Measurements of diffraction were carried out on a Rigaku AFC-5R diffractometer, using graphite-monochromated Mo Kα radiation (λ = 0.71073 Å). The unit cell dimensions were obtained by least squares of 20 2θ values.

Five reference reflections monitored showed no significant intensity deterioration during data collection. Corrections were made for Lorentz and polarization factors but not for absorption. Weak reflections below background were regarded as zero reflections. The standard deviations were estimated by the equation $\sigma^2(F_o) = \sigma_p^2(F_o) + q^2F_o^2$, where q was derived from measurement of the monitored reflections and $\sigma_p(F_o)$ was due to counting statistics.[26] The crystal data and experimental details are summarized in Table V. The structure of **3a**·HBr was solved by a combination of heavy-atom and direct-method techniques.[27] The Br coordinates were derived from a Patterson synthesis. After least-squares refinement of the coordinates, weighted Fourier method revealed the remaining non-H atoms. The structure of **3a**·HCl was solved by the direct method. The atomic parameters were refined by the blocked-diagonal least-squares method. The quantity minimized was $\Sigma\omega(|F_o| - |F_c|)^2$ with $\omega = 1/\sigma^2(F_o)$. In the refinement, the zero reflections with $|F_c| > F_{lim}$ were included by assuming $|F_o| = F_{lim}$ with $\omega = \omega(F_{lim})$, F_{lim} being the observed threshold value. The hydrogen atoms were obtained by calculation and fixed. In the last cycle of refinement, H atoms were included in the calculation of structure factors and the R values were 0.12 for **3a**·HCl with 1.0σ of the maximum shift in the atomic parameters and 0.091 for **3a**·HBr with 0.4σ. The final atomic parameters of **3a**·HCl and **3a**·HBr are given in Tables VIa and VIb, respectively.

The absolute configuration of **3a**·HBr has been determined by the Bijvoet method from the anomalous scattering due to the Br atom with use of Mo radiation and found to be *S,S*.

The atomic scattering factors were taken from *International Tables for X-ray Crystallography*.[28]

Acknowledgment. We are indebted to Drs. K. Takahashi, H. Maeno, N. Inukai, and K. Murase for their valuable advice, and we express our appreciation to K. Takanobu for the HPLC analysis, to H. Kaniwa for the NMR analysis, and to T. Yahagi and E. Suzuki for the elemental analysis.

Registry No. 1a, 76093-33-9; **1b**, 76093-34-0; (±)-**2**, 101979-03-7; **2a**, 101385-90-4; **2a**·(*R*)-mandelate, 101930-03-4; **2b**, 101930-07-8; **2b**·(*R*)-mandelate, 101930-08-9; **3a**, 104713-75-9; **3a**·HCl, 104757-53-1; **3a**·HBr, 104713-76-0; **3a**·(*S*)-malate, 104713-80-6; **3a**·oxalate, 104713-81-7; **3b**, 104757-56-4; **3b**·HCl, 104831-94-9; **3c**, 104713-77-1; **3c**·HCl, 104757-54-2; **3c**·oxalate, 104713-82-8; **3d**, 104713-78-2; **3d**·HCl, 104757-55-3; (±)-**3e**, 101930-02-3; (±)-**3e**·malonate, 104641-99-8; (±)-**3f**, 101930-21-6; **4**, 99-61-6; (±)-**6**, 104713-79-3; **6a**, 101930-01-2; **7**, 101469-91-4; (*S*)-malic acid, 97-67-6; benzylamine, 100-46-9; (*R*)-mandelic acid, 611-71-2; diketene, 674-82-8; methyl 3-aminocrotonate, 14205-39-1; calcium, 7440-70-2.

Supplementary Material Available: Table VIII of anisotropic thermal parameters of non-hydrogen atoms, Table IX of bond distances and angles, Table X of torsion angles, and Table XI of calculated and observed values of Bijvoet pairs (9 pages); Table VII of lists of structure factors (28 pages). Ordering information is given on any current masthead page.

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Substituted-Vinyl Hydroxytriarylethylenes, 1-[4-[2-(Diethylamino)ethoxy]phenyl]-1-(4-hydroxyphenyl)-2-phenylethylenes: Synthesis and Effects on MCF 7 Breast Cancer Cell Proliferation

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A series of triarylethylene compounds related to 4-hydroxyclomiphene (**2**) in which the vinyl Cl substituent was replaced by ethyl (**5**), Br (**6**), H (**7**), CN (**8**), or NO_2 (**9**) substituents were synthesized to facilitate studies of the molecular actions of synthetic nonsteroidal antiestrogens. The relative binding affinities of these compounds for the estrogen receptor (ER) and the antiestrogen binding site (AEBS) in MCF 7 human mammary carcinoma cells were measured and correlated with the effects of these drugs on cell proliferation kinetics. Affinities for ER and AEBS were highly correlated, illustrating that vinyl substituents influence binding to ER and AEBS in a parallel manner. All compounds except **7** had biphasic effects on cell proliferation kinetics, indicating the presence of at least two distinct mechanisms by which hydroxytriarylethylenes inhibit breast cancer cell proliferation. In the concentration range 10^{-10}–10^{-8} M, cell proliferation was inhibited by 60–70%, these effects were estrogen-reversible, and the degree of growth inhibition was in the order Cl > Et > Br > NO_2 > CN > H, which paralleled the order of affinities for ER. There was no further inhibition of cell growth between 10^{-8} and 10^{-6} M, but at concentrations $>10^{-6}$ M there was a further dose-dependent decrease in cell growth mediated by mechanisms yet to be defined but apparently distinct from ER-mediated events. In both concentration ranges, growth inhibition was accompanied by accumulation of cells in the G_1 phase of the cell cycle. These data, obtained with a novel series of hydroxytriarylethylenes, have enabled clear definition of two distinct mechanisms of growth inhibition by triarylethylene antiestrogens. They also indicate that among the vinyl substitutions examined to date the Cl substituent yields the most active molecule both in terms of affinity for ER and AEBS and potency as a growth inhibitory agent.

Triarylethylene antiestrogens such as tamoxifen (1)[1] are of considerable interest due primarily to their ability to antagonize the growth of some hormone-dependent tumors, particularly breast cancer.[2] Although the mecha-

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(1) For convenience, the "trans" configurations of 1 and other triarylethylenes are illustrated.

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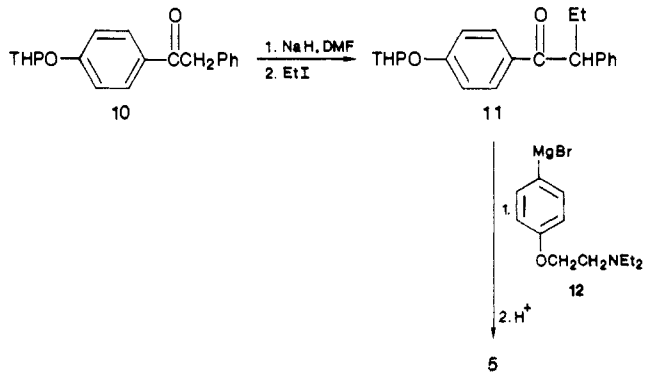
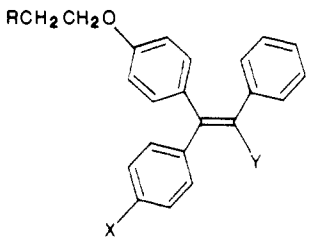
Scheme I



nisms by which 1 and structurally related compounds exert these effects are not fully understood, interactions with estrogen receptors (ER) and possibly antiestrogen binding sites (AEBS), both of which are found in cancer cells susceptible to antiestrogens, may be mechanistically important.[3] Thus, affinity for ER paralleled growth-inhibiting activity in cultures of MCF 7 human breast cancer cells treated with 1 and its analogues[4] while molecules with high affinity for AEBS had more potent antiproliferative effects than analogues with low or no affinity for this site but equal affinities for ER.[5]

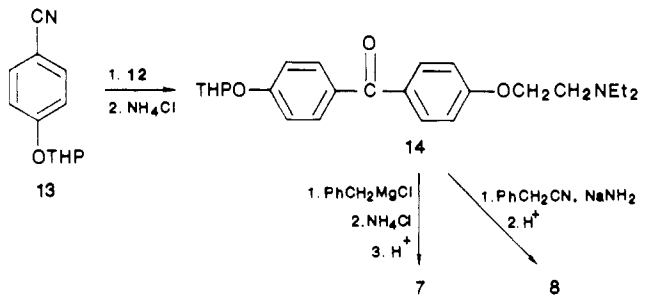


	R	X	Y
1	Me_2N	H	Et
2	Et_2N	OH	Cl
3	Me_2N	OH	Et
4	C_4H_8N	OH	NO_2

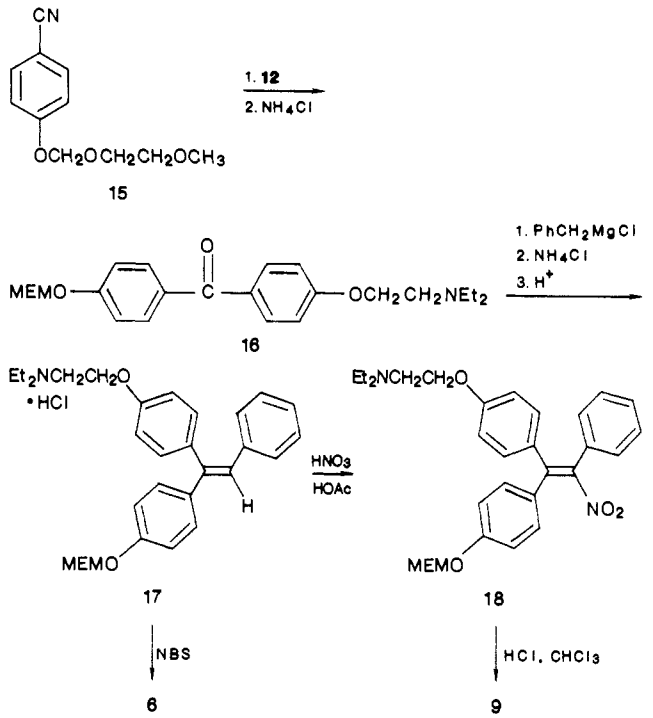
Among triarylethylene antiestrogens, affinity for ER is maximized by the presence of an appropriately placed phenolic substituent. Thus **2–4** each have ER affinities at least 10 times greater than do their respective nonhydroxylated counterparts.[6] Differences in ER affinity among **2** through **4** were suggested to be due mainly to the nature of the Y substituent.[6c] We therefore aimed to study how these and other variations in Y influenced affinity for ER and AEBS in an attempt to establish relationships between these parameters and the degree of inhibition of breast cancer cell growth.

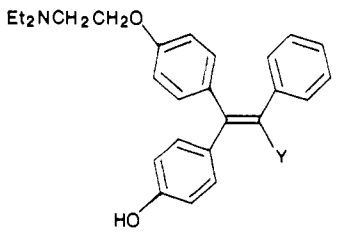
This paper reports the synthesis of novel hydroxytriarylethylenes **5–9** and the evaluation of **2** and **5–9** as outlined above in the well-characterized, hormone-responsive, human breast cancer cell line MCF 7.[7]

Scheme II



Scheme III





5, Y = Et
6, Y = Br
7, Y = H
8, Y = CN
9, Y = NO_2

(2) For reviews on nonsteroidal antiestrogens, see the following: (a) *Nonsteroidal Antiestrogens*; Sutherland, R. L., Jordan, V. C., Eds.; Academic: Sydney, 1981. (b) Sutherland, R. L.; Murphy, L. C. *Mol. Cell. Endocrinol.* **1982**, *25*, 5–23. (c) *Estrogen Receptors in Human Breast Cancer*; McGuire, W. L., Carbone, P. P., Vollmer, E. P., Eds.; Raven: New York, 1976. (d) Jordan, V. C. *Pharmacol. Rev.* **1984**, *36*, 245–276.

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Results and Discussion

Chemistry. Compound **5** was prepared in 42% yield by reaction of THP ether 11 with [4-[2-(diethylamino)-ethoxy]phenyl]magnesium bromide (**12**) followed by acid-catalyzed dehydration/deprotection of the tertiary carbinol intermediate (Scheme I). While **2** and numerous other triarylethylenes have been prepared by analogous addition of Grignard reagents to deoxybenzoins,[6c,8] we wanted to determine whether a different approach, using a non-enolizable ketone, would result in improved yields of hydroxytriarylethylenes.

Thus, **12** was allowed to react with the THP ether of *p*-cyanophenol (**13**) to afford a ketimine intermediate, which on refluxing in a mixture of tetrahydrofuran, methanol, and aqueous ammonium chloride (pH 6) afforded benzophenone **14** in 60% yield after column chromatography (Scheme II). Under conventional conditions for hydrolysis of stable ketimines (dilute aqueous methanolic hydrochloric acid at reflux), the ketimine was not hydrolyzed as readily and the protecting group was lost. Reaction of **14** with benzylmagnesium chloride gave **7** in 73% yield after dehydration/deprotection under acidic conditions. Alternatively, **14** underwent condensation with benzyl cyanide[9] to afford **8** in 32% yield.

Use of hydrolytically stable MEM ethers in the synthesis of **6** and **9** is outlined in Scheme III. Benzophenone **16** was prepared in 67% yield by a slight modification[10] of the procedure used to prepare **14**. This was treated with benzylmagnesium chloride followed by aqueous acid-catalyzed dehydration of the resulting carbinol to furnish **17** in 93% yield. Prior attempted use of methoxymethyl (MM) ethers for the application summarized in Scheme III was not successful. Significant deprotection occurred during acid-catalyzed formation of the MM-protected analogue of **17**, although this was not quantitative as was the case with formation **7** from its THP-protected precursor.

When **17** was stirred at room temperature with a 20% molar excess of *N*-bromosuccinimide in dry chloroform for 40 h, TLC analysis of the reaction mixture showed the presence of **6** and deprotected **17** in a ratio of about 10:1; no MEM-**6** or **17** were detected. Deprotection probably occurred due to evolution of an acidic gas, which was noted shortly after the reaction was started. Previously, it was found that MEM-**2** could be similarly deprotected by brief treatment with anhydrous hydrogen chloride in dry chloroform.[6c]

This method of deprotection was thus used to prepare **9** from **18**, which in turn had been prepared by treatment of **17** with fuming nitric acid in acetic acid. The overall yield was 59%. Lewis acids such as zinc bromide or titanium tetrachloride, reagents first used for MEM-ether cleavage,[11] were not suitable in this case. Addition of excess solid anhydrous zinc bromide to a solution of **18**·HCl in methylene chloride resulted in adsorption of most of the starting material onto the suspended Lewis acid and a low recovery of **9**. Similarly, attempted use of titanium tetrachloride resulted in a purple reaction mixture from which a yield of less than 5% of **9** was obtained. An effective method for deprotecting other amino phenols, using trifluoroacetic acid in methylene chloride, had previously been reported.[11b] Deprotection of **18** (or its precursor MEM ethers) under these conditions at 0 °C proceeded very slowly as determined by TLC and resulted in extensive decomposition of product and/or starting material at higher temperatures.

Table I. Chromatographic and Relevant ^{1}H NMR Spectral Properties of Geometric Isomers of Hydroxytriarylethylenes **2** and **5–9**

	TLC:[a] R_f		OCH_2 triplet, ppm (% rel intensity)[b]	
no.	trans	cis	trans	cis
2[c]	0.20	0.22	3.93 (91)	4.10 (9)
5	0.27	0.32	3.90 (43)	4.14 (57)
6	0.19	0.22	3.90 (54)	4.10 (46)
7	0.22	0.27	4.00 (49)	4.11 (51)
8[d]	0.06	0.10	3.96 (50)	4.05 (50)
9	0.09	0.09	3.98 (48)	4.05 (52)

[a] Solvent system: benzene–piperidine (9:1, v/v). Support: plastic-backed silica gel 60 F_{254} sheets (EM Reagents catalog no. 5775). [b] ^{1}H NMR spectra were recorded with use of pyridine-d_5 solvent and tetramethylsilane as internal standard. [c] This compound was purified by fractional crystallization. [d] Data are for the isomer mixture prior to fractional crystallization.

Configurational Analysis. The absolute configurations of **1** and its cis isomer[12] were determined by crystallography and correlated with ^{1}H NMR spectral data.[13] In ^{1}H NMR spectra, the *O*-methylene triplet in **1** and related *trans*-triarylethylenes is between 3.8 and 4.0 ppm and it is between 4.0 and 4.2 ppm in *cis*-triarylethylenes.[8c,13a] The relative integrated intensities of these spin systems were about equal in the spectra of each of the hydroxytriarylethylenes (**5–9**), suggesting each of these to be approximately equal mixtures of respective geometric isomers. Under TLC conditions similar to those used in this study, *trans*-hydroxytriarylethylenes always had lower R_f values than did corresponding cis isomers.[8c,14] The R_f values in Table I have been assigned on this basis and also on the basis of results obtained with the constituent isomers of **8**, which were separated by fractional crystallization and column chromatography. Thus, the isomer of **8** with R_f 0.06 had an OCH_2 triplet at 3.95 in its ^{1}H NMR spectrum; this was centered at 4.07 in the spectrum of the isomer with R_f 0.10. These assignments are consistent with an earlier finding[6c] that, **2**, after fractional crystallization, was a 10:1 mixture favoring the trans isomer, on the basis of spectral features presented for purposes of comparison in Table I.

Biological Properties. The binding affinities of **2** and **5–9** for the nuclear ER and microsomal AEBS in MCF 7

(7) (a) Soule, H. D.; Vazquez, J.; Long, A.; Albert, S.; Brennan, M. *J. Natl. Cancer Inst. (U.S.)* **1973**, *51*, 1409–1416. (b) Sutherland, R. L.; Hall, R. E.; Taylor, I. W. *Cancer Res.* **1983**, *43*, 3998–4006.

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(9) Buu-Hoi, N. P.; Lecocq, J. *J. Chem. Soc.* **1947**, 641–644.

(10) Pickard, P. L.; Tolbert, T. L. *J. Org. Chem.* **1961**, *26*, 4886–4888.

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(12) Unambiguous use of *E* and *Z* affixes is not possible in **5–9**. We refer to isomers in which the Y substituent and the ring bearing the side chain are on opposite sides of the double bond as trans isomers; these moieties are on the same side of the double bond in cis isomers.

(13) (a) Bedford, G. R.; Richardson, D. N. *Nature (London)* **1966**, *212*, 733–734. (b) Kilbourn, B. T.; Mais, R. H. B.; Owston, P. G. *Chem. Commun.* **1968**, 291. (c) Kilbourn, B. T.; Owston, P. G. *J. Chem. Soc. B* **1970**, 1–5.

(14) Ruenitz, P. C.; Bagley, J. R.; Mokler, C. M. *J. Med. Chem.* **1982**, *25*, 1056–1060.

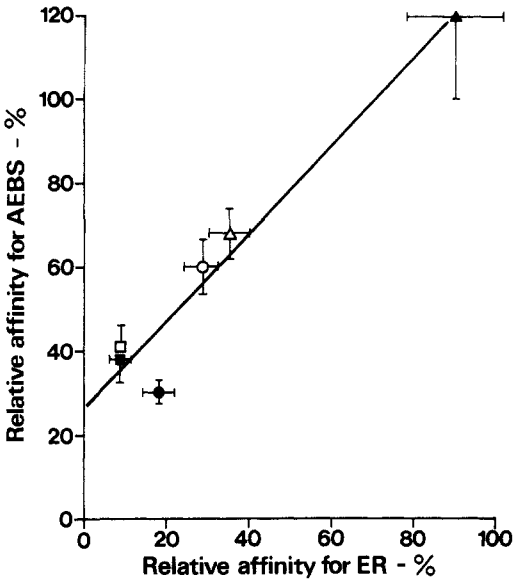

Figure 1. Relationship between relative affinities of compounds **2** and **5–9** for the salt-extractable nuclear estrogen receptor and the microsomal antiestrogen binding site from MCF 7 cells. Relative binding affinities were measured in competitive binding assays where increasing concentrations of these compounds were compared with estradiol and tamoxifen, respectively, for their ability to compete with [^{3}H]estradiol for binding to ER or [^{3}H]tamoxifen for binding to AEBS. Binding affinities were expressed in relation to those for estradiol and tamoxifen, respectively, which were arbitrarily assigned values of 100%. Compounds are represented by the following symbols: **2** (▲), **5** (△), **6** (○), **7** (□), **8** (■), **9** (●). Data points represent the mean ± SEM of three to six determinations. The relationship between affinities for the two binding sites was calculated by using least-squares linear regression analysis, and the regression line is shown.

cell extracts were determined in competitive binding assays employing estradiol and tamoxifen as the respective tritiated ligands. All compounds demonstrated affinity for both saturable, high-affinity binding sites and modifications that changed the affinity for ER-induced qualitatively similar changes in affinity for AEBS such that the changes in affinity for both sites were highly correlated (relative affinity for AEBS = 1.049 × relative affinity for ER − 26.46, $r^2 = 0.942$, $p < 0.01$, Figure 1). These data indicate that variations in Y markedly affect affinity for both ER and AEBS, and thus this portion of the molecule is a major structural determinant of binding to both sites. This novel finding can be contrasted with previous structure–affinity studies where structural modifications in the phenolic ring[6,15] and the amino ether side chain[5,15] have been shown to have major effects on affinity for ER and AEBS, respectively.[5,6,15] Modifications in these positions did not result in parallel changes in affinity for the two binding sites. For example, a phenolic substituent at X markedly increased affinity for ER[6] but invariably decreased affinity for AEBS[15] while a series of structural modifications in the amino ether side chain that caused major changes in affinity for AEBS had minimal effects on affinity for ER.[15]

The data presented in Figure 1 also demonstrate that among these compounds there was a 10-fold range of affinities for ER and a fourfold range of affinities for AEBS. This gave an appropriate range of affinities to allow investigation of affinity–activity relationships. In both cases the three most potent compounds were in the order **2** > **5** > **6** while **7** and **8** had similar but markedly lower affinities for both ER and AEBS (Figure 1). A complete correlation between affinities for the two binding sites did not occur because of the properties of the NO_2-substituted compound **9**, which had an intermediate affinity for ER but the lowest affinity for AEBS. The differences in affinity of the various compounds do not seem to be due to steric effects since substituents with large size (CN, NO_2, C_2H_5) had both low and high affinity. Instead, inductive and resonance effects transmitted through the vinyl group to the remainder of the molecule may be responsible as evidenced by the parallel effects of vinyl substitution on binding affinity and the directive influence of benzene nucleus substitution; i.e., increasing affinity for ER and AEBS parallels increasing ortho- and para- and decreasing meta-directive influence. Affinity might therefore be influenced in part by altered hydroxyl group acidity[6c] although other factors must be important since substitutions at Y influence binding of nonhydroxylated triphenylethylenes to AEBS in a similar fashion.[15b] One such factor may be the degree of lipophilicity conferred to the ligands by the Y substituents, since compounds in which Y was nonpolar had high TLC R_f values (Table 1) and higher affinities than did compounds in which Y was relatively polar.

The effects of **2** and **5–9** on MCF 7 cell growth are recorded in Figure 2. With the exception of **7**, all compounds had distinct biphasic dose–response curves resulting in 60–70% growth inhibition in the nanomolar concentration range, no further effect between 10^{-8} and 10^{-6} M, and a further concentration-dependent decrease in cell proliferation at micromolar concentrations of all drugs. Further experiments demonstrated that the effects of 10^{-8} and 10^{-6} M concentrations of these compounds on cell growth could be completely negated by the simultaneous addition of equimolar concentrations of estradiol to the culture medium (data not shown). Effects in the micromolar range were partially reversed or unaffected by the simultaneous addition of estradiol. These data are compatible with triarylethylene antiestrogens affecting MCF 7 cell proliferation rates by two distinct mechanisms, one of which involves the ER and is estrogen-reversible and the other of which is only apparent at concentrations $>10^{-6}$ M, being mediated by an as yet undefined mechanism. Potential candidates for involvement in this latter mechanism include interactions with pathways controlled by calmodulin[16] and/or protein kinase C[17] since tamoxifen has recently been shown to have interactions with these molecules at micromolar concentrations. The availability of our hydroxylated triarylethylenes with their high affinities for ER has allowed clear distinction of these two mechanisms and confirmed our earlier observations with **3**.[4c] Previous studies[5,18] with compounds having lower affinities for ER have yielded dose–response curves similar to that seen here with **7** where, presumably because of lower affinity for ER, the dose–response curve for the

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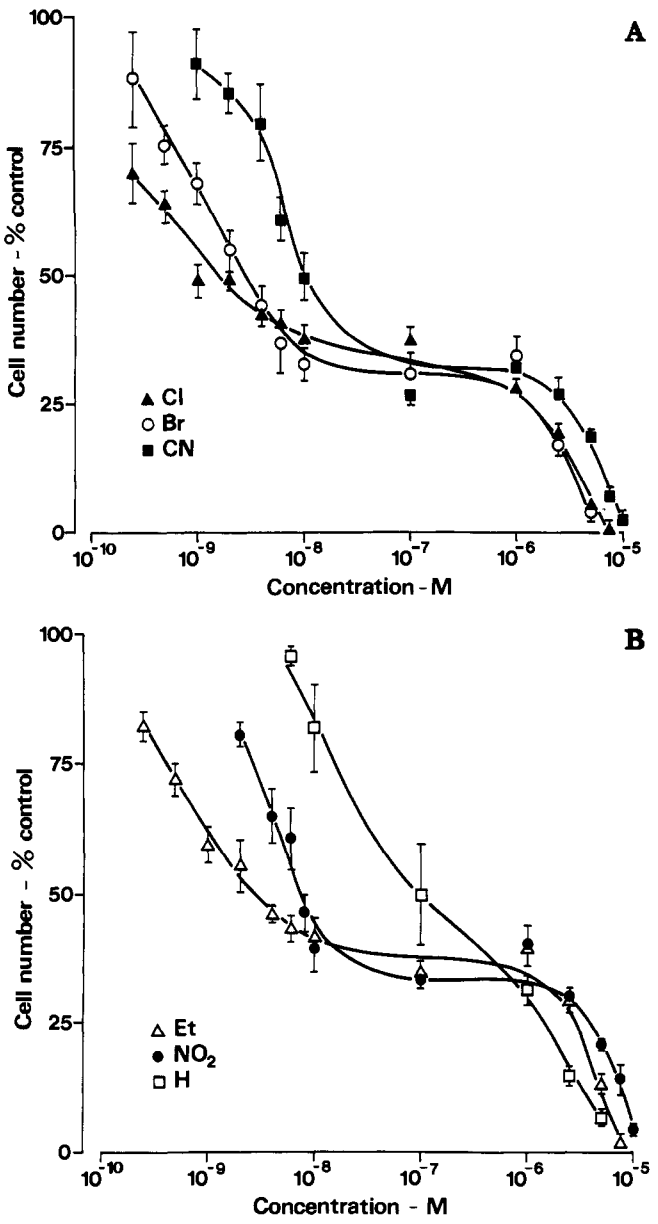

Figure 2. Effects of compounds **2** and **5–9** on the growth of MCF 7 cells. Cells (5×10^4) in exponential growth phase were plated into 25-cm^2 tissue culture flasks in 5 mL of RPMI 1640 culture medium supplemented with 5% fetal calf serum. When cell numbers had reached 10^5/flask (ca. 24 h later), varying concentrations of drug were added from ethanolic stock solutions such that the final concentration of ethanol was 0.1% (v/v). Cells were harvested 4 days later when mean ± SEM cell numbers in control flasks were $1.56 \pm 0.05 \times 10^6$ cells/flask; i.e., control cultures had passed through approximately four population doublings. Estimates of cell numbers were made on a hemocytometer and expressed as the percentage of the number of cells in control flasks. Since cell numbers at the ends of experiments were 15.6-fold greater than at drug inoculation, complete arrest of cell proliferation occurred when cell numbers were 6.4% of control. Data points are the mean ± SEM of six to twelve estimates from two to four separate experiments. (A) and (B) represent the dose–response curves for compounds **2** (▲), **6** (○), and **8** (■); and **5** (△), **7** (□), and **9** (●), respectively.

ER-mediated mechanism is moved to the right thus converging on the second mechanism and leading to a loss of the biphasic response (Figure 2B).

The relative potencies of the test compounds in the nanomolar, estrogen-reversible concentration range were in the order Cl > Et > Br > NO_2 > CN > H with IC_{30} values of 0.25, 0.58, 0.85, 3.4, 5.4, and 20 nM, respectively. Potency was thus highly correlated with affinity for ER, which was in the same order (Figure 1), and this is compatible with ER being intimately involved in mediating the growth-inhibitory effects of antiestrogens in this concentration range. Thirty-percent inhibition of cell growth (IC_{30}) is approximately half-maximal for this inhibitory mechanism (Figure 2). To investigate how this response is related to occupancy of ER, IC_{30} values were compared with apparent K_d values for ER, given that half-maximal receptor saturation occurs at a ligand concentration equal to K_d. The apparent K_d values for ER were determined from the relative binding affinities of these compounds for ER as presented in Figure 1 and a K_d value of 0.1 nM previously measured at 0–4 °C for the affinity of estradiol for the ER of MCF 7 cells[4c] and were in the following order: E_2 (0.10 nM) > Cl (0.11) > Et (0.29), > Br (0.36) > NO_2 (0.56) > CN (1.11) = H (1.11). The relationship between K_d and IC_{30} was not linear, and low-affinity ligands, i.e., those with NO_2, CN, and H substituents, had potencies 6–18-fold lower than predicted from the corresponding K_d values for ER while the high-affinity ligands (Cl, Et, and Br) were only 2–2.4-fold less potent. The nonlinear coupling between binding and response may indicate differences in intrinsic activity of the ligand–ER complex or that properties of these molecules other than their affinities for ER also play a role in determining overall antiestrogenic and antiproliferative activity. Any influence of affinity for AEBS on potency in this dose range was impossible to assess because of the close correlation with affinity for ER.

The order of potencies in the micromolar range was significantly different from that seen at lower concentrations supporting a different mechanism of growth inhibition (Figure 2). In particular, **7** became markedly more potent at these concentrations while **9** was relatively less potent. IC_{90} values were in the order Br (3.6 μM) > H (3.8) > Cl (4.2) > Et (5.4) > CN (7.1) > NO_2 (8.5). Potency in this range was not highly correlated with affinity for either ER or AEBS but was more closely related to the latter.

Cell cycle phase distribution data were obtained in the same experiments and are summarized in Figure 3. All compounds induced a dose-dependent decrease in the proportion of cells in the S, or DNA-synthetic, phase of the cell cycle (Figure 3), and this was accompanied by a concomitant accumulation of cells in the G_1 phase (data not shown). Such data are consistent with our previous finding that tamoxifen and its analogues inhibit cell growth primarily by increasing the net G_1 transit time and thus inhibiting the entry of cells into the DNA-synthetic phase of the cell cycle.[3,18] As was observed with effects on cell number, dose–response curves for drug effects on S phase were biphasic with the exception of compound **7**, i.e., there was a dose-dependent decrease in S-phase cells between 10^{-10} and 10^{-8} M, no further change between 10^{-8} and 10^{-6} M, and a further concentration-dependent decrease in S phase at concentrations $>10^{-6}$ M. Drug effects at concentrations $<10^{-6}$ M were reversed by the simultaneous administration of estradiol while at concentrations $>10^{-6}$ M effects were incompletely reversed by estradiol (data not shown). Relative potencies in inducing the changes in the cell-cycle kinetic parameters within the concentration range 10^{-10}–10^{-8} M were in the same order as the effects on cell number, i.e., Cl > Et > Br > NO_2 > CN > H, and were correlated with affinity for ER. It was of considerable interest that growth inhibition in the micromolar range was also accompanied by a depletion of cells in S phase and an accumulation of cells in G_1 phase of the cell cycle. Relative potencies for depletion of the proportion of S-phase cells at concentrations $>10^{-6}$ M were

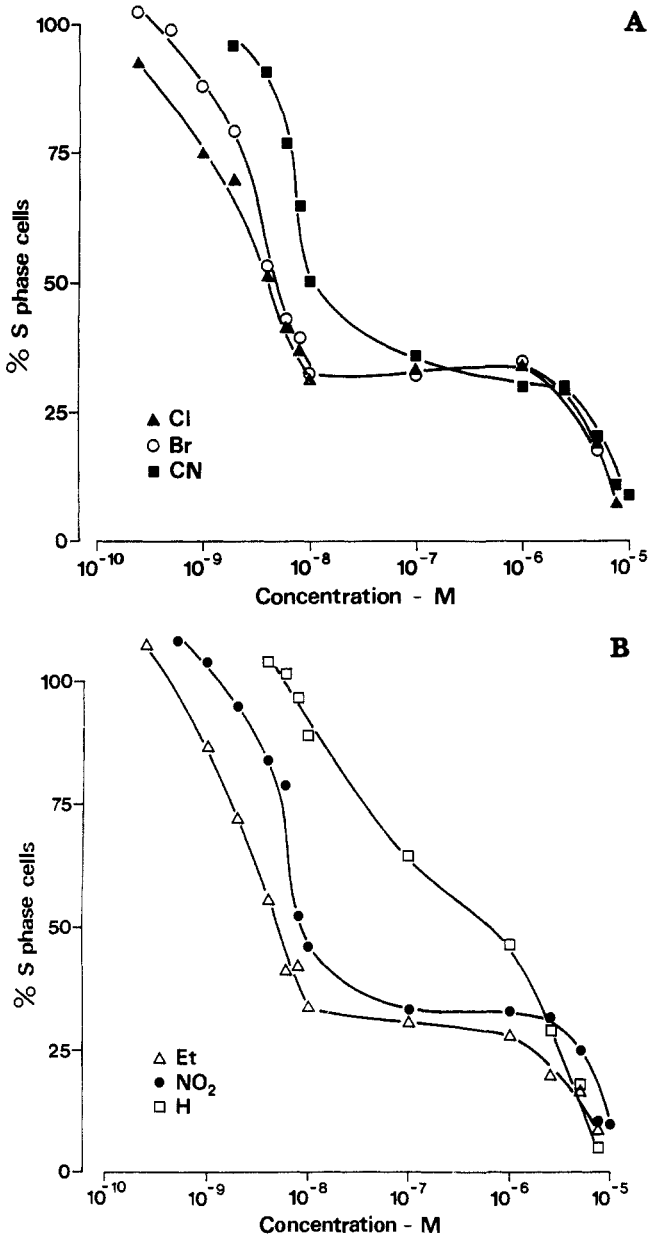

Figure 3. Effect of treatment of MCF 7 cells with compounds **2** and **5–9** on the percentage of cells in the S (DNA synthetic) phase of the cell cycle. Cells from the experiments described in Figure 2 were stained for analytical DNA flow cytometry and analyzed, and the proportion of cells in various phases of the cell cycle was calculated as detailed in ref 18. Data are expressed as a percentage of the S-phase cells measured in control cultures, i.e., 36.1 ± 1.0%, and are the means of two to four observations from two to four separate experiments. Symbols are as presented in Figure 2.

identical to those seen with changes in cell number, i.e., Br > H > Cl > Et > CN > NO_2.

Although potency in the micromolar range was more closely related to affinity for AEBS than ER, the large disparity between affinity for AEBS, which was in the 0.8–3.3-nM range, and the concentrations of drug required for half-maximal responsiveness for this second mechanism of growth inhibition, i.e., 2–7.5 μM, argues against the direct involvement of AEBS in this process. Perhaps a more plausible explanation is that those properties of the triarylethylene molecule that favor strong interactions with AEBS also facilitate interactions with regulatory molecules controlling this pathway. We are currently attempting to establish a relationship between affinity for AEBS and inhibition of some calmodulin-regulated processes since, as stated above, 1 has been shown to half-maximally inhibit a calmodulin-dependent enzyme (cAMP phosphodiesterase) at a concentration of 2 μM.[16] This and the observation that calmodulin antagonists inhibit breast cancer cell growth[19] and induce G_1 arrest[20] suggest that inhibition of calmodulin-activated cellular processes by triarylethylenes, as previously suggested Jordan,[2d] is a potential mechanism by which these antiestrogens inhibit cell proliferation in the estrogen irreversible concentration range.

In summary, this paper has described the synthesis of several novel hydroxytriarylethylene antiestrogens and tested their effects on MCF 7 human breast cancer cell proliferation kinetics. The high affinity of these compounds for ER plus their spectrum of affinities for ER and AEBS and their differing potencies in inhibiting MCF 7 cell growth have extended knowledge of structure–affinity and structure–activity relationships among this class of compounds and allowed clear definition of at least two distinct mechanisms of action as antiproliferative agents. Furthermore, the Cl-substituted compound **2** described here is one of the most potent antiestrogenic molecules yet synthesized and provides a structural basis for further modifications needed to produce even more potent molecules within this class.

Experimental Section

Melting points are uncorrected. Elemental analyses were obtained from Atlantic Microlab, Inc., Atlanta, GA. ^{1}H NMR spectra were recorded on a JEOL JNM/MX-90Q(I) instrument, using tetramethylsilane as internal standard. UV and IR spectra were recorded on Bausch and Lomb Spectronic 2000 and Perkin-Elmer 467 spectrophotometers, respectively. Reaction progress and purity of products were checked by analytical TLC with use of strips of various dimensions cut from plastic-backed silica gel 60 F_{254} sheets (20 × 20 cm, 0.20-mm thickness, EM Reagents catalog no. 5775). Unless indicated, the developing solvent for TLC was benzene–triethylamine (9:1, v/v). Developed strips were viewed under light of 254- and 366-nm wavelengths. Coarse silica (Baker, 60–200 mesh) was used for conventional column chromatography while fine silica (Brinkmann, 230–400 mesh) was used for flash column chromatography.[14] Reactions involving air-sensitive materials were carried out under dry nitrogen.

Starting Materials. Tetrahydropyranyl ethers **10** and **13** were prepared by reaction of benzyl 4-hydroxyphenyl ketone and 4-cyanophenol, respectively, with dihydropyran in the presence of a trace of sulfuric acid.[14] After workup, **10** was purified by crystallization from 95% ethanol as colorless needles (74% yield; mp 99–102 °C (softens)). Compound **13** was purified by flash column chromatography (benzene–hexane, 3:1, v/v), providing a residual concentrate, which on stirring in ice-cold petroleum ether (30–60 °C) afforded colorless crystals: mp 68.5–70 °C, lit.[21] mp 69–70 °C. (Methoxyethoxy)methyl ether **15** was prepared in 86% yield (bp 112–125 °C (0.15 mmHg)) by reaction of 4-cyanophenol with NaH in THF, followed by alkylation with MEM chloride.[11] [(Diethylamino)ethoxy]bromobenzene (colorless oil: bp 95–105 °C (0.20 mmHg), lit.[22] bp 110–113 °C (0.30 mmHg)) was prepared in 94% yield by reaction of 4-bromophenol with 2-(diethylamino)ethyl chloride hydrochloride in the presence of a NaH–DMF medium in a manner analogous to the synthesis of the dimethylamino congener.[14]

1-[4-[(Tetrahydropyran-2-yl)oxy]phenyl]-2-phenylbutan-1-one (11). A 50% mineral oil suspension of NaH (216

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(20) (a) Chafouleas, J. G.; Bolton, W. E.; Hidaka, H.; Boyd, A. E.; Means, A. R. *Cell (Cambridge, Mass.)* **1982**, *28*, 41–50. (b) Sutherland, R. L.; Watts, C, K. W., unpublished observations with MCF 7 cells.
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mg, 4.5 mmol) was washed with hexane (2 × 5 mL). To this was added dropwise a solution of **10** (1.2 g, 4 mmol) in DMF (20 mL). After hydrogen evolution had ceased, the reaction flask was placed in an ice bath and ethyl iodide (0.8 g, 5.1 mmol) was added in portions with a disposable pipet. After addition, the ice bath was removed and gradually over a 10-min period the color of the reaction mixture changed from dark yellow to colorless. Analysis by TLC (benzene–chloroform, 1:1) indicated completion of reaction. The reaction mixture was poured into water (50 mL), and the emulsion was extracted with ether (3 × 50 mL) and dried (Na_2SO_4). After evaporation of solvent, the residual oil (IR (neat) 1666 cm^{-1} (C=O)) was further concentrated in vacuo (25 °C (0.10 mmHg)) overnight to yield crude **11** (1.3 g, 99%) as a white solid, which was nearly homogeneous by TLC (R_f 0.41; trace impurity, R_f 0.54): ^1H NMR ($CDCl_3$) δ 0.90 (t, 3, J = 8 Hz, CH_2CH_3), 1.42–2.36 (m, 8, CH_2CH_3, 3′-H, 4′-H, and 5′-H), 3.30–3.90 (m, 2, 6′-H), 4.28 (t, 1, J = 8 Hz, 1′-H), 7.22 (s, 5, Ar H), 7.92 (d, 2, J = 9.5 Hz, H ortho to C=O). Compound **11** was used directly in the synthesis of **5** without further purification.

1-[4-[2-(Diethylamino)ethoxy]phenyl]-1-(4-hydroxyphenyl)-2-phenylbut-1-ene (5). The preparation of [4-[2-(diethylamino)ethoxy]phenyl]magnesium bromide (**12**) employed in the syntheses of compounds **5–9** is described as follows.

To a stirred mixture of Mg turnings (134 mg, 5.14 mmol) in THF (2 mL) was added a few drops of ethyl bromide and ethylene bromide, plus a crystal of iodine. Within a few minutes a mild exothermic reaction ensued. Then a solution of 4-[2-(diethylamino)ethoxy]bromobenzene (1.3 g, 4.8 mmol) in THF (7 mL, containing a few drops of the above alkyl bromides) was added at such a rate as to maintain an intense reaction. After completion of addition, the reaction mixture was refluxed for 1 h and then cooled to room temperature.

To the above solution was added a solution of **11** (1.3 g, 4 mmol) in THF (7 mL) over a period of 10 min, and the reaction mixture was refluxed for 19 h. The mixture was cooled and quenched with saturated aqueous NH_4Cl (0.3 mL), and the resulting suspension was stirred for 30 min. This was filtered through Super-Cel, and filtrate was concentrated and dissolved in MeOH–THF–10% HCl (20:10:10, v/v/v), and the mixture was stirred at room temperature for 1 h. After concentration in vacuo, the residue was dissolved in water–acetone (50:20, v/v) and the aqueous phase was extracted with ether (2 × 50 mL, discarded) and then with chloroform (3 × 50 mL). The combined organic extracts were washed with saturated aqueous $NaHCO_3$ (50 mL) and water (2 × 50 mL) and dried (Na_2SO_4). The amber glass left after evaporation of solvent was eluted through a column of coarse silica (70 g) with benzene–triethylamine (15:1, v/v) to yield **5** (0.72 g, 42%) as a white foam. Crystallization from benzene–hexane gave analytically pure **5**: mp 155–157 °C (softens), 157–160 °C (melts); UV (EtOH) λ_{max} 287 nm (ϵ = 14.0 mM^{-1} cm^{-1}); ^1H NMR (pyridine-d_5) δ 0.82–1.20 (m, 9, CH_2CH_3), 2.32–2.96 (m, 8, NCH_2 and allylic CH_2), 3.90 and 4.14 (2 t, 2, J = 6 Hz, OCH_2CH_2N), 6.68–7.62 (m, 13, Ar H), 8.70 (s, 1, OH). Anal. ($C_{28}H_{33}NO_2$) C, H, N.

4-[2-(Diethylamino)ethoxy]-4′-[(tetrahydropyran-2-yl)oxy]benzophenone (14). The Grignard reagent **12** was prepared as described above from the aryl bromide (4.8 g, 18 mmol) and Mg (0.52 g, 22 mmol) in THF (25 mL). After cooling to room temperature, a solution of **13** (3.0 g, 15 mmol) in THF (25 mL) was added over a period of 15 min. The reaction mixture was refluxed for 5 h, cooled, and quenched with saturated aqueous NH_4Cl (12 mL). Methanol (15 mL) was added, and this mixture was stirred under reflux for 3.5 h, at which time TLC analysis showed complete hydrolysis of the intermediate with R_f 0.37 to a product with R_f 0.59. The reaction mixture was cooled and filtered through Super-Cel. The filtrate was concentrated in vacuo to a brown oil, which was partitioned between chloroform (150 mL) and water (50 mL). The chloroformic extract was further washed with water (50 mL) and brine (50 mL) and dried (Na_2SO_4). The crude product left after evaporation of solvent was purified by flash column chromatography on fine silica (60 g) with an initial elution of benzene–hexane (3:1, v/v) followed by benzene–hexane–triethylamine (15:3:1, v/v/v). Compound **14** (3.5 g, 60%), which was homogeneous by TLC, was recovered as an amber oil: IR (neat) 1646 cm^{-1} (C=O); ^1H NMR ($CDCl_3$) δ 1.14 (t, 6, J = 8 Hz, NCH_2CH_3), 1.32–2.06 (m, 6, 3′-H, 4′-H, and 5′-H), 2.36–3.02 (m, 6, NCH_2CH_3 and OCH_2CH_2N), 3.24–4.12 (m, 4, OCH_2CH_2N), 5.42 (s, 1, 2′-H), 6.66–7.86 (m, 8, Ar H). When hot ethanolic solutions of equimolar amounts of this base and citric acid were combined and allowed to cool, the monocitrate salt separated as white crystals: mp 121–124 °C. Anal. ($C_{24}H_{31}NO_4 \cdot C_6H_8O_7$) C, H, N.

1-[4-[2-(Diethylamino)ethoxy]phenyl]-1-(4-hydroxyphenyl)-2-phenylethene (7). To Mg turnings (0.25 g, 10 mmol) and an iodine crystal was added a solution of benzyl chloride (1.2 g, 9.3 mmol) in ether (15 mL). An immediate reaction ensued and was maintained during the addition period (10 min) without external heating. After addition, the reaction flask was placed in a preheated oil bath and the mixture was refluxed for 1.5 h. After cooling a solution of **14** (1.5 g, 3.7 mmol) in THF (20 mL) was added over a 15-min period. The reaction mixture was refluxed for 0.5 h, cooled, and quenched with saturated aqueous NH_4Cl (1.3 mL). The resulting suspension was stirred for 30 min and filtered through Super-Cel, and the filtrate was concentrated in vacuo. The crude tertiary carbinol was dissolved in MeOH–THF–10% HCl (20:20:10, v/v/v) and stirred at room temperature for 1 h. The residue left after concentration in vacuo was partitioned between ether (50 mL) and 10% HCl (50 mL). The aqueous phase was extracted again with ether (50 mL) and then with chloroform (50 mL) and chloroform–methanol (40:10, v/v). The combined chloroformic extracts were washed with saturated aqueous $NaHCO_3$ (50 mL) and water (2 × 50 mL) and dried (Na_2SO_4). Evaporation of solvent left a beige foam (1.3 g), which was purified by column chromatography on 70 g of coarse silica. Elution with benzene–triethylamine (20:1, v/v) gave **7** (1.1 g, 73%) as a white foam. Recrystallization from benzene–hexane afforded analytically pure **7**: mp 121–124 °C (softens); UV (EtOH) λ_{max} 314 nm (ϵ = 18.9 mM^{-1} cm^{-1}); ^1H NMR (pyridine-d_5) δ 0.99 (t, 6, J = 7 Hz, NCH_2CH_3), 2.54 (q, 4, J = 7 Hz, NCH_2CH_3), 3.82 (t, 2, J = 6 Hz, OCH_2CH_2N), 4.11 (dt, 2, J = 6 Hz, OCH_2CH_2N), 6.85–7.58 (m, 14, vinyl and Ar H), 8.66 (s, 1, OH). Anal. ($C_{26}H_{29}NO_2$) C, H, N.

2-Phenyl-3-(4-hydroxyphenyl)-3-[4-[2-(diethylamino)ethoxy]phenyl]acrylonitrile (8). To a stirring suspension of $NaNH_2$ (0.64 g, 16.3 mmol) in ether (4 mL) over a period of 10 min. The mixture was refluxed for 2 h and cooled, and a solution of **14** (2.1 g, 5.3 mmol) in THF (20 mL) was added in a rapid dropwise manner. The reaction mixture was refluxed for 8 h and then stirred overnight at ambient temperature. The reaction mixture was concentrated in vacuo, and the residue was suspended in MeOH–THF–10% HCl (25:25:10, v/v/v) and stirred at room temperature for 1 h. Concentration in vacuo left a residue, which was partitioned between 10% HCl (50 mL) and ether (50 mL). The aqueous phase was further extracted with ether (2 × 50 mL), and the organic extracts were discarded. There followed extraction with chloroform (2 × 50 mL), and these organic extracts were combined and washed with saturated aqueous $NaHCO_3$ (80 mL) and water (2 × 50 mL). Drying (Na_2SO_4) and evaporation of solvent left a viscous purple oil (1.2 g), which was chromatographed on a column of coarse silica (70 g) by gradient elution with benzene–acetone–triethylamine (16:4:1 to 10:4:1, v/v/v) to give a brown gum, which was chromatographed over coarse silica (70 g) with chloroform–methanol (25:1, v/v) to give **8** (0.70 g, 32%) as a pale yellow solid: IR (Nujol) 2200 cm^{-1} (C≡N). Two crystallizations from benzene–ethanol–hexane gave 0.37 g of the slower migrating isomer by TLC (Table I) in pure form: mp 199–201 °C; UV (EtOH) λ_{max} 343 nm (ϵ = 14.4 mM^{-1} cm^{-1}); ^1H NMR (pyridine-d_5) δ 0.95 (t, 6, J = 7 Hz, NCH_2CH_3), 2.48 (q, 4, J = 7 Hz, NCH_2CH_3), 2.74 (t, 2, J = 6 Hz, NCH_2CH_2O), 3.96 (t, 2, J = 6 Hz, OCH_2CH_2N), 6.62–7.69 (m, 13, Ar H), 8.64 (s, 1, OH). Anal. ($C_{27}H_{28}N_2O_2$) C, H, N.

The mother liquor was chromatographed on 70 g of coarse silica gel. Elution with benzene–triethylamine (10:1, v/v) afforded 0.11 g of the faster migrating isomer by TLC (Table I) in pure form. This had UV, IR, and NMR features nearly identical with those of the crystalline isomer, except that the OCH_2 triplet in its ^1H NMR spectrum was centered at 4.05 ppm.

4-[2-(Diethylamino)ethoxy]-4′-[(methoxyethoxy)methyl]benzophenone (16). The Grignard reagent **12** was prepared as described above from the aryl bromide (3.9 g, 14.5 mmol) and Mg (0.42 g, 17 mmol) in THF (30 mL). After cooling, a solution of **15** (2.5 g, 12 mmol) in THF (25 mL) was added over a period of 20 min. The reaction mixture was refluxed for 5 h,

cooled, and quenched with MeOH (10 mL). The residue left after concentration in vacuo was partitioned between 1% HCl (100 mL) and ether (50 mL). The aqueous phase was further extracted with ether (50 mL) and then alkalinized with 12 N NaOH. Extraction with chloroform (150 mL) precipitated a white emulsion, which was allowed to settle out overnight. The chloroformic extract was washed with water (3 × 50 mL) and dried (Na_2SO_4). Evaporation of solvent left a golden oil (5 g): TLC, R_f 0.24; IR (neat) 1605 cm^{-1} (C=N). This was dissolved in THF–methanol (50:50, 20 mL), saturated aqueous NH_4Cl (10 mL) was added, and the mixture was refluxed for 6 h. TLC analysis showed the presence of a single component with R_f 0.41.[23] The reaction mixture was concentrated in vacuo and partitioned between 10% HCl (100 mL) and ether (50 mL). The aqueous phase was extracted with chloroform (2 × 50 mL), and the combined chloroformic extracts were washed with saturated aqueous $NaHCO_3$ (50 mL) and water (3 × 50 mL) and dried (Na_2SO_4). Evaporation of solvent left **16** (3.2 g, 67%) as a pale yellow oil, which was homogeneous by TLC: IR (neat) 1645 cm^{-1} (C=O); ^1H NMR ($CDCl_3$) δ 1.05 (t, 6, J = 8 Hz, NCH_2CH_3), 2.55 (q, 4, J = 8 Hz, NCH_2CH_3), 3.32 (s, 3, OCH_3), 3.40–3.58 (m, 2, $CH_2OCH_2CH_2$), 3.74–3.94 (m, 2, CH_2OCH_3), 4.08 (t, 2, J = 6 Hz, OCH_2CH_2N), 5.30 (s, 2, OCH_2O), 6.94 (d, 4, J = 9 Hz, Ar H ortho to alkoxy groups), 7.76 (d, 4, J = 9 Hz, Ar H ortho to C=O). The citrate salt, prepared as stated above, crystallized from ethanol–ether: mp 97–100 °C (softens). Anal. ($C_{23}H_{31}NO_5 \cdot C_6H_8O_7 \cdot H_2O$), C, H, N.

1-[4-[2-(Diethylamino)ethoxy]phenyl]-1-[4-[(methoxyethoxy)methyl]phenyl]-2-phenylethene Hydrochloride (17). Benzylmagnesium chloride was prepared from benzyl chloride (2.8 g, 22 mmol) and Mg (0.58 g, 24 mmol) in ether (30 mL) and mixed with **16** (3.5 g, 8.7 mmol) in THF (30 mL) in the manner described for the synthesis of compound **7**. However, the dehydration, workup, and purification steps differed. The crude tertiary carbinol, left after NH_4Cl quenching (3 mL), filtration through Super-Cel, and concentration in vacuo, was dissolved in ethanol–10% HCl (30:10, mL) and stirred at room temperature for 15 min. Water (100 mL) was added. The mixture was extracted with ether (150 mL, discarded) and then with chloroform (150 mL). The dried (Na_2SO_4) chloroform extract was concentrated in vacuo and purified by flash column chromatography (60 g of fine silica). Elution with chloroform–methanol (95:5, v/v) gave **17** as a colorless gum (4.1 g, 93%): ^1H NMR (free base of **17** in $CDCl_3$) δ 1.06 (t, 6, J = 8 Hz, NCH_2CH_3), 2.63 (q, 4, J = 8 Hz, NCH_2CH_3), 2.86 (t, 2, J = 6 Hz, NCH_2CH_2O), 3.35 (s, 3, OCH_3), 3.42–3.66 (m, 2, CH_2OCH_3), 3.70–4.18 (m, 4, $OCH_2OCH_2CH_2$ and OCH_2CH_2N), 5.27 (s, 2, OCH_2O), 6.64–7.50 (m, 14, vinyl and Ar H). A small sample of **17** in ethanol was mixed with a solution of excess citric acid monohydrate in ethanol to yield, on crystallization from ethanol–ether, white crystals: mp 110.5–111 °C. Anal. ($C_{30}H_{37}NO_4 \cdot C_6H_8O_7$), C, H. N.

Halogenation and Nitration of 17. A. Preparation of 1-[4-[2-(Diethylamino)ethoxy]phenyl]-4-(4-hydroxyphenyl)-2-bromo-2-phenylethene (6). To a solution of **17** (1.5 g, 2.9 mmol) in chloroform (25 mL) was added, portionwise, *N*-bromosuccinimide (0.6 g, 3.4 mmol; recrystallized from water, mp 173–180 °C). Within 10 min after completion of addition, a weak exotherm (ca. 30 °C) was noted by the appearance of condensate on the interior of the reaction flask. Insertion of wet litmus paper into the mouth of the flask indicated a faint evolution of acidic gas (presumably HBr). TLC analysis showed trace spots in the "phenol" region (R_f ca. 0.2). The reaction mixture was stirred at room temperature for 24 h. TLC analysis (three developments) showed that some **17** (or MEM-**6**) still remained along with a mixture of **6** and **7** (10:1, respectively) and impurities. Full deprotection was noted after an additional 16 h of stirring. The reaction mixture was concentrated in vacuo to a brown semisolid, which was dissolved in MeOH (10 mL). Water (75 mL) and 10% HCl (10 mL) were added, and the aqueous mixture was extracted with ether (2 × 50 mL, discarded) and chloroform (2 × 50 mL). The combined chloroformic extracts were washed with saturated aqueous $NaHCO_3$ (50 mL) and dried (Na_2SO_4). Evaporation of solvent left a tan foam (1.0 g), which was chromatographed on coarse silica (70 g) with benzene–piperidine (9:1, v/v) to give a golden oil (0.8 g). This in turn required chromatography (70 g of coarse silica) with chloroform–methanol–ammonia (135:5:0.5, v/v/v) to give a white foam. This was recrystallized three times from benzene–hexane to afford 244 mg (18%) of a white powder: mp 146–148.5 °C; UV (EtOH) λ_{max} 310 nm (ϵ = 9.91 mM^{-1} cm^{-1}); ^1H NMR (pyridine-d_5) δ 0.84–1.08 (m, 6, NCH_2CH_3), 2.32–2.64 (m, 4, NCH_2CH_3), 2.64–2.95 (m, 2, OCH_2CH_2N), 3.90 and 4.10 (2 t, 2, J = 6 Hz, OCH_2CH_2N), 6.70–7.66 (m, 13, Ar H), 8.70 (s, 1, OH). Anal. ($C_{26}H_{28}BrNO_2$) C, H, N; C: calcd, 66.95; found, 67.52.

B. Preparation of 1-[4-[2-(Diethylamino)ethoxy]phenyl]-1-(4-hydroxyphenyl)-2-nitro-2-phenylethene (9). A stirring solution of **17** (1.4 g, 2.8 mmol) in glacial acetic acid (30 mL) was cooled to 15–20 °C with a cold water bath. Fuming nitric acid (0.18 mL, 4.3 mmol) was added, and the reaction mixture was stirred for 4 h at ambient temperature. To this were added THF (125 mL), water (100 mL), and then, in portions, solid $NaHCO_3$ to neutralization. The mixture was extracted with chloroform (2 × 125 mL), and the combined organic extracts were washed with water (4 × 100 mL) and dried (Na_2SO_4). Evaporation of solvent left a viscous, orange oil (1.2 g), which was chromatographed on coarse silica (70 g). Elution with benzene–triethylamine (50:1, v/v) gave MEM ether **18** (0.9 g, 56%) as a yellow oil.

Into an ice-chilled solution of **18** (0.9 g, 1.6 mmol) in chloroform (35 mL) was passed a stream of HCl gas. After 15 min, TLC analysis showed complete absence of **18**. The mixture was concentrated in vacuo, and the residue was dissolved in 10% HCl–ethanol (30:5, 25 mL). This was extracted with chloroform (2 × 50 mL), and the combined organic extracts were washed with saturated aqueous $NaHCO_3$ (25 mL) and water (2 × 25 mL) and dried (Na_2SO_4). Evaporation of solvent left a yellow solid (0.7 g), which was eluted over coarse silica (40 g) with benzene–acetone–triethylamine (20:5:1, v/v/v) to yield **9** (0.41 g, 59% based on deprotection of **18**) as a yellow solid that was homogeneous (one spot) by TLC (R_f 0.09 (benzene–piperidine, 9:1, v/v), 0.29 (chloroform–methanol–ammonia, 95:5:0.5, v/v/v), 0.44 (benzene–acetone–triethylamine, 5:5:1, v/v/v)). This was crystallized from benzene–ethanol–hexane to give yellow crystals: mp 172.5–173.5 °C; UV (EtOH) λ_{max} 288 nm (ϵ = 13.9 mM^{-1} cm^{-1}); ^1H NMR (pyridine-d_5) δ 0.98 (dt, 6, J = 7 Hz, NCH_2CH_3), 2.35–2.95 (m, 6, NCH_2CH_3 and OCH_2CH_2N), 3.98 and 4.05 (m, 2, OCH_2CH_2N), 6.78–7.64 (m, 13, Ar H), 8.71 (s, 1, OH). Anal. ($C_{26}H_{28}N_2O_4$) C, H, N.

Preparation and Storage of Stock Solutions. Stock solutions of all compounds at a concentration of 10^{-2} M were prepared in ethanol. Serial 10-fold dilutions in ethanol yielded stock solutions in the range 10^{-7}–10^{-2} M, and these were stored at −20 °C. Those compounds that were pure trans or cis isomers reverted to approximately 50:50 mixtures when stored under these conditions. Thus the data reported herein are for approximately equimolar mixtures of the geometric isomers of all six compounds. Five-microliter aliquots of compounds prepared and stored in this way were added directly to tissue culture flasks containing 5 mL of medium to give final concentrations of drug in the range 10^{-10}–10^{-5} M and a final ethanol concentration of 0.1%.

Biochemical and Cell Biology Methods. The relative binding affinities for the ER and AEBS were measured as described in detail in ref 4c, 5, and 15. Cell culture techniques, drug effects on MCF 7 cell growth, and analytical DNA flow cytometry were as detailed in ref 18.

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(23) A more direct approach to **16** wherein the Grignard reaction was quenched with excess saturated aqueous NH_4Cl followed by immediate refluxing provided, after workup, a crude product requiring column chromatographic purification. This afforded a lower yield (51%) of product spectroscopically identical to that prepared as described.

experiments, and Warnell Hall, who prepared the manuscript.

Registry No. (*E*)-**2**, 104575-08-8; (*Z*)-**2**, 104575-09-9; (*E*)-**5**, 76579-59-4; (*Z*)-**5**, 76579-58-3; (*E*)-**6**, 104575-10-2; (*Z*)-**6**, 104575-11-3; (*E*)-**7**, 104575-21-5; (*Z*)-**7**, 104575-12-4; (*E*)-**8**, 104575-22-6; (*Z*)-**8**, 104575-13-5; (*E*)-**9**, 104575-23-7; (*Z*)-**9**, 104575-14-6; **10**, 104575-15-7; **11**, 70354-02-8; **13**, 67106-71-2; **14**, 104575-16-8; **15**, 104575-17-9; **16**, 104575-18-0; **17**, 104575-19-1; **18**, 104575-20-4; MEM chloride, 3970-21-6; 4-$BrC_6H_4OCH_2CH_2NEt_2$, 1823-62-7; benzyl 4-hydroxyphenyl ketone, 2491-32-9; 4-cyanophenol, 767-00-0; 4-bromophenol, 106-41-2; 2-(diethylamino)ethyl chloride hydrochloride, 869-24-9; benzyl chloride, 100-44-7; benzyl cyanide, 140-29-4; ethyl iodide, 75-03-6.

Inhibition of Cathepsin D by Substrate Analogues Containing Statine and by Analogues of Pepstatin

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Five new cathepsin D inhibitors were synthesized and tested as inhibitors of bovine cathepsin D. The compounds were derived by replacing a Phe-Phe dipeptidyl unit of a good cathepsin D substrate, Boc-Phe-Leu-Ala-Phe-Phe-Val-Leu-OR, with statine ((3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid, Sta) or with Sta-Phe. The best inhibitor, Boc-Phe-Leu-Ala-(*S*,*S*)-Sta-Val-Leu-OMe, inhibited cathepsin D with a K_i value of 1.1 nM. In general, the more effective inhibitors were consistent with the proposal that statine functions as a replacement for a dipeptidyl unit. Thirty-five known pepstatin analogues also were evaluated as cathepsin D inhibitors. Substituents in the P_4 and P_3' positions are important for maximal inhibition of this aspartic proteinase, and the P_4 substituent appears more important for inhibition of cathepsin D than for inhibition of other aspartic proteinases.

Cathepsin D (EC 3.4.23.5), an aspartic proteinase first isolated from bovine spleen by Press et al.,[1] is thought to be involved in intracellular tissue degradation.[2] As a result, several attempts to develop synthetic substrates[3] and inhibitors[4] of cathespin D have been reported. At present, the most potent reversible inhibitor of cathepsin D reported (K_i = 0.5 nM)[5] is the pentapeptide inhibitor pepstatin [isovaleryl-L-valyl-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoic acid; Iva-Val-Val-Sta-Ala-Sta (1, Figure 1)] first isolated by Umezawa et al.[6]

The importance of the central statine [4(*S*)-amino-3(*S*)-hydroxy-6-methyl-heptanoyl (sta, **2**)] residue for maximum inhibition of pepsin[5,7a-d] and other aspartic proteinases[8] by pepstatin analogues has focused attention on the possible interactions between this novel amino acid and the active site of the inhibited enzyme. Powers, noting similarities between the structures of pepstatin and good substrates of aspartic proteinases, suggested that statine might replace a dipeptide unit when the inhibitor was bound at the active site of the enzyme.[9] Marciniszyn et al.[10] and Marshall,[11] noting similarities between the structures of statine and possible reaction pathway intermediates, suggested that statine might be a transition-state analogue inhibitor of aspartic proteinases. Boger extended these ideas via molecular modeling methods, describing the close structural similarities between the conformation of pepstatin in the crystal structure of the pepstatin–*Rhizopus chinensis* pepsin complex[12] and a realistic tetrahedral intermediate for hydrolysis of substrate.[13] This new concept that statine could replace a dipeptide tetrahedral intermediate portion of the substrate was used by Boger et al. to synthesize potent renin inhibitors in which the Leu-Val or Leu-Leu units in peptides derived from various angiotensinogen sequences were replaced with statine analogues.[14a,b] Independently, Szelke et al. developed remarkably potent renin inhibitors by incorporating a hydroxyethylene isostere of Leu-Val (**3**) in place of the scissile dipeptide moiety,[15] and the close agreement in potency of the statine-derived and hydroxyethylene-derived inhibitors is consistent with the statine $\simeq$ dipeptide tetrahedral intermediate hypothesis. Rich

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