Experimental Section

Biological Activity. The biological activities of the inhibitors of mannosidase were taken from the literature. Where several values were cited for activity of some compounds, the range of values was taken. The activities of the compounds studied are summarized in Table I. Several compounds had been tested for their inhibitory activity in rat liver mannosidase instead of the jack bean enzyme. Howard et al.[36] reported the activity of swainsonine (4) in mannosidases from several biological sources, including rat liver and jack bean, and found relatively small differences in IC_{50}.

Molecular Orbital Calculations. Molecular orbital calculations were performed on a MicroVAX II computer, using the AMPAC[37] semiempirical molecular orbital and the GAUSSIAN 80 ab initio molecular orbital packages. The AMPAC molecular orbital method appears to provide a better description of hydrogen bonding and to yield more realistic geometries than MINDO/3.[38,39] It was used to optimize the geometry of each half-chair form of the mannopyranosyl cation. The AMPAC starting geometries for the two possible half-chair forms of the mannosyl cation were adapted from the crystal structure of α-D-mannose.[40] The starting geometry of the second half-chair form was derived from the geometry of the first half-chair form with the appropriate ring flip applied via the modelling capabilities of CHEM-X.[41] This optimum geometry obtained from the semiempirical MO calculation was used as the starting geometry for a fully geometry-optimized ab initio MO calculation to obtain the relative energies of the two half-chair forms. These calculations utilized the GAUSSIAN 80[42] package and employed the minimal STO-3G basis set.

Molecular Modelling. The molecular modelling was carried out on a MicroVAX II computer using the CHEM-X[41] software. Crystal structures were available for swainsonine (3)[43] and nojirimycin,[44] α-D-mannose (1),[40] and castanospermine (3).[45] The geometries of the other inhibitors were obtained by modification of castanospermine, swainsonine, and nojirimycin and by the use of the molecular modification and building capabilities of CHEM-X. The derived geometries were optimized by means of a molecular mechanics calculation (in CHEM-X). Errors in the molecular structures derived by these means are usually small and do not significantly affect the results of the molecular superimposition studies.

The superimpositions were accomplished by choosing several pairs of topographically equivalent atoms on the two molecules being studied and by using a least-squares procedure to move one of the structures to ensure optimum overlap of the nominated atoms. The choice of topographically equivalent atoms for the superimpositions was usually clear, with hydroxyl groups of inhibitors and cation being superimposed, as were the pyranose ring oxygen atom and the heterocyclic nitrogen atoms of the inhibitors. In most cases the inhibitors studied were relatively rigid, cyclic compounds and superimpositions were performed with rigid geometries. Relaxation of geometries during the superimpositions would be expected to improve the quality of the fit, but the extra computational effort was not justified.

Where the molecules being superimposed exhibited conformational flexibility in a side chain, an energy-weighted superimposition was carried out allowing relaxation of the flexible torsion angles in the side chain. In this procedure the molecular geometry used for superimposition was a compromise between the lowest energy conformations and those most consistent with good overlap between equivalent functional groups on the two molecules. In all cases this resulted in a geometry which overlapped well with the template but which still adopted an acceptably low energy (<40 kJ/mol above the global minimum) conformation. In effect, the superimposition was weighted to account for the conformational energy of the flexible groups.

Note Added in Proof. One of the referees noted that there was no published work showing that mannosidase inhibitors exhibited activity against HIV. However, Montefiori et al. have recently shown that deoxymannojirimycin (11) dramatically attenuates the infectivity of HIV-1 in micromolar concentrations, although bromoconduritol (a glucosidase inhibitor) and swainsonine were inactive in vivo. Montefiori, D. C.; Robinson, W. E.; Mitchell, W. M. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 9248–52.

Registry No. 1, 27710-20-9; 2, 19130-96-2; 3, 79831-76-8; 4, 72741-87-8; 5, 111467-60-8; 6, 96648-51-0; 7, 107982-28-5; 8, 95189-02-9; 9, 121421-09-8; 10, 100937-52-8; 11, 84444-90-6; 12, 53185-12-9; 13, 107244-34-8; 14, 121348-72-9; α-D-mannosidase, 9025-42-7.

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New Anticancer Agents: Chiral Isomers of Ethyl 5-Amino-1,2-dihydro-2-methyl-3-phenylpyrido[3,4-*b*]pyrazine-7-carbamate

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Racemic ethyl 5-amino-1,2-dihydro-2-methyl-3-phenylpyrido[3,4-*b*]pyrazine-7-carbamate (**1a**) has shown antitumor activity in a variety of in vivo experiments. The preparation of the *R* (**9**) and *S* (**10**) isomers gave compounds with significant differences in potency in several biological tests.

A series of 1,2-dihydropyrido[3,4-*b*]pyrazine-7-carbamates (e.g., **1a,b**)[1] have shown activity against P388 leukemia in mice.[2,3] In addition, **1a** has demonstrated activity against L1210 leukemia, M5076 ovarian sarcoma, mouse

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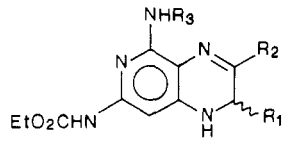

1a, $R_1 = CH_3$, $R_2 = C_6H_5$, $R_3 = H$
b, $R_1 = H$, $R_2 = C_6H_5N(CH_3)CH_2$, $R_3 = H$
c, $R_1 = CH_3$, $R_2 = C_6H_5$, $R_3 = (R)$-(1-$C_{10}H_7$)$CH(CH_3)NHCO$
d, $R_1 = R_3 = H$, $R_2 = C_6H_5$
e, $R_1 = CH_3$, $R_2 = 4$-HOC_6H_4, $R_3 = H$

colon tumor 36, and the vincristine-, adriamycin-, and melphalan-resistant P388 leukemias.[4] This compound is a potent inhibitor of the proliferation of cultured L1210 cells and the polymerization of tubulin to microtubules. Combinations of **1a** with [^{3}H]colchicine gave a decrease in the binding of the latter to tubulin. In contrast, the binding of [^{3}H]vincristine to tubulin was increased in the presence of **1a**. These results suggest that **1a** binds to tubulin at or near the colchicine site and that this binding enhances the binding of vincristine at another site. The latter is consistent with the synergistic activity of combinations of **1a** and vincristine against P388 leukemia in mice.

The above biological studies were performed with racemic **1a**. Prior to preclinical development, it was desirable to prepare and evaluate the 2*R* (**9**) and 2*S* (**10**) isomers.

Chemistry. The target compounds (**9** and **10**) were prepared from readily available chiral reagents by modification of procedures previously reported (Scheme I).[1] Amination of **2** with [*S*-(*R**,*S**)]-(+)-norephedrine (**3**) in refluxing ethanol in the presence of triethylamine gave the 4-[(2-hydroxy-1-methyl-2-phenylethyl)amino]pyridine **5**. The alcohol group of the latter was oxidized with the chromium trioxide–pyridine complex in methylene chloride to give the 4-[(1-methyl-2-oxo-2-phenylethyl)amino]-pyridine **7**, which on reductive cyclization in acetic acid in the presence of Raney nickel provided **9**. Similarly, reaction of **2** with [*R*-(*R**,*S**)]-(−)-norephedrine (**4**) gave **6**, which was oxidized to give **8**, and the latter reductively cyclized to give **10**. To ensure that homogeneous samples were prepared, all intermediates and final products were purified by flash chromatography on silica gel. The equal and opposite optical activity of each pair of intermediates (**5** and **6**, **7** and **8**) and the final products (**9** and **10**) provided support for the preparation of enantiomeric pure samples (Table I).

The addition of (*R*)-(−)-1-(1-naphthyl)ethyl isocyanate to the 5-amino group of **1a** produced a mixture of diastereomers (**1c**), which were not separated by reverse-phase HPLC. Although the ^{1}H NMR spectrum in $CDCl_3$ showed a number of overlapping peaks, the pyridine ring protons of **1c** were resolved and indicated a 1:1 mixture of diastereomers. Examination of the spectrum of the [*S*-(*R**,*S**)] isomer **11**, prepared from **10**, indicated that a trace amount of the [*R*-(*R**,*S**)] isomer might be present. Computer-assisted determination of the areas for the 8-CH of this product before and after addition of **1c** (5%) indicated that the enantiomeric purity of **11** and therefore **10** was greater than 99%. The possible contamination of **10** with **9** can probably be attributed to the presence of a trace amount of **3** in **4** rather than to slight racemization during the synthesis.

Biological Evaluation. Preliminary results for **1a**,[1] **1d**,[1] **9**, and **10** against P388 leukemia in mice show significant antitumor activity over a wide dose range (0.5–30 mg/kg) (Table II).[5] Also, differences in potency were observed in the assays for determination of the concentrations required to inhibit proliferation and to cause mitotic arrest, both to 50%.[2] The values listed are considered to be a measure of the tightness of binding of agent to tubulin.[3] A comparison of $MI_{0.5}$ values reveals that **9** does not contribute to the activity observed for the racemic mixture (**1a**) and that the greater activity of **1a** over **1d** might be attributed to an increase in the basicity of the ring 1-NH group. The lower activity of **9** relative to **10**, however, must be accounted for by the orientation of the methyl group, which might affect the binding of agent to tubulin. In addition, the orientation of the methyl group might influence transport or metabolism. The latter surmise is supported by a preliminary experiment, which suggested that both **9** and **10** inhibit the polymerization of partially purified pig brain tubulin at comparable concentrations.[6] Since earlier studies in mice showed that **1a** was hydroxylated, probably to give **1e**, the synthesis and evaluation of the *R* and *S* isomers of **1e** is currently being investigated. These studies might provide additional information on the mechanism of action of these agents.

Experimental Section

Melting and decomposition temperatures were determined in capillary tubes in a Mel-Temp apparatus. The ^{1}H NMR spectra were determined on DMSO-d_6 solutions with a Nicolet NT300NB spectrometer with tetramethylsilane as internal standard. Mass spectra were taken with a Varian Mat 311A spectrometer operating in the fast-atom-bombardment mode to provide the $(M + 1)^+$ molecular ion. The progress of reactions was followed by thin-layer chromatography (TLC) on plates of silica gel from Analtech, Inc. HPLC chromatograms were developed with mixtures of acetonitrile and 0.1 M acetate buffer (pH 3.6) on an ALC-242 liquid chromatograph equipped with an UV detector (254 nm), an M-6000 pump, and a μC_{18} Bondapak ODS column (10 μm). Flash chromatography was performed with silica gel 60 (230–400 mesh) from E. Merck. Raney nickel no. 2800 was obtained from Davison Specialty Chemical Co. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within 0.4% of the theoretical value.

Ethyl [*R*-(*R,*S**)]-6-Amino-4-[(2-hydroxy-1-methyl-2-phenylethyl)amino]-5-nitropyridine-2-carbamate (5).** A solution of **2** (8.96 g, 34.4 mmol), [*S*-(*R**,*S**)]-(+)-norephedrine (**3**) (6.49 g, 42.9 mmol), and Et_3N (6.0 mL, 4.4 g, 43 mmol) in EtOH (110 mL) was refluxed for 24 h and evaporated in vacuo, and the resulting foam was triturated with H_2O (200 mL). The residue was collected by filtration, washed with additional H_2O (200 mL), dried in vacuo (P_2O_5), and purified by flash chromatography (270 g, $CHCl_3$) to give a yellow foam: yield, 12.5 g (87%); mp 270–80 °C dec with softening from 75 °C and foaming from 100 °C; TLC (98:2 $CHCl_3$–MeOH) R_f 0.25.

Similarly, **6** was prepared from **2** (15.2 g, 58.2 mmol) and [*R*-(*R**,*S**)]-(−)-norephedrine (**4**) (9.68 g, 64.0 mmol): yield, 17.9

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Scheme I

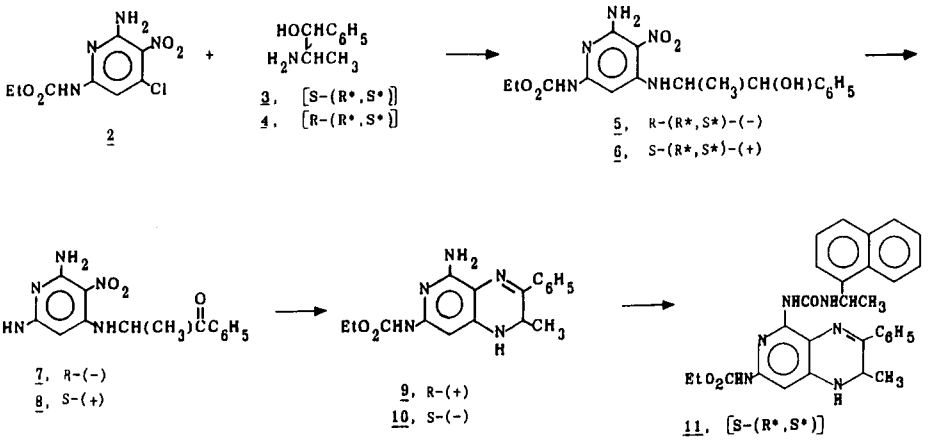

Table I. Properties of Compounds 5–10

compd	$[\alpha]^{25}_D$,[a] deg	ultraviolet spectra,[b] pH 7, λ_{max} ($\epsilon \times 10^{-3}$)	FAB mass spectra $(M + 1)^+$	^{1}H NMR spectra,[c] selected peaks, δ	formula	anal.
5	−172 ± 1.7 (c = 1, MeOH)	220 (30.6), 258 (16.2), 353 (16.8)	376	3.75 (m, 2′-CH), 4.89 (t, 1′-CH), 6.82 (3-CH)	$C_{17}H_{21}N_5O_5 \cdot 0.3CHCl_3 \cdot 0.3H_2O$	C, H, N
6	+169 ± 1.7 (c = 1.2, MeOH)	220 (31.9), 258 (16.8), 353 (17.4)	376	3.76 (m, 2′-CH), 4.89 (br s, 1′-CH), 6.82 (3-CH)	$C_{17}H_{21}N_5O_5 \cdot 0.4CHCl_3$	C, H, N
7	−2.6 ± 0.1 (c = 1, dioxane)	216 (26.3), 234 (21.3), 251 (24.1), 351 (16.3)	374	5.44 (m, 2′-CH), 6.76 (3-CH)	$C_{17}H_{19}N_5O_5 \cdot 0.3CHCl_3 \cdot 0.5H_2O$	C, H, N
8	+2.5 ± 0.1 (c = 1.3, dioxane)	216 (26.3), 234 (21.3), 251 (24.1), 352 (16.2)	374	5.43 (m, 2′-CH), 6.74 (3-CH)	$C_{17}H_{19}N_5O_5 \cdot 0.3CHCl_3 \cdot 0.3H_2O$	C, H, N
9	+675 ± 14 (c = 0.5, MeOH)	238 (34.0), 374 (15.4)	326	1.09 (d, 2-CH_3, J = 6.0 Hz), 4.81 (m, 2′-CH), 6.55 (8-CH)	$C_{17}H_{19}N_5O_5 \cdot 0.3H_2O$	C, H, N
10	−688 ± 18 (c = 0.5, MeOH)	238 (33.6), 374 (15.1)	326	1.09 (d, 2-CH_3, J = 6.0 Hz), 4.81 (m, 2′-CH), 6.55 (8-CH)	$C_{17}H_{19}N_5O_2 \cdot 0.3CHCl_3$	C, H, N

[a] Sample weights were corrected to correspond to anhydrous material. [b] Sample dissolved in MeOH followed by dilution with pH 7 phosphate buffer. [c] Peaks for H_2O and $CHCl_3$ were observed at δ 3.2 and 8.3, respectively.

Table II. Biological Activities

compd	L1210 IC_{50}, nM[a]	L1210 $MI_{0.5}$, nM[b]	P388,[c] 10^6 tumor cell implant, ip, qd 1–5 dose, mg/kg	% ILS[d]
1a[e]	0.200	0.580	1	71
1d	4.70	2.80	2	51[f]
9	17.0[g]	27.0[g]	30	88
10	0.094[g]	0.140[g]	0.5	64

[a] Nanomolar concentration of agent that inihibits proliferation of cultured lymphoid leukemia L1210 cells to 50% control growth during 48 h. [b] Nanomolar concentration of agent that causes a mitotic index (fraction of cells in mitosis divided by total cells) of 0.5 for cultured lymphoid leukemia L1210 cells during an exposure period of 12 h. [c] Lymphocytic leukemia P388. [d] Increase in life span at the highest nontoxic dose tested. [e] Racemic mixture of 9 and 10. [f] Schedule: qd 1–9. [g] Values are from multiple determinations.

g (73%); mp 270–5 °C dec with softening from 75 °C and foaming from 95 °C; TLC (97:3 $CHCl_3$–MeOH) R_f 0.35.

Ethyl (*R*)-6-Amino-4-[(1-methyl-2-oxo-2-phenylethyl)amino]-5-nitropyridine-2-carbamate (7). Chromium trioxide (6.4 g, 64 mmol) was added to a vigorously stirred solution of pyridine (10.3 mL, 10.1 g, 128 mmol) in dry dichloromethane (265 mL), the mixture was stirred for 0.5 h, and the resulting nearly clear dark-red solution was treated with a solution of **5** (4.0 g, 9.6 mmol) in dry CH_2Cl_2 (210 mL). After stirring for 1 h, the cloudy supernate was filtered (Celite), the remaining gummy residue was extracted with CH_2Cl_2 (2 × 100 mL), and the combined filtrate and extracts were evaporated in vacuo to give a brown semisolid. After evaporating toluene (50 mL) in vacuo from the semisolid, the residue was dried in vacuo (P_2O_5) for 1 h and extracted with $CHCl_3$ (100 mL). After filtration, the clear brownish filtrate was concentrated (20 mL) in vacuo, applied to a flash column (250 g, $CHCl_3$), and eluted to give **7** as a yellow foam: yield, 3.1 g (76%); mp 150–5 °C with softening and foaming from 100 °C; TLC (97:3 $CHCl_3$–MeOH) R_f 0.58.

Similarly, **6** (4.00 g, 9.45 mmol) was converted to **8**: yield, 2.06 g (53%); mp 150–5 °C with softening from 85 °C and foaming from 100 °C; TLC (97:3 $CHCl_3$–MeOH) R_f 0.58.

Ethyl (*R*)-5-Amino-1,2-dihydro-2-methyl-3-phenylpyrido[3,4-*b*]pyrazine-7-carbamate (9). A solution of **7** (1.50 g, 3.59 mmol) in glacial HOAc (75 mL) was stirred under 1 atm of H_2 in the presence of Raney nickel (4.5 g weighed wet, washed 4 × H_2O and 1 × HOAc) for 3.5 h. Although the uptake of H_2 was 90% of the theoretical amount, TLC of the reaction supernate indicated the absence of **7**. After the catalyst was removed by filtration through Celite, the clear green filtrate was evaporated in vacuo to give a semisolid. A solution of this residue in H_2O (50 mL) was neutralized to pH 7 with 1 N NaOH to deposit an oily semisolid, which was extracted into $CHCl_3$ (3 × 100 mL). After each extraction, the pH was readjusted to 7–8. The organic layers were combined, dried over Na_2SO_4, evaporated in vacuo, and purified by flash chromatography (100 g, $CHCl_3$–MeOH, 99:1). The product was precipitated from EtOH with H_2O and dried in vacuo (P_2O_5) to give **9** as a fluffy, yellow solid: yield, 0.73 g (62%); mp >155 °C with gradual decomposition; TLC (1:1 cyclohexane–ethyl acetate) R_f 0.38.

Similarly, **8** (0.937 g, 2.26 mmol) was reductively cyclized to give **10**: yield, 0.336 g (41%); mp >155 °C with gradual decomposition; TLC (1:1 cyclohexane–ethyl acetate) R_f 0.38.

Ethyl [*S*-(*R,*S**)]-1,2-Dihydro-2-methyl-5-[[[[1-(1-naphthyl)ethyl]amino]carbonyl]amino]-3-phenylpyrido[3,4-*b*]pyrazine-7-carbamate (11).** A solution of **10** (25.0 mg, 0.069 mmol) in anhydrous, deoxygenated (N_2) toluene (20 mL) containing (*R*)-1-(1-naphthyl)ethyl isocyanate (25 μL, 28 mg, 0.14 mmol) was heated under N_2 in a stoppered flask at 95° for 5 h, followed by evaporation of the solvent in a stream of N_2. The residue was dissolved in EtOH, the solution was evaporated in vacuo, and the resulting semisolid was purified by chromatography (5 g, $CHCl_3$): yield, 29.9 mg (71.5%); mp, gradual decomposition above 220 °C; ^{1}H NMR ($CDCl_3$) δ 1.23 (d, 2-CH_3, J = 6.0 Hz), 1.33 (t, CH_3CH_2O), 1.80 (d, CH_3CHN), 4.19 (q, CH_2O), 4.60 (d, 1-NH), 4.81 (dq, 2-CH), 5.83 (quin, CH_3CHN), 6.45 (5-NH), 6.78 (8-CH), 7.88 (m, aromatic CH), 8.16 (7-NH), 9.71 (d, $NHCHCH_3$).

Peaks at δ 7.25 and 1.63 were attributed to $CHCl_3$ and H_2O, respectively. Computer-assisted determination of the areas of the 8-CH peak and an adjacent upfield small peak before and after the addition of 5% by weight of a 1:1 mixture of diastereomers (see below) indicated that the enantiomerical purity of the [*S*-(*R**,*S**)] isomer was greater than 99%. Anal. ($C_{30}H_{30}N_6O_3 \cdot 0.5CHCl_3 \cdot 1.2H_2O$) C, H, N.

The diastereomers (**1c**) were prepared as described above from **1a** (12.5 mg, 0.038 mmol) and (*R*)-1-(1-naphthyl)ethyl isocyanate (12.5 μL, 14.0 mg, 0.071 mmol): yield, 16.2 mg (76%); mp, gradual decomposition above 220 °C; ^1H NMR ($CDCl_3$) δ 1.25 (q, 2-CH_3), 1.33 (t, CH_3CH_2O), 1.80 (d, CH_3CHN), 4.19 (m, CH_2O), 4.56 (br s, 1-NH), 4.82 (m, 2-CH), 5.82 (quin, CH_3CHN), 6.39 (d, 5-NH), 6.77 (d, 8-CH), 7.87 (m, aromatic CH), 8.15 (7-NH), 9.70 (t, $NHCHCH_3$). Both the 5-NH and 8-CH were observed as two resolved peaks and the latter indicated a 1:1 mixture of diastereomers. Anal. ($C_{30}H_{30}N_6O_3 \cdot 0.27CHCl_3 \cdot 0.4H_2O$) C, H, N.

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Effect of Triphenylacrylonitrile Derivatives on Estradiol-Receptor Binding and on Human Breast Cancer Cell Growth

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In a study of a series of 26 triphenylacrylonitrile derivatives (TPEs), we investigated the influence of several possibly interrelated factors on the proliferation of human breast cancer cell lines. (1) *Chemical substituents*: the test compounds were for the most part para-hydroxylated with increasingly bulky hydrophobic and/or basic side chains [isopropyloxy or (diethylamino)ethoxy] or standard reference compounds. (2) *Relative binding affinities (RBAs)*: they competed diversely for [^3H]estradiol (E_2) binding to calf uterus cytosol and little, if at all, for binding to the [^3H]tamoxifen-labeled antiestrogen binding site (AEBS) in lower speed supernatant. A multiparametric comparison of RBAs recorded for calf, rat, and mouse uterus cytosol estrogen receptor (ER) revealed a possible influence of species-specific receptor conformation and/or environment on binding. (3) *Estrogen/antiestrogen potency*: their stimulation and inhibition of the proliferation of the ER-positive human breast cancer cell line (MCF_7) was measured. Compounds with only hydroxy substituents stimulated proliferation more markedly than methylated derivatives and had a maximum effect at 10^{-11}–10^{-6} M. Stimulation was related to the RBA for ER. Compounds with isopropyloxy or (diethylamino)ethoxy side chains only weakly stimulated MCF_7 cell growth and more powerfully antagonized E_2-promoted growth. The extent of inhibition depended upon the bulk of the side chain and could be reversed by 10^{-7} M E_2. Within the same concentration ranges, the test compounds were without effect on the BT_{20} ER-negative cell line. (4) *Cytostatic and/or cytolytic activity*: most compounds could arrest the proliferation of both MCF_7 and BT_{20} cells at concentrations above 3×10^{-6} M. This activity was thus independent of ER. Nevertheless, those compounds with a charged hydrophobic side chain, which were the most powerful antagonists of E_2-promoted cell growth, were also the most cytotoxic. The overall results for all molecules on all parameters were submitted to a multivariate analysis (correspondence analysis) which revealed the progressive influence of increasing substitution by hydroxy and more bulky groups on the generation of antagonist activity and cytotoxicity.

The growth of benign and neoplastic breast tumor tissue is under the influence of several hormones (insulin, hcG, prolactin, steroid hormones) and of different growth factors such as epidermal, transforming, and insulin growth factors (for a review, see ref 1). Receptors for these hormones and factors have been identified in a much investigated cell line MCF_7 derived from a human breast cancer metastasis. Several studies have suggested that the antiproliferative action of triphenylethylene derivatives (TPEs) on the growth of estrogen-dependent tumors might be mediated by the estrogen receptor (ER).[2-6] The observations that have led to this hypothesis are (1) these TPEs can have considerable affinity for ER apart from actions on other molecular targets,[5,7-14] (2) the compounds with the highest affinity for ER are growth inhibitory at the lowest concentrations,[2,13,15-18] (3) this inhibition is abolished in the presence of estradiol,[5,13,17,19-21] and (4) ER-negative cells are less sensitive to TPEs.[6,11,20,22-24]

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