purified by column chromatography with 75% C_6H_{14} and 25% EtOAc to give 8 g (74.7%) of a straw-colored oil. Anal. ($C_{22}H_{31}NO_4$) C, H, N.

3,5-Disubstituted Isoxazoles. 5-[7-(2-Chloro-4-methoxyphenoxy)heptyl]-3-methylisoxazole (32). A solution of 2.91 g (0.03 mol) of 3,5-dimethylisoxazole in 70 mL of THF was cooled to −70 °C, at which point 18.8 mL of 1.55 M *n*-butyllithium (0.03 mol) in C_6H_{14} was added under N_2 over a 10-min period. The solution was stirred for an additional 30 min at −70 °C and then 9.6 g (0.03 mol) of 1-bromo-6-(2-chloro-4-methoxyphenoxy)hexane[3] in 15 mL of THF was added dropwise over a period of 1 h. The mixture was allowed to warm to room temperature and then left overnight. The mixture was concentrated to dryness in vacuo and the resulting oil crystallized on standing and was recrystallized from a mixture of $(C_2H_5)_2O$–C_6H_{14} to give 6 g of a white solid (59.2%); mp 45–46 °C. Anal. ($C_{18}H_{24}ClNO_3$) C, H, N.

4-[[7-(3-Methyl-5-isoxazolyl)heptyl]oxy]benzoic Acid (2). A solution of 10.0 g (0.033 mol) of nitrile 1 in 60 mL of 20% aqueous HCl and 60 mL of glacial HOAc was heated to reflux for 30 h. After the solution cooled, the resulting solid was collected by filtration and dried to give a white solid, which was recrystallized from C_2H_5OH; 8.5 g (94.6%); mp 129–130 °C. Anal. ($C_{18}H_{23}NO_4$) C, H, N.

Ethyl 4-[[7-(3-Methyl-5-isoxazolyl)heptyl]oxy]benzoate (39). A solution of 8.5 g (0.027 mol) of 1 in 90 mL of ethanol and 1.1 mL of concentrated H_2SO_4 was heated to reflux for 6 h. On cooling, solid separated and was collected. Recrystallization from ethanol gave 8.0 g (92%); mp 60–61 °C. Anal. ($C_{20}H_{27}NO_4$) C, H, N.

3-Methyl-5-[7-(4-Carboxamidophenoxy)heptyl]isoxazole (46). To a solution of 4.47 g (0.015 mol) of nitrile 2 in 18 mL of 95% EtOH and 6 mL of 30% H_2O_2 was added 0.6 mL of 6 N NaOH at ambient temperature.[6] The temperature rose to 50 °C with much foaming. Cooling was necessary to maintain a temperature of 40–50 °C and then the solution was kept at this temperature for 4 h and left at room temperature overnight. The solution was neutralized with 34 mL of 5% H_2SO_4 and chilled. The resulting solid was collected, washed with cold EtOH, and dried. The material was recrystallized from CH_3OH to give 4.4 g (72%); mp 153–154 °C. Anal. ($C_{18}H_{24}N_2O_3$) C, H, N.

1-[4-[[7-(3-Methyl-5-isoxazolyl)heptyl]oxy]phenyl]ethanone (47). To 1.092 g (0.045 mol) of magnesium was added dropwise 2.825 mL (0.045 mol) of methyl iodide in 15 mL of ether. The addition was carried out at such a rate that a gentle reflux was maintained. After the mixture was stirred for $^1/_2$ h, an additional 0.5 mL of methyl iodide was added until all the magnesium had reacted. The solution was then heated to reflux for $^1/_2$ h. After the solution cooled, 8.46 g (0.03 mol) of 4-[[7-(3-methyl-5-isoxazolyl)heptyl]oxy]benzonitrile (1) in 12 mL of benzene was added dropwise. After the addition was complete the mixture was refluxed for 3 h. To the cooled mixture was added 60 mL of a saturated NH_4Cl solution with stirring. The supernatant liquid was removed by decantation and the residual gum stirred with 50 mL of dilute HCl for 1 h. The aqueous solution was heated to reflux for 1 h during which time a gum separated, which eventually solidified. The solid was collected and dried. Recrystallization from C_2H_5OH gave 5.35 g (60%); mp 69–71 °C. Anal. ($C_{19}H_{25}NO_3$) C, H, N.

1,1-Dimethylethyl 4-[[7-(3-Methyl-5-isoxazolyl)heptyl]oxy]benzoate (41). Compound 41 was prepared in 23% yield by the general procedure described, after recrystallization from C_6H_{14};mp 76 °C. Anal. ($C_{22}H_{31}NO_4$) C, H, N.

Ellipticine Derivatives with an Affinity to the Estrogen Receptor, an Approach to Develop Intercalating Drugs with a Specific Effect on the Hormone-Dependent Breast Cancer

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In order to obtain breast tumor directed agents, we have prepared mixed compounds using estradiol or (*E*)-clomiphene as specific vectors for the breast tissue and a DNA intercalator from the ellipticine series as the cytotoxic agent. Among the newly synthesized ellipticine derivatives, only the 2-[3-aza-5-(3,17β-dihydroxy-1,3,5-estratrien-17α-yl)-4-oxopentamethylene]ellipticinium bromide (24) shows the desired properties, DNA intercalation and affinity for estrogen receptor. Competition experiments with estradiol on the hormone-dependent human MCF-7 breast cancer cell line demonstrate that a transport by the estrogen receptor system is not involved in the antitumor activity of derivative 24.

Clinical use of most antitumor drugs is limited by their high toxicity for fast-growing cells in healthy tissues. With the object of overcoming this problem, several cytotoxic agents, such as nitrogen mustards,[1,2] intercalating drugs,[3-5] toxins,[6-8] have been coupled to carriers exhibiting some selectivity toward the tumors themselves[2,5] or to the tissues from which these tumors derive.[1,9] This strategy is aimed at concentrating cytotoxic agents into cells bearing binding sites for the carrier.

The growth of endometrium and of nearly 30% of breast cancers is stimulated by estrogens. Antiestrogens antagonize estrogen effects, and clinical studies have shown that these compounds could be used to treat estrogen-dependent tumors with some success. The mechanism of action of estrogens[10] and probably antiestrogens[11,12] is

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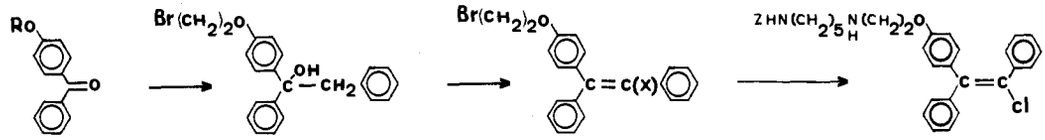
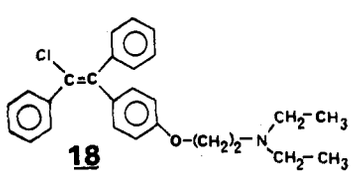
Scheme I



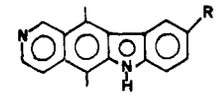
1 R = H
2 R = $(CH_2)_2Br$
3
4 X = H
5 X = Cl
6

mediated by intracellular receptors, their ultimate targets being presumably localized in the nuclear matrix. Therefore, both estrogens and antiestrogens are potentially good vectors to convey DNA-directed agents into the nuclei of receptor-rich cells. In fact, the presence of intracellular steroid-binding proteins in some tumor cells has been used more or less successfully as a basis for building tumor-directed conjugates containing an (anti)estrogenic and a cytotoxic moiety.[1,13]

In an attempt to obtain breast tumor directed compounds, we have connected with a spacer either a derivative of (*E*)-clomiphene [1-[4-[2-(diethylamino)ethoxy]-phenyl]-1,2-diphenyl-2-chloroethylene, **18**], a potent antiestrogen[14] or estradiol with ellipticine (**19**) or ellipticine derivatives (**19a,b**) (see formula). Ellipticines (5,11-di-



18



19 R = H
19a R = OCH_3
19b R = OH

methyl-6*H*-pyrido[4,3-*c*]carbazoles) are DNA intercalators and some of them, e.g., 9-hydroxyellipticine,[15] show good antitumor properties. Since the achievement of such an approach is dependent on the maintenance of the intrinsic biological properties of each of the two components of the conjugates, we first investigated the binding to DNA and to the estrogen receptor of the new composite molecules. Then the cytotoxic properties of the compounds were determined on L1210 tumor cells in vitro because of the good predictive value of this system for drug activity on human cancers. In this test only the estradiol–ellipticine derivative **24** showed the same cytotoxic potency as ellipticine. Therefore, the possibility of enhancing the antitumor activity of this compound through a receptor-mediated mechanism was tested on estrogen receptor rich tumor cells (MCF-7) in vitro.

Chemistry

Preparation of Triarylethylene Derivatives (Schemes I–III). It seems that the main structural requirements for a high binding affinity of triarylethylenes to the estrogen receptor are as follows: (i) the presence of (*N*-alkyl-2-aminoethoxy)phenyl or at least alkoxyphenyl groups,[16] (ii) substitution of the double bond by a bulky group such as chlorine in clomiphene, (iii) a trans configuration for two unsubstituted phenyl groups.[17] Therefore the (*E*)-1-[4-[[3-aza-8-(benzyloxycarboxamido)octamethylene]oxy]phenyl]-2-chloro-1,2-diphenylethylene (**6**) was used as starting material for the vector component and substituted by a chloroacetamido group allowing the quaternization of the 6*H*-pyridocarbazole ring. Indeed, quaternized ellipticines exhibit both higher DNA affinity and antitumor potency.[18] The length of the linking chain was chosen to theoretically permit simultaneous interactions with DNA and estrogen receptor.

Compound **6** was prepared in four steps from 4-hydroxybenzophenone (**1**) following the method of Palopoli et al.[19] (Scheme I). Reaction of 1,2-dibromoethane with **1** gave 4-(2-bromoethoxy)benzophenone (**2**), which was condensed with the Grignard reagent of benzyl chloride. The obtained triaryl alcohol **3** led by dehydration to a mixture of (*Z*)- and (*E*)-2-(bromoethoxy)triarylethylenes, which gave **5** [1-[4-(2-bromoethoxy)phenyl]-2-chloro-1,2-diphenylethylene] by direct chlorination. *Z* and *E* isomers of **5** were separated by successive crystallizations and the structures of the pure isomers were unambiguously attributed by ^1H NMR and X-ray crystallography.[20] Owing to the preferential interaction of "*trans*"-triarylethylenes with the estrogen receptor, only these isomers were used in the following steps. Compound **6** (*E* configuration) was obtained by substitution of **5E** by 5-(benzyloxycarboxamido)pentylamine in hot amyl alcohol.

The three kinds of triarylethylene ellipticine derivatives were synthesized with use of **6** as common starting material (Schemes II and III). Removal of the *Z* protective group of **6** followed by a condensation with chloroacetyl chloride led to **8**, which reacted in hot DMF with either two 9-methoxy- or two 9-hydroxyellipticine rings to give **9** and **9′** (respectively). Compound **9** and **9′** contain two antitumor residues for one triarylethylene vector. Compounds **12** (short spacer) as well as **17** and **17′** (long spacer) contain only one intercalating residue. Reaction of chloroacetyl chloride with **6** gave **10**. Quaternization of 9-methoxyellipticine followed by removal of the *Z* group gave the short-chain derivative **12**. Alternative protection of the central nitrogen of the chain of **6** by a *tert*-butyloxycarbonyl (Boc) group and removal of the terminal *Z* group led to **14**, which was allowed to react with chloroacetyl chloride to give **15**. 9-Methoxy- and 9-hydroxyellipticine were quaternized by **15** to give respectively **16** and **16′**. The long-chain compounds **17** and **17′** were obtained by acidolysis of the protective Boc group in HCl–dioxane.

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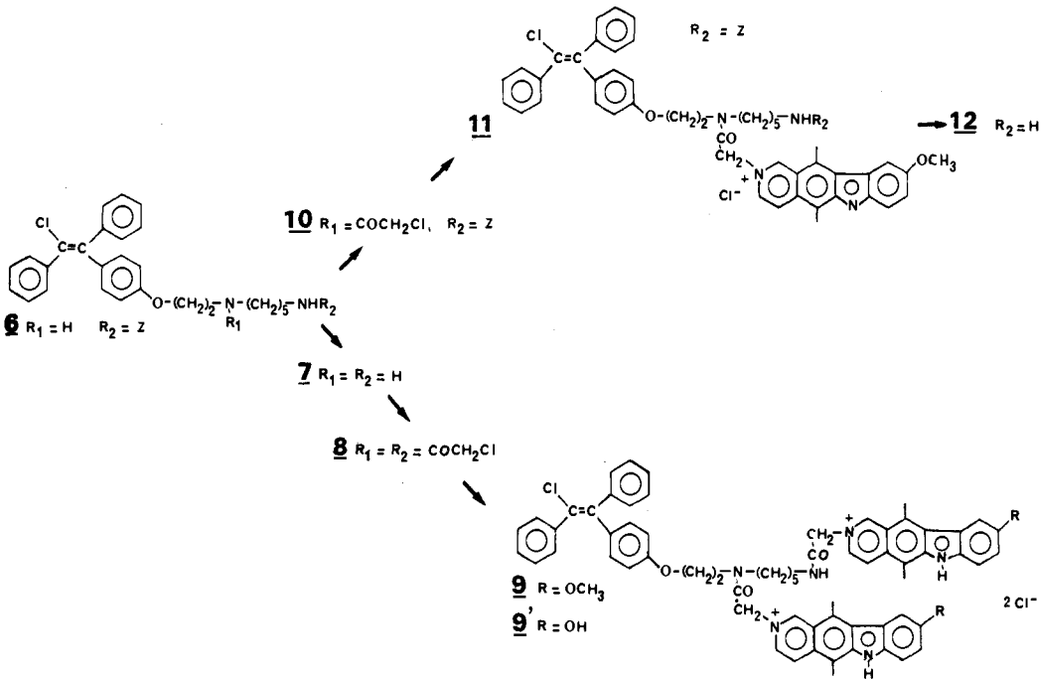
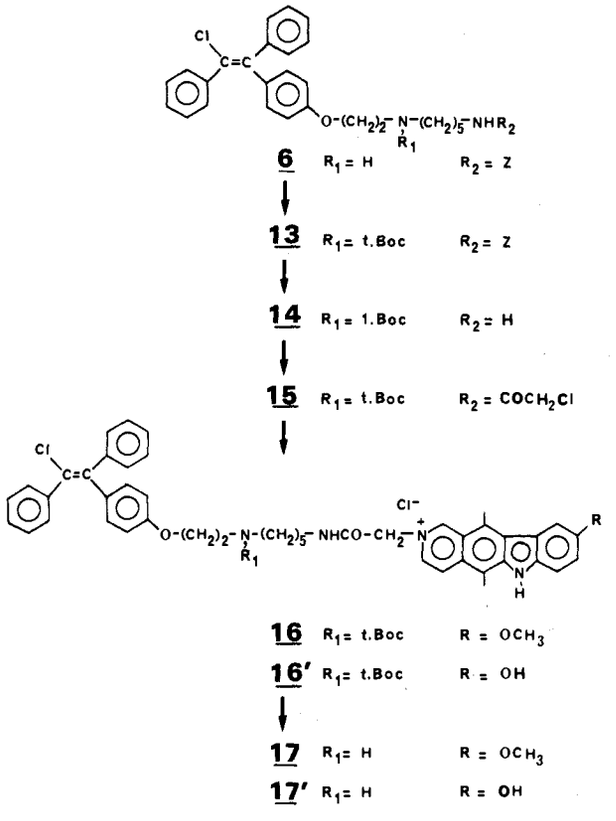
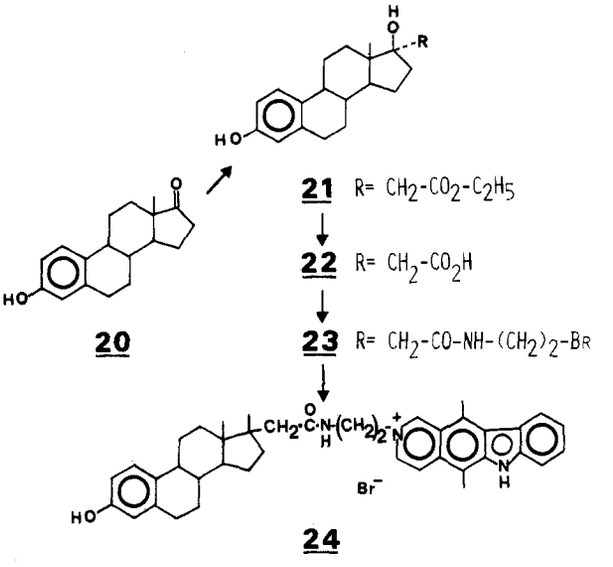
Scheme II



Scheme III



Scheme IV



Preparation of Estradiol Derivatives (Scheme IV). The 3-OH and 17β-OH groups of the estradiol molecule play a crucial role in receptor binding, but most of the other positions on the steroid ring can be substituted without a drastic loss of affinity.[21,22]

In a first step, ethyl bromoacetate was added to estrone **20** in the presence of zinc to give one diastereoisomer **21**, whose configurational purity was checked by ^{1}H NMR. This reaction is oriented by the steric hindrance of the CH_3 in position 13 of the steroid and specifically leads to the 17β-OH isomer.[23] Compound **21** was converted by saponification to **22**, which was subsequently condensed with 2-bromoethylamine to yield **23**. ^{1}H NMR spectra indicated that no dehydration occurred during the preparation of the β-hydroxy ester **21** and acid **22**. Quaternization of ellipticine by **23** in hot DMF led to the estradiol ellipticine derivative **24**.

Biological Properties

Interactions with DNA. The DNA binding constants of clomiphene (*Z* and *E* isomers) (**18**), 2-methyl-9-methoxyellipticinium iodide (**19a**), and compounds **9**, **12**, **17**, **17′**,

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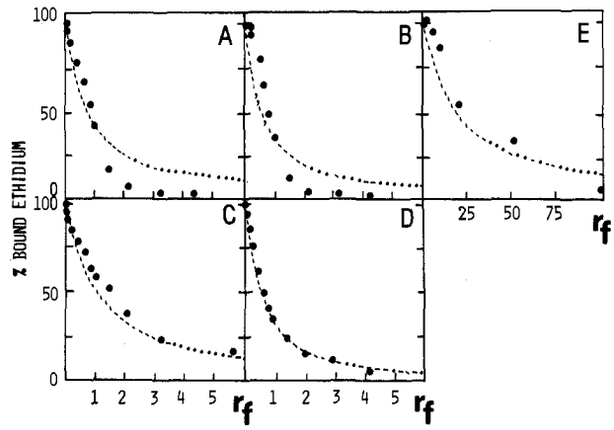

Figure 1. Displacement of ethidium (total molar concentration 5×10^{-6} M) bound to sonicated calf thymus DNA (base pairs concentration 2.5×10^{-6} M) by competition with long-linked compound **17** (A), short-linked compound **12** (B), estradiol ellipticine **24** (C), 9-methoxy-2-methylellipticinium (**19a**) (D), and clomiphene (*Z* and *E* isomers) (**18**) (E; note the change of scale) in 0.02 M sodium acetate buffer, pH 5.5, $T = 20$ °C. Experimental data were compared with theoretical curves (dashed lines) computed for $n = 2$ (number of base pairs covered by a ligand) and following K_{app} values (A) 1×10^7 M^{-1}, (B) 1.2×10^7 M^{-1}, (C) 5×10^6 M^{-1}, (D) 1.1×10^7 M^{-1}, (E) 2×10^5 M^{-1} from McGhee and Von Hippel equation. rf is the ratio of total molar concentration of the competitor to the DNA concentration expressed in base pairs.

and **24** were measured through a fluorometric assay by competition with ethidium bromide in 0.02 M sodium acetate buffer at pH 5.5. At this pH the amino groups of the linking chains in triarylethylene derivatives are positively charged and can bind to the DNA phosphate backbone through Coulombic interactions. Low solubility prevented determination of estradiol binding by the same procedure. Nevertheless it has been previously reported that steroid hormones bind only weakly to DNA (K_{app} = 3000 M^{-1}).[24]

Gaugain et al.[25] have shown that the experimental DNA displacement curves of ethidium bromide by a competitor can generally be fitted with curves computed from the McGhee and Von Hippel equation.[26] This computation involves the DNA binding constant (K_{app}) and the number (n) of base pairs covered on the polymer by the competitor. Since several pairs of values (K_{app}, n) lead to similar computed curves, the fitting process is unreliable if n is not accurately determined. In such a case, only rf_{50} values (ratio of total molar concentration of competitor to the concentration of DNA expressed in base pairs leading to a 50% displacement of bound ethidium bromide), determined under similar experimental conditions, are usually reported in order to compare the DNA affinities of various ligands. Indeed, the lowest the rf_{50} value of a given compound, the highest may be its DNA affinity.

Competition curves in Figure 1 show that each compound is able to displace DNA intercalated ethidium bromide. Experimental data achieved with ellipticinium ions **19** or **19a** (Figure 1D) and estradiol ellipticine derivative **24** (Figure 1C) could be fitted with computed curves by use of integer values of n. This led to K_{app} values reported in Table I. By contrast, the sigmoidal profile of competition curves obtained from triarylethylene derivatives and clomiphene (Figure 1A,B,E) prevented such a direct analysis. Therefore, only rf_{50} values are given for these compounds in Table I. It can be seen that the DNA affinities of most of the new compounds average those of related ellipticinium ions (Table I). Large decreases in affinity for **9** and **9′** were attributed to steric hindrance resulting from the bulk of these molecules. In addition, we observed that both isomers of clomiphene **18** bind rather tightly to DNA ($rf_{50} = 20$). In a review devoted to nonintercalating DNA ligands, Baguley reported a similar DNA affinity for the triarylethylene tamoxifen.[27]

Table I. DNA Binding Affinities Obtained by Competition with Ethidium Bromide (5×10^{-6} M)[a]

compd	rf_{50}	$10^{-6}K_{app}$, M^{-1}
2-methylellipticinium acetate (**19**)	2.50	1.5
9-methoxy-2-methylellipticinium acetate (**19a**)	0.64	11.0
clomiphene (**18**); *E* and *Z* isomers	20.00	
9	10.00	
9′	4.80	
12	0.70	
17	0.86	
17′	0.32	
24	1.50	5.0

[a] ([DNA]) = 2.5×10^{-6} M in base pairs; sodium acetate buffer 0.02 M, pH 5.5, $T = 20$ °C). rf_{50} is the total concentration of ligand (expressed as the ratio of ligand concentration to DNA concentration) displacing 50% of bound ethidium bromide. Binding constants K_{app} of intercalators **19**, **19a**, and **24** were estimated by fitting experimental data with computer curves, with $n = 2$.

Compounds **9**, **9′**, **12**, **17**, **17′**, and **24** were expected to interact with DNA through intercalation of their ellipticinium part between adjacent base pairs. Lengthening of the DNA helix consequent on drug intercalation causes the viscosity of a solution of rodlike DNA to be increased. For a classical intercalator such as ellipticine, a plot of log (η/η_0) vs. log $(1 + 2r)$ (where η and η_0 are the intrinsic viscosities of the DNA solution in the presence and in the absence of the ligand respectively and r is the ratio of the molar concentration of bound molecules to the molar concentration of DNA expressed in nucleotides) is expected to give a straight line with a slope between 2.3 and 3.[28] Extension of the DNA helix induced by intercalation is also visualized by electron microscopy with use of monodisperse DNA and is measured as a function of the ligand concentration.[29] K, the apparent DNA affinity, and ΔL_{max}, the DNA helix lengthening at saturation, are determined by using the relation $\Delta L/C = K(\Delta L_{max} - \Delta L)$, where ΔL is the extension at a given ligand concentration, directly measured on micrographs, and C is the free drug concentration. ΔL_{max} is expected to be 50%. This method is required to study poorly soluble ligands like estradiol ellipticine **24**.

Viscometry experiments with rodlike DNA showed that none of triarylethylene derivatives induced DNA lengthening. Therefore, compounds **9**, **9′**, **12**, **17**, and **17′** are unlikely to intercalate between DNA base pairs. On the contrary we observed, by electron microscopy, that increasing amounts of estradiol ellipticine **24** produced extension of nicked circles of PM2 DNA (3 μM in nucleotides). In the presence of a large excess of **24** (>40 μM), samples underwent a precipitation. However, up to a 26% lengthening was measured. Therefore, it was concluded that estradiol ellipticine **24** showed intercalation properties. An apparent binding affinity of 2×10^4 M^{-1} and a DNA

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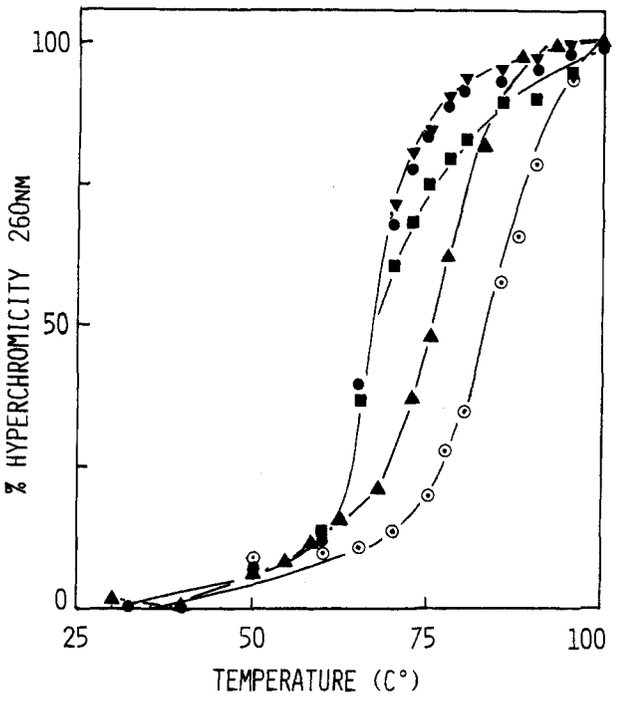

Figure 2. Thermal denaturation of calf thymus DNA (base pairs concentration 3.7×10^{-5} M) in sodium acetate buffer, 0.02 M, pH 5.5, monitored at 260 nm in the presence of (⊙) 9-methoxy-2-methylellipticinium acetate (**19a**) (T = 83 °C), (■) clomiphene (**18**) (T = 67 °C), (●) triarylethylene ellipticine derivative **17** (T = 66 °C), (▲) estradiol ellipticine derivative **24** (T = 76 °C). The curves ▼ corresponds to melting of the uncomplexed DNA (T = 66 °C). Concentration of ligand was equal to 1.5×10^{-5} M.

lengthening at saturation of 53% were derived from experimental measurements. As already reported by Butour et al.,[29] this procedure, in comparison to fluorometric methods, underestimates DNA binding affinities of intercalating agents. Finally, triarylethylene derivatives, just as clomiphene, did not protect DNA against thermal denaturation while the intercalating drugs **19** and **24** did (Figure 2).

Quaternization with a chain bearing a triarylethylene group seems to preclude the ellipticinium residue from intercalation within DNA although dimerization of ellipticine with an aminoalkyl chain branched upon the pyridinic nitrogen allows mono- and bisintercalation.[30] These apparent discrepancies in the mode of DNA binding seem to indicate that external binding of triarylethylene derivatives is thermodynamically more favorable as already reported in the case of quinolinium rings substituted by an aromatic residue.[31] Such features could be related to the DNA recognition process of these molecules. Indeed, from X-ray crystallography data, it has been proposed that the ethidium ion intercalates between DNA base pairs with its phenyl group lying into the narrow groove.[32] Since clomiphene is able to displace DNA intercalated ethidium cation, it is assumed to interact with DNA through the minor groove as may do many other nonintercalating DNA ligands. Furthermore, crystallographic[33] and ^{1}H NMR[34] studies have shown that ellipticine derivatives intercalate into RNA minihelices with the quaternizable nitrogen of the pyridine ring pointing toward the major groove. Assuming that the same requirement for DNA binding occurs for the compounds **9**, **9′**, **12**, **17**, and **17′**, the position of the linking chain on the pyridine nitrogen impedes simultaneous intercalation of the pyridocarbazole ring with the proper geometry and external binding of the triarylethylene moiety in the minor groove. Nevertheless, when DNA intercalation is hindered, intercalators can interact with DNA through external binding. Therefore, the binding of the nonintercalating triarylethylene ellipticine derivatives to DNA is presumably driven by external interactions of both triarylethylene and ellipticinium ion moiety. This results in rf_{50} values similar to that of ellipticine **19** and higher than that of clomiphene **18**. This assumption is supported by the DNA intercalating ability of the composite molecule **24** in which the triarylethylene residue is replaced by a steroid moiety almost devoid of DNA affinity.

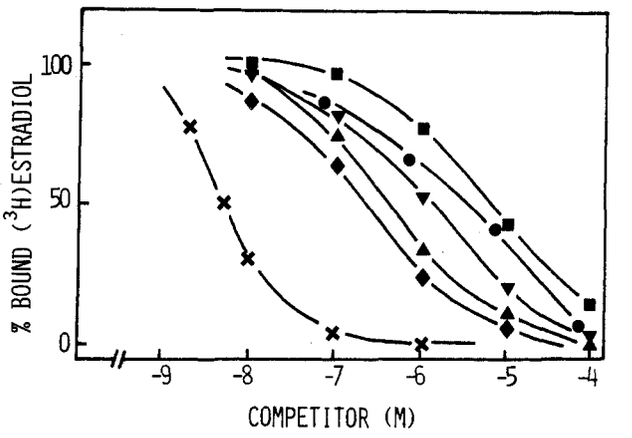

Figure 3. Displacement of [^{3}H]estradiol (5 nM) from calf uterine receptor in TE buffer (incubation 24 h at 0 °C and then 5 h at 20 °C) by increasing concentrations of unlabeled estradiol (x), (*E*)-clomiphene (♦), triarylethylene ellipticine derivatives **9′** (●), **12** (▼), and **17** (▲) and estradiol ellipticine derivative **24** (■).

Interactions with the Estrogen Receptor. The affinity constants of the various derivatives for the estrogen receptor were measured by competition with [^{3}H]estradiol as described in the Experimental Section. Affinity (RAC) of the competitors for the estrogen receptor, relative to that of estradiol, was calculated with the following equation:[35]

$$\mathrm{RAC} = K_{\mathrm{comp}}/K_{\mathrm{est}} \sim \frac{R}{(R+1)\mathrm{RA}}$$

where R and RA are the ratio of the molar concentrations of free and bound estradiol and the ratio of the molar concentration of competitor to the concentration of unlabeled estradiol displacing the same amount of bound [^{3}H]estradiol, respectively.

Displacement curves of bound [^{3}H]estradiol by estradiol, (*E*)-clomiphene **18E**, and compounds **9′**, **12**, **17**, and **24** are displayed in Figure 3. The mixed molecules inhibited estradiol binding even after preincubation with DNA in excess (3×10^{-4} M in nucleotides). They specifically interacted with the estrogen receptor since no significant inhibition of the binding of the progesterone agonist, [^{3}H]R_{5020} (17α,21-dimethyl-19-norpregna-4,9-diene-3,20-dione), to the progesterone receptor occurred in their presence.

Relative affinities for the estrogen receptor are listed in Table II. As already reported, halogenation of the

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Table II. Relative Binding Affinities of Triarylethylene and Estradiol Derivatives for Cytoplasmic Uterine Receptor Obtained by Competition with [^{3}H]Estradiol (5 nM)[a]

compd	rel affinity[b]
estradiol	1.0
(*E*)-clomiphene (18)	0.016
(*Z*)-clomiphene (18)	0.0003
4, *E* and *Z* isomer	<0.0001
5, *E* or *Z* isomer	0.0004
7, *E* isomer	0.011
9 and 9′	0.001
12	0.003
17 and 17′	0.008
24	0.0005

[a] Incubation time 24 h at 0 °C and then 5 h at 20 °C. Estradiol affinity is taken equal to 1. [b] The relative affinity constants were determined from the molar concentration of competitors able to displace 50% of bound [^{3}H]estradiol. The results are the mean of triplicate experiments with SD < ±15%.

Table III. Inhibitory Activity of Triarylethylene Ellipticine and Estradiol Ellipticine Derivatives on L1210 Cell Growth[a]

compd	ED_{50}, μM	C_0, μM
9-hydroxy-2-methylellipticinium acetate (**19b**)	0.08	0.04
9′	4.00	>10.0
12	5.00	9.0
17′	4.00	>10.0
2-methylellipticinium acetate (**19**)	0.85	1.4
24	0.50	0.15

[a] ED_{50} is the dose that reduces by 50% the growth rate of the cells [1 × 10^5 cells/mL in RPMI medium, 37 °C] after 24 h of drug exposure. C_0 is the mean lethal concentration required to reduce cloning efficiency to a factor of 0.37 after 24-h drug exposure.

ethylene bond in triarylethylenes increased estrogen receptor binding. This is shown by the difference in affinity between **4Z** and **4E** (RAC ≪ 10^{-4}) and their chloro derivatives **5Z** and **5E** (RAC = 4 × 10^{-4}). Differences between *Z* and *E* isomers only appear after introduction of an aminoalkyl chain as shown by the higher affinity for the estrogen receptor of (*E*)-clomiphene as compared to the *Z* isomer. Mixed compounds exhibit RAC between 0.0005 and 0.0008. Interestingly, triarylethylene derivatives retain up to 50% of the affinity of their precursor. The affinity appears to be modulated by the length of the connecting chain and the bulk of the molecule. The important loss of affinity observed with **24** could be related to the 17α substitution or the short length of the spacer as well.

Antitumor Activity. L1210 murine leukemia has been widely used in screening tests for new antitumor drugs because of its predictive value for activity in human cancer.[36] Besides, in the ellipticine series, a good correlation was observed between in vivo and in vitro assays on L1210 cells.[37] Therefore, L1210 cells in culture were used as a first screening to evaluate the cytotoxicity of the molecules.

Triarylethylene ellipticine derivatives were found to exhibit only a weak cytostatic effect (Table III). Figure 4 shows that compound **17′** has no effect on the cloning efficiency of the cells after 24-h treatment. Thus it seems that a continuous exposure of the L1210 cells to triarylethylene ellipticine derivatives **9**, **9′**, **12**, **17**, and **17′** results in a decreased average rate of cell division rather than in the induction of a lethal event. By contrast, compound **24** exerts both a cytostatic and a cytotoxic effect (Figure 4). L1210 cell growth was 50% inhibited at a dose of about 5 × 10^{-7} M as compared to about 8.5 × 10^{-7} M for ellipticine and 63% of the cloning efficiency (control 100%) was inhibited at about 1.5 × 10^{-7} M of this drug. Therefore, the estradiol ellipticine derivative induces irreversible lesions in the cells.

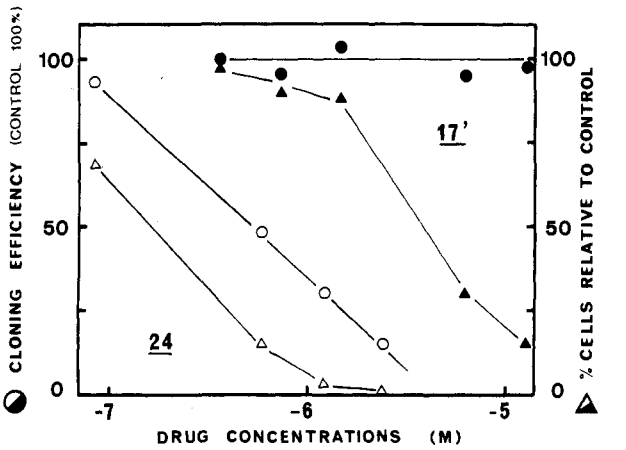

Figure 4. Effect of triarylethylene ellipticine 17′ (black symbols) and estradiol ellipticine 24 (open symbols) on the growth (circles) and the efficiency of cloning (triangles) of L1210 cells. Cells were incubated without (control) or with drug for 24 h as described in the Experimental Section and then counted and tested for cloning efficiency.

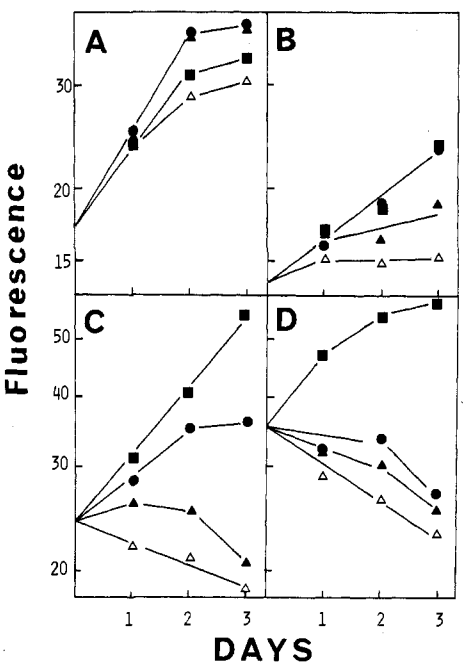

Figure 5. Effect of estradiol ellipticine 24 (A, B) and ellipticine 19 (C, D) on MCF-7 cells. Cells were grown in the absence (■) or in the presence of drug, 7 × 10^{-6} M (●), 3 × 10^{-5} M (▲), and 7 × 10^{-5} M (△), either in medium containing 10% fetal calf serum (A, C) or in serum free medium (B, D).

The human breast cancer cell line MCF-7 contains estrogen receptors[38] and its growth is regulated by estrogens.[39] This system is therefore adequate to check targeting efficiency. None of the triarylethylene derivatives was tested since they are devoid of cytotoxicity. In contrast, because of its cytotoxicity and its putative selectivity, the estradiol derivative **24** was assayed on the MCF-7 cell

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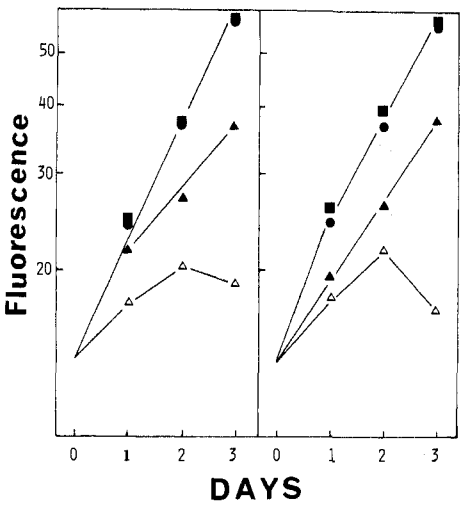

Figure 6. Effect of estradiol on the toxicity of the estradiol ellipticine derivative **24** on MCF-7 cells. The cells were grown in serum-free medium in the absence (■) or in the presence of drug at 7×10^{-6} M (●), 3×10^{-5} M (▲), and 7×10^{-5} M (△) without (left) or with 5×10^{-8} M estradiol (right).

system. We first compared the effect of different drug concentrations on the cell growth rate either in medium containing 10% calf serum or in serum-free medium. Figure 5A,B shows that **24** almost completely blocked cell growth at 7×10^{-5} in serum-free medium while this dose had almost no effect in serum-containing medium. When tested in the same conditions, ellipticine was also found to be more active in serum-free medium but at doses about 10 times lower than those of estradiol ellipticine (Figure 5C,D). Finally, Figure 6 shows that estradiol at 5×10^{-8} M had no effect on the toxicity of **24** at the different concentrations tested. Taken together, these results indicate that the estradiol ellipticine derivative **24** exerts a toxic effect that is related to the pyridocarbazole moiety of the molecule, but the uptake of the drug seems to be unrelated to interactions with the estrogen receptor.

Conclusion

Triarylethylene ellipticine derivatives display a great loss of antitumor activity as compared to ellipticines despite the retention of their binding properties to DNA. Since ellipticines[40] and triarylethylenes are lipophilic molecules, the loss of activity of the compounds **9**, **9'**, **12**, **17**, and **17'** could be due to an enhanced retention in the cell membrane. However, it is more probable that the lack of antitumor properties of these molecules is due to their peculiar mode of DNA binding since the closely related intercalating estradiol ellipticine derivative **24** retains the ellipticine antitumor efficiency. On the other hand, estrogen receptors appear not to be involved with the toxic effect than **24** exerts on MCF-7 cells since addition of estradiol did not modify the cytotoxicity of this compound. This could be due to the low affinity of **24** for the estrogen receptor, affording only a negligible fraction of the protein to be filled with the ligand.

Experimental Section

Melting points were determined on a Kofler apparatus and were not corrected.

Structures of the compounds were established by their NMR spectra recorded on a Bruker 270-MHz spectrometer.

Analyses indicated only by symbols of the elements mean that analytical results obtained for these elements were within ±0.4% of the theoretical values.

4-(2-Bromoethoxy)benzophenone (2). A 0.5-mol sample of freshly prepared sodium methoxide in 200 mL of anhydrous ethanol was added within 15 min, under nitrogen gas, to a stirred solution of 101.7 g (0.5 mol) of 4-hydroxybenzophenone (**1**) in 200 mL of ethanol. After 30 min, 170 mL (2 mol) of 1,2-dibromoethane was added and the mixture was refluxed for 17 h. The brownish oil obtained following concentration in vacuo was blended with an emulsion of 5% aqueous sodium hydroxide and ether. The organic phase was washed twice with 5% aqueous sodium hydroxide and then with a saturated solution of NaCl and dried over sodium sulfate. Evaporation of the solution left **2**, which crystallized from ether: yield 76 g (50%); mp 77 °C. Anal. ($C_{15}H_{13}O_2Br$) C, H, Br.

1-[4-(2-Bromoethoxy)phenyl]-1,2-diphenylethanol (3). Benzyl chloride (20 mL, 0.175 mol) in 300 mL of anhydrous ether was added within 2 h under nitrogen to a stirred suspension of 4.25 g (0.175 mol) of magnesium turnings in ether (100 mL). The mixture was refluxed for 1 h until the metal had disappeared and then it was diluted with 75 mL of benzene and added in 30 min to 53.4 g (0.175 mol) of **2** dissolved in 105 mL of benzene. The temperature of the medium rose to a gentle reflux. After 1.5 h the liquor was poured into 470 mL of a cold stirred 10% ammonium chloride solution. The organic phase was washed with water and dried over sodium sulfate before it was evaporated. Compound **3** was crystallized from the syrup in benzene–hexane: yield 53.3 g (77%); mp 106 °C. Anal. ($C_{22}H_{21}O_2Br$) C, H, Br.

1-[4-(2-Bromoethoxy)phenyl]-1,2-diphenylethylene (4). A suspension of 26.7 g of **3** (0.0675 mol) in 225 mL of 7.8 N HCl–ethanol was slowly warmed to a gentle reflux. After 7 h the clear solution was concentrated and then mixed with water and ether. Sodium hydroxide (10 N) was added dropwise under nitrogen to the stirred mixture until the appearance of a white solid. After evaporation of the ether, the solid was filtered in vacuo. Compound **4** was crystallized from ether as an equimolecular mixture of both *Z* and *E* isomers, which was not resolved: yield 25.5 g (100%); mp 92–98 °C. Anal. ($C_{22}H_{19}OBr$) C, H, Br.

1-[4-(2-Bromoethoxy)phenyl]-2-chloro-1,2-diphenylethylene (5*Z* and 5*E*). A 48-mL sample of 1.41 M chlorine solution in CCl_4 (0.0675 mol of chlorine) was added within 2 h to 25.6 g (0.0675 mol) of **4** dissolved in 150 mL of carbon tetrachloride. The mixture was stirred for 2 h and then gently refluxed for 2.5 h before it was evaporated. Compound **5** (*Z* and *E* isomers) spontaneously crystallized from the oily residue. The *E* isomer (mp 138 °C) was obtained pure from dichloromethane: yield 17.9 g (64%). The *Z* isomer (mp 103 °C, 0.5 g) was obtained after successive crystallizations in benzene–hexane, starting from 5 g of a mixture of both isomers (mp 80 °C). Anal. (*Z* and *E* isomers) ($C_{22}H_{18}OBrCl$) C, H, Br, Cl.

(*E*)-1-[4-[[3-Aza-8-(benzyloxycarboxamido)octamethylene]oxy]phenyl]-2-chloro-1,2-diphenylethylene (6). A 5-g sample (0.0232 mol) of 5-(benzyloxycarboxamido)pentylamine (prepared following the procedure described in ref 41) in 15 mL of hot amyl alcohol was added under nitrogen to 15 mL of a boiling solution of **5** (4.8 g of *E* isomer, 0.0116 mol) in amyl alcohol. The mixture was gently refluxed for 5 h. A white solid appeared, which was dissolved in dichloromethane, filtered, and then chromatographed over a silica gel column (chloroform–methanol, 9:1). Compound **6** spontaneously crystallized: yield 4.91 g (75%); mp 68 °C. Anal. ($C_{35}H_{37}N_2O_3Cl$) C, H, N, Cl.

(*E*)-1-[4-[(8-Amino-3-azaoctamethylene)oxy]phenyl]-2-chloro-1,2-diphenylethylene Dihydrobromide (7). A solution of 1.13 g (0.002 mol) of **6** in dichloromethane (2 mL) was added to 0.006 mol of hydrobromic acid in acetic acid (1 mL, 35% w/w). The solution was stirred for 1.5 h and then evaporated to give a brownish syrup. Compound **7** was crystallized from an anhydrous mixture of ether, acetone, and methanol: yield 0.97 g (82%); mp 120 °C. Anal. ($C_{27}H_{33}N_2OBr_2Cl$) C, H, N, Br, Cl.

(*E*)-1-[4-[[3-Aza-8-(chloroacetamido)-3-(chloroacetyl)-octamethylene]oxy]phenyl]-2-chloro-1,2-diphenylethylene (8). A 0.170-mL sample (0.002 mol) of freshly distilled chloroacetyl chloride in 2 mL of 1,2-dichloroethane was slowly added to a cold emulsion of **7** (0.5 g, 0.000 84 mol) in 1,2-dichloroethane (4 mL)

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and 5 N aqueous sodium hydroxide (0.5 mL). After 3 h, 5 mL of water was added to the reaction mixture, and the organic and aqueous phases were separated. The organic phase was thoroughly washed with water and then with a saturated solution of NaCl and dried over sodium sulfate. The yellowish foam obtained following evaporation was chromatographed over silica gel (chloroform–methanol, 9:1) to give **8**: yield 0.23 g (46%); oil. Anal. ($C_{31}H_{33}N_2O_3Cl_3$) C, H, N, Cl.

(***E***)-**2-[11-[4-(2-Chloro-1,2-diphenylethenyl)phenoxy]-3,9-diaza-9-[(9-methoxy-2-ellipticinio)acetyl]-2-oxoundecamethylene]-9-methoxyellipticinium Dichloride** (**9**). A 0.48-g sample (0.001 73 mol) of 9-methoxyellipticine and 0.49 g of **8** (0.000 83 mol) were warmed at 95 °C in 15 mL of DMF for 5 h. The solvent was evaporated in vacuo and the resulting solid was purified by column chromatography on Sephadex LH 20 in methanol. Compound **9** was crystallized from a mixture of methanol–2-propanol: yield 0.30 g (32%); mp 252 °C. Anal. ($C_{67}H_{65}N_6O_5Cl_3$) C, H, N, Cl.

(***E***)-**2-[11-[4-(2-Chloro-1,2-diphenylethenyl)phenoxy]-3,9-diaza-9-[(9-hydroxy-2-ellipticinio)acetyl]-2-oxoundecamethylene]-9-hydroxyellipticinium Dichloride** (**9′**). This compound was prepared as for the previous derivative **9**. From 0.001 73 mol of **8**, 0.32 g of **9′** was obtained: yield 16%; mp 258 °C. Anal. ($C_{65}H_{61}N_6O_5Cl_3$) C, H, N, Cl.

(***E***)-**1-[4-[[3-Aza-8-(benzyloxycarboxamido)-3-(chloroacetyl)octamethylene]oxy]phenyl]-2-chloro-1,2-diphenylethylene** (**10**). A 0.103-mL sample (0.0013 mol) of freshly distilled chloroacetyl chloride in 3 mL of ether was added to a cold stirred mixture of **6** (0.376 g, 0.000 66 mol) in 4 mL of dichloromethane–ether (1:3) and aqueous 1 N sodium hydroxide (2.3 mL). After 30 min the organic phase was separated, washed with water to neutrality, dried over sodium sulfate, and then evaporated. Compound **10** was obtained by chromatography over silica gel (chloroform–methanol, 9:1): yield 0.211 g (58%); foam. Anal. ($C_{37}H_{38}N_2O_4Cl_2$) C, H, N, Cl.

(***E***)-**2-[3-Aza-8-(benzyloxycarboxamido)-3-[2-[4-(2-chloro-1,2-diphenylethenyl)phenoxy]ethyl]-2-oxooctamethylene]-9-methoxyellipticinium Chloride** (**11**). A mixture of **10** (0.38 g, 0.000 59 mol) and 9-methoxyellipticine (0.163 g, 0.000 59 mol) in 5 mL of DMF was stirred at 100 °C for 4 h. The solvent was removed in vacuo and the residue chromatographed over Sephadex LH 20 with chloroform–methanol (1:1) as eluent. **11**: yield 0.497 g (92%); red solid. Anal. ($C_{52}H_{56}N_4O_5Cl_2$) C, H, N, Cl.

(***E***)-**2-[8-Amino-3-aza-3-[2-[4-(2-chloro-1,2-diphenylethenyl)phenoxy]ethyl]-2-oxooctamethylene]-9-methoxyellipticinium Chloride** (**12**). A solution of 0.377 g of **11** (0.000 41 mol) in methanol–dioxane (4 mL, 1:1) was added with vigorous stirring to an hydrogen-activated (39 mL) suspension of 10% palladium on carbon (0.149 g) in methanol (4 mL). The mixture was gradually acidified to pH 1 with HCl in dioxane and the reaction was terminated when hydrogen uptake had ceased (3 h). The catalyst was removed by filtration. Compound **12**, which crystallized spontaneously, was recrystallized in a mixture of methanol–ether: yield 0.104 g (65%); mp 206 °C. Anal. ($C_{47}H_{49}N_4O_3Cl_3$) C, H, N, Cl.

(***E***)-**1-[4-[[3-Aza-8-(benzyloxycarboxamido)-3-(*tert*-butyloxycarbonyl)octamethylene]oxy]phenyl]-2-chloro-1,2-diphenylethylene** (**13**). A 0.207-g sample (0.000 95 mol) of di-tert-butyl carbonate in 0.5 mL of dioxane was added within 10 min to a cold stirred mixture of **6** (0.492 g, 0.000 86 mol), dioxane (3 mL), and water (0.5 mL). After 20 min, the solution was warmed to room temperature, stirred for 40 min, and then evaporated in vacuo to give 0.63 g of syrup. Compound **13** was obtained following chromatography over silica gel with chloroform–methanol (9:1) as eluent: yield 0.46 g (69%); oil. Anal. ($C_{40}H_{45}N_2O_5Cl$) C, H, N, Cl.

(***E***)-**1-[4-[[8-Amino-3-aza-3-(*tert*-butyloxycarbonyl)octamethylene]oxy]phenyl]-2-chloro-1,2-diphenylethylene** (**14**). A solution of **13** (0.562 g, 0.000 84 mol) in 5 mL of methanol–ether (1:1) in the presence of 10% palladium on carbon catalyst (0.05 g) was hydrogenated at room temperature. The reaction was terminated after 1.5 h when hydrogen uptake had ceased (34 mL). After filtration, the solution was concentrated in vacuo to leave 0.399 g (96%) of the crude amine **14**. Anal. ($C_{32}H_{39}N_2O_3Cl$) C, H, N, Cl.

A 0.388-g sample of **14** diluted with an anhydrous mixture of ether and methanol was converted into its hydrochloride with HCl in methanol. The solvent was evaporated to give an hygroscopic foam: yield 0.375 g (95%).

(***E***)-**1-[4-[[3-Aza-8-(chloroacetamido)-3-(*tert*-butyloxycarbonyl)octamethylene]oxy]phenyl]-2-chloro-1,2-diphenylethylene** (**15**). A 0.155-mL sample (0.002 mol) of freshly distilled chloroacetyl chloride in 3 mL of ether was added within 10 min to a strongly stirred mixture of **14** (0.532 g, 0.001 mol), ether (3 mL), and 1 N sodium hydroxide (3.5 mL) cooled in an ice bath. The heavy emulsion gradually became clear (final pH 9). After 30 min the organic phase was taken, washed with water to neutrality, and then dried over sodium sulfate. The residue obtained after evaporation of the solvent was dissolved in chloroform and chromatographed over a silica gel column (chloroform–methanol, 9:1): yield 0.389 g (64%); foam. Anal. ($C_{34}H_{40}N_2O_4Cl_2$) C, H, N, Cl.

(***E***)-**2-[3,9-Diaza-11-[4-(2-chloro-1,2-diphenylethenyl)phenoxy]-2-oxo-9-(*tert*-butyloxycarbonyl)undecamethylene]-9-methoxyellipticinium Chloride** (**16**). A 0.437-g sample (0.000 72 mol) of **15** was stirred with 0.198 g (0.000 72 mol) of 9-methoxyellipticine in 7 mL of hot DMF (90 °C) for 2.5 h. The solvent was removed in vacuo and the residue, dissolved in 9 mL of chloroform–methanol (1:1), was purified by column chromatography on Sephadex LH 20 (chloroform–methanol, 1:1). **16**: yield 0.472 g (74%); orange solid. Anal. ($C_{52}H_{56}N_4O_5Cl_2$), C, H, N, Cl.

(***E***)-**2-[3,9-Diaza-11-[4-(2-chloro-1,2-diphenylethenyl)phenoxy]-2-oxo-9-(*tert*-butyloxycarbonyl)undecamethylene]-9-hydroxyellipticinium Chloride** (**16′**). This compound was obtained similarly to **16** from 0.000 72 mol of **15** and 0.000 72 mol of 9-hydroxyellipticine and was crystallized from chloroform–methanol–ether (1:1:1): yield 0.450 g (72%); mp 148 °C. Anal. ($C_{51}H_{54}N_4O_5Cl_2$) C, H, N, Cl.

(***E***)-**2-[3,9-Diaza-11-[4-(2-chloro-1,2-diphenylethenyl)phenoxy]-2-oxoundecamethylene]-9-methoxyellipticinium Chloride Hydrochloride** (**17**). A 0.1-mL sample of HCl–dioxane (23.4%, w/w; 0.000 12 mol) was added to 0.105 g (0.000 12 mol) of **16** in 0.5 mL of dioxane. After 15 h, the solvent was removed and the residue thoroughly washed with dioxane. Compound **17** was crystallized from a mixture of acetone–methanol–water: yield 0.082 g (82%); mp 217 °C. Anal. ($C_{47}H_{49}N_4O_3Cl_3$) C, H, N, Cl.

(***E***)-**2-[3,9-Diaza-11-[4-(2-chloro-1,2-diphenylethenyl)phenoxy]-2-oxoundecamethylene]-9-hydroxyellipticinium Chloride Hydrochloride** (**17′**). Compound **17′** was obtained in 43% yield from compound **16′** and 9-hydroxyellipticine by the preceding method: mp 200 °C. Anal. ($C_{46}H_{47}N_4O_3Cl_3$) C, H, N, Cl.

Ethyl 3,17β-Dihydroxy-1,3,5-estratriene-17α-acetate (**21**). A 1.9-g sample (0.0114 mol) of ethyl bromoacetate in 2 mL of dry toluene was mixed with estrone **20** (5 g, 0.0185 mol) and zinc (3.6 g, 0.055 mol) in dry THF (75 mL). After the reaction was started, the reflux was maintained for 4 h first by slow addition of 7.4 g (0.0443 mol) of ethyl bromoacetate in dry toluene (8 mL) and then by warming. Thereafter the medium was treated with 3% sulfuric acid and then with ethyl acetate and the organic layer was separated. The organic phase was successivly washed with 10% sodium hydrogen carbonate and with a saturated solution of NaCl before it was dried over sodium sulfate and evaporated. Chromatography over silica gel (ether–petroleum ether, 15:85) gave 5 g (75%) of the ester **21** as an oil. Anal. ($C_{22}H_{30}O_4$) C, H.

3,17β-Dihydroxy-1,3,5-estratriene-17α-acetic Acid (**22**). A 5-g sample (0.0114 mol) of ester **21** was saponified at room temperature with 4 g of NaOH in a mixture of ethanol (200 mL) and water (10 mL). After 4 h the reaction mixture was poured into 1.5 L of water and the resulting solution was washed twice with ether before it was acidified to pH 1 with HCl. A 4.8-g sample of crude acid **22** was extracted with ether from the acidic water. Final purification of **22** followed the same procedure except that in the first step water was replaced by 10% sodium hydrogen carbonate (1 L) and gave 3.8 g of product (76%): mp 142 °C. Anal. ($C_{20}H_{28}O_4$) C, H.

***N*-(2-Bromoethyl)-3,17β-dihydroxy-1,3,5-estratriene-17α-acetamide** (**23**). A 2.5-mL sample of water containing 0.86 g (0.0045 mol) of water-soluble carbodiimide, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride, 1.06 g (0.0052

mol) of 2-bromoethylamine hydrobromide, and 0.183 g (0.0018 mol) of triethylamine was added with stirring to 1 g (0.003 mol) of **22** in 10 mL of THF. After 6 h the mixture was acidified with 1 N HCl and extracted with ethyl acetate. The organic phase was successively washed with a saturated solution of $NaHCO_3$, water, and a saturated solution of NaCl and then it was dried over sodium sulfate before evaporation. One gram of **23** (93%) was crystallized from methanol: mp 241 °C. Anal. ($C_{22}H_{30}NO_3Br$) C, H, N, Br.

2-[3-Aza-5-(3,17β-dihydroxy-1,3,5-estratrien-17α-yl)-4-oxopentamethylene]ellipticinium Bromide (24). A 0.000 25-mol sample of estradiol derivative **23** (0.110 g) and ellipticine (0.065 g) were warmed in 3 mL of DMF at 90 °C for 7 h. The solution was then filtered and its volume reduced in vacuo. Estradiol ellipticine derivative **24** (0.072 g, 42%) was precipitated from the solution on addition of acetone: mp 270 °C. Anal. ($C_{39}H_{44}N_3O_3Br$) C, H, N, Br.

The concentrations of the ellipticine derivatives were measured spectrophotometrically with absorption coefficients $\epsilon^{309} = 6.9 \times 10^4$ M^{-1} cm^{-1} for **9** and **9'**, $\epsilon^{310} = 3.4 \times 10^4$ M^{-1} cm^{-1} for **12**, $\epsilon^{316} = 3.5 \times 10^4$ M^{-1} cm^{-1} for **17** and **17'**, and $\epsilon^{305} = 7.1 \times 10^4$ M^{-1} cm^{-1} for **24**.

Fluorometric Determination of the Apparent Affinity Constant (K_{app}) of Compounds for DNA. Measurements were performed at 20 °C, in 0.02 M sodium acetate buffer, pH 5.5, with a Perkin-Elmer MPF 44 fluorometer. Ethidium bromide was excited at 546 nm and fluorescence recorded at 590 nm. DNA affinity of ethidium bromide was taken equal to 2.5×10^6 M^{-1}.[42]

Final concentrations of sonicated calf thymus DNA (in base pairs) and of ethidium bromide were 2.5×10^{-6} and 5×10^{-6} M, respectively. Microliter portions of freshly prepared solutions of competitor were added. The concentration of bound ethidium bromide per base pair was deduced from the fluorescence measurements and plotted vs. rf (ratio of the total molar concentration of the competitor to the molar concentration of DNA base pairs). For true monointercalator complete displacement curves could be computed as described by Gaugain et al.[25] for different K_{app} assuming $n = 2$ (n = number of DNA base pairs covered by one ligand molecule) and compared with experimental data. rf_{50} was defined as the rf value displacing 50% of DNA-bound ethidium bromide. Only rf_{50} values were given for nonintercalating ligands because experimental data achieved with these compounds could not be fitted with use of theoretical curves.

Thermal Denaturation of Calf Thymus DNA. Thermal denaturation of sonicated calf thymus DNA (3.7×10^{-5} M viscometer base pairs) was scanned at 260 nm between 30 and 100 °C in 0.02 M sodium acetate buffer, pH 5.5, with a Unicam SP8-100 spectrophotometer. Ligand concentration was 1.5×10^{-5} M. The temperature was regulated with a Haake temperature controller.

DNA Lengthening Measurements. Viscosity measurements were done at 25 °C in a semimicro dilution viscometer mounted in a highly accurate temperature-controlled water bath. Flow times were measured at ±0.1 ms simultaneously with photoelectric sensors and an electronic timer. The intrinsic viscosity of sonicated calf thymus DNA was measured in the presence of increasing concentrations of ligand. Log $(\eta)/(\eta)_0$ was plotted as a function of log $(1 + 2r)$, where η and η_0 are the intrinsic viscosities of the DNA solution measured in the presence and in the absence of bound agent and r is the number of bound ligands per nucleotide.

Determination by electron microscopy of the DNA lengthening induced by the drug was done following the procedure described by Butour et al.[29] Relaxed DNA circles of uniform length were obtained by random introduction of nicks in supertwisted molecules of PM2 bacteriophage DNA with use of γ ray irradiation. Nicked DNA (1 μg/mL) was incubated for 10 min in acetate buffer, 0.05 M, pH 5, with increasing amounts of **24**. Then the drug-treated molecules were adsorbed onto charged carbon coated grids, which were subsequently contrasted by rotatory platinium shadowing and observed. After calibration of the microscope with a grating replica, absolute measurements were done on enlarged tracings with a curvimeter. For each ligand concentration, DNA extension was the mean of a minimum of 20 determinations done on separate circles. The accuracy of the measurements was found to be better than 2%.

Determination of the Apparent Affinity for the Estrogen Receptor. Calf uterine cytosol was prepared in TE buffer (10 mM Tris-HCl, 1.5 mM EDTA, pH 7.4) as described previously.[43] It was adjusted to 2 mg of protein/mL (estrogen receptor ~3 nM) and then incubated for 24 h at 0 °C and then 5 h at 20 °C with a constant concentration (5 nM) of [^{3}H]estradiol (50 Ci/mmol) and increasing concentrations of unlabeled competitors. Aliquots (300 μL) of cytosol were then added to an equal volume of dextran-coated charcoal suspension (0.5% charcoal Norit A, 0.05% dextran T 70 in TE buffer). The mixture was agitated for 2.5 h at 0 °C and then charcoal was pelleted by centrifugation. Aliquots of supernatant (200 μL) were counted in 3 mL of ethanol and 10 mL of a PPO/POPOP/toluene mixture with 25–30% efficiency. The specific saturable binding of [^{3}H]estradiol to the estrogen receptor in the presence of the various competitors was expressed as a percentage of saturable [^{3}H]estradiol measured in the absence of competitor and was plotted against the concentration of competitor on a logarithmic scale. Relative affinities of competitors were calculated according to Korenman[35] from concentrations of unlabeled estradiol and of competitors that inhibited 50% of the [^{3}H]estradiol specific binding.

Determination of the Apparent Affinity for the Progesterone Receptor. It was determined from experiments similar to those done for determination of the affinity for the estrogen receptor. [^{3}H]R_{5020} (17α,21-dimethyl-19-norpregna-4,9-diene-3,20-dione; 4 nM; 90 Ci/mmol) was used instead of [^{3}H]estradiol. After a 24-h incubation at 0 °C, the binding of [^{3}H]R_{5020} to the progesterone receptor was assayed by using a 0.5-h charcoal treatment.

In Vitro Antitumor Activity on Leukemia L1210 Cell Line. Exponentially growing L1210 cells (1×10^5/mL) were incubated in RPMI 1640 medium supplemented with fetal calf serum (10%), penicillin (200 IU/mL), streptomycin (50 μg/mL), and β-mercaptoethanol (60 μM) and containing different drug concentrations. The cells were maintained in nonagitated suspension for 24 h at 37 °C in 5% CO_2 atmosphere. Cell number in the different cultures was then counted with a Cytograf Model 6300A (Biophysics Systems, Inc.). The average number of cells in each duplicate treated cultures was expressed as a percentage of the average number of cells in the triplicate untreated controls. The ED_{50} value, defined as the dose inhibiting 50% of the cell growth after 24 h of drug exposure, was calculated by plotting the percentage inhibition vs. the drug concentration on a logarithmic scale.

The 24-h drug-treated cells were then diluted in RPMI medium to a concentration of 250 and 500 cells/mL. Cell suspension (2.5 mL) was then added to 0.4 mL of 2.4% Noble Agar (Difco Laboratories),[44] which had been kept at about 50 °C beforehand and then poured into 35-mm diameter petri dishes (Corning). The dishes were then incubated for 14 days in a humidified incubator at 37 °C in 5% CO_2 atmosphere. Colonies of 1–2 mm diameter were counted with a Biothran III counter (New Brunswick Scientific Co. Inc.). Triplicate assays were carried out at each drug concentration. The cloning efficiency in the controls was about 65%. Dose–response curves were used to determine the mean lethal concentration (C_0), defined as the concentration required to reduce cloning efficiency to a factor of 0.37.

In Vitro Antitumor Activity on Hormone-Dependent Human Breast Cancer Cell Line MCF-7. MCF-7 cells, obtained from Dr. M. Rich (Michigan Cancer Foundation, Detroit), were maintained in monolayer culture in Dulbecco's modified Eagle's medium supplemented with antibiotics and 10% fetal calf serum. Serum-free cultures were carried out as described by Barnes and Sato.[45] Prior to drug treatment, the cells were grown for 4 days in serum-free medium. Then 3×10^5 cells were plated in 16-mm diameter wells of a 24-well Costar dish containing 2 mL of medium at the given drug concentrations. At the indicated times, the cells were trypsinized and, after centrifugation, re-

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suspended in 0.05 M Tris-HCl buffer, pH 7.5, containing NaCl (0.05 M) and EDTA (0.001 M). The samples were frozen and stored at −20 °C until the last day. All the samples were then treated together for fluorometric estimation of the DNA concentration according to the method described by Karsten and Wollenberger.[46] Fluorescence measurements were done on a single photon counting apparatus built in the laboratory of one of us (J.B.L.). The ethidium bromide fluorescence was calibrated with use of DNA extracted from untreated MCF-7 cells (~3 × 10^4 cells). Then the DNA content of the samples was compared to that of untreated cells stopped at the zero time of the experiments.

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Synthesis and Neuroleptic Activity of 3-(1-Substituted-4-piperidinyl)-1,2-benzisoxazoles[1]

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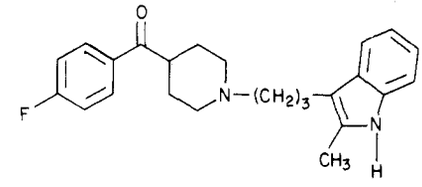
The synthesis of a series of 3-(1-substituted-4-piperidinyl)-1,2-benzisoxazoles is described. The neuroleptic activity of the series was evaluated by utilizing the climbing mice assay and inhibition of [^{3}H]spiroperidol binding. Structure–activity relationships were studied by variation of the substituent on the benzisoxazole ring with concomitant variation of four different 1-piperidinyl substituents. Maximum neuroleptic activity was realized when there was a 6-fluoro substituent on the benzisoxazole ring. The 1-piperidinyl substituent appeared less significant, although in most cases, the (1,3-dihydro-2-oxo-2*H*-benzimidazol-1-yl)propyl group imparted maximum potency. The most potent compound in both assays was 6-fluoro-3-[1-[3-(1,3-dihydro-2-oxo-2*H*-benzimidazol-1-yl)propyl]-4-piperidinyl]-1,2-benzisoxazole (11b).

The synthesis in our laboratory of the 4-benzoylpiperidine[3] I (HP 291) and the subsequent discovery of its interesting neuroleptic profile[4] provided the impetus to study structural variants of this system. It is apparent that HP291 can be viewed as a butyrophenone in which the 2-, 3-, and 4-carbons are constrained in a six-membered ring. Since this modification had led to an altered and perhaps more desirable profile with respect to the butyrophenones, it was of interest to study the introduction of further rigidity into the system. One interesting possibility was replacement of the 4-benzoyl group of the piperidine with a bicyclic system such as a 1,2-benzisoxazole to afford II. This system would have one less degree of rotational freedom than a benzoylpiperidine; additionally, it would maintain a somewhat similar conjugative interaction as manifested by the carbonyl–aryl relationship of the benzoyl group. Precedent exists, albeit in another therapeutic area, for such a bioisosteric relationship between benzoyl and



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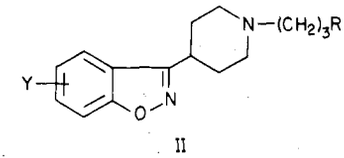

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the 1,2-benzisoxazole moiety.[5] Since such an interchange might alter other factors, structure–activity relationships were studied by the variation of the benzisoxazole substituent (Y) in combination with four different substituents at the piperidine nitrogen.

Chemistry. The stepwise process to the 1,2-benzisoxazoles began with the synthesis of 4-aroylpiperidines, all of which possessed a 2-halo or 2-hydroxy substituent in the aromatic ring (Scheme I, Table I). Thus, 4-chloro-*N*-methylpiperidine (1) was reacted, utilizing Grignard chemistry, with 2-halobenzonitriles to yield the desired ketones **3a** and **3d** (method A). Alternatively, an

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