Antiestrogens. 3.[1] Estrogen Receptor Affinities and Antiproliferative Effects in MCF-7 Cells of Phenolic Analogues of Trioxifene, [3,4-Dihydro-2-(4-methoxyphenyl)-1-naphthalenyl][4-[2-(1-pyrrolidinyl)ethoxy]-phenyl]methanone

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Benzothiophenes 3 and 4, derived from the acrylophenone antiestrogen trioxifene (2), are characterized by high estrogen receptor (ER) affinity and low residual estrogenicity compared to tamoxifen (1a). In order to characterize further the growth suppression mechanism for these structural types we have prepared structural variants of 2 bearing hydroxy groups positioned to maximize ER affinity. Thus, dihydronaphthalenes 5 and 6 and benzofluorenes 7 and 8 were prepared and studied in MCF-7 human breast cancer cells, in comparison with 3 and 4. All compounds were powerful suppressants of cell growth, with 50% inhibition ranging from 4.5 to 160 nM. Greatest potency was seen with diphenols 6 and 8. These compounds had intracellular ER affinities ranging from 0.2 to 4.1% of that of estradiol, suggestive of a potential for partial agonist effects. Simultaneous exposure of cells to 0.1 μM concentrations of estradiol and 3 or 4 did not affect the degree of growth inhibition seen with 0.1 μM 3 or 4 alone. Partial reversal of inhibition occurred when 0.1 μM 5–8 were each accompanied by 0.1 μM estradiol. Under these conditions complete reversal of growth inhibition has been found with 1a, 1b, and other triarylethylenes. Calmodulin, a putative target for triarylethylenes, and which is antagonized by 1a, was shown to interact weakly with 7 and 8 and not at all with 3–6. These results suggest that MCF-7 cell growth suppression by 3–8 may be due to interaction with unidentified receptors besides ER and extend earlier findings indicating that events occurring after interaction of these compounds with ER differ from those of triarylethylene antiestrogens.

Introduction

Triarylethylene antiestrogens, such as tamoxifen (1a, Chart I), are of current medical and experimental interest due to their ability to suppress the growth of estrogen receptor (ER) positive cancers, especially breast cancer.[2] A potential limiting factor in this application is the partial agonist effect characteristic to these compounds. Thus, 1a is known not only to antagonize the growth promoting effect of estrogens in tissues and cancer cells containing ER,[3] but also to stimulate the growth of these when administered in the absence of estrogens.[4] Consequently, efforts have been directed toward identification of analogues of 1a,b with high ER affinity and reduced estrogenic activity.[5] In this regard, increased estrogen antagonism has been observed experimentally in benzothiophenes 3 and 4.[6,7] Each of these compounds has ER affinity approximately equal to that of estradiol and ca. 2 orders of magnitude greater than that of 1a, or of trioxifene (2), the prototype upon which 3 and 4 are based.[1] In the immature rat, 3 and 4 had greater antiuterotrophic effects and reduced uterotrophic effects compared to those of 1a,b.

Results of studies using human breast cancer cells in culture were in many ways consistent with the above findings. Thus, 3 was 100–1000 times as potent as 1a in suppressing MCF-7 human breast cancer cell growth, an effect paralleled by the relative affinities of 1a and 3 for cytosolic ER derived from these cells.[8] Also, 3 and 4, unlike 1a,b, did not induce characteristics in MCF-7 cells believed to be linked to residual estrogenicity.[9]

On the basis of the above findings we hypothesized that carbocyclic analogues of phenolic benzothiophenes would be effective inhibitors of breast cancer cell growth. Thus, we sought to compare the effects of four such analogues with 3 and 4, with regard to ER affinities and antiproliferative activity in cells. In these analogues, we wanted to assess the effects of ring fusion involving the carbonyl carbon and the adjoining phenyl ring. With regard to chemical synthesis, we wanted to devise new methods which would obviate problems previously encountered in

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(2) (a) Lippman, M. E. In *Williams Textbook of Endrocrinology*, 7th ed.; Wilson, J. D., Foster, D. W., Eds.; W. B. Saunders: Philadelphia, 1985; pp 1309–1319. (b) Rutqvist, L. E., et al. Adjuvant Tamoxifen in Early Breast Cancer: Occurrence of New Primary Cancers. *The Lancet* **1989**, *1* (Jan 21), 117. (c) Early Breast Cancer Trialists' Collaborative Group. Effects of Adjuvant Tamoxifen and of Cytotoxic Therapy on Mortality in Early Breast Cancer. An Overview of 61 Randomized Trials Among 28,896 Women. *New England J. Med.* **1988**, *319*, 1681.

(3) (a) Jordan, V. C.; Haldemann, B.; Allen, K. E. Geometric Isomers of Substituted Triphenylethylenes and Estrogenic Action. *Endocrinology (Baltimore)* **1981**, *108*, 1353. (b) Marth, C.; Daxenbichler, G.; Buehring, G. C.; Hofstadter, F.; Dapunt, O. Inhibition of the Estradiol-induced Growth of Cultured Human Breast Cancer Cells by the Anti-estrogens Tamoxifen, Desmethyl-tamoxifen, 4-Hydroxytamoxifen and Enclomiphene. *Biochem. Pharmacol.* **1984**, *33*, 3951. (c) Berthois, Y.; Katzenellenbogen, J. A.; Katzenellenbogen, B. S. Phenol Red in Tissue Culture Media is a Weak Estrogen: Implications Concerning the Study of Estrogen-responsive Cells in Culture. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 2496.

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Scheme I

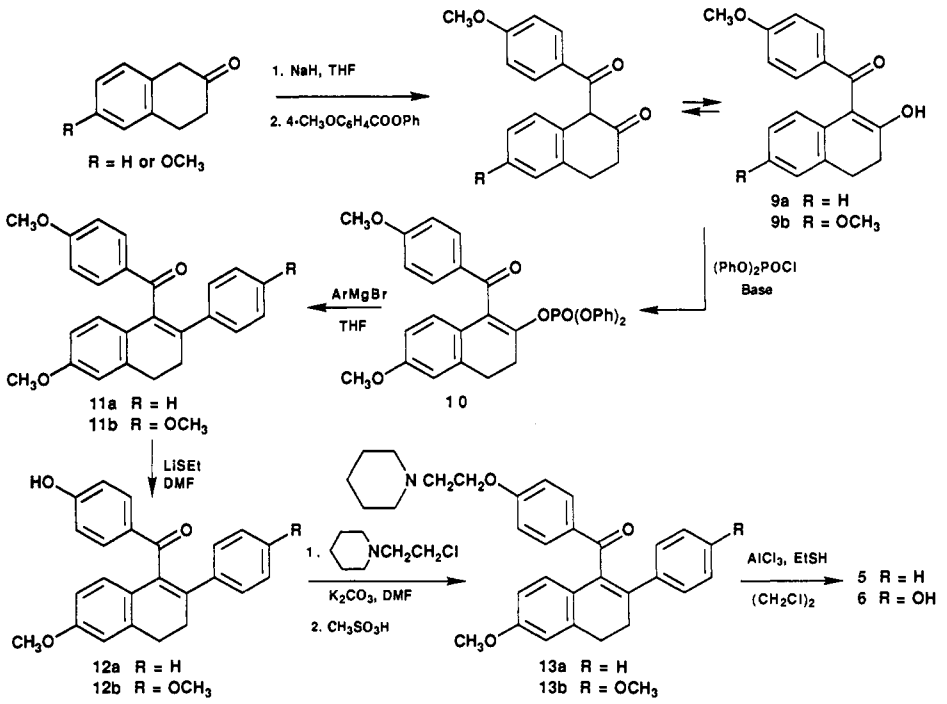
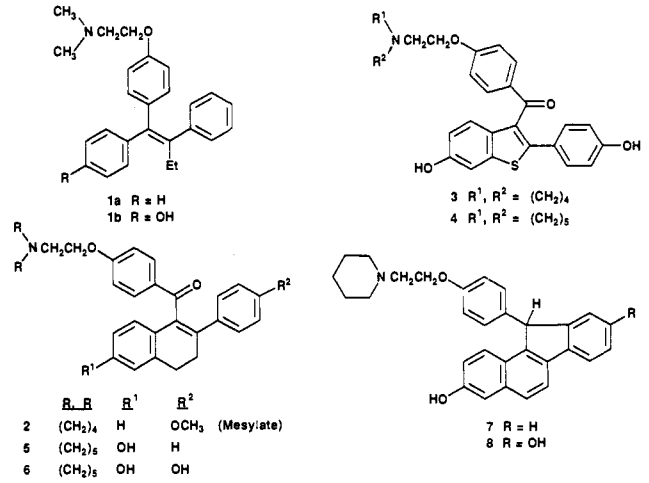


Chart I



the preparation of **2**.[10] We therefore report the synthesis of dihydronaphthalenes **5** and **6**, and their benzo[*a*]-fluorene counterparts, **7** and **8**. During these studies it became apparent that the potent growth inhibitory effects seen with **3–8** could only be partially reversed, if at all, by estradiol, indicating that these effects may have been due to interaction with other receptors besides ER. A number of alternative intracellular targets have been suggested to account for the estrogen-*irreversible* component of growth suppression of MCF-7 cells observed with tamoxifen and structurally-related compounds.[11] Of these, considerable evidence points to interference with calmodulin-dependent processes as the molecular basis for these effects, and so we hypothesized that **3–8** might interact with calmodulin as well. Thus, we have examined the antagonistic effects of **3–8**, compared to that of **1a**, on a calmodulin-dependent enzyme.

Chemistry

Synthesis of Dihydronaphthalene Derivatives 5 and 6 (Scheme I). The synthesis which we reported previously[10] for the 1-acyl-2-aryl-3,4-dihydronaphthalene nucleus embodied in **2** involved the addition of an aryl Grignard reagent to the 1-acyl-2-tetralone **9a**. In that reaction, we encountered the multiple problems of (1) proton abstraction, (2) addition to the incorrect (exocyclic) carbonyl group of **9a**, and (3) dehydration of the Grignard adduct to a mixture of 1,4- and 3,4-dihydronaphthalenes. For the synthesis of further analogues with additional aromatic oxygen functionalities, a more regiochemically defined and higher yielding process was sought. The

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(10) Jones, C. D.; Suarez, T.; Massey, E. H.; Black, L. J.; Tinsley, F. C. Synthesis and Antiestrogenic Activity of [3,4-Dihydro-2-(4-methoxyphenyl)-1-naphthalenyl][4-[2-(1-pyrrolidinyl)ethoxy]phenyl]methanone, Methanesulfonic Acid Salt. *J. Med. Chem.* **1979**, *22*, 962.

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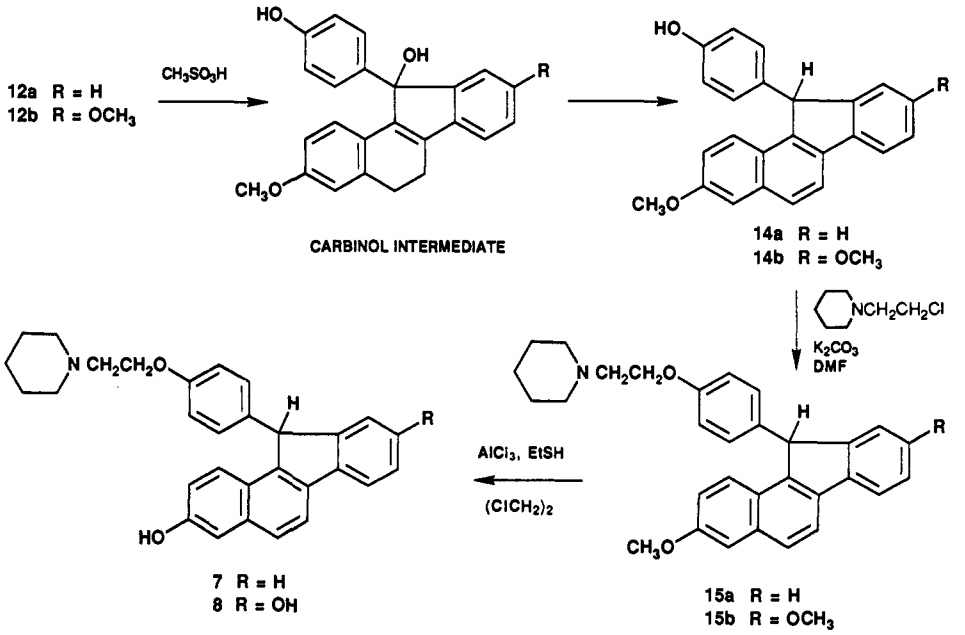
Scheme II



synthetic route used to obtain the new analogues is delineated in Scheme I. The highly enolized diketone **9b** was readily prepared by acylation of the sodium salt of 6-methoxy-2-tetralone. Subsequent derivatization using sodium hydride followed by the addition of diphenyl chlorophosphate gave the enol phosphate derivative tentatively assigned structure **10**. Addition of phenyl- or (4-methoxyphenyl)magnesium bromide to **10** provided the desired dihydronaphthalenes **11a** and **11b**, respectively. Structural assignments for **11a** and **11b** are supported by similarity of their proton NMR spectra with published data for [3,4-dihydro-2-(4-methoxyphenyl)-1-naphthalenyl](4-methoxyphenyl)methanone.[10] Thus, the replacement of the phosphate group follows the regiochemical path defined by formal addition–elimination of organometallics to enol phosphate derivatives of 1,3-dicarbonyl compounds.[12] Utilizing this enol phosphate protocol, all three of the aforementioned synthetic difficulties have been overcome, and the desired 3,4-dihydronaphthalene derivatives (**11a** and **11b**) have been obtained in quite excellent yields.

Compounds **11a** and **11b** were next subjected to demethylation conditions similar to those employed previously in the synthesis of **2**.[10] As expected, selective cleavage by LiSEt of the methoxy group para to the carbonyl group occurred with good (ca. 70–90%) selectivity, thereby producing **12a** and **12b**. The regioselectivity of this transformation was determined by comparing the NMR spectrum of **12b** with that of the corresponding potassium phenolate anion using the method of Highet and Highet.[13] Aromatic protons which shifted upfield were clearly those of the aromatic ring that bears the carbonyl group. Subsequently, the basic side chain derivatives (**13a** and **13b**) based on the piperidinylethoxy amine moiety were prepared under conventional conditions. Finally, the remaining methoxy group(s) were selectively cleaved by the use of $AlCl_3$–EtSH reagent,[1,14] to provide the desired hydroxylated dihydronaphthalene derivatives **5** and **6**.

Synthesis of Benzo[*a*]fluorene Derivatives 7 and 8 (Scheme II). An interesting transformation occurred when 1-acyl-2-aryl-3,4-dihydronaphthalene derivatives were exposed to strong acidic conditions (Scheme II). Cyclization of the 2-aryl moiety onto the carbonyl group took place readily and was accompanied by rapid dehydration of the (presumed) carbinol intermediate to provide the 11-aryl-11*H*-benzo[*a*]fluorene nuclear skeleton embodied in structures **14a** and **14b**. Important support for this structural assignment is the presence of a singlet integrating for one proton at approximately δ 5.3 ppm, which is attributed to the Ar_3C–H. Subsequently, we prepared the hydroxy analogues **7** and **8** so that their activity could be compared to the dihydronaphthalenes (**5** and **6**) described above. One can easily envisage the cyclization of the methoxylated dihydronaphthalenes **11a** and **11b** to the benzo[*a*]fluorene system. However, such an approach would result in an early loss of the carbonyl group and the benefit of its directive effect during the subsequent demethylation. Therefore, the demethylated phenolic dihydronaphthalenes **12a** and **12b** were cyclized to the respective benzo[*a*]fluorenes (**14a** and **14b**) by means of methanesulfonic acid. Compounds **14a** and **14b** were converted to **15a** and **15b**, respectively, by attachment of the aminoethoxy side chain. The synthesis of **7** and **8** was completed by cleavage of the remaining methoxy groups by $AlCl_3$–EtSH reagent by procedures closely analogous to those described above for the dihydronaphthalenes.

Biological Results

Effects of Compounds 3–8. A. In Cells. Effects of antiestrogens on cell growth correlates with clinical antitumor activity. Thus **1a** was cytostatic at 1–10 μM concentrations in this cell line, and **1a** plus its active metabolites was found at similar intratumor levels in breast cancer patients being successfully treated with **1a**.[15] We

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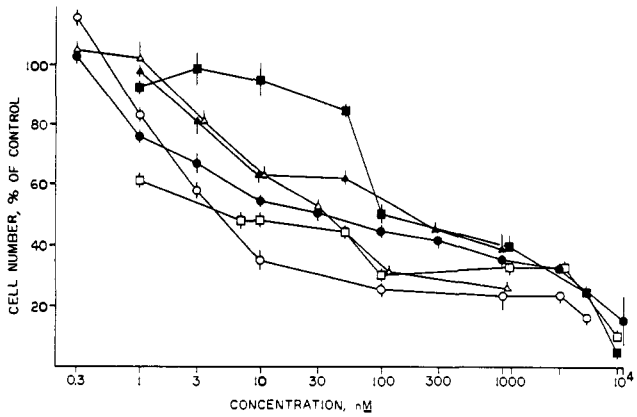

Figure 1. Inhibition of MCF-7 breast cancer cell proliferation by **3** (▲), **4** (△), **5** (●), **6** (○), **7** (■), and **8** (□). Data shown are expressed as percentages of viable cell numbers in control flasks and are the means ± SEM of at least three estimates.

Table I. Interaction of **3–8** and Estradiol with Estrogen Receptors in MCF-7 Cells[a]

compd	IC_{50}, nM	RBA[b]
3	120	2.5
4	170	1.8
5	1650	0.2
6	73	4.1
7	740	0.4
8	125	2.4
estradiol	3	100

[a] Competitive binding assays were carried out in triplicate as described in the Experimental Section. [b] Each relative binding affinity (RBA) is (IC_{50} of estradiol/IC_{50} of test compound) × 100.

incubated test compounds for 5 days with cells which had been brought into log phase growth during a 5-day preincubation period. Cells were harvested and counted, and concentrations required for 50% inhibition of proliferation (IC_{50} values) were calculated. Respective IC_{50} values for **3–8** were 160, 34, 30, 4.5, 100, and 5.0 nM (Figure 1). Thus, highly effective growth inhibition was seen in both the benzo[*a*]fluorene- and dihydronaphthalene-derived compounds.

"Reversal" of antiestrogen-mediated cell growth antagonism by estradiol has been suggested to indicate the degree to which antagonism is mediated through ER. Thus, the antiproliferative effect seen in the presence of 2.5 μM **1a** is not observed when 0.1 μM estradiol is present.[16a] Using the above assay, the effects of **3–8** were only

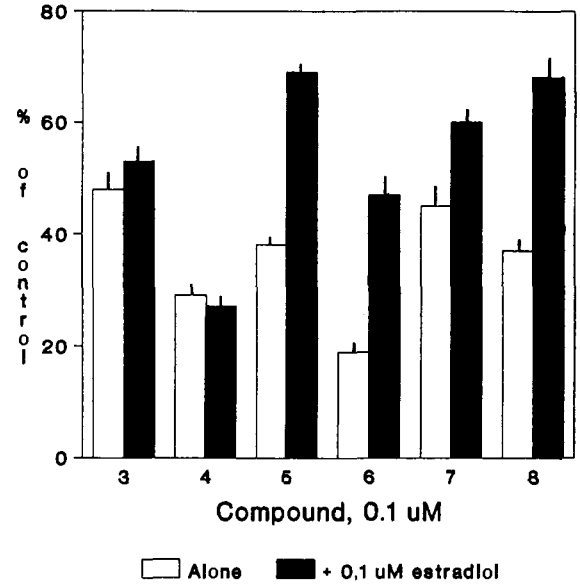

Figure 2. Effect of estradiol on MCF-7 cell growth inhibitory effects of **3–8**. Cells were prepared as described in the Experimental Section. Data are averages ± SEM of triplicate flasks.

partially reversible in the presence of 0.1 μM estradiol (Figure 2).

The ability of antiestrogens to interact with ER in whole cells has been suggested to provide a measure of partial agonist activity.[16] Thus, the ability of increasing concentrations of the test compounds to displace specifically bound [^{3}H]estradiol in these cells was determined. Concentrations required for 50% displacement of specifically bound radioactivity (IC_{50} values) were calculated from the plotted data, and are shown in Table I. The IC_{50} values of **3–8** were, in turn, 120, 170, 1650, 73, 740, and 125 nM. That of unlabelled estradiol was 3 nM. The significance of these results will be discussed below.

B. On Calmodulin. Antiestrogens such as **1a** have been shown to interact with calmodulin, which may account in part for their growth suppressive effects.[11,18] The standard method for evaluation of such interactions is to assess the effect of various concentrations of test compounds on calmodulin-stimulated cyclic nucleotide phosphodiesterase activity (see Experimental Section). Compared to **1a**, compounds **3–8** were poor calmodulin antagonists (Table I). Only with **1a** was marked concentration-dependent inhibition observed, which was comparable to that previously reported.[18]

Discussion

Our results reaffirm the powerful growth suppressant effects of **3** and **4** on hormone sensitive MCF-7 cells, in agreement with earlier studies.[8,9,19] Comparison of Dreiding models suggest that the overall planarity of the benzo[*a*]fluorene 6,6,5,6 ring system is greatly increased relative to that defined by the corresponding atoms in **2–6**. This observation, combined with the idea that such a planar moiety can mimic the structural framework of steroidal estrogens, suggested that the novel benzo[*a*]-fluorene series would retain hormonal activity. The finding

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Table II. Inhibition of Calmodulin Activation of Cyclic Nucleotide Phosphodiesterase by Compounds 1a and 3–8

compd[a]	inhibition,[b] %
1a	44
3	3
4	6
5	6
6	2
7	22
8	18

[a] The compounds were added as solutions in dimethyl sulfoxide to give incubation mixture concentrations of 10 μM containing 1% v/v dimethyl sulfoxide. [b] Results are averages of triplicate determinations, corrected for "direct" inhibition of cyclic nucleotide phosphodiesterase.

that 7 and 8 exhibit antihormonal activity generally similar to that of their less planar counterparts is in keeping with such a model. In the benzo[a]fluorene series, introduction of the second phenolic hydroxy group greatly improved activity (cf. 7 and 8). This difference was less pronounced in dihydronaphthalenes 5 and 6. All of these compounds were superior to 1a, which under conditions similar to those used in this study had an IC_{50} of approximately 1 μM.[16] They were somewhat less potent than analogous hydroxylated triarylethylenes 1b and 4-hydroxyclomiphene, which had respective IC_{50} values of 3 and 1 nM.[20]

The most important factors affecting MCF-7 cell growth are estrogens. Estradiol has been shown to reverse completely the growth-inhibitory effect of 1 μM concentrations of 1a and its hydroxylated analogues.[16,20] Although incubation of MCF-7 cells with 10 nM estradiol after exposure to 10 nM 3 restored the rate of growth,[8] our data show (Figure 2) that simultaneous exposure of cells to equimolar amounts of 3 and estradiol did not reverse the inhibitory effect of 3. This was also the case for 4. Partial restoration of growth was evident in experiments with 5–8. These results suggest that events occurring after interaction of 3–8 with ER are different than those of 1a and related hydroxytriarylethylenes. Earlier animal studies have indicated mechanistic differences between 1a and benzothiophene-derived antiestrogens. Thus, while 1a is a full estrogen agonist in the mouse, 3 had partial antiuterotrophic activity in this species.[6] In the rat, 3 prevented the uterotrophic effect of 1a.[6,19] Studies of the interaction of 1a and 3 with rat uterine ER suggested differences in the nature of respective ligand–receptor complexes.[21]

Alternatively, the failure of estradiol to reverse completely the inhibitory effects of 3–8 may signify interaction with another receptor besides ER. Calmodulin is known to regulate many cellular processes and interference with the function of this protein may contribute to the antiproliferative effects of antiestrogens. Indeed, estrogen-insensitive inhibition of MCF-7 cell growth has been correlated with antagonism of calmodulin in a diverse collection of structural types, including 1a.[11] The present studies (Table II) suggest that antiproliferative effects of 3–8 are not mediated primarily via interactions with calmodulin. This is especially true for 3–6, with which meaningful calmodulin antagonism was not observed.

Observable interaction of ligands with intracellular ER has been suggested to indicate residual estrogenicity in antiestrogens. Thus for full estrogens the ratio of whole cell to cytosolic ER affinity approaches 1.0, but for estrogen antagonists, these ratios are much lower: those of 1a and 1b were 0.06 and 0.03.[17] Previously, 3 and 4 were found to have cytosolic ER affinities of 75% and 175% that of estradiol.[7,21] The present results (Table I) indicate these compounds to have respective intracellular ER affinities 2.5% and 1.8% of that of estradiol. Hence, their affinity ratios are 0.03 and 0.01. These findings, suggestive of partial agonist activity of 3 and 4, are consistent with recent studies in which these benzothiophenes were found to stimulate breast cancer cell proliferation in the absence of estrogens.[22] However, the benzothiophenes were unable to induce progesterone receptors[8] or to stimulate "invasiveness" in MCF-7 cells,[9b,22a] properties attributed to residual estrogenicity of 1a and 1b. Together, these findings amplify differences in partial agonist expression of 3 and 4, compared to triarylethylene-based antiestrogens, which are not evident simply from comparison of ER affinity ratios. Thus, assessment of the degree of residual estrogenicity of 3–8, suggested by their intracellular ER affinities (Table I), would require evaluation of their effects on cancer cell growth similar to those referenced above.

A drawback to clinical applications of 3 and 4 has been the relatively low in vivo activity of these antiestrogens.[6b,19,23] Biopharmaceutic studies with 4 suggested this to be due to the ease by which glucuronide conjugation occurred.[24] Whether 5–8, which the present results show to be potent antiproliferative drugs as well, are less prone to such metabolic loss of effectiveness will require further study. Alternatively, studies of alternative routes of administration (e.g. transdermal) and/or suitable prodrug modifications in this series, with the aim of minimizing exposure to conjugating enzymes, may improve in vivo effectiveness. In this regard, unmetabolized 1b was retained in breast tumors of patients who received the drug by local percutaneous application, although 1b cannot be used orally due to metabolic loss.[25]

In summary, an efficient method for preparation of carbocyclic analogues of benzothiophene-based anti-

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estrogens is reported. The new dihydronaphthalene- and benzo[a]fluorene-based compounds were highly effective in inhibiting the growth of MCF-7 cells in culture. The low observable ER affinity of these compounds in whole MCF-7 cells, relative to that of estradiol, was nonetheless comparable to that of triarylethylene antiestrogens. Although estradiol did not fully reverse the antiproliferative effects of 3–8, calmodulin, a putative alternative "receptor" for 1a and other triarylethylene antiestrogens, was shown not to be affected significantly by 3–8.

Experimental Section

Methods. Melting points were determined with a Thomas-Hoover capillary melting point apparatus and are uncorrected. Infrared spectra were recorded on a Nicolet 10MX Fourier transform spectrophotometer and ultraviolet spectra were taken on a Cary 219 instrument. Proton NMR spectra were determined at 300 MHz on a General Electric QE-300 spectrometer using tetramethylsilane as internal standard with values given in parts per million (ppm). Mass spectra were obtained on a Finnegan MAT731 spectrometer in the EI mode with samples introduced directly into the ion source or in the FD mode using carbon dendrite emitters for the spectral determination. Although only selective spectral data are presented herein, all new compounds exhibited IR, UV, and NMR spectra consistent with the structures assigned to them. Microanalyses were performed at Eli Lilly and Co., and results are within 0.4% of the calculated values except as noted. Most of the starting materials are commercially available or well-known in the literature. Preparative high-pressure liquid chromatography (HPLC) was carried out using a Waters Prep 500A machine with one normal-phase silica gel cartridge unless specified otherwise. Yields have not been maximized and in many cases were considerably higher than the amounts of pure products which were eventually obtained would seem to indicate.

Synthesis of 3,4-Dihydro-6-methoxy-1-(4-methoxybenzoyl)-2(1*H*)-naphthalenone (9b) by Acylation of 6-Methoxy-2-tetralone. To a suspension of phenyl 4-methoxybenzoate (6.5 g, 0.0284 mol) in 40 mL anhydrous THF at 25 °C under a dry nitrogen atmosphere was added (1.5 g, 0.0625 mol) NaH (60% suspension in mineral oil). Next, 6-methoxy-2-tetralone (5.0 g, 0.0284 mol, Aldrich Chem. Co.) in 20 mL of THF was added dropwise with stirring. Evolution of hydrogen gas ensued and the reaction mixture gradually became yellow. Stirring was continued at ambient temperature for 1 h. The reaction mixture was heated to 55–60 °C for 1.5 h and then allow to cool to 25 °C. It was next poured over a mixture of 250 mL of 1 N HCl, ice (200 mL), and EtOAc (200 mL). The EtOAc was washed with brine, dried over $MgSO_4$, and evaporated to provide 12.3 g of an orange oil. Analysis by TLC (SiO_2, toluene–EtOAc 9:1) indicated the presence of much phenol, as well as a yellow product corresponding to the desired material. Most of the phenol was removed under vacuum (pressure below 0.1 Torr) by heating the product in a distillation setup in a 100 °C oil bath for 16 h. The resulting thick oily residue was then crystallized from MeOH with the aid of a seed crystal (obtained by slow evaporation of a dilute isooctane solution of the crude product). The diketone **9b**, mp 92–4 °C, was obtained in a yield of 5.01 g (57%): ^1H NMR ($CDCl_3$) δ 2.61 (t, 2, J = 6.8 Hz, $-CH_2-$ α to aromatic), 2.94 (t, 2, J = 6.8 Hz, $-CH_2-$ α to enol), 3.78 (s, 3, OCH_3), 3.85 (s, 3, OCH_3), 6.47 (m, 1, H-7), 6.67 (d, 1, $J_{7,8}$ = 8.7 Hz, H-8), 6.74 (d, 1, $J_{5,7}$ = 2.4 Hz, H-5), 6.83 (d, 2, J = 8.8 Hz, aromatic ortho to OCH_3), 7.52 (d, 2, J = 8.8 Hz, aromatic ortho to C=O). Anal. ($C_{19}H_{18}O_4$) C, H, O.

3,4-Dihydro-1-(4-methoxybenzoyl)-6-methoxy-2-naphthalenyl Diphenyl Phosphoric Acid Ester (10). To a solution of the diketone **9b** (1.50 g, 0.0048 mol) at 5 °C under N_2 in 15 mL of CH_2Cl_2 was added diphenyl chlorophosphate (1.36 g, 0.0051 mol) and 4-(dimethylamino)pyridine (5 mg). Triethylamine (0.514 g, 0.0051 mol) in CH_2Cl_2 (20 mL) was then added dropwise over 10 min, while keeping the reaction temperature below +5 °C. The resulting mixture was stirred overnight, and then it was poured over brine and ice and the crude product was extracted by EtOAc (50 mL). The organic layer was washed well with brine, dried over anhydrous K_2CO_3, and evaporated to obtain 2.92 g of a yellow oil. Silica gel chromatography which utilized 10% EtOAc in toluene gave **10** as a yellow oil, 2.17 g (83%). This material gave a strong peak in its field desorption mass spectrum at m/e 542 and was essentially a single component by NMR spectroscopy. Nevertheless, it failed to crystallize and did not give an acceptable combustion analysis for carbon. Anal. ($C_{31}H_{27}PO_7$) H, O; C: calcd, 68.63; found, 65.37.

[3,4-Dihydro-6-methoxy-2-phenyl-1-naphthalenyl](4-methoxyphenyl)methanone (11a) via Addition of Grignard Reagent to Enol Phosphate Diphenyl Ester. Sodium hydride (60% in mineral oil, 128 mg, 0.0053 mol) was suspended in anhydrous THF (15 mL) under a nitrogen atmosphere, and the mixture was cooled to 5 °C in an ice bath. A solution consisting of **9b** (1.50 g, 0.0048 mol) and diphenyl chlorophosphate (1.36 g, 0.0051 mol, Aldrich Chem. Co.) in THF (10 mL) was added at a rate so that the temperature of the reaction mixture remained below 10 °C. Following the initially rapid evolution of hydrogen gas, the reaction mixture was stirred for 30 min with continued cooling from the ice bath. Analysis of a small sample by TLC (SiO_2, toluene-EtOAc 9:1) showed essentially quantitative formation of the enol phosphate intermediate. The reaction mixture was maintained near 0 °C and phenylmagnesium bromide (3.6 mL of a 2 M solution in ether, 0.0072 mol) in 5 mL of THF was added dropwise over approximately 5 min. The resulting mixture was stirred at 0 °C for 1 h, and then it was allowed to warm to room temperature. By TLC analysis, loss of enol phosphate had accompanied the formation of a major product which migrated at high R_f. The reaction was worked up by pouring it over a large excess of iced NH_4Cl solution and extracting the crude product with 2 × 30-mL portions of EtOAc. The extracts were washed with brine and dried over anhydrous $MgSO_4$. After filtration and evaporation of the solvents, a brown oil (2.9 g) was obtained. The oil was easily purified by chromatography over silica gel. Pooling of appropriate fractions gave 1.6 g (89%) of a yellow oil which crystallized. Recrystallization from MeOH gave 1.23 g (69%) of **11a** as off-white crystals which had a melting point of 101.5–104 °C. Anal. ($C_{25}H_{22}O_3$) C, H, O.

[3,4-Dihydro-6-methoxy-2-(4-methoxyphenyl)-1-naphthalenyl](4-methoxyphenyl)methanone (11b). This compound was prepared by a route similar to that given above for synthesis of **11a**. Compound **9b** (25 g, 0.0806 mol) and (4-methoxyphenyl)magnesium bromide (prepared from 4-bromoanisole and Mg in anhydrous THF) were used. The resulting brown oily product was purified by chromatography over silica gel. The column was eluted initially with 1 L of isooctane–toluene (1:1), followed by a gradient consisting of 4 L of toluene that changed linearly to an equal volume of toluene–EtOAc (9:1). Appropriate fractions were combined and concentrated to the pale yellow oily product that was obtained in a yield of 30.8 g (96%): ^1H NMR (DMSO-d_6) δ 2.69 (m, 2, CH_2), 2.96 (m, 2, CH_2), 3.64 (s, 3, OCH_3), 3.71 (s, 3, OCH_3), 3.74 (s, 3, OCH_3), 6.6–6.9 (m, 7, aromatic), 7.16 (d, 2, J = 8.6 Hz, aromatic ortho to C=C), 7.75 (d, 2, J = 8.7 Hz, aromatic ortho to C=O). Anal. ($C_{26}H_{24}O_4$) C, H, O. After long standing, a sample crystallized. Recrystallization from a small amount of MeOH provided yellow crystals, mp 172–173 °C.

[3,4-Dihydro-6-methoxy-2-phenyl-1-naphthalenyl](4-hydroxyphenyl)methanone (12a). To EtSH (6.85 g, 0.11 mol) in anhydrous THF (100 mL) at −30 °C under a dry nitrogen atmosphere in a 1-L single-neck round-bottomed flask was added slowly via syringe 1.5 M n-BuLi (64.9 mL, 0.097 mol). This addition was only mildly exothermic. After addition was complete, the reaction was warmed to 25 °C and dry DMF (100 mL) was added. The resulting mixture was evaporated carefully on a rotary evaporator to reduce the total volume to ca. 75 mL. The residue was then warmed to 40 °C and the dimethoxy compound **11a** (24.0 g, 0.065 mol) was added. After heating the reaction mixture at 80 °C for 2.5 h, TLC analysis (SiO_2, toluene–EtOAc 9:1) showed the **11a** to be nearly gone. Two spots were present at lower R_f. These were attributed to the desired product and the corresponding diphenol (lowermost spot). The reaction mixture was cooled and poured into 500 mL of ice 1 N HCl solution. The crude product was extracted into EtOAc. The EtOAc phase was washed with saturated aqueous NaCl solution, dried over anhydrous $MgSO_4$, and evaporated to a yellow oil. The product was purified by HPLC using a gradient consisting of 3.5 L of toluene changing linearly to an equal volume of toluene–EtOAc 9:1. Following

evaporation of the appropriate fractions, a yellow oil was obtained (21.3 g, 92%). This material, which gave a single spot by TLC, crystallized on standing and was subsequently recrystallized from EtOAc–isooctane to yield 14.7 g, (64%) of white crystalline **12a**, mp 180–181 °C. Anal. ($C_{24}H_{20}O_3$) C, H.

[3,4-Dihydro-6-methoxy-2-(4-methoxyphenyl)-1-naphthalenyl](4-hydroxyphenyl)methanone (12b). The trimethoxylated compound **11b** (29.0 g, 0.0725 mol) was also demethylated by the procedure given above for synthesis of **12a**. Chromatography under similar conditions gave a 73% yield of **12b** as a yellow oil which soon crystallized: ^{1}H NMR (DMSO-d_6) δ 2.67 (br s, 2, CH_2), 2.96 (m, 2, CH_2), 3.64 (s, 3, OCH_3), 3.71 (s, 3, OCH_3), 6.6–6.8 (m, 7, aromatic), 6.86 (s, 1, aromatic H-5), 7.16 (d, 2, J = 8.7 Hz, aromatic ortho to C=C), 7.65 (d, 2, J = 8.6 Hz, aromatic ortho to C=O), 10.34 (s, 1, OH). Determination by NMR of the site of the selective demethylation was carried out by treatment of the **12b** in DMSO solution with several equivalents of KOD in DMSO as described in ref 13. The sharp doublet signal at δ 7.65 (corresponding to the two aromatic protons that are ortho to the carbonyl group) shifted 0.33 ppm upfield and widened into a broad singlet. Similarly, a resonance at δ 6.60 (attributable to the two protons meta to the C=O) was shifted even more (0.6 ppm) upfield to δ 6.0 where it appeared as a slightly broadened doublet. The remaining signals in the spectrum for **12b** in DMSO-d_6 were essentially unaltered by addition of KOD. The shifts described above demonstrate that the OH and C=O groups are on the same aromatic ring (i.e. selective demethylation occurred at the methoxy group which is para to the carbonyl group). Anal. ($C_{25}H_{22}O_4$) C, H, O. A small sample recrystallized from EtOAc–isooctane exhibited a melting point of 172–173 °C.

(3,4-Dihydro-6-methoxy-2-phenyl-1-naphthalenyl)[4-[2-(1-piperidinyl)ethoxy]phenyl]methanone, Methanesulfonic Acid Salt (13a). Compound **12a** (12.0 g, 0.034 mol), anhydrous K_2CO_3 (23.3 g, 0.168 mol), *N*-(2-chloroethyl)piperidine hydrochloride (7.44 g, 0.040 mol, Aldrich Chem. Co.) and 2-butanone (300 mL) were combined under a nitrogen atmosphere, and the resulting mixture was refluxed for 4 h. Analysis by TLC (SiO_2, MeOH) showed that a single lower R_f spot was formed. Ethyl acetate (200 mL), ice, and water were added and the organic layer was separated, washed with brine, and dried over anhydrous $MgSO_4$. After concentration to an oil, the product was purified by HPLC using 2% Et_3N in ether as the elution solvent. The appropriate fractions gave, on evaporation of the solvent and vacuum drying of the residue overnight, an oil which weighed 15.6 g. The oil was dissolved in 30 mL of dry acetone and cooled in an ice bath, and a freshly-prepared solution of methanesulfonic acid (3.23 g, 0.0337 mol) in 8 mL acetone was added. No crystals appeared on standing overnight, but evaporation to near dryness followed by crystallization from acetone-MeOH 5:1 at −78 °C was successful. White crystals, which slowly formed, were washed with −78 °C acetone and dried under vacuum to provide **13a** (15.8 g, 83%, mp 193–194 °C. Anal. ($C_{32}H_{37}NO_6S$) C, H, N, O, S.

[3,4-Dihydro-6-methoxy-2-(4-methoxyphenyl)-1-naphthalenyl][4-[2-(1-piperidinyl)ethoxy]phenyl]-methanone, Methanesulfonic Acid Salt (13b). By using an analogous procedure as that used for synthesis of **13a**, the basic side chain was appended to **12b** (12.0 g, 0.031 mol), thereby providing **13b**. Following chromatography, the crude free base was obtained as a pale brown oil. The oil was dissolved in acetone, cooled in an ice bath and methanesulfonic acid in acetone was added. Crystallization of **13b** was difficult, but after repeated efforts the product was obtained from MeOH–acetone in 76% yield as off-white crystals, mp 162–163.5 °C. Anal. ($C_{33}H_{39}NO_7S$) C, H, N.

[3,4-Dihydro-6-hydroxy-2-phenyl-1-naphthalenyl][4-[2-(1-piperidinyl)ethoxy]phenyl]methanone (5) by Demethylation with $AlCl_3$–EtSH. In a 500-mL three-neck round-bottom flask equipped with mechanical stirrer, condenser, and dry nitrogen inlet, $AlCl_3$ (8.3 g, 0.062 mol) was suspended in 1,2-dichloroethane (100 mL) and cooled to near 0 °C. Ethanethiol (5.5 g, 0.089 mol) was slowly added via syringe, and the resulting mixture was stirred for 15 min at 0 °C. Then **13a** (5.0 g, 0.0089 mol) was added as a solid in several portions. The addition resulted in the formation of an intense reddish-brown solution. After vigorous stirring of the reaction mixture for 1.5 h, TLC (SiO_2, $CHCl_3$–MeOH) showed loss of the starting material and the formation of a single lower R_f spot. The contents of the flask were cooled to 0 °C, and 100 mL of THF was added gradually. The resulting mixture was then poured over ice (200 mL), H_2O (500 mL), and concentrated HCl (10 mL). After thorough mixing, solid $NaHCO_3$ was very carefully added until pH 8.4 was reached. The crude product was then extracted with 3 × 200 mL of EtOAc. The extract was dried over anhydrous $MgSO_4$ and evaporated to a pale yellow oil (4.6 g) that crystallized. Recrystallization from MeOH–acetone gave compound **5**, (3.27 g, 81%), mp 122–4 °C. Anal. ($C_{30}H_{31}NO_3$) C, H, N.

[3,4-Dihydro-6-hydroxy-2-(4-hydroxyphenyl)-1-naphthalenyl][4-[2-(1-piperidinyl)ethoxy]phenyl]methanone (6). By the same method, **13b** (5.0 g, 0.0084 mol) was demethylated to provide **6**. Additional EtOAc was required during the workup to dissolve the crude product that was an odorous yellow oil (4.1 g). Chromatography on silica gel with gradient elution consisting of 3.5 L of 5% MeOH in CH_2Cl_2 progressing to an equal volume of 30% MeOH in CH_2Cl_2 gave 3.9 g of pure material. Compound **6** slowly crystallized from EtOAc as yellow crystals, 3.38 g (86%), mp 206–208 °C. Anal. ($C_{30}H_{31}NO_4$) C, H, N.

4-(3-Methoxy-11*H*-benzo[*a*]fluoren-11-yl)phenol (14a). Synthesis of Benzo[*a*]fluorene Derivatives by Acid-Catalyzed Cyclization. The dihydronaphthalene derivative **12a** (15.0 g, 0.042 mol) and methanesulfonic acid (200 mL) were well mixed and stirred at room temperature under a nitrogen atmosphere. The starting material was essentially absent after 16 h as determined by TLC (SiO_2, toluene–EtOAc 9:1). The acidic reaction mixture was poured into brine and the product was extracted with 3 × 200-mL portions of EtOAc. After drying the organic extract over anhydrous magnesium sulfate and evaporation of the solvent, the crude product was obtained as 16.5 g of a purple oil. Crystalline **14a**, mp 207–208 °C, was obtained from 2-propanol in a yield of 9.97 g (70%). Anal. ($C_{24}H_{18}O_3$) C, H.

4-(3,9-Dimethoxy-11*H*-benzo[*a*]fluoren-11-yl)phenol (14b). Cyclization of **12b** (8.4 g, 0.022 mol) was carried out in 90 mL of methanesulfonic acid by the same procedure used above for **12a** except for a longer reaction time. After 3 days, the starting material was almost completely consumed as indicated by TLC (SiO_2, toluene–EtOAc 9:1). Workup by the procedure described for **14a** gave a dark, tarry oil. The oil was purified by chromatography over silica gel using a gradient system consisting of 3.5 L of toluene that changed linearly to 3.5 L of 10% EtOAc in toluene. Appropriate fractions yielded 3.1 g of somewhat oily tan-colored crystals which were recrystallized from 2-propanol to obtain 1.81 g (23%) of white crystalline **14b**, mp 196–199 °C. NMR (DMSO-d_6) δ 3.72 (s, 3, OCH_3), 3.82 (s, 3, OCH_3), 5.31 (s, 1, CH), 6.6 to 7.0 (m, 12, aromatic), 9.24 (s, 1, OH). Anal. ($C_{25}H_{20}O_3$) C, H.

3-Methoxy-11-[4-[2-(1-piperidinyl)ethoxy]phenyl]-11*H*-benzo[*a*]fluorene (15a). A mixture of the phenol **14a** (8.0 g, 0.0237 mol), anhydrous K_2CO_3 (16.3 g, 0.118 mol), dry DMF (75 mL), and *N*-(2-chloroethyl)piperidine hydrochloride (4.58 g, 0.0249 mol) were refluxed under a nitrogen atmosphere for 1 h. The reaction mixture was cooled and poured into a mixture of brine (1 L) and ice (500 g). The product was extracted by 3 × 200 mL of EtOAc. The organic extracts were washed with brine, dried over anhydrous $MgSO_4$, and evaporated to provide 10.6 g of an oil which was chromatographed over silica gel using 2% Et_3N in ether as eluant. Concentration of the appropriate fractions from the column gave 9.5 g of **15a** which was a single component by TLC (SiO_2, MeOH). Recrystallization from isopropyl alcohol provided **15a**, 8.74 g (82%), mp 130–133 °C. Anal. ($C_{31}H_{31}NO_2$) C, H, N.

3,9-Dimethoxy-11-[4-[2-(1-piperidinyl)ethoxy]phenyl]-11*H*-benzo[*a*]fluorene (15b). By the same procedure used for synthesis of **15a**, 1.80 g (0.0049 mol) of **14b** was reacted with *N*-(2-chloroethyl)piperidine hydrochloride (0.95 g, 0.0051 mol) in the presence of K_2CO_3 (3.37 g, 0.0244 mol) to provide **15b** (1.93 g 82%): mp 162–164 °C after recrystallization from EtOAc–isooctane. NMR (DMSO-d_6) δ 1.33 (br m, 2, 4-CH_2 of piperidine ring), 1.45 (br m, 4, 3-CH_2 groups of piperidine ring), 2.36 (br m, 4, 2-CH_2 groups of piperidine ring), 2.57 (t, 2, NCH_2CO), 3.70 (s, 3, OCH_3), 3.81 (s, 3, OCH_3), 3.94 (t, 2, $NCCH_2$), 5.38 (s, 1, CH), 6.7 to 8.0 (m, 12, aromatic). Anal. ($C_{32}H_{33}NO_3$) C, H, N.

11-[4-[2-(1-Piperidinyl)ethoxy]phenyl]-11*H*-benzo[*a*]-fluoren-3-ol (7). Demethylation in the benzo[*a*]fluorene series

was accomplished by the same procedure described above for dihydronaphthalene derivatives **13a** and **13b**. In the demethylation of **15a** (2.0 g, 0.0045 mol) by $AlCl_3$ (4.15 g, 0.0312 mol) and EtSH (2.77 g, 0.0447 mol), the temperature of the reaction was maintained near 0 °C, so as to minimize side reactions. After 2.5 h, the reaction was complete as judged by TLC (SiO_2, 2% Et_3N in ether). Workup was accomplished by the procedure described above for preparation of **5**. The off-white crystalline **7** was obtained as the free base in a yield of 1.71 g (87%), following recrystallization from acetone. Compound **7** exhibited mp 216–217 °C and gave acceptable elemental analysis. Anal. ($C_{30}H_{29}NO_2$) C, H, N.

11-[4-[2-(1-Piperidinyl)ethoxy]phenyl]-11*H*-benzo[*a*]fluorene-3,9-diol (8). Demethylation of compound **15b** (1.0 g, 0.0021 mol) was carried out in the same manner as described above in the preparation of **7**. The reaction temperature was carefully maintained at or below 0 °C throughout the course of the reaction which was complete after 2.5 h, by TLC (SiO_2, 2% Et_3N in ether). The crude product was isolated by the usual procedure as 1.0 g of an odorous oil. The oil was purified by chromatography over silica gel using a gradient elution system which consisted of 2% MeOH in $CHCl_3$ (2 L) and an equal volume of 30% MeOH in $CHCl_3$. The desired diphenol **8** was a tan foam that amounted to 0.70 g (74%) and did not exhibit a definite melting point, but gave acceptable elemental analysis. Anal. ($C_{30}H_{29}NO_3$) C, H, N.

Inhibition of MCF-7 Cell Growth by Compounds 3–8. Cells in their 148th passage were obtained from American Type Culture Collection, Rockville, MD. Experiments were run in 25-cm^2 flasks. Each flask contained 5 mL of growth medium (RPMI 1640 containing 10% v/v fetal bovine serum, HEPES buffer (20 mM), $NaHCO_3$ (14 mM), glutamine (5 mM), gentamicin sulfate (20 μg/mL), and insulin (10 μg/mL). Flasks were seeded with approximately 50000 cells each and were incubated at 37 °C in humidified 95% oxygen–5% carbon dioxide atmosphere for 4 days. The medium was replaced with fresh medium every other day. On the fifth day, the medium was replaced and test compounds were added as solutions in 5-μL aliquots of DMSO to get final concentrations ranging from 0.3 to 10 μM. Control flasks received 5 μL of DMSO. Incubations were continued for five days. During the course of incubations, medium was not replaced. Subsequently, the cells were harvested with 0.05% trypsin–0.02% EDTA in phosphate buffered saline. Viable cell counts were made under phase contrast on hemocytometers.

Whole Cell Estrogen Receptor Binding Assay. Experiments were run in six-well tissue culture dishes. To each well was added ca. 2×10^5 MCF-7 cells plus 5 mL of growth medium. Medium was replaced every other day during this period. After 4 days of growth, the medium was aspirated out of the wells and replaced with serum-free medium containing 2 nM [^{3}H]estradiol (prepared by adding 67 μL of 1.5 μM [^{3}H]estradiol (58 Ci/mmol specific activity) to 50 mL of serum-free medium). Incubations were carried out in triplicate. To the medium in each member of the first set of wells was added 5 μL of DMSO. Solutions of the test compound were added in 5-μL aliquots of DMSO to give final test compound concentrations ranging from 0.01 to 3.0 μM in sets 2–8. To the medium in each member of the last set of wells was added 5 μL of a 120 μM solution of estradiol to give a final concentration of 0.4 μM. Solutions were mixed by swirling the multiwell dishes. Incubations were run for 60 min at 37 °C. Then the medium was aspirated out, and the monolayer was washed thrice with ice-cold phosphate buffered saline. Finally, 1 mL of ethanol was added to each well and incubated at room temperature for 20 min. An aliquot of 800 μL of ethanol from each well was transferred to a miniscintillation vial and ^{3}H was determined in 4 mL of Ecoscint liquid scintillation fluid.

Antagonism of Calmodulin Activation of Cyclic Nucleotide Phosphodiesterase. Activity was assayed by adaptation of an established procedure.[26] Incubation mixtures contained, in a final volume of 1.0 mL, 40 mM imidazole, 2.5 mM $CaCl_2$, 5 mM $MgCl_2$, 0.2 unit of 5′-nucleotidase, 0.01 unit of cyclic nucleotide phosphodiesterase, and 10 units of calmodulin (CM), in 40 mM Tris buffer, pH 7.5. Test drugs were added as solutions in dimethyl sulfoxide (10 μL) to give final concentrations ranging from 0–100 μM. Reactions were started by addition of cyclic AMP (final concentration, 1 mM). Incubations were run at 30 °C for 30 min. Then 1.0 mL of 35% trichloroacetic acid was added to precipitate protein, and inorganic phosphate was determined in 1.0-mL aliquots of the supernatants.[27] Control incubations contained no cyclic AMP. CM-independent activity was determined in incubations in which CM was omitted.

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Registry No. **1a**, 10540-29-1; **3**, 63676-25-5; **4**, 84449-90-1; **5**, 138630-65-6; **6**, 138630-66-7; **7**, 138666-17-8; **8**, 138630-67-8; **9b**, 138630-68-9; **10**, 138630-69-0; **11a**, 138630-70-3; **11a** diphenol, 138630-71-4; **11b**, 138630-72-5; **12a**, 138630-73-6; **12b**, 63620-02-0; **13a**, 138630-75-8; **13a** free base, 138630-74-7; **13b**, 138630-77-0; **14a**, 138630-78-1; **14b**, 138630-79-2; **15a**, 138630-80-5; **15b**, 138630-81-6; phenyl 4-methoxybenzoate, 4181-97-9; 6-methoxy-2-tetralone, 2472-22-2; 4-bromoanisole, 104-92-7; *N*-(2-chloroethyl)piperidine hydrochloride, 2008-75-5; bromobenzene, 108-86-1.

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