87394-51-2; **2o** (free base), 84951-44-0; **2p**, 87394-52-3; **2p** (free base), 87394-64-7; **2q**, 87394-53-4; **2q** (free base), 87394-65-8; **2r**, 87394-54-5; **3**, 87394-41-0; **3** (free base), 87394-66-9; 4, 39512-50-0; 2-chloro-3-fluoropyridine, 17282-04-1; piperazine, 110-85-0; 2-chloro-3-iodopyridine, 78607-36-0; benzyl bromide, 100-39-0; 2-chloro-3-nitropyridine, 5470-18-8; 5-chloro-2-pyridinol, 4214-79-3; trifluoromethanesulfonyl chloride, 421-83-0; 5-chloro-2-[[(trifluoromethyl)sulfonyl]oxy]pyridine, 87412-10-0.

Estrogen Receptor Binding and Estrogenic/Antiestrogenic Effects of Two New Metabolites of Nitromiphene, 2-[*p*-[2-Nitro-1-(4-methoxyphenyl)-2-phenylvinyl]phenoxy]-*N*-ethylpyrrolidine

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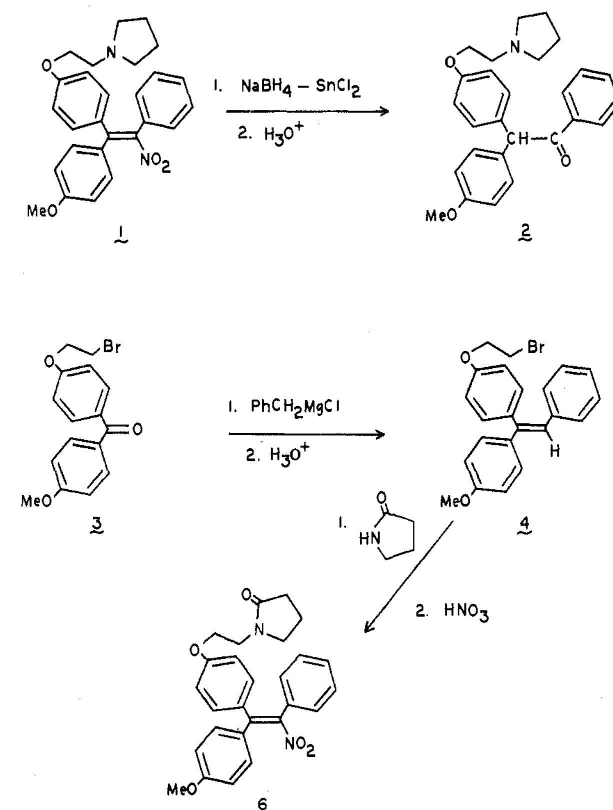
Reduction of the triarylethylene antiestrogen 2-[*p*-[2-nitro-1-(4-methoxphenyl)-2-phenylvinyl]phenoxy]-*N*-ethylpyrrolidine (1) with sodium borohydride–stannous chloride afforded 2-(*p*-methoxyphenyl)-*p*′-(2-pyrrolidin-1-ylethoxy)deoxybenzoin (2). Incubation of 1 with rat cecal content suspension under aerobic or anaerobic conditions also resulted in the generation of 2. The lactam analogue of 1 (6) was prepared by condensation of 4-(2-bromoethoxy)-4′-methoxybenzophenone (3) with benzylmagnesium chloride, followed by dehydration, amidation with 2-pyrrolidinone, and nitration. A metabolite with chromatographic and spectral properties identical with those of 6 was found in extracts from incubation mixtures of 1 with phenobarbital-induced rat liver 9000*g* supernatant. Compound 2 did not exhibit appreciable binding to the rat uterine cytosol estrogen receptor at concentrations of up to 1×10^{-6} M and had no estrogenic or antiestrogenic activity in the 3-day rat uterotropic assay. By contrast, 6 had estrogen receptor affinity somewhat greater than that of 1 and slightly greater estrogenic activity accompanied by reduced antiestrogenic activity in comparison with those of 1.

Triarylethylene antiestrogens, including 1[1] (CI 628, nitromiphene), appear to exert their effects through binding to cytosol estrogen receptors. The resulting antiestrogen–estrogen receptor complexes are much less effective in promoting estrogenic responses than is that involving estradiol.[2] The ability of triarylethylene antiestrogens to antagonize the growth-promoting effect of estradiol in target tissue has focused attention on the potential application of these compounds as therapeutic alternatives to surgery in estrogen-dependent cancers.[3] Also, compounds such as 1 have been of value in studies of the molecular mechanism of action of estrogens in target tissues.[4,5]

As is the case with other triarylethylenes, 1 undergoes biotransformation to a phenolic metabolite, *O*-demethyl-1. This metabolite binds more strongly to estrogen receptors than does 1[6,7] and appears to contribute substantially to the biological effects seen on administration of 1.[7] In addition to the *O*-methyl group, the structure of 1 contains several other moieties, in particular the pyrrolidine ring and the nitro group, that are major sites of biotransformation in other drugs. Thus, the pyrrolidine rings in nicotine and tremorine undergo hepatic *N*-methylene oxidation to afford the lactam metabolites cotinine and oxotremorine, respectively.[8-10] And numerous aromatic and heteroaromatic nitro compounds have been demonstrated to undergo reduction to the corresponding amino compounds in the presence of gastrointestinal microflora.[11-13]

Scheme I



(1) For convenience, the "trans" configurations of 1 and other triarylethylenes are shown in Scheme I. Actually, each of these compounds is an approximately equal mixture of both geometric isomers.

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Table I. Spectral Properties of 1 and Authentic and Metabolic 2 and 6

	1	2		6	
		authentic	metabolic	authentic	metabolic
	Ultraviolet Spectral Data[a]				
λ_{max}, nm (ϵ, mM^{-1}, cm^{-1})	282 (15.5) 239 (21.6)	272 (9.0) 232 (37.3)	274 232	283 (14.1) 237 (19.7)	282 232
	Mass Spectral Data,[b] *m/z* (Relative Abundance)				
$M^{+\cdot}$	444 (1)	415 (8)	415 (2)	458 (21)	458 (25)
M − benzoyl		310 (29)	310 (9)		
side chain	98 (6) 84 (100)	98 (52) 84 (100)	98 (41) 84 (100)	112 (100)	112 (100)

[a] Ultraviolet spectra were obtained with ethanol as solvent. [b] Low-resolution mass spectra were obtained at 70 eV (probe temperature, 220 °C).

We wanted to find out whether 1 underwent analogous biotransformations, yielding 2 (after hydrolysis of the enamine reduction product) and 6.

Unconjugated triarylethylene metabolites generally retain estrogen receptor affinities at least as great as those of the parent drugs.[7,14,15] In addition, synthetic modification of the basic side chains[16] or the triarylethylene moieties[17,18] of compounds related to 1 has resulted in analogues with potent estrogenic and/or antiestrogenic activity. We thus were prompted to assess the estrogen receptor affinities and estrogen agonist and antagonist effects of 2 and 6.

Results

Chemistry. Ketone 2 was prepared from 1[19] by reduction with sodium borohydride–stannous chloride in ethanol[20] (Scheme I). In contrast to the conventional procedure, we found it necessary to combine the sodium borohydride with 1, prior to adding the stannous chloride. Conjugate reduction was not observed under these conditions, although such reduction of some α,β-unsaturated nitroolefins with sodium borohydride has been reported.[21,22] Addition of stannous chloride discharged the yellow color of the reaction mixture during the course of the reaction. The reducing agent is probably metallic tin, generated in situ and consumed as quickly as it is produced.[23] Although conversion of nitroolefins to ketones has often been carried out by treatment with zinc dust in refluxing acetic acid,[24] 1 decomposed rapidly when heated above 75 °C in this solvent.

Spectral data suggested that 2 did not enolize in protic or aprotic solvents. The infrared spectrum (CCl_4) featured strong carbonyl absorption at 1690 cm^{-1}, with no absorbance in the 3200–3700-cm region. The NMR spectrum

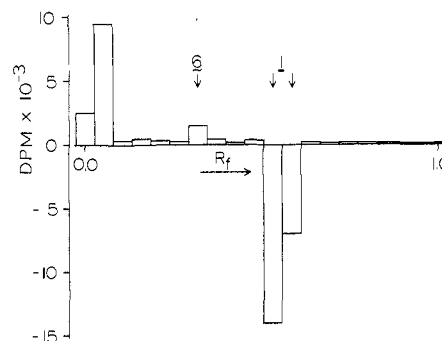

Figure 1. Differential radiochromatographic analysis of extracts from incubation of [^{14}C]1 with liver 9000*g* supernatant from phenobarbital-treated rats. Arrows indicate R_f values of standard compounds. Data are averages of four experiments and are plotted after substracting disintegrations per minute in chromatograms from control (no cofactor added) incubation mixture extracts.

Table II. Recoveries of 1 and 2 from Incubation Mixtures Containing [^{14}C]1[a]

incubation mixtures contained	incubation atmosphere	nmols found: 1	nmols found: 2
cecal contents[b]	air	48	144
cecal contents + 2 mM EDTA[c]	air	76	128
cecal contents	N_2[e]	37	172
microsomes[f]	air or N_2[e]	*d*	0

[a] Results are averages of two to four experiments with separate animals. [b] Cecal contents were homogenized in 2 vol of ice-cold 0.1 M potassium phosphate buffer, pH 7.4 (KPPB 7.4). Incubation mixtures contained 2 mL of this suspension and 4 mL of 0.2 M KPPB 7.4. Reaction was started by addition of 0.08 μCi (0.36 μmol) of [^{14}C]1 in 20 μL of *N,N*-dimethylacetamide. Incubation mixtures were shaken for 1 h at 70 cpm (37 °C) and subjected to analysis as described in the Experimental Section. [c] Cecal content suspension was preincubated for 1 h with 2 mM EDTA prior to adding [^{14}C]1. [d] Recovery was not determined. [e] Serum-capped incubation flasks were evacuated with a water aspirator and returned to atmospheric pressure by introduction of pure nitrogen. This procedure was repeated six times prior to adding [^{14}C]1. [f] Incubation mixtures containing microsomal suspensions were prepared and used as described in the Experimental Section.

($CDCl_3$) featured a singlet at 5.92 ppm, which integrated for one proton, and the ultraviolet spectrum (EtOH) exhibited maxima and respective absorptivities markedly different from those of 1 and 6 (Table I).

Compound 6 was prepared in three steps starting from benzophenone 3.[19] Reaction of 3 with benzylmagnesium chloride, followed by acid-catalyzed dehydration, gave triarylethylene 4. This was treated with 2-pyrrolidinone, in a mixture of tetrahydrofuran and powered KOH, using

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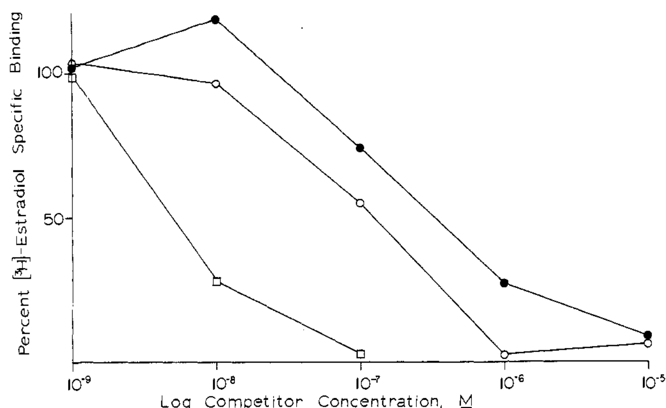

Figure 2. Effect of increasing concentrations of estradiol (□), oxonitromiphene, 6 (○), and nitromiphene, 1 (●), on the binding of [^{3}H]estradiol to uterine cytosol from mature rats. Specifically bound radioactivity (disintegrations per minute) is plotted as a percentage of that in control incubations.

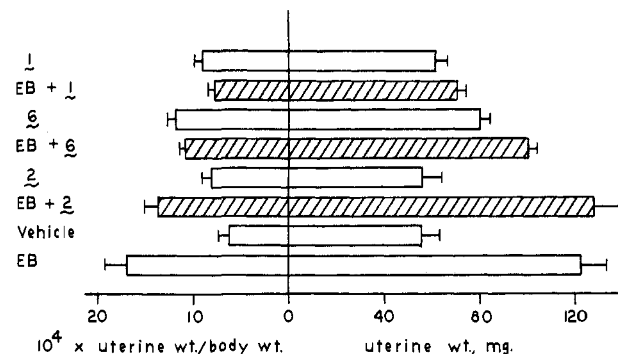

Figure 3. Uterotropic effect of 1, 2, and 6 in immature rats when administered without, and with, estradiol benzoate (EB). The dose of each triarylethylene was 50 μg per animal per day for 3 days; that of EB was 0.5 μg per animal per day for 3 days. Animals in the control group received vehicle only. Standard errors are indicated by brackets. There were six to seven animals per treatment group.

tetrabutylammonium bisulfate as phase-transfer catalyst. The resulting lactam (**5**) was treated with fuming nitric acid to afford **6** as an approximately equal mixture of *E* and *Z* isomers, as evident by NMR spectral analysis. These isomers were indistinguishable by TLC (Figure 1).

Metabolism Studies. Incubation of **1** with rat cecal content suspension for 1 h resulted in consumption of most of the substrate. The major metabolite, isolated chromatographically, was indistinguishable from authentic **2** by TLC (R_f 0.45) and had spectral properties similar to those of authentic **2** (Table I). Results of a preliminary study of factors affecting the formation of **2** (metabolism of [^{14}C]**1**) are summarized in Table II. Conversion was slightly less under aerobic conditions than when anaerobic conditions were used and was decreased modestly in the presence of 2 mM EDTA. Use of a cecal content suspension that had previously been frozen at −80 °C and stored for 5 weeks at −17 °C resulted in no detectable conversion of **1** to **2**. Formation of **2** was also not seen using liver microsomal incubation mixtures under aerobic or anaerobic conditions.

Incubation of [^{14}C]**1** with rat liver 9000*g* supernatant resulted in consumption of substrate, accompanied by the appearance of two labeled metabolites (Figure 1). The major of these has not been characterized, but it may be *O*-demethyl-**1**, a previously reported metabolite with similar chromatographic properties.[25] The other metabolite was isolated chromatographically from preparative incubations to which unlabeled **1** was added and was found to have spectral features identical with those of authentic **6** (Table I). The average recovery of this metabolite from incubation mixtures was 24 nmol (g of liver)$^{-1}$ 30 min^{-1} (average of four experiments). Under the same conditions, use of the microsomal suspension rather than the 9000*g* supernatant resulted in a 59% decrease in the amount of **6** recovered.

Estrogen Receptor Binding Studies. The ability of **1**, **2**, and **6** to compete with [^{3}H]estradiol for cytoplasmic receptors from mature rat uterus was determined by adaption of the dextran-coated charcoal adsorption technique.[5,14,26] Results are summarized in Figure 2. In contrast to **1** and **6**, **2** did not displace estradiol significantly at concentrations of up to 1×10^{-6} M. Thus, data for this compound are not shown.

Estrogenic and Antiestrogenic Effects of Compounds 1, 2, and 6. Uterotropic activity was determined by using the 3-day immature uterine weight test,[27,28] in comparison with estradiol benzoate. The ability of these compounds to antagonize estradiol benzoate induced uterine weight gain was determined similarly. Results are in Figure 3. As previously reported,[5] **1** had modest estrogenic activity and decreased greatly the growth-promoting effect of estrogen. Compound **6** had increased estrogenic activity and had less antiestrogenic activity than did **1**, while compound **2** had neither estrogenic nor antiestrogenic activity.

Discussion

Previous results suggested that the ability of the polar side chain in triarylethylenes to interact with complimentary binding groups in the estrogen receptor through *hydrogen bonding* was a determinant of antiestrogenic activity.[16] Our finding that lactam **6** had such activity, though not as great as that of **1**, is consistent with this hypothesis. The decreased antagonistic effect of **6** in comparison with that of **1** may have been the result of its greater partial agonist effect: **1** and **6** stimulated uterine weight gain by 11 and 43%, respectively (Figure 3). Several studies have suggested that antiuterotropic effects of triarylethylenes are limited by their inherent estrogenic effects.[18,27,29]

Hepatic biotransformation of pyrrolidine and homologous rings in drug molecules is thought to involve initial microsomal *N*-methylene hydroxylation, followed in many cases by further oxidation to lactams via a second nonmicrosomal (soluble) enzyme.[30] In accord with this, our results suggest that both microsomal and soluble enzymes participate in conversion of **1** to **6**, since conversion was decreased when soluble enzymes were omitted from incubation mixtures. Formation of **6** did not represent a major route of metabolism of **1** (Figure 1). In contrast, analogous triarylethylenes bearing dialkylamino side chains instead of pyrrolidine rings underwent liver microsomal *N*-methylene hydroxylation (N-dealkylation) as the predominant metabolic route.[31,32]

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Compound 2 had no detectable effects in vivo under our conditions, presumably due to its lack of affinity for the cytosol estrogen receptor. This lack of affinity may have been due to its failure to enolize (see above) to a tautomer containing the *trans*-stilbene (or 1,1-diarylethylene) moiety characteristic of most nonsteroidal estrogens and antiestrogens.[33,34] Alternatively, it may have been due to the absence in the receptor of a complimentary binding group for the carbonyl (or enolic hydroxyl) group of 2.

Our results suggest that facile conversion of 1 to 2 in the presence of rat cecal content suspension was due to the presence of *Escherichia coli*. This microorganism is less sensitive to oxygen with regard to its ability to reduce nitro groups than are other intestinal bacteria, but it is inhibited by substances, such as EDTA, that chelate essential divalent metal cations, especially manganese.[35,36]

The extent to which 1 is converted to 2 and 6 in vivo remains to be determined. The presence of significant amounts of 6 would have interesting pharmacodynamic implications, since 6 had a greater partial agonist effect than did 1 under our conditions. The comparative pharmacokinetics of 1 and 6 may also be of interest, since 1 and other triarylethylene antiestrogens with *basic* side chains have been shown to bind with antiestrogen specific binding sites, found in a wide variety of tissues, which may influence their distribution and duration of action.[37-39] Triarylethylenes with nonbasic side chains did not have affinity for these binding sites.[39]

Experimental Section

All new compounds gave elemental analysis data within ±0.3% of calculated values, as determined by Atlantic Microlab, Inc., Atlanta GA. Melting points are uncorrected. Solvents and reagents were used as purchased, except that tetrahydrofuran was dried immediately before use by distillation from lithium aluminum hydride under a dry nitrogen stream. Rapid preparative column chromatography was performed according to the method of Still[40] by using 0.040–0.063 mm silica gel 60 (Brinkmann). Conventional column chromatography utilized Brinkmann silica gel 60, 0.063–0.200 mm. Thin-layer chromatography (TLC) was done using 5 × 20 cm silica gel GF_{254} plates (0.25-mm thickness, Analtech). The developing solvent was benzene–triethylamine (85:15, v/v). Synthesis of $[^{14}C]$1 (0.23 mCi/mmol) was described elsewhere.[41]

2-(4-Methoxyphenyl)-*p*′-[2-(1-pyrrolidinyl)ethoxy]deoxybenzoin (2). Nitromiphene (1) was synthesized according to the procedure outlined by Black et al.[19] and purified as the dihydrogen citrate salt (mp 82–86 °C; lit.[19] mp 85 °C, acetone) from 2-butanone–ether. Isolation of 1 as the free base, for use in the following reaction, was accomplished by liberation from an aqueous methanolic solution of the citrate salt by 10% Na_2CO_3, extraction with methylene chloride, drying (Na_2SO_4), and careful evaporation in vacuo (<50 °C) of solvent.

A mixture of 1 (2.7 g, 6.1 mmol), $NaBH_4$ (118 mg, 3.0 mmol), and ethanol (125 mL) was stirred at 45–50 °C for 2 h. The yellow solution was cooled to room temperature, $SnCl_2{\cdot}2H_2O$ (7.0 g, 30.5 mmol) was added in portions, and heating was continued for 5 h. The nearly colorless reaction mixture was cooled, diluted with ice-cold water (200 mL), and stirred for 45 min. There followed careful alkalinization to pH 10 with 10% Na_2CO_3 (250 mL) and extraction with chloroform (2 × 200 mL). The combined organic extracts were washed with brine (200 mL), water (3 × 150 mL), and dried (Na_2SO_4). Concentration in vacuo left crude 2 (1.4 g), which exhibited an acetophenone carbonyl signal (1690 cm^{-1}) in its infrared spectrum. In like manner, 2.4 g of 1 yielded 1.1 g of 2, which was combined with the first crop in stirring, chilled acetone (50 mL). Filtration of insoluble material and evaporation of solvent left 2.0 g of a viscous, tan oil. This was purified by rapid preparative column chromatography (60 g of silica; benzene–triethylamine, 30:1), followed by conventional column chromatography (50 g of silica; same eluent) to yield 0.55 g (12%) of 2 as a colorless oil: ^1H NMR ($CDCl_3$) δ 1.60–1.92 (4 H, m, pyrrolidinyl 3,4-CH_2), 2.44–2.68 (4 H, m, pyrrolidinyl N-CH_2), 2.82 (2 H, t, J = 6 Hz, >NCH_2CH_2O), 3.66 (3 H, s, OCH_3), 4.02 (2 H, t, J = 6 Hz, CH_2O), 5.92 (1 H, s, benzyl CH), 6.72–8.15 (13 H, 7, Ar H).

The dihydrogen citrate salt of 2 was isolated from 2-butanone–ether as a gummy residue. Trituration in ether gave a white powder, which was recrystallized from methanol–ether, mp 84–88 °C (softens). Anal. ($C_{27}H_{29}NO_3{\cdot}C_6H_8O_7{\cdot}H_2O$) C, H, N.

1-[4-(2-Bromoethoxy)phenyl]-1-(4-methoxyphenyl)-2-phenylethene (4). A solution of benzyl chloride (9.4 g, 74.3 mmol) in ether (100 mL) was added slowly to magnesium turnings (2.0 g, 82.3 mmol) and an iodine crystal. When vigorous reaction had begun, the remainder of the solution was added at such a rate as to maintain reflux. After completion of the addition, the reaction mixture was refluxed for 2 h and then cooled to room temperature. Compound 3, an intermediate in the synthesis of 1[19] (mp 106–108 °C; lit[19] mp 111.5–113 °C), was dissolved in 100 mL of tetrahydrofuran. This solution was added in a rapid dropwise manner to the ethereal solution of the Grignard reagent. The reaction mixture was stirred at reflux for 5 h. It was then cooled in an ice bath, quenched with 10% aqueous HCl (80 mL), and stirred for 30 min, during which time the unreacted magnesium was consumed. The ethereal and aqueous layers were separated, and the latter was further extracted with ether (100 mL). The combined organic extracts were washed with water (100 mL), and the solvent was removed in vacuo to leave an oily residue, which was dissolved in EtOH-THF-4 N HCl (50:50:10, v/v). The mixture was stirred overnight (16 h) at room temperature and then concentrated in vacuo, and the residue was dissolved in ether (200 mL). The yellow solution was substantially decolorized by washing with 10% NaOH (50 mL), water (100 mL), again with 10% NaOH (50 mL), and finally with water (3 × 100 mL). It was dried (Na_2SO_4) and concentrated in vacuo to leave a yellow oil (10 g). Unreacted 3 (0.5 g) was removed by filtration after stirring in ether (50 mL). The crude product was purified by rapid preparative column chromatography (80 g of silica; hexane–chloroform, 4:1) to yield 7.5 g (65%) of 4 as a viscous, colorless oil. Crystallization from ethanol afforded the analytical sample as white, flocculant crystals: mp 98–99.5 °C; ^1H NMR ($CDCl_3$) δ 3.56 (2 H, t, J = 6 Hz, CH_2Br), 3.80 (3 H, s, OCH_3), 4.24 (2 H, t, J = 6 Hz, CH_2O), 6.75–7.33 (14 H, m, =CH and Ar H). Anal. ($C_{23}H_{21}BrO_2$) C, H, Br.

2-[*p*-[1-(4-Methoxyphenyl)-2-phenylvinyl]phenoxy]-*N*-ethylpyrrolidin-2-one (5). A solution of 4 (5.8 g, 14.2 mmol) and 2-pyrrolidinone (1.2 g, 14.2 mmol) in dry tetrahydrofuran (25 mL) was added to a suspension of crushed KOH (1.2 g, 86.3%, 18.5 mmol)[42] and powdered tetrabutylammonium hydrogen sulfate (1.0 g, 3.0 mmol) in tetrahydrofuran (10 mL) over 30 min at room temperature under nitrogen. After completion of the addition, the reaction mixture was stirred at 40–50 °C for 5 h. The precipitate was filtered away and then washed with tetrahydrofuran, and the filtrate was concentrated in vacuo to leave an oil, which was partitioned between 100 mL each of methylene chloride and

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water. The organic extract was further washed with water (2 × 50 mL) and dried (Na_2SO_4). The solvent was removed in vacuo to leave 5.5 g of a viscous, plum-red oil. Purification by rapid preparative column chromatography (60 g of silica; 900 mL of petroleum ether–ethyl acetate, 9:1, then 500 mL of benzene–triethylamine, 10:1) furnished 3.0 g (51%) of **5** as a pale yellow oil: IR (liquid film) 1690 (lactam C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.77–2.54 (4 H, m, pyrrolidinyl 3,4-CH_2), 3.52 (2 H, t, J = 7 Hz, pyrrolidinyl 5-CH_2), 3.63 (2 H, t, J = 6 Hz, NCH_2CH_2O), 3.78 (s, 3 H, OCH_3), 4.05 (2 H, t, J = 6 Hz, CH_2O) 6.68–7.35 (14 H, m, =CH and Ar H). This material was used directly in the next reaction. A later repeat of the above reaction provided, after trituration in ether, the analytical sample as a white, crystalline powder, mp 117–118 °C. Anal. ($C_{27}H_{27}NO_3$) C, H, N.

2-[*p*-[2-Nitro-1-(4-methoxyphenyl)-2-phenylvinyl]phenoxy]-*N*-ethylpyrrolidin-2-one (6). To a solution of **5** (2.8 g, 6.8 mmol) in glacial acetic acid (25 mL) was added fuming nitric acid (90%, 1.0 mL, 13.6 mmol). The reaction mixture was stirred at room temperature for 2 h, during which time its color changed from dark green to orange. Careful concentration in vacuo (water bath <55 °C) yielded a red oil, which was partitioned between methylene chloride (50 mL) and 10% $NaHCO_3$ (50 mL). The organic extract was washed with water (3 × 50 mL) and dried (Na_2SO_4). Removal of solvent in vacuo left 2.9 g of a yellow solid, which exhibited a lactam carbonyl signal (1690 cm^{-1}) in its infrared spectrum (Nujol). Crude **6** was subjected to rapid preparative column chromatography (60 g of silica; benzene–triethylamine, 20:1). After attempts to crystallize the compound failed, it was rechromatographed as above, yielding 2.5 g (81%) of analytically pure **6**: mp 43–48 °C (softens); ^1H NMR ($CDCl_3$) δ 1.80–2.58 (4 H, m, oxopyrrolidinyl 3, 4-CH_2), 3.35–3.87 (4 H, m, oxopyrrolidinyl 5-CH_2 and >NCH_2CH_2O), 3.72 and 3.80 (3 H, ds, E/Z ≈ 1, OCH_3), 4.02 and 4.10 (2 H, dt, $E/Z \approx 1$, J = 6 Hz, CH_2O), 6.55–7.40 (13 H, m, Ar H). Anal. ($C_{27}H_{26}N_2O_5$) C, H, N.

Preparation of Liver Fractions. Male Sprague–Dawley rats (3-months old, 300–400 g) were maintained on a normal laboratory diet consisting of Purina Lab Chow and water ad libitum. Pretreated animals received sodium phenobarbital (80 mg/kg, ip) once daily of 3 days prior to sacrifice. Animals were killed by stunning and decapitation. Livers were dissected free of connective tissue, minced, and homogenized in 3 vol of ice-cold 1.15% KCl. The homogenate was centrifuged at 9000*g* for 20 min (4 °C). When microsomal suspensions were required, the 9000*g* supernatant was centrifuged at 105000*g* for 60 min (4 °C). The supernatant was removed, and the microsomal pellet was homogenized in a volume of ice-cold 1.15% KCl equal to one-half the volume of supernatant removed. Protein concentration was determined colorimetrically[43] and adjusted to a final concentration of 10 mg/mL by the addition of 1.15% KCl.

Studies with Liver Homogenates. Incubations were run in 25-mL Erlenmeyer flasks. Standard conditions were as follows. To 2 mL of 0.2 M potassium phosphate buffer, pH 7.4, were added 1 mL of 0.025 M $MgCl_2$ in 1.15% KCl and 1 mL of a solution of NADP (1.7 mg), glucose 6-phosphate (11.7 mg), and glucose-6-phosphate dehydrogenase (3 units). After 10 min at room temperature, 1 mL of 9000*g* supernatant equivalent to 0.25 g of rat liver (or 1 mL of microsomal suspension containing 10 mg of protein) was added, followed by the addition of 20 μL of a solution of 0.7 μmol (0.16 μCi) of [^{14}C]1 in *N,N*-dimethylacetamide (initial concentration was 0.14 mM). The mixture was shaken at 70 cpm for 0.75 h.

Analysis of Incubation Mixtures. To the mixture was added 25 mL of peroxide-free ether,[44] and the mixture was shaken for 20 min. The mixture was centrifuged at 200*g* for 10 min. The organic layer (20 mL) was concentrated under a stream of N_2 at 40 °C. The residue was dissolved in 100 μL of ethyl acetate and applied to a TLC plate. Appropriate reference compounds were then applied. After development, these were located under UV light (254 nm). Silica gel segments (1.5 cm in width) were removed, and each was shaken for 2 min with 1 mL of ethanol. Mixtures were centrifuged at 300*g* for 15 min. Supernatants were poured into scintillation vials, 8 mL of Scintiverse I (Fisher Scientific Co.) was added to each, and radioactivity (in disintegrations per minute) was measured by liquid scintillation spectrometry. Quench corrections were made by the external standard method.

Isolation of Compounds 2 and 6 from Incubation Mixtures. Methods for preparation and extraction of incubation mixtures were exactly as described above (see also Table II), except that unlabeled **1** was used, and all quantities were scaled up by a factor of 15. Extracts were subjected to preparative TLC. Compound **2** or **6** was chromatographed separately for comparison purposes. Chromatographic zones of appropriate R_f were located under ultraviolet light (254 nm), removed, and eluted with ethanol. Mixtures were filtered, and the filtrates were concentrated in vacuo. Residual silica gel was removed by dissolving each concentrate in about 0.5 mL of ethyl acetate, transferring the solution to a glass vial, and evaporating the solvent under a stream of nitrogen gas.

Receptor-Binding Assay. The [^{3}H]estradiol (58 Ci/mmol) used in this assay was obtained from Amersham Corp.; radiochemical purity was checked by TLC. Uteri from Sprague–Dawley rats (200–250 g) were homogenized (1 uterus/2 mL) in ice-cold 10 mM Tris buffer, pH 7.4, which contained 1.5 mM EDTA and 3 mM sodium azide (TEA buffer). The homogenate was centrifuged at 100000*g* for 1 h at 4 °C. Incubation mixtures contained 200-μL aliquots of the supernatant, 10 μL of a solution of 1.1 × 10^{-7} M [^{3}H]estradiol in *N,N*-dimethylacetamide, and 10 μL of unlabeled competitor in 1:1 *N,N*-dimethylacetamide–TEA buffer. Six concentrations of competitor were used ranging from 1 × 10^{-10} to 1 × 10^{-5} M. Control incubations contained 10 μL of solvent alone, and nonspecific binding was determined in similarly prepared incubations, which contained 1 × 10^{-5} M estradiol. Incubations were performed in triplicate, in 5-mL polypropylene centrifuge tubes, at 2–4 °C for 4 h. Then, a suspension of 400 μL of dextran-coated charcoal [0.1% dextran (Sigma Chemical Co. no. D-1390), 1% acid-washed Norit A in TEA buffer] was added, and the incubation was continued for 15 min at 2–4 °C. Tubes were then centrifuged at 1000*g* for 10 min, and 40-μL aliquots were dissolved in 5 mL of Scintiverse. Bound [^{3}H]estradiol was determined by liquid scintillation spectrometry.

Uterotropic Assay for Estrogenic Activity. Immature Wistar female rats (21 days old) were obtained from Harlan Sprague–Dawley, Inc., Indianapolis, IN. They were divided randomly into groups of at least six animals. To 0.1-mL aliquots of fresh solutions of estradiol benzoate (0.25 mg/mL) and **1**, **2**, or **6** (25 mg/mL) in *N,N*-dimethylacetamide was added 5 mL of peanut oil. The resulting solutions (0.1 mL) were administered subcutaneously once daily for 3 days. Control animals received vehicle alone. On the 4th day, the animals were killed by decapitation. The uteri were dissected, and fat and connective tissue were removed. After uteri were blotted lightly to remove intraluminal fluid, they were weighed to the nearest 0.1 mg. Body weights were also recorded.

Uterotropic Assay for Antiestrogenic Activity. This was carried out exactly as described above, except that animals receiving the test compounds also received 0.5 μg/0.1 mL of estradiol benzoate, administered separately at different injection sites. One group of control animals received 0.5 μg of estradiol benzoate and vehicle; the other received two injections of vehicle.

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Registry No. **2**, 87198-64-9; **2** citrate, 87261-93-6; **3**, 13278-76-7; **4**, 87261-94-7; **5**, 87261-95-8; **6**, 87261-96-9; benzyl chloride, 100-44-7; 2-pyrrolidinone, 616-45-5.

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