# 17α-Substituted analogs of estradiol for the development of fluorescent estrogen receptor ligands

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*For the successful development of a high-affinity fluorophore–estradiol conjugate, the fluorophore must be attached to the estradiol molecule at a position that interferes least with its binding to the receptor. We have concentrated on 17α substituents as models for fluorophore attachment, based on literature precedent and on our earlier work with small 17α side chains. In this report, we describe syntheses and estrogen receptor binding affinities of 19 analogs of estradiol substituted in the 17α position with larger side chains (of six to 11 carbons), some of which may be synthetically modified to link a fluorophore. These analogs were synthesized either by nucleophilic cleavage of estrone-17β-oxirane 3-benzyl ether and subsequent debenzylation (4 to 18), by cross-coupling of alkynes (21 to 24), by alkylation of 17α-ethynylestradiol 3,17-*bis*(tetrahydropyranyl ether) and subsequent acidic hydrolysis (25 to 28), or by reacting estrone either with appropriate aryl/alkynyllithium reagents (29, 30, and 32) or with benzylmagnesium bromide (31). Relative binding affinities of these newly synthesized analogs were determined for estrogen receptor (rat uterus) using a standard competition assay. The results suggest that analogs with reduced mobility and/or more polarizable electron density in the side chain generally bind more strongly to the receptor. The relative affinities of several selected compounds were also determined in the presence of 4% dimethylformamide; some compounds bearing larger, nonpolar 17α substituents showed dramatically improved affinities, while affinities for compounds with shorter nonpolar side chains remained largely unchanged. These binding affinity results should be useful in designing new high-affinity fluorescent ligands for the estrogen receptor.* (*Steroids* **56**:375–387, 1991)

**Keywords:** steroids; estradiol analogs; estrogen receptor; relative binding affinities

## Introduction

Estrogen receptors (ERs) and progesterone receptors are known to be valuable in predicting endocrine responsiveness and aggressiveness of breast cancer.[1,2] Cytochemical or flow cytometric detection of these receptors would offer new information on tumor cell heterogeneity with respect to the presence of receptor and on its significance for the above predictions, as well as making receptor determinations faster and requiring less tissue.

For these reasons, a great variety of steroid–fluorophore conjugates and at least one naturally fluorescent ER ligand have been proposed for the in situ detection of receptors. Unfortunately, histochemical methods based on these conjugates have turned out to have a number of pitfalls.[3–5] In particular, low affinity and poor or insufficient specificity of the conjugate for the receptor, along with low stability of the conjugates on storage or on contact with tissues or tissue extracts, have been constant problems. Most of these conjugates were prepared by linking fluorescein to estradiol either directly via an appropriate "spacer"[6–10] or indirectly via bovine serum albumin[11,12]; in general, the fluorophore was attached at carbon-6 (ref. 6) (involving a carboxymethyl oxime), at carbon-7 (ref. 9) (involving an alkyl side chain), or at the carbon-17 hydroxyl[10] (involving a hemisuccinate). Finally, one of the more widely used fluorescent conjugates was generated by linking fluorescein with estrone hydrazone.[6,13]

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Since functionalization at these sites interferes directly with the binding of estrogens to receptor, we have avoided these approaches. Rather, recognizing that 17α-derivatives such as methylestradiol or ethynylestradiol have excellent affinities,[14] we concentrated on 17α linkages. We have previously reported[15] a series of estradiol derivatives bearing three- or four-carbon 17α side chains with or without functional groups and with varying degrees of unsaturation. Based on the relative binding affinities (RBAs) of these compounds, we concluded that steric interference of conformationally mobile saturated 17α side chains with the receptor has a profound influence on the ability of these compounds to bind effectively, since compounds having more constrained and unsaturated side chains showed better affinities. We report an extension of our earlier study involving syntheses and RBAs of medium length (six to 11 carbons) 17α-substituted estradiol analogs in our continuing attempt to define appropriate side chains for linking fluorophores with estradiol.

## Experimental

### *Chemistry*

Melting points (mp) were determined in open capillary tubes either immersed in a heated sulfuric acid bath or with an Electrothermal melting point apparatus, and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer 283B or Beckman IR 4220 spectrophotometer. All the compounds exhibited expected and unexceptional IR spectra, which have therefore been omitted from the experimental details.

$^1$H Nuclear magnetic resonance spectra were taken in deuterated chloroform, unless noted otherwise, on a Varian T60 or Jeol FX 90Q spectrophotometer using tetramethylsilane as internal standard. Salient resonances are reported below and, in the subsequent individual experimental texts, with chemical shift values expressed in parts per million (δ) relative to the standard and with coupling constants (J) in Hz. Variable chemical shifts of exchangeable protons are not reported for spectra using $CDCl_3$ (which may contain trace DCl) as solvent; however, individual chemical shifts and multiplicity for hydroxyl functional groups are reported for spectra using anhydrous DMSO-$d_6$ (dimethylsulfoxide) or acetone-$d_6$, since individual resonances having characteristic downfield-shifted chemical shifts and predicted multiplicities can be observed in these solvents.[16] In general, all the estradiol derivatives reported here exhibited steroid nucleus protons and 17α-substituent aliphatic methylene protons as broad, overlapping resonances between δ 1.0 and 2.8 of appropriate integration intensity. Characteristic resonances were observed for C-18 methyl protons and for aromatic A ring protons: δ 0.85–0.92 (s, 3H, 18$CH_3$), 6.45–6.70 (s with secondary coupling, 1H, C-4H, J = 8–9), 6.55–6.75 (d with secondary coupling, 1H, c-2H, J = 8–9), and 7.10–7.20 (d, 1H, C-1H). For compounds containing 3-benzyl ethers, two additional characteristic resonances were observed: δ 5.0 (s, 2H, $OCH_2Ph$) and 7.3 (bs, 5H, aromatic-H). Compounds containing 17α aliphatic ω-methyl protons showed a broad resonance (3H) at the base of the C-18 methyl singlet. Thus, in the following experimental texts, only other recognizable proton resonances (e.g., acetyl singlets; methylenes adjacent to nitrogen, iodine, and oxygen; vinyl; aryl) are specifically reported.

Mass spectra were obtained on a Hewlett-Packard Model 5982 quadrupole mass spectrometer with a Hewlett-Packard Model 5933 data system. The relative intensity of the salient fragment ions to the base peak (100) is given in parentheses. Accurate mass EI analyses were obtained at 70 eV on a Finnigan-MAT model 212 double-focusing mass spectrometer in conjunction with an INCOS data system. The ion source temperature was 250 C, and the accelerating voltage was 3 kV. Samples were introduced by means of a moving belt interface (Finnigan-MAT) operated at 2.5 cm/s, at a setting of 5 W for heating the tip of the interface. A resolution of 4,000 was used, and mass determination was made by computer comparison with ions of PFK. Elemental analyses were performed by Midwest Microlabs (Indianapolis, IN, USA). The homogeneity of all the compounds was routinely checked by thin-layer chromatography (TLC; Type G, Sigma Chemical Co., St. Louis, MO, USA). Preparative TLC was also performed on silica gel plates (Type G, Sigma), prepared as slurry in water or purchased from Analtech (Newark, DE, USA). The desired compounds were visualized by UV, iodine, or potassium permanganate spray, and were subsequently isolated from their silica gel bands by extraction with ethyl acetate. Estrone, estrone 3-benzyl ether, and 17α-ethynylestradiol were purchased from Steraloids (Wilton, NH, USA). All other reagents and solvents were purchased from Aldrich (Milwaukee, WI, USA).

Hexamethylphosphoric triamide was dried over sodium at ambient temperature until the solution became blue; it was then distilled under reduced pressure. Dimethylsulfoxide and *N,N*-dimethylformamide (DMF) were dried over calcium hydride at ambient temperature and a decanted portion was distilled under reduced pressure. Anhydrous tetrahydrofuran (THF) containing less than 1% water was further purified by distillation from the blue sodium benzophenone ketyl, from butyllithium, or from lithium aluminum hydride. Diisopropyl amine and pyridine were stored over sodium hydroxide, and a decanted portion was distilled immediately prior to use. The titer of the alkyllithium solutions was determined as reported.[17]

The reaction conditions described below are not necessarily optimized.

**17α-Octylestradiol (4).** A solution of 17α-(oct-2′-ynyl)-estradiol 3-benzyl ether (**13** vide infra, 150 mg, 0.32 mmol) in absolute ethanol (6 ml) underwent alkyne hydrogenation and O-benzyl hydrogenolysis in the presence of hydrogen with palladium-carbon (10%, 10 mg) at atmospheric pressure and room temperature for 24 hours. The catalyst was removed by filtration through Celite and rinsed with ethanol (10 ml); the washings were combined with the filtrate. The com-

bined filtrate was concentrated in vacuo to afford a colorless oil. Purification by preparative TLC (50% ether/hexane) followed by recrystallization of the chromatographed solid from ether/hexane afforded the title compound **4:** yield, 120 mg (98%); mp, 150 to 151 C; $M^+$ m/z 384 (100), fragment ions at m/z 366 (51), m/z 253 (40), m/z 228 (57), and m/z 213 (69). Analysis calculated for $C_{26}H_{40}O_2$: C, 81.20; H, 10.48. Found: C, 81.02; H, 10.61.

**17α-(Hexylaminomethyl)estradiol 3-benzyl ether (5).** A mixture of oxirane 3-benzyl ether[15,18,19] (**2,** 150 mg, 0.4 mmol), boric acid (120 mg, 1.94 mmol), and *n*-hexylamine (0.5 ml, 3.8 mmol) in dry DMSO (4 ml) was stirred at 70 to 73 C for 92 hours, then cooled; water (25 ml) and ethyl acetate (30 ml) were added, and the mixture was stirred for 5 minutes. The layers were separated and the organic phase was washed with water (2 × 25 ml). The aqueous phase was extracted with ethyl acetate (2 × 25 ml), and the combined organic phase was washed with brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. Purification by preparative TLC (10% methanol/benzene) followed by recrystallization of the chromatographed solid from ether/hexane afforded the title compound **5:** yield, 183 mg (80%); mp, 72.5 to 74 C; $M^+$ m/z 475 (8), fragment ions at m/z 360 (30), m/z 114 (100), and m/z 91 (64). Analysis calculated for $C_{32}H_{45}NO_2$: C, 80.79; H, 9.54; N, 2.95. Found: C, 81.03; H, 9.55; N, 3.05.

**17α-(Hexylaminomethyl)estradiol (6).** A solution of **5** (96.7 mg, 0.2 mmol) in absolute ethanol (10 ml) was hydrogenated in the presence of palladium–carbon (10%, 35 mg) at atmospheric pressure and room temperature for 24 hours. Spent catalyst was removed by filtration as described above, and the filtrate was again hydrogenated using fresh catalyst (15 mg) for 24 hours. The catalyst was removed and washed as above and the combined filtrate was concentrated in vacuo. Recrystallization from methylene chloride/hexane afforded the title compound **6:** yield, 76.1 mg (97%); mp, 144 to 146 C; δ ($CD_3OD$) 3.0 (bs, 4H, $CH_2NCH_2$), 4.9 (s, 2H, OH and NH); $M^+$ m/z 385 (15), fragment ions at m/z 116 (20) and m/z 114 (100).

**17α-(*N*-Acetylhexylaminomethyl)estradiol 3-benzyl ether (7).** Acetic anhydride (0.1 ml, 1.06 mmol) was added drop-wise to a solution of **5** (100 mg, 0.21 mmol) in pyridine (4 ml); the mixture was stirred at room temperature for 15 minutes. Water (30 ml) and ethyl acetate (25 ml) were added, and the reaction mixture was stirred for 10 minutes. The organic phase was washed with water (20 ml) containing a few drops of diluted HCl, and finally with water to neutrality, dried over anhydrous sodium sulfate, and concentrated in vacuo. Purification by preparative TLC (10% methanol/benzene) followed by recrystallization of the chromatographed solid afforded the title compound **7:** yield, 96.7 mg (98%); mp, 99 to 100 C; δ 2.2 (s, 3H, $COCH_3$) and 3.0–3.9 (m, 4H, $CH_2NCH_2$); $M^+$ m/z 517 (8), fragment ions at m/z 142 (27) and m/z 91 (100). Analysis calculated for $C_{34}H_{47}NO_3$: C, 78.87; H, 9.15; N, 2.71. Found: C, 78.94; H, 8.96; N, 2.93.

**17α-(*N*-Acetylhexylaminomethyl)estradiol (8).** Hydrogenolysis of **7** was carried out as described for the preparation of **6.** Recrystallization from ether/hexane afforded the title compound **8:** yield, 98%; mp, 172.5 to 174 C; δ 2.2 (s, 3H, $COCH_3$) and 3.2–3.8 (m, 4H, $CH_2NCH_2$); $M^+$ m/z 427 (21), fragment ions at m/z 142 (100), m/z 114 (54), and m/z 87 (93). Analysis calculated for $C_{27}H_{41}NO_3$: C, 75.84; H, 9.66; N, 3.29. Found: C, 76.05; H, 9.81; N, 3.53.

**17α-(Hexyloxymethyl)estradiol 3-benzyl ether (9).** Under nitrogen, sodium (400 mg, 17.4 mmol) was dissolved in freshly distilled *n*-hexanol (12 ml) with heating. Solid oxirane 3-benzyl ether (**2,** 600 mg, 1.6 mmol) was then added, and the mixture was heated at 90 to 95 C for 21 hours, cooled, and slowly poured into water (75 ml). The heterogeneous mixture was extracted with ether (100 ml) and the ether layer was washed with water (2 × 50 ml). Each aqueous layer was extracted with ether (2 × 50 ml), and the combined organic phase was washed with brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. Purification by column chromatography on silica gel (25 g, 28 to 200 mesh) using benzene and benzene/ethyl acetate as eluent followed by recrystallization of the chromatographed solid from hexane afforded the title compound **9:** yield, 496 mg (65%); mp, 51 to 52 C; δ 3.1–3.6 (m, 4H, $CH_2OCH_2$); $M^+$ m/z 476 (32), fragment ions at m/z 361 (48) and m/z 91 (100). Analysis calculated for $C_{32}H_{42}O_3$: C, 80.63; H, 9.30. Found: C, 80.34; H, 9.55.

**17α-(Hexyloxymethyl)estradiol (10).** Compound **9** was debenzylated by hydrogenolysis using the procedure described for the preparation of **4.** Recrystallization from benzene/hexane afforded the title compound **10:** yield, 94%; mp, 119 to 120 C; δ 3.2–3.7 (m, 4H, $CH_2OCH_2$); $M^+$ m/z 386 (43), fragment ions at m/z 271 (100) and m/z 253 (70). Analysis calculated for $C_{25}H_{38}O_3$: C, 77.67; H, 9.91. Found: C, 77.83; H, 10.17.

**17α-(Hexylthiomethyl)estradiol 3-benzyl ether (11).** In a flame-dried flask, freshly distilled *n*-hexanethiol (0.17 ml, 1.9 mmol) was dissolved in dry THF (3 ml); the solution was cooled in an ice bath. To this cold solution, methyllithium (1.4 ml, 1.9 mmol, 1.4 M solution in ether) was added under nitrogen, and the mixture warmed to room temperature. Oxirane 3-benzyl ether (**2,** 560 mg, 1.5 mmol) was then added as solid followed by the addition of freshly distilled DMF (7 ml). The reaction mixture was stirred at 100 C for 20 hours, cooled, and poured into deoxygenated water (35 ml) containing trace acetic acid. The aqueous emulsion was extracted with deoxygenated chloroform (3 × 25 ml) and the combined organic phase was washed with water (2 × 15 ml), followed by brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. Purification by column chromatography on silica gel (20 g, 28 to 200 mesh) using benzene as eluent afforded the

title compound **11** as an oil (530 mg, 72%) that was suitable for the following debenzylation step. An analytic sample was obtained by preparative TLC (5% ethyl acetate/benzene) followed by drying under high vacuum at 50 C for 48 hours: $M^+$ m/z 492 (2.8), fragment ions at m/z 360 (23.3) and m/z 91 (100). Analysis calculated for $C_{32}H_{44}O_2S$: C, 78.00; H, 9.00; S, 6.51. Found: C, 77.92; H, 8.96; S, 6.61.

**17α-(Hexylthiomethyl)estradiol (12).** Under argon, a solution of **11** (419 mg, 0.85 mmol) in *iso*-propanol (0.065 ml) and dry THF (15 ml) was placed in a dry flask fitted with a dry ice–acetone condenser and a drying tube, and was cooled to −78 C (dry ice–acetone bath). Ammonia (50 ml) was then distilled into the flask followed by the addition of lithium (16 mg, 2.3 mmol). The blue reaction mixture was stirred for 10 minutes, allowed to warm to room temperature, and stirred for 20 minutes. Ammonia was allowed to evaporate under a gentle stream of nitrogen; a mixture of 1.2 N HCl (25 ml) saturated with sodium chloride and ethyl acetate (25 ml) was then added, and the mixture was stirred for 5 minutes. The organic phase was washed with deoxygenated water (2 × 15 ml) and the aqueous phase was extracted with ethyl acetate (2 × 15 ml). The combined organic phase was washed with brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. Purification by preparative TLC (10% ethyl acetate/benzene) followed by recrystallization of the chromatographed solid from ether/hexane afforded the title compound **12:** yield, 291 mg (85%); mp, 112 to 113 C; $M^+$ m/z 402 (2), fragment ions at m/z 253 (13) and m/z 270 (100). Analysis calculated for $C_{25}H_{38}O_2S$: C, 74.58; H, 9.51; S, 7.96. Found: C, 74.56; H, 9.55; S, 7.78.

**17α-(Oct-2′-ynyl)estradiol 3-benzyl ether (13).** In a flame-dried flask, methyllithium (4.3 ml, 6.45 mmol, 1.5 M solution in ether) was added to a solution of 1-heptyne (1.1 ml, 8 mmol) in dry THF (4 ml) at 10 C under nitrogen. The mixture was brought to room temperature; solid **2** (1.0 g, 2.67 mmol) was added, followed by the addition of dry hexamethylphosphoric triamide (3 ml). The reaction mixture was stirred at 45 to 50 C for 22 hours, cooled, poured into water (70 ml), and extracted with ethyl acetate (3 × 50 ml). The combined organic phase was washed with water (2 × 30 ml) followed by brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. Purification by column chromatography on neutral alumina (38 g) using increasing amounts of ether in hexane afforded the title compound **13:** yield, 1.03 g (82%). An analytic sample was obtained by preparative TLC (5% ethyl acetate/benzene) followed by recrystallization of the chromatographed solid from hexane: mp, 54.5–56 C; $M^+$ m/z 470 (24), fragment ions at m/z 361 (36) and m/z 91 (100). Analysis calculated for $C_{33}H_{40}O_2$: C, 84.21; H, 8.99. Found: C, 84.13; H, 9.12.

**17α-(Oct-2′-ynyl)estradiol (14)**

*Method A.* Compound **13** was debenzylated with lithium in ammonia using the procedure described for the preparation of **12** (but with less than 2 g atom equivalents of lithium and no isopropanol to prevent reduction of the triple bond); purification by preparative TLC (10% ethyl acetate/benzene) followed by crystallization from ether/hexane afforded the title compound **14:** yield, 53%; mp, 66 to 68 C; $M^+$ m/z 380 (26), fragment ions at m/z 271 (100) and m/z 53 (45). Analysis calculated for $C_{26}H_{36}O_2$: C, 82.06; H, 9.45. Found: C, 81.49; H, 9.60.

*Method B.* Treatment of deprotected oxirane **3**[15,19,20] with heptynyllithium using the procedure described for the preparation of **13** (but with a 10-fold excess of the acetylide reagent) and purification as in method A afforded the title compound **14** in 70% yield. The physical and analytic properties were identical to those described above for method A.

***Z*-17α-(Oct-2′-enyl)estradiol 3-benzyl ether (15).** A solution of **13** (300 mg, 0.64 mmol) in pyridine (8 ml) was hydrogenated for 2 hours at 1 atm pressure using palladium–barium sulfate (5%, 30 mg). The catalyst was removed by filtration through Celite and washed with ethyl acetate (5 ml); the combined filtrate was concentrated in vacuo. Purification by preparative TLC (40% ether/hexane) afforded the title compound **15** as an oil: yield, 290 mg (96%); δ 5.6 (t, 2H, vinylic-H, J = 6); $M^+$ m/z 472 (6), fragment ions at m/z 361 (27) and m/z 91 (100). Analysis calculated for $C_{33}H_{44}O_2$: C, 88.85; H, 9.38. Found: C, 83.89; H, 9.11.

***Z*-17α-(Oct-2′-enyl)estradiol (16).** Compound **15** was debenzylated with lithium in ammonia using the procedure described for the preparation of **12.** Recrystallization from ethyl acetate afforded the title compound **16:** yield, 82%; mp, 171 to 172 C; δ ($CD_3OD$) 5.6 (t, 2H, vinylic-H, J = 6); $M^+$ m/z 382 (4), fragment ions at m/z 271 (100) and m/z 253. Analysis calculated for $C_{26}H_{38}O_2$: C, 81.62; H, 10.01. Found: C, 81.89; H, 10.05.

**17α-(3′-Cyclohexylprop-2′-ynyl)estradiol 3-benzyl ether (17).** Treatment of **2** with cyclohexylethynyllithium (generated from methyllithium and cyclohexylacetylene) using the procedure described for the preparation of **13** and purification by column chromatography on neutral alumina followed by recrystallization of the chromatographed solid from ether/hexane afforded the title compound **17:** yield, 87%; mp, 88.5 to 90 C; $M^+$ m/z 482 (41), fragment ions at m/z 361 (70) and m/z 91 (100). Analysis calculated for $C_{34}H_{42}O_2$: C, 84.60; H, 8.77. Found: C, 84.57; H, 8.93.

**17α-(3′-Cyclohexylprop-2′-ynyl)estradiol (18).** Compound **17** was debenzylated with lithium in ammonia using the procedure described for the preparation of **12.** Purification by preparative TLC (10% ethyl acetate/benzene) followed by recrystallization of the chromatographed solid from ether/hexane afforded the title compound **18:** yield, 83%; mp, 121 to 122 C; $M^+$ m/z 392 (19), fragment ions at m/z 271 (100) and m/z 253 (50).

Analysis calculated for $C_{27}H_{36}O_2$: C, 82.61; H, 9.24. Found: C, 82.46; H, 9.09.

**17α-(Bromoethynyl)estradiol (19b).** Tetramethylethylenediamine (0.153 ml, 1.02 mmol) was added to a suspension of cuprous bromide (145 mg, 1.01 mmol) in dry THF (5 ml), and the mixture was stirred to homogeneity. Bromine (0.034 ml, 0.663 mmol) in dry THF (1 ml) was added dropwise, and the mixture was stirred for 8 minutes. To the resulting green suspension, a solution of pyridine (0.055 ml, 0.681 mmol) in dry THF (2 ml) was added, and the stirring continued for 10 minutes. To this, a solution of 17α-ethynylestradiol 3-acetate (from 17α-ethynylestradiol [**19a**], acetic anhydride, and pyridine; 100 mg, 0.338 mmol) in dry THF (2 ml) was added; the reaction mixture was stirred for 52 hours. The heterogeneous mixture was transferred to a column (silica gel, 10 g, 28 to 200 mesh) and eluted with 1 : 1 ether/hexane (50 ml). Removal of solvents in vacuo and purification by preparative TLC (10% ethyl acetate/benzene) followed by recrystallization of the chromatographed solid from benzene/petroleum ether afforded 17α-(bromoethynylestradiol 3-acetate: yield, 108 mg (76.7%); mp, 174 to 175 C; δ 2.28 (s, 3H, $COCH_3$).

17α-(Bromoethynyl)estradiol 3-acetate prepared in this manner was dissolved in methylene chloride/methanol (4.5 ml, 1 : 2) and the solution was cooled to 0 C. At this temperature, sodium hydroxide (27 mg, 0.68 mmol) in water (1.7 ml) was added; the mixture was stirred for 2 hours, diluted with water (10 ml), acidified with diluted HCl to pH 5 to 6, and extracted with methylene chloride (3 × 15 ml). The organic phase was washed with brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. Purification by preparative TLC (10% ethyl acetate/benzene) followed by recrystallization of the chromatographed solid from acetone/hexane afforded the title compound **19b:** yield, 85 mg (67%); mp, 169 to 170 C (literature[21]: mp, 169.6 C).

**17α-(3′-Iodoprop-2′-ynyl)estradiol (20b).** 17α-(Prop-2′-ynyl)estradiol (**20a**)[15] was iodinated using the procedure described for the preparation of **19b** (but with iodine and cuprous iodide and no pyridine). Purification by preparative TLC (10% ethyl acetate/benzene) followed by recrystallization of the chromatographed solid from ethyl acetate/hexane afforded the title compound **20b:** yield, 78%; mp, 147 to 150 C (previous softening at 95 to 100 C); $M^+$ m/z 436 (100), fragment ions at m/z 271 (71), m/z 253 (37) and m/z 213 (29). Analysis calculated for $C_{21}H_{25}IO_2$: C, 57.80; H, 5.73; I, 29.13. Found: C, 57.66; H, 5.77; I, 29.09.

**17α-(Nona-1′,3′-diynyl)estradiol (21).** 1-Heptyne (0.11 ml, 0.84 mmol) was added to a stirred suspension of cuprous chloride (3.2 mg, 0.032 mmol), hydroxylamine hydrochloride (4 mg, 0.06 mmol), and *n*-butylamine (0.17 ml, 2.0 mmol). After this mixture was stirred for 2 minutes, a solution of 17α-bromoethynylestradiol (**19b,** 121 mg, 0.32 mmol) in ethanol (1.5 ml) was added dropwise over 15 minutes. During addition of the steroid, the color of the reaction mixture changed from pale yellow to blue, at which time the addition was stopped and solid hydroxylamine hydrochloride (6 mg, 0.09 mmol) was added to regenerate the pale yellow color; addition of the steroid was then continued. The reaction mixture was stirred for 10 minutes, aqueous HCl (0.12 N, 30 ml) was added, and the heterogeneous mixture was extracted with ethyl acetate (25 ml). The organic phase was washed with water; the aqueous layer was separately extracted with ethyl acetate (3 × 20 ml). The combined organic phase was washed with brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. Purification by preparative TLC (10% ethyl acetate/benzene) followed by recrystallization of the chromatographed solid from ethyl acetate/hexane afforded the title compound **21:** yield, 85.3 mg (67.4%); mp, 182 to 184 C; δ (acetone-$d_6$) 2.90 (s, 1H, 17-OH) and 7.90 (bs, 1H, phenolic OH); $M^+$ m/z 390 (22), fragment ions at m/z 375 (20) and m/z 347 (30), m/z 333 (100), m/z 253 (79), and m/z 133 (54). Analysis calculated for $C_{27}H_{34}O_2$: C, 83.03; H, 8.78. Found: C, 82.93; H, 8.73.

**17α-(4′-Phenylbuta-1′,3′-diynyl)estradiol (22).**

*Method A.* Compound **19b** and phenylacetylene (1.2 equivalents) were cross-coupled using the procedure described for the preparation of **21.** Purification by preparative TLC (ethyl acetate/benzene/methanol 11 : 87 : 2) followed by recrystallization of the chromatographed solid from ethyl acetate/hexane afforded the title compound **22:** yield, 90%; mp, 158 to 160 C; δ (acetone-$d_6$) 5.70 (s, 1H, 17-OH), 7.3–7.8 (m, 5H, Ph), and 9.0 (s, 1H, phenolic OH); $M^+$ m/z 396 (100), fragment ions at m/z 353 (61), m/z 259 (75), and m/z 133 (68). Analysis calculated for $C_{28}H_{28}O_2$: C, 84.81; H, 7.12. Found: C, 85.02; H, 7.23.

*Method B.* Under nitrogen, a solution of 1-bromo-2-phenylacetylene[22–24] (170 mg, 0.94 mmol) in ethanol (1.5 ml) was added dropwise to a stirred mixture of 17α-ethynylestradiol (**19a,** 200 mg, 0.68 mmol), cuprous chloride (1.3 mg, 0.013 mmol), hydroxylamine hydrochloride (81 mg, 1.16 mmol), and *n*-butylamine (0.35 ml, 4.1 mmol) at 10 C. The reaction mixture was stirred at room temperature for 2 hours and worked-up as described for the synthesis of **21.** Purification by preparative TLC (ethyl acetate/benzene/methanol 11 : 87 : 2) followed by recrystallization of the chromatographed solid from ethyl acetate/hexane afforded the title compound **22:** yield, 65 mg (95% with 24% conversion); recovered 17α-ethynylestradiol (150.2 mg, 74.6%).

*Method C.* Under nitrogen, tetramethylethylenediamine (34.8 mg, 0.3 mmol) was added to a solution of cuprous chloride (99 mg, 1.0 mmol) in dry acetone (1.5 ml), and the greenish blue mixture was stirred for 30 minutes. In a separate flask, **19a** (296 mg, 1.0 mmol) and phenylacetylene (1.02 g, 10 mmol) were dissolved in dry acetone (3.5 ml); to this, the above mixture was slowly added. A stream of dry air was bubbled through the reaction mixture for 9 hours at room temperature. The mixture was concentrated in vacuo and the residue

was filtered. The filter cake was washed with water (100 ml) and dried. Purification by preparative TLC (20% ethyl acetate/benzene) followed by recrystallization of the chromatographed solid from ethyl acetate/hexane afforded the title compound **22:** yield, 79 mg (61% with 33% conversion); recovered 17α-ethynylestradiol (200 mg, 67%).

**17α-(Nona-2′,4′-diynyl)estradiol (23).** 17α-(Prop-2′-ynyl)estradiol (**20a**) and 1-bromo-1-hexyne (1.4 equivalents) were cross-coupled using the procedure described for the preparation of **21.** Purification by preparative TLC (10% ethyl acetate/benzene) followed by recrystallization of the chromatographed solid from ethyl acetate/hexane afforded the title compound **23:** yield, 90%; mp, 117 to 118 C; δ (acetone-$d_6$) 3.55 (s, 1H, 17-OH) and 7.90 (s, 1H, phenolic OH); $M^+$ m/z 390 (18), fragment ions at m/z 271 (100), m/z 270 (38), and m/z 253 (73). Analysis calculated for $C_{27}H_{34}O_2$: C, 83.03; H, 8.78. Found: C, 83.26; H, 8.70.

**17α-(5′-Phenylpenta-2′,4′-diynyl)estradiol (24).** Compound **20a** and 1-bromo-2-phenylacetylene (1.4 equivalents) were cross-coupled using the procedure described for the preparation of **21.** Purification by preparative TLC (10% ethyl acetate/benzene) followed by recrystallization of the chromatographed solid from ethyl acetate/hexane afforded the title compound **24:** yield, 65%; mp, 215 to 217 C; δ (acetone-$d_6$) 3.60 (s, 1H, 17-OH), 7.35 (m, 5H, Ph), and 7.7 (bs, 1H, phenolic OH); $M^+$ m/z 410 (25), fragment ions at m/z 340 (38) and m/z 271 (100), m/z 253 (73), and m/z 133 (61). Analysis calculated for $C_{29}H_{30}O_2$: C, 84.84; H, 7.37. Found: C, 84.75; H, 7.39.

**17α-(6′-Iodohex-1′-ynyl)estradiol (25).** *n*-Butyllithium (0.32 ml, 0.5 mmol, 1.6 M solution in hexane) and dry THF (10 ml) were placed in a flame-dried flask under nitrogen and cooled to −20 C. At this temperature, a solution of 17α-ethynylestradiol 3,17-bis(tetrahydropyranyl ether)[25] (92.8 mg, 0.2 mmol) in dry THF (20 ml) was added dropwise, and the reaction mixture was allowed to warm to room temperature and was stirred for 1.5 hours. A large excess of 1,4-diiodobutane (248 mg, 0.8 mmol) was added in one portion, and the mixture was stirred for 8 hours. Saturated aqueous ammonium chloride (0.5 ml) was then added; the reaction mixture was diluted with ethyl acetate (50 ml) and washed with brine (3 × 25 ml), dried over anhydrous sodium sulfate, and concentrated in vacuo to yield a diastereomeric mixture of 17α-(6′-iodohex-1′-ynyl)estradiol 3,17-bis(tetrahydropyranyl ether) as an oil that was directly hydrolyzed.

To a solution of oily tetrahydropyranyl ethers in ethanol (95%, 5 ml), *p*-toluenesulfonic acid monohydrate (15 mg, 0.08 mmol) and water (1 ml) were added. The reaction mixture was stirred at 60 C for 4 hours, diluted with water (100 ml), and extracted with ethyl acetate (3 × 30 ml). The combined organic phase was washed with water to neutrality, dried over anhydrous sodium sulfate, and concentrated in vacuo. Purification by preparative TLC (15% ethyl acetate/benzene) followed by recrystallization of the chromatographed solid from benzene/hexane afforded the title compound **25:** yield, 30 mg (31%); mp, 151 to 152 C; δ 3.18 (t, 2H, $CH_2I$, J = 7); $M^+$ m/z 478 (31.4), fragment ions at m/z 463 (100), m/z 460 (10), m/z 351 (15), and m/z 213 (56.9). Exact mass ($M^+$) calculated for $C_{24}H_{31}IO_2$: 478.1320; observed, 478.1342.

**17α-(6′-Iodohex-1′-ynyl)-2-(4′-iodobutyl)estradiol (25a).** This compound was isolated as a byproduct from the reaction above during the preparative TLC purification. Recrystallization of the chromatographed solid from benzene/hexane afforded **25a:** yield, 13 mg (10%); mp, 95 to 96 C; δ 2.52 (t, 2H, $CH_2Ar$, J = 7); 3.17 (t, 4H, $CH_2I$, J = 7), 6.34 (s, 1H, C-4H), and 6.94 (s, 1H, C-1H); $M^+$ m/z 660 (53.9), fragment ions at m/z 642 (63.3), m/z 627 (20), and m/z 532 (100). Exact mass ($M^+$) calculated for $C_{28}H_{38}I_2O_2$: 660.0870; observed, 660.0898.

**17α-(8′-Iodooct-1′-ynyl)estradiol (26).** The title compound was prepared as described for the two-step preparation of **25** (but with methyllithium in place of butyllithium and with 1,6-diiodohexane as alkylating agent); purification by preparative TLC (15% ethyl acetate/benzene) followed by recrystallization of the chromatographed solid from benzene/hexane afforded the title compound **26:** yield, 45%; mp, 182 to 183 C; δ ($CDCl_3$ + $CD_3OD$) 3.15 (t, 2H, $CH_2I$, J = 7); $M^+$ m/z 506, fragment ions at m/z 491 (100), m/z 488 (11), and m/z 213 (48.3). Exact mass ($M^+$) calculated for $C_{26}H_{35}IO_2$: 506.1632; observed, 506.1683.

**17α-(Oct-1′-ynyl)estradiol (27).** The title compound was prepared as described for the two-step preparation of **25,** but with methyllithium in place of butyllithium and with *n*-hexyliodide as alkylating agent. Purification by preparative TLC (10% ethyl acetate/benzene) followed by recrystallization of the chromatographed solid from benzene/hexane afforded the title compound **27:** yield, 60%; mp, 162 to 163 C; $M^+$ m/z 380 (25.3), fragment ions at m/z 365 (100) and m/z 213 (36.3). Exact mass ($M^+$) calculated for $C_{26}H_{36}O_2$: 380.2706; observed, 380.2642.

**17α-(Iodoethynyl)estradiol (28).**

*Method A.* The title compound was prepared as described for the two-step preparation of **25,** but with methyllithium in place of butyllithium and with iodine. Purification by preparative TLC (15% ethyl acetate/benzene) and recrystallization of the chromatographed solid from benzene/hexane afforded the title compound **28:** yield, 66%; mp, 189 to 190 C (decomposition) (literature[26,27]: no mp); $M^+$ m/z 422 (100), fragment ions at m/z 295 (41) and m/z 213 (58.8). Analysis calculated $M^+$ for $C_{20}H_{23}IO_2$: 422.0696; observed, 422.0680.

*Method B.* 17α-Ethynylestradiol was iodinated in one step using cuprous iodide and iodine as described for the preparation of **20b.** Purification by dry column (silica gel, 65 g) and preparative TLC (10% ethyl acetate/benzene) followed by recrystallization of the chromato-

graphed solid from ethyl acetate/hexane afforded the title compound **28:** yield, 90%; mp, 192 to 193 C (decomposition).

**17α-(2'-Cyclohexylethynyl)estradiol (29).** Methyllithium (12 ml, 18 mmol, 1.5 M solution in ether) and dry THF (20 ml) were placed in a flame-dried flask, and the mixture was cooled to 10 to 15 C. Cyclohexylacetylene (2 g, 18.5 mmol) was added dropwise, and the resulting solution was warmed to room temperature and stirred for 1 hour. Estrone (270 mg, 1.0 mmol) in dry THF (80 ml) was added; the mixture was stirred at room temperature for 12 hours, heated at reflux for 3 hours, cooled, and quenched with saturated aqueous ammonium chloride (5 ml). The reaction mixture was then diluted with ethyl acetate (100 ml) and the organic phase was washed with brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. Purification by preparative TLC (10% ethyl acetate/benzene) followed by recrystallization of the chromatographed solid afforded the title compound **29:** yield, 30 mg (7.9%); mp, 183 to 184 C; δ ($CDCl_3$ + DMSO-$d_6$) 3.34 (s, 1H, 17-OH) and 8.36 (s, 1H, phenolic OH); $M^+$ m/z 378 (17.8), fragment ions at m/z 363 (100), m/z 360 (5), and m/z 345 (3).

**17α-(2'-Phenylethynyl)estradiol (30).** The title compound was prepared as described for the preparation of **29,** but with excess phenylethynyllithium. Purification by preparative TLC followed by recrystallization of the chromatographed solid from ethyl acetate/hexane afforded the title compound **30:** yield, 40%; mp, 156 C (literature[28]: mp, 156 to 158 C).

**17α-Benzylestradiol (31).** The title compound was prepared as reported[28]: mp, 214 to 215 C (literature[28]: mp, 214.5 to 216.5 C).

**17α-Phenylestradiol (32).** Phenyllithium (5 ml, 10 mmol, 2.0 M solution in cyclohexane/ether) and dry THF (20 ml) were placed in a flame-dried flask, and the mixture was cooled to 10 to 15 C. Estrone (270 mg, 1 mmol) in dry THF (80 ml) was added dropwise; the mixture was stirred at room temperature for 2 hours, heated at reflux for 8 hours, then cooled and quenched with saturated aqueous sodium chloride (3 ml). The reaction mixture was then diluted with ethyl acetate (100 ml), and the organic phase was washed with water, dried over anhydrous sodium sulfate, and concentrated in vacuo. Purification by preparative TLC (10% ethyl acetate/benzene) followed by crystallization from ethyl acetate/hexane and recrystallization from THF/hexane afforded the title compound **32:** yield, 150 mg (43%); δ (DMSO-$d_6$) 5.07 (s, 1H, 17-OH), 7.33 (s, 5H, Ph), and 8.89 (s, 1H, phenolic OH); $M^+$ m/z 348 (100), fragment ions at m/z 330 (42) and m/z 213 (51). Purity of **32** was confirmed by high-performance liquid chromatography (HPLC) on a C-18 reverse-phase column using 70 : 30 mixture of acetonitrile to water; under these conditions, no starting material was detected.

### *Receptor affinity determinations*

The RBAs of the newly synthesized compounds with respect to [$^3$H]estradiol were determined for ER from rat uterus, as described earlier.[15] In brief, cytosol ER was prepared from uteri of mature ovariectomized Harlan-Sprague-Dawley rats. For RBA determinations in the absence of DMF, triplicate sets of tubes received 200 μl cytosol, 25 μl 2 × $10^{-8}$ M [$^3$H]estradiol (102 Ci/mmol, DuPont NEN), and 25 μl unlabeled test compound at five or more concentrations. Test compounds were dissolved in ethanol to make a 1 mM solution and subsequently diluted in the assay buffer (10 mM Tris-HCl, pH 7.4 at 0 C, 1.5 mM EDTA, 0.5 mM dithiothreitol). After 18 hours at 0 to 4 C, all tubes received 500 μl DCC suspension (10 mM Tris-HCl, pH 8.0 at 0 C, 0.25% Norit A, 0.0025% dextran), and were mixed for 15 minutes and centrifuged. Supernatants (500 μl) were counted in a liquid scintillation counter.

For RBA determinations in the presence of DMF, triplicate sets of tubes received 200 μl cytosol, 40 μl 1.25 × $10^{-8}$ M [$^3$H]estradiol (102 Ci/mmol, DuPont NEN), and 10 μl unlabeled test compound at five or more concentrations in DMF, giving a final concentration of 4% DMF. The assay was completed as above.

Sets of tubes also containing $10^{-6}$ M unlabeled estradiol were used to determine nonspecific binding, which was subtracted from all values. Results were plotted and RBAs determined as described earlier. The RBA values reported here are the average of three or more determinations.

## Results and discussion

### *Chemistry*

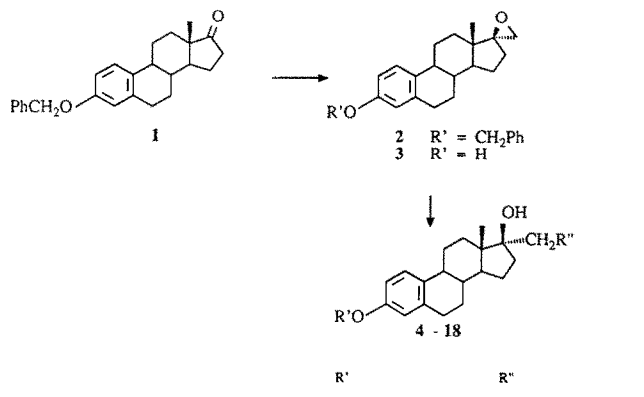
For the successful development of an effective fluorophore-estradiol conjugate using a 17α linkage, the fluorophore must be attached to the estradiol molecule via some spacer arm. Earlier,[15] we reported synthesis and receptor affinities of analogs of estradiol substituted in the 17α position with three- and four-carbon chains (with or without functional groups and with varying degrees of unsaturation). It was shown that steric bulk and conformational mobility of the side chain apparently play a significant role in decreasing the ability of analogs to bind to the receptor. Direct attachment of fluorophore via such short chains would indeed increase the steric bulk very close to the steroid nucleus. We have therefore synthesized analogs of estradiol with 17α substituents having longer (six to 11 carbon) chains to model more appropriate spacer arms, and have compared their RBAs for ER. A variety of convenient synthetic approaches to these 17α spacer models was used that led to preparation of substituents incorporating 2'-heteroatoms, unsaturation specifically at 1' or 2', ω-iodides, and aromatic and other conjugated unsaturated moieties, as well as saturated alkyl analogs.

Three general methods of constructing 17α substituents have been used in the current study: nucleophilic opening of 17β-oxirane, acetylenic cross-coupling and

alkylation of terminal 17α-alkynes, and organometallic additions to 17-ketone. For protection of the phenolic hydroxyl, we used benzyl and tetrahydropyranyl ethers to take advantage of the facile and varied conditions available for their removal.

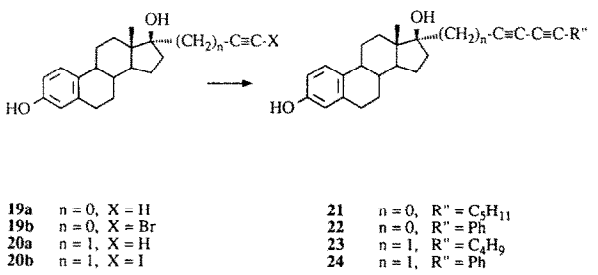
The 17β-oxirane is an excellent synthon for the synthesis of 17α-substituted analogs of estradiol, since the epoxide can be cleaved with various carbon and heteroatom nucleophiles. This synthetic sequence enabled us to incorporate heteroatoms and carbon unsaturation at the 2′ position in the side chain. Thus, estrone 3-benzyl ether (**1**) was first converted to the corresponding 17β-oxirane 3-benzyl ether (**2**)[15,18,19] using dimethylsulfonium methylide; selective debenzylation of **2** afforded **3**[15]; C-20 opening of epoxide **2** or **3** with various nucleophiles, followed by selective removal of the 3-benzyl protecting group in the former case, afforded one series of 17α-substituted analogs of estradiol (**4** to **18;** Figure 1), as described below.

For the conversion of **2** to 17α-(hexylaminomethyl)estradiol 3-benzyl ether (**5**), attempts to use a reported procedure[29,30] for the preparation of analogous compounds containing a 3-methyl ether, using short chain alkylamine in ethanol with acetic acid, proved ineffective. Complex mixtures were avoided by a modification using hexylamine in DMSO with boric acid to afford **5** in excellent yield. Debenzylation of **5** using palladium-catalyzed hydrogenolysis afforded **6.** Alternatively, acetylation of **5** with acetic anhydride and pyridine gave the acetamide **7,** which, on hydrogenolysis, afforded the debenzylated acetamide **8** in near quantitative yield. Oxygen and sulfur analogs were readily prepared in high yield by similar reactions: 17α-(hexyloxymethyl)estradiol 3-benzyl ether (**9**) was prepared from **2** and sodium hexyloxide in *n*-hexanol at 90 to 95 C, and 17α-(hexylthiomethyl)estradiol 3-benzyl ether (**11**) was prepared from **2** and excess lithium hexylmercaptide in DMF at 100 C. Debenzylation of **9** by hydrogenolysis afforded the desired 17α-(hexyloxymethyl)estradiol (**10**). However, because of the incompatibility of a thio-ether with palladium-catalyzed hydrogenolysis, alternative reductive debenzylation of **11** was accomplished using lithium in ammonia/THF/isopropanol to afford 17α-(hexylthiomethyl)estradiol (**12**).




| | R' | R" |
|---|---|---|
| 4 | H | -$(CH_2)_6CH_3$ |
| 5 | $CH_2Ph$ | -NH-$(CH_2)_5CH_3$ |
| 6 | H | -NH-$(CH_2)_5CH_3$ |
| 7 | $CH_2Ph$ | -N(Ac)-$(CH_2)_5CH_3$ |
| 8 | H | -N(Ac)-$(CH_2)_5CH_3$ |
| 9 | $CH_2Ph$ | -O-$(CH_2)_5CH_3$ |
| 10 | H | -O-$(CH_2)_5CH_3$ |
| 11 | $CH_2Ph$ | -S-$(CH_2)_5CH_3$ |
| 12 | H | -S-$(CH_2)_5CH_3$ |
| 13 | $CH_2Ph$ | -C≡C-$(CH_2)_4CH_3$ |
| 14 | H | -C≡C-$(CH_2)_4CH_3$ |
| 15 | $CH_2Ph$ | -CH=CH-$(CH_2)_4CH_3$ (*cis*) |
| 16 | H | -CH=CH-$(CH_2)_4CH_3$ (*cis*) |
| 17 | $CH_2Ph$ | -C≡C-*c*-$C_6H_{11}$ |
| 18 | H | -C≡C-*c*-$C_6H_{11}$ |

**Figure 1** Synthesis of 17α-substituted estradiol derivatives from estrone-17β-oxirane.




| | | | |
|---|---|---|---|
| **19a** | n = 0, X = H | **21** | n = 0, R" = $C_5H_{11}$ |
| **19b** | n = 0, X = Br | **22** | n = 0, R" = Ph |
| **20a** | n = 1, X = H | **23** | n = 1, R" = $C_4H_9$ |
| **20b** | n = 1, X = I | **24** | n = 1, R" = Ph |

**Figure 2** Synthesis of conjugated diynes.

An acetylide ring opening[15] of the 17β-oxirane **2** with heptynyllithium in THF afforded 17α-(oct-2′-ynyl)estradiol 3-benzyl ether (**13**); complete hydrogenation and hydrogenolysis of **13** using excess hydrogen in the presence of 10% Pd-carbon gave 17α-octylestradiol (**4**) in high yield over two steps. Selective hydrogenation of **13** in pyridine in the presence of 5% Pd-barium sulfate gave the *cis*-hydrogenated 3-benzyl ether **15;** no debenzylation was observed under these conditions. Selective reductive debenzylation of **13** and **15** using less than two g atom equivalents of lithium in ammonia afforded the desired 17α-(oct-2′-ynyl)estradiol (**14**) and *Z*-17α-(oct-2′-enyl)estradiol (**16**), respectively.* Analog **14** was also prepared directly from **3** (ref. 15) using a large excess of heptynyllithium.17α-(3′-Cyclohexylprop-2′-ynyl)estradiol 3-benzyl ether (**17**) was prepared from **2** and lithium cyclohexylacetylide; subsequent selective debenzylation by lithium in ammonia, as above, afforded 17α-(3′-cyclohexylprop-2′-ynyl)estradiol (**18**).

For preparation of conjugated diynes (**21** to **24;** Figure 2), we used either Cadiot-Chadkiewicz–directed cross-couplings[31,32] of alkynes with bromoalkynes catalyzed by copper (I) or simple oxidative cross-coupling of alkynes using air oxidation in the presence of tetramethylethylenediamine(TMEDA)–copper (I) complex.[33] Thus, dynes **21** to **24** were synthesized by the

* *E*-17α-(Oct-2′-enyl)estradiol was prepared (data not shown) by reacting **14** with sodium in lithium/isopropanol. The desired compound was isolated in more than 98% purity (contaminated with less than 2% of fully saturated side chain analog **4,** as envisaged by mass spectrum) and was used as such for RBA determinations.

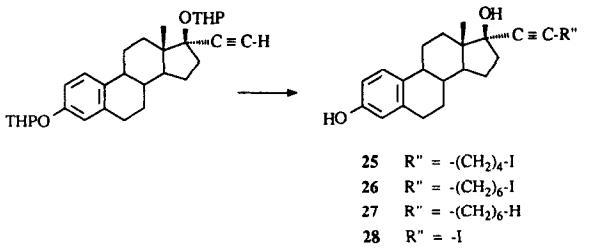



**Figure 3** Synthesis of 17α-(1′-alkynyl)-substituted estradiol derivatives.

following procedures: by directed cross-coupling of 17α-(2′-bromoethynyl)estradiol (**19b**) with appropriate terminal alkynes, by directed cross-coupling of 17α-ethynylestradiol (**19a**) or 17α-(prop-2′-ynyl)estradiol (**20a**)[15] with appropriate bromoalkynes, or by simple cross-coupling of **19a** with an appropriate terminal alkyne. Haloalkynes (**19b** and **20b**; Figure 2) were prepared in excellent yield by direct halogenation of **19a** and **20a**, respectively; bromination in dry THF using a complex made from bromine and TMEDA–cuprous bromide† converted **19a** to **19b**. Iodination in THF using the analogous complex made from iodine and TMEDA-cuprous iodide converted **20a** to 17α-(3′-iodoprop-2′-ynyl)estradiol (**20b**). Formation of 17α-(nona-1′,3′-diynyl)estradiol (**21**) and 17α-(4′-phenylbuta-1′,3′-diynyl)estradiol (**22**) by directed cross-coupling of **19b** with heptyne and phenylacetylene, respectively, was effected in excellent yield, provided that oxidation of the copper (I) coupling catalyst was suppressed by the addition of sufficient hydroxylamine hydrochloride. For comparison, **22** was also prepared by the other two coupling procedures in good yield but much lower conversions. Formation of 17α-(nona-2′,4′-diynyl)estradiol (**23**) and 17α-(5′-phenylpenta-2′,4′-diynyl)estradiol (**24**) was effected in good yield by directed cross-coupling of **20a** with 1-bromo-1-hexyne and 1-bromo-2-phenylacetylene,[22–24] respectively.

Analogs bearing a 17α-(1′-alkynyl) substituent (**25** to **28**, Figure 3) were synthesized by acetylide alkylation starting with 17α-ethynylestradiol (**19a**). Thus, **19a** was converted to the corresponding 3,17-bis(tetrahydropyranyl ether) as reported[25]; acetylide anion was formed using excess methyllithium, and was subsequently alkylated using excess iodoalkanes or α,ω-diiodoalkanes, and the products were directly deprotected by aqueous acidic hydrolysis to afford **25** to **28**. These alkylation and deprotection sequences proceeded in modest to good overall yields. Methyllithium was found to be superior to butyllithium for acetylide formation, since an excess of butyllithium for generating acetylide led to formation of the C-2 alkylated byproduct **25a**. As noted elsewhere,[34] use of 1,2-diiodoethane in this sequence produced only 17α-iodoethynylestradiol (**28**); apparently, in this reaction, C-iodination of the lithium acetylide (through elimination of ethylene) is favored over C-alkylation. Compound **28** was also prepared in excellent yield by iodination of **19a** in THF using the complex described in the preceding paragraph made from iodine and TMEDA–cuprous iodide.

Finally, several additional compounds (**29** to **32**; Figure 4) were prepared in good yield directly from estrone by the addition of excess organometallic, with subsequent mildly acidic aqueous work-up. The method is amenable to a variety of Grignard-type additions. Thus, analogs **29** to **31** were prepared and isolated in conventional fashion, as simpler alkyl, allyl, and alkynyl analogs had already been reported.[15,35] However, when 17α-phenylestradiol (**32**) was prepared from estrone and excess phenyllithium, monitoring the progress of the reaction and the purity of the isolated product required use of reverse-phase HPLC because estrone and 17α-phenylestradiol were indistinguishable by TLC in a variety of solvent systems.

### *Receptor binding*

In our continuing effort to develop effective estradiol–fluorophore conjugates, we have synthesized a number of estradiol derivatives having substituents at the 17α position that may serve as models of a desirable "spacer" between the steroid nucleus and the fluorophore. Subsequently, useful spacer models (selected from active analogs in our current studies) may be employed for attaching fluorophores to estradiol without substantial loss of ER affinity. It is even hoped that suitable spacer chains might be definable that actually enhance binding, by serendipitous, effective interactions of the spacer with some subsite of the receptor. We have concentrated on the 17α position of estradiol because the ER appears to be relatively tolerant of substituents in this region of the steroid skeleton.

Earlier, we reported[15] synthesis and ER binding af-

† Preparation of **19b** suffered from occasional unexplainable irreproducibility, especially during scale-up attempts of the bromination step. During these failed attempts, apparently the dimer (from alkyne self-coupling of the ethynylestradiol system) formed instead of the normal bromalkyne **19b**. Several variations of the bromination procedure did not eliminate the sporadic problem. In contrast, our reported preparations of **20b** and **28** using the analogous iodination reaction are reproducibly high-yield reactions. We are currently investigating the scope and limitations of these convenient alkyne halogenations and will report the results in detail elsewhere. In our hands, an alternative preparation[21] of **19b** using *N*-bromosuccinimide and catalytic silver nitrate afforded product mixtures containing satisfactory amounts of **19b** which, however, proved difficult and tedious to separate.

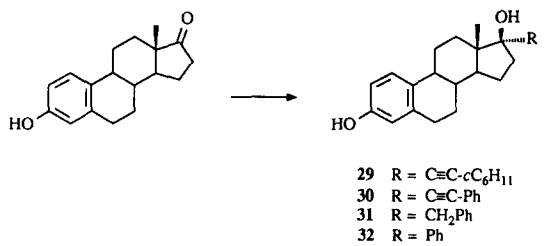



**Figure 4** Synthesis of 17α-substituted estradiol derivatives from estrone.

finities of a series of estradiol derivatives bearing relatively short side chains in the 17α position; our binding affinity data on these analogs suggested that, although overall polarity of the ligand and modifications of the dual hydrogen bonding ability of the 17β-hydroxyl do appear to affect analog binding affinity, it is adverse steric interference of mobile and bulky 17α side chains with the receptor that seems to exert the most profound limiting effects on the binding affinities of these compounds. This preliminary conclusion was based on a limited number of compounds, most of which contained as 17α substituents three- and four-carbon side chains with or without polar functional groups and with varying degrees of unsaturation.

In this study, we have evaluated several different synthetic methodologies to prepare estradiol analogs having medium-sized 17α substituents (with side chains of six to 11 carbons), incorporating either heteroatoms and/or rings and unsaturations (of varying number and location) into the side chain. Note that appropriate 2′ and 3′ heteroatom functionalities might effectively hydrogen bond with the 17β-hydroxyl and, thus, somewhat restrict the mobility of the side chain while also effecting some changes in hydrogen bonding character of this hydroxyl group toward the receptor; heteroatoms positioned significantly further along the side chain are unlikely to hydrogen bond to this hydroxyl. Rings and unsaturation are expected to restrict side chain mobility and the latter may also afford π-interactions with residues in the receptor subsite. At this time, we report RBAs of our new series of analogs (see Tables 1 and 2) and offer partial rationalization of the effects of these varied, medium-length 17α substituents on RBAs toward receptor.

**Table 1** Relative binding affinities[a] of 17α-substituted estradiols for estrogen receptor

| Compound no. | 17α-R | RBA |
|---|---|---|
| Table 1a | | |
| — | $CH_2—CH_2—CH_3$ | 4.9[b] |
| — | $CH_2—CH_2—CH_2—CH_3$ | 4.7[b] |
| 4 | $CH_2—CH_2—(CH_2)_5—CH_3$ | <0.1 |
| 6 | $CH_2—NH—(CH_2)_5—CH_3$ | <0.1 |
| 8 | $CH_2—N(Ac)—(CH_2)_5—CH_3$ | <0.1 |
| 10 | $CH_2—O—(CH_2)_5—CH_3$ | 0.2 |
| 12 | $CH_2—S—(CH_2)_5—CH_3$ | <0.1 |
| Table 1b | | |
| 20a | $CH_2—C\equiv CH$ | 49[b] |
| 16 | $CH_2—CH=CH—(CH_2)_4—CH_3$ | 0.5 |
| 14 | $CH_2—C\equiv C—(CH_2)_4—CH_3$ | 1.1 |
| 18 | $CH_2—C\equiv C—cC_6H_{11}$ | 1.1 |
| 23 | $CH_2—C\equiv C—C\equiv C—(CH_2)_3—CH_3$ | 1.1 |
| 24 | $CH_2—C\equiv C—C\equiv C—Ph$ | 0.4 |
| 31 | $CH_2—Ph$ | 4.3 |
| Table 1c | | |
| 19a | $C\equiv CH$ | 104[b] |
| — | $C\equiv C—CH_3$ | 32[b] |
| 27 | $C\equiv C—(CH_2)_5—CH_3$ | 0.9 |
| 21 | $C\equiv C—C\equiv C—(CH_2)_4—CH_3$ | 2.5 |
| 29 | $C\equiv C—cC_6H_{11}$ | 3.2 |
| 30 | $C\equiv C—Ph$ | 5.8 |
| 22 | $C\equiv C—C\equiv C—Ph$ | 7.1 |
| Table 1d | | |
| 28 | $C\equiv C—I$ | 81 |
| 20b | $CH_2—C\equiv C—I$ | 28 |
| 25 | $C\equiv C—(CH_2)_3—CH_2I$ | 4.7 |
| 26 | $C\equiv C—(CH_2)_5—CH_2I$ | 0.8 |
| — | $C\equiv C—CH_2—O—THP$ | 4.0[b] |
| Table 1e | | |
| 32 | Ph | 12.0 |

[a] In buffer without DMF.
[b] From ref. 15.

**Table 2** Relative estrogen receptor affinities of 17α-substituted estradiols with or without *N,N*-dimethylformamide

| Compound no. | 17α-R | RBAs | | |
|---|---|---|---|---|
| | | No DMF | 4% DMF | Ratio |
| 19a | $C\equiv CH$ | 104[a] | 123 | 1.2 |
| — | $C\equiv C—CH_3$ | 32[a] | 40 | 1.3 |
| 20a | $CH_2—C\equiv CH$ | 49[a] | 38 | 0.8 |
| 25 | $C\equiv C—(CH_2)_3—CH_2I$ | 4.7 | 31 | 6.6 |
| 26 | $C\equiv C—(CH_2)_5—CH_2I$ | 0.8 | 1.6 | 2.0 |
| 27 | $C\equiv C—(CH_2)_5—CH_3$ | 0.9 | 7.6 | 8.4 |
| 29 | $C\equiv C—cC_6H_{11}$ | 3.2 | 9.9 | 3.1 |
| 30 | $C\equiv C—Ph$ | 5.8 | 33 | 5.7 |
| 32 | Ph | 12.0 | 30 | 2.5 |
| 31 | $CH_2—Ph$ | 4.3 | 19 | 4.4 |
| 22 | $C\equiv C—C\equiv C—Ph$ | 7.1 | 17 | 2.4 |

[a] From ref. 15.

From Table 1a, it can be seen that estradiol analogs having an extended, completely saturated, and thus mobile, medium-length side chain at the 17α position generally show low affinity toward ER; for example, when 17α-octylestradiol (**4**, RBA < 0.1%) is compared with 17α-propylestradiol (RBA 4.9%) and 17α-butylestradiol (RBA 4.7%),[15] a dramatic reduction in affinity is observed. Substituting various heteroatoms at position 2′ in the eight-carbon side chain (compounds **6, 8, 10,** and **12**) does not significantly improve the binding affinity, although 17α-(hexyloxymethyl)estradiol (**10,** RBA 0.2%) shows a modest increase in affinity. In contrast, 17α-benzylestradiol (**31,** RBA 4.3%, Table 1b), with a seven-carbon side chain containing a compact, highly unsaturated ring, exhibits an affinity comparable to the 17α three- and four-carbon saturated side chain analogs.

In Table 1b, estradiol analogs having 17α side chains containing seven to 11 carbon atoms with $sp^2$ alkenyl unsaturation (**16** and its trans isomer,‡ as well as **31**) or sp alkynyl unsaturation (compounds **14, 18, 23,** and **24**) at the 2′ position show substantially greater affinity toward ER than analog **4** with its saturated 17α octyl side chain (Table 1a). For analogs of directly comparable chain length, sp unsaturation at 2′ has the most profound effect; thus, 17α-(oct-2′-ynyl)estradiol (**14,**

‡ The RBA of *E*-17α-(oct-2′-enyl)estradiol (contaminated with <2% of **4**) was very similar to that of the corresponding *cis*-isomer **16.** This is consistent with our observation that increasing the unsaturation at 2′ enhances binding affinity.

RBA 1.1%) is twice as active as 17α-(oct-2′-enyl)estradiol (**16,** RBA 0.5%), which is already more than five times more active than **4.** Increasing the chain length of the 17α substituent by one carbon is apparently offset by adding a second constraint on mobility, such as tying the chain into a saturated ring or adding a second triple bond, since **18** and **23** have ER affinities equivalent to **14.** Increasing the 17α side chain to 11 carbons containing a conjugated system of two triple bonds along with a highly unsaturated phenyl ring, as in compound **24** (RBA 0.4%), leads to substantially reduced ER affinity. However, the shorter seven-carbon 17α benzyl side chain with its nonflexible phenyl group at the 2′ position makes analog **31** (RBA 4.3%) the most active compound we have prepared, having a 17α side chain of medium length with a methylene group at the 1′ position.

Table 1c contains estradiol analogs having 17α substituents with no heteroatoms, but containing both short and medium-length carbon chains (two to 10 carbon atoms) with sp unsaturation at the 1′ position: 17α-ethynylestradiol (**19a**), 17α-(prop-1′-ynyl)estradiol, and compounds **21, 22, 27, 29,** and **30.** These analogs illustrate several interesting relationships among each other and with some comparable analogs in Table 1b. Thus, as expected, 17α short-chain analogs are substantially more active than their medium-length analogs. However, for 17α substituent chains of eight or more carbons, an increased number of alkyne linkages and saturated or aromatic rings leads to increased ER affinity, even with chain lengths of nine or 10 carbons. These results are consistent with our earlier observations from Table 1b that for medium-length 17α carbon chains, restricting degrees of freedom of the side chain potentiates binding affinity considerably. The effect appears to be substantially more pronounced in the eight- to 10-carbon 1′-alkynyl compounds (Table 1c) than in their 2′ regioisomers (Table 1b), since the effects of increased chain length appear to be more than offset by decreased mobility.

Direct comparisons of structurally similar compounds in Tables 1b and 1c should also be noted. For 1′-alkynyl and 2′-alkynyl side chains, no dramatic contrast in ER affinity is discernible for both 17α short-chain and 17α medium-chain substituents containing a single alkyne moiety as the side chain's only restriction of mobility. Consider the effects of increased chain length on analogs containing 1′ or 2′ alkynyl functionality: 17α-ethynylestradiol (**19a,** RBA 104%) has greater affinity than both regioisomers of 17α-propynylestradiol (1′ alkyne, RBA 32%; 2′ alkyne, RBA 49%), which have greater affinity than the corresponding regioisomers of 17α-octynylestradiol (1′ alkyne **27,** RBA 0.9%; 2′ alkyne **14,** RBA 1.1%). From the trends in the preceding set of regioisomers, one might assume that a 2′ alkyne is more effective as a side chain structural component than its 1′ regioisomer. However, when additional rigidity is added, this apparent trend is reversed; in two conjugated diyne regioisomers of 17α-nonadiynylestradiol (2′,4′ diyne **23,** RBA 1.1%; 1′,3′ diyne **21,** RBA 2.5%), activity of the former is comparable to 1′ and 2′ octynyl compounds above, while activity of the latter is increased more than two-fold. Even more dramatic comparisons are the nine- and eight-carbon 17α-cyclohexylalkynyl compounds (2′ alkyne **18,** RBA 1.1%; 1′ alkyne **29,** RBA 2.5%), and the 11- and 10-carbon 17α-phenylalkdiynyl compounds (2′,4′ diyne **24,** RBA 0.4%; 1′,3′ diyne **22,** RBA 7.1%). The one-carbon shorter side chain in both sets of analogs is dramatically more active toward ER. Apparently, analogs with extended linear segments at the 1′ position (Table 1c) have substantially better ER affinity than comparable 2′ analogs (Table 1b). It appears that analogs containing a rigid, linearly extended segment of the 17α side chain (with the linear segment longer than approximately 5 angstroms, i.e., **21, 22, 30,** and **32** [Table 1e]) are more readily accommodated in the receptor site when this linear segment is an extension of the 17α bond than when the linear segment is separated from the steroid nucleus by a methylene group. Two factors may be operating simultaneously, and it is difficult to know which is more significant: (1) reduced rotational mobility of side chains with unsaturation between positions 1′ and 2′ and (2) the direction of the linearly extended segment of the side chain from the 17α bond of the steroid nucleus through position 1′ (C-17/C-1′/C-2′ bond angle) of 109 degrees (as in Table 1b) or 180 degrees (as in Table 1c). Compatible with these observations are the respectable affinities of 17α-phenylethynylestradiol (**30,** RBA 5.8%, Table 1c) and 17α-phenylestradiol (**32,** RBA 12%, Table 1e). Both of these analogs (with six- and eight-carbon side chains) are significantly more active than **31** (Table 1b) with its seven-carbon benzyl side chain containing a 1′ methylene group.

Table 1d contains analogs with 17α alkynyl substituents having heteroatoms far from the steroid nucleus, at or near the end of the chain. This series of analogs, including several ω-iodoalkynyl compounds (**28, 20b, 25,** and **26**) and 17α-(3′-tetrahydropyranyloxyprop-1′-ynyl)estradiol, was prepared because of the synthetic flexibility offered by such heteroatom systems in preparing modified side chains containing fluorophores, via carbon–carbon or carbon–oxygen bond formation reactions using ω-iodides or via a host of other suitable ether formation reactions. Their affinities toward ER provide some interesting observations. The presence of a terminal iodide in analogs **28, 20b, 25,** and **26** does not appear to affect the binding affinity considerably at either sp or $sp^3$ carbon, when one compares these analogs to appropriate examples in Tables 1b and 1c; it should be noted that iodine is larger than methyl, but also contains polarizable electron density. We have already observed the excellent binding characteristics of aryl and conjugated arylalkynyl systems in Table 1c. Perhaps the polarizable π-electron density of these conjugated systems and the polarizable electron density of ω-iodides both lead to increased binding interactions with residues in this subsite of the receptor. Thus, despite the bulk of iodine, one might explain the excellent binding of 17α-iodoethynylestradiol (**28,** RBA 81%), compared with 17α-ethynylestradiol (**19a,** RBA

104%) and 17α-(prop-1′-ynyl)estradiol (RBA 32%),[15] and the binding of **20b** (RBA 28%), compared with **20a** (Table 1b, RBA 49%).§ Similarly, 17α-(6′-iodohex-1′-ynyl)estradiol (**25,** RBA 4.7%) is five-fold more active than the nearly comparable octynyl analog **27** (Table 1c, RBA 0.9%); however, lengthening the 1′-iodoalkynyl side chain by two carbons substantially decreases binding affinity, as illustrated by six-fold reduced affinity of 17α-(8′-iodooct-1′-ynyl)estradiol (**26,** RBA 0.8%) compared with **25.** Finally, because its 17α substituent may be equivalent to a 10-carbon side chain containing both a 1′-alkyne and a distant, saturated six-membered ring, the respectable affinity of 17α-(3′-tetrahydropyranyloxyprop-1′-ynyl)estradiol (Table 1d, RBA 4.0%) should perhaps be compared with RBAs of analogs **22** and **29** in Table 1c. At this time, we cannot suggest whether the oxygen plays a role in enhancing binding or whether short flexible spacers are desirable to separate the 1′ alkyne and the saturated ring.

During the course of this work, Vessieres et al.[36] reported the RBA of 17α-(2′-phenylethynyl)estradiol (**30**) to be 25% toward estrogen receptor from lamb uterus; our observation of the RBA of this compound (Table 1c) is 5.8% toward ER from rat uterus. The difference in affinities could have resulted from one or both of two experimental variations: the different source of receptor and the different cosolvent used during assay. Regarding the latter, Vessieres et al. used a competition assay developed by Katzenellenbogen et al.[37] that uses dimethylformamide (7% final DMF concentration in the assay solution) to solubilize the test compounds, while the RBA data reported here in Table 1 were derived in a similar competition assay, but using ethanol (<0.1% final ethanol concentration in the assay solution) to solubilize test compounds. It has been suggested that DMF reduces nonspecific binding and also helps in solubilizing and mobilizing lipophilic steroids. Consequently, we subjected analog **30** and a selected series of other nonpolar 17α-substituted compounds from the present study to competition assays using DMF to solubilize the test compounds (4% final concentration of DMF in the assay solution). The results are listed in Table 2. All but one compound showed potentiation of binding affinities, albeit to varying degrees. Relative binding affinity data in Table 2 confirm many of the structure–activity relationships discussed above using data from Table 1, i.e., compounds with less flexible and 1′-unsaturated side chains tend to bind more strongly to the receptor. 17α-(2′-Phenylethynyl)estradiol (**30**) showed an RBA of 33% (Table 2), agreeing well with the RBA reported by Vessieres et al.[36] The effect of the length of the side chain on RBA appears to be much more pronounced in the presence of DMF; analog **25,** with its six-carbon iodohexynyl side chain, is almost 20-fold more active than its corresponding eight-carbon iodoalkyne analog **26** and almost five-fold more active than its corresponding noniodinated analog **27.** Besides **25,** the most active compounds using DMF are still derivatives having compact and electron-rich substituents at the 17α position; it appears that the presence of DMF may enhance specificity and binding affinities of estradiol analogs substituted with medium-length 1′-alkynyl or aryl substituents (**25** to **27, 29** to **32**), while affinities of analogs with shorter 1′- or 2′-alkynyl substituents (**19a, 20a,** and 17α-(prop-2′-ynyl)estradiol) remain largely unchanged.

We conclude that polarity, several kinds of steric (size) and stereochemical factors, and suitable electronic character of the 17α side chain may all play a critical role in determining the binding capability of estradiol analogs. From the examples reported here, it appears that compact substituents with electron-rich character that are fused to the steroid skeleton via fully or partially rigid "spacers" bind strongly to the estrogen receptor. Such aryl and arylalkynyl systems may serve well to link fluorophores, since the conjugated system of the spacer may serve as an integral part of the fluorophore. We are continuing our efforts to develop fluorescent estrogens using this approach, and have also begun to apply this kind of modeling of potential spacers to prepare fluorescent progestins and androgens.

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§ Some of the excellent binding affinity observed for analogs with iodine directly bonded to the alkyne terminus may be due to prior deiodination of analogs **20b** and **28** to **20a** and **19a,** respectively, since it has been reported that iodoethynylestradiol undergoes facile, partial deiodination[27] under in vitro assay conditions.

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