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High affinity 17α-substituted estradiol derivatives: Synthesis and evaluation of estrogen receptor agonist activity

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ABSTRACT

We synthesized four derivatives of 17β-estradiol (E2) with an azide substitution on a 17α-side chain of varying length, namely 17α-(azidopropargyl)-3,17β-estradiol (**5**), its 17β-azido derivative (diazide **7**), 17α-(5-azido-pent-1-ynyl)-3,17β-estradiol (**6**) and 17α-(azidopentyn-2-yl)-3,17β-estradiol (**10**). While most of the derivatives had low (**7**) or marginal (**6** and **10**) relative binding affinity (RBA) for both types of estrogen receptor (ERα and ERβ), the RBAα and RBAβ of **5** were practically identical to those of E2. The estrogenic activity of the derivatives was assessed using estrogen-responsive breast (MCF-7) and endometrial cancer (Ishikawa) cells. While **5** was a potent and effective inducer of alkaline phosphatase in Ishikawa cells and **7** was less potent but as effective as **5**, **6** was marginally active and **10** was totally inactive in this respect. In the presence of 0.1 nM E2, however, **6** exhibited some ER antagonist activity at the highest concentration tested (1 μM). Similar results were obtained as regards the potency and efficacy of stimulation of MCF-7 cell proliferation and induction of luciferase gene expression in MCF-7:D5L cells, a clone stably transfected with an estrogen-responsive form of the gene. These data suggest that, while **5**, **6**, **7** and **10** interact with either type of ER in isolation, only **5** and **7** exhibit substantial ER agonist activity in the different estrogen-target cells examined, which could provide for photoaffinity labelling of the receptor in the cell as well as in isolation.

1. Introduction

Estrogens are known to affect growth, development and maintenance of many tissues and organs [1–3]. The effects of estrogens are mediated by the two estrogen receptor (ER) subtypes, ERα and ERβ, which are ligand-activated transcription factors capable of regulating membrane-initiated kinase-mediated signaling as well as transcription from estrogen-dependent promoters in a species-, tissue-, cell- and promoter-dependent manner [4–7]. Estradiol (E2), the most potent of the endogenous estrogens, regulates the development of the reproductive system in females. As a consequence, a plethora of estradiol derivatives have been presented to women as oral contraceptives and/or hormone replacement therapy. Prominent among the numerous synthetic estradiol analogs and derivatives are the 17α-substituted ones, since they often exhibit good binding affinities for the ER [8,9]. 17α-Ethynyl estradiol (EE2), in particular, is known to display excellent binding affinities for the

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receptor, and this is also the case with 17α-alkynyl-substituted estradiol derivatives containing three carbon side chains (17α-propynyl, 17α-iodopropargyl and 17α-fluoropropargyl estradiols) [10]. However, the elongation of 17α-alkynyl side chains has been reported to result in reduction of the affinity of these derivatives for the ER [10–13].

In the context of an ongoing effort towards the synthesis of photoreactive compounds displaying high binding affinity to the ER, we introduced the alkynylol (propargyl alcohol, 4-pentyn-1-ol, 4-pentyn-2-ol) substituents in the molecular framework of estradiol. Subsequently, the primary (or secondary) hydroxy groups of these compounds were transformed to azides, providing the corresponding novel 17α-alkynylazido estradiol derivatives. We focused on the azide group because it represents a characteristic example of an electron rich triple bond that can be further utilized as intermediate for the synthesis of various amino-substituted estrogen derivatives. For example, similar azide derivatives have been used in the synthesis of the pure steroidal antiestrogen ICI 164,384 [14] and several estradiol derivatives bearing biotin, fluorescein and acridinium probes [15]. However, previous endeavors involving the introduction of azide functionalities in estradiol derivatives have not been studied with respect to their ER-binding affinity and estrogenic activity. Herein we report on the synthesis and biological evaluation of a number of azide derivatives of estradiol using purified preparations of ERα and ERβ to determine receptor binding affinities and cell-based assays to assess agonist/antagonist activities in target tissues of central interest as the breast and the uterus.

2. Experimental

2.1. General remarks

All anhydrous reactions were carried out under argon atmosphere. Solvents were dried by distillation prior to using. Solvent mixtures employed in chromatography were reported as volume-to-volume ratios. Starting materials were purchased from Aldrich (analytical reagent grades) and used without further purification. Analytical thin-layer chromatography (TLC) was conducted on Merck glass plates coated with silica gel 60 F_{254} and spots were visualized with UV light or/and an alcoholic solution of anisaldehyde. Flash column chromatography was performed using Merck silica gel 60 (230–400 mesh ASTM). When analyses are indicated by symbols of the elements, analytical results obtained for those elements were ±0.4% of their theoretical values.

2.2. Apparatuses

Melting points were determined on a Büchi melting point apparatus and are uncorrected. ^{1}H and 2D NMR spectra were recorded at 400 MHz on a Bruker DRX-400 spectrometer in the indicated solvents. The coupling constants are recorded in Hertz (Hz) and the chemical shifts are reported in parts per million (δ, ppm) downfield from tetramethylsilane (TMS), which was used as an internal standard (by asterisk are indicated the overlapped peaks). Infrared spectra were obtained on a Nicolet Magna 750, series II spectrometer.

2.3. General procedure for the synthesis of 17α-alkynylol derivatives of estradiol

To an ice-cold solution of estrone (0.5 g, 1.84 mmol) and potassium ethoxide (2.25 g, 26.8 mmol) in anhydrous THF (10 mL), 14.8 mmol of an alkynylol was added. The reaction mixture was allowed to reach the room temperature and stirred for additional 2 h. Then was poured into ice water, neutralized with acetic acid and extracted with EtOAc (2 × 20 mL). The combined organic layers were washed with brine, dried over $MgSO_4$ and concentrated under vacuum. Flash column chromatography on silica gel using EtOAc–hexane (3:7) as eluant provided the title products.

2.4. 17α-(Hydroxypropargyl)-3,17β-estradiol (1)

Yield: 70%; mp: 200–202 °C; IR (neat) ν_{max} 3380, 2225 cm^{-1}; ^{1}H NMR (acetone-d6) δ, 0.97 (s, 3H, CH_3), 1.39–1.59 (m, 5H, H-8, H-11, H-15), 1.75 (m, 1H, H-14), 1.79–1.86 (m, 4H, H-6, H-12), 2.02 (dt, J = 7.4 Hz, 1H, H-16), 2.23 (m, 1H, H-9), 2.26–2.38 (m, 3H, H-11, H-16) 2.78–2.81 (m, 2H, H-7), 4.17 (s, 2H, H-22), 6.59 (d, J = 2.4 Hz, 1H, H-4), 6.68 (dd, J = 8.1, 2.5 Hz, 1H, H-2), 7.18 (d, J = 8.1 Hz, 1H, H-1). Anal. ($C_{21}H_{26}O_3$) C, H.

2.5. 17α-(5-Hydroxy-pent-1-ynyl)-3,17β-estradiol (2)

Yield: 75%; mp: 120–121 °C; IR (neat) ν_{max} 3470 cm^{-1}; ^{1}H NMR (acetone-d6) δ, 0.84 (s, 3H, CH_3), 1.23–1.42 (m, 5H, H-8, H-11, H-15), 1.61–1.72 (m, 4H, H-12, H-23), 1.85 (m, 2H, H-6), 1.90 (m, 1H, H-14), 2.05–2.12 (m, 2H, H-16), 2.28 (t, J = 7 Hz, 2H, H-22), 2.31 (m, 1H, H-9), 2.69–2.75 (m, 2H, H-7), 3.62 (m, 2H, H-24), 6.49 (d, J = 2.4 Hz, 1H, H-4), 6.57 (dd, J = 8.4, 2.2 Hz, 1H, H-2), 7.08 (d, J = 8.4 Hz, 1H, H-1), 7.92 (br s, 1H, OH). Anal. ($C_{23}H_{30}O_3$) C, H.

2.6. 17α-(4-Hydroxy-pent-1-ynyl)-3,17β-estradiol (8)

Yield: 78%; mp: 129–131 °C; IR (neat) ν_{max} 3378, 3296 cm^{-1}; ^{1}H NMR (acetone-d6) δ, 0.93 (s, 3H, CH_3), 1.28 (d, J = 2.5 Hz, 3H, H-24), 1.33–1.51 (m, 5H, H-8, H-11, H-15), 1.74 (m, 1H, H-14), 1.79–1.86 (m, 4H, H-6, H-12), 2.01 (dt, J = 4.7 Hz, 1H, H-16), 2.21 (m, 1H, H-9), 2.25–2.34 (m, 5H, H-11, H-16, H-22), 2.76–2.82 (m, 2H, H-7), 3.9 (m, 1H, H-23), 6.49 (d, J = 2.5 Hz, 1H, H-4), 6.65 (dd, J = 8.5, 2.5 Hz, 1H, H-2), 7.16 (d, J = 8.5 Hz, 1H, H-1), 8.01 (br s, 1H, OH). Anal. ($C_{23}H_{30}O_3$) C, H.

2.7. General procedure for the synthesis of the methanesulfonates

To an ice-cold solution of 17α-(5-hydroxy-pent-1-ynyl)-3,17β-estradiol **2** (1.52 mmol) in anhydrous THF (6 mL), Et_3N (2.28 mmol) and MsCl (2.28 mmol) were added. The reaction mixture was allowed to proceed the room temperature, stirred for additional 2 h and concentrated under reduced pressure. Flash column chromatography on silica gel using hexane–ethyl acetate (7:3) as eluant gave the desired product.

2.8. 17α-(Methanesulfonyloxypropargyl)-3,17β-estradiol (3a)

Yield: 55%; mp: 88–90 °C; IR (neat) ν_{max} 3319 cm^{-1}; ^1H NMR ($CDCl_3$) δ, 0.92 (s, 3H, CH_3), 1.31–1.56 (m, 5H, H-8, H-11, H-15), 1.74 (m, 1H, H-14), 1.78–1.85 (m, 4H, H-6, H-12), 2.01 (dt, J = 4.7 Hz, 1H, H-16), 2.20 (m, 1H, H-9), 2.25–2.36 (m, 2H, H-16), 2.76–2.80 (m, 2H, H-7), 2.98 (s, 3H, -S-CH_3), 4.12 (s, 2H, H-22), 6.59 (d, J = 2.4 Hz, 1H, H-4), 6.64 (dd, J = 8.1, 2.5 Hz, 1H, H-2), 7.18 (d, J = 8.1 Hz, 1H, H-1). Anal. ($C_{22}H_{28}O_5S$) C, H, S.

2.9. 17α-(Methanesulfonyloxypropargyl)-3,17β-estradiol-17β-methanesulfonate(3b)

Yield: 25%; mp: 79–81 °C; ^1H NMR ($CDCl_3$) δ, 1.12 (s, 3H, CH_3), 1.31–1.56 (m, 5H, H-8, H-11, H-15), 1.74 (m, 1H, H-14), 1.78–1.85 (m, 4H, H-6, H-12), 2.03 (dt, J = 4.7 Hz, 1H, H-16), 2.21 (m, 1H, H-9), 2.28–2.36 (m, 3H, H-11, H-16), 2.78–2.85 (m, 2H, H-7), 2.99 (s, 6H, -S-CH_3), 4.07 (s, 2H, H-22), 6.59 (d, J = 2.4 Hz, 1H, H-4), 6.64 (dd, J = 8.1, 2.5 Hz, 1H, H-2), 7.23 (d, J =8.1 Hz, 1H, H-1). Anal. ($C_{23}H_{30}O_7S_2$) C, H, S.

2.10. 17α-(5-Methanesulfonyloxypent-1-ynyl)-3,17β-estradiol (4)

Yield: 55%; mp: 101–103 °C; IR (neat) ν_{max} 3360 cm^{-1}; ^1H NMR ($CDCl_3$) δ, 0.84 (s, 3H, CH_3), 1.23–1.39 (m, 5H, H-8, H-11, H-15), 1.61–1.72 (m, 4H, H-12, H-23), 1.88 (m, 3H, H-6, H-14), 2.05–2.12 (m, 2H, H-16), 2.28 (t, J = 7 Hz, 2H, H-22), 2.31 (m, 1H, H-9), 2.68–2.74 (m, 1H, H-7), 2.92 (s, 3H, -SCH_3), 3.61 (m, 2H, H-24), 6.49 (d, J = 2.4 Hz, 1H, H-4), 6.58 (dd, J = 8.4, 2.2 Hz, 1H, H-2), 7.12 (d, J = 8.4 Hz, 1H, H-1), 7.88 (br s, 1H, OH). Anal. ($C_{24}H_{32}O_5S$) C, H, S.

2.11. 17α-(4-Methanesulfonyloxypent-1-ynyl)-3,17β-estradiol (9)

Yield: 65%; mp: 112–114 °C; IR (neat) ν_{max} 3365 cm^{-1}; ^1H NMR ($CDCl_3$) δ, 0.93 (s, 3H, CH_3), 1.37 (d, J = 2.5 Hz, 3H, H-24), 1.39–1.53 (m, 5H, H-8, H-11, H-15), 1.72 (m, 1H, H-14), 1.80–1.87 (m, 4H, H-6, H-12), 2.01 (dt, J = 7.4 Hz, 1H, H-16), 2.21 (m, 1H, H-9), 2.24–2.34 (m, 5H, H-11, H-16, H-22), 2.76–2.82 (m, 2H, H-7), 3.05 (s, 3H, -SCH_3), 3.9 (m, 1H, H-23), 6.49 (d, J = 2.5 Hz, 1H, H-4), 6.65 (dd, J = 8.5, 2.5 Hz, 1H, H-2), 7.16 (d, J = 8.5 Hz, 1H, H-1), 8.01 (br s, 1H, OH). Anal. ($C_{24}H_{32}O_5S$) C, H.

2.12. General procedure for the synthesis of the alkynylazides

To a solution of the estradiol methensulfonate derivative (0.21 mmol) in anhydrous DMF (2 mL), sodium azide (0.5 mmol) was added. The reaction mixture was warmed to 50 °C and stirred for 2 h. Then, the mixture was partitioned between water and EtOAc and the organic phase was separated, washed with brine, dried over magnesium sulfate (anhydrous), filtered, and evaporated to dryness. Flash column chromatography purification on silica gel using hexane–ethyl acetate (4:1) as eluant afforded the desired product.

2.13. 17α-(Azidopropargyl)-3,17β-estradiol (5)

Yield: 88%; mp: 141–143 °C; IR (neat) ν_{max} 3562, 3388, 2124 cm^{-1}; ^1H NMR ($CDCl_3$) δ, 0.94 (s, 3H, CH_3), 1.37–1.55 (m, 5H, H-8, H-11, H-15), 1.74 (m, 1H, H-14), 1.80–1.86 (m, 4H, H-6, H-12), 2.05 (dt, J = 7.4 Hz, 1H, H-16), 2.21 (m, 1H, H-9), 2.32–2.41 (m, 3H, H-11, H-16), 2.84–2.92 (m, 2H, H-7), 4.01 (s, 2H, H-22), 6.95 (d, J = 2.2 Hz, 1H, H-4), 7.08 (dd, J = 8.4, 2.2 Hz, 1H, H-2), 7.27 (d, J = 8.4 Hz, 1H, H-1); ^{13}C NMR ($CDCl_3$) δ, 12.69 (C-18), 22.89 (C-15), 26.42 (C-11), 27.24 (C-6), 29.61 (C-7), 32.76 (C-12), 34.43 (C-21), 38.70 (C-16), 39.36 (C-8), 43.48 (C-9), 47.63 (C-13), 49.59 (C-14), 79.86 (C-17), 82.23 (C-21), 91.27 (C-20), 112.75 (C-2), 115.28 (C-4), 126.54 (C-1), 132.33 (C-10), 138.22 (C-5), 154.32 (C-3). MS, M^+ 351 m/z (70), fragment ions at m/z 308 (25) and m/z 213 (100). Anal. ($C_{21}H_{25}N_3O_2$) C, H, N.

2.14. 17α-(5-Azido-pent-1-ynyl)-3,17β-estradiol (6)

Yield: 88%; mp: 108–110 °C; IR (neat) ν_{max} 3388, 2119 cm^{-1}; ^1H NMR ($CDCl_3$) δ, 0.89 (s, 3H, CH_3), 1.23–1.37 (m, 5H, H-8, H-11, H-15), 1.42 (t, J = 7 Hz, 2H, H-24), 1.62–1.72 (m, 4H, H-12, H-23), 1.85 (m, 2H, H-6), 1.90 (m, 1H, H-14), 2.07–2.12 (m, 2H, H-16), 2.17 (t, J = 7 Hz, 2H, H-22), 2.31 (m, 1H, H-9), 2.69–2.76 (m, 1H, H-7), 6.48 (d, J = 2.2 Hz, 1H, H-4), 6.61 (dd, J = 8.4, 2.2 Hz, 1H, H-2), 7.11 (d, J = 8.4 Hz, 1H, H-1), 7.97 (br s, 1H, OH). Anal. ($C_{23}H_{29}N_3O_2$) C, H, N.

2.15. 17-Azido-17-(3-azido-prop-1-ynyl)-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-3-ol (7)

Yield: 86%; mp: 117–118 °C; IR (neat) ν_{max} 3516, 2124, 2077 cm^{-1}; ^1H NMR ($CDCl_3$) δ, 0.89 (s, 3H, CH_3), 1.35–1.57 (m, 5H, H-8, H-11, H-15), 1.73 (m, 1H, H-14), 1.76–1.84 (m, 4H, H-6, H-12), 2.01 (dt, J = 7.4 Hz, 1H, H-16), 2.20 (m, 1H, H-9), 2.31–2.41 (m, 3H, H-11, H-16), 2.85–2.91 (m, 2H, H-7), 4.01 (s, 2H, H-22), 7.01 (d, J = 2.4 Hz, 1H, H-4), 7.04 (dd, J = 8.4, 2.4 Hz, 1H, H-2), 7.34 (d, J = 8.4 Hz, 1H, H-1); ^{13}C NMR ($CDCl_3$) δ, 13.31 (C-18), 22.91 (C-15), 26.42 (C-11), 27.24 (C-6), 29.61 (C-7), 32.76 (C-12), 34.43 (C-21), 37.36 (C-8), 38.72 (C-16), 43.48 (C-9), 45.06 (C-13), 49.59 (C-14), 68.71 (C-17), 82.01 (C-21), 90.98 (C-20), 112.69 (C-2), 115.28 (C-4), 126.54 (C-1), 132.33 (C-10), 138.52 (C-5), 154.48 (C-3). MS, M^+ 376 m/z (10), fragment ions at m/z 348 (100) and m/z 213 (45). Anal. ($C_{21}H_{24}N_6O$) C, H, N.

2.16. 17α-(Azidopentyn-2-yl)-3,17β-estradiol (10)

Yield: 82%; mp: 119–120 °C; IR (neat) ν_{max} 3379, 2115 cm^{-1}; ^1H NMR ($CDCl_3$) δ, 0.97 (s, 3H, CH_3), 1.18 (d, J = 2.5 Hz, 3H, H-24), 1.33–1.51 (m, 5H, H-8, H-11, H-15), 1.74 (m, 1H, H-14), 1.79–1.86 (m, 5H, H-6, H-12, H-23), 2.01 (dt, J = 4.7 Hz, 1H, H-16), 2.21 (m, 1H, H-9), 2.25–2.34 (m, 5H, H-11, H-16, H-22), 2.76–2.82 (m, 2H, H-7), 6.49 (d, J = 2.5 Hz, 1H, H-4), 6.59 (dd, J = 8.5, 2.5 Hz, 1H, H-2), 7.17 (d, J = 8.5 Hz, 1H, H-1), 8.06 (br s, 1H, OH). Anal. ($C_{23}H_{29}N_3O_2$) C, H, N.

2.17. Cell culture and assessment of cell proliferation

Cell proliferation was assessed using MCF-7 human mammary adenocarcinoma cells, as already described [16]. Briefly, cells

Table 1 – RBA values of the 17α-substituted estradiol derivatives

Compound	RBAα[a]	RBAβ[a]
Estradiol	100	100
5	83.46 ± 9.30	76.49 ± 12.80
6	0.03 ± 0.01	~0.01
7	2.93 ± 0.30	4.24 ± 0.96
10	0.03 ± 0.01	<0.01

[a] The RBA values (mean ± S.E.M. of at least three independent experiments) for ERα (RBAα) and ERβ (RBAβ) were calculated by [(IC_{50} estradiol/IC_{50} derivative) × 100], where IC_{50} values are estradiol and derivative concentrations capable of inhibiting binding of the fluorescent estrogen ES2 (1 nM) to ERα and ERβ by 50%. IC_{50} values of estradiol for ERα and ERβ were 3.42 ± 0.99 and 2.87 ± 0.64, respectively. The RBAα and RBAβ of estradiol were set equal to 100.

that have been cultured and sub-cultured as recommended by the supplier (ATCC), were plated in 96-flat-bottomed-well microplates at a density of 10,000 cells/well in Dulbecco's MEM devoid of phenol-red and supplemented with 10% dextran coated charcoal (DCC)-treated fetal bovine serum (FBS). Serial dilutions of the test compounds were added to the cells 24 h after plating, and after incubation for 6 days with both medium and test compounds being renewed every 48 h, the number of viable cells was determined using the conventional conversion of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) to coloured formazan. Cells that received 0.1 nM E2 (Sigma) served as stimulated proliferation controls, whereas those that received vehicle (DMSO to a final concentration ≤ 0.2%) only, served as basal proliferation controls. The pure antiestrogen ICI 182,780 (Tocris) was used to inhibit the estrogenic response as stated in the text.

2.18. *Binding to isolated human ERα and ERβ*

The relative binding affinity (RBA) values were assessed as previously described [16]. Briefly, the concentrations of **5**–**7** and **10** that inhibited ES2 (a fluorescein-labelled estrogen from Invitrogen) binding of isolated human ERα or ERβ (Invitrogen) by 50% (IC_{50}), as assessed using a Beacon 2000 Fluorescence Polarization Reader (Invitrogen), were used to derive the RBA values of Table 1, as described in the legend to the table.

2.19. *Induction of luciferase gene expression*

Steroid induction of estrogen-responsive element (ERE)-dependent luciferase gene expression was assessed using MCF-7:D5L cells as previously described [16]. Briefly, MCF-7:D5L cells that have been cultured and sub-cultured as reported above for the parental MCF-7 cells, were plated in 96-flat-bottomed-well microplates at a density of 10,000 cells/well in phenol-red-free MEM supplemented with 5% DCC-FBS, and 72 h later the cells were exposed to the test compounds for 16 h before assayed for luciferase activity using the commercial Steady-Glo Luciferase Assay System (Promega).

2.20. *Induction of alkaline phosphatase activity*

Steroid induction of alkaline phosphatase (AlkP) activity was carried out using estrogen-responsive Ishikawa endometrial adenocarcinoma cells, as already described [16]. Briefly, cells that have been cultured and sub-cultured as described by the supplier (ECACC), were plated in 96-flat-bottomed-well microculture plates at a density of 12,000 cells per well in phenol-red-free MEM supplemented with 5% DCC-FBS and 24 h later were exposed to test compounds for 72 h before being assayed for AlkP activity.

2.21. *Statistics*

Data were analysed using the SPSS 10.0 statistical package for windows and compared using one-way ANOVA with a Tukey Post Hoc test for multiple comparisons. Differences were considered statistically significant for values of $P < 0.05$.

3. Results and discussion

3.1. *Chemistry*

The syntheses of the target azide derivatives are outlined in Figs. 1 and 2. More specifically, estrone was reacted with propargyl alcohol, 4-pentyn-1-ol and 4-pentyn-2-ol in the presence of potassium ethoxide to provide the corresponding 17α-alkynylol derivatives of estradiol (**1**, **2**, **8**). Then, the primary and secondary hydroxy functionalities of these compounds were mesylated by reaction with methanesulfonylchloride using triethylamine as base. It is noteworthy however that in the coarse of mesylation reaction of 17α-(hydroxypropargyl)-3,17β-estradiol (**1**), the simultaneous partial mesylation of the 17β-tertiary hydroxyl group was also observed, affording the corresponding dimethylsulphonate product **3b** (25% yield). It is evident that under these specific experimental conditions the 17β-tertiary hydroxy groups of triols (**2** and **8**) and the phenol functionalities were not reacted. Finally, the displacement of the aforementioned methanesulphonate groups by reaction with sodium azide in anhydrous DMF, resulted in the production of the desired target azides (**5**, **6**, **7**, **10**) in very good overall yields.

3.2. *Binding to isolated human ERα and ERβ*

The RBA values of the new alkynylazido derivatives of E2 are shown in Table 1. Azide **5** binds very strongly to both ERα and ERβ and, in addition, exhibits lack of preference for any ER subtype. In fact, the RBA of **5** are practically similar to those of E2 (Table 1) and EE2 [8,9]. When compared to the known 17α-alkynyl-substituted estradiol derivatives containing three carbon side chains, **5** exhibits equivalent (17α-(fluoropropargyl estradiol, RBA = 83%) or considerably higher (17α-propynyl estradiols, RBA = 32 and 49%, respectively; 17α-iodopropargyl estradiol, RBA = 28%) RBAα [10].

Substitution of the azide moiety for the 17β-hydroxy group of **5** to produce diazide **7** dramatically decreased the RBA for either type of ER, in accordance with findings that two hydroxyl groups with an O—O distance of 10.5–12.5 Å are nec-

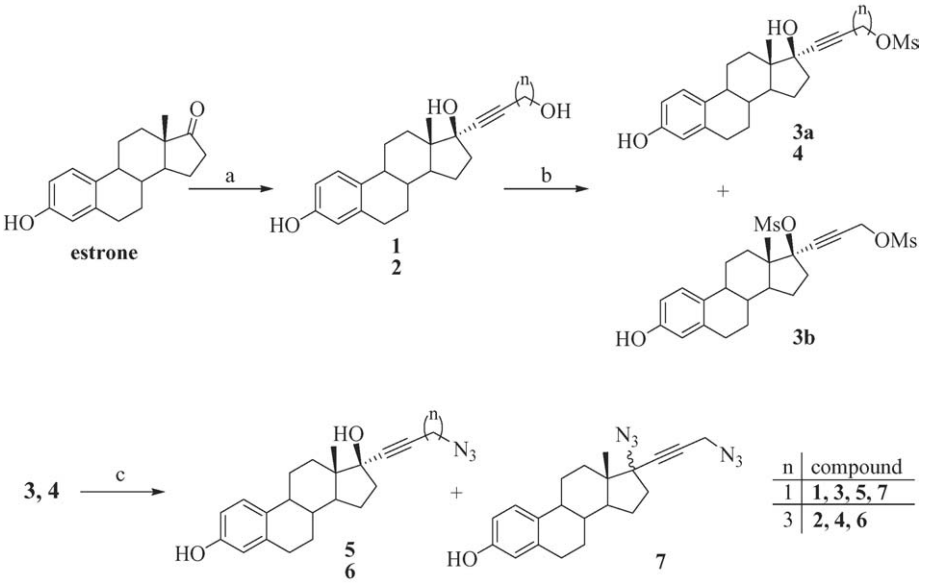

Fig. 1 – Synthesis of the azides 5, 6 and 7. Reagents and conditions: (a) KOEt, propargyl alcohol or pentyn-1-ol, THF, 0 °C; (b) MsCl, Et_3N, THF; (c) NaN_3, DMF, 50 °C.

essary for high affinity binding to either type of ER [17,18]. Indeed, in the crystal structure of E2-ERα complex, the 3- and 17β-OH groups of E2 (with an O—O distance of ~10.8 Å) form hydrogen bonds with Glu353/Arg394 and His524, respectively, of ERα [17]. Similarly, in the crystal structure of the genistein-ERβ complex, the 4′- and 7-OH of genistein (with an O—O distance of ~12.1 Å) form hydrogen bonds with Glu305/Arg346 and His475, respectively, of ERβ [18]. It is possible that **5** and **7** are oriented similarly to E2 inside the ER binding pocket, with the respective His acting as hydrogen bond acceptor and donor, respectively. Although **5**, **6** and **10** possess appropriately spaced OH groups, the RBA values of the two latter azides are comparatively much lower, which is suggestive of steric hindrance affecting their overall binding capacity. In accordance with this finding, 17α-alkynyl derivatives with long side chains have been reported to possess low affinities for the ER [10–13]. Contrary to **5** and **7**, the azides **6** and **10** exhibit considerable ERα-binding selectivity, probably reflecting a more pronounced steric hindrance for binding to ERβ. Significantly, it has been reported that the binding pocket of ERα is considerably more spacious than that of ERβ [17,18].

3.3. *Induction of alkaline phosphatase activity in Ishikawa cells*

The AlkP expression of human Ishikawa endometrial adenocarcinoma cells is positively regulated by estrogens in a ER-dependent manner and is considered as a very sensitive means to assess the ER agonist and antagonist activity of plant-derived compounds and pharmaceutical or commodity chemicals as well as steroids [16,19]. In this context, only azides **5** and **7** were effective inducers of AlkP activity in the range of concentrations tested (1 pM to 1 μM; Fig. 3A). In fact, **5** was only ~10-fold less potent than E2 in inducing AlkP in Ishikawa cells, while **7** was 63-fold less potent than **5** (Table 2).

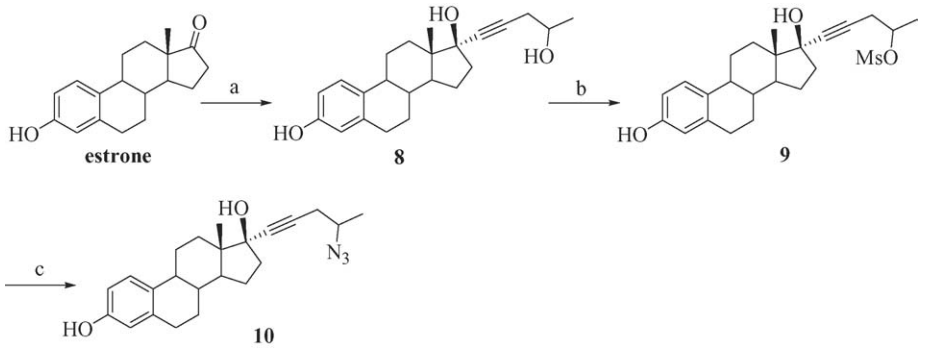

Fig. 2 – Synthesis of the azide 10. Reagents and conditions: (a) KOEt, pentyn-2-ol, THF, 0 °C; (b) MsCl, Et_3N, THF; (c) NaN_3, DMF, 50 °C.

Notably, the efficacy of the AlkP response of **5** was significantly higher (ANOVA; $p < 0.05$) than that of E2 (Fig. 3B), probably reflecting that the latter is far more prone to metabolic inactivation (e.g. by conversion to estrone) as compared to the former. The efficacy of the AlkP response of **7** was comparable to that of E2; by contrast, the other azides were either marginally active (**6**) or totally inactive (**10**), even at very high concentrations (Fig. 3A). On the other hand, while the AlkP response of the cells in the presence of 0.1 nM E2 was either enhanced (**5**) or not significantly affected (**7**) by the simultaneous presence of 1 μM of the estrogenic azides, **6** was significantly (ANOVA; $p < 0.05$) repressive and **10** was inactive at this concentration under these conditions (Fig. 3B). The induction of AlkP by E2 was fully inhibited by the pure antiestrogen ICI 182,780, indicating that the response was ER-mediated. These data suggest that **5** and **7** are pure ER agonists in Ishikawa cells; and that contrary to **6**, which may bind to ER (Table 1) and repress the AlkP expression of the cells at ≥1 μM, **10** is inactive in this respect.

3.4. *Induction of an artificial luciferase reporter gene stably inserted in MCF-7 cells*

The luciferase expression of MCF-7:D5L cells is a very sensitive means to assess the ER agonist and antagonist activity of phytoestrogens, xenoestrogens and medicinal estrogens and antiestrogens ([16]; data not shown). These cells, which are a subclone of human MCF-7 mammary adenocarcinoma cells, have been engineered to express the gene under the control of an ERE enhancer located upstream of the β-globin promoter [16]. Treatment of MCF-7:D5L cells with **5**, **6**, **7** or **10** in the absence or presence of 0.1 nM E2 elicited an ER-dependent luciferase response similar to the AlkP response of Ishikawa cells (data not shown). In addition, the potencies of **5**, **6**, **7** or **10** in this system were similar to those observed using Ishikawa cells (Table 2).

3.5. *Stimulation of the proliferation of MCF-7 cells*

Compounds **5** and **7** stimulated the proliferation of MCF-7 cells in estrogen-deprived media with a relative potency comparable to that observed for the AlkP and luciferase responses

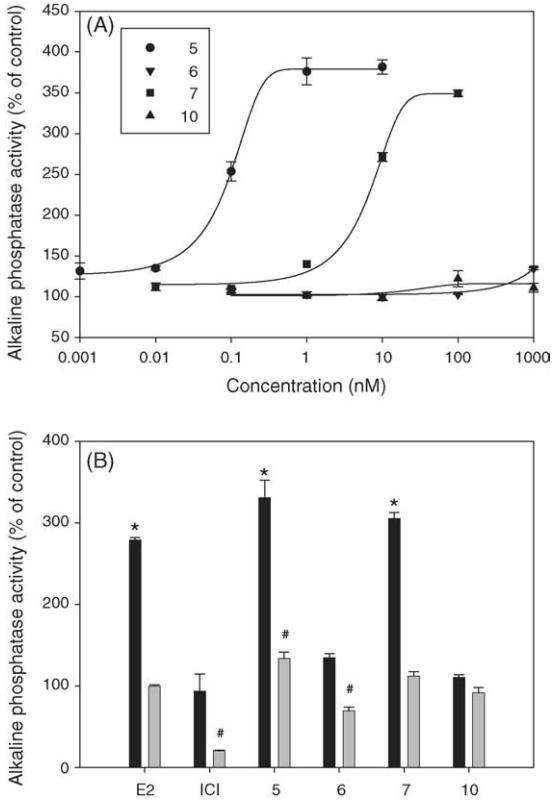

Fig. 3 – (A) Dose–response curves of the induction of AlkP activity in Ishikawa cells by serial 1/10 dilutions of the azides 5, 6, 7 and 10. (B) The AlkP response of Ishikawa cells exposed to E2 (0.1 nM), the estrogen antagonist ICI 182,780 (1 μM), or the azides 5, 6, 7 and 10 (1 μM) for 3 days, in the absence (black bars; steroid-free medium as control) or presence of 0.1 nM E2 (grey bars; estradiol-supplemented medium as control). Data are mean ± S.E.M. of three independent experiments. The significance of the difference in AlkP activity between control and treated cells in the absence (indicated by '*') or presence (indicated by '#') of E2 was determined using one-way ANOVA. E2, 17β-estradiol; ICI = ICI 182,780.

Table 2 – Estrogenic responses of the 17α-substituted derivatives relative to estradiol

Compound	AlkP activity		Luciferase activity		Cell proliferation	
	EC25[a] (nM)	Relative potency[b]	EC25[a] (nM)	Relative potency[b]	EC25[a] (nM)	Relative potency[b]
5	0.07 ± 0.01	8.78 ± 0.99	0.10 ± 0.02	5.04 ± 0.83	0.04 ± 0.01	6.71 ± 2.25
6	n-a	n-a	n-a	n-a	n-a	n-a
7	4.14 ± 0.28	0.14 ± 0.04	5.07 ± 1.15	0.10 ± 0.02	4.28 ± 1.06	0.05 ± 0.01
10	n-a	n-a	n-a	n-a	n-a	n-a
Estradiol	5.9[c]	100	4.8[c]	100	1.9[c]	100

n-a = non-applicable.

[a] EC_{25} values are test compound concentrations required to achieve 25% of the effect of 0.1 nM estradiol in Ishikawa cells (AlkP activity), MCF-7:D5L cells (luciferase activity) and MCF-7 cells (cell proliferation as assessed using MTT). Values are mean ± S.E.M. of at least three independent experiments.

[b] Relative potency was calculated by $[100 \times EC_{25}\ \text{estradiol}]/[EC_{25}\ \text{derivative}]$.

[c] pM.

(Table 2). Again, **10** was largely ineffective, whereas **6** was a weak antagonist in this system also.

On the whole, the above data suggest that, while **5**, **6**, **7** or **10** interact with either type of ER in isolation, only **5** and **7** exhibit substantial ER agonist activity in the different estrogen-target cells examined. In the light of these findings and in view of the fact that azide derivatives usually are the primary choice for photoaffinity labelling studies [20], **5** and **7** could provide a means to: (i) explore the plasticity of E2-ER interface in the cell as well as in isolation, (ii) identify estrogen target proteins in the different cellular compartments and (iii) track the protein partners of the receptor in different cell contexts with the help of a chemical cross-linking approach.

3.6. *Conclusions*

We synthesized four new 17α-alkynyl-substituted estradiols bearing an azide group at the side chain. One of them, monoazide **5**, exhibited RBA for both estrogen receptors similar to those of E2. Their RBA was strongly dependent on the presence of two appropriately spaced OH groups (the case of diazide **7**) as well as on the balkiness of the 17α- substituent (the case of monoazides **6** and **10**). The estrogenic potency of the substituted estradiols varied in a manner that is primarily dependent on the RBA for the receptor. As assessed using estrogen-responsive mammary and endometrial adenocarcinoma cells, the estrogenic potency of **5** and **7** was ∼10 and ∼1000 times lower, respectively, whereas that of **6** and **10** was marginal as compared to that of E2. The possibility of using **5** and **7** and derivatives thereof to photo-label the ER in the cell as well as in isolation is considered.

Acknowledgements

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