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Antiestrogens and Antiestrogen Metabolites: Preparation of Tritium-Labeled (±)-*cis*-3-[*p*-(1,2,3,4-Tetrahydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]-1,2-propanediol (U-23469) and Characterization and Synthesis of a Biologically Important Metabolite

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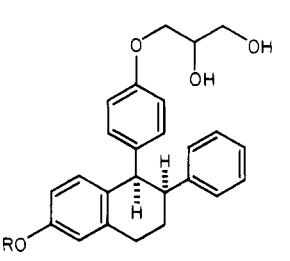
The Upjohn antiestrogen (±)-*cis*-3-[*p*-(1,2,3,4-tetrahydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]-1,2-propanediol (**2b**, U 23469) has been prepared in tritium-labeled form by reduction of an unsaturated dihydronaphthalene precursor with carrier-free tritium gas over a palladium catalyst followed by alkylation with 3-iodo-1,2-propanediol. After extensive chromatographic purification, the final material was obtained with a specific activity of 13 Ci/mmol and a radiochemical purity of 94%. In vivo studies with immature rats show that [^{3}H]**2b** is slowly converted to a more polar metabolite that is selectively accumulated in the nuclear fraction of the uterus where it is bound to the estrogen receptor. Chromatographic comparisons indicate that this metabolite is the demethylated analogue **2c**, a compound that has an affinity for estrogen receptor more than 300 times greater than that of **2b**. These studies suggest that the demethylated analogue **2c** may be a biologically important metabolite of **2b** that is involved in the action of this antiestrogen.

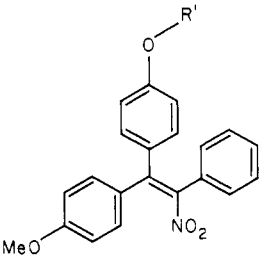
Antiestrogens are compounds that block, at least in part, the action of estrogens in target tissues.[1] While the pursuit of compounds with such activity was initially prompted by the search for effective contraceptive agents for the human female, interest has refocused on these compounds because of their potential for controlling the growth of estrogen-dependent neoplasms, particularly tumors of the breast.[2] In fact, recent clinical trials have shown antiestrogen treatment to be as effective as other forms of hormone additive or ablative therapy in human breast cancer.[2]

From recent studies on antiestrogens, it is clear that the molecular basis of their action is complex, involving interactions with both cytoplasmic and nuclear receptor sites.[1,3] Furthermore, the duration of action of the better-known antiestrogens is much longer than that of the estrogens with which they are normally compared, and it appears that the biological action of the antiestrogens may be mediated not only by the compounds administered but also by certain metabolites that may, in fact, be more potent than the parent compound.[3-5]

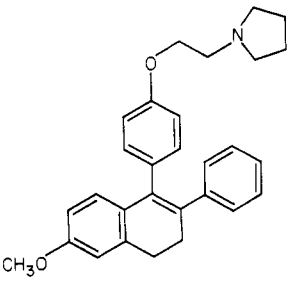
In order to facilitate studies on the molecular action of antiestrogens, we have endeavored to prepare several members of this class in high specific activity, tritium-labeled form to permit their interaction with target tissues and receptors to be followed directly. We have recently described the preparation of a Parke-Davis antiestrogen CI 628[6] in tritium-labeled form and investigated its interaction with receptor in the immature rat uterus and its in vivo metabolism.[5] Other studies with this radiolabeled antiestrogen[7] and radiosynthetic studies on a closely related antiestrogen CI 680[6,8] have appeared, as have studies utilizing [^{3}H]tamoxifen.[6,9]



U-23469 (**2b**), R = CH_3
metabolite (**2c**), R = H



CI 628, R' = CH_2CH_2-c-$N(CH_2CH_2)_2$
CI 680, R' = CH_2CH_2-CH_2NMe_2



nafoxidine; U 11,100A

In this report, we describe the preparation, in tritium-labeled form, of an Upjohn antiestrogen, (±)-*cis*-3-[*p*-1,2,3,4-tetrahydro-6-methoxy-2-phenyl-1-naphthyl)phen-

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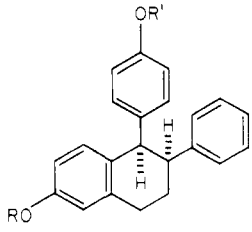
oxy]-1,2-propanediol (**2b**, U 23469),[6,10,11] shown to be ef-



	R	R'
2a	CH_3	H
2b	CH_3	$-CH_2CHOHCH_2OH$ (U-23469)
2c	H	$-CH_2CHOHCH_2OH$ (polar metabolite)
2d	H	H
2e	$-CH_2CHOHCH_2OH$	H
2f	$-CH_2CHOHCH_2OH$	$-CH_2CHOHCH_2OH$

fective in antagonizing estrogen-stimulated uterine growth[4] and the growth of hormone-dependent dimethylbenzanthracene-induced mammary tumors in rats.[3] We present the results of in vivo studies in rats that suggest that a more polar metabolite of the antiestrogen **2b** is the predominant species that interacts with the uterine estrogen–receptor system. This metabolite appears to be the demethylated analogue **2c**, and its synthesis by both chemical and microsomal demethylation is described. Our studies indicated that this metabolite (**2c**) has a much greater binding affinity for the estrogen receptor than does the antiestrogen **2b**, and thus it appears to play an important role in the biological action of this antiestrogen.

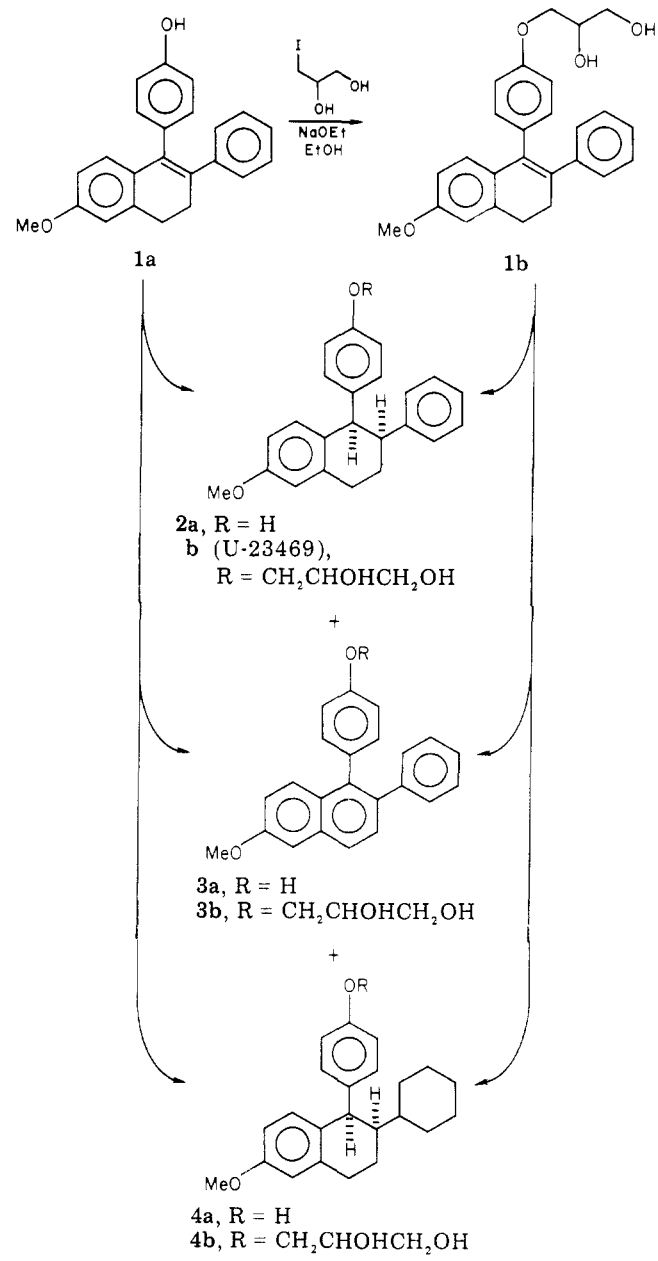
Results and Discussion

Approaches to Tritium-Labeled Antiestrogen 2b. In the original synthesis of the antiestrogen **2b** by Lednicer,[11] the hydroxydiphenyldihydronaphthalene intermediate **1a** was prepared by a series of steps from benzyl *p*-methoxyphenyl ketone. This phenol (**1a**) was reduced to the *cis*-diphenyltetrahydronaphthalene **2a** onto which was attached the 2,3-dihydroxypropyl side chain to give the antiestrogen **2b**.

Our approach to tritium-labeled **2b** involves introduction of the label by using carrier-free tritium gas in the catalytic reduction step. In order to minimize the synthetic manipulations that would be required on the high specific activity products, it was our original intent to reverse the order of the final steps in Lednicer's approach so that the 2,3-dihydroxypropyl side chain would be introduced at the dihydronaphthalene stage, followed by reduction (tritiation) as the final step (**1a** → **1b** → **2b**); see Scheme I.

Reaction of the dihydronaphthalene **1a** with a 2.5-fold excess of 3-iodo-1,2-propanediol gave the desired glyceryl ether **1b** cleanly in 91% yield. Attempts to use more nearly stoichiometric amounts of the alkylating agent gave less complete conversion, probably because of competitive polymerization via the epoxide. Catalytic hydrogenation of **1b** proved to be more difficult than anticipated. It was inert to hydrogen over palladium on charcoal in ethyl acetate or acetic acid at room temperature or over platinum oxide in these solvents and in ethanol. Reduction could be achieved using hydrogen over palladium on charcoal in acetic acid at elevated temperatures (85–95 °C); however, the side chain was partially cleaved, giving **2a**, and from ultraviolet and mass spectrometric investigations it was apparent that both overreduction, to produce an hexahydro analogue (**4b**),[12] and dehydrogenation, to produce the tetradehydro analogue **3b**,[13] had occurred. Chromatographic separation of the closely related glycols

Scheme I



was unsatisfactory, and, although the chromatographic resolution of the corresponding diacetates or acetonides was better, even multiple developments on thin-layer silica gel plates gave only marginal separation between starting material (**1b**), the desired hydrogenation product (**2b**), and the dehydrogenation product (**3b**).

The original sequence of Lednicer[11]—hydrogenation at the stage of the phenol followed by attachment of the 2,3-dihydroxypropyl side chain—proved to be more satisfactory. The phenol **1a** was more easily hydrogenated than the glyceryl ether **1b** and gave mainly the desired product **2a**, contaminated with only small amounts of starting materials **1a**, dehydrogenation product **3a**,[13] and overhydrogenated material **4a**. These materials were marginally separated on silica gel thin-layer plates, the rate of migration increasing with increasing saturation: overhydrogenated compound **4a** was well separated from hydrogenated product **2a**, starting material **2a**, and dehydrogenation product **3a**. Chromatography on a Lipidex 5000 column effected a good separation of the latter three compounds but only marginally separated the desired product (**2a**) from the overhydrogenated one (**4a**). However, since these

two compounds were very clearly separated by silica gel thin-layer chromatography, the combination of these two chromatographic procedures provided definitive means for separation of all the hydrogenation products.

The various hydrogenation products are distinguished not only on the basis of their molecular ions upon mass spectroscopy but also by their characteristic ultraviolet spectra: the dihydronaphthalene **1a** (M^+ m/e 328) shows an absorbance maximum at 307 nm (ϵ 17 800), the tetrahydro product **2a** (M^+ m/e 330) and the overreduced product **4a** (M^+ m/e 336) have similar spectra with maxima at 278 nm, and the dehydrogenation product **3a** (M^+ m/e 326) displays a maximum at 245 nm. Further structure confirmation of the dehydrogenation product **3a** comes from its proton magnetic resonance spectrum, which shows only aromatic signals (in addition to the methoxy and hydroxy resonances).

The ^1H NMR spectrum of the overhydrogenated product **4a** showed resonances for the methoxy group and the benzylic methine adjacent to the aromatic ring in the tetrahydronaphthalene system at field strengths that are the same as those in the hydrogenation product **2a**. Since one would expect these resonances to be shifted and additionally split if the naphthalene system or the hydroxylphenyl group were saturated, the overhydrogenated product is assigned structure **4a**, in which the phenyl group is reduced. The structural assignment for **4a** is additionally supported by the chemical shift of the AA'BB' pattern due to the 1,4-disubstituted phenyl system. In compounds **2a** and **2d** this pattern is centered at δ 6.36, which is somewhat upfield from the resonance position characteristic for para-substituted phenols, δ 6.84–6.90. In the overhydrogenated product **4a**, the AA'BB' pattern is at δ 6.77, more like that of an isolated phenol and indicative of the fact that the shielding effect of the adjacent phenyl group has been removed.

The antiestrogen **2b** was prepared simply by alkylation of the phenol **2a** with 3-iodo-1,2-propanediol. The diastereomeric products were purified together by silica gel column chromatography to give material that was partially solid and partially liquid. Separation of these diastereomers by chromatographic means has not been feasible. In large-scale preparations, the diastereomers are separable by fractional crystallization; however, only the crystalline isomer can be easily obtained in a pure state. The antiestrogen **2b** (U 23469), as commonly supplied, is the solid diastereomer and is termed "isomer A". The relative stereochemistry between the secondary hydroxyl group on the glyceryl side chain and the cis-related phenyl substituents on the tetrahydronaphthalene is not known, however. Crystallization is not a method that can be practicably applied to the purification of small amounts of carrier-free, tritium-labeled material, and, since the biological activity (in terms of antifertility and antiuterotrophic assays) of isomer A and the diastereomeric mixture are identical,[14] separation of the two diastereomers was not considered necessary.

Preparation of Tritium-Labeled Antiestrogen 2b. The unsaturated phenol **1a** was submitted to New England Nuclear for catalytic reduction with tritium gas. After removal of exchangeable tritium, the overall specific activity of the crude product was 12 Ci/mmol. To investigate the radiochemical purity of the crude hydrogenation product, an aliquot was mixed with authentic samples of product, starting material, and overhydrogenation and dehydrogenation products and subjected to chromatography on a Lipidex 5000 column. All the radioactivity eluted in the first peak, together with both the desired hydrogenation product **2a** and the overhydrogenated byproduct **4a**. Further analysis by silica gel thin-layer chromatography under conditions where **2a** and **4a** are separated indicated that the crude hydrogenation product contained no more than 1.5% of the overhydrogenated product.

When larger portions of the hydrogenation product were chromatographed on Lipidex 5000 without the addition of unlabeled standards, the radioactivity again eluted as a single peak. However, analysis of the individual fractions by silica gel TLC indicated that this column was effecting a partial resolution between the desired reduction product **2a** and the overhydrogenated material **4a**, the latter being enriched in the first portion of the peak. Thus, by excluding these fractions, we were able to obtain a 43% yield of [^3H]**2a** containing less than 0.3% [^3H]**4a** and having an overall radiochemical purity of greater than 90%.

A portion of the purified phenol **2a** was alkylated with 3-iodo-1,2-propanediol to produce the tritiated antiestrogen [^3H]**2b**. The crude reaction product could be freed easily from remaining unreacted phenol by chromatography on an LH-20 column. The specific activity of this material, determined by quantitation of the 278-nm absorbance in the ultraviolet, was found to be 13 Ci/mmol. UV analysis also showed that the product was free from material absorbing at 307 (**1b**) and at 245 nm (**3b**). Radiochemical purity, determined by silica gel thin-layer chromatography, was 94%.

In Vivo Metabolism of the Tritiated Antiestrogen [^3H]2b. Selective Nuclear Uptake of a Polar Metabolite in Rat Uterine Tissue. After injection of [^3H]**2b** into immature rats, thin-layer chromatographic analysis of extracts from serum indicate that a more polar metabolite is being produced (Figure 1). The metabolite constitutes only a small fraction of the extractable serum radioactivity at 1 h (Figure 1B) but is a substantial fraction (ca. 40%) at later times (Figure 1A).

It is well known that active estrogens are selectively taken up by target tissues such as the uterus where they bind to cytoplasmic estrogen receptors and are subsequently translocated to the nuclear fraction. A most significant finding was that thin-layer chromatographic analysis of the uterine nuclear fraction showed a substantial fraction of the polar metabolite at 1 h (Figure 1D), a time when it is barely detectable in the serum (Figure 1B); by 13 h, the nuclear fraction is almost totally the metabolite (Figure 1C), while the majority of extractable serum activity is still [^3H]**2b** (Figure 1A). These data suggest that the polar metabolite has a higher affinity for the estrogen receptor than does **2b** and is thus selectively taken up into the uterine nuclear fraction by virtue of its high receptor binding.

Nuclear estrogen receptors from uterus can be extracted with buffers containing 0.4 M KCl and the estrogen binding activity analyzed on high salt sucrose density gradients where it sediments as a 5S species. Figure 2A shows sucrose gradient analysis of the uterine nuclear estrogen receptor from rats at 13 h after injection of [^3H]**2b**. Binding in the 5S region of the gradient is clearly evident (closed circles), and its estrogen specificity is ascertained by the parallel gradient (open circles) in which binding to the receptor has been blocked by prior administration of a high dose of nonradiolabeled estradiol. The binding that is considered "estrogen receptor specific" is the difference between these two curves (shown in boldface). The nature of the radioactivity that is selectively bound to the uterine nuclear estrogen receptor was investigated by thin-layer chromatography of an extract of the peak fractions of the sucrose gradient. As seen in Figure 2B, at 13 h after the

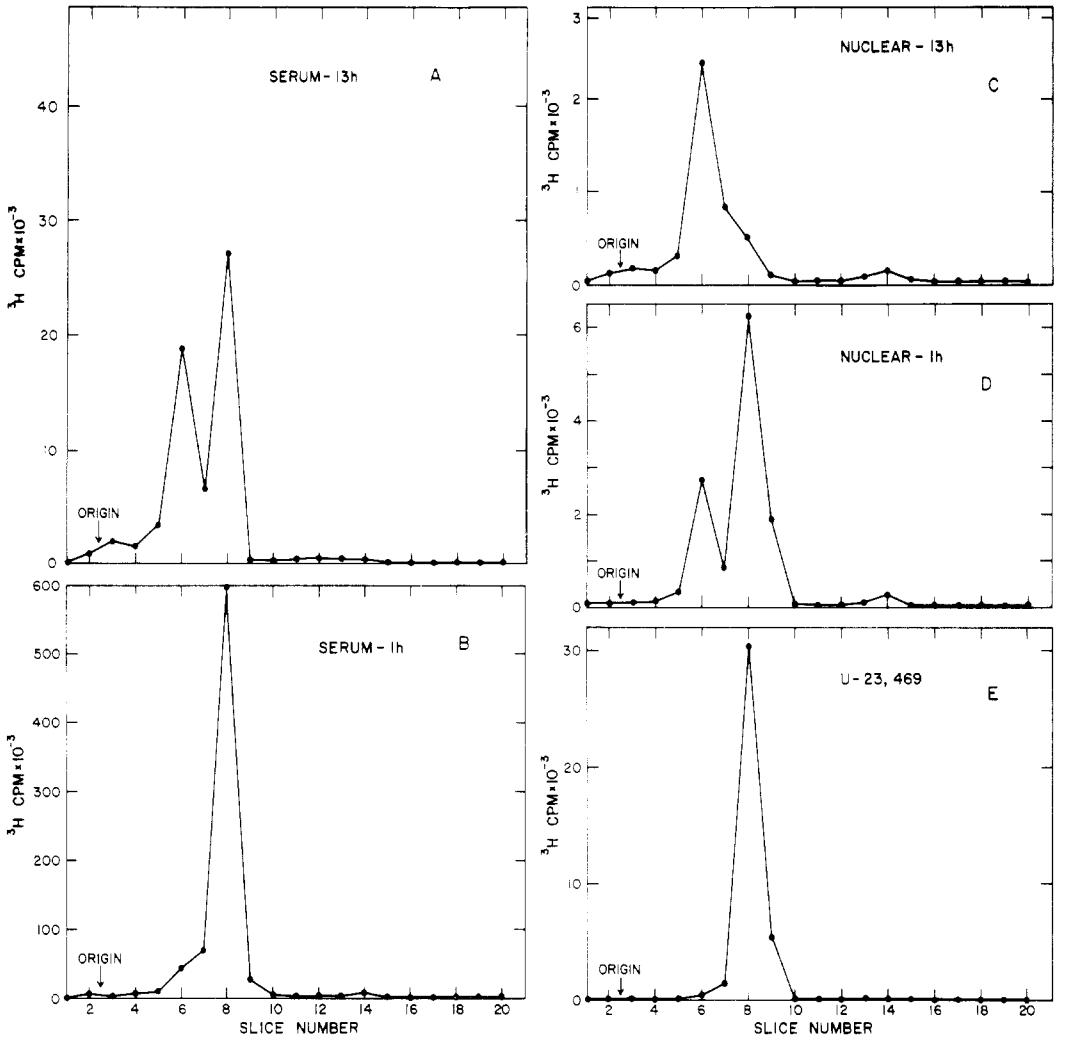


Figure 1. Thin-layer chromatographic profiles of authentic tritiated antiestrogen [^{3}H]**2b** (panel E), of serum (panels A and B), and of uterine nuclear radioactivity (panels C and D) after in vivo injection of [^{3}H]**2b**. Immature rats were injected with [^{3}H]**2b** (25 μg sc/rat) and at 1 and 13 h after injection serum was prepared and extracted with ethyl acetate; at the same times, uteri were excised and homogenized and the three-times washed nuclear pellet was then ethanol extracted. The extracts were concentrated and analyzed on thin-layer silica gel plates developed in anesthetic ether–ethanol (98:2, v/v). (For procedural details, see ref 5.)

injection of [^{3}H]**2b** the only species that is bound specifically to the uterine nuclear estrogen receptor is the polar metabolite.

Preparation of the Free Phenol Analogue 2c. Chromatographic Identity with the Polar Metabolite from the Antiestrogen 2b. The lower chromatographic mobility of the metabolite suggested that a polar group was being unmasked or added and that the glyceryl side chain remained intact. The structure of the metabolite was tentatively identified as the free phenol **2c** by chromatographic comparison of the serum and nuclear extracts with a mixture of two isomeric glyceryl ethers, **2c** and **2e**. These ethers were prepared by monoetherification of the bisphenol **2d**, which had been obtained by ether cleavage of **2b**. These isomers are easily distinguishable by ^{1}H NMR. The isomer in which the hydroxyl on the disubstituted phenyl group is unsubstituted (**2e**) displays an AA′BB′ pattern centered at δ 6.34, while the isomer in which this hydroxyl group bears the glyceryl side chain (**2c**) displays the resonances for this spin system centered at δ 6.40. A similar downfield shift of 0.06–0.08 ppm upon etherification of this phenol was noted in **2b** (δ 6.42) and the analogue in which both phenolic hydroxyls bear glyceryl ethers **2f** (δ 6.43) compared to the free phenols **2a** (δ 6.35) and **2d** (δ 6.36). Despite the fact that these isomers can be distinguished spectroscopically, we were unable to separate them on any of a large number of adsorption and partition chromatographic phases.

Since the formation of **2c** by selective etherification of **2d** or by chromatographic separation from **2e** did not prove feasible, we investigated methods for the selective cleavage of the methyl ether in **2b**. The glyceryl ether was cleaved more rapidly than the methyl ether with excess methylmagnesium bromide or boron tribromide. Sodium ethyl mercaptide gave a complex mixture of products, as did diisobutylaluminum hydride and lithium diphenylphosphide. More detailed investigations of the reaction of **2b** with boron tribromide revealed that the selectivity of ether cleavage depended upon the amount of reagent excess. With slightly more than 1 equiv of BBr_3, the desired methyl ether cleavage predominated, giving **2c** in 52% yield (74% based on consumed **2b**). Small amounts of unreacted **2b** remained, and minor amounts of the other ether cleavage products **2a** and **2d** were formed. These four products could be separated cleanly by chromatography on Sephadex LH-20. With 2 equiv of BBr_3, however, the bisphenol **2d** was the major product, and with 3.5 equiv, the sole product.

The requirement for stoichiometric amounts of BBr_3 to effect a selective ether cleavage of **2b** is awkward in terms of the preparation of **2c** in tritium-labeled form, since with the trace amounts of carrier-free material that are available

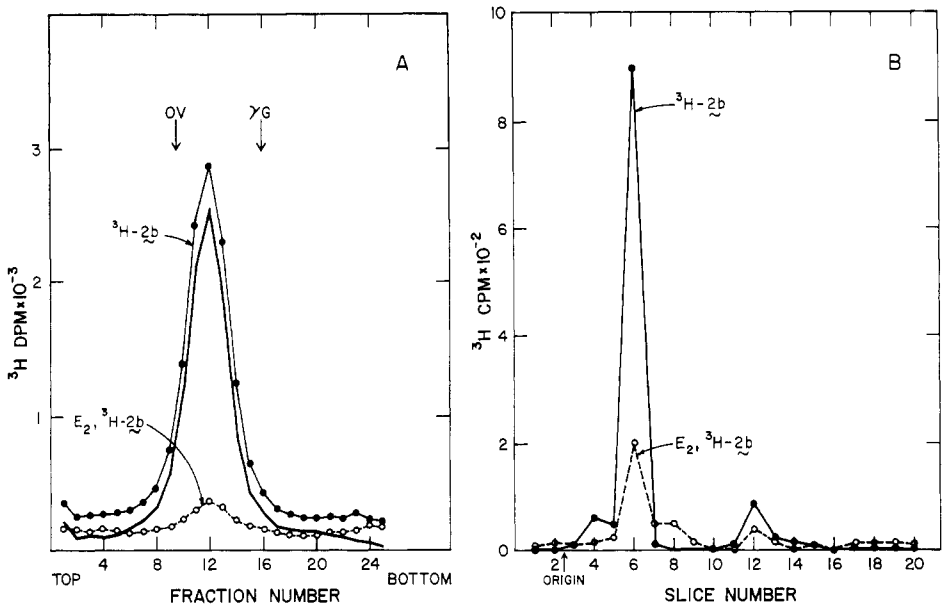


Figure 2. High salt sucrose density gradient analysis of salt-extracted nuclear receptor complexes after exposure to the tritiated antiestrogen [^{3}H]**2b** in vivo (A) and thin-layer chromatographic analysis of 5S estrogen receptor complex purified on sucrose gradients (B). Groups of rats were pretreated for 1 h in vivo with 5 μg of unlabeled estradiol (E_2) or with vehicle saline alone and each then received a subcutaneous injection of 25 μg of [^{3}H]**2b**. At 2 h after injection, uteri were excised and the three-times washed 800*g* nuclear pellet was extracted with buffer containing 0.4 M KCl for 1 h at 0 °C. (A) Extracts were treated with charcoal–dextran prior to the addition of ^{14}C-labeled marker proteins (ovalbumin, OV, and γ-globulin, γG), and a 350-μL aliquot (containing 1.2 uterine equiv) was layered onto gradients. Centrifugation was for 17 h at 4 °C at 270000*g*. (B) The peak region of the gradient (fractions 9–15, panel A) was extracted with ethyl acetate and analyzed by thin-layer chromatography. The profile of material from animals treated with [^{3}H]**2b** alone is indicated by the solid curve and that of [^{3}H]**2b** after unlabeled estradiol (E_2) pretreatment is indicated by the dashed curve. The difference between these two profiles represents radioactivity associated with estrogen-specific nuclear binding sites. Under these conditions, U 23,469 migrates in slice 12; so, the material in slice 6 corresponds to the polar metabolite. (Procedural details are given in ref 5.)

for reaction control of reagent excess is difficult. Further work with [^{3}H]**2b** confirmed our earlier experience: with excess BBr_3, glyceryl ether cleavage precedes methyl ether cleavage, even after very brief reaction times. We have been successful in generating small amounts of [^{3}H]**2c** by microsomal oxidation, however.

The liver is the organ responsible for the major portion of metabolism of most estrogens, and we have found that in the presence of liver microsomes [^{3}H]**2b** is selectively demethylated. At low (ca. 10^{-7} M) **2b** concentrations, conversions of more than 50% could be obtained. This method is not suited for preparative production of the metabolite, however, as the extent of conversion decreases when the concentration of **2b** increases.

The chromatographic identity of the polar metabolite with the phenolic glyceryl ether **2c** was established in several chromatographic systems. Figure 3 shows a thin-layer chromatogram of an extract of the rat uterine nuclear fraction 18 h after injection of [^{3}H]**2b** together with standard samples of the antiestrogens **2b** and **2c** and two other potential metabolites, the phenol methyl ether **2a** and the bisphenol **2d**. The chromatographic identity of the metabolite and **2c** is evident, as is the fact that, at most, trace amounts of the mono- and bisphenols are formed.

Estrogen Receptor Binding of the Antiestrogen 2b, the Metabolite 2c, and Related Compounds 2a and 2d. The affinity with which nonradiolabeled estrogen analogues and antiestrogens interact with the estrogen receptor can be determined indirectly by competitive binding measurements using tritium-labeled estradiol. We have used such an assay to evaluate the binding interaction of various estrogen affinity-labeling reagents[15] and breast tumor imaging agents.[16]

The receptor binding affinities of the antiestrogen U 23469 and the phenolic metabolite, as well as those of

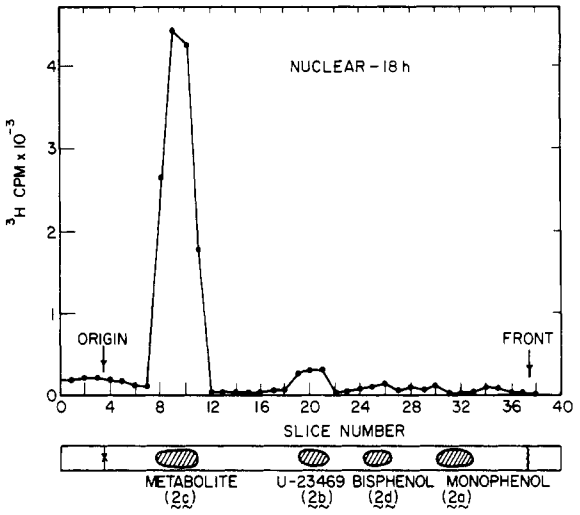
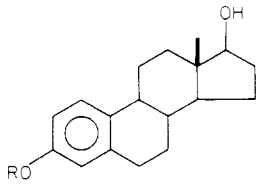
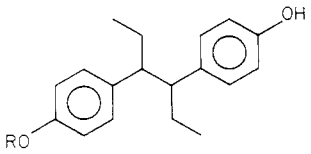


Figure 3. Thin-layer chromatographic comparison of the metabolite with the antiestrogen **2b** and analogues **2a,c,d**. An ethanol extract of the rat uterine nuclear fraction, prepared 18 h after injection of [^{3}H]**2b** as described in the legend of Figure 1, was subjected to thin-layer chromatographic analysis on silica gel (hexane–CH_2Cl_2–EtOH, 5:5:1) together with samples of the compounds noted. The position of the standard compounds, as visualized by exposure to iodine vapor, is shown in the chromatogram sketched below the profile of radioactivity.

related analogues, are presented in Table I. The antiestrogen **2b** itself has a low affinity for the receptor. The affinity increases dramatically, more than 300-fold, upon removal of the methyl ether group (**2c**). Increases in binding affinity upon unmasking of a group important in receptor interaction have been observed in other systems; for example, the binding affinity of estradiol (**5a**) vs. estradiol 3-methyl ether (**5b**) is about 30:1 and the affinity



5a, R = H
5b, R = CH_3



6a, R = H
6b, R = CH_3
6c, R = $CH_2CHOHCH_2OH$

of hexestrol (**6a**) vs. hexestrol methyl ether (**6b**) is about 15:1, but the effect of demethylation in the antiestrogen series is particularly great (cf. also **2a** vs. **2d**).

The effect of the glyceryl ether on the binding affinity of the compounds in the antiestrogen series is much more modest: compare **2a** and **2b**, **2c** and **2d**. This contrasts markedly with the effect of the introduction of one glyceryl ether into the potent synthetic estrogen hexestrol where receptor affinity is decreased about 40-fold (**6c**).

Conclusion

The Upjohn antiestrogen **2b** (U 23469) is closely related to the better-known antiestrogen nafoxidine.[6] The antiestrogen **2b** was prepared by Lednicer in an attempt to reduce the phototoxicity that was encountered with the use of nafoxidine in humans. He sought to minimize the near-ultraviolet absorbance due to the stilbene chromophore in nafoxidine, by saturation of the stilbene double bond. The *cis*- and *trans*-dihydrostilbene analogues adorned with glyceryl side chains in place of the more common substituted aminoethoxy groups were prepared. Curiously, the trans isomer proved to be estrogenic, while the cis isomer (**2b**, U 23469) retains almost all of the antagonistic properties of nafoxidine.[11] This compound (**2b**) is also not phototoxic. In in vivo studies in immature rats, we have found that this material is slowly converted to a more polar metabolite that accumulates selectively in estrogen receptor sites in uterine nuclei. Chromatographic comparison of the metabolite generated either in vivo or by in vitro oxidation with liver microsomes permitted its tentative identification as the free phenol **2c**. The receptor binding affinity of this compound is more than 300-fold greater than that of **2b**, a finding that is consistent with its in vivo behavior. A more comprehensive study of the effects of etherification on receptor binding affinity in this antiestrogen series, as compared to the estradiol or hexestrol systems, reveals that the antiestrogen system is much more sensitive to methylation but less sensitive to glyceryl ether formation.

From this study, as well as from related studies on the antiestrogens CI 628 and tamoxifen, it appears that metabolism may prove to be an important modulator of the biological potency of these compounds: CI 628 is also metabolized to a more polar material that selectively accumulates in uterine nuclear estrogen receptor sites,[5] and monohydroxytamoxifen, a known metabolite of tamoxifen,[17] has a higher receptor binding affinity than tamoxifen.[9b] It is hoped that the availability of antiestrogens in tritium-labeled form will continue to assist in the elucidation of the molecular basis of action of antiestrogens.

Experimental Section

Materials. Chemicals were obtained from the following sources: 3-chloro-1,2-propanediol, Eastman Organic Chemicals; 5% palladium on carbon and platinum oxide, Engelhard; glucose 6-phosphate (monosodium salt), glucose-6-phosphate dehydrogenase (from Torula yeast), and nicotinamide adenine dinucleotide phosphate (NADP), Sigma; 4-(dimethylamino)antipyrene, 2,4-pentanedione, and *p*-nitroanisole, Aldrich; Triton X-114, Central Solvents & Chemical Corp.

Table I. Relative Binding Affinity of U 23469, Its Metabolite, and Related Compounds to the Uterine Estrogen Receptor[a]

no.	compd	RAC × 100[b]
5a	estradiol	100
2b	U 23469	0.1
2c	(metabolite)	39.1
2a		0.24
2d		29.8
5b	estradiol 3-methyl ether	3.0
6a	hexestrol	300
6b	hexestrol methyl ether	19.3
6c	hexestrol glycerol ether	6.9

[a] Various concentrations of unlabeled compound (10^{-4} to 10^{-10} M) were incubated with 10^{-8} M [^{3}H]estradiol and lamb or rat uterine cytosol (ca. 2.5 mM receptor site concentration) for 16 h at 0 °C. Free ligands were removed by a brief treatment with dextran-coated charcoal. For details, see ref 15. [b] The affinity relative to that of estradiol is expressed by RAC × 100, which is the ratio of association constants ($K_a^{comp}/K_a^{estradiol}$) × 100.

The phenol **1a** (mp 160–162 °C, lit.[11] 160–162 °C) was prepared according to the route described by Lednicer.[11] Our material appears to contain 5–8% of naphthalenic impurities that result from aromatization of this relatively sensitive dihydronaphthalene system during its preparation.[13] 1-Iodo-2,3-propanediol (mp 42–45 °C, lit.[18] 48–49 °C) was prepared by heating 1-chloro-2,3-propanediol with sodium iodide in nitromethane under reflux for 18 h.

Chemical Methods. Analytical thin-layer chromatography was performed using 0.25-mm silica gel glass-backed plates with F-254 indicator (precoated TLC plates, silica gel 60 F-254, Merck), and compounds were visualized by ultraviolet light (254 nm), iodine, or by using a ceric sulfate–sulfuric acid spray reagent. Preparative thin-layer chromatography was done using 2-mm silica gel glass-backed plates with F-254 indicator (precoated PLC plates, silica gel F-254, Merck). Silica gel column chromatography was performed on 0.05–0.2-mm silica gel (Brinkmann). Sephadex LH-20 (Pharmacia Fine Chemicals) and Lipidex-5000 (Packard) were packed in the glass column after suspension in organic solvents and sonication for 10 min. Elution from the Lipidex column was monitored by an Instrumentation Specialties Co. (ISCO) Model UA-5 absorbance monitor at 254 nm. Melting points were determined on a Fisher-Johns apparatus and were not corrected.

Infrared spectra were taken as KBr pellets or Nujol mulls, using a Beckman Model IR-12 instrument. Data were presented in reciprocal centimeters, and only the important diagnostic bands were reported. Proton magnetic resonance (^{1}H NMR) spectra were recorded on Varian Associates spectrometers, Models A-60, HA-100, and HR-220. Selected chemical shifts were reported in parts per million downfield from a tetramethylsilane internal standard (δ scale). Mass spectra were obtained at 70 eV (unless stated otherwise) on Varian MAT CH-5 and 731 (high-resolution mass spectra) spectrometers; only major ion fragments are reported in the form m/e (relative intensity). UV spectra were obtained in absolute ethyl alcohol on a Cary-15 instrument. Elemental analyses were provided by the Microanalytical Service Laboratory of the University of Illinois.

A standard procedure for product isolation was used in all reactions, except where mentioned otherwise: quenching by addition to water, exhaustive extraction with a solvent, drying over an anhydrous salt, filtration, and evaporation of solvent under reduced pressure.

Radioactivity was measured in a Nuclear Chicago Isocap 300 liquid scintillation counter, in minivials using 5 mL of a xylene-base cocktail containing 0.55% 2,5-diphenyloxazole, 0.01% *p*-bis[2-(5-phenyloxazolyl)]benzene, and 25% Triton X-114. Tritium counting efficiency was 27–53%. Radiochemical purity was determined by chromatography on plastic-backed silica gel thin-layer plates (Eastman chromogram sheet no. 6061, without fluorescent indicator). The labeled material was spotted on top of unlabeled carrier. After development, the carrier spot was visualized by exposure to iodine vapor, and the chromatogram was cut into

strips, which were then placed in minivials for radioactivity determination.

Biochemical Methods. Complete details for the biochemical methods listed are given in the reference indicated: animal injection, preparation and extraction of serum and uterine nuclear fractions, thin-layer chromatography, sucrose density gradient sedimentation, ref 5; receptor affinity measurements by competitive binding, ref 16. A synopsis of these methods is given in the legends to the figures and in the footnotes in Table I. Details of the generation of [^{3}H]**2c** by microsomal oxidation are given in the chemical synthesis section.

1-[*p*-(2,3-Dihydroxypropoxy)phenyl]-2-phenyl-6-methoxy-3,4-dihydronaphthalene (1b). Freshly cut sodium (80 mg, 3.46 mmol) was dissolved in 5 mL of ethanol, and 401 mg (1.22 mmol) of 1-(*p*-hydroxyphenyl)-2-phenyl-6-methoxy-3,4-dihydronaphthalene (**1a**) in 15 mL of ethanol was added dropwise over a 10-min period. After refluxing the mixture for 1.5 h, 626 mg (3.10 mmol) of 3-iodo-1,2-propanediol in 10 mL of ethanol was added, and reflux was continued for 21 h. The reaction mixture was concentrated under reduced pressure, redissolved in chloroform, and washed five times with water. The organic layer was dried ($MgSO_4$), filtered, and concentrated in vacuo to give 666 mg of oil. Separation by column chromatography on 60 g of silica gel gave 91 mg of the starting phenol **1a** (23%) and 346 mg of alkylated compound **1b** (71%; yield based on consumed phenol was 91%): IR (KBr) 3430 (OH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.84 (s, 3 H, CH_3O–), 6.5–7.3 (12 H, aromatic protons), 7.5–7.8 (0.25 H, aromatic protons of naphthalenic impurities corresponding to 8%); MS 402 (M$^+$, 100), 400 (M$^+$ of naphthalenic impurity, 16); UV λ_{max} 302 nm (ϵ 16 700).

Hydrogenation of 1-[*p*-(2,3-Dihydroxypropoxy)phenyl]-2-phenyl-6-methoxy-3,4-dihydronaphthalene (1b). The diol **1b** (70 mg, 0.17 mmol) in 2 mL of acetic acid was added to a suspension of 84 mg of 5% palladium on charcoal that had been presaturated with hydrogen in 2 mL of acetic acid. After stirring at 90 °C for 13 h under hydrogen, the resulting reaction mixture was acetylated with acetic anhydride in pyridine and separated on 2-mm silica gel thin-layer plates (benzene–ether, 5:1; developed four times). The material of R_f 0.85–0.82 (1.5 mg) had UV absorption at 280, 255 (shoulder), 230 (shoulder) nm and a mass spectrum of 372 (43), 368 (24), 330 (11), 326 (45), and 209 (100), indicating a mixture of acetates in **2a** and **3a**. The material from R_f 0.76–0.70 was the diacetate of **2b** (39 mg, 46%): UV λ_{max} 280, 250, 230 nm. The other products, R_f 0.82–0.78 (0.6 mg) and 0.63–0.58 (2.9 mg), were isolated but not identified.

Hydrogenation of 1-(*p*-Hydroxyphenyl)-2-phenyl-6-methoxy-3,4-dihydronaphthalene (1a). The phenol **1a** (71 mg, 0.22 mmol) in 2 mL of acetic acid was added to the suspension of 40 mg of 5% palladium on charcoal that had been presaturated with hydrogen in 2 mL of acetic acid, and stirring under hydrogen was continued at room temperature for 8 h. Evaporation of the solvent after filtration gave 60 mg of colorless solid, which was separated on a 1 × 47 cm column by Lipidex 5000. Elution with hexane–chloroform (7:3) gave the desired phenol **2a** (37.3 mg): mp 185–187 °C (lit.[10] 188.5–190 °C); IR (KBr) 3440 (OH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.78 (s, 3 H, CH_3O–), 4.21 (d, 1 H, J = 5 Hz, benzylic proton of the phenol); MS 330 (M$^+$, 98), 195 (100); UV λ_{max} 278 nm. Also obtained was the dehydrogenated phenol **3a** (3.5 mg): mp 153–157 °C; IR (KBr) 3420 (OH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.94 (s, 3 H, CH_3O–); MS 326 (M$^+$, 23), 57 (100); UV λ_{max} 245 nm. Anal. Calcd for $C_{23}H_{18}O_2$: M_r 326.1306. Found (high-resolution mass spectrum): 326.1303.

A larger sample of the overhydrogenated compound **4a** was obtained by hydrogenation with platinum oxide in acetic acid at room temperature and purified by silica gel chromatography: IR (Nujol) 3400 (OH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.77 (s, 3 H, CH_3O–), 4.10 (d, 1 H, J = 4 Hz, benzylic proton of the phenol); MS 336 (M$^+$, 100), 300 (30); UV λ_{max} 279 nm. Anal. Calcd for $C_{23}H_{28}O_2$: M_r 336.2089. Found (high-resolution mass spectrum): 336.2086.

Alkylation of 1-(*p*-Hydroxyphenyl)-2-phenyl-6-methoxy-1,2,3,4-tetrahydronaphthalene (2a). Sodium (42 mg, 1.8 mmol) was dissolved in 1 mL of ethanol, 37 mg (0.11 mmol) of the phenol **2a** in 4 mL of ethanol was added, and the reaction was refluxed for 2 h. 3-Iodo-1,2-propanediol (440 mg, 2.2 mmol) in 5 mL of ethanol was added over a 40-min period, and refluxing was continued for 38 h. After evaporation of the solvent, product isolation ($CDCl_3$, $MgSO_4$) gave 50 mg of crude product. Separation by chromatography on 10 g of silica gel afforded 15 mg of unreacted phenol (40%) and 14 mg of the diol product (**2b**, 51% based on consumed material).

Preparation of ^{3}H-Labeled 1-(*p*-Hydroxyphenyl)-2-phenyl-6-methoxy-1,2,3,4-tetrahydronaphthalene ([^{3}H]2a). (This reaction was carried out at New England Nuclear Corp.) A 25-mg sample of 5% Pd/C catalyst in 1 mL of acetic acid was reduced in an atmosphere of tritium gas. After removal of tritium gas, 52 mg of the phenol **1a** was dissolved in 1 mL of acetic acid and injected into the reaction flask. Tritium gas (25 Ci) was added, and the reaction mixture was stirred overnight at room temperature. Labile tritium was removed in vacuo using methanol as solvent. After filtration from the catalyst, the product was taken to dryness, in vacuo, and then redissolved in 10 mL of benzene–methanol (9:1); 1934 mCi of radioactivity was incorporated into the compound (37.2 Ci/mg or 12 Ci/mmol). The labeled compound was stored in benzene–methanol but was evaporated to dryness and redissolved in hexane–chloroform prior to purification. A significant portion of the material failed to dissolve in chloroform. However, it contained less than 1% of total radioactivity and was discarded.

Analysis of radiochemical purity of the crude radiochemical by silica gel TLC (hexane–THF–acetone–ethyl acetate, 15:1:1:1) indicated that 89.6% of the radioactivity cochromatographed with the desired phenol **2a** and only 1.1% with the overhydrogenated compound **4a**. A mixture of the unlabeled compounds **1a**, **2a**, **3a**, and **4a** (15 mg total) and 10 μCi of labeled mixture was applied to a 1 × 47 cm column of Lipidex 5000 and eluted with hexane–chloroform (70:30) at a flow rate of 50 mL/h. Fractions (10 mL) were collected and portions were analyzed for UV absorbance at 254 nm and for radioactivity. Elution positions were the following: **2a** and **4a**, fraction 16; **1a**, fraction 20; **3a**, fraction 25.

A single peak of radioactivity was observed to coelute with **2a** and **4a**; thus, it appeared that Lipidex 5000 did not separate the hydrogenation product **2a** from the overhydrogenated product **4a**. Radiochemical analysis of individual fractions by TLC, however, showed that these materials were being partially resolved by this column; e.g., fraction 14 contained 8.6% **4a**, but fractions beyond 15 contained less than 0.5% **4a**. Thus, chromatography on Lipidex 5000 appeared to provide a method for obtaining the desired reduction product **2a**, free from all the possible contaminants (**1a**, **3a**, and **4a**). Selection of the later peak fractions from preparative scale separation of 774 mCi of labeled material on a similar Lipidex 5000 column gave 333 mCi (43%) of [^{3}H]**2a** free from [^{3}H]**4a** and with radiochemical purity in excess of 90%.

^{3}H-Labeled 1-[*p*-(2,3-Dihydroxypropoxy)phenyl]-2-phenyl-6-methoxy-1,2,3,4-tetrahydronaphthalene ([^{3}H]2b; ^{3}H-labeled U 23469). The radiolabeled phenol **2a** (614 mCi) was dissolved in 2 mL of ethanol, mixed with 1 mL of ethanolic sodium ethoxide [prepared from 42 mg (1.8 mmol) of sodium], and refluxed for 5 h. Iodopropanediol (461 mg, 2.3 mmol) in 2 mL of ethanol was added dropwise over 30 min, and refluxing was resumed for 37 h. At this point, radiochemical analysis by silica gel TLC (3% ethanol in ether) showed that 62% of the radioactivity cochromatographed with the desired product **2b**, while 27% remained with the starting phenol **2a**. The solvent was removed under a gentle stream of nitrogen; the crude product was redissolved in chloroform, washed five times with water, and then dried ($MgSO_4$). This material was purified on a 1 × 55 cm Sephadex LH-20 column, eluting with cyclohexane–ethanol, 9:1, at 8 mL/h. Radiochemical analysis of every second fraction showed two major peaks, the first being unreacted phenol [^{3}H]**2a** (170 mCi in combined peak fractions) and the second the antiestrogen [^{3}H]**2b**. Radiochemical analysis by TLC of fractions from the second peak indicated purities ranging from 93 to 98.5%; combination of the peak fractions gave 261 mCi of [^{3}H]**2b** with a radiochemical purity of 94% and a specific activity of 13 Ci/mmol. The ultraviolet spectrum of this compound is identical with that of unlabeled **2b**, and the specific activity of the [^{3}H]**2b** was determined by comparison of the absorbance at 278 nm with the absorption of various known concentrations of unlabeled **2b**.

For long-term storage, 2.5-mCi aliquots were diluted to 2.5 mL with benzene–ethanol, 9:1, and placed in glass tubes under liquid nitrogen. The radiochemical purity of this material after 2 years of storage was 93%.

Reaction of the Antiestrogen 2b with Boron Tribromide. Preparation of 1-[*p*-(2,3-Dihydroxypropoxy)phenyl]- (2c) and 1-(*p*-Hydroxyphenyl)-2-phenyl-6-hydroxy-1,2,3,4-tetrahydronaphthalene (2d). To a solution of 200 mg (0.495 mmol) of **2b** in 10 mL of dry dichloromethane at 0 °C was added 0.65 mL (0.676 mmol) of 1.04 M boron tribromide in dichloromethane. After being stirred for 2 h, the reaction mixture was quenched with water. Product isolation (ethyl acetate, $MgSO_4$) gave 261 mg of residue, which showed four spots on silica gel TLC (hexane–dichloromethane–ethanol, 5:5:1): R_f 0.59, monomethyl ether **2a**; 0.47, diphenol **2d**; 0.35, starting material **2b**; 0.21, demethylated product **2c**.

Separation of these four components on a preparative scale was carried out on a column of Sephadex LH-20 (1 × 57 cm) eluting with cyclohexane–ethanol (4:1) at 13.5 mL/h. Elution was monitored by a UV detector using a 280-nm filter: peak A was monomethyl ether **2a** (10.5 mg, 6%); peak B was U 23469 (**2b**; 61.2 mg, 31%); peak C was the diphenol **2d** (8.8 mg, 6%) (the order of elution of **2b** and **2d** on LH-20 is the reverse of that on silica gel); peak D was the demethylated product **2c** (100 mg, 52%, 74% based on consumed **2b**).

A pure sample of **2c** was obtained by recrystallization from ethanol: mp 218–220 °C; IR (KBr) 3415 cm^{-1}; ^1H NMR (C_5D_5N) δ 1.71 and 2.24 (each m, 1 H, together CH_2), 3.00 (m, 2 H, benzylic CH_2), 3.33 (m, 1 H, benzylic CH), 4.15 (d, 1 H, J = 5 Hz, bisbenzylic CH); MS 391 (31), 390 (100, M^+); UV λ_{max} 279 nm (3460). Anal. Calcd for $C_{25}H_{26}O_4$: M_r 390.1831. Found (high-resolution mass spectrum): 390.1829.

Larger amounts of boron tribromide provided diphenol **2d** as the major product. Thus, 99 mg (0.245 mmol) of **2b** and 0.50 mL of 1.04 M boron tribromide in 6 mL dichloromethane, after 2.8 h at 0 °C, product isolation, and silica gel preparative TLC (two developments, dichloromethane–ethanol, 20:1), gave 45 mg (58%) of the diphenol **2d**: mp 220–223 °C (after recrystallization from benzene); IR (KBr) 3420 cm^{-1}; ^1H NMR (acetone-d_6) δ 1.76 and 2.20 (each m, 1 H, together 2 H), 3.00 (m, 2 H, benzylic CH_2), 3.32 (m, 1 H, benzylic CH), 4.19 (d, 1 H, J = 5 Hz, bisbenzylic CH); MS 317 (20), 316 (79 M^+), 212 (100), 211 (100); UV λ_{max} 281 nm (ϵ 3680). Anal. Calcd for $C_{22}H_{20}O_2$: M_r 316.1463. Found (high-resolution mass spectrum): 316.1462. This reaction also gave 22 mg (23%) of **2c**.

Reaction of 3-Iodo-1,2-propanediol with 1-(*p*-Hydroxyphenyl)-2-phenyl-6-hydroxy-1,2,3,4-tetrahydronaphthalene (2d). Preparation of 2e and 2f. A sample (52 mg, 0.16 mmol) of diphenol (**2d**) was dissolved in 3 mL of ethanol, mixed with 2 mL of ethanolic sodium ethoxide (from 41 mg, 1.8 mmol of sodium), and stirred under reflux for 3 h. Then, 406 mg (2.01 mmol) of 3-iodo-1,2-propanediol in 4 mL of ethanol was added, and refluxing was continued for 15 h. Product isolation (chloroform, $MgSO_4$) gave 51 mg of residue, which was separated by silica gel TLC (ether) to give 12 mg of the starting material **2d** (38%), 11 mg of monoalkylated mixture **2c** and **2e** (28%), and 2 mg of dialkylated compound **2f** (4.3%). The monoalkylated mixture displayed a ^1H NMR spectrum ($CDCl_3$) showing δ 6.34 (AA'BB', J = 9 Hz) for **2e** and δ 6.40 (AA'BB', J = 9 Hz) for **2c**. The dialkylated compound **2f** gave MS 465 (16), 464 (55, M^+), 28 (100); ^1H NMR ($CDCl_3$) δ 4.25 (d, J = 4.4 Hz), 6.43 (center of AA'BB', J = 8.5 Hz).

Preparation of ^3H-Labeled 1-[*p*-(2,3-Dihydroxypropoxy)phenyl]-2-phenyl-6-hydroxy-1,2,3,4-tetrahydronaphthalene ([^3H]2c) by Microsomal Demethylation of [^3H]2b. Liver microsomes were prepared from 51-day-old male Holzman rats. After the rats were decapatated, the livers were perfused in situ with cold PBS (150 mM NaCl, 10 mM Na_xPO_4, pH 7.4), minced, and homogenized in the cold using 3 mL of PBS plus 1 mM EDTA/g of liver in a Teflon–glass homogenizer. The homogenate was centrifuged at 9000g for 10 min; the pellet was discarded, and the supernatant was centrifuged at 100000g for 1 h. The pellet was resuspended in homogenization medium and recentrifuged at 100000g for 1 h. The pellet of washed microsomes was resuspended in homogenization medium (3 mL/g of liver) by gentle homogenization. The final suspension contained 10 mg/mL of microsomal protein. The microsome suspension could be stored indefinitely at liquid nitrogen temperatures. The enzymatic activity of the microsomal preparation was verified by assaying for the dealkylation of dimethylaminoantipyrene (*N*-demethylase activity) according to the procedure of Schenkman[19] and for the demethylation of *p*-nitroanisole (*O*-demethylase activity) by the method of Netter and Seidel.[20] The microsomes remained active for about 30 min at 37 °C and they cleaved ca. 60 nmol (mg of microsomal protein)$^{-1}$ 30 min^{-1}.

Incubation conditions for the demethylation of [^3H]**2b** contained 50 mM sodium phosphate buffer, pH 7.4; 0.3 mM NADP; 5 mM $MgCl_2$; 10 mM glucose 6-phosphate; 2 units of glucose-6-phosphate dehydrogenase; and 3 mg of microsomes in a total of 1.5 mL. The substrate was added in 1 μL of ethanol, and the incubation was allowed to proceed for 30 min at 37 °C with agitation. The mixture was then cooled to 0 °C, and the microsomes were pelleted at 100000g for 1 h. The pellet, containing 96% of the radioactivity, was resuspended in 0.25 mL of 0.5 M sodium phosphate, pH 7.4, and extracted with 0.5 mL of ethanol. After 30 min at 0 °C, centrifugation at 850g for 15 min gave a clear supernatant containing 97% of the radiolabeled materials from the microsomal pellet.

Under these conditions, up to 50% of 1.8×10^{-10} mol of [^3H]**2b** was converted into material that cochromatographed with the metabolite **2c**. The extent of conversion varied inversely with the amount of [^3H]**2b** added to the incubation: Below 10^{-8} mol, conversion efficiencies of 30–50% were achieved, but this fell to 15% and below with amounts of 10^{-7} mol and greater. The small amounts of [^3H]**2c** that could be generated by microsomal demethylation could be purified to >90% radiochemical purity by silica gel TLC (Et_2O, two developments). The chemical purity of these samples, however, is not known.

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(13) Careful ^{1}H NMR analysis of the starting phenol **1a** and the alkylation product **1b** indicate that these materials contain a small amount (estimated to be 5–8%) of naphthalenic impurities. These materials are not effectively removed by recrystallization and preparative scale chromatography on silica gel. They are presumed to be the result of aromatization of the sensitive dihydronaphthalene system during the cyclization of the precursor **5**. Because of the presence of this naphthalenic material in **1a**, it is difficult to determine whether the compounds **3a** and **3b**, noted after the hydrogenation step, are the result of dehydrogenation or simply result from carry over of the naphthalenic impurities in our sample of **1a**.
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Electrostatic Potentials of Deoxydinucleoside Monophosphates. 1. Deoxydinucleoside Monophosphates and Actinomycin Chromophore Interactions

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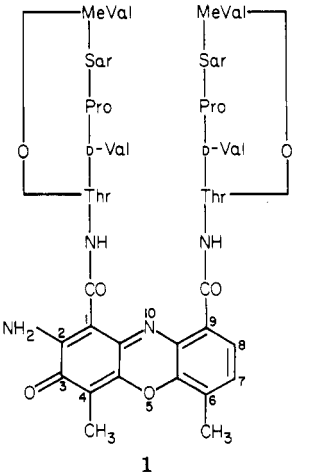
We present electrostatic potential maps for the ten Watson–Crick base-pair combinations of dinucleoside monophosphates in a conformation appropriate for intercalation of drugs to occur. These maps reveal interesting differences among the base-pair combinations and suggest reasons for the base-pair specificities often observed upon intercalation. Simple electrostatic calculations on the intercalation energy of substituted actinomycin chromophores correlate qualitatively with the relative biological activity of these compounds, although the correlation with binding affinity is not as satisfactory.

The importance of drug–DNA interactions has long been recognized. An important step in our molecular level understanding of such processes was made with Lerman's suggestion[1] that flat, planar drugs could "intercalate" between the bases of the DNA. Because the base–base distance along the helical axis in normal double-stranded DNA is only 3.4 Å, there must be a considerable conformational change in the DNA backbone (sugar and phosphate) for a drug to be able to intercalate. The resulting conformation of the backbone must have a base–intercalator separation of ~3.4 Å and thus a base–base separation of ~6.8 Å. Specific stereochemical models for DNA intercalation have been developed by Sobell[2] and Alden and Arnott.[3] The former is based on a high-resolution X-ray structure of the deoxyguanosine–actinomycin D complex and the latter on general stereochemical considerations. Although they differ somewhat in the values proposed for the backbone structure, both studies conclude that the DNA base–base distance can be increased to ~6.8 Å without appreciable "strain".[4]

Another very interesting realization has been the base specificity of different intercalators. Sobell,[2] Krugh,[5] and Müller and Crothers[6] have documented specific examples of base specificity; in particular, Sobell and Krugh have noted that actinomycin D (1) prefers to intercalate between G-C H-bonded base pairs.

We have been interested in the electrostatic potentials surrounding globular proteins in the hope of developing simple models for protein–substrate interactions.[7] Interesting applications of the electrostatic potential have been made in the analysis of the structure–activity relationships of hallucinogens,[8] cholinergics,[9] and narcotic analgetics.[10] Theoretical and experimental studies on small polar molecule gas-phase interactions are consistent with the view that the electrostatic component of the interaction energy is the dominant one.[11] We thus chose to develop a simple electrostatic model for DNA–intercalator interactions. Our interest was particularly stimulated by these interactions,



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