Nonsteroidal estrogens bearing acyl azide functions: potential electrophilic and photoaffinity labeling agents for the estrogen receptor

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In an effort to develop novel affinity labeling agents for the estrogen receptor, we have synthesized two nonsteroidal ligands, a 1-aroyl-2-aryl tetralin system (1) and a 2-aryl-3-aroylbenzo[b]thiophene system (2). These agents, patterned after the Lilly antiestrogens trioxifene and LY 117018, respectively, embody acyl azide functions as part of a benzoyl chromophore. The acyl azide group has weak acylating activity, suitable for electrophilic affinity labeling, but this function is also photoreactive and, in its particular embodiment within these ligands, it could provide an efficient photochemical route to the highly reactive singlet acyl nitrene. The tetralin system (1) was prepared in nine steps from 6-methoxy-1-tetralone, and the benzothiophene system (2) was prepared in four steps from a known substituted benzo[b]thiophene precursor. In competitive binding assays, both compounds show reasonable binding affinity for the rat and lamb uterine estrogen receptor: estradiol = 100%, 1 = 3%, and 2 = 12%. When assayed by indirect receptor consumption assays, both compounds appear to have substantial capacity for irreversible binding (electrophilic reaction) with the receptor. This reactivity, which suggests that acylation of the receptor has occurred, is photoreversible. The nature of this ligand-receptor interaction is being investigated further. (*Steroids* **57**:222–232, 1992)

Keywords: estrogen receptor, affinity labeling, photoaffinity labeling, nonsteroidal estrogens, antiestrogens, steroids

Introduction

Affinity labeling agents for steroid hormone receptors are useful in identifying regions of the receptor that are involved in ligand binding.[1] Such information is particularly important in designing site-specific mutagenesis studies on the receptor in which the effect of specific amino acid replacements at sites known to be in contact with a specific ligand can be explored, in terms of both alteration in ligand binding as well as other functions of receptor (dimerization, DNA binding, transcriptional activation).

We have had a longstanding interest in developing affinity labeling agents for the estrogen receptor.[2,3] In the past, we have prepared a number of photoreactive and electrophilic derivatives of steroidal and nonsteroidal estrogens as receptor labeling reagents.[4–7] While some of these compounds bound and reacted irreversibly with the receptor, they did so with only modest affinity and selectivity.[7] We found, however, that the aziridine function could be incorporated into nonsteroidal ligands to target them for the estrogen receptor and give electrophilic affinity labeling agents that were highly efficient and selective.[8,9] Recently, we have reported that two aziridine-based electrophilic affinity labeling reagents, tamoxifen aziridine and ketononestrol aziridine, covalently label the human estrogen receptor at cysteine-530, a residue near the C-terminus of the hormone binding domain.[10] More recently, we have described the preparation of two estrogens that embody photoreactive aryl azide groups[11] and documented their selective covalent labeling of the estrogen receptor.[12] These compounds are the first efficient and selective electrophilic and photoaffinity labeling agents for the estrogen receptor.

We describe the preparation and biochemical evaluation of two novel systems that both incorporate an acyl azide, a functional group with the potential for both electrophilic and photochemical reactivity (Scheme 1). The first of these is structurally based on the 1-aroyl-2-aryl tetralin system (azidotetralin, **1**); the

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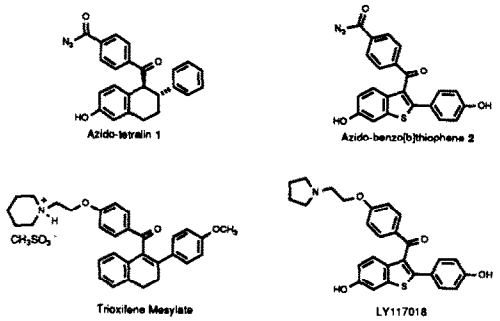


Scheme 1

second is based on the 2-aryl-3-aroylbenzo[*b*]thiophene system (azidobenzo[*b*]thiophene, **2**). These compounds are reactive analogs of the potent antiestrogens trioxifene mesylate[13] and LY117018,[14] respectively, prepared by Jones and co-workers (Eli Lilly, Inc.) and known to demonstrate high binding affinity for the estrogen receptor.[15–17]

Both acyl azides demonstrate rapid and specific electrophilic inactivation of the estrogen receptor (**1** = 29% to 36%; **2** = 45% to 54%). This inactivation can be reversed by UV light ($t_{1/2} \cong 7$ minutes at >315 nm). Therefore, these compounds may be useful as unique electrophilic labeling reagents for the estrogen receptor with the potential for photoreversal of the inactivation.

Experimental

General methods

All melting points are uncorrected. Analytic thin-layer chromatography (TLC) was performed using Merck silica gel 60 F-254 pre-coated (0.2 mm) plastic- or glass-backed plates. Reversed-phase TLC used Whatman PLK C18F Linear-K plates (1,000 μm). Visualization methods included UV light (short and/or long wavelength), saturated $CeSO_4$ in 65% H_2SO_4, phosphomolybdic acid, or iodine vapor. Flash chromatography was performed according to Still et al.[18] using Woelm 32 to 63 μm silica gel.

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded at 90, 200, 300, or 500 MHz. Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra were recorded in the proton decoupled mode at 75, 90, or 125 MHz. All two-dimensional (2D) NMR spectra were recorded at 500 MHz. Infrared (IR) spectra were recorded in $CHCl_3$ solution and important diagnostic bands are reported in cm^{-1}. Mass spectra were obtained by electron impact or fast atom bombardment (FAB). Analytic gas chromatograph was performed using a Hewlett-Packard Ultra 1 (cross-linked methyl silicone gum) capillary column (12 m × 0.2 mm × 0.33 μm film thickness). Elemental analyses were performed by the Microanalytical Service Laboratory of the University of Illinois School of Chemical Sciences.

Chemicals and solvents were obtained from the following suppliers and were used as received, unless otherwise noted: Aldrich, Alfa, Mallinckrodt, Sigma, Fisher, and Matheson. Tetrahydrofuran (THF) was distilled from sodium/benzophenone immediately prior to use. Dichloromethane and chlorotrimethylsilane were distilled from CaH_2. Tert-amyl alcohol was dried over activated 4 Å molecular sieves and fractionally distilled under reduced pressure. N,N,N′,N′-tetramethylethylenediamine (TMEDA) was dried over activated 4 Å molecular sieves and fractionally distilled under reduced pressure from n-BuLi immediately prior to use. Hexamethylphosphoramide (HMPA) was dried over activated 4 Å molecular sieves. Hydrogen chloride gas was dried by passage through concentrated sulfuric acid.

In most cases a standard product isolation procedure was followed (an aqueous quench, exhaustive extraction with an organic solvent, occasionally washing the extract with an aqueous phase followed by drying the extract over an anhydrous salt, filtration and solvent removal by rotary evaporation). This is indicated by the phrase "product isolation," followed by a listing of the quenching medium, extraction solvent, any aqueous washes, and drying agent in parentheses. All reactions were performed under a nitrogen atmosphere, unless otherwise noted.

Chemical syntheses

6-Methoxy-1-tetralone toluenesulfonylhydrazone (4). 6-Methoxy-1-tetralone **3** (2.0 g, 11.35 mmol) and an equimolar amount of p-toluenesulfonylhydrazine (2.11 g, 11.35 mmol) were dissolved in absolute ethanol (35 ml). A catalytic amount of p-toluenesulfonic acid monohydrate (0.10 g) was added and the mixture was refluxed. After 15 minutes, a white solid began to fall out of solution and, after 45 minutes, reaction was complete. The solid was collected by filtration and washed with a small amount of cold hexane to give 3.26 g (9.47 mmol) of the toluenesulfonylhydrazone **4** as a white crystalline material. A second crop (0.256 g, 0.743 mmol) was also recovered for a 90% overall yield (3.52 g, 10.2 mmol) of a solid, mp (dec.) 199 to 201 C. IR ($CHCl_3$): 3,017.0, 1,597.3, 1,498.9, 1,388.9, and 1,226.9 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 1.88 (quintet, 2H, J = 6.03 Hz, -C$\underline{H}_2$ at C-3), 2.41 (s, 3H, Ts-C$\underline{H}_3$), 2.43 (t, 2H, J = 6.5 Hz, -C$\underline{H}_2$ at C-2), 2.68 (t, 2H, J = 5.8 Hz, -C$\underline{H}_2$ at C-4), 3.80 (s, 3H, ArOC$\underline{H}_3$), 6.59 (d, 1H, J = 2.54 Hz, Ar*H* at C-5), 6.75 (dd, 1H, J = 8.68, 2.54 Hz, Ar$\underline{H}$ at C-7), 7.32 (d, 2H, J = 7.93 Hz, Ar$\underline{H}$ *ortho* to -CH_3 on pendant aryl ring), 7.92 (d, 2H, J = 8.2 Hz, Ar$\underline{H}$ *meta* to -CH_3 on pendant aryl ring), and 7.94 (d, 1H, J = 8.95 Hz, Ar$\underline{H}$ at C-8); ms (70 eV) m/z (relative intensity): 344 (M^+, 1.3), 189 (100), 161 (21.7), and 91 (20.5). Analysis calcd for $C_{18}H_{20}N_2O_3S$: C, 62.77; H, 5.85; N, 8.13; S, 9.31. Found: C, 62.85; H, 5.92; N, 8.22; S, 9.40.

6-Methoxy-1-tributylstannyl-3,4-dihydronaphthalene (6). 6-Methoxy-1-tetralone tosylhydrazone **4** (6.0 g, 17.4 mmol) was added via Gooch tubing to a well-stirred TMEDA solution (200 ml) containing n-BuLi (69.7 mmol) at −35 C over a 15-minute period. After 1 hour the orange mixture was returned to room temperature, where it gradually turned black and evolved nitrogen. After 1.5 hours, the mixture was cooled to 0 C, quenched with tributyltin chloride (22.7 g, 69.7 mmol), and stirred for an additional 1 hour at 0 C. Product isolation (H_2O, EtOAc, aq. $CuSO_4$, brine, $MgSO_4$) gave a dark yellow oil. Partial purification by vacuum distillation (compound obtained as a pot residue) followed by flash chromatography (40/60 : diethylether/hexane) gave vinylstannane **6** as a yellow oil (5.49 g, 12.2 mmol, 70% yield). ^{1}H NMR ($CDCl_3$): δ 0.87 (t, 9H, J = 7.2 Hz, Sn-(CH_2)$_3$-C$\underline{H}_3$), 0.90 (m, 6H, Sn(CH_2)$_2$-C$\underline{H}_2$-CH_3), 1.30 (m, 6H, Sn-CH_2-C$\underline{H}_2$-CH_2-CH_3), 1.45 (m, 6H, Sn-C$\underline{H}_2$(CH_2)$_2$-CH_3), 2.25 (m, 2H, -C$\underline{H}_2$ at C-3), 2.70 (t, 2H, J = 8.0 Hz, -C$\underline{H}_2$ at C-4), 3.79 (s, 3H, -OCH_3), 6.11 (t, 1H, J = 4.2 Hz, -C$\underline{H}$ at C-2), 6.70 (m, 2H, Ar$\underline{H}$ at C-5 and C-7), and 6.90 (m, 1H, Ar$\underline{H}$, at C-8); ms (70 eV) m/z (relative intensity): 449 (M^+, 0.15), 393 (4.5), 337 (3.9), 291 (84), 234 (100), 177 (94), and 121 (50).

4-(Ethoxycarbonyl)benzoic acid (9).[19] Dimethylterephthalate **8** (3.94 g, 20.3 mmol) was stirred in EtOH/H_2O : 3/1 (200 ml) and heated until solution was achieved. The solution was then allowed to cool just to the point at which the ester began to crystallize, at which time a 10% NaOH solution (2 ml) was added.

Solution was achieved, and an additional 8 ml of 10% NaOH was added over 30 minutes at 25 C. After 2.5 hours, the solution was diluted with an equal volume of water and cooled to 0 C by addition of ice. The aqueous phase was brought to pH 1 by the addition of 6 ml of 3 *M* HCl and extracted exhaustively with EtOAc. After being dried over $MgSO_4$, the solvent was removed in vacuo to afford 3.17 g (16.3 mmol) of a white solid (80%). Recrystallization from hexane/ether to remove a slight amount of terephthalic acid gave 1.87 g (9.61 mmol) of the ester **9** as a pure white powder (47%), mp 166 to 169 C (literature[19]: 169 to 171 C). IR ($CHCl_3$): 1,701.4 cm^{-1} (acid carbonyl), 1,716.0 (ester carbonyl), 3,027, 3,019, and 1,422; ^1H NMR ($CDCl_3$) δ: 1.43 (t, 3H, J = 6.98 Hz, $-OCH_2C\underline{H}_3$), 4.41 (q, 2H, J = 6.98 Hz, $-OC\underline{H}_2CH_3$), and 8.14 (s, 4H, Ar<u>H</u>); ms (70 eV) m/z (relative intensity): 194 (M^+, 18.0), 166 (38.3), 149 (100), 121 (20.2), and 65 (34.0). Analysis calculated for $C_{10}H_{10}O_4$: C, 61.85; H, 5.19. Found: C, 61.71; H, 5.11.

4-(Ethoxycarbonyl)benzoyl chloride (10).[19] 4-(Ethoxycarbonyl)-benzoic acid **9** (1.87 g, 9.61 mmol) and thionyl chloride (1.94 g, 16.3 mmol) were refluxed for 30 minutes, then cooled to 23 C; acid chloride **10** (1.49 g, 6.99 mmol, 73%) was then obtained following purification by short-path distillation as a clear liquid, bp 90 C at 0.08 mm Hg (literature[20]: bp 133 to 134 C at 6 mm Hg). IR ($CHCl_3$): 1,782.4 cm^{-1} ((C=O)Cl), 1,722.6 ((C=O)OEt), 3,017, 1,609, 1,576, 1,499, 1,447, and 1,476; ^1H NMR ($CDCl_3$): δ 1.43 (t, 3H, J = 7.3 Hz, $-OCH_2CH_3$), 4.43 (q, 2H, J = 7.3 Hz, $-OCC\underline{H}_2CH_3$), and 8.17 (s, 4H, Ar<u>H</u>); ms (70 eV) m/z (relative intensity): 212 (M^+, 3.5), 177 (100), 167 (17.2), 149 (24.8), 104 (14.1), 76 (18.9), 65 (11.9), and 50 (17.0). Analysis calculated for $C_{10}H_9O_3Cl$; C, 56.49; H, 4.27; Cl, 16.67. Found: C, 55.69; H, 4.32; Cl, 16.49.

1-[(4′-Ethoxycarbonyl)benzoyl]-3,4-dihydro-6-methoxynaphthalene (11). Vinylstannane **6** (3.93 g, 8.74 mmol), acid chloride **10** (1.84 g, 8.66 mmol), and benzylchlorobis(triphenylphosphine) palladium(II) (0.0033 g, 0.0043 mmol) were stirred in HMPA (15 ml) at 65 C under anhydrous conditions. After 24 hours, product isolation (H_2O, EtOAc, brine, $MgSO_4$) gave a pale yellow oil. Flash chromatography (3 : 2, hexane/ether) followed by crystallization from hexane/ethanol (95 : 5) afforded the α,β-unsaturated ketone **11** as a white solid (1.04 g, 3.10 mmol) 35%, mp 108 to 109 C. IR ($CHCl_3$): 1,653 cm^{-1} (ketone carbonyl), 1,717 (ester carbonyl), 3,024, 1,252, 1,107, and 1,024; ^1H NMR ($CDCl_3$): δ 1.41 (t, 3H, J = 7.0 Hz, $-OCH_2C\underline{H}_3$), 2.47 (dt, 2H, J = 4.4, 7.8 Hz, $-C\underline{H}_2$ at C-3), 2.86 (t, 2H, J = 7.8 Hz, $-C\underline{H}_2$ at C-4), 3.80 (s, 3H, $-OC\underline{H}_3$), 4.40 (q, 2H, J = 7.0 Hz, $-OC\underline{H}_2CH_3$), 6.42 (t, 1H, J = 4.4 Hz, -C<u>H</u> at C-2), 6.69 (dd, 1H, J = 8.5, 2.4 Hz, Ar<u>H</u> at C-7), 6.77 (d, 1H, J = 2.4 Hz, Ar<u>H</u> at C-5), 7.27 (d, 1H, J = 8.5 Hz, Ar<u>H</u> at C-8), 7.88 (d, 2H, J = 7.8 Hz, Ar<u>H</u> *ortho* to ketone), and 8.10 (d, 2H, J = 7.8 Hz, Ar<u>H</u> *ortho* to ester); ms (70 eV) m/z (relative intensity): 336 (M^+, 100), 308 (7.8), 263 (9.3), 245 (11.3), 177 (18.8), and 159 (32.6). Analysis calculated for $C_{21}H_{20}O_4$: C, 74.98; H, 5.99. Found: C, 75.11; H, 6.08.

1-[(4′-Ethoxycarbonyl)benzoyl]-6-methoxy-2-phenyltetralin (12a,b). To a stirred solution of phenyllithium (3.30 mmol, 1.65 ml) in Et_2O (30 ml) at −60 C was added cuprous bromide-dimethyl sulfide complex (0.340 g, 1.65 mmol) in dimethylsulfide (2 ml). After 20 minutes, ketone **11** (0.371 g, 1.10 mmol) was slowly added in THF (5 ml). The reaction mixture immediately darkened in color. After 2.5 hours at −20 C, product isolation (0.1 M HCl, EtOAc, Na_2SO_4) gave a mixture of diastereomers **12a,b** (approximately 1 : 1) as a dark oil (1.10 mmol). Purification by flash chromatography (3/2 : hexane/ether) and crystallization from hexane/ethanol (90 : 10) afforded the phenylated *cis*-derivative **12a** (2.28 g, 0.55 mmol, 50%) as a white powder, mp 106 to 110 C. IR ($CHCl_3$): 1,717 cm^{-1} (ester carbonyl), 1,653 (ketone carbonyl), 3,025, 1,506, and 1,215; ^1H NMR ($CDCl_3$): δ 1.36 (t, 3H, J = 7.2 Hz, $-OCH_2C\underline{H}_3$), 2.0 (m, 1H, -C<u>H</u> at C-3), 2.84 (m, 1H, -C<u>H</u> at C-3), 3.0 (m, 1H, -C<u>H</u> at C-4), 3.15 (m, 1H, -C<u>H</u> at C-4), 3.40 (m, 1H, -C<u>H</u> at C-2), 3.77 (s, 3H, $-OC\underline{H}_3$), 4.33 (q, 2H, J = 7.2 Hz, $-OC\underline{H}_2CH_3$), 5.11 (d, 1H, J = 5.7 Hz, -C<u>H</u> at C-1), 6.60 (dd, 1H, J = 8.5, 2.7 Hz, Ar<u>H</u> at C-7), 6.74 (d, 1H, J = 8.5 Hz, Ar<u>H</u> at C-8), 6.77 (d, 1H, J = 2.7 Hz, Ar<u>H</u> at C-5), 7.1 (m, 5H, Ar<u>H</u> on C-2 phenyl), 7.51 (d, 2H, J = 8.4 Hz, Ar<u>H</u> *ortho* to ketone), and 7.85 (d, 2H, J = 8.4 Hz, Ar<u>H</u> *ortho* to ester); ^{13}C NMR ($CDCl_3$): δ 15 ($-OCH_2\underline{C}H_3$), 23, (C-3), 30 (C-4), 45 (C-2), 52 (C-1), 56 ($-O\underline{C}H_3$), and 62 ($-O\underline{C}H_2CH_3$); ms (70 ev) m/z (relative intensity): 414 (M^+, 4.0), 336 (0.7), 237 (100), 159 (4.6), and 91 (38.3). Analysis calculated for $C_{27}H_{26}O_4$: C, 78.24; H, 6.32. Found: C, 78.13; H, 6.32.

1-[(4′-Ethoxycarbonyl)benzoyl]-6-hydroxy-2-phenyltetralin (13a,b). To a stirred solution of tetralin **12a** (0.710 g, 1.71 mmol) in CH_2Cl_2 (50 ml) was added a $BF_3{\cdot}SMe_2$ complex (12 ml). After 24 hours, product isolation ((10%) $NaHCO_3$, CH_2Cl_2, Na_2SO_4) gave a diastereomeric mixture **13a,b** from which the pure *trans*-hydroxy **13b** crystallized (hexane/ethanol 95 : 5) as a white powder (0.633 g, 1.58 mmol, 92%), mp 155 to 158 C. IR (KBr): 3,430 cm^{-1} (Ar-OH), 1,750 (ester carbonyl), 1,695 (ketone carbonyl), 1,500, 1,280, and 1,110; ^1H NMR ($CDCl_3$): δ 1.39 (t, 3H, J = 7.2 Hz, $-OCH_2C\underline{H}_3$), 2.1 (m, 2H, $-C\underline{H}_2$ at C-3), 2.85 (m, 1H, -C<u>H</u> at C-4), 3.0 (m, 1H, -C<u>H</u> at C-4), 3.45 (m, 1H, -C<u>H</u> at C-2), 4.38 (q, 2H, J = 7.2 Hz, $-OC\underline{H}_2CH_3$), 4.88 (d, 1H, J = 9.2 Hz, -C<u>H</u> at C-1), 6.56 (dd, 1H, J = 8.4, 2.6 Hz, Ar<u>H</u> at C-7), 6.67 (d, 1H, J = 2.6 Hz, Ar<u>H</u> at C-5), 6.73 (d, 1H, J = 8.4 Hz, Ar<u>H</u> at C-8), 7.2 (m, 5H, Ar<u>H</u> at C-2 phenyl), 7.72 (d, 2H, J = 8.4 Hz, Ar<u>H</u> *ortho* to ketone), and 8.00 (d, 2H, J = 8.4 Hz, Ar<u>H</u> *ortho* to ester); ms (70 eV) m/z (relative intensity): 400 (M^+, 5.0), 355 (2.0), 223 (100), 145 (7.2), 91 (42.5), and 43 (12.5). Analysis calculated for $C_{26}H_{24}O_4$: C, 77.98; H, 6.04. Found: C, 78.55; H, 6.15.

1-(4′-Carboxybenzoyl)-6-hydroxy-2-phenyltetralin (14a,b). To a well-stirred solution of hydroxytetralin **13b** (0.400 g, 0.999 mmol) in H_2O (20 ml) at room temperature was added (10%) NaOH (45 ml). Phase mixing was accomplished by drop-wise addition of ethanol (10 ml). After 10 minutes, the reaction mixture was poured into water (100 ml) and acidified to pH 1.0 by addition of 3 *M* HCl. Product isolation (EtOAc, H_2O, brine, Na_2SO_4) gave a solid that was triturated with hexane/ethanol (95 : 5) to afford *trans*-acid **14b** (0.210 g, 0.564 mmol, 56%) as a yellow solid, mp (dec.) 200 to 205 C. IR (KBr): 3,300 cm^{-1} (-COOH), 1,700 (acid carbonyl), 1,660 (ketone carbonyl), 1,510, 1,410, 1,290, and 1,220; ^1H NMR (acetone-d_6): δ 2.85 (m, 2H, $-C\underline{H}_2$ at C-3), 3.05 (m, 2H, $-C\underline{H}_2$ at C-4), 3.45 (m, 1H, -C<u>H</u> at C-2), 5.05 (d, 1H, J = 9.5 Hz, -C<u>H</u> at C-1), 6.57 (dd, 1H, J = 8.4, 2.4 Hz, Ar<u>H</u> at C-7), 6.70 (m, 2H, Ar<u>H</u> at C-5 and C-8), 7.2 (m, 5H, Ar<u>H</u> at C-2 phenyl), 7.87 (d, 2H, J = 8.2 Hz, Ar<u>H</u> *ortho* to ketone), and 8.04 (d, 2H, J = 8.2 Hz, Ar<u>H</u> *ortho* to acid); ms (70 eV) m/z (relative intensity): 372 (M^+, 3.2), 328 (2.8), 213 (100), 185 (2.7), 157 (2.1), and 149 (4.6).

1-[(4′-Azidocarbonyl)benzoyl]-6-hydroxy-2-phenyltetralin (1). To a stirred solution of acid-tetralin **14b** (0.100 g, 0.269 mmol) in *N,N*-dimethylformamide (DMF) (0.5 ml) at 0 C was added *N*-methylmorpholine (0.0272 g, 0.269 mmol), followed by the slow addition of diphenylphosphoryl azide (0.0739 g, 0.269 mmol). After 2 hours, an additional 0.0046 g of *N*-methylmorpholine was added, and the solution was returned to room temperature. After 30 minutes, product isolation (H_2O, EtOAc, Na_2SO_4) gave a yellow oil. The material was purified by flash chromatography (40/60 : EtOAc/hexane) followed by recrystallization from hex-

ane/ether (70 : 30) to afford *trans*-tetralin **1** as a pale yellow solid (31.8 mg, 0.080 mmol, 28%), mp 109 to 111 C. IR ($CHCl_3$): 2,137.4 cm^{-1} (doublet, —N=N=N), 1,686 (azide carbonyl), 2,932, 1,500, 1,441, and 1,252; ^1H NMR ($CDCl_3$): δ 2.1 (m, 2H, -CH$_2$ at C-3), 2.85 (m, 1H, -CH at C-4), 3.05 (m, 1H, -CH at C-4), 3.40 (m, 1H, -CH at C-2), 4.86 (d, 1H, J = 9.2 Hz, -CH at C-1), 6.57 (dd, 1H, J = 8.4, 2.7 Hz, ArH at C-7), 6.68 (d, 1H, J = 2.7 Hz, ArH at C-5), 6.72 (d, 1H, J = 8.4 Hz, ArH at C-8), 7.15 (m, 5H, ArH on C-2 phenyl), 7.71 (d, 2H, J = 8.4 Hz, ArH *ortho* to ketone), and 7.98 (d, 2H, J = 8.4 Hz, ArH *ortho* to acyl azide); ms (FAB) m/z: 398 (M + 1), 309, 223, 155, 135, and 119. Analysis calculated for $C_{24}H_{19}O_3N_3$: C, 72.53; H, 4.82; N, 10.57. Found: 72.26; H, 5.41; N, 9.54.

1-[(4′-Ethoxycarbonyl)benzoyl]-6-methoxy-2-(4-methoxyphenyl) benzo[*b*]thiophene (17). Acid chloride **10** (0.751 g, 3.53 mmol) was dissolved in CH_2Cl_2 (40 ml) to which benzo[*b*]thiophene **16**[14] (0.955 g, 3.53 mmol) and $AlCl_3$ (3.53 g, 26.5 mmol) were added. The solution turned deep red on addition of the $AlCl_3$. After 12 hours, product isolation (H_2O, EtOAc, H_2O, brine, $MgSO_4$) gave a pale yellow oil. Crystallization from hexane/ethanol afforded ketone **17** (1.15 g, 2.58 mmol, 73%) as a pale yellow solid, mp 134 to 138 C: ^1H NMR ($CDCl_3$): δ 7.90 (d, 2H, J = 8.2 Hz, ArH *ortho* to -CO_2Et), 7.78 (d, 2H, J = 8.3 Hz, ArH *meta* to -CO_2Et), 7.68 (d, 1H, J = 8.9 Hz, ArH on C-4), 7.33 (d, 1H, J = 2.1 Hz, ArH on C-7), 7.27 (d, 2H, J = 8.6 Hz, ArH *meta* to -OMe on pendant aryl ring), 7.01 (dd, 1H, J = 8.9, 2.3 Hz, ArH on C-5), 6.71 (d, 2H, J = 8.7 Hz, ArH *ortho* to -OMe on pendant aryl ring), 4.35 (q, 2H, J = 7.1 Hz, -OCH$_2$CH$_3$), 3.90 (s, 3H, -OCH$_3$), 3.72 (s, 3H, -OCH$_3$), and 1.38 (t, 3H, J = 7.1 Hz, -OCH$_2$CH$_3$); MS, m/z: 446 (M^+, 100), 431 (4), and 297 (5). Analysis calculated for $C_{26}H_{22}O_5S$: C, 69.94; H, 4.97; S, 7.18. Found: C, 69.49; H, 5.14; S, 7.36.

1-[(4′-Ethoxycarbonyl)benzoyl]-6-hydroxy-2-(4-hydroxyphenyl) benzo[*b*]thiophene (18). To a well-stirred solution of bis-methyl ether **17** (0.050 g, 0.112 mmol) in CH_2Cl_2 (6 ml) was added $BF_3{\cdot}SMe_2$ complex (2 ml). After 24 hours, product isolation (H_2O, CH_2Cl_2, H_2O, brine, $MgSO_4$) gave **18** as a dark oil. Purification by flash chromatography (silica gel, 40 × 200 mm, 40/60 : EtOAc/hexane) followed by crystallization from $CHCl_3$ and subsequent trituration from hexane afforded bis-phenol **18** (0.0204 g, 0.0487 mmol, 44%) as a yellow solid, mp 105 to 107 C (decomposition). ^1H NMR ($CDCl_3$): δ 7.90 (d, 2H, J = 8.5 Hz, ArH *ortho* to -CO_2Et), 7.76 (d, 2H, J = 8.5 Hz, ArH *meta* to -CO_2Et), 7.68 (d, 1H, J = 9.0 Hz, ArH on C-4), 7.28 (d, 1H, J = 1.9 Hz, ArH on C-7), 7.20 (d, 2H, J = 8.3 Hz, ArH *meta* to -OH on pendant aryl ring), 6.91 (dd, 1H, J = 8.9, 1.8 Hz, ArH on C-5), 6.63 (d, 2H, J = 8.6 Hz, ArH *ortho* to -OH on pendant aryl ring), 4.36 (q, 2H, J = 7.3 Hz, -OCH$_2$CH$_3$), and 1.38 (t, 3H, J = 7.0 Hz, -OCH$_2$CH$_3$); MS (m/z): 418 (M^+, 100) and 269 (7). Analysis calculated for $C_{24}H_{18}O_5S$: C, 68.89; H, 4.34; S, 7.66. Found: C, 67.83; H, 4.76; S, 7.67.

1-(4′-Carboxybenzoyl)-6-hydroxy-2-(4-hydroxyphenyl)benzo[*b*] thiophene (19). (The most efficient route to compound **19** involved the two-step procedure from **17,** as described below.) To a well-stirred solution of bis-methyl ether **17** (1.15 g, 2.58 mmol) in CH_2Cl_2 (50 ml) was added $BF_3{\cdot}SMe_2$ complex (20 ml). After 12 hours the reaction was quenched with H_2O (50 ml), and bis-phenol **18** was isolated by extraction with CH_2Cl_2 (2 × 50 ml). The crude sample of compound **18** in CH_2Cl_2 was passed through a plug of silica gel (5 cm); the solvent was removed under reduced pressure, and the resultant oil was taken up in H_2O (20 ml) and EtOH (10 ml), to which a 10% NaOH solution (20 ml) was slowly added over a 5-minute period. After an additional 15 minutes, the mixture was poured into H_2O (50 ml) and acidified with 3 N HCl to a pH less than 3, at which point crystals formed in the solution. Filtration followed by drying afforded the bis-phenolic acid **19** (0.611 g, 1.57 mmol, 61% [two steps]) as an off-white solid, mp 110 to 115 C (decomposition). ^1H NMR (acetone-d_6): δ 8.15 (s, 1H, -OH), 7.94 (d, 2H, J = 8.3 H, ArH *ortho* to -CO_2H), 7.90 (s, 1H, -OH), 7.79 (d, 2H, J = 8.3 Hz, ArH *meta* to -CO_2H), 7.63 (d, 1H, J = 8.8 Hz, ArH on C-4), 7.38 (d, 1H, J = 2.2 Hz, ArH on C-7), 7.20 (d, 2H, J = 8.6 Hz, ArH *meta* to -OH on pendant aryl ring), 6.98 (dd, 1H, J = 8.8, 2.2 Hz, ArH on C-5), and 6.68 (d, 2H, J = 8.5 Hz, ArH *ortho* to -OH on pendant aryl ring); MS, m/z 390 (M^+, 100) and 344 (4), 269 (15).

3-[(4′-Azidocarbonyl)benzoyl]-6-hydroxy-2-(4-hydroxyphenyl) benzo[*b*]-thiophene (2). Carboxylic acid **19** (0.100 g, 0.256 mmol) and *N*-methylmorpholine (0.0259 g, 0.256 mmol) were dissolved in DMF (0.5 ml) and cooled to 0 C. Diphenylphosphoryl azide (0.0705 g, 0.256 mmol) in DMF (0.08 ml) was added in one portion, and the mixture was stirred for 1 hour. An additional aliquot of *N*-methylmorpholine (0.01 ml) was added, and the mixture was stirred for 4 hours. Product isolation (H_2O, EtOAc, H_2O, brine, Na_2SO_4) gave the acyl azide **2** as an oil. Purification by flash chromatography (silica gel, 20 × 400 mm, 3/2 : hexane/EtOAc) followed by crystallization ($CHCl_3$) afforded the acyl azide **2** (0.0160 g, 0.0385 mmol, 15%) as a pale orange solid, mp 144 to 146 C (decomposition). IR (KBr): ν 3,400 (broad), 2,170 (doublet, azide), 1,670, 1,600; ^1H NMR (acetone-d_6): δ 8.72 (s, 1H, -OH), 8.61 (s, 1H, -OH), 7.90 (d, 2H, J = 8.4 Hz, ArH *ortho* to -C(O)N_3), 7.80 (d, 2H, J = 8.3 Hz, ArH *meta* to -C(O)N_3), 7.67 (d, 1H, J = 8.8 Hz, ArH on C-4), 7.40 (d, 1H, J = 2.1 Hz, ArH on C-7), 7.19 (d, 2H, J = 8.6 Hz, ArH *meta* to -OH on pendant aryl ring), 7.00 (dd, 1H, J = 8.8, 2.2 Hz, ArH on C-5), and 6.68 (d, 2H, J = 8.6 Hz, ArH *ortho* to -OH on pendant aryl ring); MS (FAB) m/z: 416 (M + 1). Analysis calculated for $C_{22}H_{13}O_4N_3S$: C, 63.61; H, 3.15; N, 10.12; S, 7.72. Found: C, 63.81; H, 3.27; N, 9.35; S, 7.28.

Biologic Procedures

Materials. The following compounds were obtained from the sources indicated: tritium-labeled estradiol ([6,7-^{3}H]E_2) (estra-1,3,5(10)-triene-3,17β-diol), 49–51 Ci/mmol, Amersham Corp; dextran, grade C, Schwarz/Mann; (ethylenedinitrilo)tetraacetic acid, tetrasodium salt (EDTA), Eastman Kodak Co.; Triton X-114, Chem Central-Indianapolis; 1,4-bis(5-phenyloxazol-2-yl)-benzene (POPOP), Aldrich Chemical Co.; DMF, Fisher Scientific; unlabeled estradiol, activated charcoal, Trizma Base, 3-[*N*-morpholino]propanesulfonic acid (MOPS), Sigma Chemical Co.; and 2,5-diphenyloxazole (PPO), Research Products International Corp.

Rat and lamb uterine cytosols were prepared and stored as previously described.[20] Experiments were performed in TEA buffer (0.01 M Tris-HCl, 0.0015 M EDTA, 0.02% sodium azide, pH 7.4, at 25 C) or in MOPS buffer (0.01 M MOPS, 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[5] and was used at 1 part to 10 parts of cytosol solution.

Liquid scintillation counting was carried out on a Nuclear Chicago Isocap 300 instrument using a xylene-based cocktail containing 0.55% 2,5-diphenyloxazole, 0.01% p-bis[2-(5-phenyl-oxazoyl)]benzene, and 25% Triton X-114.[21]

Relative binding affinity. Assays were performed as previously reported[5] using lamb or rat uterine cytosol diluted to approximately 1.5 nM of receptor. Cytosol was incubated with buffer or several concentrations of unlabeled competitor together with 10 nM ^{3}H-tracer at 0 C for 18 to 24 hours. The unlabeled competitor was prepared in 1 : 1 DMF/TEA to ensure solubility.

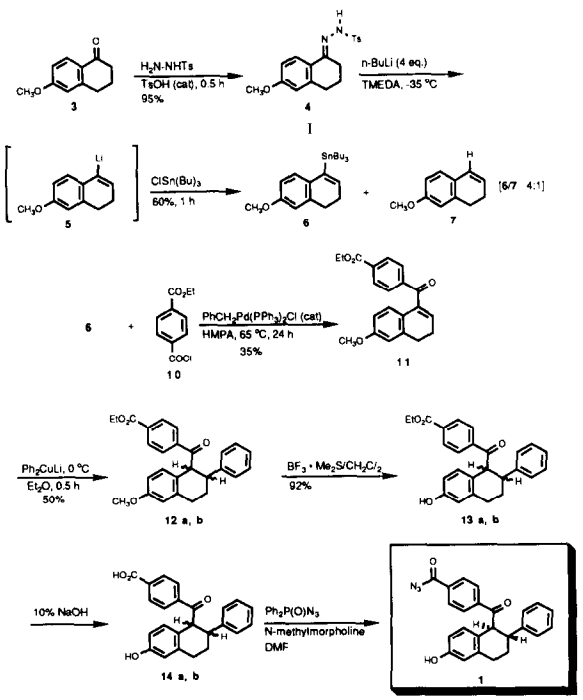
Electrophilic inactivation. Fresh solutions of the acyl azides or estradiol were added to uterine cytosol to give the concentrations of compounds as indicated. To protect the binding sites with estradiol, additional incubations were performed with an excess of estradiol for 1 hour at 4 C before the addition of the acyl azides. The mixtures were incubated at 22 C, and aliquots were removed at different times and assayed after charcoal-dextran treatment for surviving reversible estrogen-binding activity by exchange with 30 nM [^{3}H]E_2 in the presence and absence of a 100-fold excess of E_2 for 22 hours at 22 C, as detailed previously.[21]

Photoreactivation. Solutions of cytosol and the acyl azides with or without excess E_2 were set up exactly as for the electrophilic inactivation. They were incubated, in the dark, for 1 hour. Photolysis was carried out at greater than 315 nm (450-W mercury vapor lamp, Hanovia L679A, surrounded by a solution filter of saturated aqueous copper(II) sulfate) at 2 to 4 C using Pyrex reaction vessels or at 300 nm using a Rayonet photochemical reactor employing 16 300-nm bulbs. Aliquots were removed at various times and assayed by exchange, for surviving reversible estrogen binding activity. Complete details of the photolysis procedure are as previously described.[22]

Results

Chemical synthesis

Preparation of azidotetralin 1. The azidotetralin reagent was prepared in nine steps by a semiconvergent synthesis (Scheme 2). The first principal component, vinylstannane **6,** was readily prepared from 6-methoxy-1-tetralone **3** using a modification of the Shapiro reaction.[23] While it was difficult to purify **6** completely from the 3,4-dihydro-6-methoxynaphthalene side product **7,** this was inconsequential as olefin **7** does not interfere with the subsequent reaction and is readily removed later.



Scheme 2

(4-Ethoxycarbonyl)benzoyl chloride **10,**[19] the second major component, was prepared by initial *mono*-hydrolysis of dimethylterphthalate **8** with sodium hydroxide in ethanol/water, with accompanying transesterification to afford (4-ethoxycarbonyl)benzoic acid **9.** Treatment with thionyl chloride gave the requisite acid chloride **10** in excellent yield.

The coupling of components **6** and **10** was achieved using a Pd(II)-catalyzed reaction[24] to afford the benzoyl tetralin **11.** Introduction of the phenyl substituent at C-2 was achieved by conjugate addition of lithium diphenylcuprate to the α,β-unsaturated ketone **11,** giving a mixture of *cis* and *trans* diastereomers **12a,b** (approximately 1 : 1 by NMR). The best results were achieved using the preformed cuprous bromide-dimethyl sulfide complex to generate the cuprate.[25] One diastereomer of the phenylated tetralin derivative **12a** was obtained in crystalline form. The other diastereomer has never been crystallized, and actually remains as only a very minor component in the mother liquor after crystallization. Thus, the *cis* and *trans* isomers apparently equilibrate in solution, with the more crystalline isomer being the major isolated product. This is not surprising, since the benzoyl benzyl system in **12a** and **12b** is highly activating, giving these compounds a substantial enolic content. A combination of NMR experiments (^{1}H NMR, 2D-COSY, and 2D-HETCOR) was used to assign the crystalline diastereomer **12a** the *cis* configuration. This assignment is based primarily on the vicinal coupling constant between the two benzylic methine hydrogens, which is 5.7 Hz for the *cis* compound and 9.2 Hz for the *trans* isomer.

Deprotection of the methyl ether in compound **12** was accomplished using excess $BF_3{\cdot}S(CH_3)_2$ complex[26] in nearly quantitative yield to afford a mixture of *cis* and *trans* diastereomers **13a,b.** On crystallization, only the *trans* diastereomer **13b** was isolated. Energy minimization calculations* predict that the *trans* hydroxyester **13b** should be more stable than the *cis* isomer **13a** by 7.1 kcal/mol. The dihedral angle between H_A and H_B in the *trans* isomer **13b** is predicted to be 172.4° (Figure 1), consistent with the larger (9.2 Hz) coupling constant. In the *cis* isomer **13a,** the same angle is predicted to be 62.8° (Figure 2), consistent with the smaller (5.7 Hz) coupling constant. The stereoview of the *cis* isomer (Figure 2) clearly shows the steric hindrance between the bulky aryl substituents in this minimum energy conformation; these interactions are absent in the *trans* isomer (Figure 1).

Saponification of the ethyl ester **13** in NaOH/H_2O afforded the free acids **14a,b** as a diastereomeric mixture, from which the pure *trans* isomer **14b** (J = 9.5 Hz for benzylic methines) was crystallized. The most efficient means of generating acyl azide **1** involved

* Molecular mechanics calculations were done with the MAXIMIN option of the SYBYL molecular modeling system (Tripos Associates, St. Louis, MO, USA).

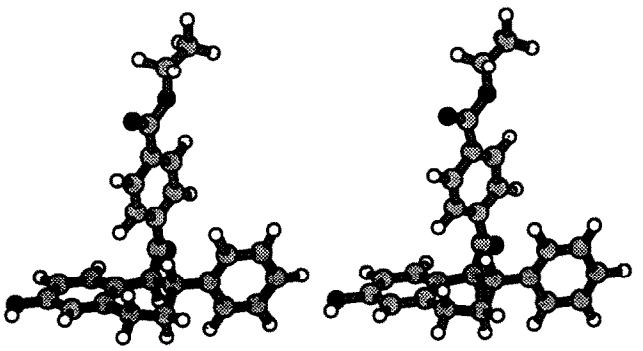


Figure 1 Relaxed stereoview of the *trans* isomer, ester **13b.** The structure is determined by molecular mechanics minimization (see text).

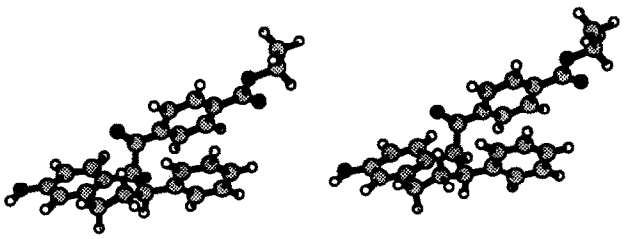


Figure 2 Relaxed stereoview of the *cis* isomer, ester **13a.** The structure is determined by molecular mechanics minimization (see text).

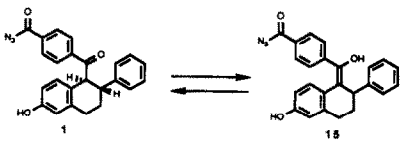
treatment of the free acid **14b** with diphenylphosphoryl azide in the relatively weak base *N*-methylmorpholine (Scheme 1).[27] Surprisingly, only a single diastereomer was observed in solution on aqueous work-up. It crystallized as a pale yellow solid, which was identified as the *trans* isomer **1** (J = 9.2 Hz). The color of the compound may be due to a contribution from the highly conjugated enol tautomer **15** (Scheme 3).

Preparation of Azidobenzo[*b*]thiophene 2. The synthesis of azidobenzo[*b*]thiophene **2** was facilitated by a highly efficient Lewis acid-catalyzed Friedel-Crafts acylation reaction between the electron-rich C-3 center of 6-methoxy-2-(4-methoxyphenyl)benzo[*b*]thiophene **16** and (4-ethoxycarbonyl)benzoyl chloride **10** (Scheme 4), as was demonstrated by Jones and co-workers (Eli Lilly, Inc.) in related systems.[14] Benzo[*b*]thiophene **16** may be prepared in multigram quantities via a facile two-step process (nucleophilic displacement, cyclization/rearrangement) from 3-methoxybenzenethiol and 1-bromo-4′-methoxyacetophenone.[14,28] Treatment of the coupled benzo[*b*]thiophene **17** with $BF_3 \cdot SMe_2$ complex resulted in cleavage of the bis-methyl ether protecting groups to generate bis-phenol (ester) **18,** which was saponified to carboxylic acid **19.** It was not necessary to isolate ester **18;** it may be treated as an intermediate and directly converted to carboxylic acid **19** (i.e., one-pot process, 61% yield for the two steps). Acyl azide **2** was prepared by treatment of carboxylic acid **19** with diphenylphosphoryl azide and *N*-methylmorpholine.

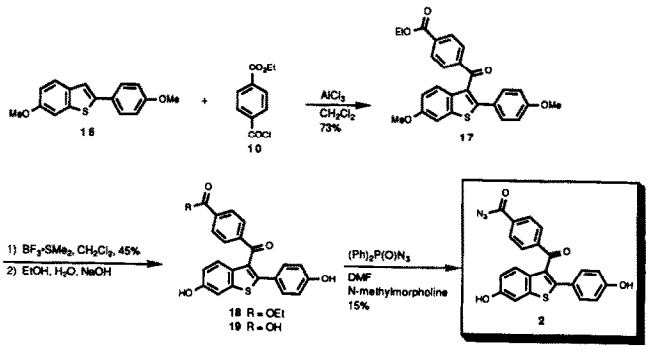
Biochemical evaluation of the acyl azides with the estrogen receptor

Estrogen receptor binding studies with acyl azides 1 and 2. The relative binding affinity (RBA) of acyl azides **1** and **2** for the estrogen receptor was determined by a competitive radiometric binding assay using [^{3}H]estradiol as the tracer.[5] The values (Table 1) are reported relative to estradiol, which is assigned a value of 100%. The compounds in the benzo[*b*]thiophene series all have significantly higher binding affinity for the estrogen receptor than the corresponding compounds in the tetralin series. Ethyl ester **18** (RBA = 70%), in fact, demonstrates the highest binding affinity for the estrogen receptor of any of the compounds in either of the two series. In each series, the carboxylic acids **14b** and **19** have substantially lower binding affinities than their corresponding esters **13b** and **18,** possibly because of their charged nature. While the binding affinity of neither azidotetralin **1** (RBA = 3%) nor azidobenzo[*b*]thiophene **2** (RBA = 12%) is extremely high, it is certainly sufficient to qualify them both for further biochemical analysis as potential affinity labeling reagents of the estrogen receptor. The binding data on the two acyl azides, however, should be interpreted with caution because of their hydrolytic lability (see below).

Electrophilic inactivation assay of acyl azides 1 and 2. Because acyl azides are active acylating agents, they were first evaluated for their capacity for electrophilic inactivation of the estrogen receptor.[8,21] These assays were performed by incubating estrogen receptor with the acyl azides **1** and **2** in the dark, in three buffer systems: TEA buffer (that is, 10 mM in the primary



Scheme 3



Scheme 4

Table 1 Estrogen receptor binding affinity of acyl azides and related compounds

Compound	R	RBA[a] (estradiol = 100%)
Tetralin system		
13b	OEt	13
14b	OH	0.4
1	N_3	0.6 (3)
Benzo[*b*]thiophene system		
18	OEt	70
19	OH	0.8
2	N_3	4 (12)
Trioxifene methanesulfonate		8[b]
LY117018		45 (52)[c]

[a] The RBA is determined by a competitive radiometric binding assay using [^{3}H]estradiol as tracer, charcoal-dextran as adsorbant of free ligand, and lamb uterus as a source of receptor. For further details, see ref. 5. Binding data in parentheses are from assays using rat uterus as a source of estrogen receptor.
[b] The RBA of trioxifene methanesulfonate is reported in the literature[13] to be 170. In repeated binding experiments, we obtain the RBA value reported in Table 2.
[c] The RBA of LY117018 in rat is from the literature (ref. 14).

amine, Tris), in TEA buffer plus 5% DMF (0.68 M DMF, which generally contains a small quantity of the secondary amine dimethylamine from hydrolysis), and MOPS buffer (a tertiary amine). After differing periods of time (0 to 22 hours), excess azide is removed by charcoal-dextran adsorption, and the surviving sites are assayed by an exchange assay with [^{3}H]estradiol (22 hours at 22 C).[22] The loss of reversible binding capacity is considered to represent irreversible binding to receptor by the acyl azides, and the site specificity of this process is ascertained by comparing irreversible binding with the acyl azides alone and in the presence of an excess of unlabeled estradiol. The data are reported in Table 2, and a time course for both compounds in MOPS buffer is shown in Figure 3.

Both acyl azides demonstrate rapid and substantial irreversible binding to the estrogen receptor, with the azidobenzo[*b*]thiophene system **2** being the more effective. The data in Table 2 are also consistent with competition for reaction of these acyl azides with the estrogen receptor by amines. Thus, in TEA buffer in the presence of DMF (which generally contains some dimethylamine), the inactivation is very low; in TEA buffer alone, it is substantially improved, while in MOPS buffer (which contains only tertiary amines), the extent of inactivation is 10% greater than in TEA.

When a fresh aliquot of the azidobenzo[*b*]thiophene **2** is added after 30 minutes to an incubation of cytosol and the thiophene **2,** no increased inactivation is seen (Figure 3B), suggesting that the limiting factor is not simply consumption of the existing ligand. Perhaps acid **19,** generated by acyl azide hydrolysis, binds to the receptor, protecting it from further inactivation. The ester derivative **13b** of the tetralin series demonstrates low to negligible inactivation of estrogen receptor.

Approximately 16% of the initial inactivation reverses in the dark with time for both acyl azides in either TEA or MOPS buffer (Figure 4). This reversal is complete in 1 to 2 hours, and the remaining inactivation is stable for at least 22 hours at 25 C. While it is not certain why there is this partial, spontaneous reactivation of sites after treatment of the estrogen receptor with the acyl azides, it may represent the fact that covalent attachment is occurring at two different sites, one that is stable (and persists for 22 hours) and a second that is spontaneously labile. Nevertheless, the persistence of the major portion of these compounds bound irreversibly to the estrogen receptor indicates that the acyl azides may have potential as electrophilic molecular probes for the estrogen receptor.

Despite their reactivity toward the estrogen receptor, both azides **1** and **2** demonstrate excellent hydrolytic stability (>95% stable at 13 hours as evaluated by high-performance liquid chromatography) in the buffered media alone (TEA: 0.01 M Tris, 0.0015 M EDTA, 0.02% sodium azide). However, they are both rapidly consumed in buffered media containing rat serum albumin (data not shown) and would be similarly consumed in uterine cytosol preparations. Melvin and Schuster[29] similarly found that while 4-acetylbenzoyl

Table 2 Electrophilic inactivation efficiencies

	Percent (%) of specific inactivation				
	Azidotetralin, **1**		Azidobenzo[*b*]thiophene, **2**		
Buffer	Initial	Stable	Initial	Stable	Tetralin ester, **13b**
MOPS	35	16 ± 4 (5)	52	33 ± 7 (5)	ND
TEA	27 ± 9 (5)	11 ± 4 (6)	46 ± 4 (6)	28 ± 2 (6)	0–9%
TEA + 5% DMF	10 ± 6 (3)	ND	2 ± 4 (3)	ND	ND

Inactivation efficiency is determined by an inactivation exchange assay. For further details, see refs. 8, 23, and 36. Numbers in parentheses indicate number of determinations.
Abbreviation: ND, not done.

azide has a long half-life in Tris buffer alone (17 hours), it is quite rapidly consumed (half life = 1.8 hours) in Tris buffer containing albumin.

Photoinactivation: photoreactivation of the estrogen receptor with acyl azides 1 and 2. The rapid irreversible binding of the acyl azides with estrogen receptor complicates assay of their photoreactivity.[6,22] In fact, from the extent of receptor inactivation that is achieved by an electrophilic process by 1 hour, the effect of further reaction by irradiation is to reverse the irreversible binding. An example of such photoreactivation with acyl azide **2** is shown in Figure 4. This photoreactivation process is rapid ($t_{1/2}$ = 3.5 minutes at 300 nm and 7 minutes at 315 nm) and appears to represent the release of the acyl azide adduct with the receptor by a light-induced deacylation process. (The extent of specific exchange increases because total exchange increases after irradiation, whereas nonspecific exchange remains constant; see Discussion.)

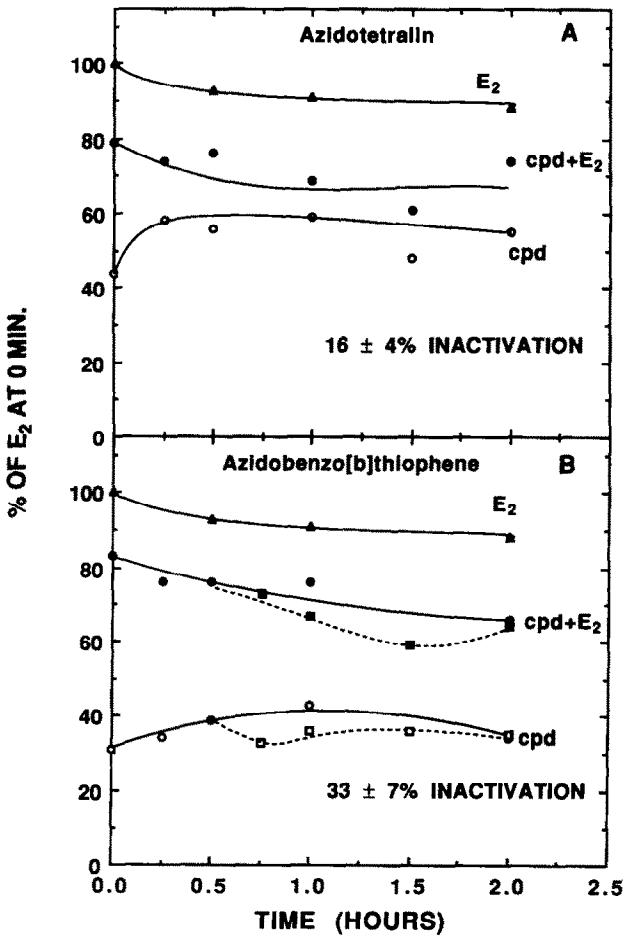


Figure 3 Electrophilic inactivation assay. Time course of the irreversible binding of the tetrahydronaphthalene acyl azide **1** (A) and the benzo[*b*]thiophene acyl azide **2** (B), determined in MOPS buffer. The assay is run by incubating estrogen receptor preparations with the compound alone at 30 nM/RBA (910 nM for **1** and 250 nM for **2**) (○) or in the presence of a 100-fold excess of unlabeled estradiol (pd + E_2, ●) or with estradiol alone (E_2, △) for the indicated time. Excess ligand is removed by adsorption onto charcoal-dextran, and the surviving reversibly bound receptor is assayed by an exchange assay (see Experimental). The specific irreversible binding is the difference between the curves for compound alone and compound plus estradiol. In panel B, data from an additional experiment in which a fresh aliquot of acyl azide **2** was added at 0.5 hours is also shown (■, □, and dotted lines).

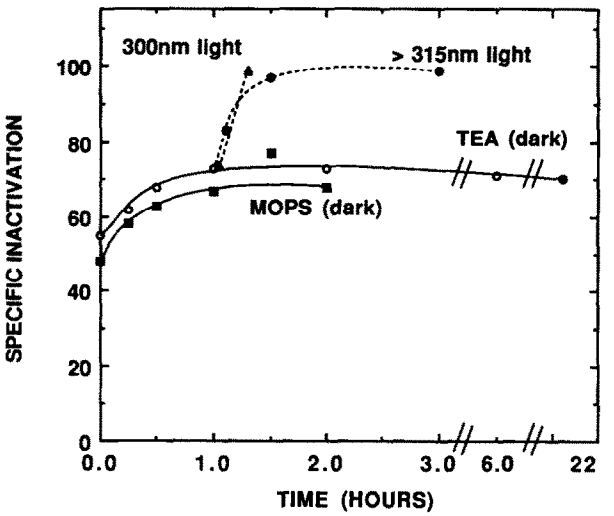


Figure 4 Photoreactivation of irreversibly bound estrogen receptor with the acyl azide **2**. The assays were performed as described in Figure 3, with the exception that some samples were exposed to either 300 nm or more than 315 nm light beginning at 1 hour (broken lines).

Discussion

We have synthesized two nonsteroidal estrogens patterned after two antiestrogens prepared by the Eli Lilly Co. that embody an acyl azide function and thus have the potential for both electrophilic and photoreactive covalent labeling of the estrogen receptor. Both compounds have substantial affinity for the estrogen receptor, the benzo[*b*]thiophene system being higher. Both also show substantial site-specific irreversible reaction that is rapid, and in part spontaneously reversible, and is further reversed by irradiation. Thus, these compounds have potential utility as unique electrophilic affinity labels for the estrogen receptor that may be photoreversible.

The acyl azide function is generally considered a poor choice for incorporation into affinity labeling reagents for two reasons: hydrolytic lability and undesired photochemistry. First, acyl azides are weak acylating agents and, although moderately stable in water, they are quite reactive toward nucleophilic species, as abound in biologic preparations. This reactivity can be a disadvantage in photoaffinity labeling, since reagent may be destroyed before it binds to the desired site and is irradiated. On the other hand, their acylating activity means that acyl azides are potential electrophilic affinity labeling agents, as their reaction with nucleophilic residues could result in the acylation of active site residues.

The second problem concerns the photochemical behavior of acyl azides. On photolysis, acyl azides usually undergo a photochemical Curtius rearrangement that leads to a less reactive isocyanate. This rearrangement competes effectively with the formation of the reactive nitrene.[30] The Curtius rearrangement does not proceed from the singlet or triplet nitrene,[31] but is thought to occur via a photoexcited singlet azide through a concerted mechanism.[32] Recent studies by Schuster and co-workers,[33,34] however, have demonstrated that the incorporation of a triplet sensitizer (e.g., benzoyl group) into the acyl azide function provides an efficient, although multistep, route to the singlet acyl nitrene. Excitation of the acyl azide to the excited acyl azide singlet is followed by rapid intersystem crossing (ISC) to the triplet azide, promoted by the internal triplet sensitizer. This rapid ISC effectively eliminates the competing Curtius rearrangement. Loss of nitrogen then generates a triplet nitrene, which undergoes another ISC to the more reactive singlet nitrene.

Schuster and co-workers have recently prepared four new acyl azides and evaluated their photochemical behavior.[29] These systems incorporated a benzoyl internal triplet sensitizer, and they displayed efficient photochemical generation of singlet acyl nitrene and insertion into unactivated C-H bonds. Although stable in water, they were, however, rapidly consumed in protein-containing solutions.

We incorporated intramolecular triplet sensitization via the benzoyl function into the two acyl azide systems **1** and **2** we have prepared (Scheme 1) so that they would have potential as both electrophilic and photoaffinity labeling reagents for the estrogen receptor. The tetralin acyl azide **1** mimics the structure of the Lilly antiestrogen trioxifene; however, it lacks the unsaturation at C-1, as we felt that the extended chromophore might interfere with the triplet sensitizing activity of the benzoyl group. It also contains a C-6 hydroxyl group in place of the C-6 methoxy, to improve the binding to the estrogen receptor. Acyl azide **2** is fully conjugated, and this is very closely related in structure to LY117018, a potent antiestrogen with a favorable binding profile for the estrogen receptor.

While not high-affinity ligands for the estrogen receptor, both acyl azides demonstrate sufficient affinity for further study. The azidotetralin system **1** has an affinity that is still somewhat less than that of the related antiestrogen trioxifene, even though one would expect a more favorable interaction with the estrogen receptor by the C-6 hydroxy group in **1** as compared with the methoxy group in trioxifene. The azidobenzo[*b*]thiophene system **(2)** also has lower affinity than LY117018. The results of the binding experiments on the acyl azide systems **1** and **2** should be interpreted with caution, however, as these compounds are hydrolytically unstable in protein-containing solutions, and partial hydrolysis to the acids **14b** and **19,** respectively, could account for their lower affinity.

After adding the acyl azides **1** and **2** to uterine cytosol preparations, there is a rapid, selective loss of binding sites that can be blocked by an excess of a nonreactive ligand such as estradiol. Although it is more rapid, this behavior is suggestive of electrophilic covalent labeling and has been observed before with other well-characterized, affinity labeling agents for the estrogen receptor, such as the aziridine derivatives of tamoxifen,[9] hexestrol,[35] and nafoxidine.[36] There are two features of the irreversible binding by the acyl azides that are curious, however: (1) a minor portion appears to be spontaneously reversible, perhaps due to acylation of a nucleophilic residue on the receptor that gives hydrolytically labile acyl linkages (e.g., cysteine [thiol ester], tyrosine [aryl ester], or glutamate or aspartate [anhydride], and (2) irradiation of these electrophilic receptor adducts actually reverses the receptor ligand acyl linkage.

There is precedent for photochemical hydrolysis of esters.[37] In addition, there are other examples in which photolytic and acylation-deacylation reactions occur in supplementing or antagonistic ways. In earlier studies on photoaffinity labeling of rat alphafetoprotein,[38] we found that a steroidal diazoketone underwent direct photocovalent attachment to the binding protein on irradiation, but also rearranged to a species that appeared to generate an acyl azide,[3] which subsequently (in the dark) underwent further electrophilic reaction with the protein.[38] Recent studies by Porter and coworkers[39,40] have shown that irradiation can isomerize a cinnamyl chymotrypsin ester from a hydrolytically stable *trans* isomer to a hydrolytically labile *cis* isomer.

The *acylating* reactivity of the acyl azides is intriguing and potentially useful in characterizing binding site residues in the estrogen receptor because it may differ in selectivity from the *alkylating* activity of the aziridine affinity labeling agents noted above. The aziridines are weak electrophiles and, in the two cases we have characterized, tamoxifen aziridine and ketononestrol aziridine,[10] they label only a cysteine residue (cys-530) in the hormone-binding domain of the human estrogen receptor. The acyl azides are electrophiles of a different character and, based on chemical reactivity alone (ignoring spatial constraints that may operate in ligand-binding site interaction), they may show a preference for labeling the basic and nucleophilic residue of lysine.

We are planning further studies of the estrogen receptor labeling behavior of the acyl azides **1** and **2.** These may involve their preparation in high specific activity tritium-labeled form for radiometric detection of labeled receptor at the tracer level and indirect Edman sequencing of labeled peptides.[10] Alternatively, if larger quantities of purified estrogen receptor were available, peptide purification and sequencing could be based on the unique chromophoric nature of these species, especially the benzo[*b*]thiophene system, whose UV spectrum is clearly distinct from that of all amino acid residues. Finally, the fact that the putative covalent link between these acyl azides and the estrogen receptor can be reversed on irradiation holds interesting possibilities for photo-

chemical control of estrogen receptor occupancy by ligands.

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