A synthesis of 7α-substituted estradiols: synthesis and biological evaluation of a 7α-pentyl–substituted BODIPY fluorescent conjugate and a fluorine-18–labeled 7α-pentylestradiol analog

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In an effort to assist in the preparation of ligands for the study of the estrogen receptor (ER), we have developed a new synthesis of 7α-substituted estradiols. The key step in the synthesis involves a copper-catalyzed, α-selective, 1,6-conjugate addition of 4-pentenyl magnesium bromide to a suitably protected 6-dehydrotestosterone derivative. Desaturation and then reductive aromatization of the resulting 7α-pentenyl androgen gave the 7α-pentenylestradiol in good yields. The α-stereoselectivity of this addition in the testosterone series, compared with the 19-nortestosterone series, is significantly improved by the presence of the C-19 methyl group, which shields the beta face from attack. A key intermediate was functionalized further by substitution with fluorine-18 to provide a potential imaging agent for positron emission tomography, and by conjugation with a BODIPY (Molecular Probes Inc., Eugene, OR, USA) fluorophore to make a fluorescent probe for the estrogen receptor. The synthesis and biological evaluation of these analogs is presented, as well as a discussion of the improvements in the synthetic procedure. (*Steroids* **58**:157–169, 1993)

Keywords: steroids; estrogen receptor; fluorescence; breast tumor imaging; fluorine-18; 7α-substituted estrogen

Introduction

The synthesis of substituted estradiol derivatives to explore estrogen receptor (ER) structure and function remains a challenge to the organic/medicinal chemist. Selection of the appropriate site of substitution, as well as the type of substituent and the synthesis of the desired compounds with good diastereoselectivity, contribute to this challenge. Compounds developed through this approach can provide information at the molecular level about how ER functions and at the clinical level can open up new approaches for the diagnosis and treatment of hormone-dependent breast cancer.

We have had a long-standing interest in the design and synthesis of substituted estrogens as affinity labels,[1–4] fluorescent probes,[5–9] and radiopharmaceuticals for the study of estrogen-responsive breast tumors.[10–12] Several 16α-fluoroestradiols, both with and without substituents at C-11β, have been synthesized in our laboratory,[11,13,14] and have been used to successfully image both primary and metastatic breast tumors.[15,16] The tolerance of the 11β position to substitution led us to synthesize other radiopharmaceuticals with the radionuclide positioned on the pendant C-11β substituent itself.[17] Although these

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Received June 22, 1992; accepted December 24, 1992.

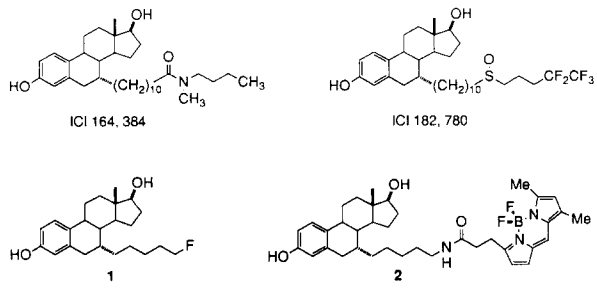


Figure 1 Examples of 7α-substituted estrogens.

latter compounds proved to be less effective than those previously synthesized, they established the tolerance limits for substituents at this site, and thus suggested the need for exploring other sites for substituent attachment.

Some time ago, Bucourt et al.[18] noted that long hydrocarbon chains placed at C-7α of estradiol were well tolerated by ER. More recently, Bowler et al. have described a series of estrogens substituted at C-7α with a variety of undecyl amides and various sulfoxides that display potent and pure antiestrogenic activity.[19] In addition, Mühlenbruch et al.[20] and DaSilva and VanLier[21] have described other estrogen derivatives substituted with large groups at the 7α position. The high receptor-binding affinity (RBA) of these compounds convinced us to pursue the 7α position as a site of attachment of affinity labels, radionuclides, and fluorescent probes.

In this report we describe a new synthesis of 7α-alkyl–substituted estradiols. The improvements, with respect to previous syntheses, are discussed. The synthesis of two analogs, a fluorine-substituted derivative (**1**) and a fluorescent conjugate (**2**) (Figure 1), are given to demonstrate the utility of this synthesis. Both of these compounds, as well as the others we have synthesized, display excellent binding affinity to ER. The fluoropentyl derivative (**1**) was prepared in fluorine-18 (^{18}F)-radiolabeled form and its biodistribution studied in immature rats. The fluorescence properties of the BODIPY (Molecular Probes Inc., Eugene, OR, USA) conjugate (**2**) were studied by fluorescence spectroscopy, both in solution and when bound to cell-free preparations of ER.

Experimental

Chemical syntheses

General. Melting points (mp) were determined on a Thomas Hoover melting point apparatus and are uncorrected. Analytical thin-layer chromatography (TLC) was performed using 0.25-mm silica gel plates with F-254 indicator (Merck). Visualization was achieved by ultraviolet (UV) light, phosphomolybdic acid, or anisaldehyde spray reagents. Flash chromatography was performed as previously described,[22] using Woelm silica gel (32–64 μm). Ozonolyses were performed on a Welsbach T-816 ozonator. High-performance liquid chromatography (HPLC) was performed isocratically with a Spectra-Physics SP8700 liquid chromatograph, using a Whatman Partisil M-9 (9 mm × 50 cm) preparative silica gel column at a flow rate of 5 ml/min. The solvents are given parenthetically. The HPLC eluent was monitored for UV absorbance at 254 nm; for the radiochemical preparations, HPLC eluent was also monitored with an NaI(Tl) radioactivity detector. Radioactivity was measured in a Capinec dose calibrator.

Proton nuclear magnetic resonance (NMR) spectra were obtained on a Varian XL-200 (200 MHz) or General Electric QE-300 (300 MHz) spectrometer with $CDCl_3$ as solvent unless otherwise noted; chemical shifts are reported downfield from a tetramethylsilane internal standard (δ scale). Infrared (IR) spectra were obtained on a Perkin Elmer 1320 spectrometer in the indicated phase: predominant and diagnostic peaks are reported. Low-resolution electron impact mass spectra (EIMS) were obtained on a Finnigan MAT CH-5 spectrometer. High-resolution mass spectra were obtained on a Finnigan MAT 731 spectrometer. Fast-atom bombardment mass spectra (FABMS) were obtained on a Varian ZAB spectrometer with a xenon gun as the ionizer and dithiothreitol as matrix. Elemental analyses were performed by the Microanalytical Services Laboratory of the University of Illinois. Reagent grade chemicals were obtained from Aldrich (Milwaukee, WI). BODIPY succinimidyl ester was obtained from Molecular Probes Inc. (Eugene, OR).

Unless otherwise noted, a standard procedure for product isolation was used. This involved quenching the reaction mixture with an aqueous solution, exhaustive extraction with an organic solvent, and drying of the organic phase over an anhydrous salt. The standard product purification was flash column chromatography on 32–64 μm silica gel. These procedures are indicated by the phrases "standard workup" (quenching solvent, extraction solvent, drying agent) and "flash column chromatography" (solvent system).

7α-(4-Pentenyl)testosterone 17-acetate (4a). 5-Bromopentene (2.10 g, 14.1 mmol) was added slowly to a hot solution of magnesium turnings (342 mg, 14.2 mmol) in tetrahydrofuran (THF) (5 ml) under argon. Upon completion of the addition, the resulting slurry was diluted with Et_2O and THF (10 : 5), and cooled to −40 C. Copper (I) iodide (1.34 g, 7.04 mmol) was added in one portion as a solid, and the slurry was stirred at −40 C for 45 minutes. 6-Dehydrotestosterone 17-acetate (**3**) (1.156 g, 3.52 mmol)[23] was added as a solution in 1 : 1 Et_2O : THF over a period of 2 hours. The solution was maintained at −35 to −40 C for two hours and quenched with 5 N HCl. Workup involved filtration of the copper salts and standard workup (EtOAc, $MgSO_4$) and yielded a yellow oil as a mixture of two diastereomers (**4a** + **4b**) (4 : 1 α : β). Separation of the diastereomers was accomplished by flash column chromatography (5/95 EtOAc/hexane) and yielded a single diastereomer (**4a**) as a pale yellow oil (790 mg, 56%). The β-diastereomer (**4b**) was isolated as a white solid (190 mg, 13%) ^{1}H NMR (α-diastereomer) (300 MHz) δ 0.83 (s, 3H, C18-C$\underline{H}_3$), 1.19 (s, 3H, C19-C$\underline{H}_3$), 2.04 (s, 3H, C17β-OCOC$\underline{H}_3$), 4.60 (t, J = 7.6, 1H, C17α-C$\underline{H}$), 4.96 (dd, 2H, C5′-C$\underline{H}_2$), 5.72 (s, 1H, C4-C$\underline{H}$), 5.75 (m, 1H, C4′-C$\underline{H}$). MS (70 eV) m/z relative intensity (RI) 398 (15), 338 (7), 329 (8). High-resolution EIMS calculated for $C_{26}H_{38}O_3$: 398.2821. Found: 398.2922. 7β-diastereomer: mp 104–105 C; ^{1}H NMR (300 MHz) δ 0.83 (s, 3H, C18-C$\underline{H}_3$), 1.15 (s, 3H, C19-C$\underline{H}_3$), 2.04 (s, 3H, C17β-OCOC$\underline{H}_3$), 4.54 (t, J = 7.7, 1H, C17α-C$\underline{H}$), 4.98 (dd, 2H, C5′-C$\underline{H}_2$), 5.71 (s, 1H, C4-C$\underline{H}$), 5.72 (m, 1H, C4′-C$\underline{H}$). High-resolution EIMS calculated for $C_{26}H_{38}O_3$: 398.2821. Found: 398.2923.

7α-(4-Pentenyl)androsta-1,4-dien-17β-ol 17-acetate (5). A 3-necked, round-bottomed flask was charged with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (378 mg, 1.66 mmol), enone (**4a**) (510 mg, 1.20 mmol), and 1,4-dioxane (50 ml). The

solution was heated to 120 C for 5 hours. The solution was cooled, and the dioxane was removed under reduced pressure. Standard workup (H_2O, EtOAc, $MgSO_4$) and flash column chromatography (40/60 EtOAc/hexane) gave a pale oil, (**5**) (200 mg, 40%). ^{1}H NMR (300 MHz), δ 0.87 (s, 3H, C18-C$\underline{H}_3$), 1.24 (s, 3H, C19-C$\underline{H}_3$), 2.05 (s, 3H, C17β-OCOC$\underline{H}_3$), 4.60 (t, J = 8.5 Hz, 1H, C17α-C$\underline{H}$), 4.97 (dd, J_1 = 9.0 Hz, J_2 = 1.2 Hz, 2H, C5′-C$\underline{H}_2$), 5.77 (m, 1H, C4′-C$\underline{H}$), 6.06 (s, 1H, C4-C$\underline{H}$), 6.23 (d, 1H, C2-CH), 7.05 (d, 1H, C1-C$\underline{H}$). MS (70 eV) m/z (RI), 396 (6), 215 (8), 122 (100). High-resolution EIMS calculated for $C_{26}H_{36}O_3$: 396.2664. Found: 396.2665.

7α-(4-Pentenyl)estradiol (6). A three-necked flask was charged with lithium (1.5 g, 53.7 mmol), biphenyl (2.37 g, 15.4 mmol), and diphenylmethane (1.29 g, 7.68 mmol) in THF (50 ml). The solution was heated to reflux, and a solution of dienone (**5**) (1.52 mg, 3.84 mmol) in THF was added dropwise over 1 hour. The solution was refluxed for 45 minutes, then cooled to room temperature. The excess lithium was quenched with methanol and water, and the organic phase was evaporated under reduced pressure. A 200-ml portion of 3 : 1 benzene : hexane was added to the resulting aqueous solution and extracted with Claisen's alkali.[24] The aqueous layer was washed with hexane and neutralized with acetic acid. Standard workup (EtOAc, sat, $NaHCO_3$, $MgSO_4$) and flash column chromatography (15/85 EtOAc/hexane) gave a clear oil (**6**) that crystallized from deuteriochloroform (780 mg, 50%). ^{1}H NMR (300 MHz), δ 0.78 (s, 3H, C18-C$\underline{H}_3$), 3.76 (t, J = 8.4 Hz, 1H, C17α-C$\underline{H}$), 5.00 (dd, 2H, C5′-C$\underline{H}_2$), 5.80 (m, 1H, C4′-C$\underline{H}$), 6.55 (d, J = 2.3 Hz, 1H, C4-C$\underline{H}$), 6.63 (dd, J = 8.4 Hz, J_2 = 2.4 Hz, 1H, C2-C$\underline{H}$), 7.15 (d, J = 8.5 Hz, 1H, C1-C$\underline{H}$). EIMS (70 eV) m/z (RI), 340 (56), 269 (49), 157 (100). High-resolution EIMS calculated for $C_{23}H_{32}O_2$: 340.2402. Found: 340.2404. Analysis calculated for $C_{23}H_{32}O_2$: C, 81.13; H, 9.47. Found: C, 81.09; H, 9.59. Crystals for x-ray analysis were grown from deuteriochloroform and stored in mother liquor at 0 C. Cell parameters at −65 C: a = 16.395(5) Å, b = 16.765(5) Å, c = 17.734(5) Å, Z = 8. Refinement of 185 variables against 1,342 data gave residuals R = 0.167 and R_w = 0.204.

7α-(5-Hydroxypentyl)estradiol (7). A flask was charged with primary olefin (**6**) (50 mg, 0.15 mmol) in THF (5 ml) and stirred with 9-borabicyclo[3.3.1]nonane (9-BBN) (1.5 ml of a 0.5-M solution, 0.75 mmol) at room temperature for 16 hours. KOH (3N, 0.25 ml), followed by 30% H_2O_2 (0.25 ml) was added to the solution by syringe, and stirring was continued for 5 hours. Standard workup (H_2O, EtOAc, $MgSO_4$) and flash column chromatography (30/70 EtOAc/hexane) gave **7** as a clear oil (30 mg, 57%).^{1}H NMR (300 MHz), δ 0.79 (s, 3H, C18-C$\underline{H}_3$), 2.73 (dd, 2H, C6-C$\underline{H}_2$), 3.52 (dd, J = 6.0 Hz, 2H, C5′-C$\underline{H}_2$), 3.69 (t, J = 8.1 Hz, 1H, C17α-C$\underline{H}$), 6.53 (d, J = 2.4 Hz, 1H, C4-C$\underline{H}$), 6.60 (dd, J_1 = 8.4 Hz, J_2 = 2.6 Hz, 1H, C2-C$\underline{H}$), 7.11 (d, J = 8.4 Hz, 1H, C1-C$\underline{H}$). EIMS (70 eV) m/z (RI), 358 (82), 271 (25), 157 (100), 145 (50). High-resolution EIMS calculated for $C_{23}H_{34}O_3$: 358.2508. Found: 358.2504.

7α-(4-Pentenyl)estradiol 3-benzyl ether (8). A solution of estradiol (**6**) (180 mg, 0.53 mmol) in 2-butanone (50 ml) was stirred over potassium carbonate with benzyl bromide (668 mg, 5.28 mmol) at reflux for 12 hours. Standard workup (H_2O, EtOAc, $MgSO_4$) and flash column chromatography (35/65 (EtOAc/hexane) gave **8** as a clear oil that foamed under vacuum (198 mg, 83%). ^{1}H NMR (300 MHz), δ 0.77 (s, 3H, C18-C$\underline{H}_3$), 3.74 (t, J = 8.3 Hz, 1H, C17α-C$\underline{H}$), 5.00 (dd, 2H, C5′-C$\underline{H}_2$), 5.02 (s, 2H, C3-OC$\underline{H}_2$Ph) 6.71 (d, J = 2.4 Hz, 1H, C4-C$\underline{H}$), 6.60 (dd, J_1 = 7.6 Hz, J_2 = 2.4 Hz, 1H, C2-C$\underline{H}$), 7.23 (d, J = 6.5 Hz, 1H, C1-C$\underline{H}$). 7.30–7.45 (m, 5H, C3-OCH$_2$C$_6\underline{H}_5$). EIMS (70 eV) m/z (RI), 430 (17), 91 (100). High-resolution EIMS calculated for $C_{30}H_{38}O_2$: 430.2872. Found: 430.2866.

7α-(5-Hydroxypentyl)estradiol 3-benzyl ether (9). A flask was charged with primary olefin (**8**) (100 mg, 0.23 mmol) in THF (5 ml) and stirred with 9-BBN (4.6 ml of a 0.5-M solution, 2.32 mmol). The solution was stirred at room temperature for 16 hours. KOH (3N, 0.75 ml) followed by 30% H_2O_2 (0.75 ml) was added to the solution by syringe and stirred for 5 hours. Standard workup (H_2O, EtOAc, $MgSO_4$) and flash column chromatography (40/60 EtOAc/hexane) yielded **9** as a clear oil (84.1 mg 81%). ^{1}H NMR (300 MHz), δ 0.78 (s, 3H, C18-C$\underline{H}_3$), 2.72 (dd, 2H, C6-C$\underline{H}_2$), 3.61 (t, J = 6.6 Hz, 2H, C5′-C$\underline{H}_2$), 3.75 (t, J = 8.1 Hz, 1H, C17α-C$\underline{H}$), 5.03 (s, 2H, C3-OC$\underline{H}_2$Ph), 6.72 (d, J = 2.5 Hz, 1H, C4-C$\underline{H}$), 6.79 (dd, J_1 = 8.5 Hz, J_2 = 2.49 Hz, 1H, C2-C$\underline{H}$), 7.21 (d, J = 8.6 Hz, 1H, C1-C$\underline{H}$) 7.30–7.45 (m, 5H, C3-OCH$_2$C$_6\underline{H}_5$). EIMS (70 eV) m/z (RI), 448 (78), 357 (20), 339 (20), 91 (100). High-resolution EIMS calculated for $C_{30}H_{40}O_3$: 448.2978. Found: 448.2975.

7α-(5-Methanesulfonyloxypentyl)estradiol 3-benzyl ether (10). A solution of alcohol (**9**) (300 mg, 0.67 mmol) in THF was cooled to 0 C. Methanesulfonyl chloride (51 μl, 0.67 mmol) and triethylamine (93 μl, 0.67 mmol) were added, and the solution stirred at 0 C for 6 hours. Standard workup (H_2O, EtOAc, $MgSO_4$) and flash column chromatography (40/60 EtOAc/hexane) gave **10** as a clear oil (275 mg, 78%) as well as 40 mg of recovered starting material. ^{1}H NMR (300 MHz), δ 0.78 (s, 3H, C18-C$\underline{H}_3$), 2.72 (dd, 2H, C6-C$\underline{H}_2$), 3.00 (s, 3H, -OSO$_2$C$\underline{H}_3$), 3.75 (t, J = 8.1 Hz, 1H, C17α-C$\underline{H}$), 4.20 (t, J = 6.6 Hz, 2H, C5′-C$\underline{H}_2$), 5.03 (s, 2H, C3-OC$\underline{H}_2$Ph), 6.71 (d, J = 2.47 Hz, 1H, C4-C$\underline{H}$), 6.79 (dd, J_1 = 8.6 Hz, J_2 = 2.61 Hz, 1H, C2-C$\underline{H}$), 7.21 (d, J = 8.58 Hz, 1H, C1-C$\underline{H}$) 7.30–7.45 (m, 5H, C3-OCH$_2$C$_6\underline{H}_5$). EIMS (70 eV) m/z (RI), 526 (11), 157 (4), 91 (100). High-resolution EIMS calculated for $C_{31}H_{42}SO_5$: 526.2753. Found: 526.2765.

7α-(5-Methanesulfonyloxypentyl)estradiol (11). A solution of benzyl ether (**10**) (12.5 mg, 0.023 mmol) in ethanol : ethyl acetate 1 : 1 was stirred under hydrogen in the presence of catalytic $PdCl_2(CH_3CN)_2$ (~1 mg) and acetic acid for 20 minutes. Filtration of the reaction solution, followed by evaporation of solvent, gave an oil that was purified by HPLC (50/50 CH_2Cl_2 with 5% 2-propanol/hexane) to give a clear oil (**11**) that foamed under vacuum (9.7 mg, 94%). ^{1}H NMR (300 MHz), δ 0.78 (s, 3H, C18-C$\underline{H}_3$), 2.66–2.86 (dd, 2H, C6-C$\underline{H}_2$), 2.99 (s, 3H, -OSO$_2$C$\underline{H}_3$), 3.75 (q, J = 5.7 Hz, 1H, C17α-C$\underline{H}$), 4.20 (t, J = 6.5 Hz, 2H, C5′-C$\underline{H}_2$), 4.77 (s, 1H, C3-O$\underline{H}$), 6.55 (d, J = 2.4 Hz, 1H, C4-C$\underline{H}$), 6.64 (dd, J = 8.5 Hz, J_2 = 2.6 Hz, 1H, C2-C$\underline{H}$), 7.15 (d, J = 8.5 Hz, 1H, C1-C$\underline{H}$). EIMS (70 eV) m/z (RI), 436 (63), 377 (9), 157 (100). High-resolution EIMS calculated for $C_{24}H_{36}SO_5$: 436.2283. Found: 436.2290.

7α-(5-Fluoropentyl)estradiol (1). Benzyl ether mesylate (**10**) (12.7 mg, 0.024 mmol) was dissolved in THF (500 μl) and added to a solution of tetrabutylammonium fluoride (120 μl of a 1-M solution). The reaction solution was heated in a sealed Reactivial at 80 C for 1 hour. The reaction solution was cooled and filtered through a plug of silica and concentrated to dryness. The resulting residue was dissolved in EtOAc and EtOH and stirred under hydrogen in the presence of catalytic $PdCl_2(CH_3CN)_2$ (~1 mg) and acetic acid (10 μl) for 20 minutes. Filtration of the reaction solution, followed by evaporation of solvent, gave an oil that was purified by HPLC (40/60 CH_2Cl_2 with 5% 2-propanol/hexane) to give a clear oil (**1**) that foamed under vacuum (5.4 mg 63%).^{1}H NMR (300 MHz), δ 0.78 (s, 3H, C18-C$\underline{H}_3$), 2.67–2.85 (dd, 2H, C6-C$\underline{H}_2$) 3.75 (t, J = 8.5 Hz, 1H, C17α-C$\underline{H}$), 4.42 (dt, J_F = 47.3 Hz, J_2 = 6.0 Hz, 2H, C5′-C$\underline{H}_2$), 4.77 (s, 1H, C3-O$\underline{H}$), 6.55 (d, J = 2.3 Hz, 1H, C4-C$\underline{H}$), 6.64 (dd, J_1 = 8.4 Hz, J_2 = 2.5 Hz, 1H, C2-C$\underline{H}$), 7.15 (d, J = 8.4 Hz, 1H, C1-C$\underline{H}$).

^{19}F NMR (282 MHz, −222.63 (Septet). EIMS (70 eV) m/z (RI), 360 (100), 157 (54). High-resolution EIMS calculated for $C_{23}H_{33}FO_2$: 360.2465. Found: 360.2461.

7α-(5-Phthalimidopentyl)estradiol 3-benzyl ether (12). A solution of mesylate (**10**) (13.0 mg, 0.025 mmol) in DMF (500 μl) was refluxed with potassium phthalimide (9.0 mg, 0.025 mmol) for 48 hours. Standard workup (water, EtOAc, brine, $MgSO_4$) and flash column chromatography (50/50 EtOAc/hexane) afforded **12** as a clear oil that foamed under vacuum (6.0 mg, 71%). ^1H NMR (300 MHz), δ 0.77 (s, 3H, C18-C$\underline{H}_3$), 2.72–2.90 (dd, 2H, C6-C$\underline{H}_2$), 3.66 (t, J = 7.2 Hz, 2H, C5′-C$\underline{H}_2$), 3.74 (t, J = 8.4 Hz, 1H, C17α-C$\underline{H}$), 5.02 (s, 2H, C3-OC$\underline{H}_2$Ph), 6.695 (d, J = 2.4 Hz, 1H, C4-C$\underline{H}$), 6.79 (dd, J_1 = 8.6 Hz, J_2 = 2.40 Hz, 1H, C2-C$\underline{H}$), 7.19 (d, J = 8.6 Hz, 1H, C1-C$\underline{H}$) 7.30–7.45 (m, 5H, C3-OCH$_2$C$_6\underline{H}_5$), 7.68–7.84 (m, 4H, Phth C$\underline{H}$). EIMS (70 eV) m/z (RI), 577 (2), 486 (12), 468 (7), 91 (100). High-resolution EIMS calculated for $C_{38}H_{43}NO_4$: 577.3192. Found: 577.3188.

7α-(5-Phthalimidopentyl)estradiol (13). A solution of benzyl ether (**12**) (4.2 mg, 7.4 μmol) in ethanol : ethyl acetate 1 : 1 was stirred under hydrogen in the presence of catalytic $PdCl_2(CH_3CN)_2$ (~1 mg) and acetic acid (10 μl) for 20 minutes. Filtration of the reaction solution, followed by evaporation of solvent, gave a clear oil that was purified by HPLC chromatography (45/55 CH_2Cl_2 with 5% 2-propanol/hexane) to give **13** as a clear oil that foamed under vacuum (3 mg, 83%).^1H NMR (300 MHz), δ 0.77 (s, 3H, C18-C$\underline{H}_3$), 2.67–2.90 (dd, 2H, C6-C$\underline{H}_2$), 3.65 (t, J = 7.2 Hz, 2H, C5′-C$\underline{H}_2$), 3.74 (t, J = 8.4 Hz, 1H, C17α-C$\underline{H}$), 4.60 (s, 1H, C3-O$\underline{H}$), 6.54 (d, J = 2.5 Hz, 1H, C4-C$\underline{H}$), 6.67 (dd, J_1 = 8.6 Hz, J_2 = 2.4 Hz, 1H, C2-C$\underline{H}$), 7.15 (d, J = 8.5 Hz, 1H, C1-C$\underline{H}$), 7.68–7.84 (m, 4H, Phth C$\underline{H}$). EIMS (70 eV) m/z (RI), 487 (36), 469 (15), 373 (25), 157 (100). High-resolution EIMS calculated for $C_{31}H_{37}NO_4$: 487.2723. Found: 487.2725.

7α-(5-Azidopentyl)estradiol 3-benzyl ether (14). To a solution of mesylate (**10**) (58 mg, 0.11 mmol) in DMF (10 ml) was added sodium azide (14.3 mg, 0.22 mmol) and catalytic tetrabutylammonium hydrogen sulfate (~1 mg). The solution was heated to reflux for 4 hours. After standard workup (H_2O, EtOAc, $MgSO_4$) and flash column chromatography (40/60 EtOAc/hexane), **14** was isolated as a clear oil (52 mg, 99%). ^1H NMR (300 MHz), δ 0.78 (s, 3H, C18-C$\underline{H}_3$), 2.72–2.90 (dd, 2H, C6-C$\underline{H}_2$), 3.23 (t, J = 6.9 Hz, 2H, C5′-C$\underline{H}_2$), 3.74 (t, J = 8.4 Hz, 1H, C17α-C$\underline{H}$), 5.02 (s, 2H, C3-OC$\underline{H}_2$Ph), 6.71 (d, J = 2.5 Hz, 1H, C4-C$\underline{H}$), 6.79 (dd, J_1 = 8.6 Hz, J_2 = 2.7 Hz, 1H, C2-C$\underline{H}$), 7.23 (d, J = 8.5 Hz, 1H, C1-C$\underline{H}$) 7.30–7.45 (m, 5H, C3-OCH$_2$C$_6\underline{H}_5$). EIMS (70 eV) m/z (RI), 473 (49), 445 (7), 354 (57), 157 (33), 91 (100). 8 FTIR cm^{-1}, 3094 (OH), 2928 (CH), 2093 (N_3). High-resolution EIMS calculated for $C_{30}H_{39}N_3O_2$: 473.3042. Found: 473.3046.

7α-(5-N-BOC-aminopentyl)estradiol 3-benzyl ether (15). A solution of lithium aluminum hydride (8.2 mg, 0.216 mmol) in THF was cooled to 0 C under nitrogen. Azide (**14**) (51 mg, 0.11 mmol) was added by syringe, and the solution stirred for 20 minutes. Excess $LiAlH_4$ was quenched with water and 1 N NaOH, and the solution extracted with EtOAc, dried, and concentrated. The resulting oil was redissolved in methylene chloride and excess BOC anhydride and sodium carbonate were added. The solution was stirred for 5 hours, followed by standard workup (H_2O, CH_2Cl_2, $MgSO_4$). Flash column chromatography (70/30 EtOAc/hexane) gave **15** as a clear oil that foamed under vacuum (22 mg, 37%).^1H NMR (300 MHz), δ 0.78 (s, 3H, C18-C$\underline{H}_3$), 1.45 (s, 9H, BOC C$\underline{H}_3$), 2.70–2.93 (dd, 2H, C6-C$\underline{H}_2$), 3.08 (q, 2H, C5′-C$\underline{H}_2$NHBOC), 3.75 (t, J = 8.1 Hz, 1H, C17α-C$\underline{H}$), 4.47 (bs, 1H, -N$\underline{H}$-BOC), 5.03 (s, 2H, C3-OC$\underline{H}_2$Ar), 6.71 (d, J = 2.35 Hz, 1H, C4-C$\underline{H}$), 6.79 (dd, J_1 = 2.6 Hz, J_2 = 8.5 Hz, 1H, C2-C$\underline{H}$), 7.20 (d, J = 8.61 Hz, 1H, C1-C$\underline{H}$), 7.29–7.45 (m, 5H, C3-CH$_2\underline{Ar}$). FABMS, m/z (RI), 548 (M + $\underline{H}$, 35), 492 (45), 474 (44), 448 (60). High-resolution FABMS calculated for $C_{35}H_{50}NO_4$ (M + H): 548.3739. Found: 548.3730.

7α-(5-N-BOC-aminopentyl)estradiol (16). Steroid (**15**) was dissolved in ethanol (1 ml) with EtOAc as cosolvent (0.50 ml). The solution was stirred over hydrogen in the presence of catalytic $PdCl_2(CH_3CN)_2$ (~1 mg) and acetic acid (10 μl) for 20 minutes. Removal of palladium by filtration of the reaction solution, followed by evaporation of solvent, gave a clear oil that was purified by HPLC chromatography (80/20 CH_2Cl_2 with 5% 2-propanol/hexane) to give **16** as a clear oil (10 mg, 60%).^1H NMR (300 MHz), δ 0.78 (s, 3H, C18-C$\underline{H}_3$), 1.44 (s, 9H, BOC C$\underline{H}_3$), 2.67–2.84 (dd, 2H, C6-C$\underline{H}_2$), 3.03 (t, J = 6.9 Hz, C5′-C$\underline{H}_2$NHBOC), 3.34 (s, 1H, N$\underline{H}$BOC), 6.63 (t, J = 7.2 Hz, 1H, C17α-C$\underline{H}$), 4.72 (MeOH-d4), 6.52 (d, J = 2.0 Hz, 1H, C4-C$\underline{H}$), 6.61 (dd, J_1 = 2.1 Hz, J_2 = 8.5 Hz, C2-C$\underline{H}$), 7.12 (d, J = 8.5 Hz, 1H, C1-C$\underline{H}$). FABMS, m/z (RI), 458 (M + H,15). High-resolution FABMS calculated for $C_{28}H_{44}NO_4$ (M + H): 458.3270. Found: 458.3272.

7α-[5-(4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-3-indacenepropionyl)-aminopentyl]estradiol (2). A solution of azide (**14**) (14.3 mg, 0.03 mmol) was stirred over hydrogen with catalytic $PdCl_2(CH_3CN)_2$ (~3 mg) and acetic acid (10 μl) in ethanol, with ethyl acetate as cosolvent. The solution was stirred for 30 minutes, and the resulting suspension filtered over Celite. The solution was concentrated and dried under vacuum for 1 hour. The resulting oil was redissolved in THF/N,N-dimethylformamide (DMF) (500 μl each) and added to a solution containing the commercially available BODIPY N-hydroxysuccinimidyl ester (2.5 mg, 0.013 mmol). The solution was stirred with heating at 80 C for 2 days. Triethylamine (1 μl) was added, and the solution heated for 24 hours, at which time BODIPY N-hydroxysuccinimidyl ester was consumed. Flash column chromatography (50/50 EtOAc/hexane) followed by HPLC chromatography (75/25 CH_2Cl_2 with 5% 2-propanol/hexane) gave 1.0 mg (32%) of desired product (**2**). The low yield is presumed due to the instability of the compound to silica gel. ^1H NMR (300 MHz), δ 0.78 (s, 3H, C18-C$\underline{H}_3$), 2.25 (s, 3H, BODIPY-C$\underline{H}_3$), 2.25 (s, 3H, BODIPY-C$\underline{H}_3$), 2.61 (t, J = 7.4 Hz, 2H, BODIPY-C$\underline{H}_2$CH$_2$CO$_2$NH), 2.62–2.83 (dd, 2H, C6-C$\underline{H}_2$), 3.16 (t, J = 6.4 Hz, 2H, C5′-C$\underline{H}_2$NH-BODIPY), 3.25 (t, J = 7.4 Hz, 2H, BODIPY-CH$_2$C$\underline{H}_2$CO$_2$NH), 3.74 (t, J = 8.6 Hz, 1H, C17α-C$\underline{H}$), 5.65 (m, 1H, N$\underline{H}$-BODIPY), 6.12 (s, 1H, BODIPY-C$\underline{H}$), 6.25 (d, J = 3.9 Hz, 1H, BODIPY-C$\underline{H}$), 6.56 (d, J = 2.5 Hz, 1H, C4-C$\underline{H}$), 6.64 (dd, J_1 = 2.8, J_2 = 8.5 Hz, 1H, C2-C$\underline{H}$), 6.77 (d, J = 3.9 Hz, 1H, BODIPY-C$\underline{H}$), 7.02 (s, 1H, BODIPY-C$\underline{H}$), 7.15 (d, J = 8.43 Hz, 1H, C1-C$\underline{H}$). FDMS m/z 631 (M+, 100), 612 (M-F). High-resolution FABMS calculated for $C_{37}H_{49}BN_3O_3F_2$(M + H): 632.3835. Found: 632.3833.

Biological procedures

Materials. The following compounds were obtained from the sources indicated: tritium-labeled estradiol ([6,7-^{3}H]estra-1,3,5(10)-triene-3,17β-diol), 49-51 Ci/mmol, from Amersham Corp.; ethylenedinitrilotetraacetic acid tetrasodium salt (EDTA) and dextran C from Eastman Kodak Co.; sodium azide, spectrophotometric grade acetonitrile, and 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP) from Aldrich Chemical Co.; N,N-dimethylformamide (DMF) from Fisher Scientific; unlabeled estradiol, activated charcoal, Trizma base, from Sigma Chemical Co.; 2,5-diphenyloxazole (PPO) from Research Products International Corp.; Triton X-114 from Chem Central, Indianapolis, IN; spectrophotometric grade ethyl acetate and cyclohexane from Mal-

linckrodt, Inc.; and absolute ethanol from Midwest Grain Products Co.

Rat and lamb uterine cytosols were prepared and stored as previously described.[1,25] All experiments were performed at 0–4 C in TEA buffer (0.01 M Tris-HCl, 0.0015 M EDTA, 0.02% sodium azide, pH 7.4, at 25 C). The charcoal-dextran slurry used to remove unbound ligand was prepared as previously reported[1] and was used at 1 part to 10 parts of cytosol solution.

Tritium radioactivity was measured in a Nuclear Chicago Isocap 300 using a xylene-based scintillation cocktail, previously described.[26]

Sprague-Dawley rats for the uptake studies were obtained from Sasco Laboratory Animals, Omaha, NE, USA.

Radiochemical synthesis procedure. Cyclotron-produced [^{18}F]fluoride ion was generated by the ^{18}O(p,n)^{18}F reaction at the Mallinckrodt Institute of Radiology of Washington University Medical School. An efficient [^{18}O]H_2O target has been designed and is capable of delivering Curie amounts of ^{18}F activity in a one-hour bombardment of highly enriched (95–99% oxygen-18) water.[27] [^{18}F]fluoride ion thus produced is highly solvated and poorly nucleophilic so that azeotropic removal of water (with acetonitrile)[28] was required before resolubilization of activity in tetrahydrofuran. All radiochemical syntheses were conducted with no carrier added. Tetrabutylammonium hydroxide was used as the phase transfer agent in the resolubilization of [^{18}F]fluoride; therefore, the radioactive species was in the form n-$Bu_4N^{18}F$.

Resolubilized activity (185 mCi) was transferred to a vial containing 1.5 mg of the mesylate precursor (**10**). After 20 minutes at 70 C, the reaction was filtered through silica gel (to remove unreacted fluoride), evaporated to dryness, and the residue purified by HPLC chromatography (30/70 CH_2Cl_2 with 5% 2-propanol/hexane). The purified activity was collected, concentrated to dryness and redissolved in ethanol. $PdCl_2(CH_3CN)_2$ in ethanol and acetic acid was added, and hydrogen was bubbled through the solution for 10 minutes. Again, the reaction solution was filtered, concentrated, and purified by HPLC chromatography (60/40 hexane/CH_2Cl_2 with 5% 2-propanol). The deprotection step proved to be incomplete, resulting in two radioactive products, final product **1**, and its benzyl-protected precursor. Collection of the desired product and concentration gave sufficient activity for the tissue distribution study. A typical radiochemical synthesis takes 90–120 minutes from the end of bombardment and gives a decay-corrected yield of 5–15%. Effective specific activities[28] were determined by a competitive radiometric binding assay[11] on the purified material after full radiochemical decay.

Receptor binding affinity (RBA). Assays were performed as previously reported,[1] using rat or lamb uterine cytosol diluted to ~1.5 nM of receptor. Cytosol was incubated with buffer or several concentrations of unlabeled competitor together with 10 nM [^{3}H]estradiol, at 0 C for 18–24 hours. Free ligand was removed by adsorption to dextran-coated charcoal. Unlabeled competitors were prepared in 1 : 1 DMF : TEA to ensure solubility.

In vivo biodistribution studies. In vivo uptake studies were performed as previously reported.[26] Immature female Sprague-Dawley rats (25 days, ~50 g) were injected (i.v., tail vein), under ether anesthesia, with 50 μCi of ^{18}F-labeled steroid in a 1 : 1, physiological saline : ethanol solution. To ascertain whether the uptake was mediated by a high-affinity, limited-capacity system, one set of animals was treated with 15 μg unlabeled estradiol coinjected with the radiopharmaceutical. Animals were killed by decapitation, at the times indicated, and samples of tissue and blood were weighed. Radioactivity in blood, organs, and standard samples was determined with a Beckman Gamma 8000 automatic well-type gamma counter.

Spectrofluorometric assays. Spectrofluorometric analysis was performed on a Spex Fluorolog 2 spectrofluorometer (model IIIC) equipped for photon counting; excitation was at 490 nm, with emission scanned from 500 to 600 nm. Fluorescence intensities were measured at the maximum of the emission band. Measurements of protein incubations were performed with the cuvette holder cooled to ~4 C. Fluorescence quantum yields were obtained from lamp-corrected spectra and were calculated after the procedure of Pesce et al.[29] using fluorescein in 0.1 N NaOH (Φ_f = 0.9) as the standard.[30] UV measurements were determined on a Hewlett-Packard 8451A Diode Array spectrophotometer. All solvents were spectrophotometric grade.

Results and discussion

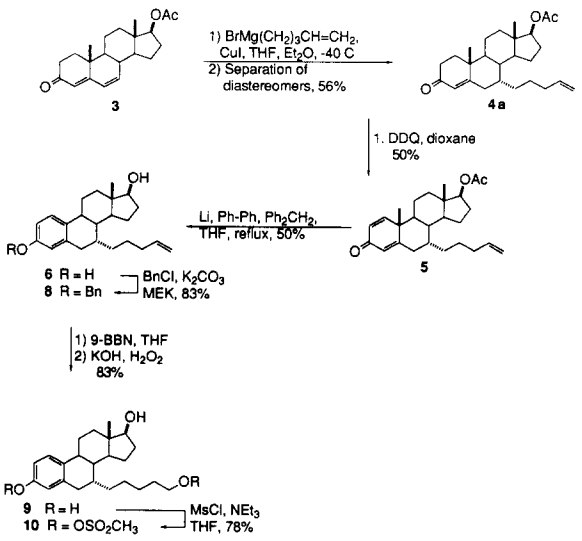
Synthesis of 7α-(5-hydroxypentyl)estradiol 3-benzyl ether (9)

Several syntheses of 7α-substituted estradiols have been described.[18–21] In each case, the key step was the introduction of the substituent at C-7 with high diastereoselectivity. Traditionally, a copper-catalyzed 1,6-conjugate addition, first reported by Bucourt et al.,[18] has been used to introduce the C-7 substituent. They described this addition to 6-dehydro-19-nortestosterone as proceeding with good diastereoselectivity,[18] finding a 9 : 1 α : β selectivity using a 2-tetrahydropyranyl (THP)-protected 3-bromopropanol and a 5 : 1 selectivity using a THP-protected 11-bromoundecanol as the nucleophilic precursor. Others have found lower and more variable selectivity, for example, Bowler et al., who reported virtually no diastereoselectivity in the addition of a silyl protected 11-bromoundecanol.[19]

Alternatively, Kirk and Miller described a radical addition of an allyl substituent to 6-dehydrotestosterone using an allyl silane and $TiCl_4$.[31] They reported low yields, poor selectivity, and the generation of many side products, including a dimer. Nickisch and Laurent,[32] using dehydrotestosterone, were able to get excellent yields of the 7α-diastereomer, along with a cyclobutyl product, using a similar procedure at lower temperatures.

In an effort to better control the diastereofacial outcome of the copper-catalyzed 1,6-conjugate addition to steroidal dienones, we selected 6-dehydrotestosterone as our starting material (Scheme 1). Previous work has shown that the level of diastereoselectivity of this addition is a function of both the dienone structure as well as the size of the dialkyl cuprate. We anticipated, in any case, that the 19-methyl group would increase the steric bulk on the β-face and thus provide increased α-selectivity in the products. While traditional oxidative aromatization methods were precluded due to the presence of the 19-methyl group, the transformation of such androgens to their corresponding estrogens by a sequence involving a reductive aromatization reaction was precedented.

Baran et al. were able to reductively aromatize 11β-hydroxy-1,4-androstadiene-17-ketal in adequate yields (50%).[33] Earlier work in our own laboratory with 11β-substituted estradiol analogs[13] had led us to repeat Baran's work, and our success in improving the yield and



Scheme 1 Synthesis of 7α-pental analogs from testosterone.

consistency of the reductive aromatization process prompted us to apply this sequence to the synthesis of 7α-substituted estradiols as well.

The 4-pentenyl group was selected as the nucleophilic component in the addition for a number of reasons. Previous investigators have used protected alcohols as nucleophilic precursors.[18–21] The alcohols are later deprotected, reprotected, and finally revealed toward the end of each synthesis. We felt that the protecting group manipulations could be better addressed by the use of a terminal olefin as a masked primary alcohol. In this manner, a hydroboration/oxidation step would provide the required alcohol at the end of the synthesis, eliminating the need for protecting group manipulation. A second consideration for the selection of an olefin was the harshness of the subsequent two steps in the synthesis: dichlorodicyanoquinone (DDQ) dehydrogenation and reductive aromatization with lithium in refluxing THF. Not many protecting groups will withstand these harsh oxidation and reduction conditions.

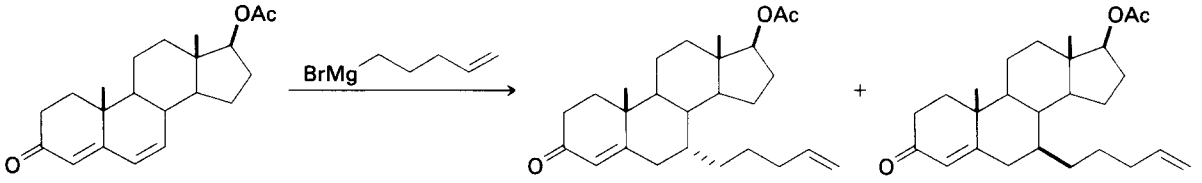
In selecting the length of the chain, we considered the size of the 7α substituent formed when coupled with the bulky fluorescent probe. When appended to a 5-aminopentyl group, the BODIPY fluorophore gives a 7α conjugate that has a substituent ~16 atoms long. This is roughly the dimensions of Wakeling's undecyl linked butyl amide, ICI 164,384.[19]

A variety of conditions were investigated, including Bowler's method, to optimize the stereoselectivity of addition (Table 1). We found that the addition of 1 vol of diethyl ether as cosolvent provided an optimum yield, with diastereoselectivity as high as 5 : 1 in favor of the α-diastereomer. Separation of the diastereomers is easily accomplished by flash column chromatography to provide **4a** in high yields. Initially, the stereochemistry of addition was assigned based on the elution order of the diastereomers, by analogy with those assigned by Wakeling et al.[19]

In other syntheses that use 19-nortestosterone, the absence of the C-10-methyl group (C-19) does facilitate aromatization of the A ring,[18] so this conversion was achieved in good yields under relatively mild conditions.[18] Aromatization of our pentenyl testosterone acetate (**4a**) required two steps, under more vigorous conditions: dehydrogenation to the cross-conjugated 1,4-dien-3-one with DDQ in refluxing dioxane, followed by reductive aromatization.[33] These reactions went in adequate yields, 50% and 62%, respectively. The resulting 7α-pentenylestradiol proved to be crystalline, and a single crystal was isolated from deuteriochloroform and examined by single crystal x-ray analysis to verify the stereochemistry at C-7 (see below).

Completion of the synthesis (Scheme 1) of the precursor involved protection of 3-phenol as the benzyl ether, conversion of the terminal olefin to the primary alcohol (**9**) with 9-borabicyclo[3.3.1]nonane (9-BBN) followed by basic peroxide, and selective methanesul-

Table 1 Copper-catalyzed 1,6-conjugate addition: optimization of yield and facial selectivity



Grignard Eq	Temperature (C)	Copper salt	Added salt	THF/ether ratio	Yield (%)	α : β
12.0	−30 to −50	CuBr	LiBr	2 : 1	12	ND
6.0	−20	CuI		4 : 1	5	ND
12.0	−30	CuI		1 : 9	57	1.38 : 1
12.0	−40	CuI		1 : 4	64	1.50 : 1
12.0	−30	CuI		1 : 1	80	3.55 : 1
4.0	−30	CuI		1 : 1	70	4.56 : 1

ND, not determined.

fonylation of the primary alcohol to give the precursor, 7α-(5-methanesulfonyloxypentyl)estradiol 3-benzyl ether (**10**) in good yields. Our synthesis proceeds from 6-dehydrotestosterone to **10** in six steps, with an overall yield of 8.5%.

The strength of our approach lies in the increased stereocontrol and improved yields achieved in the 1,6-conjugate addition. Additionally, the use of an olefin as a masked alcohol provides for fewer protecting group manipulations in the synthesis. The limitation of our synthesis is the relatively low yields in the aromatization (and demethylation) of the A ring.

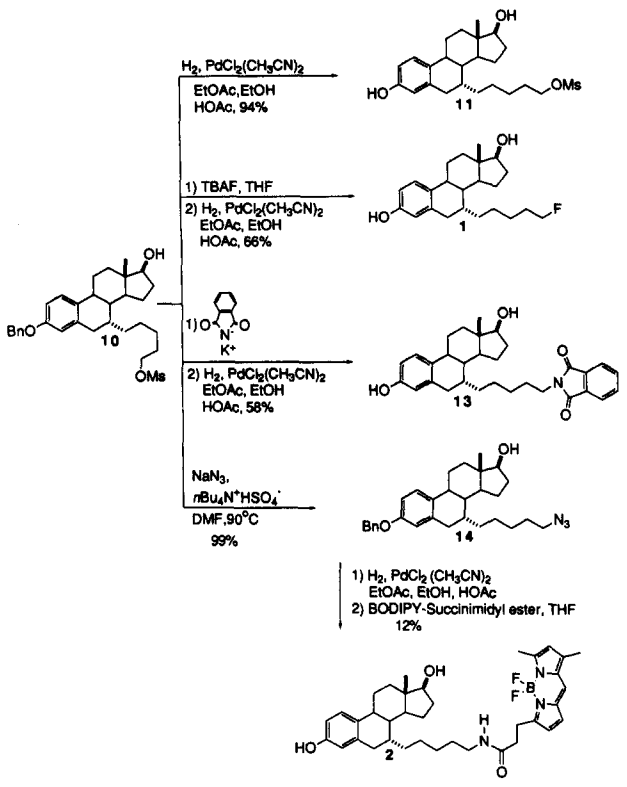
Single crystal x-ray analysis of 7α-pentenylestradiol (6)

Crystals grown from deuteriochloroform were extremely sensitive to solvate loss. Room temperature exposure caused immediate changes in transparency, so the data crystal was stored in deuteriochloroform at 0 C. The x-ray data were collected at −65 C, with the crystal frozen in oil. Exposure to temperatures below −70 C significantly increased the mosaic spread of the diffraction pattern. All of the crystals tested were twinned in the plane normal to the b-axis. Owing to poor crystal quality, bond length constraints were required to ensure successful convergence of the least-squares refinement. Chemically equivalent O—C and C—C bond lengths were constrained to variable average values, and for the pentenyl groups and solvate molecules, bond angles were also constrained.

There are two independent molecules in the unit cell (Figure 2). The molecules are oriented in square columns; the C-3 and C-17 hydroxyl groups hydrogen bond with each other at the corners of these columns. The steroid backbone acts as a pseudo-plane of symmetry, with the C-18β methyl group and C-7α-pentenyl on the outside and inside of the column. Solvent molecules are located in the hydrophobic region of the column. This crystal packing helps explain the sensitivity of the crystals, because the removal of the solvent molecules from the interior of the columns probably results in the collapse of the crystal. The α-stereochemistry at C-7 can be determined with confidence, based on the known stereochemistry at carbons C-8, C-9, C-13, C-14, and C-17. As expected, the stereochemical assignment made by Bowler et al.,[19] on the basis of elution order and NMR, holds true for this molecule as well.

Synthesis of 7α-pentyl derivatives

Derivatization of alcohol **9** involved formation of the mesylate (**10**) (Scheme 1) and displacement of the mesylate-leaving group and hydrogenolysis of the benzyl ether with hydrogen and $PdCl_2(CH_3CN)_2$. Therefore, simple deprotection of the mesylate gave **11** in quantitative yield (Scheme 2), and fluorination with tetrabutylammonium fluoride followed by deprotection gave the 7α-(5-fluoropentyl)estradiol (**1**). Displacement with potassium phthalimide and deprotection gave the phthalimide derivative (**13**).



Scheme 2 Synthesis of 7α-pentylestradiol derivatives.

For the fluorescent conjugate synthesis, 7α-(5-aminopentyl)estradiol was prepared. Synthesis of the phthalimide derivative, as stated earlier, proceeded in excellent yields; however, treatment with hydrazine to remove the phthalimide failed to produce the primary amine. As an alternative, we synthesized the aliphatic azide (**14**) in excellent yield by displacement with sodium azide in the presence of tetrabutylammonium hydrogen sulfate in refluxing THF. A variety of reducing agents were then investigated to convert the azide to the amine; two methods proved successful. Reduction with lithium aluminum hydride in THF gave the primary amine benzyl ether in low yield (35%). Alternatively, both the azide and the benzyl ether could be efficiently cleaved with hydrogen and $PdCl_2(CH_3CN)_2$ to give the amino phenol. Due to its instability, we had difficulty obtaining full characterization of this material: FAB mass spectroscopy of the reaction mixture showed the appropriate molecular ion (M + 1), but concentration or chromatography of the sample resulted in product decomposition. In situ protection of the primary amine as the t-butyl carbamate (**16**) enabled isolation, purification, and characterization.

BODIPY fluorescent probe (2)

The fluorescent probe selected for attachment is the BODIPY group (Figure 3). This fluorescent moiety has been used to label a variety of different ligands,[34] as well as proteins,[35] and its excellent fluorescent proper-

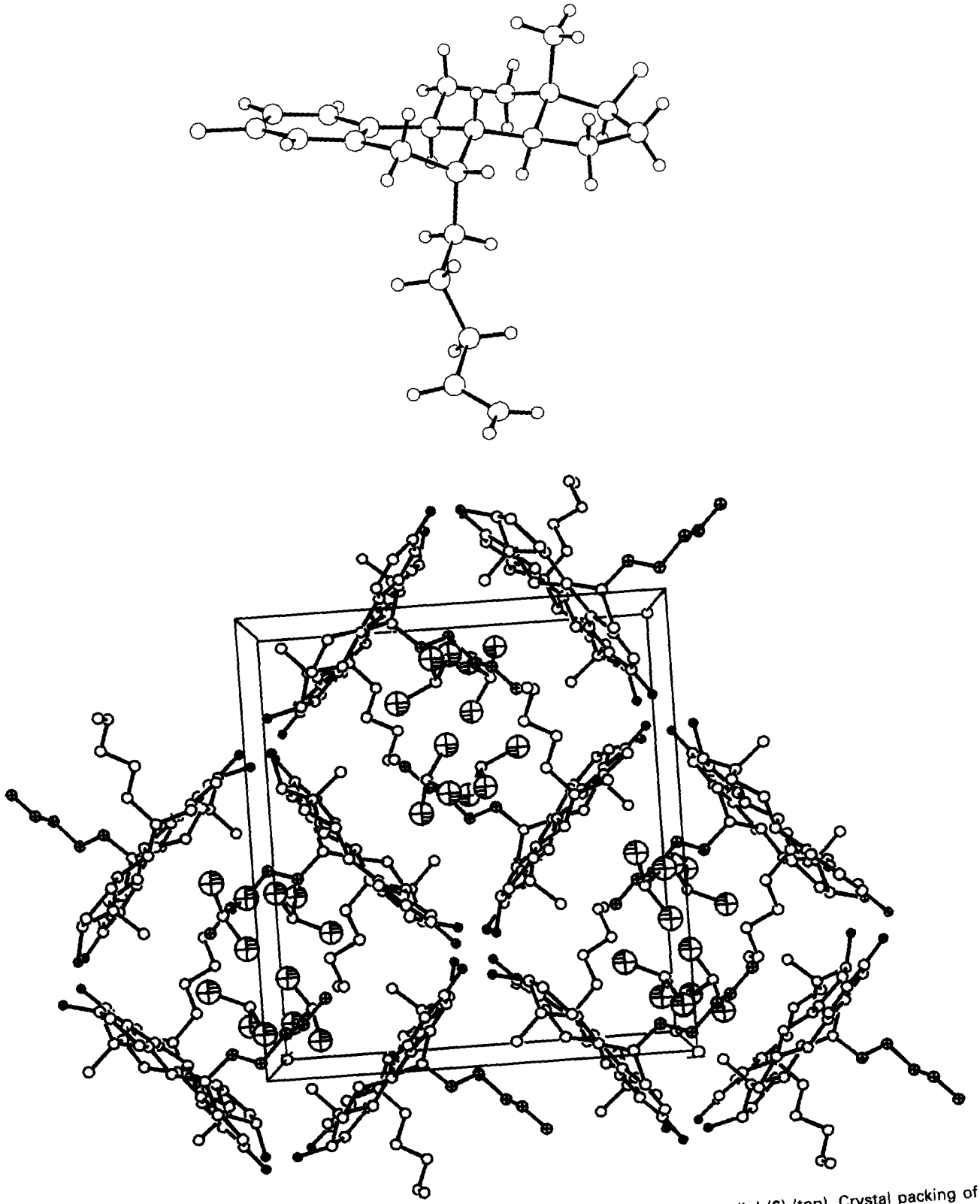


Figure 2 Ball and stick representation derived from x-ray crystal structure of 7α-pentenylestradiol (**6**) (top). Crystal packing of 7α-pentenylestradiol (**6**) in the unit cell (bottom).

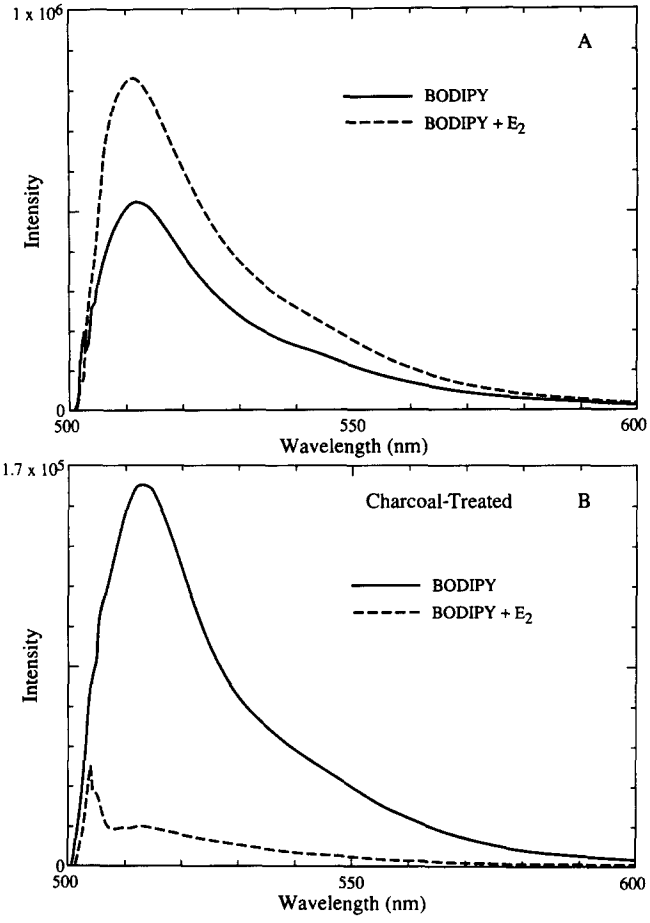


Figure 3 Fluorescence emission of 5 nM of 7α-pentyl-BODIPY-estradiol conjugate (**2**). Measurement was performed in a preparation of yeast-expressed estradiol receptor containing 1.3 nM receptor. Excitation = 490 nm. (A) Equilibrium incubation. (B) After charcoal adsorption.

ties have been described.[35] Its high quantum yield, narrow emission band, and relatively small size make it a useful probe.

Synthesis of the conjugate (**2**) involved in situ generation of 7α-(5-aminopentyl)estradiol by hydrogenation of the azide benzyl ether (**14**) with hydrogen and $PdCl_2(CH_3CN)_2$. The resulting amine was stirred in THF with the N-hydroxysuccinimidyl ester of the BODIPY acid (Scheme 2), and the conjugate (**2**) was isolated and purified by HPLC.

Estrogen receptor binding affinity of 7α-pentyl estradiol conjugates

The estrogen receptor binding affinity of these 7α-substituted estrogens was determined by a competitive radioreceptor binding assay, using rat or lamb uterine cytosol as a source of estrogen receptor, tritium-labeled estradiol as radiotracer, and dextran-coated charcoal as adsorbant for free ligand. The affinities are expressed relative to estradiol on a percent scale, as relative binding affinity (RBA) (Table 2). All analogs synthesized display high RBA, including the bulky BODIPY conjugate. It appears that hydrophobic functional groups are better tolerated than hydrophilic (OH) groups. The RBA values we obtain for these derivatives based on 7α-pentylestradiol compare favorably with a series of derivatives based on the longer 7α-undecyl substituent[18,19,21] and to some related 7α-butanoic acid derivatives.[20]

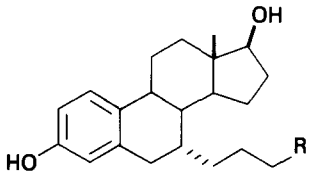
Radiochemical synthesis of 7α-(5-[^{18}F] fluoropentyl)estradiol (1)

We were encouraged by the exceptionally high binding affinity of 7α-(5-fluoropentyl)estradiol (**1**) to synthesize this compound in radiolabeled form. The short half-life of fluorine-18 (110 minutes) precludes extensive synthetic manipulation of the ^{18}F-containing compound before in vivo use. For that reason, the reactive mesylate precursor was used in the fluoride ion displacement step in the radiochemical synthesis. This strategy has proved successful in the rapid introduction of [^{18}F]fluoride into a variety of compounds.[11,17,26] Therefore, displacement with fluoride followed by deprotection and purification by normal phase HPLC gave adequate quantities of 7α-(5-[^{18}F]fluoropentyl)estradiol (**1**) for tissue distribution studies.

Tissue distribution of 7α-(5-[^{18}F] fluoropentyl)estradiol (1) in immature rats

The tissue distribution of 7α-(5-[^{18}F]fluoropentyl)estradiol (**1**) in immature female rats is shown in Table 3. Distribution is given at 1 and 3 hours with a normal dose (~50 μCi) of radioligand and at 1 hour with radioligand plus coinjection of a blocking dose (15 μg) of unlabeled estradiol. This last experiment was designed to block receptor-mediated uptake and establish the level of nonspecific uptake.

Table 2 RBAs for 7α-substituted estradiol derivatives (estradiol = 100)



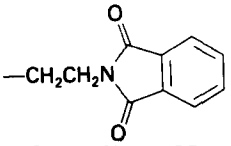
R	Compound	RBA
—CH=CH$_2$	**6**	132
—CH$_2$CH$_2$OH	**7**	36.3
—CH$_2$CH$_2$OMs	**11**	74.1
—CH$_2$CH$_2$F	**1**	63.1
	**13**	26.9
—CH$_2$CH$_2$NH—BODIPY	**2**	22.9

Table 3 Biodistribution of 7α-(5-[^{18}F]fluoropentyl)estra-3,17β-diol (**1**)

Tissue	1 h	1 h blocked[a]	3 h
Blood	0.197 ± 0.032	0.199 ± 0.033	0.148 ± 0.014
Liver	0.617 ± 0.108	0.741 ± 0.169	0.393 ± 0.104
Spleen	0.139 ± 0.016	0.165 ± 0.020	0.059 ± 0.007
Kidney	0.234 ± 0.032	0.260 ± 0.053	0.085 ± 0.013
Muscle	0.094 ± 0.016	0.106 ± 0.030	0.024 ± 0.007
Fat	1.252 ± 0.068	1.308 ± 0.271	0.388 ± 0.058
Bone	1.056 ± 0.191	1.223 ± 0.204	1.668 ± 0.243
Uterus	0.649 ± 0.156	0.246 ± 0.061	0.105 ± 0.027
Ovaries	0.618 ± 0.085	0.416 ± 0.114	0.131 ± 0.020
Thymus	0.142 ± 0.038	0.158 ± 0.032	0.035 ± 0.013
Uterus/blood	3.28 ± 0.48	1.26 ± 0.33	0.72 ± 0.18
Uterus/muscle	6.88 ± 1.05	2.39 ± 0.59	4.80 ± 1.59

Female Sprague-Dawley rats (~48 g), five animals per time point, were injected i.v. with 50 μCi of **1** (effective specific activity 745 Ci/mmol) in 25% ethanol saline. Tissue distribution values are % injected dose/g and are presented as mean ± SD.
[a] Coinjection of 50 μCi of **1** and 15 μg estradiol

It is clear that there is some selective uptake in target tissues. This can be seen by comparing the uterine uptake in unblocked versus blocked rats at 1 hour; in unblocked tissue the uptake is about three times greater than in blocked tissue. However, the level of nontarget tissue uptake, especially in fatty tissue and in the blood, is high. This is to be expected, because the addition of a five-carbon chain would increase the lipophilicity of the molecule considerably.[36] The selectivity of uptake is also reflected in the ratio of uptake of target tissues versus nontarget tissues (Table 3, bottom). For this compound, uterus-to-blood and uterus-to-muscle ratios at 1 hour were ~3 and 7, respectively. This indicated some selective uptake, but not as high as we have seen with other fluoroestrogens; a good ratio, for reference, would be greater than 50 to 1.[13,14] Additionally, the compound is rapidly cleared from target tissues, so that by 3 hours, the level of uptake in uterine tissue has dropped lower than blood levels. The low selectivity and rapid clearance may again be due to the high lipophilicity of this molecule.

Metabolic defluorination is measured by the level of activity in bone[37,38] as percentage injected dose per organ. The level of defluorination for 7α-(5-[^{18}F]fluoropentyl)estradiol (**1**) was moderate (5–10%), indicating the relative stability of a molecule of this type to metabolism.

Table 4 Fluorescence properties of 7α-(5-BODIPY-pentyl)estradiol (**2**) in various solvents

Solvent	Emission[a] λ_{max}(nm)	Quantum yield[b]
Cyclohexane	513	0.236 ± 0.071
EtOAc	509	0.429 ± 0.017
CH_3CN	508	0.357 ± 0.046
EtOH	509	0.444 ± 0.032
TEA buffer[c]	509	0.012

[a] Excitation λ = 490 nm.
[b] Quantum yield values determined using fluorescein in 0.1 N aqueous NaOH as a standard. Values represent the mean ± SD of two to four determinations, except for the buffer value, which was a single determination.
[c] TEA buffer = Tris, EDTA, NaN_3 buffer adjusted to pH 7.4.

Fluorescence studies of 7α-(5-BODIPY amidopentyl)estradiol (2)

The fluorescence properties of the BODIPY conjugate (**2**) were measured in a variety of solvents (Table 4). The wavelength of emission for the BODIPY group is quite insensitive to solvent, ranging only from 513 nm in cyclohexane to 508 nm in acetonitrile. Quantum yields range from 0.45 in ethyl acetate to 0.012 in TEA buffer (Table 4). This low value in buffer may represent, in part, the insolubility of this compound in water.

To measure the fluorescence of the BODIPY conjugate (**2**) when bound to estrogen receptor, the conjugate was incubated with a human estrogen receptor preparation from a yeast expression system (Figure 3) both with and without a blocking dose of estradiol. The ER preparation from the yeast expression system is richer in ER than uterine cytosol preparations of ER; it was used in the fluorescence studies because lower levels of background fluorescence and nonspecific binding were encountered. (Nonspecific binding is less of an issue in the competitive binding experiments in which [^{3}H]E_2 is used as a tracer, and uterine ER preparations can be used.) Fluorescence of the probe was measured under three conditions: at equilibrium, after treatment with charcoal, and after extraction with ethyl acetate. The fluorescence of the derivative in the equilibrated solution, both with and without added estradiol, is extensive. This indicates that the derivative is associated with protein, because the BODIPY conjugate (**2**) fluoresces only poorly in buffer alone (Table 4).

At equilibrium, the fluorescence intensity is greater in the sample where ER is blocked by estradiol (+E_2) than in the sample where the BODIPY conjugate is

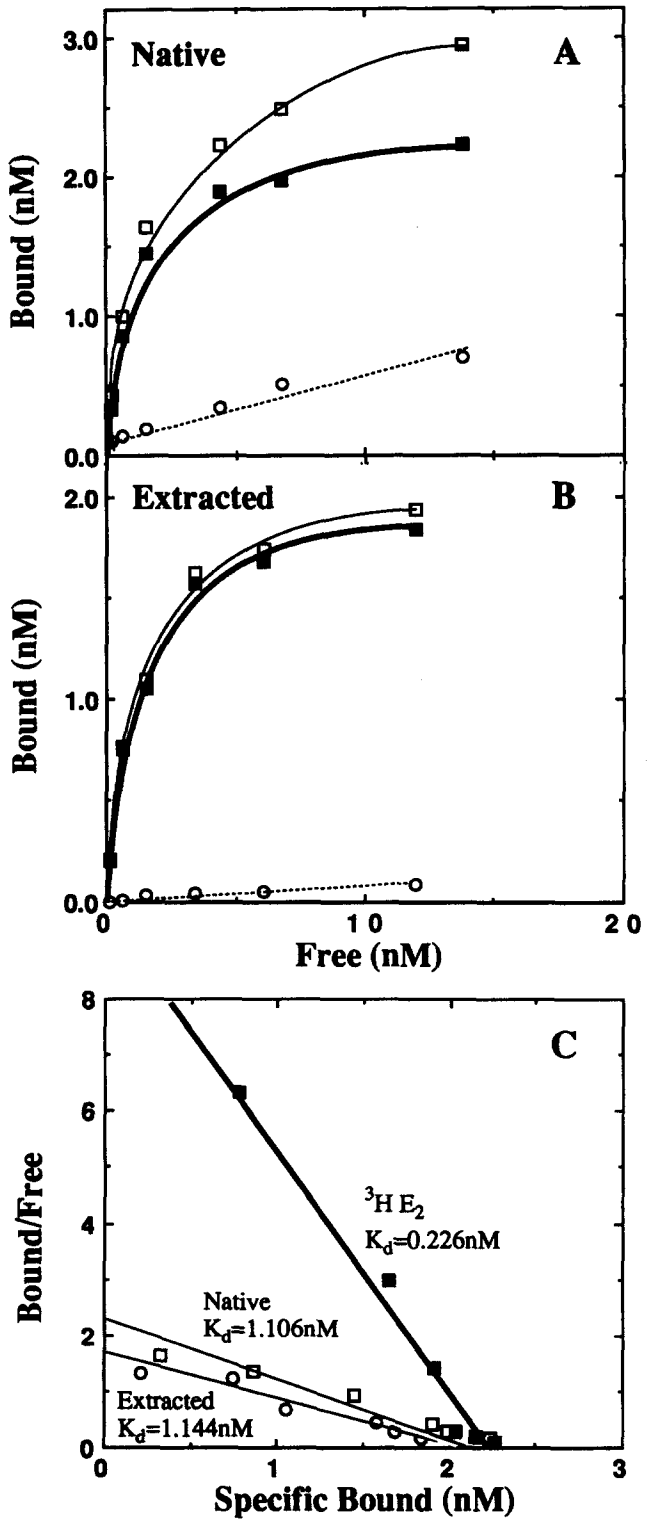


Figure 4 Direct binding curves for the BODIPY conjugate (2) to yeast-expressed estrogen receptor measured by fluorometry. Several concentrations of ligand were incubated with an estrogen receptor preparation in the absence (—— solid line) (total) or presence (---- dashed line) (non-specific) of an excess of estradiol at 0 C for 3 hours and then treated with charcoal-dextran. The specific binding to ER (—— bold line) is the difference between the total and NS. The concentration of bound ligand was determined in the native protein incubation (A) or in ethanol (B) after extraction with ethyl acetate. Scatchard plots of specific binding are shown in C (□, native; ○, extracted) along with data for [^{3}H]E$_2$ bound to the same protein preparation (■, bold line). Whether measured in the native state or after extraction, the data show the BODIPY conjugate (2) binds to 91–107% of the receptor sites bound by [^{3}H]E$_2$ with a K$_d$ relative to [^{3}H]E$_2$ of 20%.

bound to receptor (Figure 3A). Because both incubations contain the same concentration of ligand, this implies that the BODIPY conjugate fluoresces more intensely when it is bound nonspecifically (i.e., to low affinity sites on proteins other than ER) than when it is specifically bound, presumably to ER. Several other fluorescent ligands for hormone receptors also exhibit this behavior.[39,40]

After charcoal treatment, most of the nonspecifically bound ligand has been removed (Figure 3B). Thus, the fluorescence from the sample blocked with estradiol (+E$_2$) is very low, and the fluorescence in the unblocked sample (BODIPY) comes mostly from ligand bound to ER.

We incubated several concentrations (1–30 nM) of the BODIPY conjugate with an ER preparation, and then treated them with charcoal. By reference to the appropriate standard curves, we could convert the fluorescence intensity to fluorophore concentrations, and we could then construct a binding curve (Figure 4A) from these native incubations. At each concentration, the specific binding is measured as the difference spectra between the unblocked and blocked samples.

These same samples were then extracted with ethyl acetate, which was evaporated to dryness under a stream of nitrogen, and the residue was dissolved in ethanol and scanned for fluorescence intensity measurement. Now, in a constant environment (ethanol), the ligand from all incubations exhibited a constant fluorescence per concentration (regardless of the fraction of free or ER- or nonspecifically bound fluorophore in the incubation itself). The fluorescence intensities could be converted to concentrations, and a binding curve constructed from the extracted samples (Figure 4B). These curves can be converted to Scatchard plots to give a direct measurement of K$_d$ of fluorophore binding to ER and total ER site concentrations (Figure 4C). A similar set of binding curves was generated using the same protein solution and [^{3}H]E$_2$. The data, converted to a Scatchard plot, is also shown in Figure 4C.

Whether measured under native or extracted conditions, the fluorescence assay shows that the BODIPY conjugate binds to estrogen receptor, effectively filling the same number of sites as measured by the binding of [^{3}H]E$_2$. By this direct assay, the K$_d$ of the BODIPY conjugate is 1.11–1.14 nM, 20% that of [^{3}H]E$_2$ (0.23 nM). This agrees well with the 21.7 ± 1.8% RBA measured indirectly by the competitive binding assay.

The fluorescence intensity of the BODIPY conjugate (2) is greater when the ligand is bound nonspecifically (to the hydrophobic sites on the nonreceptor proteins) than when bound to ER. However, after treatment with charcoal-dextran, less than 5% of the ligand remains bound nonspecifically. Although preliminary, these ex-

periments show that the binding of the BODIPY conjugate (**2**) to ER is quite specific and indicate that this derivative as a fluorescent probe may be worth further study.

In conclusion, we have reported a new synthetic route to the 7α-alkyl–substituted estradiols. The key steps, 1,6-conjugate addition to dehydrotestosterone and the subsequent reductive aromatization, provide the desired 7α-substituted estradiol moiety in good yields. The use of a monosubstituted olefin as a masked alcohol reduces the number of protecting group manipulations required in the synthesis, and results in an improved overall yield of the sequence. This route provides a new strategy for the synthesis of a number of 7α-substituted analogs for the study of estrogen receptor structure and function.

As is known for the antiestrogens and other derivatives in this series, most of the 7α-substituted estrogens bind to the estrogen receptor with high affinity. Two in particular were studied further: 7α-(5-[^{18}F]fluoropentyl)estradiol (**1**) was prepared as a potential breast tumor imaging agent; it had good affinity for the estrogen receptor and showed selective uptake by target tissues in the immature rat. The BODIPY fluorescent derivative (**2**) of 7α-(5-aminopentyl)estradiol also bound to the estrogen receptor with high affinity, and could be used in a fluorometric assay of the receptor.

Acknowledgments

We are grateful for support of this research through grants from the National Institutes of Health (PHS 5R01 CA25836 and 5R37DK15556 to J.A.K.) and the Department of Energy (DE FG02 84ER60218 to M.J.W.). The assistance of Kathryn E. Carlson (University of Illinois) and Tom Bonasera (Washington University) is gratefully acknowledged.

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