References

Buhler, J.-M., Iborra, F., Sentenac, A., and Fromageot, P. (1976), *J. Biol. Chem. 251,* 1712.
Buhler, J.-M., Sentenac, A., and Fromageot, P. (1974), *J. Biol. Chem. 249,* 5963.
Burgess, R. R. (1976), in RNA Polymerase, Losick, R., and Chamberlin, M., Ed., Cold Spring Harbor, N.Y., Cold Spring Harbor Press, pp 69–100.
Burgess, R. R., and Jendrisak, J. J. (1975), *Biochemistry 14,* 4634.
Castellino, F. J., and Barker, R. (1968), *Biochemistry 7,* 2207.
Davis, B. J. (1964), *Ann. N.Y. Acad. Sci. 121,* 404.
Dunker, A. K., and Rueckert, R. R. (1969), *J. Biol. Chem. 244,* 5074.
Gershman, L. C., and Dreizen, P. (1969), *Biophys. J. 9,* A235.
Greenleaf, A. L., and Bautz, E. K. F. (1975), *Eur. J. Biochem. 60,* 169.
Jendrisak, J. J., and Burgess, R. R. (1975), *Biochemistry 14,* 4639.
Jendrisak, J. J., Petranyi, P. W., and Burgess, R. R. (1976), in RNA Polymerase, Losick, R., and Chamberlin, M., Ed., Cold Spring Harbor, N.Y., Cold Spring Harbor Press, pp 779–791.
Jovin, T. A., Chrambach, A., and Naughton, M. A. (1964), *Anal. Biochem. 9,* 351.
Laemmli, U. K. (1970), *Nature (London) 227,* 680.
O'Farrell, P. O. (1975), *J. Biol. Chem. 250,* 4007.
Ornstein, L. (1964), *Ann. N.Y. Acad. Sci. 121,* 321.
Schwartz, L. B., and Roeder, R. G. (1975), *J. Biol. Chem. 250,* 3221.
Shapiro, A. L., Vinuela, E., and Maizel, J. V. (1967), *Biochem. Biophys. Res. Commun. 28,* 815.
Steinmetz, M. A., and Deal, W. C., Jr. (1966), *Biochemistry 5,* 1399.
Swaney, J. B., Van de Woulde, G. F., and Bachrach, H. L. (1974), *Anal. Biochem. 58,* 337.
Tanford, C., Kawahara, K., and Lapanji, S. (1967), *J. Am. Chem. Soc. 89,* 729.
Ullmann, A., Goldberg, M. E., Perrin, D., and Monod, J. (1968), *Biochemistry 7,* 261.
Valenzuela, P., Weinberg, F., Bell, G., and Rutter, W. J. (1976), *J. Biol. Chem. 251,* 1464.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Woods, E. F., Himmelfarb, S., and Harrington, W. F. (1963), *J. Biol. Chem. 238,* 2374.

Estrogen Photoaffinity Labels. 1. Chemical and Radiochemical Synthesis of Hexestrol Diazoketone and Azide Derivatives; Photochemical Studies in Solution†

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ABSTRACT: Two photosensitive estrogen derivatives, hexestrol diazoketopropyl ether (**5**) and hexestrol azide (**8a**), have been synthesized in radiolabeled form, and their photochemical behavior in solution has been studied. The radiolabeled compounds were prepared in good yields according to improved synthetic procedures; they are stable and were obtained with specific activities in the range of 50–100 Ci per mmol and radiochemical purities in excess of 95%. A simpler model system, phenyl diazoketopropyl ether, was used to study the photochemical behavior of the diazoketopropyl ether group. Direct irradiation of this compound at 254 nm in methanol led to 33% insertion product (methoxyketone) and 67% Wolff rearrangement product (ester). Irradiation of [^{3}H]hexestrol diazoketopropyl ether (**5**) in methanol gives mainly nonpolar photoproducts (presumed to be the methoxy ketone and ester); however, irradiation in aqueous medium leads to large amounts of free hexestrol (52%). Photolysis of hexestrol azide (**8a**) in either methanol or water gives the corresponding amine in low yield as the only identifiable photoproduct.

We have attempted to take a systematic approach to the problem of developing photoaffinity labeling reagents for the uterine estrogen receptor. Our previous publications have covered in detail the rationale of our approach (Katzenellenbogen et al., 1976, 1977a) and have described the synthesis of several photosensitive derivatives of steroidal and nonsteroidal estrogens, all in nonradiolabeled form (Katzenellenbogen et al., 1973a). We have also fully described our investigations of the reversible binding affinity (in the dark) of these reagents

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(Katzenellenbogen et al., 1973b) and their efficiency of receptor inactivation (upon irradiation) (Katzenellenbogen et al., 1974). As the derivatives were not radiolabeled, both of these characteristics were assayed indirectly, using competition and cytosol exchange assays with labeled estradiol as tracer.

As a result of these studies, a number of derivatives were selected for further study; among these were hexestrol diazoketopropyl ether (**5**) and hexestrol azide (**8a**).[1] In this report, we describe certain simplifications in the chemical synthesis of these compounds that assisted in their radiochemical synthesis, and an investigation of their photochemical behavior in solution. The following paper details the reversible binding activity of these compounds with the estrogen receptor and describes their capacity for photoaffinity labeling of the receptor (Katzenellenbogen et al., 1977b).

Experimental Section

Materials. The following chemicals were obtained from the sources indicated: estrone and 17β-estradiol (Searle; Steraloids); *meso*-hexestrol (Mann); phenoxyacetyl chloride (Aldrich); bromoacetyl bromide (Eastman).

Ethanol-free chloroform was obtained by passing chloroform reagent over alumina. Diazomethane was freshly prepared as an alcohol-free ethereal solution from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald, Aldrich) using preparation II as given. Tetrahydrofuran (THF) was dried and purified by distillation from sodium naphthalide or sodium benzophenone ketyl before use.

Methods. Infrared data were obtained using either a Perkin-Elmer Model 137 or 521. Nuclear magnetic resonance spectra were recorded on a Varian Associates Model A-60A, A-56/60-A, or HR 220 spectrometer, using tetramethylsilane as an internal standard. Mass spectra and exact mass determinations were run on Varian MAT CH-5, SM1B, and 731 mass spectrometers.

High-pressure liquid chromatography was performed on a Varian Model 4100 instrument with a UV detector (254 nm) using either a 3.1 mm × 122 cm Corasil II (Waters) column, a 3.1 mm × 25 cm Micropak CN-10 (Varian) column, or a 3.1 mm × 50 cm ETH Permaphase (Du Pont) column. The eluting solvent was a mixture of hexane (solvent A) and 2-propanol–methylene chloride, 1:4 (solvent B); its composition was controlled by a multilinear gradient programmer.

Microanalysis was performed by the microanalytical service of the University of Illinois.

Chemical and Radiochemical Procedures. Reactions involving sensitive compounds were conducted under an atmosphere of dry nitrogen, using syringe transfer techniques, and those involving light-sensitive compounds were conducted under low illumination in vessels covered with aluminum foil.

A standard procedure for product isolation was used in all reactions: quench in water, exhaustive extraction with a solvent, drying over an anhydrous salt, filtration, and evaporation of solvent under reduced pressure. The extraction solvent and drying agent used in each procedure are given in parentheses.

Radioactivity was measured in a Nuclear Chicago Isocap 300 liquid scintillation counter, in minivials using 5 mL of either a toluene-based cocktail containing 0.54% 2,5-diphenyloxazole, 0.0039% *p*-bis[2-(5-phenyloxazolyl)]benzene, and 10% Biosolv BBS-3 (Beckman) or a xylene-based cocktail containing 0.55% 2,5-diphenyloxazole, 0.01% *p*-bis[2-(5-phenyloxazolyl)]benzene, and 25% Triton X-114 (Anderson and McClure, 1973). Tritium counting efficiency was 20–48%.

Radiochemical purity was determined by thin-layer chromatography on Eastman Chromatosheets (6061). The labeled material was spotted on top of unlabeled carrier. After development, the carrier spot was visualized (iodine vapor or UV light), and the chromatogram was cut into five strips which were then placed in minivials and eluted with 0.5 mL of THF.

Irradiations at 254 nm were conducted in a Rayonet photochemical reaction (RPR-100) equipped with four General Electric germicidal light bulbs (G8T5) at 4 °C using quartz annular photolysis tubes as described previously (Katzenellenbogen et al., 1974).

3-Bromodiazoacetone (**2**). The procedure paralleled that for the chloro analogue (Arndt and Amende, 1928; Piazza et al., 1968). Bromoacetyl bromide (4.05 g, 1.8 mL, 20 mmol) was added to a tenfold excess of distilled diazomethane in ether at 0 °C. After 3 h at 0 °C and 18 h at 25 °C, half of the solvent was removed by distillation (steam bath), and the solution remaining was filtered through Celite and evaporated to give 4.3 g of a yellow liquid. Purification by preparative TLC (chloroform–ethanol, 96:4, one development) gave 1 g (31%) of a yellow liquid: bp 124 °C dec; IR (KBr) 2120 (C=N=N), 1640 (C=O), and 1370 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.85 (s, 2 H, CH_2Br) and 5.8 (s, 1 H, $CH{=}N_2$); mass spectrum (70 eV) *m/e* (rel intensity) 164 (M^+, 5), 162 (M^+, 5), 108 (10), 107 (1), 106 (10), 95 (3), 93 (3), 69 (23), and 28 (100).

Anal. Calcd for $C_3H_3N_2BrO$: mol wt, 161.9429. Found: mol wt (high resolution mass spectrum), 161.9431.

4-O-(3′-Diazo-2′-ketopropyl)hexestrol (**5**). A solution of hexestrol (65 mg, 0.240 mmol) and 100 mg (0.725 mmol) of potassium carbonate in 2 mL of methanol–THF (4:1) was treated with 50 mg (0.302 mmol) of bromodiazoacetone in 2 mL of THF. The reaction mixture was flushed with nitrogen and then stirred at 25 °C. After 72 h, the reaction was quenched with water, and product isolation (EtOAc; $MgSO_4$) gave 43 mg (51%) of crude product. Preparative TLC (chloroform–ethanol, 96:4, three developments) furnished a yellow crystalline product **5** that was identical in all resepcts with the material prepared by the method described previously (Katzenellenbogen et al., 1973a).

[^3H]Hexestrol (86 Ci/mmol) was prepared by the method of Williams and Ronzio (1950), as described previously (Katzenellenbogen and Hsiung, 1975).

[^3H]4-O-(3′-Diazo-2′-ketopropyl)hexestrol ([^3H]**5**). A solution of [^{3}H]hexestrol (500 μCi, 5.77 nmol) in benzene–ethanol (9:1) was dried under a stream of nitrogen. THF (0.3 mL), potassium carbonate (10 mg, 0.072 mmol), and 0.2 mL of methanol were added, followed after 5 min by 5 mg (0.03 mmol) of bromodiazoacetone in 0.2 mL of THF. The reaction vessel was flushed with nitrogen and stirred at 25 °C under an atmosphere of nitrogen for 72 h. Product isolation (EtOAc; $MgSO_4$) furnished a yellow residue (some bromodiazoacetone remained) that was redissolved in THF (0.006 mL) and pu-

[1] Common names (and abbreviations) used in this paper are: estradiol, 1,3,5(10)-estratriene-3,17β-diol; hexestrol, *meso*-3,4-bis(4′-hydroxyphenyl)hexane (**1**); hexestrol diazoketopropyl ether, hexestrol 4-*O*-(3′-diazo-2′-oxopropyl) ether (**5**); hexestrol azide, 3-azidohexestrol (**8a**); THF, tetrahydrofuran; TEA buffer, 0.01 M Tris–0.0015 M EDTA–0.02% sodium azide, pH 7.4 at 25 °C; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance; UV, ultraviolet. Bold-face numbers are used in the text to designate hexestrol derivatives and related systems; the assignment of these numbers to structures is given in Schemes I–IV and to names, in the Experimental Section.

rified by liquid chromatography (Corasil II column; flow rate 80 mL/h; gradient 5–25% B at 0.80%/min). One of the forty fractions contained 194 μCi (39%) of [^{3}H]hexestrol diazoketopropyl ether with 97% radiochemical purity.

[^{3}H]-3-Nitrohexestrol (**6a**) *and [^{3}H]3,3′-Dinitrohexestrol* (**6b**). [^{3}H]Hexestrol (**1**) (1 mCi, in 9:1 benzene–ethanol, 3.13 μg, 0.0012 μmol) was dried under a stream of nitrogen. Glacial acetic acid (0.5 mL), followed by 2 μL of concentrated nitric acid, was added to the flask, and the reaction mixture was stirred. The progress of nitration was monitored by radio TLC (chloroform), and after 2.5 h the reaction mixture was diluted with 0.5 mL of distilled water and neutralized with solid sodium bicarbonate. Ether extracts were dried by passage through a small column of sodium sulfate and evaporated under nitrogen. The two labeled nitro products were separated by preparative TLC (chloroform) on silica gel sheets (Eastman 6061); they were located using unlabeled markers **6a** and **6b** that were chromatographed on the sides of the plastic-backed chromatosheet and visualized under UV light. Based on total nitrated product, [^{3}H]-3,3′-dinitrohexestrol (**6b**) was obtained in 33% chemical yield (radiochemical purity 96%), and [^{3}H]-3-nitrohexestrol (**6a**), in 67% yield (radiochemical purity 97%).

[^{3}H]-3-Aminohexestrol (**7a**). A benzene–ethanol solution of [^{3}H]-3-nitrohexestrol (**6a**) (1.088 mCi, 3.96 μg, 12.6 nmol) was dried under a stream of nitrogen. The residue was redissolved in 3 mL of acetone, 2 mL of 1 N sodium hydroxide, and 1 mL of water, and the reaction mixture was heated at reflux. Sodium dithionite (20 mg, 0.11 mmol) was added, followed by an additional 5-mg portion after 30 min. After 1 h, the reaction mixture was cooled, solvent volume was reduced under a stream of nitrogen, and the reaction mixture was neutralized with 10% acetic acid. Product isolation (ethyl acetate; Na_2SO_4) and removal of ethyl acetate under nitrogen gave the radiolabeled amine **7a** in 84% chemical yield (85% radiochemical purity) (4% MeOH in $CHCl_3$, three developments). The labeled amine was stored at 4 °C in 9:1 benzene–ethanol and used in subsequent reactions without further purification.

[^{3}H]-3-Azidohexestrol (**8a**). A benzene–ethanol solution of [^{3}H]-3-aminohexestrol (**7a**) (1.5 mCi, 5.7 μg, 20.0 nmol) was dried under a stream of nitrogen. The residue was dissolved in 2 mL of acetone and 0.75 mL of 2 N hydrochloric acid and diazotized with 20 mg (0.29 mmol) of sodium nitrite in 3 mL of water at 0 °C for 40 min before quenching with a cold solution of 200 mg of sodium azide in 0.75 mL of distilled water. Product isolation (ether; Na_2SO_4) and removal of ether under nitrogen gave the crude azide, which was stored in benzene–ethanol until purification.

The solution of crude azide was reduced to dryness, taken up in 5 μL of THF, and purified by liquid chromatography on an ETH column: 25% solvent B in solvent A, increasing at 1%/min, 40 mL/h. [^{3}H]-3-Azidohexestrol (**8a**), obtained in a 13% chemical yield (200 μCi; 97% radiochemical purity; 1% MeOH in chloroform), was stored in the dark at 4 °C as a solution in 9:1 benzene–ethanol.

[^{3}H]-3,3′-Diaminohexestrol (**7b**). This compound was prepared from [^{3}H]-3,3′-dinitrohexestrol (**6b**) by the procedure described for compound **7a** (94% chemical yield; 75% radiochemical purity; 4% MeOH in chloroform, three developments).

[^{3}H]-3,3′Diazidohexestrol (**8b**). This compound was prepared from [^{3}H]-3,3′-diaminohexestrol (**7b**) by the procedure described for compound **8a** (29% chemical yield; 98% radiochemical purity; 5% MeOH in chloroform or 1:1 benzene–ethyl acetate).

Phenoxydiazoacetone (**9**). Phenoxyacetyl chloride (3.0 g, 0.0176 mol) was added to a large excess of distilled diazomethane in ether at 0 °C. Reaction and product isolation (ether; $MgSO_4$) according to the procedure described for compound **5** furnished a yellow oil which was purified by preparative TLC (chloroform–ethanol, 97:3) to yield 2.03 g of a yellow crystalline product (after vacuum drying at −78 °C): mp 26–28 °C; IR (KBr) 2100 (C=N=N), 1640 (C=O), and 1360 (C–O) cm^{-1}; ^{1}H NMR (see Figure 1A) ($CDCl_3$) δ 4.5 (s, 2 H, CH_2), 5.7 (s, 1 H, CH), 6.8–7.5 (m 5 H, aromatic); mass spectrum (70 eV) *m/e* (rel intensity) 176 (M^+, 5), 148 (5), 132 (7), 131 (14), 120 (9), 119 (5), 107 (8), 94 (60), 77 (38), 28 (100).

Anal. Calcd for $C_9H_8N_2O_2$: C, 61.36; H, 4.58; N, 15.9. Found: C, 61.48; H, 4.57; N, 16.21.

Irradiation of Phenoxydiazoacetone (**9**). A 1% solution of the diazoketone (**9**) (200 mg in 20 mL of methanol) in quartz photolysis tubes was flushed with a stream of nitrogen, sealed with a serum cap, and irradiated at 254 nm. At the end of 1 h of irradiation, the photolysis tubes were removed from the photochemical reactor, and the solvent was evaporated in vacuo. An ^{1}H NMR spectrum of the reaction mixture (Figure 1B) revealed the presence of two products plus starting material. Products were identified as phenoxymethoxyacetone (**10**) and methyl 3-phenoxypropionate (**11**) in the ratio of 1:2.

α-Phenoxy-α′-methoxyacetone (**10**). To a solution of the diazoketone (**9**) (300 mg, 1.7 mmol) in 10 mL of methanol at 0 °C was added 75 mg (0.44 mmol) of *p*-toluenesulfonic acid. The mixture (in Pyrex photolysis tubes) was irradiated (>315 nm) at 0 °C. After 1 h the solvent was evaporated, and the crude reaction mixture was purified by preparative chromatography (chloroform–ethanol, 97:3, two developments) to give a brown oil: IR ($CHCl_3$) 1735 (C=O, ketone), 1600, 1500, and 1250 cm^{-1}; ^{1}H NMR (see Figure 1C) ($CDCl_3$) δ 3.33 (s, 3 H, OCH_3), 4.20 (s, 2 H, CH_2OMe), 4.65 (s 2, H, CH_2OPh), 6.8–7.5 (5 H, aromatic); mass spectrum (70 eV) *m/e* (rel intensity) 180 (M^+, 48), 119 (51), 107 (45), 94 (41), 77 (73), 59 (46), 45 (100).

Anal. Calcd for $C_{10}H_{12}O_3$: mol wt, 180.0786. Found: mol wt (high resolution mass spectrum), 180.0786.

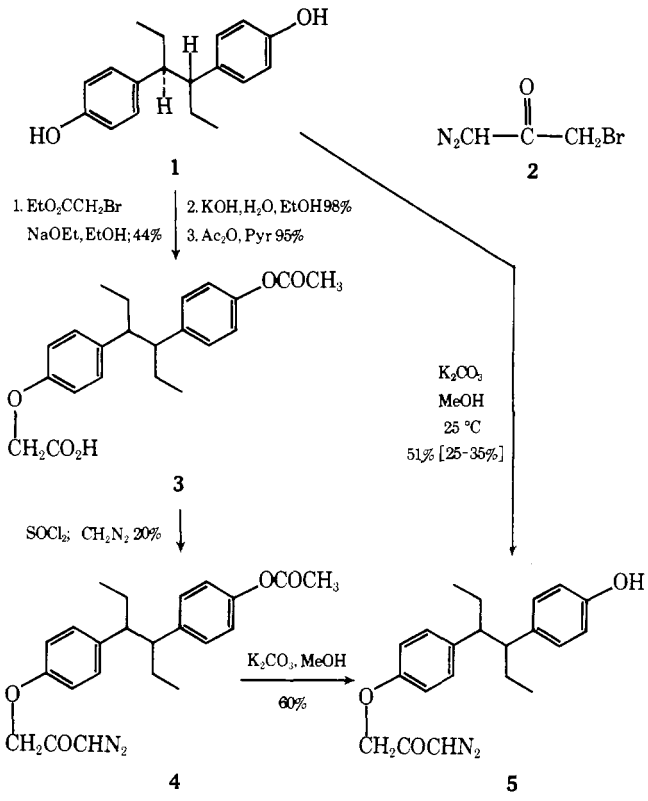
Methyl 3-Phenoxypropionate (**11**). A solution of the diazoketone (**9**) (500 mg, 2.84 mmol) in 5 mL of methanol was heated to reflux and 3 mL of a 10% slurry of silver oxide in methanol was added slowly in small portions. After 1 h, 100 mg of decolorizing carbon was added, and the mixture was filtered and evaporated to dryness. The crude product was purified by preparative chromatography (chloroform, two developments) to give 100 mg of a pale yellow oil: IR (KBr) 1740 (C=O, ester) and 1200 cm$^-$; ^{1}H NMR (see Figure 1D) ($CDCl_3$) δ 2.8 (t, 2 H), 3.7 (s, 3 H), 4.2 (t, 2 H), 6.8–7.4 (5 H, aromatic); mass spectrum (70 eV) *m/e* (rel intensity) 180 (M^+, 36), 149 (16), 107 (22), 94 (48), 87 (100).

Anal. Calcd for $C_{10}H_{12}O_3$: mol wt, 180.0786. Found: mol wt (high resolution mass spectrum), 180.0787.

Results

Chemical and Radiochemical Synthesis of Hexestrol Diazoketopropyl Ether and Hexestrol Azide. We have previously described the preparation of the diazo and azide hexestrol derivatives (Katzenellenbogen et al., 1973a). While it was straightforward, the route to the diazoketopropyl ether was long (Scheme I). We investigated the approach that appeared to be the very simplest, the direct etherification of

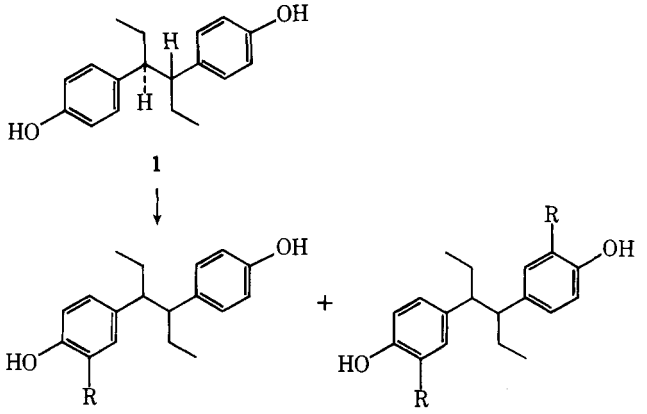
SCHEME I: Chemical and Radiochemical Syntheses of Hexestrol Diazoacetonyl Ether. Yields in Brackets Are from Radiochemical Syntheses



hexestrol (**1**) with bromodiazoacetone (**2**). Bromodiazoacetone (**2**) is a relatively stable yellow liquid and etherification of both labeled and unlabeled hexestrol proceeded without complication (Scheme I: **1**–**5**). [^{3}H]Hexestrol diazoketopropyl ether is stable indefinitely when stored at 0–4 °C in 9:1 benzene–ethanol at concentrations below 2 mCi per mL.

Our previous route to hexestrol azide (**8a**) also involved several steps (Scheme II): nitration (with separation of nitration products), reduction, and diazotization–azide coupling. In the interest of simplifying the synthesis of this compound, we were attracted by the reports of Tedder and Theaker (1958) that reactive phenols could be diazotized directly by prolonged exposure to a large excess of nitrous acid and by the methods of McKillop et al. (1969a–c) using thallation to synthesize substituted aromatics. Although neither of these methods proved suitable for the radiochemical synthesis of **8a**, we were successful in utilizing the original multistep procedure (Scheme II).

SCHEME II: Chemical and Radiochemical Syntheses of Hexestrol Azides. Yields in Brackets Are from Radiochemical Syntheses.



6a, R = NO_2, 30% [39%]
7a, R = NH_2, 92% [84%]
8a, R = N_3, 69% [13%]

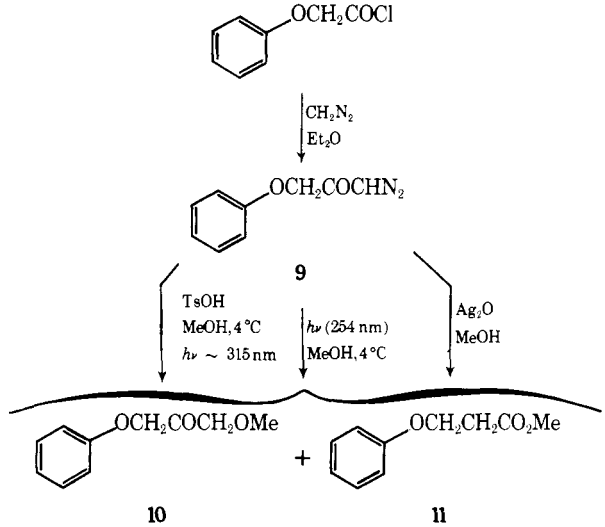
6b, R = NO_2, 14% [21%]
7b, R = NH_2, 75% [94%]
8b, R = N_3, 31% [29%]

Radiochemical Synthesis. Selective mononitration of [^{3}H]hexestrol is difficult to achieve since the addition of 1 equiv of nitric acid to the trace quantity of [^{3}H]hexestrol is not feasible. Appreciable amounts of both mono- and dinitro products could be obtained, however, by limiting the reaction time, and they can be separated by thin-layer chromatography. The corresponding amines were obtained by dithionite reduction, and because of their extreme sensitivity to oxidation, they were carried on immediately without further manipulation.

Diazotization of the mono- and diamine, followed by quenching with sodium azide, gave modest yields of the [^{3}H]hexestrol mono- and diazides, which could be purified by high-pressure liquid chromatography in the normal phase partition mode. Radiochemical purities in excess of 96% were obtained for both compounds. These derivatives were stored in the dark at 0–4 °C in 9:1 benzene–ethanol at concentrations below 2 mCi per mL. One sample, stored for nearly 2 years under these conditions, reassayed at a radiochemical purity of 70%.

Photochemical Behavior of the Hexestrol Derivatives in Solution: Diazoketopropyl Ethers. To simplify product analysis, the phenyl rather than the hexestrol derivative was used in the photochemical studies (Scheme III). Irradiation of a 1% solution of phenyl diazoketopropyl ether (**9**) in methanol at 254 nm for 1 h produced two photoproducts in a 2:1 ratio: methyl 3-phenoxypropionate (**11**), resulting from a Wolff rearrangement, and α-phenoxy-α'-methoxyacetone (**10**), resulting from the direct insertion into methanol. Figure 1 shows the ^{1}H NMR spectra of the starting diazoacetonyl ether (**9**) (panel A), the mixture of photoproducts **10** and **11** (panel B), and the spectra of pure **10** and **11** (panels C and D), prepared by the independent routes shown in Scheme III.

SCHEME III: Solution Photochemical Behavior of Phenyl Diazoacetonyl Ether



From the photoattachment studies on hexestrol diazoketopropyl ether described in the following paper (Katzenellenbogen et al., 1977b), it was evident that the free phenol (hexestrol) was also being produced as a photoproduct. The identity of the hexestrol as a photoproduct was established both by thin-layer chromatography and by isotope dilution–recrystallization (see legend, Figure 2, left panels). In order to

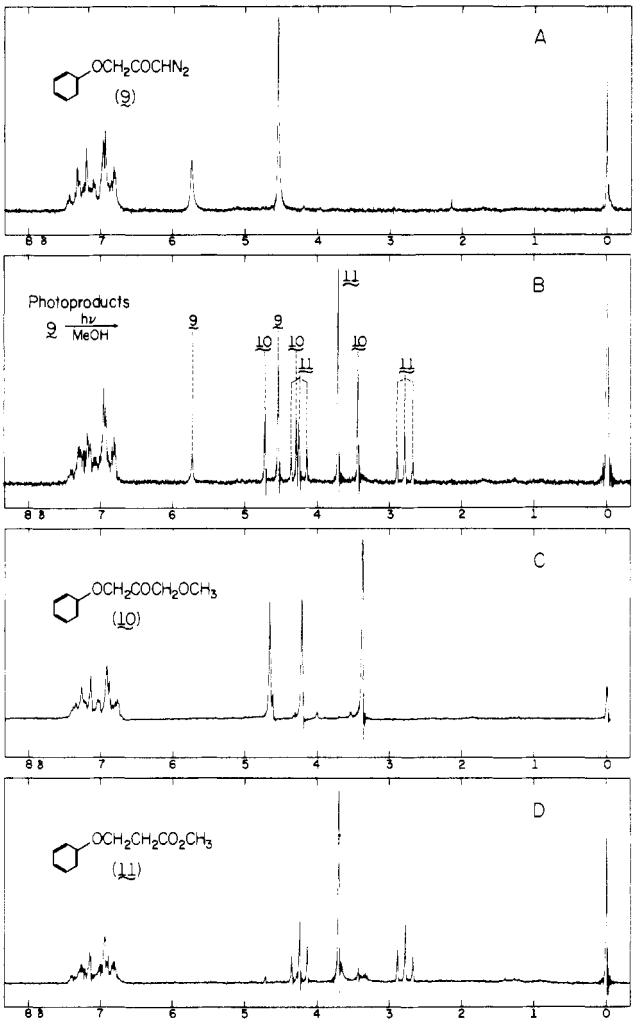


FIGURE 1: Proton magnetic resonance (60 MHz) spectra of phenyl diazoketopropyl ether **9** (A) and the two photoproducts, phenoxymethoxyacetone **10** (C) and methyl 3-phenoxypropionate **11** (D). Panel B shows the spectrum of a mixture of photoproducts resulting from an incomplete photolysis of **9**; peak assignments are noted. For details, see Experimental Section.

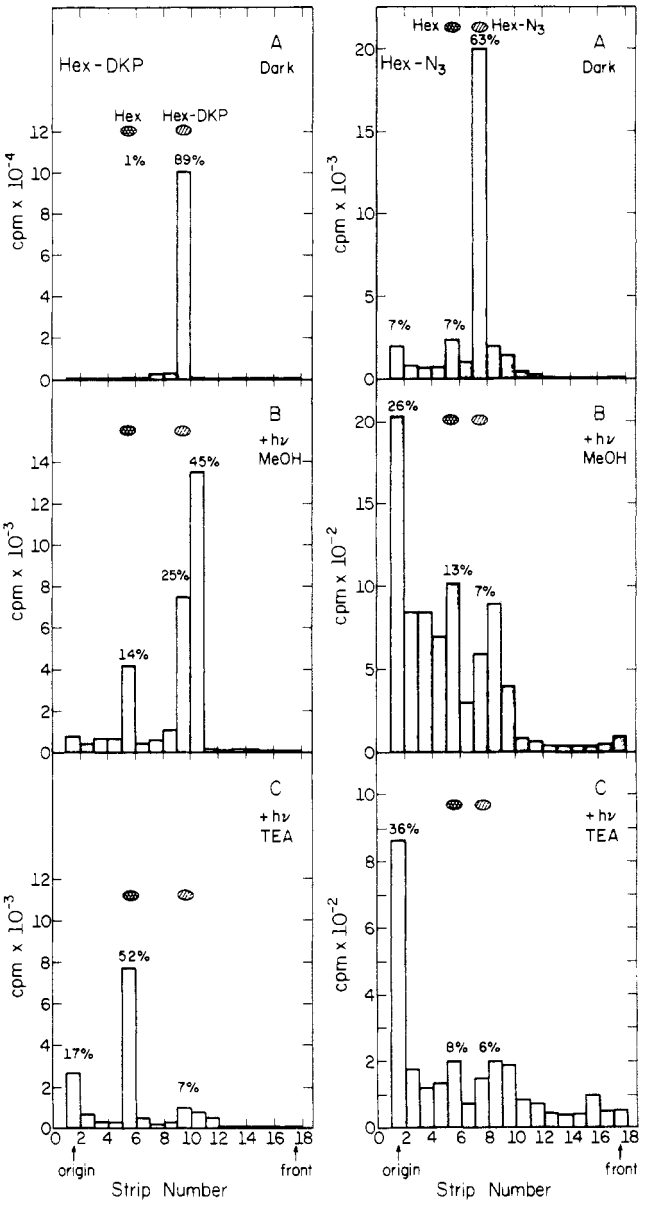


FIGURE 2: (Left) Thin-layer chromatographic analysis of [^{3}H]hexestrol diazoketopropyl ether (**5**) (A) and its photoproducts in methanol (B) and buffer (C). One microliter of a stock solution (225 μCi per mL) of [^{3}H]-hexestrol diazoketopropyl ether (**5**) was dried under a stream of nitrogen and redissolved in either 50 μL of methanol, or 5 μL of ethanol to which 45 μL of TEA buffer was added. These samples were photolyzed at 254 nm for 1.5 min in a photochemical reactor described previously (Katzenellenbogen et al., 1974). Three microliters from each sample were spotted onto a 20-cm silica gel thin-layer plate onto which had already been spotted 3 μg of unlabeled hexestrol (**1**) and 8 μg of unlabeled hexestrol diazoketopropyl ether (**5**). These were developed twice in chloroform, and radiochemical purity was determined as described in the Experimental Section after visualization with iodine vapor. Unphotolyzed [^{3}H]hexestrol diazoketopropyl ether was similarly chromatographed (A). To establish the identity of the hexestrol generated by photolysis in TEA buffer, a 10-μL aliquot of the same stock solution was dried down and redissolved in 2 μL of ethanol and 8 μL of TEA buffer, and then irradiated and chromatographed with carrier as before. The hexestrol spot was visualized under UV light and was eluted into 1.5 mL of methanol. Unlabeled hexestrol (24.9 mg) was added, to give a sample with a specific activity of 57 525 dpm per mg. This sample was recrystallized twice from THF–hexane and then twice from ethanol–water giving the following specific activities, respectively: 41 671, 45 430, 44 629, and 41 642. (Right) Thin-layer chromatographic analysis of [^{3}H]hexestrol azide (**8a**) (A) and its photoproducts in methanol (B) and buffer (C). One-microliter aliquots of a stock solution of [^{3}H]hexestrol azide (78 μCi per mL) were prepared and photolyzed for 5 min as described for [^{3}H]hexestrol diazoketopropyl ether (left panels). Unlabeled hexestrol azide (2.5 μg) was used as a marker for chromatographic separation, and development was done with 1% methanol in chloroform. The sample used in this experiment was 2 years old and had a radiochemical purity of 63%.

determine how significant this reaction was quantitatively, a sample of [^{3}H]hexestrol diazoketopropyl ether was irradiated in buffer and in methanol. Thin-layer chromatography indicated that hexestrol constituted 52% of the total photoproducts from the buffer irradiation and 14% from the methanol reaction (Figure 2, left panels). While the precise path by which the free phenol arises is not known, it should be noted that it could be formed simply by β elimination from the rearrangement product (cf. structure **11**).

Azides. Photolysis of [^{3}H]hexestrol azide gives a complex mixture of products with a wide range of polarities; hexestrol does not appear to be a major photoproduct in either methanol or buffer (Figure 2, right panels). In larger scale photolysis of unlabeled hexestrol azide in alcohol solvents, aminohexestrol appeared to be the principal photoproduct (TLC identification). One can easily imagine the formation of multiple and polar products through oxidative degradation of this species or by condensation or polymerization of its photochemical precursors, the nitrene or *o*-quinonimine (see Discussion).

Discussion

These radiochemical syntheses and solution photochemical studies on hexestrol diazoketopropyl ether (**5**) and hexestrol

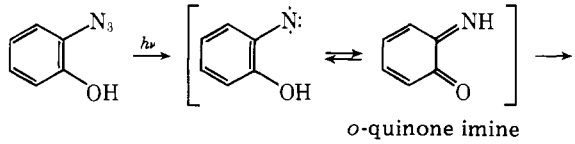
azide (**8a**) were a prelude to an investigation of their reversible and irreversible (photochemical) interactions with the proteins in estrogen receptor preparations, described in the following account (Katzenellenbogen et al., 1977b). These two compounds were selected for detailed study on the basis of their behavior in indirect (competition) assays of their reversible binding affinity with the uterine estrogen receptor and their ability to photochemically interact (inactivate) with the receptor (Katzenellenbogen et al., 1973b, 1974).

We were successful in simplifying considerably the synthesis of hexestrol diazoketopropyl ether so that its preparation in radiolabeled form was very convenient. Attempts to achieve a similar simplification of the synthesis of hexestrol azide were not successful; nevertheless, radiolabeled material could be prepared by careful execution of the earlier, multistep route. In contrast to the experiences of Wolff et al. (1975), who found some tritiated corticosteroid diazoketone derivatives to be very unstable, we have been fortunate that both of our derivatives have good radiochemical stability.

The photochemical behavior of diazocarbonyl compounds has been the subject of many studies (for reviews, see: Kirmse, 1971; Knowles, 1972; Baron et al., 1973; Cooperman, 1976). Direct irradiation produces either an α-carbonyl carbene, or, by Wolff rearrangement, a ketene (for a review, see Rodina and Korobitsyna, 1967). The percent of Wolff rearrangement observed in various α-diazocarbonyl systems appears to decrease as substituents withdraw electron charge density; the 66% rearrangement that we observe with our diazoketone is consistent with other values in the literature (Chaimovich et al., 1968; Converse and Richards, 1969; Hexter and Westheimer, 1971; Hixson and Hixson, 1972; Horner et al., 1951, 1958; Strausz et al., 1968; van Leusen and Strating, 1970; Vaughan, 1970; Weygand and Bestman, 1959; Weygand et al., 1961).

The photochemistry of aromatic azides has also been studied extensively (Smith, 1970; Knowles, 1972; Cooperman, 1976; Sundberg et al., 1972; Smalley and Suschitzky, 1970); the product of direct photolysis is the nitrene, mainly in the singlet state. While the photochemistry of azidoquinones has been studied in detail (see, for example, Moore and Weyler, 1971), the photochemical behavior of azidophenols has not been described. In addition to the normal insertion reactions of nitrene, one path open to aryl nitrenes bearing an hydroxyl group in the ortho or para position is a hydrogen transfer process to give the quinone imine (Scheme IV). The systems most analogous to

SCHEME IV: Photochemical Behavior of Aryl Azides Substituted Ortho or Para with Heteroatoms.



o-quinone imine

the azidophenols are *p*-methoxyphenyl azide studied by Horner et al. (1963), and *p*-azido-*N*,*N*-diethylaniline studied by Baetzold and Tong (1971). On irradiation, these systems produce a nitrene which can protonate to give a quinone imine or quinone diimine system. Many of the polar photoproducts produced in the irradiation of hexestrol azide may arise from nucleophilic addition to the quinone imine.

The following paper (Katzenellenbogen et al., 1977b) outlines the reversible binding characteristics and the photocovalent attachment behavior of hexestrol azide and hexestrol diazoketopropyl ether with the estrogen receptor and describes the first example of photoaffinity labeling of a steroid hormone receptor.

References

Anderson, L. E., and McClure, W. O. (1973), *Anal. Biochem. 51,* 173.

Arndt, F., and Amende, J. (1928), *Chem. Ber. 61,* 1122.

Baetzold, R. C., and Tong, L. K. J. (1971), *J. Am. Chem. Soc. 93,* 1394.

Baron, W. J., DeCamp, M. R., Henrick, M. A., Jones, M., Levin, R. H., and Sohn, M. B. (1973), in Carbenes, Vol. I, Jones, M., Jr., and Moss, R. A., Ed., New York, N.Y., Wiley-Interscience, pp 1–151.

Chaimovich, H., Vaughan, R. J., and Westheimer, F. H. (1968), *J. Am. Chem. Soc. 90,* 4088.

Converse, C. A., and Richards, F. F. (1969), *Biochemistry 8,* 4431.

Cooperman, B. S. (1976), in Aging, Carcinogenesis, and Radiation Biology, Smith, K. C., Ed., New York, N.Y., Plenum Press, pp 315–340.

Hexter, C. S., and Westheimer, F. H. (1971), *J. Biol. Chem. 246,* 3934.

Hixson, S. S., and Hixson, S. H. (1972), *J. Org. Chem. 37,* 1279.

Horner, L., Christmann, A., and Gross, A. (1963), *Chem. Ber. 96,* 399.

Horner, L., Kirmse, W., and Muth, K. (1958), *Chem. Ber. 91,* 430.

Horner, L., Spietschka, E., and Gross, A. (1951), *Justus Liebigs Ann. Chem. 573,* 17.

Katzenellenbogen, J. A. (1974), *Ann. Rep. Med. Chem. 9,* 222.

Katzenellenbogen, J. A. (1976), in Biochemical Actions of Hormones, Vol. IV, Litwack, G., Ed., New York, N.Y., Academic press, (in press).

Katzenellenbogen, J. A., Carlson, K. E., Johnson, H. J., Jr., and Myers, H. N. (1977b), *Biochemistry 16* (following paper in this issue).

Katzenellenbogen, J. A., and Hsiung, H. M. (1975), *Biochemistry 14,* 1736.

Katzenellenbogen, J. A., Johnson, H. J., Jr., Carlson, K. E., and Myers, H. N. (1974), *Biochemistry 13,* 2986.

Katzenellenbogen, J. A., Johnson, H. J., Jr., and Myers, H. N. (1973b), *Biochemistry 12,* 4085.

Katzenellenbogen, J. A., Johnson, H. J., Jr., Myers, H. N., and Carlson, K. E. (1976), *J. Toxicol. Environ. Health, Suppl. 1,* 205.

Katzenellenbogen, J. A., Johnson, H. J., Jr., Myers, H. N., Carlson, K. E., and Kempton, R. J. (1977a), in Bioorganic Chemistry IV: Electron Transfer and Energy Conversion, Cofactors, Probes, van Tamelen, E. E., Ed., New York, N.Y., Academic Press (in press).

Katzenellenbogen, J. A., Myers, H. N., and Johnson, H. J., Jr. (1973a), *J. Org. Chem. 38,* 3525.

Kirmse, W. (1971), Carbene Chemistry, New York, N.Y., Academic Press.

Knowles, J. R. (1972), *Acc. Chem. Res. 5,* 155.

Lwowski, W. (1970), *Nitrenes,* New York, N.Y., Wiley-Interscience.

McKillop, A., Bromley, D., and Taylor, E. C. (1969a), *Tetrahedron Lett.,* 1623.

McKillop, A., Fowler, J. S., Zelesko, M. J., Hunt, J. D., and Taylor, E. C. (1969b), *Tetrahedron Lett.,* 2423.

McKillop, A., Fowler, J. S., Zelesko, M. J., Hunt, J. D., Taylor,

E. C., and McGillivray, G. (1969c), *Tetrahedron Lett.*, 2427.
Moore, H. W., and Weyler, W. (1971), *J. Am. Chem. Soc. 93,* 2812.
Piazza, G., Sorriso, S., and Foffani, A. (1968), *Tetrahedron 24,* 4751
Rodina, L. L., and Korobitsyna, I. K. (1967), *Russ. Chem. Rev. 36,* 260.
Smalley, R. K., and Suschitzky, H. (1970), *Chem. Ind. (London),* 1338.
Smith, P. A. S. (1970), in Nitrenes, Lwowski, W., Ed., New York, N.Y., Wiley-Interscience, Chapter 4.
Strausz, O. P., DoMinh, T., and Gunning, H. E. (1968), *J. Am. Chem. Soc. 90,* 1660.
Sundberg, R. J., Suter, S. R., and Brenner, M. (1972), *J. Am. Chem. Soc. 94,* 513.
Tedder, J. M., and Theaker, G. (1958), *J. Am. Chem. Soc. 80,* 2573.
van Leusen, A. M., and Strating, J. (1970), *Q. Rep. Sulfur Chem. 5,* 67.
Vaughan, R. (1970), Ph.D. Thesis, Harvard University, Cambridge, Mass.
Weygand, F., and Bestman, H. (1959), *Chem. Ber. 92,* 528.
Weygand, F., Dworschak, H., Koch, K., and Konstas, S. T. (1961), *Angew. Chem. 73,* 409.
Williams, D. L., and Ronzio, A. A. (1950), *J. Am. Chem. Soc. 72,* 5787.
Wolff, M. E., Feldman, D., Catsoulacos, P., Funder, J. W., Hancock, C., Amano, Y., and Edelman, I. (1975), *Biochemistry 14,* 1750.

Estrogen Photoaffinity Labels. 2. Reversible Binding and Covalent Attachment of Photosensitive Hexestrol Derivatives to the Uterine Estrogen Receptor†

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ABSTRACT: The ability of two radiolabeled, photoreactive estrogen analogues, [^{3}H]hexestrol diazoketopropyl ether ([^{3}H]Hex-DKP) and [^{3}H]hexestrol azide ([^{3}H]Hex-N_3), to covalently label the uterine estrogen receptor is studied. Lamb uterine estrogen receptor preparations that have been partially purified (ammonium sulfate precipitation, Sephadex G-200 chromatography) and disaggregated by limited trypsinization can be electrophoresed on polyacrylamide gels under conditions where binding activity is preserved. This electrophoretic procedure was used to fractionate the proteins labeled by the two photoreactive estrogen analogues. Prior to photolysis, peaks of radioactivity indicating estrogen specific binding of [^{3}H]-Hex-N_3 and [^{3}H]Hex-DKP are evident on the gels, although dissociation of the latter compound is extensive. When preparations of uterine estrogen receptor that contain the photoreactive derivatives are irradiated and then electrophoresed, reversibly labeled proteins can be distinguished from irreversibly labeled ones (covalently bonded), by extraction of the individual gel slices with organic solvents. While no irreversible binding to receptor appears to result from irradiation with [^{3}H]Hex-DKP, irradiation with [^{3}H]Hex-N_3 does covalently label the estrogen receptor. The receptor covalently labeled with [^{3}H]Hex-N_3 has the same electrophoretic mobility as the unlabeled receptor; the covalent labeling process is estrogen-site specific, and the efficiency of labeling (15–20%) is consistent with the inactivation efficiency of Hex-N_3, previously measured by an indirect assay. This is the first example of the labeling of a steroid hormone receptor by photoaffinity labeling.

We have been investigating the technique of photoaffinity labeling as a means for studying the details of estrogen interaction with the uterine estrogen receptor (for reviews, see Katzenellenbogen, 1977; Katzenellenbogen et al., 1976, 1977a). Ideally, using this approach one should be able to covalently label the estrogen receptor in a manner that would be both selective and efficient; the covalently labeled receptor would then be available for rigorous and intensive studies that would no longer be limited to conditions consistent with normal, reversible hormone binding activity.

In principle, such a chemical approach is simple, but a number of problems are encountered in its reduction to practice: While considerable information about binding specificity of the receptor is available, little is known about the nature, distribution, and the chemical reactivity of the binding site residues; similarly, the chemical reactivity of affinity labeling reagents cannot be predicted completely. In the face of these circumstances, we considered it judicious to take an indirect but systematic approach to the development of reagents for affinity labeling the estrogen receptor. Our basic protocol has been first (1) to synthesize in nonradioactive form candidate compounds designed on the basis of the best knowledge available as to the binding specificity and chemical reactivity of the hormone binding site (Katzenellenbogen et al., 1973a), and then (2) to determine indirectly, by competition assays with radiolabeled estradiol (E_2), the binding affinity of these

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