ^1H NMR (500.13 MHz, $CDCl_3$) δ_H: 7.72 (1H, dd, $J = 8.2$ and 1.8 Hz, H-16); 7.67 (1H, br s, H-18); 7.40 (1H, d, $J = 8.2$ Hz, H-15); 5.88 (2H, m, H-2 and H-3); 3.85 (1H, d, $J = 4.4$ Hz, H-1); 2.91 (2H, m, H-12); 2.73 (1H, td, $J = 11.7$ and 2.9 Hz, H-8); 2.57 (3H, s H-21); 2.46 (1H, dq, $J = 12.9$ and 3.5 Hz, H-7β); 2.02 (1H, m, H-4α); 2.01 (1H, m, H-11α); 1.84 (1H, td, $J = 11.5$ and 2.5 Hz, H-9); 1.76 (1H, m, H-4β); 1.73 (1H, m, H-6α); 1.67 (1H, m, H-5); 1.49 (1H, m, H-6β); 1.42 (1H, m, H-11β); 1.28 (1H, qd, $J = 13.0$ and 3.7 Hz, H-7α); 0.77 (1H, s, H-19); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ_C: 198.2 (C-20); 147.1 (C-14); 137.6 (C-13); 134.4 (C-17); 130.6 (C-2); 128.9 (C-18); 127.7 (C-3); 126.6 (C-15); 125.5 (C-16); 69.5 (C-1); 43.1 (C-9); 39.4 (C-10); 38.2 (C-8); 34.3 (C-5); 31.7 (C-7); 30.7 (C-12); 30.4 (C-4); 28.9 (C-6); 26.5 (C-21); 22.0 (C-11); 10.8 (C-19); HRMS-ESI: calculated for $C_{21}H_{26}NaO_2$: 333.1825, found 333.1832.

4.2.8. 17(13→18)-Abeo-5αH-pregna-2,13,15,17-tetraen-1,20-dione (**9**)

To a solution of compound **17** (35 mg, 0.0.98 mmol) in tetrahydrofuran (15 mL) and methanol (15 mL) a 5% aqueous solution of KOH (0.6 mL) was added. After stirring for 1 h at 25 °C, the reaction mixture was neutralized with 1 M HCl and concentrated to a third of its volume. Water was added to the residue and then extracted with dichloromethane. The organic layer was dried with sodium sulfate and the solvent evaporated under vacuum.

A solution of the solid obtained above in anhydrous dichloromethane (0.7 mL) was added to a suspension of pyridinium chlorochromate (84 mg; 0.39 mmol), barium carbonate (49 mg; 0.25 mmol) and 3 Å molecular sieves (68 mg) in anhydrous

dichloromethane (1.0 mL). The reaction mixture was treated as described for **14**. Evaporation of the solvent followed by flash chromatography (hexane–ethyl acetate 90:10→80:20) gave compound **9** (24 mg, 81% from **17**) as an amorphous solid; IR (KBr): 2914; 1676.1; 1602.0; 1261.4; 710.0 cm^{-1}; ^1H NMR (500.13 MHz, $CDCl_3$) δ_H: 7.71 (1H, dd, $J = 8.2$ and 1.8 Hz, H-16); 7.69 (1H, br s, H-18); 7.38 (1H, d, $J = 8.2$ Hz, H-15); 6.72 (1H, ddd, $J = 10.2$, 4.3 and 3.2 Hz, H-3); 5.86 (1H, dt, $J = 10.2$ and 2.0 Hz, H-2); 3.05 (1H, ddd, $J = 16.8$, 12.0 and 5.1 Hz, H-12α); 2.87 (1H, ddd, $J = 16.4$, 4.1 and 3.8 Hz, H-12β); 2.68 (1H, m, H-11α); 2.67 (1H, m, H-8); 2.57 (3H, s H-21); 2.48 (1H, dq, $J = 12.8$ and 3.6 Hz, H-7β); 2.22 (2H, m, H-4); 1.94 (1H, m, H-5); 1.72 (1H, m, H-9); 1.68 (2H, m, H-6); 1.44 (1H, dddd, $J = 12.9$, 12.4, 10.2 and 3.8 Hz, H-11β); 1.25 (1H, qd, $J = 12.7$ and 4.3 Hz, H-7α); 1.14 (1H, s, H-19); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ_C: 205.7 (C-1); 198.2 (C-20); 146.5 (C-14); 145.5 (C-3); 138.0 (C-13); 134.6 (C-17); 128.9 (C-18); 128.7 (C-2); 126.1 (C-15); 125.4 (C-16); 47.4 (C-10); 43.5 (C-9); 42.6 (C-5); 39.4 (C-8); 30.9 (C-4); 30.7 (C-12); 30.4 (C-7); 28.2 (C-6); 26.5 (C-21); 26.0 (C-11); 9.7 (C-19); HRMS-ESI: calculated for $C_{21}H_{24}NaO_2$: 331.1669, found 331.1681.

4.3. Antiestrogenic activity

4.3.1. Cell culture

The human breast cancer MCF-7 and MDA-MB-231 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were routinely cultured as described previously [21] in phenol red-free [22] Dulbecco's Modified Eagle's Medium:Ham F12 (1:1) supplemented with 10% Fetal Calf Serum (FCS), 2 mM glutamine, 2 μg/mL bovine insulin, 100 IU/mL penicillin, 100 μg/mL streptomycin and 15 mM HEPES. Cells were subcultured once weekly after trypsinization (0.25% trypsin–0.025% EDTA) and seeded at a concentration of 80,000 cells/25 cm^2 flask. Medium was changed twice weekly.

4.3.2. Proliferation assays

The ERα(+) human cancer cell line MCF-7 was used to test the antiestrogenic activity of the salpichrolides **1**, **3** and **4** and analogs **5**–**9**, while the ERα(−) MDA-MB-231 cell line was used to assess the implication of ERα on the effect of analogs **5** and **9**. All compounds were dissolved in DMSO. In order to perform these experiments, 5000 cells per well were seeded in 96 well plates and incubated in phenol red-free DMEM:F12 medium supplemented with 2% charcoal-stripped FCS, 2 mM glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin and 15 mM HEPES; 24 h after seeding, the cells were treated with each compound (stock solution in DMSO) for 72 h, with medium changes every 24 h. Cells were incubated in the presence of 0.1 nM 17β-estradiol (E_2) and increasing concentrations of the compound to be tested, ranging from 0.1 nM to 1 μM. 0.2 μCi [^{3}H]-thymidine per well were added with the last change of medium. After 24 h, cells were harvested in a Nunc Cell Harvester 8 (Nunc, Rochester, NY, USA), and filters were counted in a liquid scintillation counter. As controls, cells were stimulated only with estradiol (stated as E_2) at a concentration of 0.1 nM and in the absence of any treatment (only vehicle). As a positive control for the antiestrogenic action, cells were treated with the antagonist fulvestrant (ICI 182,780, commercial name faslodex) in the presence of 0.1 nM E_2. Additional controls included cells treated only with the natural salpichrolides or the synthetic analogs at concentrations of 10 nM and 1 μM in order to evaluate any possible cytotoxic, apoptotic, antiproliferative or estrogenic action.

4.4. Molecular modeling

The starting coordinates of the ER ligand binding domain were taken from the crystal structure of the ERα/estradiol complex (pdb:1qku, chain A) [16]. The HF/6-31G** optimized structure of compound **9** was obtained using the quantum chemistry program GAUSSIAN 03 [23]. The Autodock 4.2 method [17] was applied considering as rotatables the torsion angles of the steroid side chain. A grid of 70 × 70 × 70 points with a spacing of 0.2 Å centered in the ligand binding pocket, was calculated and used to obtain 400 runs of the genetic algorithm method.

Molecular dynamics (MD) were performed with the AMBER 12 software package [18]. The starting structure of ERα-**9** complex was constructed from receptor coordinates of pdb:1qku [16] and ligand coordinates of the best solution found in cluster A. The Amber99 force field parameters were used for all receptor residues [24]. The ligand parameters were assigned according to the general AMBER force field (GAFF) and the corresponding RESP (restraint electrostatic potential) atomic partial charges at HF/6-31G** level. The complex was immersed in an octahedral box of TIP3P water molecules, initially optimized and then gradually heated to a final temperature of 300 K. Starting from these equilibrated structure, an MD production run of 20 ns was obtained. Simulation was performed at 1 atm and 300 K, maintained with the Berendsen barostat and thermostat respectively [25], using periodic boundary conditions and the particle mesh Ewald method (grid spacing of 1 Å) for treating long-range electrostatic interactions with a uniform neutralizing plasma. The SHAKE algorithm was used to keep bonds involving H atoms at their equilibrium length, allowing the use of a 2 fs time step for the integration of Newton's equations.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.05.067.

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