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Efficient and Selective Photoaffinity Labeling of the Estrogen Receptor Using Two Nonsteroidal Ligands That Embody Aryl Azide or Tetrafluoroaryl Azide Photoreactive Functions†

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ABSTRACT: 3-(4-Azido-2,3,5,6-tetrafluorobenzoyl)-6-hydroxy-2-(4-hydroxyphenyl)benzo[*b*]thiophene **1** (tetrafluoroaryl azide, TFAA) and its protio analogue 3-(4-azidobenzoyl)-6-hydroxy-2-(4-hydroxyphenyl)benzo[*b*]thiophene **2** (protioaryl azide, PAA), photoaffinity labeling (PAL) reagents for the estrogen receptor (ER), have been prepared in high specific activity tritium-labeled form (19 Ci/mmol) and shown to undergo selective and efficient photocovalent attachment to ER from rat uterus. Both azides **1** and **2** demonstrate high binding affinity for ER as determined by both a competitive binding assay (relative binding affinities: estradiol = 100; TFAA = 9.3; PAA = 66) and a direct binding assay (K_d: estradiol = 0.24 nM; TFAA = 2.64 nM; PAA = 0.37 nM). When unlabeled TFAA and PAA are irradiated at >315 nm, they demonstrate site-specific photoinactivation of ER that reaches 43% and 55%, respectively, by 30 min. Specific photocovalent attachment to ER can be effected by irradiation of the tritium-labeled azides; the covalent attachment efficiency is good (**1** = 20–30%, **2** = ca. 25%) and the selectivity of ER labeling is high. Characterization of the photolabeled proteins by SDS–polyacrylamide gel electrophoresis shows specific labeling of a major component at M_r 60 000 and a minor species at M_r 46 000, the same two species that are labeled by [^{3}H]tamoxifen aziridine, a well-characterized affinity label for ER. The ER-specific antibodies H222Spγ and D547Spγ show a clean precipitation of only these two species. In the MCF-7 human breast cancer cell line, PAA is a full estrogen agonist in terms of stimulation of cell proliferation and induction of progesterone receptor. These two azides provide the first system in which the photocovalent attachment efficiency of an aryl azide can be compared to its tetrafluorosubstituted aryl azide analogue in a complex biological receptor system. Azides **1** and **2** are the most efficient and selective PAL reagents prepared to date for ER, and they should be useful in further studies of the hormone-binding domain of this protein.

Photoaffinity labeling (PAL)[1] reagents are versatile molecular probes for studying the ligand-binding domains of complex biological receptor systems (Bayley, 1983; Schuster et al., 1989; Tometsko & Richards, 1980; Gronemeyer, 1988). PAL reagents are especially valuable for mapping the contact points of ligands with proteins whose primary structure is known but

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[1] Abbreviations: ER, estrogen receptor; TFAA, tetrafluoroaryl azide; PAA, protioaryl azide; PAL, photoaffinity labeling; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; DATD, *N*,*N*′-diallyltartardiamide; RBA, relative binding affinity.

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whose secondary or tertiary structures are still undetermined. We have been involved in the development of affinity labeling reagents for the estrogen receptor (ER) [for reviews, see Katzenellenbogen (1977), Katzenellenbogen & Katzenellenbogen (1984, 1988), and Katzenellenbogen et al. (1980)], and we have prepared a variety of potential PAL reagents containing photosensitive moieties (azides, diazoketones, nitroanisole derivatives, and ketonic estrogens) attached to or embodied within molecular skeletons (both steroidal and nonsteroidal in nature) designed for high-affinity receptor binding (Katzenellenbogen, 1977; Katzenellenbogen & Katzenellenbogen, 1984, 1988; Katzenellenbogen et al., 1973a,b, 1974, 1977a,b, 1980; Marquet et al., 1989). Unfortunately, none of these compounds has demonstrated the required combination of high binding affinity and efficient, selective photocovalent attachment necessary for use as an effective probe for ER. The most significant among these previously studied photoreactive compounds is 3-azidohexestrol, a compound that demonstrated excellent binding affinity (69%) and photoinactivation efficiency (15%) in nonradiolabeled form (Katzenellenbogen et al., 1974). In tritium-labeled form, however, 3-azidohexestrol was found to have very low photocovalent attachment to ER (ca. 10%), and its labeling selectivity was very poor, a factor that ultimately limited its use as a PAL reagent (Katzenellenbogen et al., 1977a).

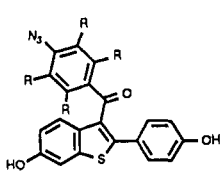
It has been known for some time that electron-withdrawing groups on aryl azides enhance the reactivity of the nitrene generated upon photolysis (Reiser & Leyshon, 1970). It is only recently, however, that both Platz and co-workers (Soundararajan & Platz, 1990; Leyva et al., 1986, 1989; Young & Platz, 1989), and Keana and co-workers (Keana & Cai, 1989, 1990; Cai & Keana, 1989) have proposed the use of polyfluorinated aryl azides as photosensitive probes for biological systems. Fluorine is an especially attractive electron-withdrawing group since it is nearly isosteric with hydrogen and therefore will not increase steric bulk in a way that might reduce the binding affinity of the PAL reagent.

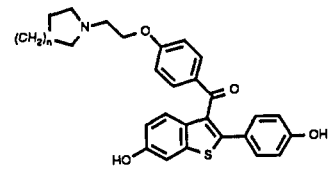
We became intrigued by the prospect of using tetrafluorosubstituted aryl azides as photoreactive groups for incorporation into ER-directed PAL reagents, and we recently prepared tetrafluoroaryl azide (TFAA) **1** as well as its protioaryl azide counterpart (PAA) **2** as PAL reagents for ER (Pinney & Katzenellenbogen, 1991). The photosensitive azide moiety is contained on a 2-aryl-3-aroylbenzo[*b*]thiophene skeleton, known to demonstrate high binding affinity for the ER, as exemplified in the potent antiestrogens LY117018 and LY139481, prepared by Jones and co-workers (Eli Lilly & Co.) [Jones et al., 1984; for pertinent biological studies concerning these antiestrogens, see Black et al. (1981, 1983), Black (1982), and Black and Goode (1980)]. We now report the preparation of both of these aryl azides in high specific activity tritium-labeled form. We have found that both azides demonstrate photocovalent attachment to ER and that they are the most efficient and selective PAL reagents for ER prepared to date. In addition, [^{3}H]TFAA and [^{3}H]PAA constitute a viable system in which to evaluate the role that fluorine substitution has on the reactivity of aryl nitrenes in a complex biological system.

Experimental Procedures

Chemical Procedures

Materials. Ethyl acetate, triethylamine, palladium on alumina, trifluoroacetic acid (TFA), sodium nitrite, and sodium azide were obtained from the Aldrich and Fisher Chemical Companies and used without further purification. Tritium gas (25 Ci, carrier-free) was obtained from DuPont (NEN). The iodoaryl azides were prepared as previously described (Pinney & Katzenellenbogen, 1991).





Methods. Normal-phase analytical thin-layer chromatography was performed on Merck 0.25-mm silica gel glass-backed plates with F-254 indicator, and reversed-phase analytical thin-layer chromatography was performed on 0.20-mm octadecylsilane-bonded silica gel glass-backed plates with F-254 indicator (Merck). Visualization was by ultraviolet light. High-performance liquid chromatography (HPLC) was performed on a Varian 5060 system with a Perkin-Elmer LC-75 Spectrophotometric Detector (variable wavelength set at 254 nm). The solvent system used was either 60% methylene chloride–isopropyl alcohol (95:5) and 40% hexane or 30% methylene chloride–isopropyl alcohol (95:5) and 70% hexane at 1.0 mL/min. Liquid scintillation counting was carried out on a Nuclear-Chicago Isocap 300 instrument using xylene-based cocktail containing 0.55% 2,5-diphenyloxazole, 0.01% 1,4-bis(5-phenyloxazol-2-yl)]benzene, and 25% Triton X-114 (Katzenellenbogen et al., 1974). The tritium gas exchange reaction was carried out on a microscale quantitative hydrogenation apparatus (Bindal, 1987), fabricated in the glass shop at the University of Illinois. The microhydrogenation reaction vessels were designed to contain 3 mL of solvent and fitted with a male standard taper 7/25 joint and were also fabricated at the University of Illinois glass shop.

Radiochemical Synthesis of [^{3}H]TFAA: (A) Preparation of 3-(4-Amino-2,3,5,6-tetrafluorobenzyl)-6-hydroxy-2-(4-hydroxyphenyl)-7-[^{3}H]benzo[b]thiophene (**5**). Tetrafluoroiodoaryl azide **3** (10.00 mg, 0.01709 mmol) was dissolved in ethyl acetate (EtOAc) (1.5 mL) and triethylamine (Et_3N) (0.067 mL) in a 3-mL microhydrogenation vessel followed by the addition of palladium/alumina (5% palladium content) (20.0 mg). The reaction vessel and the tritium gas source were both attached to the microhydrogenation apparatus (Bindal, 1987) (situated in a radioisotope-approved hood) and the reaction vessel was cooled with a dry ice/acetone bath. The system was thoroughly evacuated and then sealed off from the vacuum source. The tritium gas was released by rupture of the break seal, the reaction vessel was returned to room temperature, and the tritium gas was then transferred to the reaction vessel by repeated cycles of the Toepler pump. The contents of the vessel were stirred with an air-driven magnetic stirrer for 3.5 h under tritium gas (1.1 atm). In order to back-transfer the excess tritium gas to its original source tube, the reaction vessel was cooled with acetone/dry ice, and the tritium gas was returned with several cycles of the Toepler pump. The reaction vessel was then returned to room temperature and carefully removed from the line. The vial contents were filtered through a pad of Celite (7 mm) on a 15-mL sintered glass funnel (medium), and the solvent was carefully removed by a slow freeze/thaw process under reduced pressure with three traps (first, liquid N_2; second, isopropyl alcohol/dry ice; third, Drierite) situated in line before the pump. Labile tritium was removed by several cycles of redissolution and evaporation with ethanol in this same freeze/thaw process. The [^{3}H]tetrafluoroaryl amine **5** was immediately carried on to [^{3}H]TFAA **1**.

(*B*) *Preparation of 3-(4-Azido-2,3,5,6-tetrafluorobenzoyl)-6-hydroxy-2-(4-hydroxyphenyl)-7-[^{3}H]benzo[b]thiophene* (**1**) ([^{3}H]*TFAA*). [^{3}H]Tetrafluoroaryl amine **5** in a 10 × 45 mm screw-cap vial fitted with a small Teflon-coated magnetic stir bar was dissolved in trifluoroacetic acid (TFA) (0.30 mL). Sodium nitrite (0.0017 g, 0.0171 mmol) [as 0.1 mL of a standard solution (0.0117 g of $NaNO_2$/mL of TFA)] was added, and the reaction mixture was stirred at 0 °C in the dark for 30 min. Sodium azide (0.0111 g, 0.171 mmol) was added, and the mixture was stirred for 15 min, followed by the addition of diethyl ether (0.5 mL). After 1 h of additional stirring, the reaction was quenched by the addition of H_2O (5 mL) and the product isolated [extraction with Et_2O (2 × 5 mL), wash combined organics with brine]. The organic layer was dried by passage through a Pasteur pipette packed with $MgSO_4$, and the solvent was removed under reduced pressure (three-trap system) as previously described above. [^{3}H]TFAA was stored in toluene–ethanol (9:1) (50 mL) at −20 °C. A small amount of the crude mixture was purified in several batches by normal-phase HPLC equipped with a 30-cm Varian SI-5 silica gel analytical column, eluted with 40% hexane and 60% methylene chloride–isopropyl alcohol (95:5), or an ES Industries silica gel analytical column (30 cm × 4 mm), eluted with 70% hexane and 30% methylene chloride–isopropyl alcohol (95:5), both at a rate of 1 mL/min. The majority of the radioactivity was found in a peak with a retention time of 5.2 min (Varian column), or 9.1 min (ES Industries column), which corresponded to the retention time of unlabeled TFAA, determined by coinjection. The overall crude radiochemical yield was 8%. Most of the azide was stored in toluene–ethanol (9:1) as a crude reaction mixture; only small amounts were purified at a time for biochemical evaluation.

The specific activity of [^{3}H]TFAA was determined to be 19 Ci/mmol by HPLC analysis. The radiochemical purity was evaluated by reinjection onto HPLC and by normal-phase TLC analysis (ethyl acetate–hexane, 60:40), which indicated a single peak of radioactivity (94%) with an R_f of 0.63, identical with the R_f of an unlabeled standard run on the same plate.

Radiochemical Synthesis of [^{3}H]*PAA*: (*A*) *Preparation of 3-(4-Aminobenzoyl)-6-hydroxy-2-(4-hydroxyphenyl)-7-[^{3}H]benzo[b]thiophene* (**6**). Iodoaryl amine **4** (0.00833 g, 0.01709 mmol) was dissolved in EtOAc (1.5 mL) and Et_3N (0.067 mL) in a 3-mL microhydrogenation vessel (previously described), followed by the addition of palladium/alumina (5% palladium content) (47.0 mg). The vessel was attached to the microhydrogenator and stirred for 5 h under tritium gas (1.1 atm) as described for the tetrafluoro analogue. Product isolation was identical with that described for [^{3}H]tetrafluoroaryl amine **5**. [^{3}H]Protioaryl amine **6** was immediately carried on to [^{3}H]PAA **2**.

(*B*) *Preparation of 3-(4-Azidobenzoyl)-6-hydroxy-2-(4-hydroxyphenyl)-7-[^{3}H]benzo[b]thiophene* (**2**). [^{3}H]Protioaryl amine **6** was transferred to a 10 × 45 mm screw-cap vial equipped with a small Teflon-coated magnetic stir bar and dissolved in 5% HCl (0.25 mL) and acetone (1.0 mL). Sodium nitrite (0.00118 g, 0.0171 mmol) [as 0.1 mL of a standard solution (0.0118 g of $NaNO_2$/1.0 mL of H_2O)] was added, and the reaction mixture was stirred at 0 °C in the dark for 30 min. Sodium azide (0.0111 g, 0.171 mmol) was added in H_2O (0.3 mL) and the mixture was stirred for 15 min, at which point Et_2O (0.5 mL) was added. After being stirred for an additional 1 h, the reaction mixture was quenched, the product was isolated and stored, and small amounts were purified by HPLC as described for [^{3}H]TFAA. By HPLC, the majority of the radioactivity was found in a peak with retention time of 6.5 min (Varian column) or 12.2 min (ES Industries column), which corresponded to the retention time of unlabeled PAA (coinjection). The overall crude radiochemical yield was 32%. The majority of the [^{3}H]PAA was stored in toluene–ethanol (9:1) as a crude reaction mixture; small aliquots were purified for biochemical evaluation.

The specific activity of [^{3}H]PAA was determined to be 19 Ci/mmol by HPLC analysis. The radiochemical purity was evaluated by reinjection onto HPLC and by normal-phase TLC analysis (ethyl acetate–hexane, 60:40), which indicated a single peak of radioactivity (95%) with an R_f of 0.58, identical with the R_f of an unlabeled standard cospotted with the [^{3}H]PAA.

Biological 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 Ci/mmol, and [^{3}H]-tamoxifen aziridine (TAZ) ([*ring*-^{3}H]-(*Z*)-(1-[4-(2-[*N*-aziridinyl]ethoxy)phenyl])-1,2-diphenyl-1-butene), 20 Ci/mmol, were from Amersham Corp.; the synthetic progestin [6,7-^{3}H]R5020 (17,21-dimethyl-19-nor-4,9-pregnadien-3,20-dione; 87 Ci/mmol) and Protosol were from DuPont NEN; dextran, grade C, was from Schwarz/Mann; 2-mercaptoethanol, ethylenedinitrilotetraacetic acid tetrasodium salt (EDTA), acrylamide, *N*,*N*′-methylenebis[acrylamide], Photo-Flo 200, and *N*,*N*′-diallyltartardiamide were from Eastman Kodak Co.; Triton X-114 was from Chem Central-Indianapolis; bromophenol blue, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), sodium azide, and 1,4-bis(5-phenyloxazol-2-yl)-benzene (POPOP) were from Aldrich Chemical Co.; sodium dodecyl sulfate (SDS) was from Matheson, Coleman, and Bell; ammonium peroxydisulfate, glycerin, and *N*,*N*-dimethylformamide (DMF) were from Fisher Scientific; periodic acid was from G. Frederick Smith Chemical Co.; unlabeled estradiol, leupeptin, phenylmethanesulfonylfluoride (PMSF), activated charcoal, Trizma base, thioglycerol, Nonidet P-40, sodium lauryl sarcosine, sodium deoxycholate, ovalbumin (MW 44 600), bovine serum albumin (MW 67 000), phosphorylase B (MW 97 400), and myosin (MW 205 000) were from Sigma Chemical Co.; Coomassie Brilliant Blue R-250 was from Colab Laboratories, Inc.; and 2,5-diphenyloxazole (PPO) was from Research Products International Corp. All media (phenol red free and containing phenol red), sera, and antibiotics used to culture the MCF-7 cells were obtained from Grand Island Biological Co. Insulin was purchased from Sigma Chemical Co., rabbit antirat IgG and Zysorbin were from Zymed Co., and ER-specific antibodies H222Spγ and D547Spγ were a gift from Geoffrey L. Greene and Abbott Laboratories. The antiestrogen ICI 164,384 was kindly provided by Alan Wakeling (ICI Pharmaceuticals) and the antiestrogens LY117018 and LY139481 by C. David Jones (Eli Lilly and Co.).

Rat and lamb uterine cytosols were prepared and stored as previously described (Carlson et al., 1977). All experiments were done in TEA buffer (0.01 M Tris-HCl, 0.0015 M EDTA, and 0.02% sodium azide, pH 7.4 at 25 °C). Leupeptin (0.1 mg/mL) and PMSF (1 mM) were added to the cytosol to prevent proteolysis in samples for electrophoresis. The charcoal–dextran slurry used to remove unbound ligand was prepared as previously reported (Katzenellenbogen et al., 1973a) and was used at 1 part to 10 parts of cytosol solution.

Methods: *Relative Binding Affinity* (*RBA*). Assays were performed as previously reported (Katzenellenbogen et al., 1973a), using cytosol from MCF-7 human breast cancer cells

or from lamb or rat uterus diluted to ~1.5 nM receptor. Cytosol was incubated with buffer or several concentrations of unlabeled competitor together with 10 nM ^{3}H-tracer at 0 °C for 18–24 h. The unlabeled competitor was prepared in 1:1 dimethylformamide (DMF)–TEA to ensure solubility.

Scatchard Assay. Rat uterine cytosol was incubated at 0 °C for 4 h with various concentrations of ^{3}H-ligand in the absence or presence of a 100-fold excess of unlabeled estradiol. Aliquots of the incubation solution were counted to determine the concentration of total ^{3}H-steroid. The incubation solutions were then treated with charcoal–dextran and the bound ^{3}H-steroid was determined. Data were processed according to the method of Scatchard (1949).

Photolysis. Photolysis was routinely carried out at >315 nm [450-W mercury vapor lamp, Hanovia L679A, surrounded by a solution filter of saturated aqueous copper (II) sulfate at 2–4 °C employing Pyrex reaction vessels, as previously described (Katzenellenbogen et al., 1974)]. It was also done as above, >315 nm but at −196 °C (in liquid N_2), and at 254 nm in a Rayonet Reactor utilizing four 8-W germicidal bulbs at 0–4 °C as previously described (Katzenellenbogen et al., 1974).

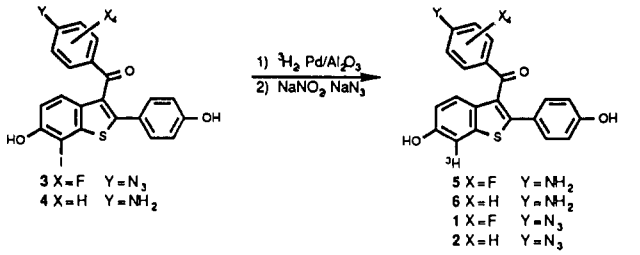
Inactivation Assay. Covalent binding of nonradioactive ligands was estimated by a photolysis–exchange assay previously described for the estrogen receptor (Katzenellenbogen et al., 1974).

Attachment Assay. Covalent binding of labeled ligands was measured directly by a filter disc assay described previously (Katzenellenbogen et al., 1975).

Electrophoresis. Samples for SDS electrophoresis were denatured in SDS denaturing solution (0.13 M mercaptoethanol, 2% SDS), in a boiling water bath for 2 min. Gels were cast in 12-cm-long × 6-mm i.d. glass tubes. The tubes had been coated with a 0.5% aqueous solution of Kodak Photo-Flo 200 and oven-dried. The catalyst solution (0.28% ammonium persulfate) was prepared fresh daily. The SDS–polyacrylamide gel electrophoresis system was modified from that of Laemmli (1970), substituting *N,N'*-diallyltartardiamide for *N,N'*-methylenebis[acrylamide] to cross-link the separation gel (Anker, 1970). The separation gel consisted of 7.5% acrylamide, 0.6% DATD, and 0.1% SDS. The gels were run at 2.5 mA/gel, at room temperature. Included in each experiment were gels of standard proteins, myosin, phosphorylase B, bovine serum albumin, and ovalbumin to establish a molecular weight curve. Following electrophoresis, the gels of the standard proteins were fixed overnight in 12.5% trichloroacetic acid. Those for radioactivity determinations were frozen on dry ice and sliced into 2.15-mm slices. Each slice was dissolved in 0.5 mL of 2% periodic acid at room temperature and then incubated with 0.5 mL of Protosol for 1 h at 50 °C; 0.1 mL of glacial acetic acid was added to quench chemiluminescence, and radioactivity was counted in 5 mL of scintillation fluid. The gels of the standard proteins were stained for 1 h at 37 °C in 0.25% Coomassie Blue in 5:1:5 methanol–acetic acid–water and destained electrophoretically in 7% acetic acid.

Immunoprecipitation. Cytosol samples, covalently labeled with ^{3}H-ligand were dissolved in 0.2% sodium lauryl sarcosine and 0.5% Nonidet P-40 (Gilmour & Lis, 1985). They were precleared by incubation with rabbit anti-rat IgG, which was removed by Zysorbin. The estrogen receptor was allowed to react with two specific antibodies, H222Spγ and D547Spγ. These antibodies were made against human ER and also detect rat ER. The immune complex was again incubated with rabbit anti-rat IgG and precipitated with Zysorbin. The pellet was washed four times in wash solution (0.1 M Tris-HCl, pH 9.0, 0.5 M LiCl, 1% sodium deoxycholate, and 1% Nonidet P-40), resuspended in SDS denaturing solution and heated in a boiling water bath for 5 min. The Zysorbin now released from the receptor was pelleted. The soluble receptor was assayed by SDS gel electrophoresis.

Scheme I



Cell Culture. MCF-7 human breast cancer cells, obtained from the Michigan Cancer Foundation (Detroit, MI), were grown in plastic T-150 flasks in Eagle's minimal essential medium containing 10% calf serum, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, gentamicin (50 μg/mL), penicillin (100 units/mL), streptomycin (100 μg/mL), and bovine insulin (6 ng/mL).

Cell Proliferation Assays. To determine the effect of PAA and estradiol on cell proliferation, MCF-7 cells grown for 1 week before experiments in phenol red free, insulin-free medium (Berthois et al., 1986) containing 10% charcoal–dextran-treated calf serum were seeded into T-25 flasks. The following day, cells from three flasks were counted with a Coulter counter (Coulter Electronics, Hialeah, FL). The medium was then changed to medium that contained PAA, E_2, or the antiestrogen ICI 164,384, alone or in combination, or control ethanol vehicle (0.1%). Medium was changed and fresh compounds were added every other day, and cell number was monitored as a function of time (Berthois et al., 1986). PAA was determined to be >80% stable in media at 37 °C for 48 h.

Progesterone Receptor Assay. MCF-7 cells were grown in phenol red free and insulin-free medium containing 10% charcoal–dextran-treated serum for 1 week before treatment for 4 days with PAA, E_2, or control vehicle (0.1% ethanol). Media and compounds were changed daily during the 4-day period. Cells were harvested and progesterone receptors were measured by using a whole cell progesterone receptor assay as previously described (Katzenellenbogen et al., 1987b). In brief, aliquots of the cell suspension [(2–5) × 10^6 cells/mL of tissue culture medium] were incubated with 10 nM [^{3}H]-R5020 in the absence and presence of 1000 nM unlabeled R5020 for 0.5 h at 37 °C. Cells were then rinsed three times with 2 mL of iced phosphate-buffered saline with 1% Tween-80 and transferred with 200 μL of phosphate-buffered saline to scintillation vials. One milliliter of ethanol was added, and samples were counted in xylene-based scintillation fluid containing 25% Triton X-114.

Results

Radiochemical Syntheses. The preparation of [^{3}H]TFAA and [^{3}H]PAA was achieved by a palladium-catalyzed hydrogenolysis of iodoaryl precursors **3** and **4** (Scheme I) using carrier-free tritium gas. Our preparation of the iodoaryl compounds **3** and **4**, as well as our development of exchange reaction conditions under deuterium gas for isotopic substitution, have been previously reported (Pinney & Katzenellenbogen, 1991). The initially formed tritiated amines **5** and

Table I: Biochemical Properties of Substituted Benzo[b]thiophenes for the Estrogen Receptor

compound	RBA[a] (estradiol = 100%)	photoinactivation efficiency[b] (%)	photocovalent attachment efficiency[c] (%)
TFAA (1)	5.9 (9.3)[d]	43	20–30
PAA (2)	36 (66), 66[e]	55	25–50
3	0.11	nd[f]	nd
5	11	nd	nd
6	13	nd	nd
7	2.0	nd	nd
LY117018	45 (52)[g]	57	0

[a] The relative binding affinity (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. Further details are presented under Experimental Procedures and in Katzenellenbogen et al. (1973b). [b] Photoinactivation efficiency is determined with the unlabeled compound, by irradiation of ER filled with the compound and measuring the specific sites that survive by an exchange assay. Correction is made for nonspecific photoinactivation. Further details are presented under Experimental Procedures and in Katzenellenbogen et al. (1974). [c] Photocovalent attachment efficiency is determined with the tritium-labeled compounds by irradiating ER filled with the compound and measuring the covalent attachment by a filter disc–solvent extraction assay. Correction is made for nonspecific photoattachment; the specific labeling efficiency is considered to be the ER sites that become covalently labeled as a percentage of those occupied at the time of photolysis. Further details are presented under Experimental Procedures and in Katzenellenbogen et al. (1975). [d] Binding data in parentheses are from assays using rat uterus as a source of ER. [e] Binding assay using MCF-7 human breast cancer cell cytosol as a source of ER. [f] nd, not determined. [g] The RBA of LY117018 in rat is from the literature (Jones et al., 1984).

6 were converted to the tritiated azides **1** and **2**, respectively, by diazotization of the amine, followed by direct displacement with sodium azide. Trifluoroacetic acid (TFA) worked well as solvent for the diazotization of ^{3}H-amine **5**, while a mixture of HCl and acetone was the system of choice for ^{3}H-amine **6**. Optimization of these conditions on small-scale reactions was described in our previous report (Pinney & Katzenellenbogen, 1991). The specific activity was determined by HPLC analysis to be 19 Ci/mmol for both [^{3}H]TFAA and [^{3}H]PAA, which is 66% of the theoretical maximum for incorporation of one tritium atom per molecule of azide.

Estrogen Receptor Binding Properties of TFAA and PAA and Related Compounds. (A) Competitive Binding Assay of TFAA and PAA and Related Compounds. The relative binding affinities (RBA) of the two aryl azides (**1** and **2**) and the two aryl amines (**5** and **6**) as well as the tetrafluoroiodoaryl azide **3**, the pentafluoroaryl azide **7**, and LY117018 (all unlabeled) for the estrogen receptor (ER) were determined indirectly by a competitive radiometric binding assay with [^{3}H]estradiol as the radiolabeled tracer (Katzenellenbogen et al., 1973a). The data, shown in Table I, are given relative to estradiol (RBA = 100%). PAA demonstrated the highest RBA (66%) of this series of compounds for the ER. Substitution by four fluorine atoms on the azidobenzoyl group results in a 7-fold reduction in the binding affinity (RBA of TFAA = 9.3%). The pentafluoroaryl azide **7** (which has been previously prepared; Pinney & Katzenellenbogen, 1991) has a fairly low RBA (2%). The iodotetrafluoroaryl azide **3** has the lowest RBA in the series (0.11%), which most likely reflects a lack of tolerance of ER for the sterically demanding iodine atom.

It is interesting to note that the binding affinity of these substituted benzo[b]thiophenes (**1** and **2**) is reduced by a factor of approximately 1.7 when the assay is performed with lamb uterine cytosol, as opposed to that obtained from the rat or MCF-7 human breast cancer cells (Table I). Similar behavior has been previously observed in the binding affinity of other estrogens and most likely reflects the fact that the nonspecific binding of these more lipophilic compounds in lamb uterine

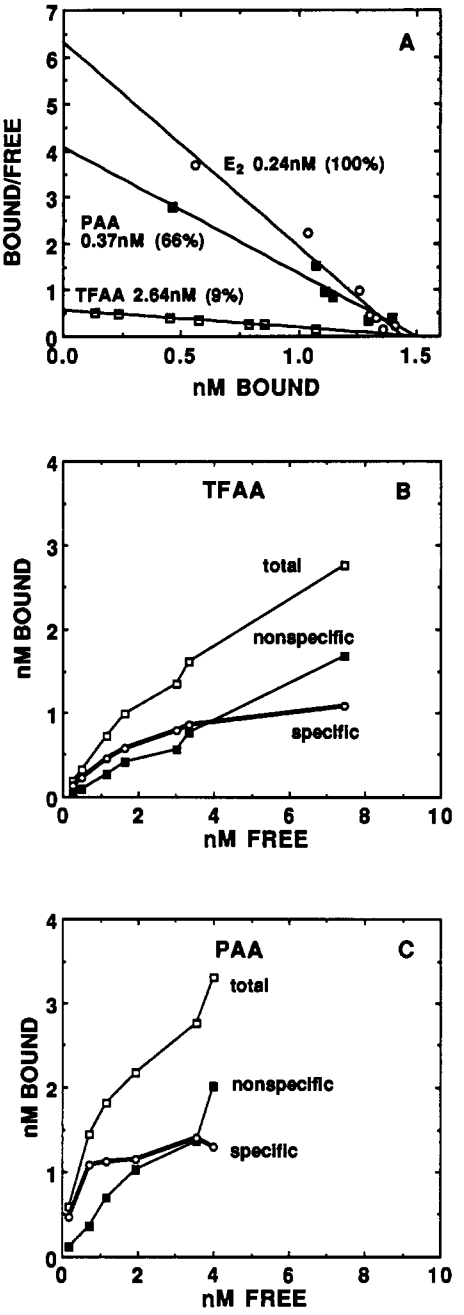


FIGURE 1: Direct binding curves for [^{3}H]TFAA and [^{3}H]PAA. Rat uterine cytosol was incubated at 0 °C for 4 h with various concentrations of tritium-labeled ligand in the absence or presence of a 100-fold excess of unlabeled estradiol. Aliquots of the incubation solution were counted to determine the concentration of total tritium-labeled ligand present. The incubation solutions were then treated with charcoal–dextran and the concentration of the bound tritium-labeled ligand was determined. Data was processed according to the method of Scatchard (1949).

cytosol is greater, and thus the concentration of free ligand is appreciably lowered (Katzenellenbogen et al., 1982).

(B) Direct Binding Assay of [^{3}H]TFAA and [^{3}H]PAA. The binding affinity of [^{3}H]TFAA and [^{3}H]PAA to ER was determined in rat uterine cytosol by using a direct charcoal adsorption assay. The binding plots for [^{3}H]TFAA (Figure 1, panel B), and [^{3}H]PAA (Figure 1, panel C) show moderate levels of nonspecific binding. A direct comparison of the specific binding curves for the aryl azides and for [^{3}H]E_2 is presented as a Scatchard plot (Scatchard, 1949) in Figure 1, panel A, and indicates that [^{3}H]TFAA has an affinity (K_d = 2.64 nM) for ER that is 9% that of [^{3}H]E_2 and that [^{3}H]PAA

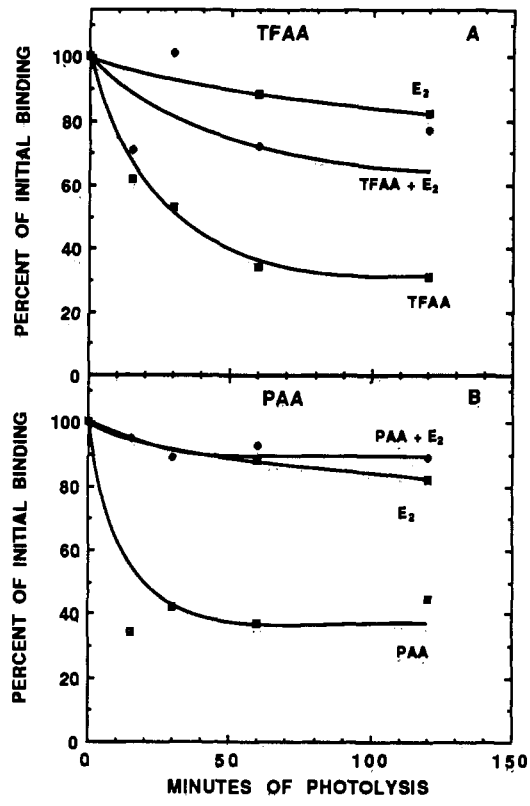


FIGURE 2: Photoinactivation of ER in rat uterine cytosol by TFAA (panel A) and PAA (panel B). Cytosol was incubated with TFAA or PAA in the absence or presence of a 100-fold excess of estradiol for 1 h at 0 °C and then photolyzed at >315 nm for various times. Following charcoal–dextran treatment to remove free ligand, the cytosol was exchanged at room temperature for 20 h against [^{3}H]-estradiol. Photoinactivation is seen as a loss in exchangeable sites and plotted as a percentage of the initial binding before photolysis.

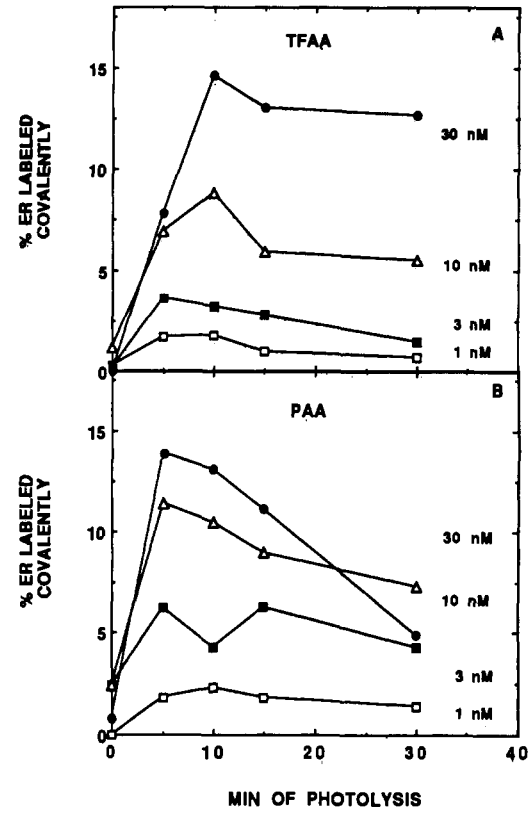


FIGURE 3: Time course of specific photoattachment of [^{3}H]TFAA (panel A) and [^{3}H]PAA (panel B) to the estrogen receptor. Rat uterine cytosol was incubated for 1 h in the dark at 0–4 °C with [^{3}H]TFAA or [^{3}H]PAA (1, 3, 10, or 30 nM) with or without a 100-fold excess of unlabeled estradiol and then photolyzed at >315 nm. Direct attachment was measured by the ethanol disc assay (see Experimental Procedures).

has an affinity (K_d = 0.37 nM) 66% that of [^{3}H]E_2. These values closely compare to those obtained for unlabeled TFAA (9.3% of E_2) and PAA (66% of E_2) through a competitive binding assay (Table I).

Photoreactive Properties of TFAA and PAA. (A) Photoinactivation of Estrogen Receptor by TFAA and PAA. A photoinactivation assay (Katzenellenbogen et al., 1974) is an indirect method by which the photoreactivity, and hence the potential photocovalent attachment reactivity, of a nonradiolabeled photoexcitable ligand can be established. Receptor–ligand complexes are irradiated and the time course of loss of reversible binding capacity is assayed by an exchange process. Suitable controls are included to monitor receptor photostability and binding-site dependence of inactivation (competition by a photoinert ligand, estradiol). The photoinactivation of ER in rat uterine cytosol at >315 nm was determined with TFAA and PAA. The results are listed in Table I, and the actual time courses of ER photoinactivation are shown in Figure 2.

Specific photoinactivation is measured as the difference between the total inactivation and the nonspecific component of inactivation (that seen when ER has been blocked by estradiol). The photoinactivation time courses were carried out for 2 h in each case; however, the specific inactivations were essentially complete at 30 min, and the values at this time were used for comparison of the relative effectiveness of each compound (Table I). Under this photolysis protocol, TFAA shows photoinactivation efficiency of 43%, while PAA demonstrates a somewhat higher photoinactivation efficiency of 55%.

(B) Efficiency and Selectivity of Covalent Labeling of Estrogen Receptor by [^{3}H]TFAA and [^{3}H]PAA. Photolysis of both [^{3}H]TFAA and [^{3}H]PAA at >315 nm in rat uterine cytosol preparations covalently labels a species that appears to be ER. The covalent attachment can be followed conveniently by a filter disc–solvent extraction assay (Katzenellenbogen et al., 1975). The estrogen specificity of the covalent photolabeling can be determined by a simultaneous measurement of the nonspecific photolabeling done in the presence of an excess of unlabeled estradiol; the difference between the labeling in the absence and presence of estradiol is considered to be estrogen-specific labeling.

Time courses showing the specific photoattachment for both [^{3}H]TFAA and [^{3}H]PAA (photolyzed without DMF and charcoal treatment, see below) are shown in Figure 3 (panel A, TFAA; panel B, PAA). The photoattachment proceeds in a time- and dose-dependent fashion. The specific attachment rises with time of photolysis until ~10 min and then appears to drop somewhat. This drop does not represent a loss of the total covalent labeling but results from the fact that the nonspecific labeling continues to rise even after the total labeling is essentially complete. This is especially evident at the higher concentrations where the level of nonspecific labeling is a large fraction of the total labeling.

With both compounds, 30 nM fully saturates the ER sites. The percent of ER that is photolabeled by each compound is less than predicted from the photoinactivation: TFAA, 15% attachment vs 43% inactivation; PAA, 14% attachment vs 55% inactivation. This is not unusual, since many processes other than covalent attachment can contribute to the loss of exchangeable sites in the indirect inactivation assay (Cridland

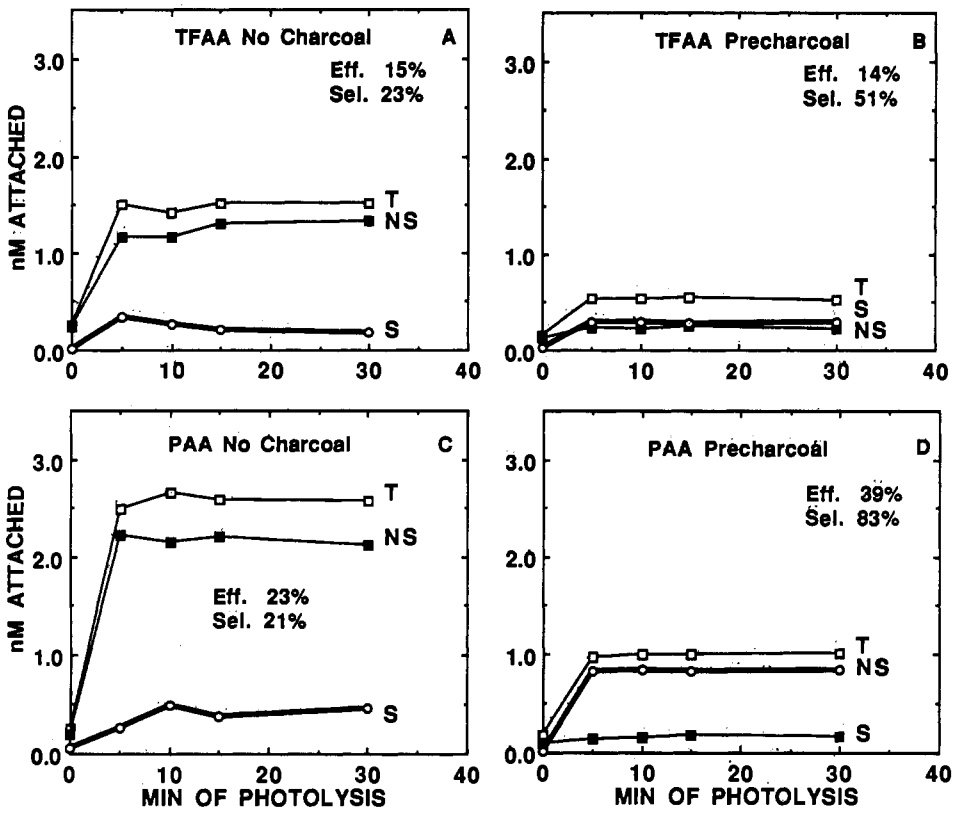


FIGURE 4: Time course of photoattachment of [^{3}H]TFAA (panel A, without charcoal pretreatment; panel B, with charcoal pretreatment) and [^{3}H]PAA (panel C, without charcoal pretreatment; panel D, with charcoal pretreatment). Rat uterine cytosol was incubated for 1 h at 0–4 °C in the dark with [^{3}H]TFAA or [^{3}H]PAA with or without a 100-fold excess of unlabeled estradiol in the presence of 5% DMF. A portion was treated with charcoal–dextran to remove free PAL reagent, and then all samples were photolyzed at >315 nm. Direct attachment was measured by the ethanol disc assay (see Experimental Procedures). T = total attachment; NS = nonspecific attachment; S = specific attachment. Efficiency represents the attachment as a percentage of the reversible binding. Selectivity is specific attachment as a percentage of total attachment.

Table II: Photocovalent Labeling of the Estrogen Receptor with [^{3}H]TFAA and [^{3}H]PAA under Various Conditions

protocol	A		B		C		D	
wavelength, temp	>315 nm, 4 °C		>315 nm, 4 °C		>315 nm, −196 °C		254 nm, 4 °C	
DMF	−	−	+	+	+	+	+	+
charcoal	−	+	−	+	−	+	−	+
TFAA (**1**) (15 nM)[a]								
% ER occupied[b]	42 ± 11	42 ± 11	72 ± 9	72 ± 9	62	62	77	77
% ER labeled[c]	8 ± 1		18 ± 4	14 ± 1	21	14	11	13
efficiency[d]	20 ● 5		31 ± 15[e]	22 ± 5[e]	33	23	14	17
selectivity[f]	23 ± 3		23 ± 1	51 ± 7	31	46	18	57
PAA (**2**) (15 nM)[a]								
% ER occupied[b]	52 ± 14	52 ± 14	79 ± 11	79 ± 11	78 ± 3	78 ± 3	66	66
% ER labeled[c]	12 ± 3	34 ± 8	21 ± 1	37 ± 2	12 ± 3	21 ± 4	21	41
efficiency[d]	23 ± 11	50 ± 4[e,g]	24 ± 1	48 ± 6[g]	16 ± 4	28 ± 8[e,g]	31	62
selectivity[f]	15 ± 7	59 ± 10	21 ± 4	83 ± 2	28 ± 9	79 ± 5	18	87

[a] Where error limits are shown, these are standard deviations (n = 3), except where otherwise indicated (footnote *e*). [b] % ER occupied is the percentage of total ER sites that are reversibly bound before photolysis by the concentration of azide used in these experiments (15 nM). [c] %ER labeled is the percentage of total ER that becomes covalently labeled by the concentration of azide used in these experiments (15 nM). [d] Photocovalent attachment efficiency is defined as the sites that become covalently labeled as a percentage of sites that are occupied. This number is determined by dividing % ER labeled by % ER occupied. [e] Error limits are the range (n = 2). [f] Photocovalent attachment selectivity is defined as the ER sites that are labeled as a percentage of the total proteins labeled. [g] These values may be overestimated (see text).

et al., 1990; Katzenellenbogen et al., 1974), whereas the direct assay measures only covalent attachment (Katzenellenbogen & Katzenellenbogen, 1984; Katzenellenbogen et al., 1975).

We define photoattachment *efficiency* as the amount of ER that becomes labeled covalently after photolysis as a percent of ER occupied by the PAL reagent reversibly, and we define photoattachment *selectivity* as the amount of ER labeled as a percent of the total protein covalently labeled upon photolysis. We find that labeling selectivity is markedly improved by the addition of a small amount of the organic solvent dimethylformamide (DMF) and by the adsorption of excess free PAL reagent by charcoal prior to photolysis. The photoattachment experiments shown in Figure 4 were done in the presence of 5% DMF, both with and without charcoal treatment before photolysis. The results of these and additional experiments are summarized in Table II. Low concentrations of DMF markedly reduce the extent of nonspecific binding of lipophilic ligands (Katzenellenbogen et al., 1982), increasing the fraction of the added ligand that is available for binding to ER (Table II, % of ER occupied, compare protocols A and B). Thus, the amount of ER that becomes labeled increases (Table II, % ER labeled) and the labeling selectivity improves (Table II, selectivity). As may be expected, the efficiency of the covalent attachment remains essentially the same with or without DMF,

within the error of the determinations (Table II, efficiency).

Beyond the effect of DMF, we can improve the selectivity of the covalent labeling further by removing excess labeling agent by charcoal adsorption before photolysis. When the photolysis is done with a sample containing both bound and free ligand (i.e., no charcoal pretreatment), the selectivity is much less [Figure 4, panel A (TFAA = 23% selective), panel C (PAA = 21% selective)] than when the free ligand is removed by charcoal treatment before photolysis [Figure 4, panel B (TFAA = 51% selective), panel D (PAA = 83% selective)]. (See also Table II, protocols A and B, selectivity, compare − and + charcoal columns.) While there is no significant change in the efficiency of TFAA labeling upon charcoal treatment (cf. Figure 4, panel A vs panel B; Table II, protocol B, − vs + charcoal), there is a statistically significant increase in labeling efficiency observed with PAA after charcoal treatment (cf. Figure 4, panel C vs panel D; Table II, protocols A and B, − vs + charcoal). Possible reasons for this apparent increase in labeling efficiency need to be considered carefully.

Charcoal treatment before photolysis greatly reduces the level of nonspecific labeling and facilitates the estimation of the specific covalent labeling of ER by PAA. This charcoal pretreatment results in an increase in estimated labeling efficiency. However, as it is unlikely that the efficiency increases simply as a result of the removal of the nonspecifically bound PAA, we believe that the specific labeling seen in the charcoal-pretreated experiment is being overestimated: charcoal pretreatment leaves more radioactivity in the total sample (T) than in the nonspecific sample (NS), the difference being specific binding. While the specific component of the radioactivity in the total sample is initially reversibly bound to ER, upon photolysis, it may redistribute so that it becomes covalently attached to both ER (true specific labeling) and to some other nonreceptor proteins (nonspecific labeling). However, from the design of the experiment, the latter component will still be registered as specific labeling to ER, because the NS sample, which initially has less radioactivity, becomes an inadequate control for redistribution processes that may occur during photolysis. Therefore, we have used the more conservative values obtained without charcoal pretreatment as the true estimates of labeling efficiency.

No attachment was detected when the incubations were kept in the dark nor when the compounds were prephotolyzed for 15 min to destroy the chromophore and then added to the cytosol, allowed to bind, and photolyzed as usual. This indicates that the specific attachment normally seen is a chromophore-dependent, photoactivated process. It does not occur in the dark, and it does not occur when the azides have been prephotolyzed. The Lilly antiestrogen, [^{3}H]LY139481 (an analog of LY117018), which does not have the photoreactive azide of PAA, also does not undergo photoattachment to ER, even though it shows substantial photoinactivation (cf. Table I).

Since Platz found higher yields of insertion products at −196 °C than at 25 °C in simple polyfluorinated aryl azide systems (Soundararajan & Platz, 1990; Leyva et al., 1986, 1989; Young & Platz, 1989), we performed photolyses with [^{3}H]-PAA and [^{3}H]TFAA at −196 °C. The ER in cytosol is stable at −196 °C and is routinely stored at this temperature. However, in our system, we found no difference in attachment efficiency whether the photolyses were done at 2–4 °C or −196 °C (Table II, protocol B vs protocol C).

The azides have UV absorbance in the 300–320-nm range, suggesting that >315 nm would be the optimal wavelength for photolysis; however, we also performed photocovalent attachment at 254 nm (Table II, protocol D). Although maximal attachment occurred sooner (2–5 min at 254 nm vs 8–10 min at >315 nm), both the efficiency and selectivity of attachment were similar to those obtained when the photolyses were carried out at >315 nm (Table II, protocol B). The estrogen receptor was 80% stable over the time course of the 254-nm irradiation.

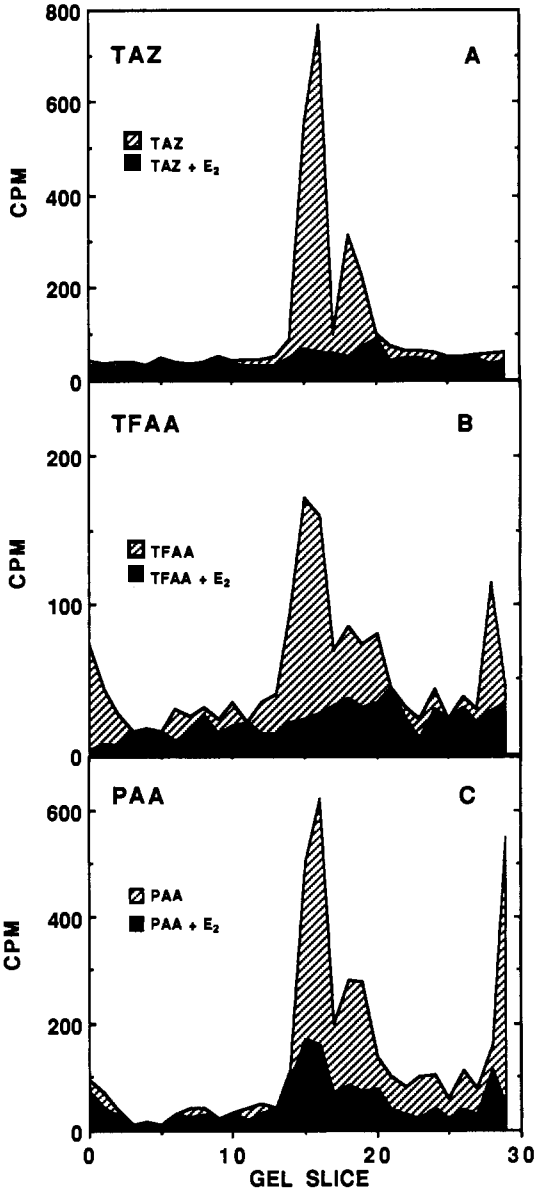


FIGURE 5: SDS–polyacrylamide gel electrophoresis of photoattached [^{3}H]TFAA (panel B), [^{3}H]PAA (panel C), and [^{3}H]tamoxifen aziridine (panel A).

Characterization of the Estrogen Receptor Covalently Labeled with the [^{3}H]Aryl Azides. ER covalently labeled with [^{3}H]TFAA or [^{3}H]PAA in the absence and presence of an excess of unlabeled E_2 was analyzed by SDS–polyacrylamide gel electrophoresis [Figure 5: TFAA (panel B); PAA (panel C)]. Both the aryl azides show two estradiol-competable peaks, a major one at a M_r of ~60 000 and a second peak at ~46 000. These correspond to the peaks observed with ER labeled with [^{3}H]tamoxifen aziridine (Figure 5, panel A), a well-characterized electrophilic affinity labeling agent for ER (Katzenellenbogen et al., 1983; Harlow et al., 1989). The major peak represents intact ER, while the second peak probably represents a proteolysis fragment. The attachment to the intact receptor as well as to the fragment is estradiol

competable. Formation of this fragment can be prevented in the cytosol from a human breast cancer cell line (MCF-7) by adding protease inhibitors, but these inhibitors are ineffective in the cytosol from rat uterus (Katzenellenbogen et al., 1987a). The labeling observed at the bottom of the gels in Figure 5, panels B and C, which runs with the tracking dye, is due to free photolysis product; the activity at the top of the gel in Figure 5B is presumed to be due to labeled protein aggregates that are difficult to disperse after photolysis.

When free ligand is not removed by charcoal treatment prior to photolysis, both aryl azides show, in addition to the two estradiol-competable peaks, a moderate level of nonspecific attachment to a protein with a M_r of ~37 000 as well as to a protein that corresponds in size to rat serum albumin (M_r 63 000–65 000; data not shown). Partial purification of the receptor, by precipitation with 40% saturated ammonium sulfate (1.7× purification) (Carlson et al., 1977), removes most of the nonspecifically labeled proteins, including the labeled rat serum albumin, but the M_r 37 000 protein copurifies with receptor (data not shown). The binding to this protein is not competable by estradiol and is evidently low affinity since most of the labeling can be avoided when excess PAL reagent is removed by charcoal treatment before photolysis.

The profiles in Figure 5 show that the aryl azides selectively label ER even in unpurified cytosol. However, in order to verify that the protein to which the aryl azides are attached is ER, the covalently labeled protein was treated with the ER-specific antibodies H222Spγ and D547Spγ (Green, 1984). The antibody-reactive proteins were precipitated, and both the precipitate and supernatant were analyzed by SDS gel electrophoresis (data not shown). Only the [^{3}H]TFAA, [^{3}H]PAA, or [^{3}H]tamoxifen aziridine labeled estradiol-competable peaks at ~60 000 and ~46 000 MW were precipitated by the antibodies.

Characterization of the Hormonal Character of PAA. The PAL agents PAA and TFAA are based structurally on the Lilly antiestrogen LY117018. The basic ether side chain, characteristic of most nonsteroidal antiestrogens, however, is absent in these compounds. We were, therefore, curious whether they were hormonally estrogen agonists or antagonists.

We have evaluated the activity of the more effective PAL agent, PAA, in the human breast cancer cells (MCF-7) in terms of its effect on growth rate and the induction of progesterone receptor, two responses that are effectively stimulated by estrogens and minimally, if at all, by antiestrogens (Read et al., 1989; Katzenellenbogen et al., 1987b). As can be seen in Figure 6, PAA is a complete estrogen agonist, stimulating these two responses as fully as estradiol. Its potency, however, is only about 0.1–0.3% that of estradiol. In addition, the stimulation of cell proliferation or progesterone receptor content by PAA is fully prevented by the antiestrogen ICI 164,384 (data not shown), as is the stimulation by E_2 (Wakeling & Bowler, 1987). It is not clear why its potency with respect to these two responses is less than its ER binding affinity relative to that of estradiol (66%, cf. Table I). It does not appear to be metabolized appreciably under the cell culture conditions used in these studies (data not shown), but its uptake by cells might be less efficient than that of estradiol.

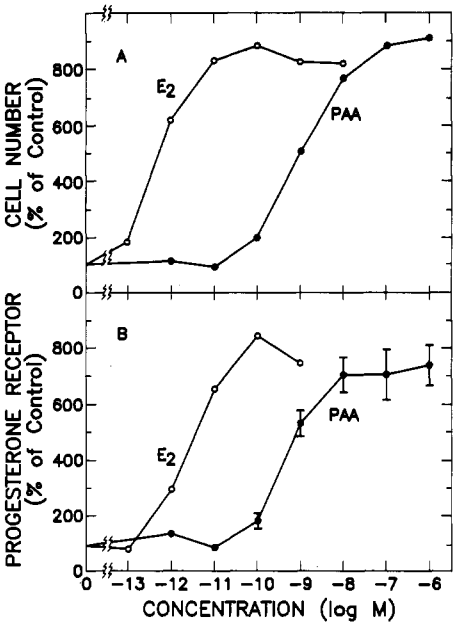


FIGURE 6: Characterization of the biological activity of PAA in MCF-7 cells. Panel A: Proliferation of MCF-7 cells treated with PAA or E_2. MCF-7 cells, grown in T-25 flasks, were treated with control vehicle (0.1% ethanol) or the indicated concentration of PAA or E_2. The number of cells per flask was determined after 7 days of growth. Data are expressed as a percentage of the control cell number and represent the mean from two experiments with three flasks of cells per treatment concentration. Panel B: Progesterone receptor levels in MCF-7 cells treated with PAA or E_2. Progesterone receptor levels were determined after 4 days of growth in the presence of the indicated concentration of PAA or E_2. Media contained 10% charcoal–dextran-treated calf serum. Fresh medium and compound were added daily during the 4-day period. The cells were then assayed for progesterone receptor, as described under Experimental Procedures. Each PAA value represents the mean ± SEM from three separate experiments; each E_2 value represents the mean of two experiments. In each experiment, duplicate T-75 flasks of cells were used for each PAA or E_2 concentration. The control (100%) value was 11 ± 2 fmol/10^6 cells.

Discussion

The search to develop an efficient photoaffinity labeling (PAL) reagent for the estrogen receptor (ER) has generated considerable interest over the past two decades, (Katzenellenbogen & Katzenellenbogen, 1984, 1988; Katzenellenbogen et al., 1973a,b, 1974, 1977a,b, 1980; Katzenellenbogen, 1977; Marquet et al., 1989; Cridland et al., 1990), yet, until this study, no truly useful photoreactive molecular probes for ER had been produced. Continuing efforts to develop simple photoreactive reagents that undergo efficient C–H insertion have recently resulted in the preparation of simple polyfluoroaryl azides that demonstrate high yields of insertion chemistry in nonnucleophilic solvents upon irradiation (Soundararajan & Platz, 1990; Leyva et al., 1986, 1989; Young & Platz, 1989; Keana & Cai, 1989, 1990; Cai & Keana, 1989). We were, therefore, encouraged to incorporate a polyfluoroaryl azide functionality within the molecular skeleton of a ligand known to demonstrate high binding affinity for ER in the hope of obtaining a new class of efficient and selective PAL reagents for ER. As a molecular skeleton, we choose the 3-aroyl-2-arylbenzo[*b*]thiophene system, which has demonstrated high binding affinity in the antiestrogens LY117018 and LY139481 prepared by Jones and co-workers (Eli Lilly and Co.) (Jones et al., 1984). We also felt that this system would enable us to compare the utility of tetrafluorosubstituted aryl azides versus traditional protioaryl azides as PAL reagents for a complex biological target such as ER.

Accordingly, we prepared tetrafluoroaryl azide (TFAA, **1**), and its protio analogue (PAA, **2**) by total synthesis (Pinney & Katzenellenbogen, 1991) and subjected them in unlabeled form for preliminary biochemical evaluation, which included determination of their relative binding affinity (RBA) for the ER and their photoinactivation efficiency. To further evaluate the utility of TFAA and PAA as PAL reagents for the ER,

we prepared them in high specific activity radiolabeled form and then determined the efficiency and selectivity of their photocovalent attachment to the ER and characterized the photolyzed ligand–receptor adducts both by SDS–polyacrylamide gel electrophoretic analysis and by precipitation by the ER specific antibodies H222Spγ and D547Spγ.

Since PAA is structurally based on the antiestrogens LY117018 and LY139481, we were interested in determining its bioactivity. In two assays commonly used to evaluate estrogen agonist and antagonist activity (growth stimulation and progesterone receptor induction in MCF-7 cells), PAA proved to be a full estrogen agonist, although its potency was less than 1% that of estradiol. It appears that in this series of antiestrogens the basic side chain plays an important role in conveying antagonist activity, as is also the case with triphenylethylene antiestrogens such as tamoxifen (Robertson et al., 1982).

PAA demonstrates high binding affinity (66% versus estradiol = 100%) for the ER, while TFAA has a somewhat diminished binding affinity (9.3%) for the ER (Table I). It is not clear whether the effect of the four aromatic fluorine atoms is a steric or an electronic influence directly on their complexes with ER or whether the effect is on the solution conformation of the TFAA ligand, which renders it less capable of binding to ER than PAA.

TFAA and PAA, both in unlabeled form, show excellent specific photoinactivation efficiency of the ER (**1** = 43%, **2** = 55%, at 30 min) (Figure 2), and in tritium-labeled form, both [^{3}H]TFAA and [^{3}H]PAA demonstrate good efficiency of photocovalent attachment to ER (Figure 4), [^{3}H]TFAA labeling 20–30% and [^{3}H]PAA ca. 25% of the occupied ER sites. The selectivity of labeling of ER in cytosol preparations was also good, being around 20% when excess azide was not removed prior to photolysis and rising to 50–80% when excess ligand was removed by charcoal adsorption.

The covalent attachment efficiency of PAA is comparable to that of TFAA. That is not what is predicted on the basis of the efficiency of solvent insertion reactions of simple protioaryl azides vs polyfluoroaryl azides (Soundararajan & Platz, 1990; Leyva et al., 1986, 1989; Young & Platz, 1989; Keana & Cai, 1989, 1990; Cai & Keana, 1989). In the latter cases, very little insertion occurred with the protioaryl azides when photolysis was conducted at room temperature, while moderate insertion yields were obtained with the fluorine-substituted analogues (Soundararajan & Platz, 1990; Leyva et al., 1986, 1989; Young & Platz, 1989; Keana & Cai, 1989, 1990; Cai & Keana, 1989). At −196 °C, insertion yields are greater, but again the fluorine-substituted system shows higher yields (Soundararajan & Platz, 1990; Leyva et al., 1986, 1989; Young & Platz, 1989). Although the photoattachment process in a complex receptor–ligand system may involve different reactions than those that have been studied in simple solvent insertion systems, it is interesting to compare the two. In our system, PAA has an attachment efficiency comparable to or slightly higher than that of TFAA at 4 °C. Only at −196 °C does TFAA appear to be more efficient than PAA, but it is not clear whether this is statistically significant.

Since the nature of the covalent linkage between PAA and TFAA and ER is not known, one needs to be cautious in advancing reasons for the comparable attachment efficiencies of fluorine-substituted vs nonsubstituted aryl azides observed in this study. What is of note is that PAA does label ER with good efficiency, while simple protioaryl azides, in general, show poor efficiency of solvent insertion reactions. The fact that PAA binds reversibly to ER with a 7-fold higher affinity than TFAA indicates that the nature of its complexes with ER may be somewhat different. Perhaps in the two complexes there are minor alterations in the orientation of the nitrene photoproduct with respect to the reactive functions in ER and these orientational factors, rather than differences in inherent nitrene reactivity, could account for the high yields of covalent attachment of PAA.

It is instructive to compare PAA, the more effective ER photoaffinity labeling agent presented in this study, with 3-azidohexestrol, an ER PAL reagent that we prepared some time ago (Katzenellenbogen et al., 1973a,b, 1974, 1977a,b) and with tamoxifen aziridine, an electrophilic affinity label for ER that we have described more recently [Harlow et al., 1989; Katzenellenbogen et al., 1983; for a description of other electrophilic affinity labeling agents for ER, similar in properties to tamoxifen aziridine, see Salituro et al. (1986) and Simpson et al. (1987)]. Of the three, tamoxifen aziridine is the most efficient (90–100%) and most selective (80–95%) in covalent labeling of ER, and 3-azidohexestrol (15–20% efficient, <30% selective) is the least.

Being an electrophilic agent, however, tamoxifen aziridine (and the other aziridine-based ER affinity labeling agents) is selective for the most nucleophilic residues in ER. Thus, in recent studies we have found that tamoxifen aziridine and ketononestrol aziridine, another aziridine-based electrophilic affinity labeling agent, react predominantly or exclusively with a cysteine residue in ER (Cys-530) (Harlow et al., 1989). Thus, the identification of ligand contact residues in ER using the aziridines is biased toward those accessible residues that are most nucleophilic. It is possible that the reactive intermediate generated by photolysis of the phenyl azides will have a higher level of reactivity so that PAA will react with a wider and different range of accessible residues in ER than do the aziridine reagents. Studies to investigate the site or sites in ER that are covalently labeled by [^{3}H]PAA are currently underway.

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Registry No. **1** (R = F), 131589-59-8; **1** (X = F; Y = N_3), 131589-63-4; **2** (R = H), 131589-60-1; **2** (X = H; Y = N_3), 131589-66-7; **3**, 131589-62-3; **4**, 131589-65-6; **5**, 131589-61-2; **6**, 131589-64-5; **7**, 131589-67-8; triethylamine, 121-44-8; progesterone, 57-83-0.

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