Synthesis and Biological Studies of Iodinated ($^{127/125}I$) Derivatives of Rhodamine 123

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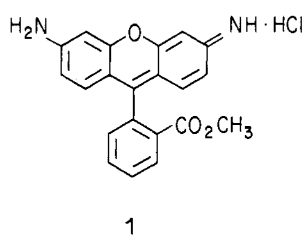
Rhodamine 123, a mitochondrial stain that preferentially accumulates in certain cancer cells, has been reduced and iodinated by using NaI in the presence of *N*-chlorosuccinimide. The various mono-, di-, and triiodo derivatives have been isolated and characterized. These nonfluorescent compounds are taken up by mammalian cells, become fluorescent within the cytoplasm (presumably following oxidation), and show the same pattern of localization as the parent compound. Iodination with no-carrier-added $Na^{125}I$ yields the same mixture of compounds. All ^{125}I derivatives accumulate preferentially in PC3 adenocarcinoma cells compared with V79 lung fibroblasts, with the differential being greatest for the monoiodo compound, followed by the di- and triiodo derivatives.

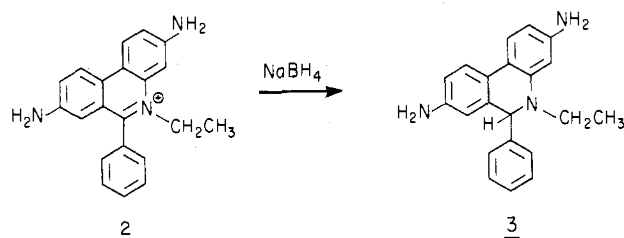
Rhodamine 123 (1), a permeant cationic dye,[1] has been used as a mitochondrial stain of viable mammalian cells[2] and as a probe for the study of mitochondrial function[3-6] and membrane potential.[7,8] The accumulation of this



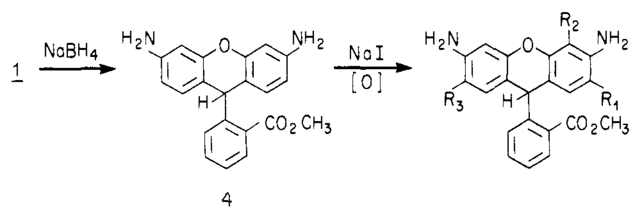
compound is greater in certain types of cancer cells than in normal cells (10–20-fold),[9] and the compound exhibits selective toxicity in certain cancer cells in vitro[10-12] and in vivo.[13] It has been postulated that the increased accumulation by tumor cells may be due to the elevated mitochondrial and plasma membrane potentials in these cells.[14]

Rhodamine 123 covalently labeled with a radioactive isotope could serve as a tumor imaging agent, as cancer cells would take up more activity than the surrounding tissues. By the same token, iodinated rhodamine 123 might function as a cancer radio- or chemotherapeutic agent. Radiolabeling of rhodamine 123 with ^{125}I and ^{131}I using the chloramine-T and Iodogen methods, respectively,[15,16] has been reported. Our attempts to prepare an iodinated derivative of rhodamine 123 by using a variety of iodinating agents such as chloramine-T, Iodogen, and peracetic acid yielded only the starting material as determined by NMR. This was not surprising since electrophilic iodination would be greatly inhibited by the presence of the delocalized positive charge on the xanthene ring system.

Scheme I



Scheme II



5 $R_1 = I$, $R_2 = R_3 = H$
6 $R_2 = I$, $R_1 = R_3 = H$
7 $R_1 = R_3 = I$, $R_2 = H$
8 $R_2 = R_3 = I$, $R_1 = H$
9 $R_1 = R_2 = R_3 = I$

According to a recent report,[17] $NaBH_4$ reduction of ethidium bromide (2), a red fluorescent nuclear stain that is not taken up by live cells, gives a blue fluorescent dihydro derivative (3, Scheme I), which is readily taken up but does not stain the nucleus. Inside the cell, 3 is converted back to 2 as indicated by the appearance of red fluorescence in the nucleus. We reasoned that $NaBH_4$ reduction of 1 would yield a compound that would be susceptible to iodination and that, by analogy with 3, would be taken up by cells and converted by cytoplasmic enzymes into cationic iodorhodamine 123. In this paper we report

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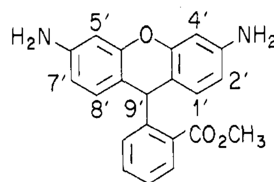
Table I. NMR Spectra and R_f Values for Dihydrorhodamine 123 and Its Iodo Derivatives



no.	position of iodine	R_f a	R_f b	NMR:[c] δ, J 1'	2'	4'	5'	7'	8'	9'
4		0.14	0.00	6.70 (d, J = 8 Hz)	6.15 (d of d, J = 2, 8 Hz)	6.30 (d, J = 2 Hz)	6.30 (d, J = 2 Hz)	6.15 (d of d, J = 2, 8 Hz)	6.70 (d, J = 8 Hz)	5.93 (s)
5	2'	0.35	0.10	7.15 (s)		6.38 (s)	6.28 (d, J = 3 Hz)	6.15 (d of d, J = 3, 8 Hz)	6.67 (d, J = 8 Hz)	5.93 (s)
6	4'	0.60	0.19	6.78 (d, J = 8 Hz)	6.35 (d, J = 8 Hz)		6.56 (d, J = 3 Hz)	6.30 (d of d, J = 3, 8 Hz)	6.67 (d, J = 8 Hz)	6.08 (s)
7	2',7'	0.72	0.27	7.22 (s)		6.43 (s)	6.43 (s)		7.22 (s)	6.05 (s)
8	4',7'	0.87	0.43	6.73 (d, J = 8 Hz)	6.32 (d, J = 8 Hz)		6.62 (s)		7.22 (s)	6.08 (s)
9	2',4',7'	0.99	0.58	7.22 (s)			6.58 (s)		7.22 (s)	6.10 (s)

[a] EtOAc/CH_2Cl_2 (5:95). [b] EtOAc/hexane (1:3). [c] $CHCl_3$, Me_4Si standard.

the synthesis and characterization of dihydrorhodamine 123 and its iodinated derivatives and their uptake, oxidation, and localization in V79 cells as determined by fluorescence microscopy. We also report the preparation of radioiodinated derivatives using carrier-free $Na^{125}I$ and the uptake of these compounds by Chinese hamster V79 lung fibroblasts and by human prostate PC3 adenocarcinoma cells.

Chemistry. Reduction of 1 with $NaBH_4$ was accomplished smoothly, giving a 90% yield of dihydrorhodamine 123 (4) after flash column chromatography (Scheme II). The structure was confirmed by NMR (Table I). This compound was sensitive to oxidation, the white solid slowly turning pink unless stored at −30 °C in the dark. Deliberate attempts to chemically oxidize 4 back to 1 by using a variety of oxidizing agents were unsuccessful and led to non-water-soluble materials. Since the aim of this work was to synthesize no-carrier-added ^{125}I derivatives, the mildest possible conditions that were also compatible with radioiodination techniques were sought, as oxidation and iodination would be competing reactions. While all the methods investigated (chloramine-T/NaI, Iodogen/NaI, Iodobeads/NaI, peracetic acid/NaI, ICl, and I_2) gave some iodinated products, the yields were low. The best method proved to be *N*-chlorosuccinimide (NClS)/NaI in CCl_4[18] (Scheme II). If the reaction was carried out with 1 equiv of NaI, the main product was the 2′-iodo derivative **5** (44% yield). Small amounts of the 4′-iodo compound **6** (~5%) and the symmetrical diiodo derivative **7** (9%) were also isolated. On the other hand, when the reaction was carried out in the absence of NaI, only starting material was observed on TLC after workup, indicating that no chlorinated byproducts were formed (the intense red color of rhodamine 123 derivatives which developed as the TLC plates stood in air enabled the visualization of very small amounts of material). When 4 equiv of NaI was used, yields of 19% **5**, 11% **6**, 17% **7**, and 12% of the unsymmetrical diiodide **8** were realized. Reverse addition of 4 to an NClS/NaI mixture resulted in yields of 25% **7**, 23% **8**, and 7% for the triiodo derivative **9**. These yields were not realized unless a $NaBH_4$ re-reduction step was included in the workup procedure. This step could be included since no reductive deiodination of the purified iodo derivatives occurred following treatment with $NaBH_4$.

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All of the iodinated derivatives were identified by their distinctive NMR spectra (Table I). None of the dihydrorhodamine derivatives were fluorescent. The dihydrorhodamine molecule seemed to become increasingly sensitive to oxidation with the addition of more iodines, and while some suggestions of a tetraiodo derivative were seen on TLCs of the reaction mixtures, it was not possible to isolate such a moiety. Attempts to oxidize the iodinated compounds and to isolate the iodinated derivatives of 1 were again unsuccessful. Radioiodination was carried out with 14.2 mCi of carrier-free $Na^{125}I$, with a 1:1:0.002 mole ratio of 4/NClS/$Na^{125}I$. After flash chromatography was performed twice, the recovery of radioactivity was 8.45 mCi, of which 4.22 mCi was **5** with the rest distributed among **6–9**. The total radiochemical yield was 60%. The radioiodinated compounds were identified by autoradiography of thin-layer chromatograms, where the radioactive samples were both spotted alone and overspotted with authentic nonradioactive samples.

Thin-layer chromatography (TLC) of the reaction mixture from various attempts at iodination of 1 without prior reduction showed the presence of an entity that had a different R_f from that of the starting material. When this material was reduced, no trace of any iodinated products could be seen by NMR. TLC of the reduced material, perhaps a more sensitive indicator than NMR due to the intense red color that develops as the compounds oxidize on the plate, likewise showed no iodinated products. We also discovered that when 1 is simply mixed with NaI in the absence of an oxidizing agent, a red precipitate is formed, which has the same R_f as the entity mentioned above, probably representing the formation of the hydroiodide of 1.

Biological Results and Discussion

When V79 cells were incubated with a 10 μM solution of dihydrorhodamine 123 for 2 h, the cell cytoplasm became fluorescent (Figure 1B), suggesting that this nonfluorescent substance is being oxidized to the highly fluorescent 1 (Figure 1A). The fluorescence was localized in the mitochondria, and the staining pattern for 4 was exactly the same as that for 1. The same behavior was observed for the iodinated compounds **5–9** (Figure 1C–G), demonstrating that the presence of one or more iodine atoms on the molecule did not affect its ability to localize in the cell cytoplasm. Similar results were obtained with the PC3 cells. No staining of the nucleus was observed for any of the derivatives, even though tetrabromo-

Table II. Uptake of [^{125}I]Dihydrorhodamine Derivatives by Chinese Hamster V79 Lung Fibroblasts and Human Prostate PC3 Adenocarcinoma Cells[a]

cells	no.	location of ^{125}I	extracellular concn, μCi/mL	cellular uptake[b] pCi/cell	cellular uptake[b] μCi/mL	pCi/cell per μCi/mL	intra- to extracellular ratio
V79	**5**	2′	0.956	0.0355	62.1	0.0371	65
V79	**6**	4′	1.272	0.0227	39.7	0.0178	31
V79	**7**	2′,7′	0.970	0.121	212	0.125	218
V79	**8**	4′,7′	1.643	0.114	200	0.0694	122
V79	**9**	2′,4′,7′	1.300	0.198	347	0.152	267
PC3	**5**	2′	0.937	0.292	1440	0.312	1540
PC3	**6**	4′	1.196	0.199	982	0.166	821
PC3	**7**	2′,7′	0.883	0.699	3450	0.792	3910
PC3	**8**	4′,7′	1.350	0.555	2740	0.411	2030
PC3	**9**	2′,4′,7′	1.151	0.401	1980	0.348	1720

[a] 2 h, 37 °C. [b] V79 cells have a volume of 5.72×10^{-10} cm^3/cell; PC3 cells have a volume of 1.61×10^{-9} cm^3/cell.

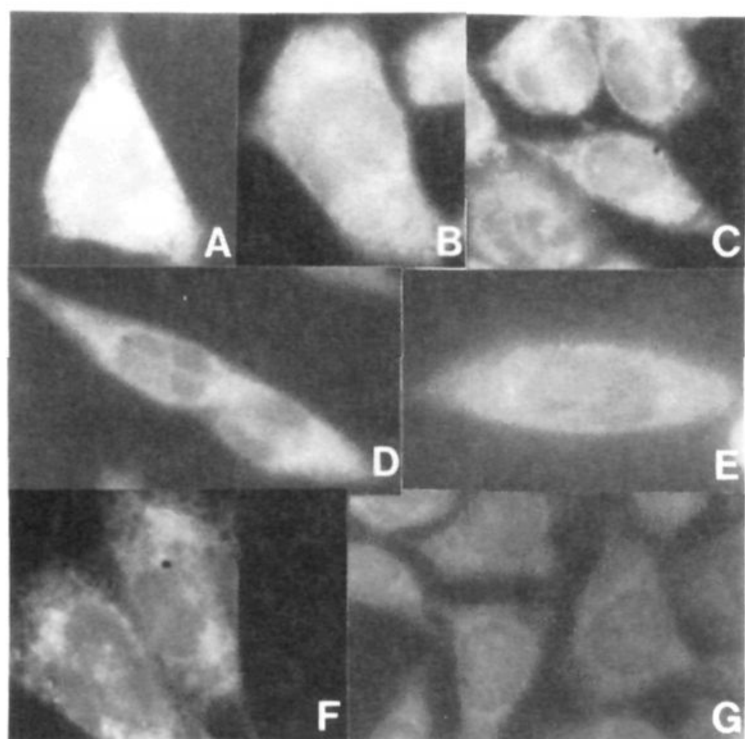

Figure 1. Fluorescence microscopy of various rhodamine derivatives following a 2-h incubation with each compound: A, rhodamine-123 (R); B, dihydrorhodamine (DR); C, 2′-iodo-DR; D, 4′-iodo-DR; E, 2′,7′-diiodo-DR; F, 4′,7′-diiodo-DR; G, 2′,4′,7′-triiodo-DR.

rhodamine 123 has been reported to intercalate with DNA.[19] In fact, we observed no changes in the visible and fluorescence spectra of **1** when it was incubated with calf thymus DNA,[20] indicating that **1** does not interact/intercalate with DNA.[21,22]

The uptake of the ^{125}I-labeled compounds in V79 and PC3 cells is presented in Table II. The last column shows the ratio of intra- to extracellular ^{125}I concentration. In both V79 and PC3 cells, when the iodine is at the 2′-position on the molecule, the uptake is about twice that of the compound with the iodine located at the 4′-position. The same phenomenon is observed when the 2′,7′- and the 4′,7′-diiodo derivatives are compared. If the R_f on TLC represents the relative polarities of the compounds, differences in lipophilicity alone cannot explain these differences in uptake as changing the iodine from the 2′- to the 4′-position increases the R_f. It appears, therefore, that binding between the rhodamine derivatives and cellular components depends to some extent on attractions involving the ring oxygen and the amino groups acting together. The presence of a bulky iodine atom would interfere sterically with this interaction.

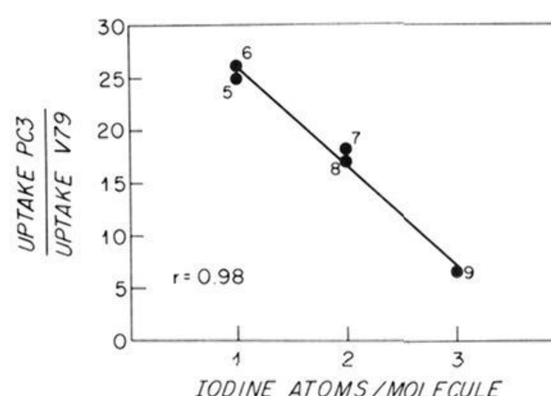

Figure 2. The relationship between the ratio of cellular uptake of PC3 carcinoma cells to V79 fibroblasts and the number of iodine atoms/molecule of dihydrorhodamine 123. The numbers refer to the structures in Table I.

The differential PC3–V79 cellular uptake of the radioiodinated dihydrorhodamine derivatives can also be seen clearly in Table II. When the intra- to extracellular concentration ratios are compared, the PC3 cells take up ~25 times as much of the monoiodo derivatives, ~17 times as much of the diiodo derivatives, and ~6 times as much of the triiodo. This assumes that the cellular uptake is affected similarly by the extracellular molar concentrations in the two cell lines, as the molar concentration of the diiodo and triiodo derivatives used in these experiments is lower than the monoiodo by factors of 2 and 3, respectively. In a study by Davis et al.,[14] the accumulation of a cationic mitochondrial probe, [^{3}H]triphenylphosphonium chloride, was 15 times greater in human breast adenocarcinoma cells than in green monkey kidney epithelium cells. Our results indicate that when the uptake is compared on a per cell basis (accounting for cell size differences),[23] there is about a 9-fold difference in uptake of the monoiodo compounds, 6-fold for the diiodo, and 2-fold for the triiodo. In fact, if the uptake ratio is plotted against the number of iodines/molecule of dihydrorhodamine (Figure 2), a linear relationship is seen: the greater the number of iodines, the less the difference in uptake. Extrapolation of the data suggests that there would be no difference in uptake whatsoever at four iodines/molecule. This difference in uptake may be related to lipophilicity: the less polar the compound, the less the difference in the uptake between normal and cancer cells.

It must be noted further that for the radioiodinated compounds, the extracellular molar concentration was probably considerably below that needed to saturate the cells. *Preliminary results*[24] *suggest that the uptake of*

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[^{125}I]-**5** in V79 cells is directly proportional to extracellular radioactive concentration, increasing sharply during the first 16 h and reaching a plateau by 18 h. At the highest concentration examined thus far, the intracellular content was ~7 pCi/cell.

Various mono-, di-, and triiodo derivatives of dihydrorhodamine have been isolated and characterized. All the derivatives accumulate preferentially in PC3 adenocarcinoma cells compared with V79 lung fibroblasts. The intra- to extracellular ratios, shown in Table II, indicate that **7** is concentrated best by the PC3 tumor cell line. However, tumor to nontumor ratio is the primary parameter to consider from a tumor imaging point of view. Therefore, compound **5**, which both exhibits the best tumor to nontumor ratio and has a high radiochemical yield, shows the most promise as a tumor imaging agent.

Experimental Section

All flash chromatography was done according to the method of Still et al.[25] Melting points were determined on a Fisher-Johns melting point apparatus and are not corrected. Thin-layer chromatograms were run on silica gel GHLF plates (Analtech). NMR spectra were obtained on a Varian T-60 spectrometer. Analyses were carried out by Galbraith Laboratories, Knoxville, TN.

Dihydrorhodamine 123 (4). Rhodamine 123 hydrochloride (1, 100 mg, 0.26 mmol; Eastman Kodak) was dissolved in water (50 mL), and CH_2Cl_2 (50 mL) was added. Excess solid $NaBH_4$ was added in portions with vigorous magnetic stirring until almost all the color was discharged. After 1 h, the pale orange organic layer was separated and the water layer extracted twice with CH_2Cl_2. The combined organic layers were passed through anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude oily reaction mixture was purified by flash chromatography using EtOAc/CH_2Cl_2 (1:9) as eluent, yielding 80 mg (88% yield) of a slightly pinkish-orange oil. The purified oil was dissolved in a small amount of CH_2Cl_2 and precipitated with hexane. The pinkish-white solid was washed with hexane and a portion submitted for analysis: mp 163–165 °C; NMR ($CDCl_3$) δ 3.93 (s, CO_2CH_3), 5.93 (s, C-9′), 6.15 (d of d, J = 2, 8 Hz, 2 H, C-7′), 6.30 (d, J = 2 Hz, 2 H, C-4′, -5′), 6.70 (d, J = 8 Hz, 2 H, C-1′, -8′), 7.0–7.17 (m, 3 H, C-3, -4, -5), 7.65 (d of d, J = 2, 8 Hz, 1 H, C-6). Anal. ($C_{21}H_{18}N_2O_3$) C, H, N.

2′-Iododihydrorhodamine 123 (5). Dihydrorhodamine 123 (4) (43 mg, 0.12 mmol) was dissolved in CCl_4 (50 mL) and CH_2Cl_2 (1 mL) and cooled in ice under red light. A solution of NaI (17 mg) in MeOH (0.5 mL) and 0.2 M NH_4OAc (0.5 mL) were added with stirring followed by a cold solution of NClS (17 mg, 0.13 mmol) in CCl_4 (50 mL) in small portions. The reaction mixture was allowed to warm to room temperature, and stirring was continued for 1 h. Water and $NaBH_4$ were added, and the organic layer was separated and filtered through anhydrous Na_2SO_4 and the solvent evaporated under reduced pressure. The red oily product was subjected to flash chromatography using EtOAc/CH_2Cl_2 (5:95), and 26 mg of **5** (44%) along with <5 mg of **6** and 7 mg of **7** (9%) was recovered. A sample of **5** was dissolved in a minimal amount of ether, precipitated with hexane, and submitted for analysis: NMR ($CDCl_3$) δ 3.88 (s, CO_2CH_3), 5.93 (s, C-9′), 6.15 (d of d, J = 3, 8 Hz, C-7′), 6.28 (d, J = 3 Hz, C-5′), 6.38 (s, C-4′), 6.67 (d, J = 8 Hz, C-8′), 7.15 (s, C-1′), 6.97–7.20 (m, 3 H, C-3, -4, -5), 7.67 (m, C-6). Anal. ($C_{21}H_{17}IN_2O_3$) C, H, N.

2′-Iododihydrorhodamine 123 (5), 4′-Iododihydrorhodamine 123 (6), 2′,7′-Diiododihydrorhodamine 123 (7), and 4′,7′-Diiododihydrorhodamine 123 (8). A solution of 4 (86.0 mg, 0.25 mmol) in CCl_4 (100 mL) was prepared with the addition of CH_2Cl_2 (3–4 mL). A solution of NaI (150 mg, 1.0 mmol) in MeOH (5 mL) and 0.2 M NH_4OAc (5 mL) were added, and the mixture was cooled in an ice bath with stirring. A cold solution of NClS (133.5 mg, 1.0 mmol) in CCl_4 (100 mL) was added all at once to the above mixture under red light, and stirring was continued in an ice bath for 0.5 h and at room temperature for 0.5 h, after which water and a small amount of solid $NaBH_4$ were added. After vigorous shaking, the organic layer was separated and the water layer washed several times with CH_2Cl_2. The combined organic layers were filtered through anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. The residue was processed by flash chromatography on a 1.5-cm column successively eluted with 1% EtOH/CH_2Cl_2 (85 mL), 5% EtOH/CH_2Cl_2 (50 mL), and 10% EtOH/CH_2Cl_2 (40 mL); 5-mL fractions were collected. Four products (**5–8**) were isolated and characterized by NMR.

5: 23 mg (21%); identical with previously prepared **5**.

6: 13 mg (11%); NMR ($CDCl_3$) δ 3.98 (s, 3 H, CO_2CH_3), 6.08 (s, C-9′), 6.30 (d of d, J = 3, 8 Hz, C-7′), 6.35 (d, J = 8 Hz, C-2′), 6.56 (d, J = 3 Hz, C-5′), 6.78 (m, 2 H, C-1′, -8′), 6.88–7.42 (m, 3 H, C-3, -4, -5), 7.22 (m, C-6). Anal. Calcd for $C_{21}H_{17}IN_2O_3$: C, 53.41; H, 3.63; I, 26.87. Found: C, 53.31; H, 3.92; I, 26.08.

7: 26 mg (17%); NMR ($CDCl_3$) δ 4.00 (s, 3 H, CO_2CH_3), 6.05 (s, C-9′), 6.43 (s, 2 H, C-4′, -5′), 7.22 (s, 2 H, C-1′, -8′), 6.98–7.23 (m, 3 H, C-3, -4, -5), 7.75 (d of d, J = 2, 7 Hz, C-6). Anal. ($C_{21}H_{16}I_2N_2O_3$) C, H, N.

8: 18 mg (12%); NMR ($CDCl_3$) δ 3.97 (s, 3 H, CO_2CH_3), 6.08 (s, C-9′), 6.32 (d, J = 8 Hz, C-2′), 6.62 (s, C-5′), 6.73 (d, J = 8 Hz, C-1′), 7.22 (s, C-8′), 6.87–7.33 (m, 3 H, C-3, -4, -5), 7.73 (d of d, J = 3, 8 Hz, C-6). Anal. Calcd for $C_{21}H_{16}I_2N_2O_3$: C, 42.17; H, 2.70; I, 42.43. Found: C, 41.20; H, 2.90; I, 42.72.

2′,4′,7′-Triiododihydrorhodamine 123 (9). *N*-Chlorosuccinimide (133.5 mg, 1.0 mmol) was dissolved in CH_2Cl_2 (3–4 mL), and CCl_4 (100 mL) was added. The solution was stirred vigorously in an ice bath under red light, and a solution of NaI (150 mg in 5 mL of 0.2 M NH_4OAc/methanol, 1:1) was added. A solution of 4 (86 mg) in CH_2Cl_2 (3–4 mL) plus CCl_4 (100 mL) was cooled in ice and distributed in ~2-mL aliquots into the NClS/NaI mixture. After the resulting mixture had been stirred in the dark for 1 h at room temperature, water and a small amount of solid $NaBH_4$ were added. The organic layer was separated, washed with water, and filtered through anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2 (2–3 mL) and adsorbed onto a small amount of silica gel, and the CH_2Cl_2 was removed under reduced pressure. The dry silica gel/reaction mixture was applied to the top of a 1.5-cm column, and flash chromatography was performed by using successively EtOAc/CH_2Cl_2, 1:99, 10:90, and 20:80. Three products (**7–9**) were recovered.

7: 37 mg (25%).

8: 35 mg (23%).

9: 10 mg (7%); NMR ($CDCl_3$) δ 3.98 (s, 3 H, CO_2CH_3), 6.10 (s, C-9′), 6.58 (s, C-5′), 7.22 (s, 2 H, C-1′, -8′), 6.80–7.30 (m, 3 H, C-3, -4, -5), 7.75 (m, C-6). Anal. Calcd for $C_{21}H_{15}I_3N_2O_3$: C, 34.84; H, 2.09; I, 52.58. Found: C, 34.61; H, 2.30; I, 53.73.

Radioiodination of 4. Compound 4 was dissolved in two drops of CH_2Cl_2, and 2 mL of CCl_4 was added to give a 2.5 mM solution. To 1.5 mL of this solution was added the contents of two vials of carrier-free Na^{125}I (14.2 mCi, 2200 Ci/mmol, Amersham). The vials were each rinsed twice with 0.2 M NH_4OAc (10 μL) and methanol (10 μL), and the resulting mixture was stoppered and stirred in an ice bath. A solution of NClS in CCl_4 (1.5 mL, 2.5 mM) was added and the reaction mixture stoppered, covered with aluminum foil, and stirred vigorously at room temperature for 1 h. One milliliter of water and several 10-μL portions of 1 M $NaBH_4$ were added, and stirring continued until most of the red color had disappeared. The CCl_4 layer was removed with a Pasteur pipet, and the water layer was washed twice with small amounts of CH_2Cl_2. Solvent was evaporated from the combined organic layers under a stream of N_2, and 1 mL of methanol and a tiny amount of solid $NaBH_4$ were added to the colored residue. The methanol was evaporated, and the now-colorless residue was taken up in EtOAc/CH_2Cl_2 (5:95) for flash chromatography in the same solvent using a 0.8 × 12 cm disposable column (BioRad). Fractions (85 drops/fraction) that showed one spot on TLC autoradiography were combined to give 4.22 mCi of **5**, 1.36 mCi of **6**, 1.49 mCi of **7**, and 0.40 mCi of **8**. Fractions showing more than one spot were combined and rechromatographed by using EtOAc/hexane (1:3). From this chromatography were isolated 0.18 mCi of **6**, 0.53 mCi of **7**, 0.16 mCi of **8**, 0.09 mCi of **9**, and 0.02 mCi of unidentified material running above **9** on TLC. The total radiochemical yield was 8.45 mCi (60%), of which 50% was **5**, 18% **6**, 24% **7**, 7% **8**, and 1% **9**.

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Cellular Uptake of Rhodamine 123 Derivatives. Chinese hamster V79 lung fibroblasts and human prostate PC3 adenocarcinoma cells were grown as monolayers at 37 °C in minimum essential medium (MEM) supplemented with L-glutamine (2 mM), penicillin (50 units/mL), streptomycin (50 μg/mL), 1% nonessential amino acids, and 15% fetal bovine calf serum (plating efficiency 60–80%).

Cell size determinations were carried out by using a Coulter Counter (Model Z_F) in conjunction with a Coulter Channelyzer (Model 256).

For the fluorescence microscopy studies, cells were grown overnight as monolayers on glass cover slips. The rhodamine 123 derivatives were dissolved in methanol (10 mM) and diluted (1:100) in 0.1 M phosphate buffered saline, pH 7.2 (PBS). The cells were rinsed gently with PBS, incubated for 1 h at 37 °C in the presence of the rhodamine 123 derivatives, washed with PBS, observed under the fluorescence microscope (Zeiss), and photographed.

Cellular Uptake of [^{125}I]Rhodamine 123 Derivatives. Logarithmically growing cells were trypsinized and suspended in calcium-free MEM (supplemented as above), the cell number was determined and adjusted to 2×10^5/mL, and 1-mL portions were dispensed into sterile tubes. Following a 4-h incubation at 37 °C on a roller shaker (Fisher Scientific), the radioiodinated dihydrorhodamine derivatives, dissolved in the same medium, were added. The cells were reincubated at 37 °C for 2 h, and their radioactive content was determined by the microfuge method.[26]

Note Added in Proof: Since the submission of this paper, we have used the radioiodination procedure described herein with carrier-free Na^{123}I (Atomic Energy Commission of Canada) and have obtained comparable results.

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Registry No. 1, 62669-70-9; 2, 1239-45-8; 3, 38483-26-0; 4, 109244-58-8; 5, 109244-59-9; 5 (I-125 labeled), 109244-63-5; 6, 109282-63-5; 6 (I-125 labeled), 109244-64-6; 7, 109244-60-2; 7 (I-125 labeled, 109244-65-7; 8, 109244-61-3; 8 (I-125 labeled), 109244-66-8; 9, 109244-62-4; 9 (I-125 labeled), 109244-67-9.

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Synthesis, Conformational Considerations, and Estrogen Receptor Binding of Diastereoisomers and Enantiomers of 1-[4-[2-(Dimethylamino)ethoxy]phenyl]-1,2-diphenylbutane (Dihydrotamoxifen)

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As part of a study into nonisomerizable antiestrogens, the diastereoisomeric dihydrotamoxifens **7** and **8** were prepared by catalytic transfer hydrogenation of (*Z*)- and (*E*)-tamoxifen and were shown by NMR spectrometry to exist in preferred conformations with hydrogen atoms in an antiperiplanar relationship. The corresponding 4-hydroxy derivatives **9** and **10** were prepared from hydrogenated precursors of (*Z*)- and (*E*)-4-hydroxytamoxifen. The relative binding affinities (RBA) of the compounds to estrogen receptors are consistent with the assigned conformations and parallel reported data on derivatives of the nonsteroidal estrogen hexestrol. The growth-inhibitory activity against the MCF-7 human breast cancer cell line in vitro was for **10** comparable to that of 4-hydroxytamoxifen, although increasing the concentration from 10^{-8} to 10^{-6} M did not significantly improve the growth inhibition. The derivative **9** analogous to (*E*)-4-hydroxytamoxifen antagonized the growth-stimulating effect of added estradiol and is therefore also an antiestrogen but at low concentration (10^{-8} M) in the absence of estradiol, MCF-7 cell growth was stimulated, indicating an estrogenic influence. The enantiomers of the dihydrotamoxifen **8** were individually prepared from the resolved enantiomers of 2-phenylbutanoic acid, the key reaction step being a lithium–ammonia reduction of the 1-(4-methoxyphenyl)-1,2-diphenyl-1-butanol to generate the triphenylbutane. The enantiomers of **8** gave identical RBA values in cytosol.

Appropriately substituted triarylethylene derivatives have antiestrogenic activity.[1] Of these, tamoxifen (**1**) is currently in clinical use for the treatment of estrogen-dependent breast cancer.[2] Its principal action is its competition with the natural hormone estradiol for binding to the estrogen receptor protein (ER), thereby reducing the ability of estradiol to stimulate nuclear transcription and consequent cell growth. On the other hand, the *E* isomer **2** of tamoxifen is an estrogen agonist.[3,4] Among the metabolites of tamoxifen detected in patients, the 4-hydroxylated derivative **3**[5,6] has a much higher potency in vitro than tamoxifen.[7,8] This derivative can undergo a facile isomerization as a consequence of conjugation between the central double bond and the hydroxyl group to give a mixture containing the *E* isomer **4**,[9–11] a process that complicates the metabolism picture. Moreover, whether the *E* isomer **4** is an estrogen agonist or antagonist is uncertain since it partially reverts to the more active *Z* isomer **3** during cell-culture experiments.[12,13]

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