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Novel estradiol based metal complexes of Tc-99m

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ABSTRACT

Aiming to contribute to the design of technetium imaging agents for estrogen receptor (ER) positive breast tumors, we have synthesized and evaluated the novel organometallic estradiol complexes (***fac*-[M(CO)$_3$(κ^3-10)]$^+$** and ***fac*-[M(CO)$_3$(κ^3-12)]** M = Re/^{99m}Tc) using two different bifunctional tridentate ligands (**4** and **8**). The rhenium complexes (**13** and **14**) were fully characterized by IR, ^{1}H NMR, ^{13}C NMR, mass spectrometry and elemental analyses. The ^{99m}Tc complexes (**15** and **16**) were obtained with high radiochemical purity and exhibited high *in vitro* radiochemical stability. To get a first insight into the relevance of these complexes for targeting ER positive tumors, ER binding affinity assays and cellular internalization studies in an ER expressing cell line, MCF-7, have also been performed suggesting a non ER mediated uptake.

1. Introduction

Breast cancer is the most malignant form of diagnosed cancer among women and still remains a major cause of death in the western world. Due to its propensity to metastasize often, even before its detection by mammography, an early detection of breast cancer is determinant for the patients' survival rate. The well documented over-expression of estrogen receptors (ER) in the majority of breast cancers makes them relevant biomarkers in diagnosis, prognosis and prediction of the therapeutic response in ER positive [ER(+)] breast tumors [1,2]. Given the high sensitivity of the nuclear techniques, namely single photon emission computerized tomography (SPECT) and positron emission tomography (PET), efforts have been undertaken to design radioactive probes directed to estrogen-receptors for breast tumor imaging [3,4].

Concerning PET-imaging, fluorine-18, a cyclotron-produced radionuclide, has been the most explored radiohalogen for *in vivo* imaging studies of estrogen receptors [5–10]. 16α-[^{18}F]-estradiol (^{18}F-FES) is the most promising candidate for *in vivo* imaging of estrogen receptor-positive tumors and is currently in phase II of a study to predict response to first line hormone therapy in women with ER(+) metastatic breast cancer [11–13].

In spite of the better resolution of PET imaging, SPECT still remains the more practical approach for routine diagnostics in nuclear medicine due to longer-lived radionuclides such as iodine-123 and techentium-99m. During the last decades several efforts have been made to synthesize estradiol derivatives that could be labeled with such γ-emitters. Several radioiodinated estradiol derivatives have been studied [3,4,14]. Among them both isomers of 11β-methoxy-(17α,20*E*/*Z*)-[^{123}I]iodovinylestradiol have been clinically assessed, with the 20*Z* isomer giving the better images of ER-positive human breast tumors. While both primary and metastatic tumors were detected with good sensitivity and selectivity, extensive correlations between imaging and clinical outcome have not been provided so far [15–19]. Efforts have also been directed to the design of steroidal estrogen derivatives labeled with ^{99m}Tc, due to its favorable nuclear decay properties, low cost and availability [20–30].

Due to the known tolerance of the estrogen receptors for large substituents at the 7α- and 17α-positions of the estradiol framework, several radioligands were prepared bearing diverse inorganic and organometallic rhenium/technetium units tethered to these positions. Many of these compounds retained high affinity for the cognate receptor, but the corresponding ^{99m}Tc-complexes did not show a receptor-mediated mechanism *in vivo*, apparently due to their high lipophilicity, bulkiness and fast metabolism [21–30]. So far, the most promising agent is a Re(I)/^{99m}Tc(I)-17α-estradiol-pyridin-2-yl hydrazine complex described and evaluated by Nayak et al. This complex showed a receptor-mediated uptake in normal target organ as well as in human breast adenocarcinoma MCF-7 tumors [31,32]. However, in

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spite of the encouraging results, further structural modifications are still needed to improve the imaging.

Aiming to contribute for the design of technetium based radiotracers as molecular probes for the estrogen receptor, we have prepared and evaluated two novel Re(I)/^{99m}Tc(I)-complexes containing 17α-substituted estradiol derivatives. These complexes were prepared by reacting the organometallic fragment *fac*-$[M(CO)_3]^+$ (M = Re, ^{99m}Tc) with 17β-estradiol bearing bifunctional chelators with different donor atom sets. Herein, we will describe the synthesis and characterization of the novel estradiol conjugates, the corresponding Re(I)/^{99m}Tc(I) tricarbonyl complexes and their *in vitro* biological behavior.

2. Experimental

2.1. General

Unless stated otherwise, all chemicals were of reagent grade and were used without further purification. ^{1}H NMR and ^{13}C NMR spectra were recorded at room temperature on a Varian Unity 300 MHz spectrometer. ^{1}H NMR and ^{13}C NMR chemical shifts are reported relatively to residual solvent signals or tetramethylsilane (TMS) as reference. Electrospray ionization/quadropole ion trap mass spectrometry (ESI/QITMS) was acquired from a Bruker HCT Mass Spectrometer. Elemental analyses were performed on an EA 110 CE Instrumental automatic analyzer. IR spectra were recorded on a Bruker Tensor 27 spectrometer, using KBr pellets. Chemical reactions were monitored by thin-layer chromatography (TLC) on Merck plates pre-coated with silica gel $60F_{254}$. Column chromatography was performed on silica gel 60 (Merck). Sodium pertechnetate, Na[$^{99m}TcO_4$], was eluted from a commercial ^{99}Mo/^{99m}Tc generator (MDS Nordion S.A.) using 0,9% saline solution.

HPLC analysis and purification of the ligands and of the Re/^{99m}Tc complexes were performed on a Perkin Elmer system, equipped with a biocompatible quaternary pump (LC 200), an UV/Vis detector (LC 290, Perkin Elmer) and a radioactivity detector (LB 509, Berthold). The ligands and the Re complexes were detected by UV (λ = 254 nm) and ^{99m}Tc complexes were identified by gamma detection. HPLC analyses of the unlabelled and labeled compounds were achieved on a reverse-phase (RP) Nucleosil Column (250 × 4 mm, 5 μm) eluted with a binary gradient system with a flow rate of 1 mL/min using the same binary system — eluents: A-methanol, B-aqueous 0.1% TFA, method 1: 0–5 min 25% A, 5–20 min 25–100% A, 20–30 min 100% A. Method 2: 0–5 min 30% A, 5–5.1 min 30–50% A, 5.1–8.1 50% A, 8.1–13.1 min 50–70% A, 13.1–16.1 min 70% MeOH, 16.1–21.1 min 70–100% A, and 21.1–30 min 100% A. HPLC purifications were performed on a semi-preparative reverse-phase VP-Nucleosil C18 column (250 × 8 mm, 7 μm) with a flow rate of 2 mL/min using the same binary system — eluents: A-methanol, B-aqueous 0.1% TFA solution, method 3: 0–6 min 70% A; 6–8 min 70–85% A; and 8–30 min 85%A.

The rhenium starting complex $[Re(CO)_3(H_2O)_3]Br$ was prepared as reported elsewhere [33]. The radioactive precursor *fac*-[$^{99m}Tc(CO)_3(H_2O)_2]^+$ was prepared using an Isolink® kit (Mallinckrodt), according to the manufacturers. Radioactivity measurements were carried out on an ionization chamber (Aloka Curiemeter, IGC-3, Japan) or a gamma-counter (Berthold, LB 2111, Germany).

2.2. Chemistry

2.2.1. N-(2-bromoethyl)-3,5-dimethyl-1H-pyrazole (2)

To a solution of 3,5-dimethylpyrazole (5.0 g, 0.05 mol) in 1,2-dibromoethane (45 mL, 0.52 mol), 40% aq. NaOH solution (15.6 mL) and tetrabutylammonium bromide (0.432 g, 1.0 mmol) were added. The reaction mixture was heated for 2 h under reflux. Thereafter, the reaction mixture was cooled to room temperature, the phases were separated, the organic phase was dried over $MgSO_4$, and the solvent was removed *in vacuum*. The residue was purified by chromatography on silica gel (ethyl acetate) to give **2** as a yellow oil (3.3 g, 31%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 2.29 (s, 3H, CH_3), 2.32 (s, 3H, CH_3), 4.26 (t, 2H, ^3J = 6.8 Hz), 4.53 (t, 2H, ^3J = 6.8 Hz), and 5.93 (s, 1H).

2.2.2. 2-[(O)-tert-Butylcarboxamidoethylaminoethyl]-3,5-dimethylpyrazole (3)

To a solution of **2** (3.29 g, 0.016 mol) in dry acetonitrile (18 mL), *ter*t-butyl-2-aminoethylcarbamate (3.87 g, 0.02 mol), K_2CO_3 (3.34 g, 0.02 mol) and KI (0.13 g, 0.8 mmol) were added. The reaction mixture was heated overnight under reflux. Afterwards, it was cooled to room temperature, and the solvent was removed *in vacuum*. The residue was purified by chromatography on silica gel ($CHCl_3$/MeOH 95/5) to give **3** as an oil (3.9 g, 85%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.38 (s, 9H, Bu^t), 2.15 (s, 3H, CH_3), 2.18 (s, 3H, CH_3), 2.67 (t, 2H, J = 6 Hz), 2.93 (t, 2H, J = 6 Hz), 3.14 (m, 2H, J = 6 Hz), 3.99 (t, 2H), 5.10 (s, 1H), and 5.73 (s, 1H).

2.2.3. N-[2-(4-Bromophenyl)ethyl]-N-{2-[(O)-tert-butylcarboxamido]ethyl}-N-[2-(3,5-dimethylpyrazol-2-yl)ethyl]amine (4)

1-Bromo-4-(2-bromoethyl)benzene (300 μL, 2 mmol), tetrabutylammonium bromide (TBAB, 2 mg, 0.01 mmol) and a solution of 40% NaOH (0.2 mL) were added to compound **3** (200 mg, 0.708 mmol). The reaction mixture was heated under reflux for 3 h. Then, the mixture was poured into water and extracted with $CHCl_3$ (10 mL), dried over $MgSO_4$, and the solvent was removed *in vacuum*. The residue was purified by chromatography on silica gel [gradient of $CHCl_3$–MeOH (100:0 to 98:2)] to give **4** as a brown oil (173 mg, 52%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.41 (s, 9H, Bu^t), 2.14 (s, 3H, CH_3), 2.19 (s, 3H, CH_3), 2.61 (m, 6H), 2.85 (m, 2H), 3.01 (m, 2H), 3.01 (m, 2H), 4.84 (s, 1H), 5.75 (s, 1H), 6.90 (d, 2H, J = 8.1 Hz), and 7.32 (d, 2H, J = 8.1 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 11.3, 13.8, 28.7, 33.3, 38.8, 47.0, 53.6, 56.3, 63.5, 79.2, 105.3, 120.0, 130.8, 131.6, 133.4, 139.4, 144.9, and 156.3. ESI/MS *m/z*: 467.0 (calcd. 467.2) $[M+H]^+$.

2.2.4. 5-(Bromopyrid-2-yl)-methanol (6)

To a solution of 5-(bromopyrid-2-yl)-carbaldehyde (700 mg, 3.76 mmol) in dry methanol (8 mL) was added, slowly, $NaBH_4$ (286 mg, 7.53 mmol), and the resulting reaction mixture was stirred overnight at room temperature. Afterwards, the solvent was removed *in vacuum*, the residue was dissolved in ethyl acetate (20 mL), and the resulting solution was washed with a solution of Na_2CO_3 (3 × 20 mL). The organic layer was separated, dried over $MgSO_4$ and the solvent was removed to give **6** as a white solid (657 mg, 93%).^{1}H NMR (CD_3OD, 300 MHz) δ: 4.64 (s, 2H), 7.48 (d, 1H, J = 8.4 Hz), 7.99 (dd, 1H, J = 2.1 Hz, J = 8.4 Hz), and 8.55 (d, 1H, J = 2.1 Hz). ^{13}C NMR (CD_3OD, 75 MHz) δ: 63.9, 118.8, 122.4, 134.0, 149.4, and 160.2. ESI/MS *m/z*: 188.1 (calcd. 188.0) $[M+H]^+$.

2.2.5. 2-Bromomethyl-5-bromopyridine (7)

A solution of triphenylphosphine (PPh_3, 2.01 g, 7.68 mmol) in dry THF (8 mL) was added, slowly, to a mixture of **6** (657 mg, 3.49 mmol) and CBr_4 (2.3 g, 6.98 mmol). The reaction mixture was stirred overnight at room temperature. Then, the mixture was filtered, and the solvent was removed *in vacuum*. The residue was purified by chromatography on silica gel (CH_2Cl_2/MeOH 98:2) to give **7** as a brown oil (507 mg, 58%). ^{1}H NMR (CD_3OD, 300 MHz) δ: 4.54 (s, 2H), 7.45 (d, 1H, J = 8.8 Hz), 7.96 (dd, 1H, J = 8.8 Hz, J = 2.4 Hz), and 8.57 (d, 1H, J = 2.4 Hz). ^{13}C NMR (CD_3OD, 75 MHz) δ: 31.8, 120.1, 125.4, 140.5, 150.2, and 156.0. ESI/MS *m/z*: 274.0 (calcd. 273.9) $[M+Na]^+$.

2.2.6. Ethyl 2-[(5-bromopyrid-2-yl)methylamino]acetate (8)

To a solution of **7** (55 mg, 0.22 mmol) in dry acetonitrile (5 mL), glycine ethyl ester hydrochloride (31 mg, 0.22 mmol), K_2CO_3 (60.7 mg, 0.44 mmol) and KI (2 mg, 0.011 mol) were added. The reaction mixture was heated overnight under reflux. Thereafter, the reaction mixture

was cooled to room temperature, and the solvent was removed *in vacuum*. The residue was purified by chromatography on silica gel ($CHCl_3$/MeOH 95:5) to give **8** as a brown oil (28 mg, 47%). ^{1}H NMR (CD_3OD, 300 MHz) δ: 1.25 (t, 3H, J = 7.2 Hz), 3.42 (s, 2H), 3.87 (s, 2H), 4.17 (q, 2H, J = 7.2 Hz), 7.38 (d, 1H, J = 8.4 Hz), 7.94 (dd, 1H, J = 2.4 Hz, J = 8.4 Hz), and 8.58 (d, 1H, J = 2.4 Hz). ^{13}C NMR (CD_3OD, 75 MHz) δ: 13.4, 49.4, 52.9, 60.7, 119.0, 124.2, 139.8, 149.8, 157.8, and 172.0. ESI/MS *m/z*: 297.0 (calcd. 297.0) $[M+Na]^+$.

2.2.7. 17α-[(2-tert-butoxycarbamido-ethyl)-(2-(3,5-dimethyl-1H-pyrazol-1-yl)-ethyl)amino)-4-ethylphenyl]-ethynyl-estra-1,3,5(10)-triene-3,17β-diol (9)

A solution of $Pd(PPh_3)_2Cl_2$ (15.1 mg, 0.02 mmol) in diisopropylamine (5 mL) was stirred for 10 min under a nitrogen atmosphere. CuI (4.1 mg, 0.02 mmol) and **4** (100 mg, 0.215 mmol) were added. After the mixture was stirred for 5 min, 17α-ethynylestradiol (63.7 mg, 0.215 mmol) was added and the solution was stirred at 55 °C for 4 h. The volatiles were removed *in vacuum*, and the residue was purified by column chromatography on silica gel (eluant: CH_2Cl_2: MeOH 100:0 to 95:5), to give **9** (80 mg, 55%) as a brown oil.^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.83 (s, 3H, CH_3), 1.20–2.37 (m, 11H), 1.34 (s, 9H, Bu^t), 2.21 (s, 3H, CH_3), 2.26 (s, 3H, CH_3), 2.52–2.63 (m, 6H), 2.77 (m, 3H), 2.86 (m, 2H), 3.03 (m, 2H), 3.93 (t, 2H), 5.77 (s, 1H), 6.55 (d, 1H, ^4J = 2.4 Hz), 6.61 (dd, 1H, ^4J = 2.4 Hz, ^3J = 8.4 Hz), 7.01 (d, 2H, J = 8.1 Hz), 7.13 (d, 1H, ^3J = 8.4 Hz), and 7.31 (d, 2H, J = 8.1 Hz). ^{13}C NMR: ($CDCl_3$, 75 MHz) δ: 10.9, 12.8, 13.2, 22.9, 26.4, 27.2, 28.4, 29.6, 33.0, 33.2, 38.9, 39.4, 43.3, 46.6, 47.5, 47.8, 49.6, 53.0, 53.4, 55.6, 80.4, 85.8, 92.4, 105.1, 112.8, 115.3, 120.5, 126.3, 128.6, 131.6, 137.8, 139.3, 140.4, 147.5, and 154.2. ESI/MS *m/z*: 681.2 (calcd. 681.4) $[M+H]^+$.

2.2.8. 17α-[(N-(2-(3,5-dimethyl-1H-pyrazol-1-yl)-ethyl)ethane-1,2-diamino)-4-ethylphenyl]-ethynyl-estra-1,3,5(10)-triene-3,17β-diol (10)

To a solution of **9** (111 mg, 0.16 mmol) in methanol (5 mL), was added, slowly, a solution of HCl (1 mL, 12 M), and the reaction mixture was stirred for 24 h at room temperature. Then, the solvent was removed *in vacuum*. The residue obtained was purified by HPLC-RP (method 1), to give **10** (32 mg, 34%). ^{1}H NMR (CD_3OD, 300 MHz) δ: 0.86 (s, 3H, CH_3), 1.15–2.32 (m, 11H), 2.16 (s, 3H, CH_3), 2.31 (s, 3H, CH_3), 2.76–2.87 (m, 7H), 3.07 (m, 2H), 3.17 (m, 2H), 3.74 (m, 2H), 4.27 (m, 2H), 5.96 (s, 1H), 6.46 (d, 1H, ^4J = 2.4 Hz), 6.52 (dd, 1H, ^4J = 2.4 Hz, ^3J = 8.4 Hz), 7.07 (d, 2H, J = 8.1 Hz), 7.16 (d, 1H, ^3J = 8.4 Hz), and 7.35 (d, 2H, J = 8.1 Hz).^{13}C NMR: ($CDCl_3$, 75 MHz) δ: 11.2, 13.3, 13.5, 23.7, 27.5, 28.4, 30.6, 34.3, 35.2, 39.8, 39.9, 40.9, 41.1, 43.5, 44.7, 45.1, 51.1, 51.9, 56.1, 70.7, 80.8, 84.6, 94.7, 109.5, 113.7, 116.0, 123.6, 127.2, 130.3, 132.4, 132.9, 137.2, 138.6, 138.7, 148.1, and 155.8. ESI/MS *m/z*: 581.8 (calcd. 581.4) $[M+H]^+$. Elemental analysis (%) for $C_{37}H_{48}N_4O_2$: Found: C, 61.09; N, 6.95; and H, 6.30. Calculated: C, 60.88; N, 6.93; and H, 6.23.

2.2.9. 17α-[2-(Ethoxycarbonylmethylaminomethyl)pyrid-5-yl-ethynyl] estra-1,3,5(10)-triene-3,17β-diol (11)

A solution of $Pd(OAc)_2$ (4.2 mg, 0.02 mmol) and PPh_3 (9.7 mg, 0.04 mmol) in diethylamine (5 mL) was stirred for 10 min under a nitrogen atmosphere. Then, CuI (7 mg, 0.37 mmol) and **8** (100 mg, 0.37 mmol) were added, and the mixture was stirred for 5 min. 17α-ethynylestradiol (110 mg, 0.37 mmol) was added and the solution was stirred at 55 °C for 4 h. The volatiles were removed *in vacuum*, and the residue was purified by column chromatography on silica gel (CH_2Cl_2/MeOH 98:2) to give **11** (112 mg, 68%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.84 (s, 3H, CH_3), 1.23 (t, 3H, J = 6.9 Hz), 1.20–2.40 (m, 13H), 2.77 (m, 2H), 3.43 (s, 2H), 3.93 (s, 2H), 4.17 (q, 2H), 6.52 (d, 1H, ^4J = 2.4 Hz), 6.59 (dd, 1H, ^4J = 2.4 Hz, ^3J = 8.4 Hz), 7.12 (d, 1H,^3J = 8.4 Hz), 7.29 (d, 1H, ^3J = 8.4 Hz), 7.68 (d, 1H, ^4J = 2.4 Hz, ^3J = 8.4 Hz), and 8.60 (d, 1H, ^4J = 2.4 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 12.9, 14.2, 23.0, 26.5, 27.2, 29.6, 33.2, 38.9, 39.5, 43.6, 47.7, 49.9, 50.8, 54.1, 60.9, 80.4, 83.4, 96.1, 112.8, 115.3, 121.7, 126.5, 132.2, 138.2, 139.3, 151.7, 153.7, 159.1, and 170.2. ESI/MS *m/z*: 489.3 (calcd. 489.3) $[M+H]^+$.

2.2.10. 17α-[2-(Hydroxycarbonylmethylaminomethyl)pyrid-5-yl-ethynyl] estra-1,3,5(10)-triene-3,17β-diol (12)

A solution of **11** (45 mg, 0.10 mmol) in THF (10 mL) was added to a solution of NaOH (92 mg) in water (4 mL), and the resulting reaction mixture was heated overnight under reflux. Then, the solution was neutralized, and the solvent was removed. The residue was redissolved in THF (10 mL), filtered, and the solvent was removed, to give **12** as a brown oil (40 mg, 94%). ^{1}H NMR (CD_3OD, 300 MHz) δ: 0.83 (s, 3H, CH_3), 1.15–2.40 (m, 13H), 2.77 (m, 2H), 3.58 (s, 2H), 4.36 (s, 2H), 6.47 (d, 1H, ^4J = 2.7 Hz), 6.53 (dd, 1H, ^4J = 2.7 Hz, ^3J = 8.4 Hz), 7.09 (d, 1H, ^3J = 8.4 Hz), 7.41 (d, 1H, ^3J = 8.4 Hz), 7.85 (dd, 1H, ^4J = 2.1 Hz, ^3J = 8.4 Hz), and 8.65 (d, 1H, ^4J = 2.1 Hz). ^{13}C NMR (CD_3OD, 75 MHz) δ: 12.2, 22.7, 26.5, 27.3, 29.5, 34.2, 38.7, 39.8, 43.7, 47.0, 47.3, 48.7, 50.2, 79.9, 83.5, 98.0, 112.6, 114.9, 122.5, 125.0, 126.2, 128.4, 131.2, 137.6, 139.8, 151.4, 154.7, and 167.6. ESI/MS *m/z*: 461.3 (calcd. 461.2) $[M+H]^+$. IR υ_{max} (KBr)/cm^{-1}: 1584 (υ(C═O)). Elemental analysis (%) for $C_{28}H_{32}N_2O_4$: Found: C, 73.08; N, 6.00; and H, 6.95. Calculated: C, 73.02; N, 6.08; and H, 7.00.

2.2.11. Synthesis of Re(I) complexes

2.2.11.1. General procedure. $[Re(H_2O)_3(CO)_3]Br$ was reacted with equimolar amounts of **10** or **12** (0.10 mol) in refluxing methanol (5 mL) for 18 h. Then, the solvent was removed *in vacuum* and the desired complexes were purified by chromatography.

2.2.11.2. fac-[Re(CO)$_3$(κ^3−10)]$^+$ (13). The complex was purified by HPLC-RP (method 3) to give **13** (33%). ^{1}H NMR (CD_3OD, 300 MHz) δ: 0.91 (s, 3H, CH_3), 1.28–2.51 (m, 12H), 2.37 (s, 3H, CH_3), 2.45 (s, 3H, CH_3), 2.51 (m, 1H), 2.82 (m, 5H), 2.92 (m, 2H), 3.55 (m, 4H), 3.76 (m, 1H), 4.10 (m, 1H), 4.27 (m, 1H), 4.55 (m, 1H), 5.50 (m, 1H), 6.22 (s, 1H), 6.47 (d, 1H, ^4J = 2.4 Hz), 6.53 (dd, 1H, ^4J = 2.4 Hz, ^3J = 8.4 Hz), 7.10 (d, 1H, ^3J = 8.4 Hz), 7.31 (d, 2H, J = 8.1 Hz), and 7.42 (d, 2H, J = 8.1 Hz). ^{13}C NMR (CD_3OD, 75 MHz) δ: 10.4, 12.3, 14.9, 22.7, 26.6, 27.5, 29.6, 31.2, 33.3, 38.8, 40.0, 42.5, 44.1, 50.0, 53.5, 62.2, 79.7, 84.8, 93.4, 108.1, 112.6, 114.9, 122.2, 126.1, 129.0, 131.3, 131.8, 137.7, 138.0, 144.2, 153.9, 154.8, 192.4, 193.7, and 194.1. ESI/MS *m/z*: 851.1 (calcd. 851.3) $[M]^+$. Elemental analysis (%) for $C_{40}H_{48}N_4O_5Re.CF_3COOH$. Found: C, 49.01; N, 4.64; and H, 5.27. Calculated: C, 49.02; N, 4.58; and H, 5.20. IR ν_{max} (KBr)/cm^{-1} 1912; 2030 (C≡O).

2.2.11.3. fac-[Re(CO)$_3$(κ^3−12)] (14). The complex was purified by column chromatography on silica gel ($CHCl_3$/MeOH/NH_4OH 80:20:2) to give **14** (57%). ^{1}H NMR (CD_3OD, 300 MHz) δ: 0.83 (s, 3H, CH_3), 1.28–2.38 (m, 13H), 2.77 (m, 2H), 3.72 (m, 2H), 4.29 (s, 1H), 4.56 (s, 2H), 6.47 (d, 1H, ^4J = 2.4 Hz), 6.53 (dd, 1H, ^4J = 2.4 Hz, ^3J = 8.4 Hz), 7.08 (d, 1H, ^3J = 8.4 Hz), 7.68 (d, 1H, ^3J = 8.4 Hz), 8.05 (dd, 1H, ^4J = 2.1 Hz, ^3J = 8.0 Hz), and 8.82 (d, 1H, ^4J = 2.1 Hz). ^{13}C RMN (CD_3OD, 75 MHz) δ: 12.3, 22.7, 26.6, 27.4, 29.6, 33.4, 38.6, 40.0, 43.9, 47.0, 50.2, 53.8, 62.2, 69.9, 79.7, 82.0, 112.6, 114.9, 122.3, 123.5, 126.1, 131.2, 137.6, 141.9, 154.0, 154.8, 158.9, 182.9, 195.2, 196.0, and 196.8. ESI/MS *m/z*: 731.3 (calcd. 732.3) $[M+H]^+$. Elemental analyses (%) for $C_{31}H_{32}N_2O_7Re$. Found: C, 51.00; N, 3.75; and H, 4.50. Calculated: C, 50.95; N, 3.83; and H, 4.41. IR ν_{max} (KBr)/cm^{-1}: 1889, 2028 (C≡O), 1612 (C═O).

2.3. Synthesis of ^{99m}Tc(I) complexes (15, 16)

2.3.1. General procedure

In a nitrogen-purged glass vial, 100 μL of 10^{-3} or 10^{-4} M ethanolic solution of **10** or **12** was added to 900 μL of a solution of *fac*-$[^{99m}Tc(OH_2)_3(CO)_3]^+$ (1–2 mCi) in PBS at pH = 7.4. The reaction

mixture was incubated for 30 min at 100 °C and analyzed by HPLC (γ detection). t_r = 23.37 min (method 1) for *fac*-[^{99m}Tc(CO)$_3$(κ^3-10)]$^+$ (**15**). Yield >96%, $\mathbf{t_r}$ = 23.93 min (method 2) for *fac*-[^{99m}Tc(CO)$_3$(κ^3-12)] (**16**).

2.4. In vitro assays

2.4.1. Partition coefficient measurements

The log $P_{o/w}$ values of complexes **15** and **16** were determined by the "shake flask" method under physiological conditions [*n*-octanol/0.1 M phosphate-buffered saline (PBS), pH 7.4] [34]. The HPLC-purified compounds (100 μL, approximately 3.7 MBq, 100 μCi) were added to a test tube containing 1 mL of *n*-octanol and 1 mL of a PBS solution. The tube was vortexed for 1 min and centrifuged for 5 min at 3500 rpm. After centrifugation, 500 μL of the *n*-octanol was transferred to another tube and further extracted with 500 μL of aqueous phase, as described for the first extraction. After separation of the phases, 50 μL aliquots of each phase were taken for radioactivity measurements (in duplicate) using a gamma-counter. The partition coefficient ($P_{o/w}$) was calculated on the basis of the ratio (activity in the *n*-octanol layer)/(activity in the aqueous layer) and is expressed as log $P_{o/w}$.

2.4.2. In vitro cellular and nuclear internalization studies

An ER (+) human breast cancer cell line, MCF-7 (ATCC), was used in this assay. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin under a humidified 5% CO_2 atmosphere. For the internalization studies, cells were plated at a density of approximately 2×10^5 cells per well in 24-well tissue culture plates and allowed to attach overnight. The cells were incubated in humidified 5% CO_2 at 37 °C for a period spanning from 15 min to 4 h with approximately 200,000 cpm of radiocomplex in 0.5 mL of assay medium (Minimum Essential Medium Eagle (MEM) without phenol red and 0.2% bovine serum albumin). Incubation was terminated by washing the cells twice with ice-cold PBS. Cell surface bound radioactive complex was removed by two steps of acid wash (50 mM glycine·HCl/100 mM NaCl, pH 2.8) at room temperature for 3 min. pH was neutralized with cold PBS with 0.2% bovine serum albumin (BSA) and, subsequently, the cells were lysed in 500 μL of lysis buffer (Tris 10 mM, $MgCl_2$ 3 mM, NaCl 10 mM, Nonidet P-40 0.5%, pH 7.5–8.0). After 30 min of incubation, the cell suspension was removed and centrifuged at 1300 *g* at 4 °C for 5 min. At different time points over the 4 h incubation period, the radioactivity associated to cell surface membrane, supernatant (activity outside the nucleus, cytoplasm) and of the precipitate (activity in the nucleus) were measured (at least 3 replicates) in a gamma-counter. Radioactivity associated to each fraction at each time point was expressed as the percentage of the total activity added to the cells and presented as an average plus the standard deviation.

2.4.3. Receptor-binding affinity

The ERα competitive binding assay was performed according to a described method with minor modifications [35]. The ERα binding buffer used for dilution of the receptor preparations consisted of 10% glycerol, 2 mM dithiothreitol, 1 mg/mL BSA and 10 mM Tris–HCl (pH 7.5). The ERα washing buffer contained 40 mM Tris–HCl and 100 mM KCl (pH 7.4). The hydoxyapatite (HAP) slurry was adjusted to a final concentration of 50% (v/v) by using a 50 mM Tris–HCl solution (pH 7.4). The reaction mixture contained 50 μL of varying concentrations of the test compound in the ERα binding buffer, 45 μL of a solution of tritiated estradiol (23.8 nM) and 5 μL (0.25 pmol) of ERα proteins solution. Non specific binding by the tritiated estradiol was determined by the addition of a 50 μM concentration of the nonradioactive E_2. The binding mixture was incubated at 4 °C for 16–18 h. At the end of the incubation, 200 μL of the HAP slurry was added and tubes were incubated on ice and vortexed three times for 15 min. An aliquot of 1 mL of washing buffer was added, mixed and centrifuged at 10,000×*g* for 10 min, and the supernatants were discarded. This wash step was repeated twice. The HAP pellets were then resuspended in 750 μL cold ethanol, vortexed three times in 20 min, centrifuged and the supernatants were transferred to scintillation vials for measurement of ^{3}H radioactivity in a liquid scintillation counter (Packard Tri-CARB 3170 TR/SL). The data obtained from triplicate measurements were expressed as the percent specific binding of [^{3}H]E_2 *vs*. the log molar concentration of the competing compound. The IC_{50} values (calculated using GraphPad Prism software) represent the concentration of the test compound required to reduce the [^{3}H]E_2 binding by 50%.

3. Results and discussion

Several ^{99m}Tc-complexes bearing estradiol derivatives have been previously studied and, for most of them, the conjugation of the metal fragment to the estradiol was performed through the 7α or 17α position [21–23,26,27,31]. Despite the promising biological data obtained for the 17α substituted estradiol derivatives none of the reported analogs was ideal for imaging, due to their low binding affinity, high lipophilicity or low specific activity of the ^{99m}Tc-complexes [32]. Several linkers between the 17α-position and the metal fragment were studied and the ethynyl group was found to be the best, as it provides an effective separation of the steroid and the metal, without introducing excessive conformational flexibility [24]. The introduction of this linker also reduced the affinity of the estrogen derivatives for α-fetoprotein and sex-hormone steroid-binding protein, resulting in more favorable *in vivo* pharmacokinetics [36].

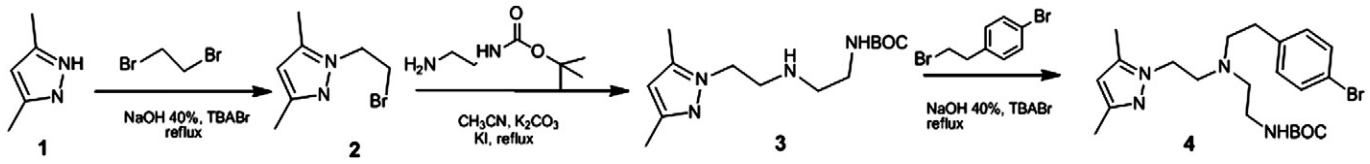
Taking into account these results, we have designed and evaluated novel estradiol derivatives bearing tridentate chelators, namely a pyrazolyl-diamine [37,38] and a pyridine-aminocarboxylate [39], which were linked to the 17α-position through an ethynyl group.

3.1. Chemistry

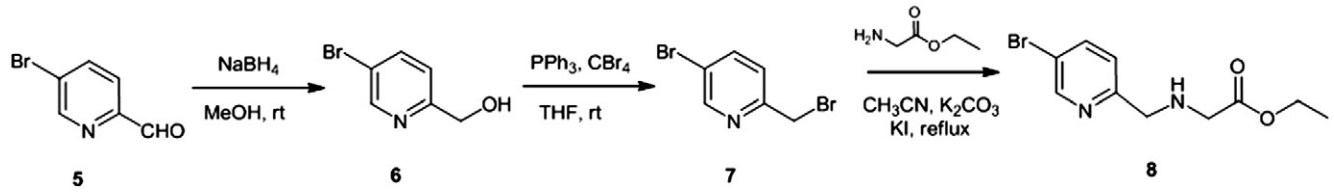
3.1.1. Synthesis of bifunctional ligands (**4** and **8**)

The bifunctional ligand *tert*-butyl-2-(4-bromoethylphenyl)-(2-(3,5-dimethyl-1*H*-pyrazol-1-ylethyl)amine)ethylcarbamate (**4**) was prepared in a three step synthesis as depicted in Scheme 1. 2-Bromoethyl-3,5-dimethyl pyrazole (**2**), obtained by *N*-alkylation of pyrazole (**1**) with excess 1,2-dibromothane, underwent a nucleophilic substitution with *tert*-butyl-2-aminoethylcarbamate, in the presence of potassium carbonate and potassium iodide, to give *tert*-butyl-2[2(3,5-dimethyl-1*H*-pyrazol-1-yl)ethylamine]ethylcarbamate (**3**) [40]. Before coupling the ligand to 17α-ethynylestradiol, the central amino group of **3** was functionalized by N-alkylation with 1-bromo-4-(2-bromoethyl)benzene. The bifunctional ligand **4** was obtained in 52% overall yield, after purification by column chromatography on silica gel (chloroform/methanol). Compound **4** was characterized by NMR spectroscopic techniques (^{1}H/^{13}C NMR) and mass spectrometry.

The bifunctional ligand **8** was synthesized from precursor 5-bromopyridine-2-carbaldehyde (**5**) that was reduced with sodium borohydride to give 5-bromopyridine-2-methanol (**6**) as depicted in Scheme 2. Bromination of the hydroxyl group of **6** under Appel conditions with triphenylphosphine and tetrabromomethane gave 5-bromo-2-bromomethylpyridine (**7**), which was subsequently reacted with ethyl glycate hydrochloride, by direct *N*-alkylation, in the presence of potassium carbonate and potassium iodide. The bifunctional ligand **8** was obtained in 47% yield, after chromatographic purification on silica gel (eluant: chloroform/methanol) and was characterized by NMR spectroscopic techniques (^{1}H/^{13}C NMR) and mass spectrometry.



Scheme 1. Synthesis of bifunctional ligand *tert*-butyl-2-(4-bromoethylphenyl)-(2-(3,5-dimethyl-1*H*-pyrazol-1-ylethyl)amine)ethylcarbamate (4).



Scheme 2. Synthesis of bifunctional ligand ethyl 2-[(5-bromopyrid-2-yl)methylamino]acetate (**8**).

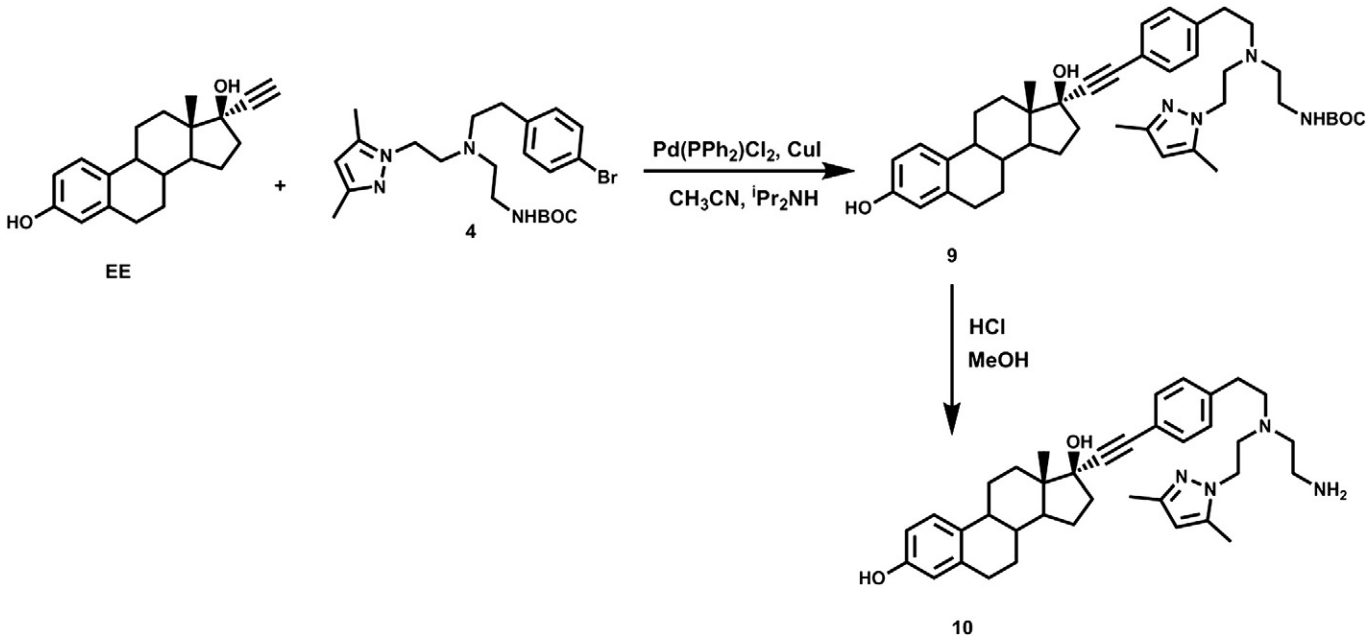
*3.1.2. Synthesis of 17α-substituted estradiol conjugates (**10** and **12**)*

The coupling of the bifunctional ligands (**4** and **8**) to 17α-ethynylestradiol (EE) was achieved by Sonogashira reaction with minor modifications, as depicted in Scheme 3 [41]. The first attempt to conjugate **4** to EE was based on a previously published procedure, which has used a mixture of tetrakis(triphenylphosphine)palladium (0) and copper iodide as catalyst and triethylamine as base [42]. However, under these conditions the yield of the reaction was very low (<5%). To improve the yield, the use of different palladium catalysts and bases was further investigated. The best results were obtained when bis(triphenylphosphine)palladium chloride and diisopropylamine were used. The resulting compound **9** was obtained in 55%, after purification by column chromatography using silica gel and a mixture of dichloromethane and methanol as eluent. Initially, a mixture of trifluoroacetic acid and dichloromethane was used to remove the BOC protective group, however, a very complex mixture was obtained. The subsequent use of a solution of hydrochloric acid in methanol removed the BOC group efficiently yielding 17α-[(*N*-(2-(3,5-dimethyl-1*H*-pyrazol-1-yl)ethyl)ethane-1,2-diamine)-4-ethylphenyl]-ethynyl-estra-1,3,5(10)-triene-3,17β-diol (**10**) in 34% yield, after HPLC purification. The conjugate **10** was characterized by NMR spectroscopic techniques ($^{1}H/^{13}C$ NMR), mass spectrometry and elemental analyses.

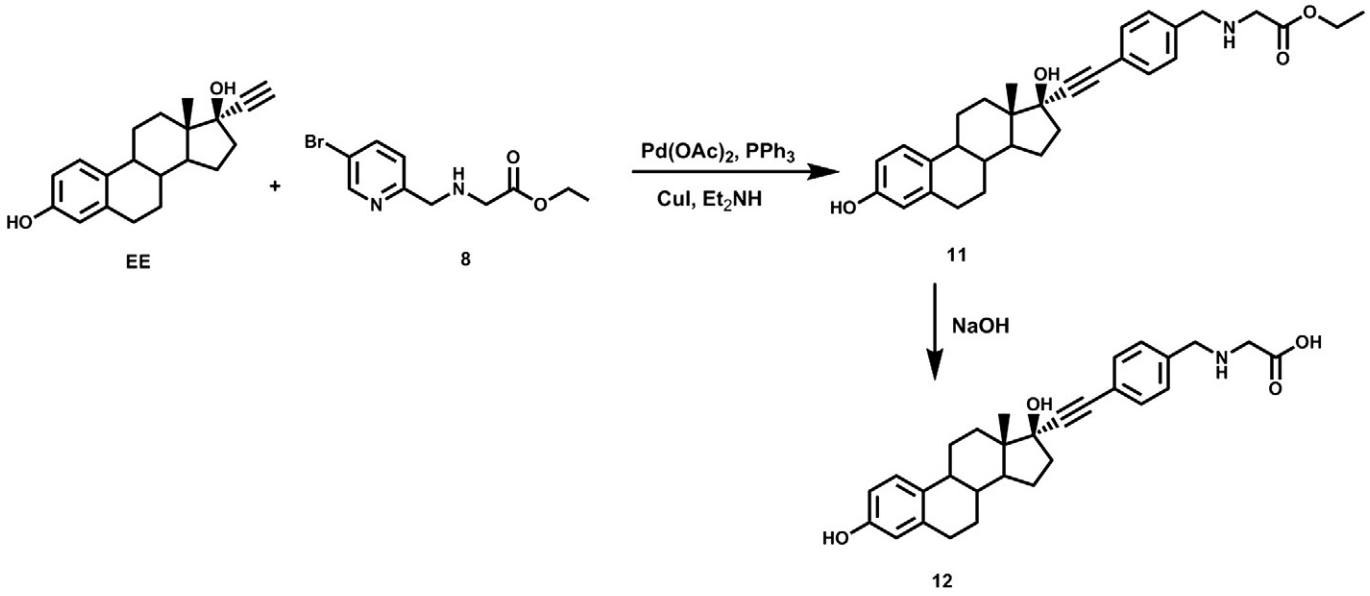
The estradiol derivative **12** was synthesized as depicted in Scheme 4. The coupling of **8** to 17α-ethynylestradiol was performed using bis(triphenylphosphine)palladium diacetate, generated *in situ*, and copper iodide as catalyst in presence of diethylamine. The conjugate **11** was obtained in 68% yield after purification by column chromatography on silica gel (eluant: dichloromethane/methanol). The ester group was removed by base catalyzed hydrolysis with aq. NaOH, and the final conjugate 17α-[2-(hydroxycarbonylmethylaminomethyl)pyrid-5-yl-ethynyl]-estra-1,3,5(10)-triene-3,17β-diol (**12**) was characterized by spectroscopic techniques (IR and $^{1}H/^{13}C$ NMR), mass spectrometry and elemental analyses.

*3.1.3. Synthesis of tricarbonyl M(I) complexes (M = Re: **13** and **14**; M = ^{99m}Tc: **15** and **16**)*

The synthesis of the rhenium complexes *fac*-$[Re(CO)_3(\kappa^3\text{-}10)]^+$ (**13**) and *fac*-$[Re(CO)_3(\kappa^3\text{-}12)]$ (**14**) was carried out by reacting *fac*-$[Re(H_2O)_3(CO)_3]Br$ with **10** and **12** in refluxing methanol (Scheme 5). Complexes **13** and **14** were analyzed by spectroscopic techniques (IR and $^{1}H/^{13}C$ NMR), mass spectrometry and elemental



Scheme 3. Synthesis of conjugate 17α-[(*N*-(2-(3,5-dimethyl-1*H*-pyrazol-1-yl)ethyl)ethane-1,2-diamine)-4-ethylphenyl]-ethynyl-estra-1,3,5(10)-triene-3,17β-diol (**10**).



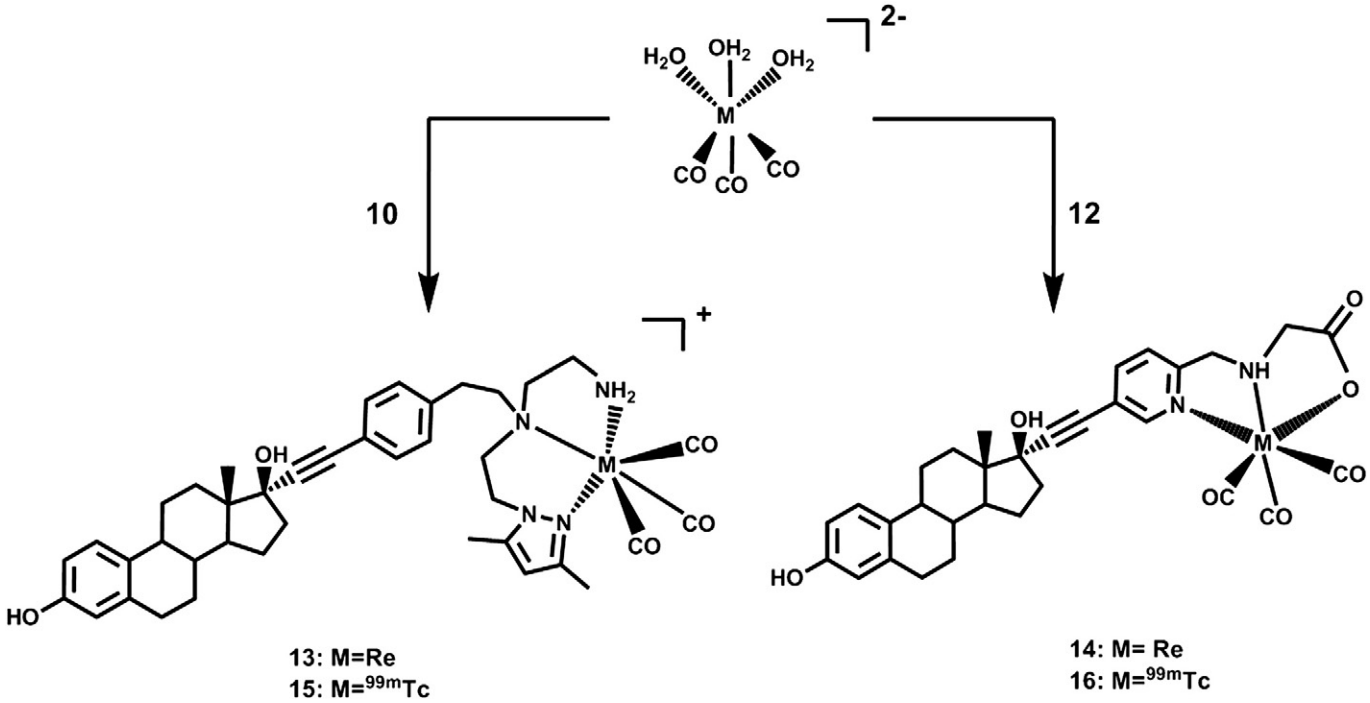
Scheme 4. Synthesis of conjugate 17α-[2-(hydroxycarbonylmethylaminomethyl)pyrid-5-yl-ethynyl]-estra-1,3,5(10)-triene-3,17β-diol (**12**).

analyses, which allowed for the unambiguous identification of their chemical structures. The IR spectra of **13** and **14** showed intense absorption bands between 1889 and 2030 cm^{-1}, easily assigned to the $\nu(C{\equiv}O)$ stretching modes of *fac*-$[Re(CO)_3]^+$ unit. The ^1H NMR data obtained corroborated a facial coordination through the pyrazolyl ring, the central and the terminal amino groups for **13** and through the pyridine ring, the central amino group and the terminal carboxylate for **14**, since all the spectra show a set of multiplets for the methylenic protons of the framework of the pyrazolyl-diamine and the pyridine-aminocarboxylate ligand, which is consistent with the diastereotopic character of the protons.

The ^{99m}Tc complexes *fac*-$[^{99m}Tc(CO)_3(\kappa^3\text{-}10)]^+$ (**15**) and *fac*-$[^{99m}Tc(CO)_3(\kappa^3\text{-}12)]$ (**16**) were obtained in aqueous solution by reaction of *fac*-$[^{99m}Tc(H_2O)_3(CO)_3]^+$ with the appropriate ligand (**10** or **12**) at 100 °C for 30 min at neutral pH (pH 7.4) (Scheme 5). The reactions were almost quantitative (radiochemical yield > 95%) and the ^{99m}Tc complexes were obtained with high radiochemical purity (> 95%). The chemical identity of **15** and **16** was ascertained by comparison of their HPLC profiles with those of the corresponding rhenium complexes **13** and **14**, respectively (Fig. 1).

3.2. In vitro assays

The *in vitro* evaluation of ^{99m}Tc complexes **15** and **16** included the determination of their lipophilicity, their radiochemical stability in physiological solutions and in human serum as well as the assessment of their internalization in MCF-7 cells. Binding affinity studies of Re(I) complexes towards the estrogen receptor are also described.



Scheme 5. Synthesis of tricarbonyl M(I) complexes (M = Re: **13** and **14**; M = ^{99m}Tc: **15** and **16**).

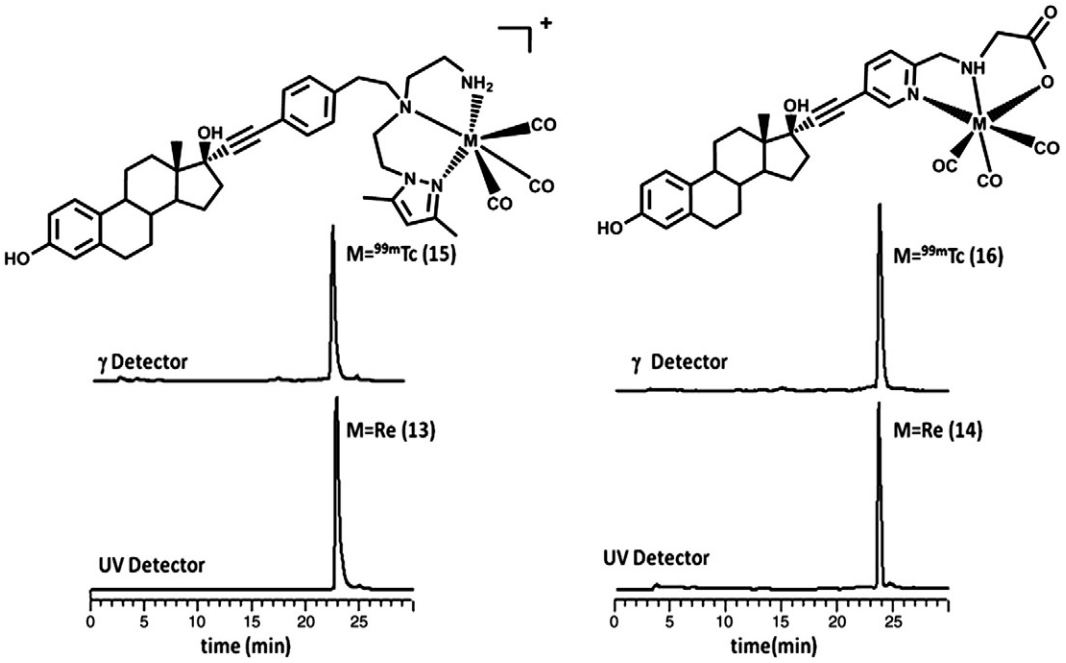


Fig. 1. Radiochromatograms of the co-elution of ^{99m}Tc complexes **15** and **16** with their Re analogs **13** and **14**.

3.2.1. Lipophilicity

The lipophilicity of the complexes **15** and **16** was assessed by measurement of the respective octanol/water partition coefficient using the "shake-flask" methodology [34]. The octanol/water partition coefficient of estradiol was reported by Pomper et al. as log P = 3.26 [5]. The values of log $P_{O/W}$ of the two ^{99m}Tc(I), complexes were found to be up to almost 100 times lower than that of estradiol (log $P_{O/W}$(**15**) = 1.08 ± 0.01, log $P_{O/W}$(**16**) = 1.25 ± 0.07). Nevertheless this reduced lipophilicity can be favorable to improve target tissue selectivity *in vivo* [32]. Comparing these values with the reported value for 17α-ethynylestradiol (log $P_{O/W}$ = 3.42) one can observe a pronounced decrease in lipophilicity, probably due the hydrophilic character of the ligands [5,43,44].

3.2.2. Radiochemical stability

The radiochemical stability of the ^{99m}Tc complexes (**15** and **16**) was evaluated by HPLC analysis of samples at certain time periods after incubation in physiological saline at 37 °C. Both complexes were shown to be radiochemically stable up to 4 h in physiological saline. The ^{99m}Tc complexes were also incubated with samples of human serum at 37 °C, after which the samples were treated with ethanol to precipitate the proteins. HPLC analyses of the ethanolic extracts have demonstrated high radiochemical stability of the complexes for up to 4 h (Fig. 2).

3.2.3. Biological assays

3.2.3.1. Cellular and nuclear internalization. To find out whether the radiolabelled complexes are effectively taken up *in vitro* by tumor cells, cellular and nuclear internalization studies were performed in an ER positive human breast cancer cell line, MCF-7. Since estrogen receptors have been identified as nuclear transcription factors, the degree of cellular/nuclear internalization of the complexes is an important parameter to predict their ability to be retained into the tumor cells by a receptor mediated process. In order, to assess specific cellular uptake similar experiments were performed in parallel by incubation of the labeled complexes in the presence of 10 μM unlabelled estradiol to assure receptors saturation. Prior to these cellular studies the stability of radioactive complexes was evaluated by incubation in the cell growth medium at 37 °C up to 4 h. HPLC analysis confirmed that both complexes were stable in these conditions during the incubation period. The internalization kinetics of both complexes into the whole cell (radioactivity retained in the cytoplasm and nucleus) with and without receptor blockade is presented in Fig. 3.

The total cellular internalization of both complexes in MCF-7 cells was time-dependent with a more evident increase for complex **15**, but the overall rate of internalization is relatively low, even after 4 h of incubation (2.0 ± 0.3 and 3.2 ± 0.2% for complexes **16** and **15**, respectively). Despite the similar lipophilic character of both ^{99m}Tc complexes, the higher rate of internalization of complex **15** possibly results from its overall positive charge as it is well-established that lipophilic cationic complexes tend to localize into tumor cells [45]. Moreover there was no evidence of a relevant decrease in the cellular uptake of any of the radioactive complexes when cells were previously treated with estradiol.

The nuclear internalization of the ^{99m}Tc-complexes in comparison with their total internalization inside the cells is presented in Fig. 4.

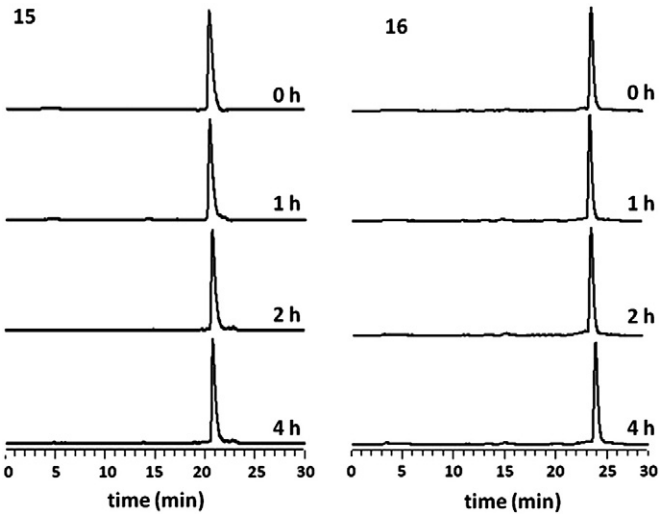


Fig. 2. Radiochemical stability of ^{99m}Tc complexes **15** and **16** in human serum.

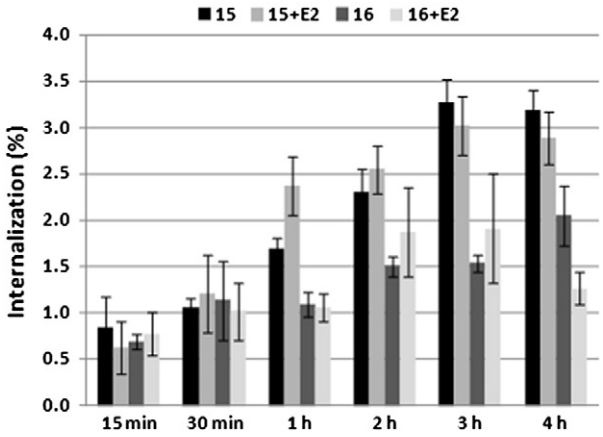


Fig. 3. Internalization of **15** and **16** with and without simultaneous incubation with unlabelled estradiol in whole MCF-7 cells (% of total activity inside cytoplasm and nucleus).

As shown in the figure, the nuclear internalization of complex **15** increases over time in parallel with the corresponding total internalization in whole cell. In addition the percentage of activity inside the nucleus was always higher than the activity in the remaining cytoplasm suggesting that after crossing the cellular membrane the complex is able to reach the nucleus and to accumulate inside. On the other hand, the nuclear internalization of complex **16** is much lower than the internalization in cytoplasm and remains almost constant over time indicating that the complex is not able to be transported or to be retained into the nucleus.

Data from these cellular studies indicate that the conjugation of the biomolecule to the bifunctional chelators **4** and **8** led to final complexes with a decreased ability to penetrate the cell membranes probably due to the evident reduction on lipophilicity when compared with the estradiol itself and other ^{99m}Tc-tricarbonyl complexes bearing a 17α-estradiol derivative [32]. Furthermore, the evidence that the internalization rate for both complexes was not decreased in cells treated with estradiol, suggests that the uptake mechanism is not an ER-mediated process.

3.2.3.2. Receptor binding affinity of Re(I) complexes. Despite the disappointing cellular internalization data, we decided to determine the relative binding affinity of the Re complexes (**13** and **14**) for the isolated recombinant human ERα trying to understand those results as these two assays measure different properties and give complementary information. While the receptor binding assay with the free ER just assesses the receptor binding, the cellular binding assays evaluate not only the receptor binding but also the ability of the complexes to enter into the cell and nucleus or any binding to other biomolecule in the cell that would interfere in the ER binding [29].

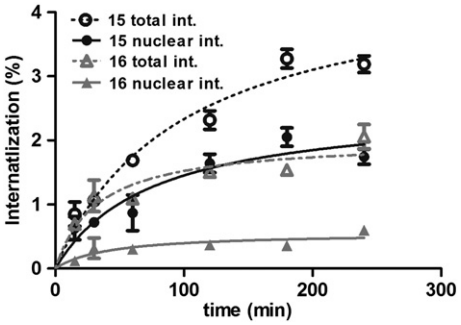


Fig. 4. Activity of 15 (black) and 16 (gray) internalized inside the nucleus *versus* total internalized activity.

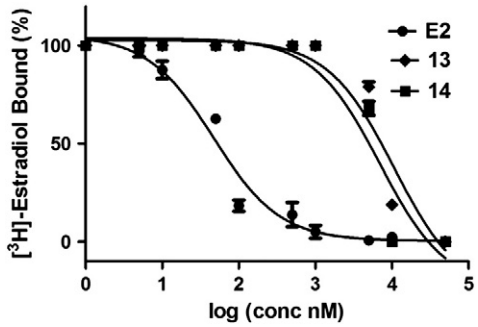


Fig. 5. Estrogen receptor binding affinity of complexes **13** and **14** in comparison with estradiol.

The binding affinities were determined by *in vitro* competitive radiometric binding assays using [^{3}H]-estradiol as tracer. Thus, affinities correspond to the inhibition of the binding of [^{3}H]-estradiol to the recombinant human ERα and were expressed as IC_{50} and relative binding affinity (RBA) where estradiol is set to 100. The IC_{50} values for each complex were calculated according to the sigmoid inhibition curves represented in Fig. 5. The binding results are presented in Table 1.

The whole set of results indicate that both complexes exhibited a low binding affinity for ERα (RBA (**13**) = 0.4 ± 0.1%, RBA (**14**) = 0.7 ± 0.2%) explaining the non-ER-mediated process observed in cellular studies. Animal studies were not undertaken due the low rate of cellular/nuclear internalization of the complexes and low ER binding observed.

4. Conclusions

We have described the synthesis of two bifunctional chelators with different donor atom sets and charge (**4** and **8**). The conjugation of **4** and **8** to 17α-ethynylestradiol was performed successfully using the Sonogashira methodology. Using these novel estradiol-ligand conjugates, the Re(I)/^{99m}Tc(I)-tricarbonyl complexes (**13**/**15** and **14**/**16**) were synthesized. The ^{99m}Tc complexes **15** and **16** were obtained in high radiochemical yield and characterized by comparing their HPLC profiles with the ones obtained for the corresponding Re analogs (**13** and **14**), which were fully characterized. The ^{99m}Tc complexes show a high stability in physiological saline, human serum and cell medium.

Cellular studies showed that **15** has a higher ability to accumulate in the nucleus when compared with **16** most probably due to its cationic overall charge. However, estradiol saturation studies indicate a non ER-mediated uptake process for both complexes. These results were corroborated with the low RBA determined for the Re analogs.

Acknowledgments

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Table 1
ER binding data for rhenium complexes.

Complexes	13	14
IC_{50} ± SD (μM)	11 ± 1	7 ± 1
RBA ± SD (%)	0.4 ± 0.1	0.7 ± 0.2

SD: Standard deviation (n = 3).

of FCT and is part of Rede Nacional de Espectrometria de Massa (RNEM).

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