Pergamon

Bioorganic & Medicinal Chemistry Letters 12 (2002) 1283–1285

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

# Synthesis of a Novel Fluorescent Probe for Estrogen Receptor

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Received 17 December 2001; accepted 20 February 2002

**Abstract**—A novel estradiol-mimetic fluorescent probe **5** was synthesized from diethylstilbestrol (DES, **1**), which is useful for probing estrogen receptor (ERα), a prognostic indicator of estrogen-dependent cancers, and for developing a homogeneous fluorescence polarization (FP) assay to identify the ligands of estrogen receptor. 

Estrogen receptors (ER) regulate the expression of genes,[1] which control the differentiation, growth and function of reproductive system in females.[2] They also play an important role in the growth of estrogen-dependent tumors such as breast cancer and other endocrinological disorders.[3] Breast cancer is the second leading cause of death in women in United States and the levels of estrogen receptors (ER) on tumor cells (estimated range: 5000–50,000 molecules/cell) have been shown to be a prognostic indicator of the disease.[4] As with most cancers, the early diagnosis of breast cancer is key for long-term survival, and the choice of treatment is aided by the determination of estrogen receptor levels on a tumor cell. Additionally, development of screening assays for estrogen receptor ligands facilitate the search for new and improved agonists and antagonists of estrogens,[5] which are potentially useful for treatment of cancers, Alzheimer's disease, cardiovascular diseases, diabetes, osteoporosis, and urinary incontinence.[6] Generally, the estrogen receptor content in a breast tumor is determined either by imaging methods, for example positron emission tomography (PET), single photon emission computerized tomography (SPECT) using radiolabeled tracers or in vitro analysis of tumor biopsy sample.[7] Limitations of these methods, coupled with the increasing need for high-throughput screening (HTS),[8] have stimulated interest in the development of new homogeneous assay technologies for determination of estrogen receptors as well as its ligand screening.

Fluorescence polarization (FP)[9] method is useful to study molecular interactions, which is based on the principle that a fluorescent molecule when excited with polarized light will emit fluorescence having a degree of polarization inversely proportional to its rate of rotation. Small fluorescent molecules (e.g., probe) rotate faster in solution than the larger molecules, (e.g., ER–probe complex) and hence, when the probe bound to ER, its rotation is slowed and the polarization value (mP) is increased. Thus, homogeneous FP technique is widely used to investigate molecular binding events in solution.[9] The probes for ER, from chemistry point of view, to be able to serve effectively in a homogeneous FP format would be those derived from its native ligand, 17β-estradiol. We[10] and others[11] have reported a variety of fluorescent probes needed for development of immunoassays for 17β-estradiol. However, in our hands, the 17β-estradiol-derived fluorescent probes,[9] which were labeled at the 6-, 7- and 17-positions of 17β-estradiol, exhibited poor binding to estrogen receptor (ERα) and were not suitable for development of homogeneous FP assay. A possible explanation for the lack of binding of these 17β-estradiol-derived probes to ER might be that the critical binding determinants were blocked when the 17β-estradiol was conjugated with a fluorescent label and thus rendering them to be less than perfect mimics of 17β-estradiol to the native protein, ER.[10] Recently, Parker et al.[12] reported an FP-based assay for estrogen receptor, which is now commercially available from PanVera Corporation (Madison, WI, USA), with undisclosed chemistry of the probe. In continuation of our interest in the development of fluorescence and chemiluminescence assays for the measurement of 17β-estradiol[9] and other steroidal hormones,[13] we decided to design a new fluorescent probe for studies of

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0960-894X/02/$ - see front matter 
PII: S0960-894X(02)00144-0

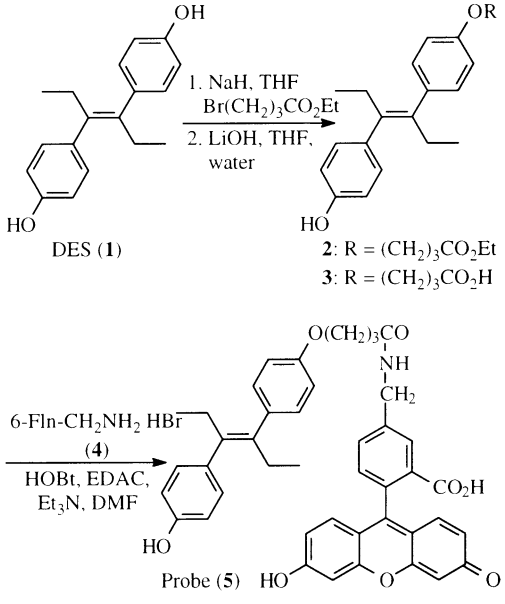
estrogen receptor (ERα) in homogeneous fluorescence polarization assay format.

We envisioned that a fluorescent conjugate derived from non-steroidal substrates, which are known to bind to estrogen receptor (ER), could be more efficient for probing the estrogen receptor. Although, the overall molecular topology of a non-steroidal ER ligand, diethylstilbestrol (DES, **1**) is different from the native ER ligand, 17β-estradiol, it has been reported that the oxygen–oxygen distance in DES (**1**, 12.1 Å) is close to the O–O distance between the 3- and 17-positions of 17β-estradiol (10.9 Å).[14] The difference of O–O distance (1.2 Å) between DES (**1**) and 17β-estradiol is well within the mobility (7 Å) of ER binding pocket,[14] and thus rendering DES (**1**) to be a promising candidate for estrogen receptor probe development. Accordingly, DES (**1**) was selectively alkylated (Scheme 1) with ethyl 4-bromobutyrate[15] using 1.1 equiv of NaH in DMF to afford the mono *O*-alkylated product **2** in 42% yield. The ester group in **2** was then hydrolyzed with LiOH in THF–water medium to afford the corresponding acid **3** in 95% yield. The acid **3** was reacted with 6-aminomethylfluorescein hydrobromide (6-Fln-$CH_2NH_2$ HBr, **4**) in the presence of HOBt, EDAC and triethylamine in DMF at room temperature for 16 h. The crude product was purified by preparative reversed-phase HPLC[16] and lyophilized to afford the fluorescent probe **5** in 20% yield as an orange powder in >99% purity.[17]

We then proceeded to assess the suitability of **5** as an estradiol-mimetic probe for ER in a homogeneous fluorescence polarization assay format using ISS PC1 photon counting spectrofluorometer. Thus, a 1.0 mM stock solution of fluorescent probe **5** in DMSO was prepared, which was diluted with 100 mM potassium phosphate buffer (pH 7.5) to obtain 1.0 nM solution, and aliquoted into individual tubes (2.0 mL/tube). Different amounts of estrogen receptor (ERα, 5800 nM) was added to the each tube to achieve final concentration of ERα ranging from 34.6 to 0.017 nM. As a control, bovine serum albumin (BSA) solution was prepared similarly to the ERα and mixed with 2.0 mL of probe **5** solution (1.0 nM) in individual tubes. All samples were then incubated at room temperature in dark for 2 h and fluorescence polarization value (mP) was measured in triplicates (Fig. 1). The titration curve showed that the probe **5** bound tightly to ERα ($K_D$, 5.8±1.2 nM). The dynamic range of fluorescence polarization for **5** was more than 350 mP. The control experiment using BSA showed only 50 mP increase FP value even at a concentration of about 40 nM of ERα.

Competitive displacement studies were then conducted with three ligands (17β-estradiol, diethylstilbestrol and tamoxifen) for their ability to displace the probe **5** from estrogen receptor (ERα) complex. Thus, solution of estrogen receptor (ERα, 36 μL, 5800 nM) was added to 30 mL of 0.2 nM probe **5** in 100 mM potassium phosphate buffer containing 0.5 mg/mL β-cyclodextrin and the mixture was aliquoted into individual tubes (2.0 mL/tube). The stock solution of 17β-estradiol (10 mM in DMSO) was diluted with the same buffer (100 mM potassium phosphate buffer containing 0.5 mg/mL β-cyclodextrin) and different amounts were added to the tubes containing ERα-probe **5** complex to achieve a final concentration of 0.1, 1.0, 2.0, 4.0, 6.0, 8.0, 10, 12, and 22 nM of 17β-estradiol, respectively. The measured fluorescence polarization value (mP) was plotted against the concentration (nM) of 17β-estradiol. A four-parameter logistic fitting in Grafit was used to obtain an inhibition constant ($IC_{50}$) of 3.40 (±1.0) nM for 17β-estradiol (Fig. 2). A similar displacement assay was carried out with diethylstilbestrol and tamoxifen as the inhibitors and the $IC_{50}$ values were determined to be 0.33 (±0.1) and 8.40 (±1.5) nM, respectively.

In summary, a novel estradiol-mimetic probe **5** was synthesized and its application in the development of homogeneous fluorescence polarization (FP) assay for




**Scheme 1.** Synthesis of fluorescent probe **5**.

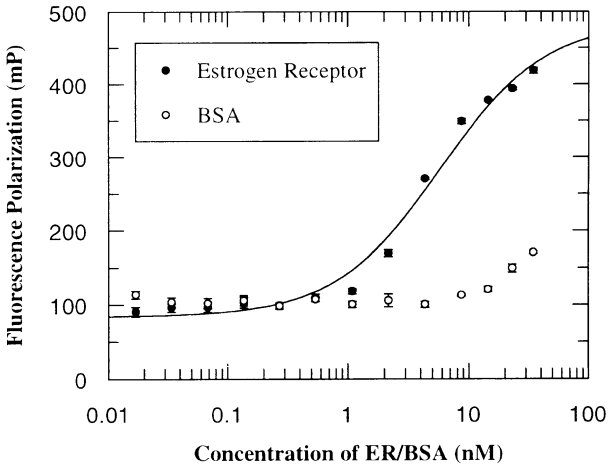



**Figure 1.** A titration curve for binding of estrogen receptor (ERα) and fluorescent probe **5**, with BSA as a control. Excitation wavelength in the ISS PC1 photon counting spectrofluorometer was set to 480 nm via a monochromator (1.0 mm slit). Polarized fluorescence was passed through a Crion 383U XM-530-F filter (530 nm, bandpass 25 nm) and the intensity was monitored by an internal PMT.

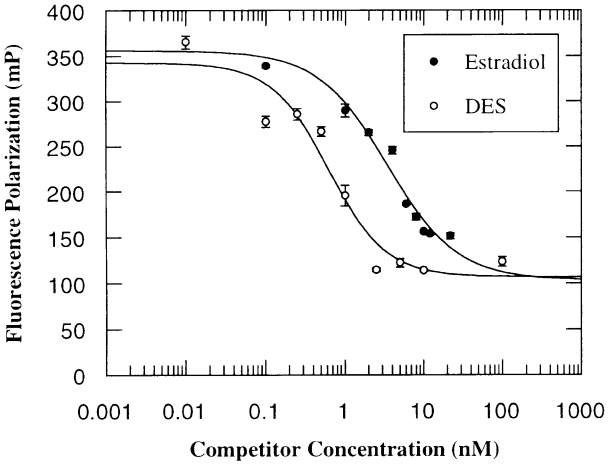



**Figure 2.** Solution competition assay using 17β-estradiol and diethylstilbestrol to displace probe **5** from estrogen receptor-α.

estrogen receptor (ERα) and screening of its ligands was demonstrated. Furthermore, the probe **5** should find wide spread use in other fluorescent techniques for estrogen receptor studies.

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16. Preparative reversed-phase (RP) HPLC was carried out on a Waters column (Symmetry, RCM, C18, particle size: 7.0 μm, pore size: 100 Å, 40×100 mm) using MeCN/0.1% aqueous trifluoroacetic acid, 50:50, 45 mL/min at 225 nm.
17. Analytical reversed-phase (RP) HPLC (Waters Symmetry, RCM, C18, particle size: 7.0 μm, pore size: 100 Å, 8×100 mm column): MeCN/0.1% aqueous trifluoroacetic acid, 50:50, 2.0 mL/min at 254 nm, $R_t$: 7.88 min, >99%.