[^{125}I]IODODESETHYL TAMOXIFEN AZIRIDINE: SYNTHESIS AND COVALENT LABELING OF THE ESTROGEN RECEPTOR WITH AN IODINE-LABELED AFFINITY LABEL

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ABSTRACT

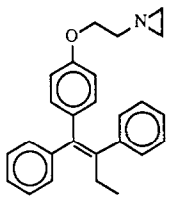
Iododesethyl tamoxifen aziridine (I-Tam-Az), an analog of the estrogen receptor-affinity label tamoxifen aziridine (Tam-Az) in which the ethyl group has been replaced by an iodine, has been prepared by two routes: (a) metallation of a bromotriarylethylene system, followed by reaction with iodine, and aziridinylation, and (b) direct iodination of a trimethylstannyl triarylethylene system that is the immediate precursor of I-Tam-Az. The latter method can be used to prepare [^{125}I]I-Tam-Az rapidly and in good yield, both at carrier-added and no-carrier-added levels; specific activities greater than 200 Ci/mmol have been obtained. In competitive radiometric binding assays with the estrogen receptor, I-Tam-Az has an apparent affinity of ca. 20%, equivalent to that of Tam-Az. It also undergoes rapid and selective time-dependent, irreversible binding to the estrogen receptor. [^{125}I]I-Tam-Az reacts covalently with estrogen receptor in uterine cytosol preparations; its attachment is rapid and efficient, but somewhat less selective than that of Tam-Az. Estrogen receptor in intact MCF-7 human breast cancer cells can also be labeled with [^{125}I]I-Tam-Az, and autoradiographic analysis of salt extracts of labeled nuclear estrogen receptor on SDS-polyacrylamide slab gels shows highly selective labeling of a 65K protein. [^{125}I]I-Tam-Az is an efficient, selective affinity label for the estrogen receptor, available at high specific activity, and should be useful in studies on estrogen receptor structure, dynamics, and chromatin interactions.

INTRODUCTION

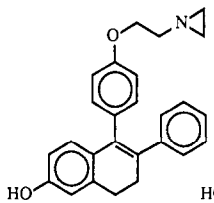
Affinity labeling agents for steroid receptors have proven to be useful in defining the molecular characteristics of receptors, in investigating their domain structure, and in studying their intracellular dynamics (1,2). We have developed a series of affinity labeling agents for the estrogen receptor based on non-steroidal ligands bearing an aziridine function on an appended side chain (3-8): Two of these agents, tamoxifen aziridine^{+} (Tam-Az) and desmethyl nafoxidine aziridine (Naf-Az), are derived from estrogen antagonists, and one, ketononestrol aziridine (KN-Az), is derived from the non-steroidal estrogen agonist hexestrol.

While all of these three agents are capable of covalent labeling of the estrogen receptor, efficiently and selectively, their sensitivity is limited by the specific activity of tritium, the radionuclide with which they are labeled; the specific activity of these agents has ranged from 20 to 60 Ci/mmol, depending, largely, on the number of tritium atoms that are incorporated during their synthesis. Since certain studies in which steroid receptor affinity labels might be useful involve very small quantities of receptor, it would be desirable to have labeling agents with higher specific activities, such as could be achieved with iodine-125 labeling. Indeed, an I-labeled photoaffinity labeling agent for the progesterone receptor has recently been described (9).

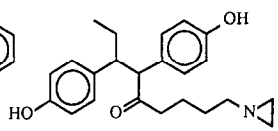
In this paper, we describe the synthesis and characterization of an iodine-labeled affinity label for the estrogen receptor. This agent, iododesethyl tamoxifen aziridine (I-Tam-Az), can be prepared in very high specific activity iodine-125-labeled form, and it labels estrogen receptor covalently with good efficiency and selectivity.



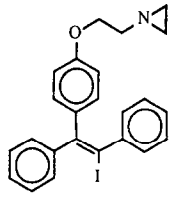
Tam-Az



Naf-Az



KN-Az



I-Tam-Az

EXPERIMENTAL

Chemical Synthesis

General. Analytical thin-layer chromatography was performed using 0.25 mm silica gel glass- or plastic-backed plates. Reverse phase thin-layer chromatography was performed using 0.25 mm thickness Merck RP-18$F_{254}S$ glass-backed plates. Visualization was by ultraviolet light, iodine, or ceric sulfate/H_2SO_4. All column chromatography was done using the flash technique as reported by Still and co-workers (10). Column packing was Woelm 32-63 μm silica gel.

Proton magnetic resonance spectra were recorded on Varian Associates spectrometers, Model EM-390 and XL-200; chemical shifts are reported in ppm relative to a tetramethylsilane internal standard (δ scale). Low resolution electron impact mass spectra were obtained on a Varian Associates MAT CH-5 spectrometer at 10 or 70 eV. Low resolution field desorption (FD) mass spectra were obtained on a Varian Associates MAT 731 spectrometer. Data are reported in the form: m/z (intensity relative to base peak = 100). High resolution electron impact mass spectrometry (HRMS) for exact mass determination was performed on a Varian Associates MAT 731 spectrometer. Melting points were determined with a Thomas-Hoover capillary melting point apparatus and are uncorrected. Elemental analyses were performed by the Microanalytical Service Laboratory of the University of Illinois School of Chemical Sciences.

Chemicals were obtained from the following sources: 2-(4-bromophenoxy)ethanol, t-butyldimethylsilyl chloride, t-butyllithium in pentane, deoxybenzoin, methanesulfonyl chloride, tetrabutylammonium fluoride, trimethylsilyl chloride, trimethylstannyl chloride from Aldrich Chemical Co. (Milwaukee, WI); mercuric trifluoroacetate from Alfa Products (Danvers, MA);

N-iodosuccinimide (NIS) from Parish (Provo, UT); Sodium [^{125}I]-iodide (in 0.1 N NaOH) from New England Nuclear (Boston, MA).

Tetrahydrofuran (THF) was distilled from sodium-benzophenone prior to use. Acetonitrile, benzene, dichloromethane, diisopropylethylamine, dimethylformamide, triethylamine, and trimethylsilyl chloride were refluxed over calcium hydride and then distilled.

A standard procedure for product isolation was utilized; it involved quenching of the reaction mixture in an aqueous solution, followed by extraction of product into an organic solvent and drying the extract over an anhydrous salt, filtration, and evaporation under reduced pressure. Such a procedure is indicated by the phrase "product isolation" followed by a parenthetical listing of the aqueous quench solution, extraction solvent, and drying agent.

Cautionary Note - Ethyleneimine, used in the preparation of I-Tam-Az and compound 6 is a volatile, reactive, carcinogenic, and acutely toxic substance (11). All manipulations involved in its preparation, purification, and use should be performed in a well-ventilated fume hood, providing protection against explosion. A full-face gas mask with a fresh ammonia (silica gel) canister is recommended in the event of inadequate ventilation. The ammonia-like odor threshold is reported as 2 ppm, but a threshold limit of 0.5 ppm in air for continuous exposure has been set. Dry ethyleneimine may be stored in the freezer, either neat (over potassium hydroxide pellets) or as a solution in an inert solvent (e.g., toluene) for several months without decomposition.

Trimethylsilyl 2-(4-Bromophenoxy)ethyl Ether (1b). 2-(4-Bromophenoxy)ethanol (1a, 9.7 g, 44.7 mmol) was dissolved in dry benzene (20 mL) and cooled to 0°C. To this solution, diisopropylethylamine (7.8 mL, 44.7 mmol) and trimethylsilyl chloride (5.67 mL, 44.7 mmol) were added. A white precipitate resulted. After 2 h the precipitate was filtered and washed with 10 mL of benzene. The organic layer was evaporated in vacuo, and the resulting liquid was vacuum-distilled. Product was collected from the fraction boiling at 119-121°C/1 mm Hg (11.5 g, 89%): NMR δ 0.1 ppm (s, 9H), 3.6 (br s, 4H), 6.3-6.9 (dd, AB pattern aromatic, 4H).

1,2-Diphenyl-1-[4-(2-hydroxyethoxy)phenyl]-ethylene (3a). A 250-mL, 3-neck round-bottom flask was fitted with a stirring bar, dropping funnel, reflux condenser, and nitrogen inlet. Magnesium turnings (672 mg, 27.6 mmol) were added to the flask, and the system was flushed with N_2 for 5 min, then flame-dried. After cooling, dry tetrahydrofuran (5 mL) was added, followed by methyl

iodide (neat, 2 μL), and the mixture was warmed to 70°C in an oil bath. When the mixture took on a silver-gray color, trimethylsilyl 2-(4-bromophenoxy)ethyl ether (**1b**, 8 g, 27.6 mmol) in dry tetrahydrofuran (30 mL) was added dropwise so that the temperature remained at 70°C. This mixture was stirred at 70°C for 3 h, then about 2/3 of the tetrahydrofuran was evaporated under positive nitrogen pressure and dry benzene (20 mL) was added. The reaction was cooled to 0°C and deoxybenzoin (3 g, 15.3 mmol) in dry benzene (20 mL) was added dropwise over a period of 1/2 h. The reaction was stirred and allowed to warm slowly to room temperature over a 3-h period, after which 15% HCl (20 mL) was slowly added. The mixture was stirred for 1-1.5 h. Product isolation (EtOAc, brine, $MgSO_4$) furnished a brown oil. Volatile side products were removed by Kugelrohr vacuum distillation at 140°C/1 mm Hg for 1.5 h. The remaining residue was purified by flash chromatography (5 x 20 cm; 30% ethyl acetate in hexane). Product was isolated as a clear oil in 76% yield: NMR ($CDCl_3$) δ 2.03 (br m, 1H, exchangeable), 3.95 (m, 2H), 4.10 (m, 2H), 6.8-7.4 (m, 15H); m/e (rel. int.) 316 (20, M^+), 271 (3), 196 (100). Anal. Calcd for $C_{22}H_{20}O_2$: C, 83.52; H, 6.37. Found: C, 83.27; H, 6.46.

1-[4-(2-t-Butyldimethylsiloxyethoxy)phenyl]-1,2-diphenylethylene (3b). Compound **3a** (1.25 g, 4 mmol) was dissolved in dry, distilled dimethylformamide (10 mL). To this solution, imidazole (816 mg, 12 mmol) and t-butyldimethylsilyl chloride (1.8 g, 12 mmol) were added. After 5 min, water (25 mL) was added to the mixture and product isolation (EtOAc, $MgSO_4$) gave an oil that was purified by flash chromatography (4 x 15 cm; 20% ethyl acetate in hexane) to give a clear oil in 95% yield: NMR ($CDCl_3$) δ 0.1 (s, 6H), 0.9 (s, 9H), 3.95 (br s, 4H), 6.5-7.2 (m, 15H). MS m/e (rel. int.) 430 (82, M^+), 373 (100), 355 (39), 179 (100). Anal. Calcd for $C_{28}H_{34}O_2Si$: C, 78.10; H, 7.95. Found: C, 78.18; H, 7.94.

2-Bromo-1-[4-(2-t-butyldimethylsiloxyethoxy)phenyl]-1,2-diphenylethylene (4). Compound **3b** (400 mg, 0.96 mmol) was dissolved in dry dichloromethane (3 mL). To this solution, bromine (1 mmol) in 50 μL dichloromethane was added. After 10 min, product isolation (satd. $NaHCO_3$, EtOAc, $MgSO_4$) gave a yellow oil which was purified by flash chromatography (2 x 10 cm; 1% diethyl ether in hexane) to give product as a clear oil in 72% yield: NMR ($CDCl_3$) 0.05 (s, 6H), 0.85 (s, 9H), 3.66 (s, 2H), 3.80 (s, 2H), 6.20-7.10 (m, 14H). MS m/z (rel. int.) 508, 510 (4.4, 5.4, M^+, Br present), 451, 453 (34, 37, Br present). Anal. Calcd for $C_{28}H_{33}O_2SiBr$: C, 66.00; H, 6.53; Br, 15.68. Found: C, 65.86; H, 6.67; Br, 15.82.

4-(2-t-Butyldimethylsiloxyethoxy)phenyl-1,2-diphenyl-2-trimethylstannylethylene (5a). Compound 4 (250 mg, 0.49 mmol) was dissolved in freshly distilled tetrahydrofuran (2 mL) under a N_2 atmosphere and chilled to -78°C. To this solution, 1.6 N t-BuLi (865 µL, 1.5 mmol) in pentane was added dropwise over a 5-min period. The clear brownish solution was stirred for 15 min at -78°C before trimethylstannyl chloride (290 mg, 1.5 mmol) in dry tetrahydrofuran (2 mL) was added. The mixture was stirred for 15 min at -78°C. Product isolation (EtOAc, $MgSO_4$) gave an oil that was purified by flash chromatography (2 x 15 cm; 5% diethyl ether in hexane) to give a clear oil in 80% yield; NMR (acetone-d_6) -0.15 (d, 9H, $Sn(CH_3)_3$), 0.1 (d, 6H, $Si(CH_3)_2$), 0.90 (d, 9H, $SiC(CH_3)_3$), 3.65 (s, 2H), 3.75 (s, 2H), 6.2-6.9 (m, 14H). HRMS Calcd for $C_{31}H_{42}O_2SiSn$: m/e 592.1963 for ^{28}Si, ^{118}Sn. Found: 592.1981.

1,2-Diphenyl-1-[4-(2-hydroxyethoxy)phenyl]-2-trimethylstannylethylene (5b). Compound 5a (133 mg, 0.23 mmol) was dissolved in dry tetrahydrofuran (2 mL). To this solution, tetrabutylammonium fluoride trihydrate (159 mg, 0.5 mmol) was added. After 10 min, product isolation (EtOAc, $MgSO_4$) gave an oil that was purified by flash chromatography (2 x 15 cm; 30% ethyl acetate in hexane) to give a clear oil in 96% yield: NMR ($CDCl_3$) -0.20 (d, 9H, $Sn(CH_3)_3$), 2.15 (broad m, 1H), 3.70-4.10 (m, 4H), 6.30-7.2 (m, 14H). MS (rel. int.) 480 (3.0, M^+, ^{120}Sn), 478 (1.5, M^+, ^{118}Sn), 476 (1.4, M^+, ^{116}Sn), 465 (100, ^{120}Sn), 463 (77, ^{118}Sn), 461 (45, ^{116}Sn), 315 (16). Anal. Calcd for $C_{25}H_{28}O_2Sn$: C, 62.66; H, 5.89. Found: C, 62.28; H, 6.05.

1,2-Diphenyl-1-[4-(2-methanesulfonyloxyethoxyphenyl]-2-trimethylstannylethylene (5c). Compound 5b (102 mg, 0.22 mmol) was dissolved in dry dichloromethane (2 mL). To this solution, triethylamine (0.25 mmol) was added, followed by methanesulfonyl chloride (0.25 mmol). After 25 min of stirring at room temperature, product isolation (EtOAc, $MgSO_4$) gave an oil that was passed through a 1-in. disposable pipet column of silica gel, eluting with 40% ethyl acetate/hexane. The product, obtained as a clear oil (one spot on TLC) in about 98% yield, was used without further purification: NMR ($CDCl_3$) -0.20 (d, 9H, $Sn(CH_3)_3$), 2.95 (d, 3H, $O-SO_2CH_3$, cis-trans), 3.85-4.20 (m, 2H), 4.20-4.45 (m, 2H), 6.30-7.20 (m, 14H).

1-[4-(2-N-Aziridylethoxy)phenyl]-1,2-diphenyl-2-trimethylstannylethylene (6). Compound 5c (110 mg, 0.22 mmol) was dissolved in dry acetonitrile (500 µL). To this solution, triethylamine (600 µL) and ethyleneimine (See Cautionary Note above) (600 µL) were added. The reaction was stoppered and allowed to stir for 24 h at room temperature. After this time, the solvent was evaporated under a gentle stream of nitrogen. The resulting

material was purified by flash chromatography (2 x 15 cm; ethyl acetate/hexane/triethylamine (40/59/1)). Product was obtained in 81% yield (one spot on TLC) and used without further purification: NMR ($CDCl_3$) -0.12 (d, 9H), 1.18 (m, 2H), 1.85 (m, 2H), 2.60 (m, 2H), 4.12 (m, 2H), 6.5-7.4 (m, 14H). HRMS Calcd for $C_{27}H_{31}NOSn$: m/e 503.1417 for ^{118}Sn. Found: 503.1426.

1-[4-(2-t-Butyldimethylsiloxyethoxy)phenyl]-1,2-diphenyl-2-iodoethylene (7a).

Procedure A (By metallation and iodination of compound 4): Compound 4 (110 mg, 0.21 mmol) was dissolved in dry tetrahydrofuran (1 mL) under a nitrogen atmosphere and chilled to -78°C. A 1.6 N solution of t-BuLi (0.63 mmol) in pentane was then added, and the mixture was stirred for 15 min prior to addition of N-iodosuccinimide (142 mg, 0.63 mmol) in dry tetrahydrofuran (1 mL). After 20 min, product isolation (EtOAc, $MgSO_4$) gave an oil that was purified by flash chromatography (2 x 15 cm; 5% diethyl ether/hexane) to give a clear oil in 54% yield: NMR ($CDCl_3$) 0.1 (s, 6H), 0.87 (s, 9H), 3.78 (br s, 4H), 6.4-7.0 (m, 14H). MS (rel. int.) 556 (.73, M^+), 430 (100), 373 (100), 115 (15), 57 (20). HRMS Calcd for $C_{28}H_{33}O_2ISi$: m/e 556.1247. Found: 556.1301.

Procedure B (By the iododestannylation of compound 5a): Vinylstannane (5a) was dissolved in a minimum amount of dry dichloromethane. To this solution, I_2 (1 eq) in tetrahydrofuran was added. After 5 min the reaction was quenched with 1 mM aqueous solution of sodium metabisulfite. Purification was carried out as described above and yielded pure product in 75% yield. NMR identical to that previously reported.

1,2-Diphenyl-1-[4-(2-hydroxyethoxy)phenyl]-2-iodoethylene (7b). Compound 7a (80 mg, 0.144 mmol) was dissolved in tetrahydrofuran (2 mL), and tetrabutylammonium fluoride (99 mg, 0.31 mmol) was added. The reaction mixture was stirred for 3 min. Product isolation (EtOAc, $MgSO_4$) gave an oil that was purified by flash chromatography (1 x 10 cm; 40% ethyl acetate in hexane). The recovered oil was triturated in hexane and filtered to give a pale yellow powder in 85% yield: mp 96-98°C. NMR ($CDCl_3$) δ 2.05 (br m, 1H), 3.80-4.20 (m, 4H), 6.40-7.40 (m, 14H). HRMS Calcd for $C_{22}H_{19}O_2I$: m/e 442.0386. Found: 442.0436.

1,2-Diphenyl-2-iodo-1-[4-(2-methanesulfonyloxyethoxy)phenyl]-ethylene (7c). Compound 7b (60 mg, 0.135 mmol) was dissolved in dry dichloromethane (1 mL). To this solution, diisopropylethylamine (24 µL, 0.14 mmol) was added, followed by methanesulfonyl chloride (10 µL, 0.14 mmol). After 30 min stirring at room temperature, product isolation (EtOAc, $MgSO_4$)

yielded an oil (88%, one spot on TLC) which was used without further purification.

1-[4-(2-N-Aziridylethoxy)phenyl]-1,2-diphenyl-2-iodoethylene (I-Tam-Az). Vinyliodide **7c** (60 mg, 0.12 mmol) was dissolved in dry acetonitrile (500 µL). To this solution, triethylamine (500 µL) and ethyleneimine (See Cautionary Note above) (500 µL, neat) were added. The mixture was stoppered and stirred for 24 h, after which the solvent was evaporated under a gentle stream of nitrogen. The remaining residue was flash chromatographed on a 10-mm column eluting first with 150 mL of a mixture of ethyl acetate/hexane/triethylamine (40/59/1) to remove starting material, then with methanol/chloroform/triethylamine (0.5/98.5/1). Product was recovered as an oil in 40% yield: NMR ($CDCl_3$) δ 1.06 (m, 1H), 1.13 (m, 1H), 1.61 (m, 1H), 1.66 (m, 1H), 2.20-2.38 (m, 2H), 3.71 (m, 1H), 3.85 (m, 1H), 6.25-7.10 (m, 14H). HRMS Calcd for $C_{24}H_{22}NOI$: m/e 467.0701. Found: 467.0742.

The title compound was also prepared using two iododestannylation procedures. One involves iododestannylation of **6** with I_2 using procedure B described for compound **7a** (yield 70%). The other method involves an appropriate upscale of the radiochemical reaction described below using unlabeled sodium iodide (yield 70%). TLC, NMR, and MS of these compounds were identical to that described above.

1-[4-(2-N-Aziridylethoxy)phenyl]-1,2-diphenyl-2-[^{125}I]iodoethylene ([^{125}I]I-Tam-Az). **Carrier-Added Preparation:** Five µL of a 1 mM solution of NaI (5 nmol) in 0.1 N NaOH was pipetted into a 15-mL conical vial. To this, 1 mCi of high pH Na^{125}I solution (10 µL) was added, and the mixture was evaporated to dryness under a gentle stream of nitrogen. The residue was redissolved in 15 µL of concentrated NaOAc/HOAc buffer (2.81 g, 50 mL glacial). To this solution was added a 0.025 M solution of chloramine-T (10 µL, 0.25 µmol) in tetrahydrofuran and **6** (0.025 µmol) in dichloromethane (50 µL). After 1 min, the reaction was quenched with 100 µL of 0.1 N sodium metabisulfite and 200 µL of water. Ethyl acetate (300-400 µL) was added, and the organic layer was drawn off and passed through a pipet containing a small pad of $MgSO_4$. The aqueous layer, $MgSO_4$, and EtOAc layer were counted for radioactivity. The organic layer was concentrated and reconstituted in 1% Et_3N-EtOH/H_2O (75/25, v/v) (30 µL) and applied to a Whatman C-18 reverse phase HPLC using a bracketing procedure. In this case 80 µL of H_2O was introduced into the injector loop; then H_2O (60 mL), an air bubble, and the sample (30 mL) were drawn into a 100-µL syringe and injected into the loop. The desired compound was isolated (R.T. = 10-11 min) and stored in the same solvent. Radiochemical yield 20-45%.

No-Carrier-Added Preparation: High pH $Na^{125}I$ solution (100 µL, 10 mCi) was pipetted into a 15-mL conical vial and evaporated to dryness under a gentle stream of nitrogen. The residue was redissolved in the buffer previously described (50 µL). The remainder of the reaction was carried out as described for the carrier reaction. Radiochemical yield: 36%.

Biochemical

General. Biochemicals were obtained from the following sources: estradiol (E_2) from Sigma (St. Louis, MO); [6,7-^{3}H]estradiol (101 Ci/mmol) from New England Nuclear (Boston, MA). Dimethylformamide was from Mallinckrodt (St. Louis, MO). Sodium molybdate was from Baker Chemical Co. (Phillipsburg, NJ). Acrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulfate, monothioglycerol, ethylenediamine tetraacetic acid (EDTA), and molecular weight standards (phosphorylase b, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; lysozyme, 14,400) were from BioRad (Richmond, CA). Fluorosol and Liquiscint scintillation cocktails were from National Diagnostics (Manville, NJ).

Radioactivity Measurements. Radioactivity was detected by scintillation counting using a Nuclear-Chicago Isocap 300. A counting efficiency for ^{125}I of 70% was taken from the manufacturer's specifications for the instrument and used throughout the course of this work. Tritium counted with an efficiency of 35-45%.

Buffers, Solutions, and Cytosol Preparations. TETG + Mo buffer contained 10 mM Tris-HCl, 1.5 mM EDTA, 10 mM thioglycerol, 10% (v/v) glycerol, and 40 mM sodium molybdate, pH 7.4 at 4°C. Two-fold concentrated sample buffer for electrophoresis contained 0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and bromphenol blue. Stock solutions of [^{125}I]I-Tam-Az were stored at -20°C in the HPLC eluate solvent, 1% Et_3N in EtOH/H_2O (75/25).

Uterine tissue from immature lambs or rats (20- to 23-day-old female Holtzman) was homogenized at 4°C in 0.01 M Tris/1.5 mM EDTA/0.02% sodium azide, pH 7.4 buffer, and the homogenate was centrifuged at 180,000 x g for 60 min. The supernatant was carefully removed, and this cytosol was diluted to a concentration of 3 mg protein/mL.

Cell Culture. MCF-7 cells, obtained from the Michigan Cancer Foundation (Detroit, MI), were maintained in Eagles Minimal Essential Medium (MEM), as previously described (12,13), in closed, 150-cm^2 Corning T-flasks at 37°C.

Competitive Binding Assays. Cytosol was incubated with various concentrations of radioinert estradiol, cis- or trans-Tam-Az, or I-Tam-Az with 1 x 10^{-8} M [^{3}H]estradiol at 0-4°C and was analyzed after 18 h by charcoal-dextran treatment (14). The relative binding affinity of each competitor is taken as the ratio of the concentration of radioinert estradiol/competitor required to inhibit 1/2 of the specific [^{3}H]estradiol binding, with the affinity of estradiol being considered 100% (14).

Time-Dependent Irreversible Binding Assays. Fresh solutions of Tam-Az or I-Tam-Az were added to uterine cytosol to give the concentrations of 150 nM and 130 nM, respectively, and a final concentration of 5% DMF. To protect the binding sites with E_2, additional incubations were performed with an excess of radioinert E_2 (3000 nM) for 1 h at 4°C prior to the addition of the aziridine. The mixture was 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 100-fold excess E_2 for 22 h at 22°C as detailed previously (15).

Covalent Attachment Assays. Fresh solutions of [^{125}I]I-Tam-Az were added to uterine cytosol to give the concentrations of compounds as indicated, and a final concentration of 5% DMF. To protect the binding sites with E_2, additional incubations were performed with 3000 nM radioinert estradiol for 1 h at 4°C prior to the addition of [^{125}I]I-Tam-Az. The mixture was incubated at 22°C, and aliquots were removed at different times and assayed for covalent attachment by an ethanol disk assay (16).

Labeling of MCF-7 Estrogen Receptors with [^{125}I]Iododesethyl Tamoxifen Aziridine. Eagles MEM (3 mL) containing 5% charcoal-dextran-treated calf serum with and without a 200-fold excess of radioinert estradiol was added to near-confluent flasks of MCF-7 cells (ca. 5 x 10^6 cells per T-25 flask) and incubated for 30 min at 37°C. After the 30-min incubation, cells were labeled with 10 or 40 nM [^{125}I]I-Tam-Az for 60 min at 37°C and then harvested (using Hank's Balanced Salt Solution containing 1 mM EDTA). The labeled cells were washed with 6 mL TETG + Mo buffer and homogenized (30 strokes in a Dounce homogenizer with the B-pestle in 1 mL TETG + Mo buffer). The homogenate was centrifuged (800 x g, 10 min) and the supernatant was collected. The crude nuclear pellet was washed twice with buffer, and the supernatants were combined. The supernatant fraction was centrifuged at 180,000 x g for 30 min, and the resulting supernatant (cytosol) and high speed pellet were assayed for radioactivity.

For salt extraction of the nuclear pellet, the washed nuclear pellet was resuspended in 0.5 mL Tris-extraction buffer (10 mM Tris-HCl, 1.5 mM EDTA, 10 mM thioglycerol, 10% glycerol, and 0.6 M NaCl, pH 8.5, at 4°C). The suspension was incubated for 1 h at 0-4°C with resuspension every 15 min, and then centrifuged at 180,000 x g for 30 min to give the nuclear salt extract and high speed pellet that were assayed for radioactivity.

SDS-Polyacrylamide Gel Electrophoresis and Autoradiography. Samples to be analyzed, after being mixed with an equal volume of 2-fold concentrated SDS-sample buffer, were incubated at 100°C for 2 min. SDS-containing 10-20% polyacrylamide gradient slab gels were used to obtain resolution of the receptor (17). The acrylamide gradient (10-20%) separating gel contained 0.373 M Tris (pH 8.8) and N,N'-dimethylene-bis-acrylamide (0.675-1.8%), and the stacking gel contained 0.4% acrylamide, 1% agarose, 0.1% SDS, and 0.125 M Tris, pH 6.8. Samples were electrophoresed (30 mA/gel) at 10°C in a 16-cm x 18-cm x 1.5-mm Protean gel apparatus (BioRad Laboratories). It took 3-4 h for the tracking dye to reach within 1 cm of the bottom of the gel. Gels were fixed and stained in Destain 1 (50% v/v methanol, 10% v/v acetic acid) plus 0.125% w/v Coomassie Blue R250 overnight, then destained in Destain 1, followed by Destain 2 (5% v/v methanol, 7% v/v acetic acid). The stained gel was then dried at 60°C under vacuum with a BioRad Gel Slab Drier. The dried gels were exposed to Kodak X-Omat AR film at -80°C for 2 days. The mobility of the estrogen receptor was compared to that of the molecular weight standards from BioRad, covering the range from 92.5K to 14.4K.

RESULTS AND DISCUSSION

Design

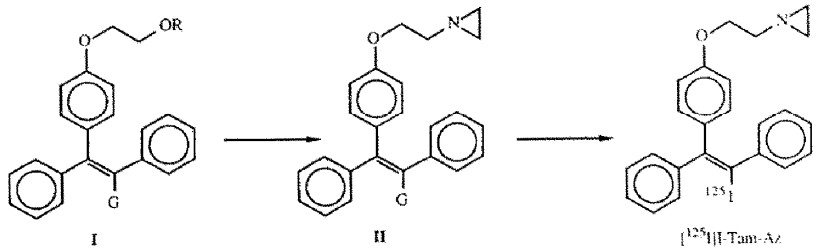
Iodine is large and lipophilic, and thus its substitution on a ligand may interfere with binding and increase non-specific binding. In fact, iodine substitution on the aromatic ring of steroidal and non-steroidal estrogens has just these effects (18-24). On the other hand, iodine is well tolerated on steroidal estrogens at certain positions, such as 16α- and as a 17α-iodovinyl substituent (25-28). There are also reports that the substitution of iodine, as well as other sizeable groups, may be well tolerated at the vinyl position of certain non-steroidal ligands for the estrogen receptor (29-37). This led us to consider

the preparation of an iodine-substituted analog of Tam-Az, in which an iodine would replace the vinylic ethyl group.

Although it is known that hydroxyl substitution on tamoxifen-type ligands for receptor causes marked increases in binding affinity (33), we were reluctant to include such hydroxyl substituents in I-Tam-Az, since in other studies we had found that iodine was rapidly lost from the corresponding position in iodoethyl stilbestrol (Allison KJ and Katzenellenbogen JA, unpublished results). Molecules such as these are known to undergo facile tautomerization to quinone methide intermediates (34,35) in which the iodine becomes readily hydrolyzable.

The approach we developed to I-Tam-Az (Scheme I) proceeded via intermediates I and II, where G is an electropositive element easily replaced by iodine, but stable to conditions necessary to introduce the aziridine functionality. Radioiodination could then be performed in the last step of the synthesis, thereby minimizing the manipulations of radioactive material. For this purpose we investigated the synthesis and iododemetallation of vinyl silanes, mercurials, and stannanes. The procedure by which we successfully prepared I-Tam-Az and [^{125}I]I-Tam-Az is outlined in Scheme II; a shorter, more efficient route to I-Tam is shown in Scheme III. A mixture of cis and trans isomers was carried through until the last step, where reversed phase HPLC was used to separate the isomers.

Scheme I



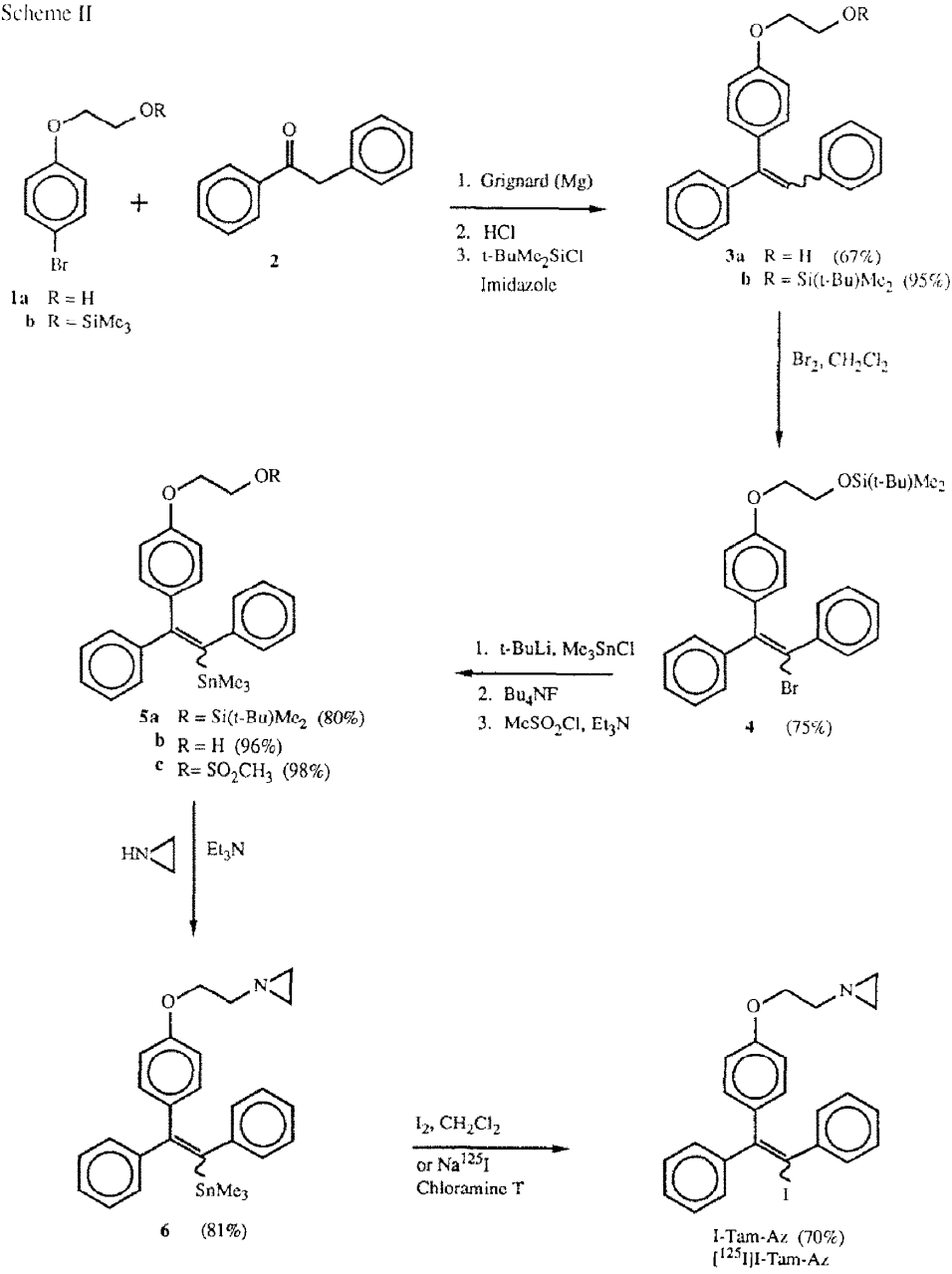
G = electropositive element, e.g., Si, Hg, Sn (Metal-metalloid)

Synthesis of I-Tam-Az. 2-(4-Bromophenoxy)ethanol (1a) was protected as the trimethylsilyl ether (1b) and converted to the Grignard reagent at 70°C in THF. To minimize the extent of enolization of desoxybenzoin (2), the tetrahydrofuran was removed from the Grignard reagent by evaporation and was replaced with benzene. The addition to desoxybenzoin proceeded in 67% yield (76% if corrected for unreacted starting material). Treatment with HCl ensured dehydration of the alcohol and deprotection of the trimethylsilyl ether. The free alcohol 3a was then protected as the t-butyldimethylsilyl ether (3b), and reaction of 3b with bromine gave the vinyl bromide 4. A small amount (<10%) of desilylation product was observed.

Bromide 4 served as a branch point in the synthesis to prepare radiolabeled [^{125}I]I-Tam-Az (Scheme II) and unlabeled I-Tam-Az (Scheme III); but by either path, the first step involved a metal-halogen exchange with t-BuLi (3 eq) at -78°C in THF to give the vinyl lithium species. To obtain unlabeled I-Tam-Az (Scheme III), the vinyl lithium was quenched with N-iodosuccinimide to give vinyl iodide 7a in 54% yield. Desilylation with tetrabutylammonium fluoride gave free alcohol 7b that was carried onto the methane sulfonate (7c). Reaction of 7c with an excess of ethyleneimine (36) in triethylamine/acetonitrile gave the desired compound (I-Tam-Az) in 40% yield (52% when corrected for recovered 7c).

The route eventually used to prepared radiolabeled I-Tam-Az is shown in Scheme II: The vinyl anion derived from the bromide 4 was quenched with trimethylstannyl chloride to give vinyl stannane 5a. No protonolized material (olefin 3b) was observed in this reaction. Desilylation of 5a and preparation of methanesulfonate 5c were followed by reaction with an excess of ethyleneimine and

Scheme II

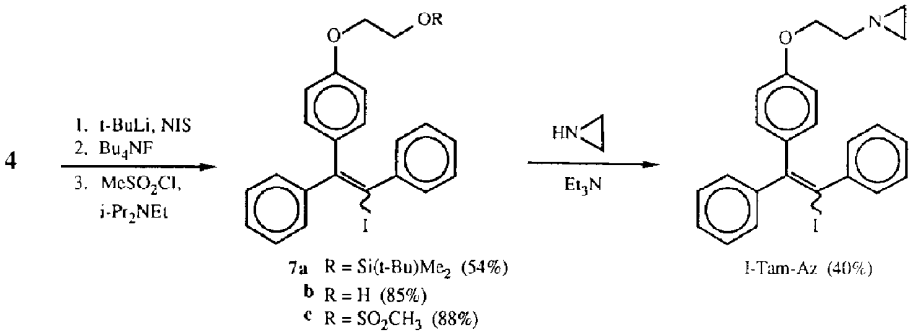

OR
O
Br
1a R = H
b R = SiMe3
+
O
2
1. Grignard (Mg)
2. HCl
3. t-BuMe2SiCl
Imidazole
3a R = H (67%)
b R = Si(t-Bu)Me2 (95%)
Br2, CH2Cl2
OSi(t-Bu)Me2
Br
4 (75%)
1. t-BuLi, Me3SnCl
2. Bu4NF
3. MeSO2Cl, Et3N
SnMe3
5a R = Si(t-Bu)Me2 (80%)
b R = H (96%)
c R= SO2CH3 (98%)
HN
Et3N
N
6 (81%)
I2, CH2Cl2
or Na125I
Chloramine T
I
I-Tam-Az (70%)
[125I]I-Tam-Az

triethylamine, to give the key vinylstannyl aziridyl intermediate 6. Unlabeled I-Tam-Az could be prepared from 6 simply by treatment with an equivalent of iodine in dichloromethane.

Prior to the preparation of I-Tam-Az via the vinyl stannane 6, we had investigated the suitability of vinyl silanes and vinyl mercurials as well as compounds with varying hydroxyl protecting groups (Schemes IV and V). Although vinyl silane 9 could be prepared in about 67% yield from bromide 8 (Scheme IV) using metallation conditions as previously described, this compound was abandoned as a possible precursor for I-Tam-Az. Double bond reduction of 9 was a significant competing side reaction under conditions investigated for the removal of the benzyl ether protecting group (H_2 over 5% and 10% Pd-C or 20% $Pd(OH)_2$-C or catalytic transfer hydrogenation; conditions: ammonium formate, methanol, catalyst acetic acid) (37). Also, quite surprisingly, vinyl silane 9 was resistant to several iododisilylation conditions.

There is literature precedent for the formation of vinyl mercurials from olefin systems such as 3a (38). Treatment of 3a with mercuric trifluoroacetate in benzene gave a mercurated compound 10 in about 67% yield (Scheme V). Other mercuration conditions ($Hg(OAc)_2$ or $Hg(NO_3)_2$) give either no reaction or unidentified products. Iodination of 10 was achieved readily by reaction with I_2 in methylene chloride. NMR investigation of the product 11, however, revealed downfield aromatic signals consistent with protons ortho to aromatic iodides. Also, the aromatic region of the NMR of 11 was different from that of the desired vinyl iodide 7b. Attempts to mercurate and iodinate an O-acetyl derivative of 3a resulted in longer reaction times and again aromatic iodination. Attempts to quench the vinyl lithium derived from 8 with mercuric chloride also failed.

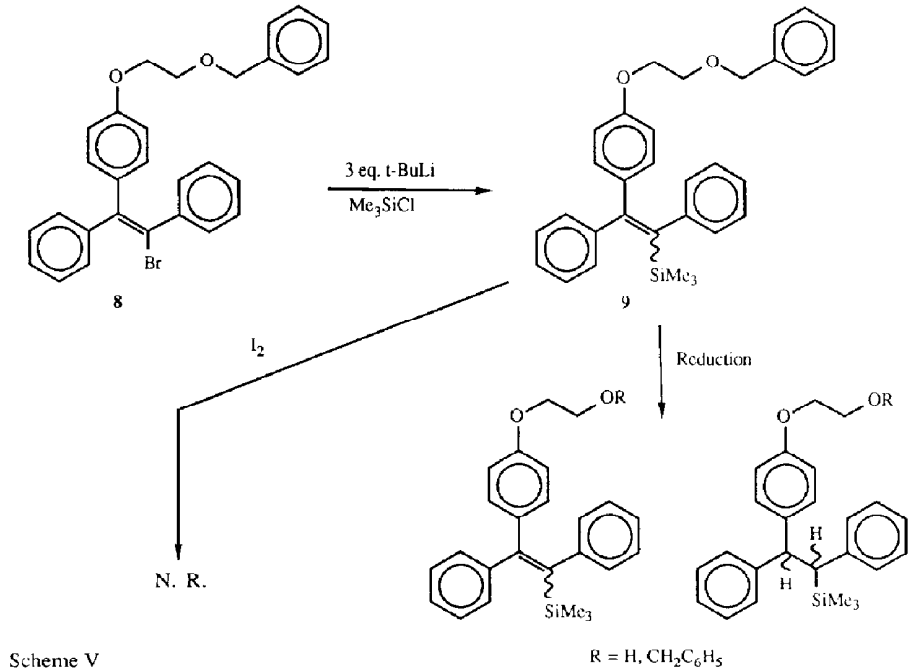
Scheme III



7a R = Si(t-Bu)Me_2 (54%)
b R = H (85%)
c R = SO_2CH_3 (88%)

I-Tam-Az (40%)

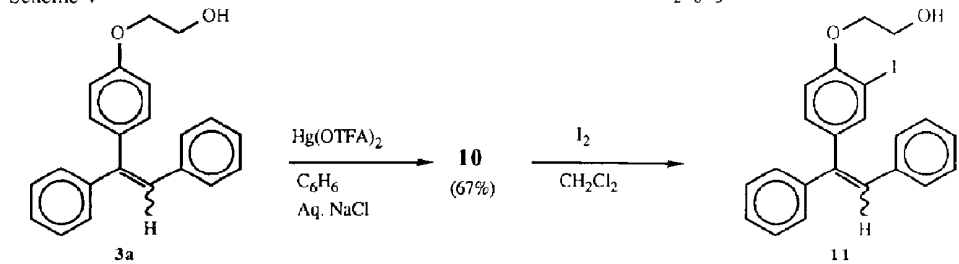
Scheme IV



8 **9**

R = H, $CH_2C_6H_5$

Scheme V



3a **11**

Synthesis of [^{125}I]I-Tam-Az. Radiolabeled I-Tam-Az was prepared by adding a methylene chloride solution of 6 to a buffered solution of Na^{125}I (containing, in some cases, carrier NaI) and chloromine-T. Sodium iodide samples, both unlabeled and radiolabeled, were pipetted as high pH aqueous solutions, which were evaporated to dryness with a stream of N_2 and reconstituted with concentrated NaOAc/HOAc buffer. Preoxidation was required, as 6 was unstable to free iodide anion. The progress of the reaction could be followed conveniently by TLC; when it was complete (5 min), the reaction mixture was simply quenched by the addition of aqueous sodium metabisulfite, and the extract was dried by passage through a short column of anhydrous $MgSO_4$ (considerable product adhered to a column of anhydrous Na_2SO_4), eluting with ethyl acetate. This solution was concentrated under a stream of nitrogen, reconstituted in a small volume of HPLC elution solvent, and injected directly onto the column.

Several different HPLC columns and solvents were explored to optimize the purification of [^{125}I]I-Tam-Az, and it was found that a Whatman C-18 column using acetonitrile/water (80/20, v/v) as eluant gave the best results (good separation of product isomers and good separation from starting material and reaction by-products). However, the product eluted in this solvent system proved to be quite unstable. (The product [^{125}I]I-Tam-Az is not stable when stored in acetonitrile, while it is stable indefinitely when stored in 5% Et_3N-EtOH). An elution solvent consisting of 1% Et_3N-EtOH/H_2O (75/25) gave good separation of the product from all by-products, and the eluted material could be simply collected and stored in the elution solvent. In this reversed phase system the trans isomer of I-Tam-Az elutes at 11 min, the cis isomer at 13 min. Unreacted vinylstannane, being much less polar, eluted after 30 min and was generally removed by flushing the column with 100% EtOH.

The radiochemical yields in both carrier-added and no-carrier added reactions ranged from ca. 20-45%; the yield tends to increase with increased levels of carrier or activity. The largest scale preparation was a 10-mCi no-carrier-added reaction, in which a 36% yield of HPLC-purified material was obtained.

The specific activity of [^{125}I]I-Tam-Az was determined by HPLC, the mass of the eluted material being estimated from the peak area of the UV trace, by comparison with a standard curve. In some cases, a volatile UV-absorbing impurity co-eluted with [^{125}I]I-Tam-Az and interfered with attempts to determine specific activity by this method. The material could be removed, however, simply by re-evaporation of the collected product and reinjection onto the same column. The material, thus repurified, eluted as a single radioactive and UV-absorbing peak. In most cases a 10-fold dilution with carrier gave material with specific activity in the range of 60-200 Ci/mmol. The material from the no-carrier-added synthesis was determined to have a specific activity of >200 Ci/mmol.

Biochemical Studies

Apparent Competitive Binding Assay Affinity. Competitive radiometric binding assays are useful for determining the relative affinity of compounds for binding proteins (14). Although not strictly applicable to ligands that are capable of irreversible binding, these assays are still useful in providing a measurement of the apparent competitive binding affinity of such ligands (3,6,7). The results of such an assay with I-Tam-Az, together with estradiol (as a standard) and the two geometric isomers of Tam-Az, are shown in Figure 1. The trans isomer of Tam-Az has an apparent competitive binding affinity of 19%, relative to estradiol; this is considerably higher than the affinity of trans-tamoxifen itself (ca. 2%). The cis isomer of Tam-Az, as the cis isomer of tamoxifen itself (data not shown), has considerably lower

affinity. I-Tam-Az, available for this assay only as a cis-trans isomer mixture, had an apparent competitive binding affinity comparable to that for trans-Tam-Az.

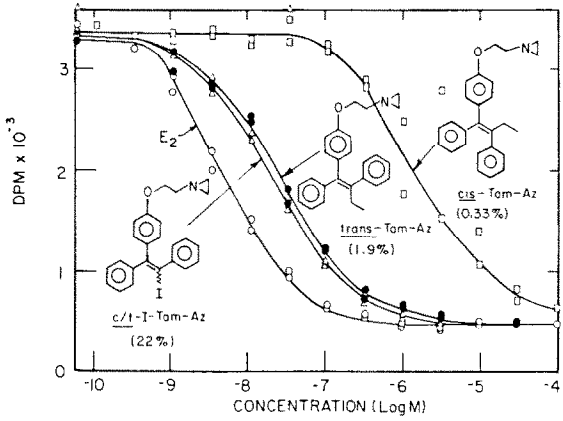


Figure 1. Assay of the apparent competitive binding affinity of estradiol, cis- and trans-Tam-Az, and I-Tam-Az for the estrogen receptor. This assay was performed as previously described (14), using lamb uterine cytosol as a source of estrogen receptor, [^{3}H]estradiol as tracer, and charcoal-dextran as adsorbant of free ligand. The percentages given in parentheses represent the affinities relative to that of estradiol (c/t, cis-trans isomer mixture).

Time-Dependent Irreversible Binding. Compounds capable of covalent attachment to receptor should demonstrate time-dependent irreversible binding. This has been shown previously for Tam-Az, Naf-Az, and KN-Az (3,6,7). This assay involves incubation of receptor with the aziridine for varying periods, removal of excess agent by charcoal-dextran adsorption, followed by assay of the surviving binding sites by an exchange assay with [^{3}H]estradiol. An example of such a time-dependent irreversible binding assay with I-Tam-Az is shown in Figure 2. Again, the irreversible binding of Tam-Az is shown as a control.

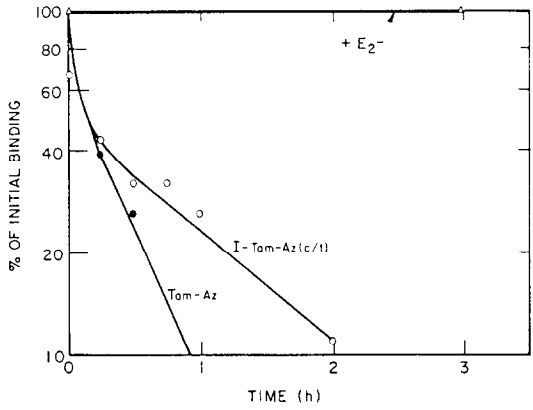


Figure 2. Time-dependent irreversible binding of [^{125}I]I-Tam-Az to the estrogen receptor. Estrogen receptor in a lamb uterine cytosol preparation was exposed to 150 nM Tam-Az or 130 nM I-Tam-Az at 22°C. At the indicated times, aliquots were removed, treated with charcoal-dextran, and allowed to exchange with 30 nM [^{3}H]estradiol in the absence and presence of 3000 nM radioinert estradiol (for the determination of total and non-specific binding, respectively). The curve labeled +E_2 was from a sample treated with 3000 nM radioinert estradiol for 60 min at 0°C prior to the assay.

It is evident that the cis-trans mixture of isomers of I-Tam-Az is capable of rapid and nearly complete time-dependent irreversible binding to receptor. The time course of this irreversible binding is somewhat slower than that for Tam-Az. Also, as was the case with Tam-Az, the irreversible binding is estrogen binding site specific, since preincubation of the receptor preparation with radioinert estradiol blocks the irreversible binding.

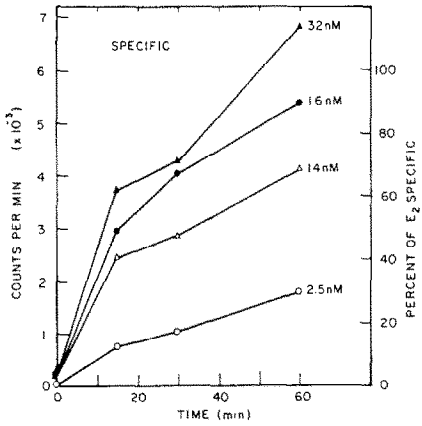
Direct Assay of Covalent Attachment of [^{125}I]I-Tam-Az to the Estrogen Receptor. With radiolabeled I-Tam-Az (available in radiolabeled form as the pure trans isomer), we were able to demonstrate directly that this agent reacts with estrogen receptor covalently. The time course of covalent attachment to receptor can be followed by a solvent extraction assay (16). A preparation of estrogen receptor is exposed to different concentrations of

[^{125}I]I-Tam-Az, and at various times, aliquots are removed and placed on paper disks for extraction with boiling ethanol; only radioactivity that has become covalently attached to receptor remains in the paper disk, associated with the solvent-precipitated protein; the remaining activity is removed by extraction. The results of such an assay are shown in Figure 3. In this figure, only the specific covalent attachment (that is, the difference between total and non-specific attachment, the latter measured in the presence of an excess of radioinert estradiol) is given.

The specific covalent attachment of [^{125}I]I-Tam-Az to the estrogen receptor proceeds in a time- and concentration-dependent manner. After 60 min, nearly all receptor is labeled when either 16 or 32 nM [^{125}I]I-Tam-Az is used. Therefore, labeling of estrogen receptor by [^{125}I]I-Tam-Az is very efficient.

The selectivity of estrogen receptor labeling by [^{125}I]I-Tam-Az can be appreciated from the results presented in Figure 4. Here the time course of both total labeling (T) and non-specific labeling (NS) is shown for the incubation utilizing 14 nM [^{125}I]I-Tam-Az; the difference between these two curves, plotted here as the boldface line labeled S, is part of the data presented in Figure 3.

It can be seen from these data that, at this concentration of ligand, the specific labeling accounts for 1/2 to 1/3 of total labeling. Although this labeling selectivity is less than that for reversibly binding ligands, and is somewhat less than we have observed previously for other covalently-reacting ligands such as Tam-Az (4), Naf-Az (6), and KN-Az (7,8), demonstration of this level of selectivity in such an unfractionated receptor preparation is still noteworthy, considering the fact that estrogen receptor represents only several parts per million of protein molecules.



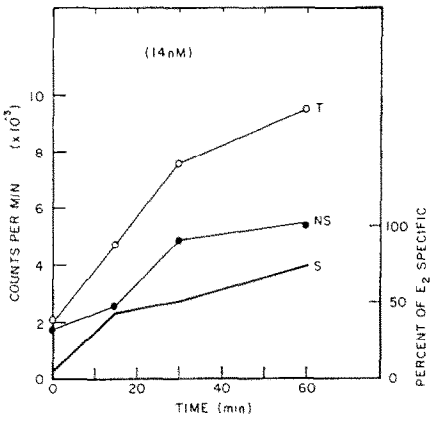


Figure 3 (left). Assay of the efficiency of specific covalent labeling of estrogen receptor with [^{125}I]I-Tam-Az. A cytosol preparation of estrogen receptor from rat uterus was incubated with the indicated concentrations of [^{125}I]I-Tam-Az at 22°C, both in the absence and presence of 3000 nM radioinert estradiol for determination of total and non-specific labeling, respectively. At the times shown, aliquots were removed and assayed for covalently bound radioactivity by solvent extraction with boiling ethanol, as described previously (16). The sample of [^{125}I]I-Tam-Az used in this assay had a specific activity of 50 Ci/mmol. In all cases, specific labeling, that is, the difference between total and non-specific labeling, is shown.

Figure 4 (right). Assay of the selectivity of covalent labeling of estrogen receptor with [^{125}I]I-Tam-Az. The total (T) and non-specific (NS) labeling curves from the incubation with 14 nM [^{125}I]I-Tam-Az shown in Figure 3 are plotted, together with the difference that represents specific binding (S).

Characterization of Estrogen Receptor Covalently Labeled by [^{125}I]I-Tam-Az by SDS-Polyacrylamide Gel Electrophoresis. [^{125}I]I-Tam-Az can be used to label estrogen receptors in intact MCF-7 human breast cancer cells in culture. Cells in culture are exposed to the agents for 1 h at 37°C, and the covalently labeled receptor is extracted from the nuclear fraction with salt and analyzed by electrophoresis on an SDS polyacrylamide slab gel. In contrast to such analysis with tritium-labeled compounds, the

radioactivity on the gel can be detected by autoradiography in a short period of time (exposure 2 days for this gel). An example of such an autoradiograph is shown in Figure 5.

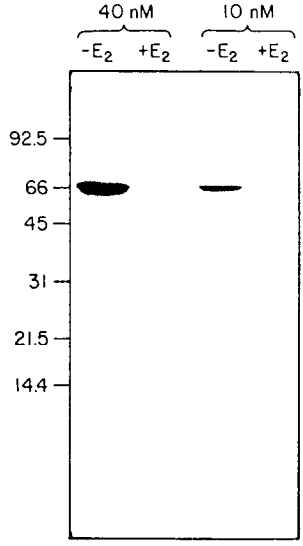


Figure 5. Autoradiograph of an SDS-polyacrylamide slab gel of [^{125}I]I-Tam-Az-labeled estrogen receptor from MCF-7 cells. Intact MCF-7 human breast cancer cells were incubated with 10 or 40 nM [^{125}I]I-Tam-Az (specific activity 200 Ci/mmol) for 60 min at 37°C in the absence (-) or presence (+) of a 200-fold excess of radioinert E_2. A salt extract of the nuclear fraction was analyzed by SDS-polyacrylamide gel electrophoresis and the distribution of radioactivity on the gel detected by autoradiography. Numbers at the left indicate the positions of the molecular weight marker proteins ; mass units are kDa.

Here, two concentrations of [^{125}I]I-Tam-Az were used for the cell labeling, both in the absence and presence of an excess of unlabeled estradiol. Very selective labeling of a single species with an M_r of 65K is clearly evident; no labeling is observed in the presence of unlabeled ligand.

CONCLUSIONS

In this report, we have described the synthesis of I-Tam-Az and [^{125}I]I-Tam-Az *via* an approach utilizing a vinyl stannane aziridine intermediate (6). The vinyl stannane proved to be of greater use than the related silane (2) because of its ease of preparation and high reactivity towards iodine; the appropriate vinyl mercurial could not be prepared.

The iodination and radioiodination of the stannane 6 proceeded smoothly and in good yield. In general in the radioiodinations, we added carrier iodide to reduce the specific activity about 10-fold. This gave I-Tam-Az with specific activity around 100-200 Ci/mmol, which is convenient to use in receptor labeling experiments. Material produced in a no-carrier-added radioiodination reaction had higher specific activity (estimated to be *ca.* 600 Ci/mmol), but was somewhat below the theoretical maximum of 2200 Ci/mmol. We are presently modifying the preparative method so as to improve this.

The *cis*-*trans* geometric isomers of all the intermediates (compounds 3-7) and of I-Tam-Az can be distinguished by proton NMR (39,40); however, their separation by chromatography is very difficult. Also, since the isomers are equilibrated in the final iododestannylation reaction, it is not worthwhile to separate them before the final step. We were able to separate small amounts of the isomers of [^{125}I]I-Tam-Az using the analytical reversed phase HPLC column system that we developed for the radiochromatography. We could assign the isomer geometry by comparisons of the peak area ratios of isomer mixtures on the chromatogram with the integral ratios in the NMR; the isomer assignments by NMR followed well-established precedents (39,40), with the *trans* isomer showing upfield shifts of the disubstituted aryl ring, due to the proximity with the *cis* phenyl substituent. Thus, we were able to obtain [^{125}I]I-Tam-Az as the pure *trans* isomer for the receptor

labeling studies. The limited capacity of the analytical HPLC column, however, prevented us from accumulating sufficient quantities of the pure isomers of unlabeled I-Tam-Az. Therefore, the competitive binding and time-dependent inactivation studies were performed using a mixture of I-Tam-Az isomers.

In terms of its receptor labeling characteristics, I-Tam-Az behaves much like Tam-Az (3-5). It demonstrates good apparent competitive binding affinity and shows rapid, selective, and complete time-dependent irreversible binding. [^{125}I]I-Tam-Az labels receptor cytosol preparations effectively, although both in terms of efficiency and selectivity, the behavior of [^{125}I]I-Tam-Az is somewhat inferior to that of Tam-Az. Nevertheless, very selective labeling of estrogen receptor in intact MCF-7 cells can be achieved, and because of the higher specific activity of [^{125}I]I-Tam-Az, autoradiographs of the slab gels can be obtained in a day or two.

[^{125}I]I-Tam-Az should prove to be very useful as a sensitive, selective, and efficient covalent labeling agent for estrogen receptor.

ACKNOWLEDGMENTS

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NOTES

*To whom correspondence should be addressed.

+The following common names and abbreviations are used: tamoxifen aziridine (Tam-Az), (Z)-1,2-diphenyl-1-[4-[2-(N-aziridinyl)-ethoxy]-phenyl]-1-butene; desmethyl nafoxidine aziridine (Naf-Az), 6-hydroxy-1-[4-[2-(N-aziridinyl)ethoxy]-phenyl]-2-phenyl-3,4-dihydronaphthalene; ketononestrol aziridine (KN-Az), (3R*,4S*)-3,4-bis(4-hydroxyphenyl)-9-(N-aziridinyl)-5-nonanone; iododesethyl tamoxifen aziridine (I-Tam-Az), (E)-1,2-diphenyl-1-[4-[2-(N-aziridinyl)ethoxy]-phenyl]-2-iodoethylene.

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