mg, 0.51 mmol) was added. After the mixture was stirred at room temperature for 1.5 h, the product was isolated in the usual manner. The product was chromatographed on silica gel eluted with 15% acetone/methylene chloride to give crude **16** (167 mg). This was purified further by HPLC using a Whatman ODS-3 M20/50 column eluted with 65% CH_3CN/H_2O to give **16** (110 mg, 54%): NMR ($CDCl_3$) δ 2.33 (9 H, s), 4.03 (3 H, s), 5.50 (2 H, s), 7.83 (2 H, s), 8.03 (1 H, s); MS, *m/z* 393 (100), 351 (80), 309 (43), 140 (100); mp 136–136.5 °C. Analysis after recrystallization from benzene/petroleum ether ($C_{18}H_{17}N_3O_{10}$) C, H, N.

Mutagenicity Assays. The mutagenicity of the nitroimidazoles was determined as described by Maron and Ames[41] with use of *Salmonella typhimurium* strain TA100 without an additional metabolic activating system. The mutagenicity of each compound was calculated as the net number of revertants/plate over the background value for each sample. The values for the mutagenicity/microgram of sample in the linear portion of the dose–response range were averaged and expressed relative to ronidazole.

Antitrichomonal Activity. *Trichomonas foetus* (Ortho strain 2) has been maintained in pure culture at Merck for several years. The organisms were cultured in Diamonds Media containing gentamycin (24 μg/mL) and calf serum (10%). Trichomonads (2×10^6 in 0.1 mL of media) were added to 1.8 mL of media. The test compound, dissolved in either water or Me_2SO, was added to the culture tube over a concentration range appropriate to bracket the dose required to reduce the number of motile organisms by 50% (EC_{50}). If Me_2SO was required to dissolve the sample, the final incubation concentration was never greater than 1% and appropriate solvent control samples were included. The number of surviving motile trichomonads were counted following a 24-h incubation at 37 °C.

(41) Maron, D. M.; Ames, B. N. *Mutat. Res.* **1983**, *113*, 173–215.

Acknowledgment. We are indebted to Evon Moore for conducting some of the in vitro mutagenicity and antitrichomonal tests.

Registry No. **1**, 104575-24-8; **2**, 104575-25-9; **3**, 104575-26-0; **4**, 18888-57-8; **5**, 104575-27-1; **6**, 104575-28-2; **7**, 104575-29-3; **8**, 104575-30-6; **9**, 104575-31-7; **10**, 21431-58-3; **11**, 104575-32-8; **12**, 104575-33-9; **13**, 104575-34-0; **14**, 104575-35-1; **15**, 104575-36-2; **16**, 104575-37-3; **17**, 551-92-8; **18**, 14766-63-3; **19**, 936-05-0; **20**, 7681-76-7; **21**, 4204-99-3; **22**, 104575-38-4; **23**, 443-48-1; **24**, 104575-39-5; **25**, 55064-41-0; **26**, 52162-47-7; **27**, 104575-40-8; **28**, 104575-41-9; **29**, 104575-42-0; $H_2NCO_2CH_3$, 589-55-0; $H_3CCHBrCHO$, 3400-55-3; $4\text{-}FC_6H_4CN$, 1194-02-1; $(CH_3)_3CCOCl$, 3282-30-2; $3,4,5\text{-}(CH_3CO_2)_3C_6H_2CO_2H$, 6635-24-1; $3,4,5\text{-}(CH_3CO_2)_3C_6H_2COCl$, 70475-59-1; $3,4,5\text{-}(CH_3O)_3C_6H_2COCl$, 4521-61-3; 1,4-dimethyl-5-nitroimidazole, 57658-79-4; 2,4(5)-dimethyl-5(4)-nitroimidazole, 49780-25-8; 1-(2-hydroxyethyl)-2-(4-fluorophenyl)-5-nitroimidazole, 4548-15-6.

Preparation, Receptor Binding, and Fluorescence Properties of Hexestrol–Fluorophore Conjugates: Evaluation of Site of Attachment, Fluorophore Structure, and Fluorophore–Ligand Spacing

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We have undertaken a staged development of certain estrogen–fluorophore conjugates, in order to prepare a fluorescent estrogen suitable for determination of the estrogen receptor content of individual cells. Since non-steroidal estrogens with bulky substituents are often more readily bound by receptor than their steroidal counterparts, we have investigated fluorophore conjugates with derivatives of the non-steroidal estrogen hexestrol ((3*R**,4*S**)-3,4-bis(4-hydroxyphenyl)hexane). On the basis of the receptor-binding affinity of model compounds, we prepared a prototypical set of three ring- and side-chain-substituted fluorescent hexestrol derivatives, whose binding and fluorescence properties ultimately led to the preparation of a series of side-chain-substituted nitrobenzoxadiazole derivatives. The compounds prepared have binding affinities for the estrogen receptors that range from ca. 1% to 5% that of estradiol, and they have very favorable fluorescence characteristics, similar to those of fluorescein.

The presence and concentration of estrogen receptors in breast tumors has been shown to be correlated with patient response to endocrine therapy, so that tumor-receptor content is now routinely assayed and used as a prognostic indicator.[1] However, the correlation between receptor and response is not perfect: The absence or very low titer of receptor is reliably related to a lack of response to hormone treatment, but the presence of receptor only increases the frequency of hormone responsiveness from ca. 30% in untested patients to ca. 60%.[1] Cell heterogeneity in tumors is one hypothesis advanced to explain this discrepancy; accordingly, nonresponsive, receptor-positive tumors might contain mixed populations of receptor-positive and receptor-negative cells (heterogeneous), while responsive, receptor-positive tumors would contain only receptor-positive cells (homogeneous).[1c,d] Whereas these two types of tumors would not be distinguishable by biochemical receptor assay of tumor homogenates, they could, in principle, be distinguished by assay methods that evaluate the receptor content of individual cells. Such considerations have prompted the development of fluorescent estrogens.[2,3]

(1) (a) DeSombre, E. R.; Carbone, P. P.; Jensen, E. V.; McGuire, W. L.; Wells, S. A.; Witliff, J. L.; Lipsett, M. B. *N. Engl. J. Med.* **1979**, *301*, 1011. (b) Allegra, J. C.; Lippman, M. E.; Thompson, E. B.; Simon, R.; Balock, A.; Green, L.; Huff, K. K.; Do, Hj. M. T.; Aitken, S. C. *Cancer Res.* **1979**, *39*, 1447. (c) Silversward, C.; Skoog, L.; Humla, S.; Gustafsson, S. A.; Nordensjold, B. *Cancer* **1980**, *16*, 59. (d) McCarty, K. S., Jr.; Reintgen, D. S.; Seigler, H. F. *Br. Cancer Res. Treat.* **1982**, *1*, 315.

(2) See for example: (a) Lee, S. H. *Am. J. Clinical Pathol.* **1983**, *79*, 484. (b) Lee, Y. J.; Notides, A. C.; Tsay, Y. G.; Kende, A. S. *Biochemistry* **1977**, *16*, 2896. (c) Pertschuk, L. P.; Tobin, E. H.; Tanapat, P.; Gaetjens, E.; Carter, A. C.; Bloom, N. D.; Mackhia, R. J.; Eisenberg, K. B. *J. Histochem. Cytochem.* **1980**, *28*, 799. (d) Joyce, B. G.; Nicholson, R. I.; Morton, M. S.; Griffiths, K. *Eur. J. Cancer Clin. Oncol.* **1982**, *18*, 1147. (e) Fisher, B.; Gunduz, N.; Saffer, E. A. *Cancer Res.* **1983**, *43*, 5244. (f) Martin, P. M.; Magdelenat, H. P.; Benyahia, B.; Rigaud, O.; Katzenellenbogen, J. A. *Cancer Res.* **1983**, *43*, 4956. (g) Van, N. T.; Raber, M. *Science (Washington, D.C.)* **1984**, *224*, 876. (h) Nelson, K.; Pavlik, E. J.; van Nagell, J. R.; Hanson, M. B.; Donaldson, E. S.; Hanigan, R. C. *Biochemistry* **1984**, *23*, 2565.

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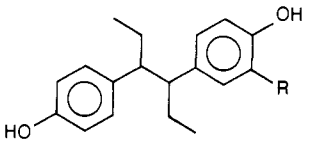
In theory, one could assay the receptor content of individual cells by fluorescence microscopy or flow cytometry using fluorescent estrogens, but in practice, there have been problems in development, and debate over the validity of such assays.[4] Since the maximum number of receptors per cell is only ca. 20 000, there are stringent requirements for a successful assay.[2f] The ideal assay would incorporate a sensitive detection method (e.g., image-intensified microscopy, laser-activated flow cytometry) coupled with a fluorescent probe with a large extinction coefficient, high quantum yield, and large Stokes shift in order to isolate the fluorochrome emission from cellular background autofluorescence. The fluorescent probe must have a high affinity for the estrogen receptor and, equally as important, low nonspecific binding to other cellular proteins.[5]

These requirements have necessitated a systematic investigation of the choice of estrogenic ligand, fluorophore, position of attachment, and spacing between ligand and fluorophore. Most known fluorophores possessing the desired properties are fairly bulky relative to the estrogen ligands; however, our previous studies have shown hexestrol, (3*R**,4*S**)-3,4-bis(4-hydroxyphenyl)hexane, a nonsteroidal estrogen, to be somewhat tolerant of bulky substituents in terms of binding affinity for the estrogen receptor. Herein, we describe the preparation of hexestrol–fluorophore conjugates, systematically optimizing the binding and fluorescence characteristics by varying the remaining choices of fluorophore, position of attachment, and spacing between ligand and fluorophore. We have found that all these parameters have a marked effect on the binding affinity of these compounds for the estrogen receptor and on their fluorescence characteristics. A full report of protein- and receptor-binding studies with these compounds will be described elsewhere.

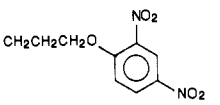
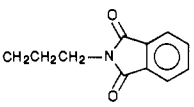
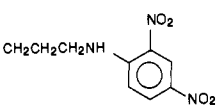
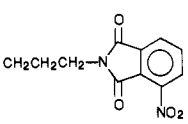
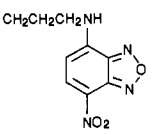
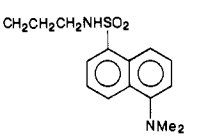
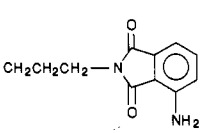
Results and Discussion

Design Considerations. We have previously described hexestrol derivatives substituted on a ring position ortho to the phenolic hydroxyl (I)[8] and derivatives substituted on the aliphatic side chain of hexestrol (II) and norhexestrol (III).[5] Members of all three of these series are readily accessible synthetically; the ring-substituted compounds can be prepared by a Claisen rearrangment of hexestrol monoallyl ether, followed by functionalization of the *o*-allyl group,[8] and the derivatives substituted on the side chain can be prepared from a desoxyanisoin derivative.[7]

Table I. Structure and Estrogen Receptor Binding Affinity of Ring-Substituted Hexestrol Derivatives (I)[a]



I

compd no.[b]	R	RBA[c]
Ia[d]	H	300
Ib[e]	$CH_2CH{=}CH_2$	54
Ic[e]	$CH_2CH_2CH_2OH$	12
Id[f]	I	37
Ie[d]	N_3	69
If	$CH_2CH_2CH_2OCH_3$	13.2
Ig	$CH_2CH_2CH_2OCH_2Ph$	5.0
Ih		1.7
Ii	$CH_2CH_2CH_2SCH_2CH_2CH_2CH_3$	3.6
Ij		5.4
Ik		1.0
Il		3.0
Im		1.5
In		1.3
Io		7.4

[a] Estrogen receptor binding affinity is determined in a competitive radioreceptor binding assay, using [^{3}H]estradiol as a tracer and dextran-coated charcoal as an adsorbant of free ligand, with cytosol preparations from rat or lamb uterus. For details, see ref 6a. [b] See ref 9. [c] The affinity is expressed on a percent scale, relative to that for estradiol (RBA = relative binding affinity; RBA-[estradiol] = 100). [d] Described in ref 6a. [e] Described in ref 8. [f] Described in ref 6b.

(3) An alternative approach to assaying estrogen receptor in individual cells involve immunocytochemistry, using antibodies to the estrogen receptor: (a) King, W. J.; Greene, G. L. *Nature (London)* **1984**, *307*, 745. (b) McCarty, K. S., Jr.; Miller, L. S.; Cox, E. B.; Konratu, J.; McCarty, K. S., Sr. *Arch. Pathol. Lab Med.* **1985**, *109*, 716.

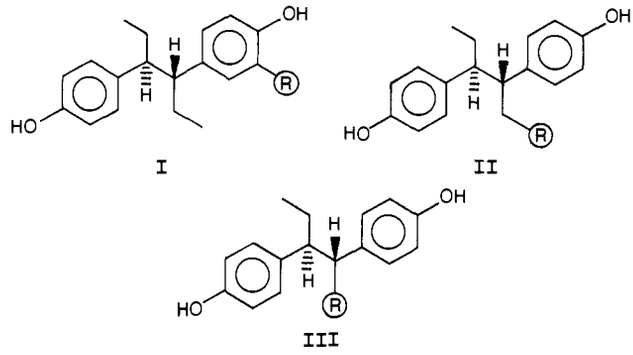
(4) (a) Chamness, G. C.; Mercer, W. D.; McGuire, W. L. *J. Histochem. Cytochem.* **1980**, *28*, 792. (b) Panko, W. B.; Mattioli, C. A.; Wheeler, T. M. *Cancer* **1982**, *49*, 2148. (c) McCarty, K. S., Jr.; Lubahn, D. B.; McCarty, K. S., Sr. *Clin. Endocrinol. Metab.* **1983**, *12*, 135. (d) Bergquist, A.; Carlström, K.; Ljungberg, O. *J. Histochem. Cytochem.* **1984**, *32*, 493. (e) Gay, G.; Jozan, S.; Marques, B.; David, J. F. *J. Receptor Res.* **1984**, *3*, 685.

(5) Katzenellenbogen, J. A.; Heiman, D. F.; Carlson, K. E.; Lloyd, J. E. In *Receptor Binding Radiotracers*; Eckelman, W. C., Ed.; CRC Press: Boca Raton, FL, 1982; Vol. 1, Chapter 6.

(6) (a) Katzenellenbogen, J. A.; Johnson, H. J.; Myers, H. M. *Biochemistry* **1973**, *12*, 4085. (b) Katzenellenbogen, J. A.; Hsiung, H. M. *Biochemistry* **1975**, *14*, 1736. (c) Katzenellenbogen, J. A.; Katzenellenbogen, B. S.; Tatee, T.; Robertson, D. W.; Landvatter, S. W. In *Estrogens and the Environment*; McLachlan, J., Ed.; Elsevier North-Holland: Amsterdam, 1980; p 33.

(7) (a) Goswami, R.; Harsy, S. G.; Heiman, D. F.; Katzenellenbogen, J. A. *J. Med. Chem.* **1980**, *23*, 1002. (b) Landvatter, S. W.; Katzenellenbogen, J. A. *Mol. Pharmacol.* **1981**, *20*, 43. (c) Landvatter, S. W.; Katzenellenbogen, J. A. *J. Med. Chem.* **1982**, *25*, 1300.

(8) Katzenellenbogen, J. A.; McGorrin, R. J.; Tatee, T.; Kempton, R. J.; Carlson, K. E.; Kinder, D. H. *J. Med. Chem.* **1981**, *24*, 435.



As part of this earlier investigation, we prepared derivatives of all three series, some of which bore relatively long or bulky side chains. As can be noted in Table I (upper section) and Table II (upper section), certain of these compounds (e.g., Ib–e, IIc, IIIc–e) still have substantial affinity for the receptor, suggesting that each type of derivative might be suitable for a hexestrol–fluorophore conjugate.

We pursued this goal through a logical sequence: First, we prepared some additional ring- and side-chain-substituted derivatives, in order to explore further the tolerance of the receptor for the bulk and functionality that might be involved in fluorophore attachment. Then, we prepared prototype fluorescent derivatives, comparing related derivatives in the ring- and side-chain-substituted series bearing three fluorophores (nitrobenzoxadiazole (NBD), 1-(dimethylamino)naphthalene-6-sulfonamide (dansyl), and aminophthalimide (AP)). Finally, we have optimized the NBD–norhexestrol spacing in a side-chain-substituted series.

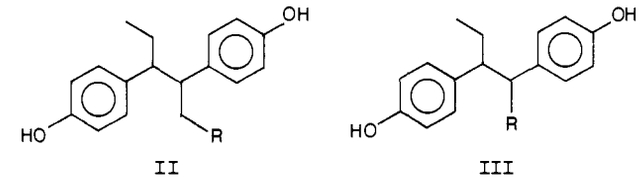
Synthesis. Ring-Substituted Hexestrol Derivatives. The initial set of new derivatives in this series (Table I, middle section) was chosen to examine further the effect of chain length, lipophilicity, and incorporation of an aromatic group, on the binding affinity of these ring-substituted hexestrols, so as to ascertain the binding potential of similar hexestrol–fluorophore conjugates.

Alcohol **1**, the precursor of all of the ring-substituted derivatives, was prepared by a modification of our earlier approach to similar systems.[8] The model ether compounds (If–h) were prepared from **1** as shown in Scheme II. Sulfur and nitrogen were envisioned as useful chain-extending groups and fluorophore attachment sites, respectively. Therefore, in order to introduce these groups into the side chain, alcohol **1** was converted into tosylate **2**. Displacement of the tosylate with sodium butanethiolate gave, after deprotection, thioether Ii. Tosylate displacement by potassium pthalimide afforded **4**, a portion of which was deprotected to give an additional model compound (Ij). The remainder was treated with hydrazine hydrate to give the terminal amine **5**, which was isolated as its dinitrophenyl derivative (Ik).

Table I (middle section) shows the receptor binding affinity values of these model compounds. Although this set is limited, the declining affinity as OMe → OCH_2Ph (If → Ig) and the low affinity of thioether Ii suggested that extending the length of the linking chain would probably not produce compounds of significantly higher affinity. Therefore, we prepared the ring-substituted hexestrol-fluorophore conjugates by derivatization of amine **5**. The NBD and dansyl derivatives (Im, In) were prepared by treatment of amine **5** with NBD-Cl and dansyl-Cl, respectively (Scheme I). In addition, we prepared the aminophthalimide Io,[10] since it closely resembled one of our higher affinity model compounds, phthalimide Ij. Condensation of amine **5** with 3-nitrophthalic anhydride in refluxing acetic acid produced the nitrophthalimide (Il) and deprotected the phenols; hydrogenation over palladium on carbon then gave Io.

(9) In order to facilitate reference to compounds in the three series of hexestrol derivatives, we have designated each series with a Roman numeral (I, ortho-substituted hexestrol; II, side-chain-substituted hexestrol; III, side-chain-substituted norhexestrol). Individual compounds within each series are indicated with subsequent lower case letters. All hexestrol derivatives have the erythro or *R**,*S** stereochemistry, as indicated on these structures.

Table II. Structure and Estrogen Receptor Binding Affinity of Side-Chain Derivatives of Hexestrol (II) and Norhexestrol (III)[a]



R	compd[b] no.	RBA[c]	compd[b] no.	RBA[c]
CH_3	IIa	300[d,e]	IIIa	133[e]
CH_2OH	IIb	16.2[f]	IIIb	8.9[f]
CH_2I	IIc	60[g]	IIIc	172[e]
CO_2CH_3	IId	20[f]	IIId	69[f]
$CO_2CH_2CH_2CH_2CH_2CH_3$	IIe	4.2[f]	IIIe	39[f]
$CH_2OCH_2CH_2OCH_2CH_2Cl$	IIf	16.6	IIIf	6.7
$CH_2SCH_2CH_2CH_2CH_3$	IIg	23.4	IIIg	61.6

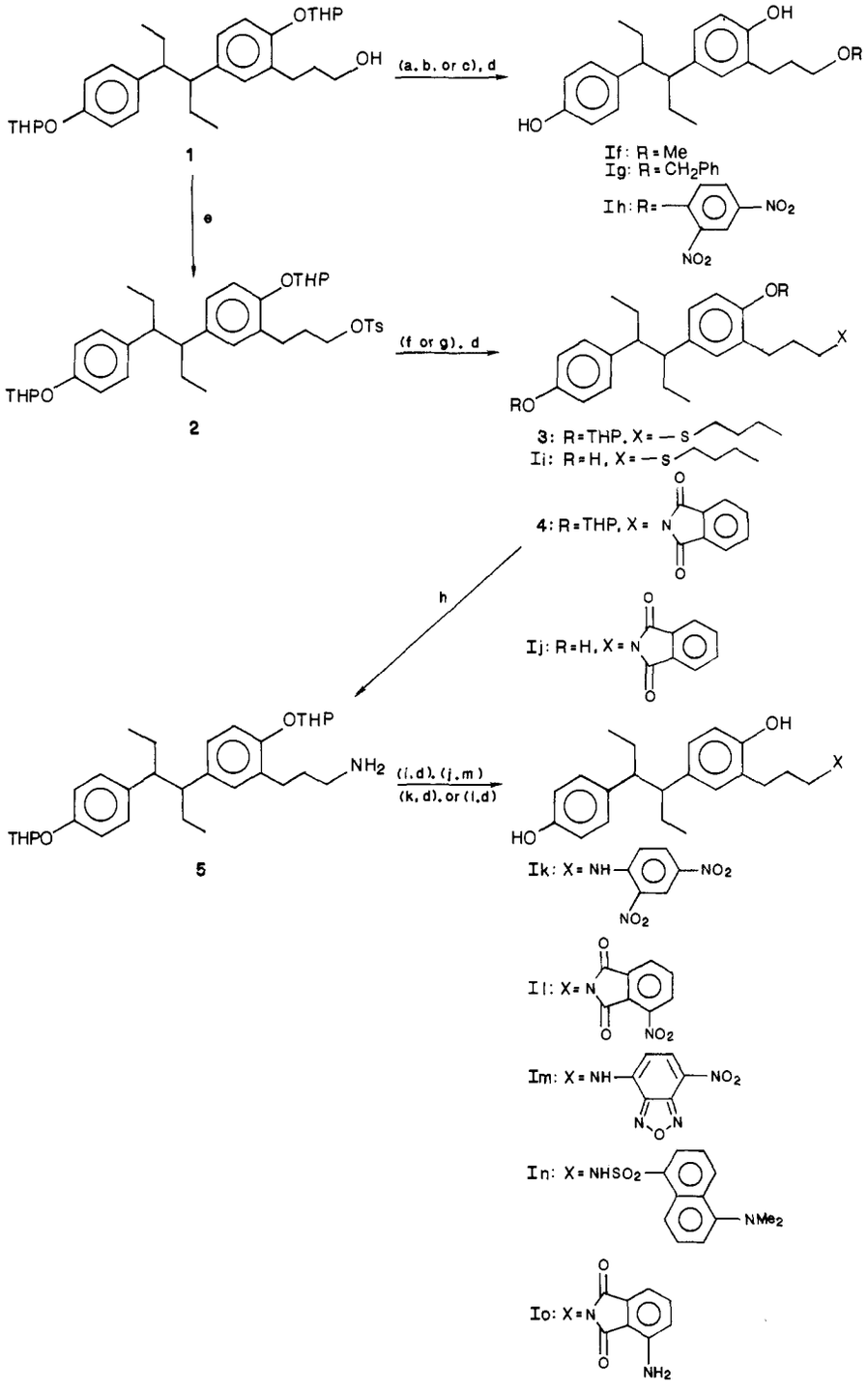
[a] See Table I, footnote *a*. [b] See Table I, footnote *b*. [c] See Table I, footnote *c*. [d] Described in ref 6a. [e] Described in ref 7c. [f] Described in ref 7b. [g] Described in ref 7a.

Side-Chain-Substituted Hexestrol and Norhexestrol Derivatives. Since previous studies have shown large differences in binding affinity of similarly functionalized hexestrol (II) and norhexestrol (III) compounds (Table II, top section),[6b] we first prepared model compounds of both series with sulfide or ether functionality (Table II, bottom section) to optimize binding affinity with respect to the type of linkage.

The synthesis of these compounds in the hexestrol series began from the known ester **6** (3*R**,4*S**), which is prepared in five steps from commercially-available desoxyanisoin.[7a] This compound was first protected as the bis(benzyl ether) (Scheme III) and then reduced to alcohol **7**. Optimized conditions for ether formation yielded the chloro ether, which was deprotected by hydrogenolysis to produce compound IIf. Thioether IIg was prepared from alcohol **7**, via bromide **8**, which was prepared by treatment of **7** with triphenylphosphine and carbon tetrabromide; displacement with sodium butanethiolate gave the sulfide, which was deprotected by hydrogenolysis to yield the desired model compound IIg.

The synthesis of the norhexestrol derivatives IIIf and IIIg proved to be more difficult, and several approaches were explored. The most successful route[7c] began with ketone **9**, a known intermediate in the preparation of ester **6** (Scheme III).[7a] Deprotection of the bis(methyl ether) **9** was effected by using sodium ethanethiolate. At this stage, the phenols were reprotected with more suitable protecting groups: ethoxyethyl or *tert*-butyldimethylsilyl ethers. Although the synthesis could be successfully completed in either case, we found the *tert*-butyldimethylsilyl ether group to be preferable because it was more stable, it gave cleaner proton NMR spectra, and it enabled diastereoisomer separation at an earlier stage of the synthesis (alcohol **12b**).

Scheme I[a]

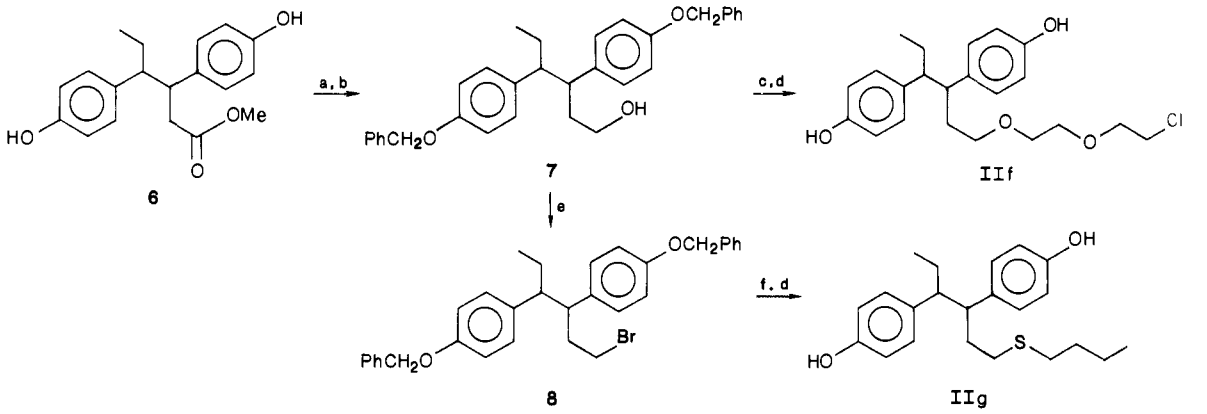


[a] (a) CH_3I, KOH, Me_2SO; (b) $PhCH_2Br$, KOH, Me_2SO; (c) DNFB, *n*-Bu_4NF; (d) TsOH; (e) TsCl, pyr; (f) NaH, *n*-BuSH; (g) potassium phthalimide, DMF; (h) NH_2NH_2, EtOH; (i) DNFB, Et_3N; (j) 3-nitrophthalic anhydride, HOAc, Δ; (k) NBD-Cl, Et_3N; (l) dansyl-Cl, Et_3N; (m) H_2, Pd/C, HOAc.

Ketones **10a/10b** were transformed into alkenes **11a/11b** by the standard methylene Wittig reaction. Hydroboration/oxidation of the olefins afforded alcohols **12a/12b** as mixtures of diastereoisomers (the desired 2*R**,3*S** isomer predominated in each case). This assignment of relative stereochemistry was confirmed by deprotection and comparison (^1H NMR, melting point) of the major product with the known (2*R**,3*S**)-norhexestrol alcohol (IIIb). Alkylation of the mixture of the ethoxyethyl-protected alcohols **12a** with dichlorodiethyl ether, followed by deprotection and HPLC separation of diastereoisomers, afforded chloro ether IIIf. Alternatively, methane sulfonylation of the alcohol mixture, displacement by potassium butanethiolate, deprotection, and HPLC separation yielded thio ether IIIg.

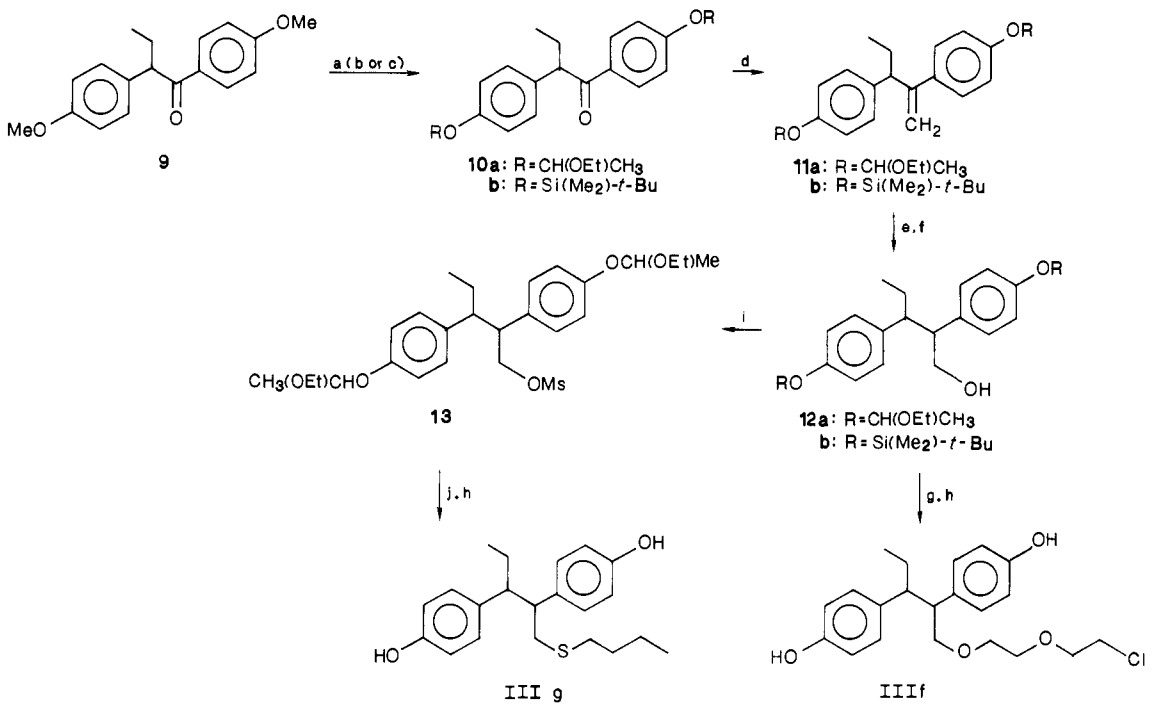
The receptor binding affinities of the new derivatives in the hexestrol and norhexestrol series are shown in the bottom section of Table II. In each case, the thioether derivatives had the greater affinity, and of the two, the binding affinity of the norhexestrol compound IIIg was considerably higher than that of the hexestrol derivative IIg. This led us to consider preparing fluorescent derivatives based on a thioether-linked norhexestrol ligand with variable chain lengths (cf. Table III).

Scheme II[a]



[a] (a) $PhCH_2Cl$, KI, DMF; (b) $LiAlH_4$; (c) KH, $Cl(CH_2)_2O(CH_2)_2Cl$, THF; (d) H_2, 5% Pd/C, HOAc; (e) PPh_3, CBr_4; (f) NaH, *n*-BuSH.

Scheme III[a]



[a] (a) NaH, EtSH, DMF, 120 °C; (b) $EtOCH{=}CH_2$, TsOH; (c) *t*-Bu(Me)$_2$SiCl, imidazole, DMF; (d) $Ph_3P{=}CH_2$, 0 °C; (e) BH_3/THF; (f) H_2O_2, NaOH; (g) KH, $Cl(CH_2)_2O(CH_2)_2Cl$, dicyclohexyl-18-crown-6, HMPA; (h) Dowex-50; (i) MsCl, Et_3N; (j) KH, *n*-BuSH, dicyclohexyl-18-crown-6.

While we had been able to prepare butyl thioether IIIg by reaction of butanethiolate with methanesulfonate **13a**, this hindered sulfonate would not react with more sterically demanding thiolates. Therefore, we converted alcohols **12a/12b** (the 2*R**,3*S** isomers) into the corresponding thiols **14a/14b** (Scheme IV) by treatment with triphenylphosphine, diisopropylazodicarboxylate and thiolacetic acid, followed by reductive hydrolysis of the thio ester. All the required aminoalkyl thioether precursors for the fluorescent norhexestrol derivatives shown in Table III can be prepared by alkylation of the sodium or potassium thiolates derived from **14a** or **14b**. Aminoethyl thioether **16a** was prepared by thiolate addition to ethylenimine;[11] aminopropyl thioether **16b** was prepared by Michael addition of the thiolate to acrylonitrile,[12] giving **15a**, followed by reduction with lithium aluminum hydride, and the higher homologues (**16c–f**) were prepared by thiolate displacement of the corresponding ω-haloalkyl nitriles, followed by hydride reduction.

The NBD derivative of each amine was prepared by reaction with NBD-Cl, followed by phenol deprotection with TsOH. The aminophthalimide (AP) derivative was also prepared from the four- and seven-carbon amino thioethers and the dansyl conjugate also from the seven-carbon amino thioether.

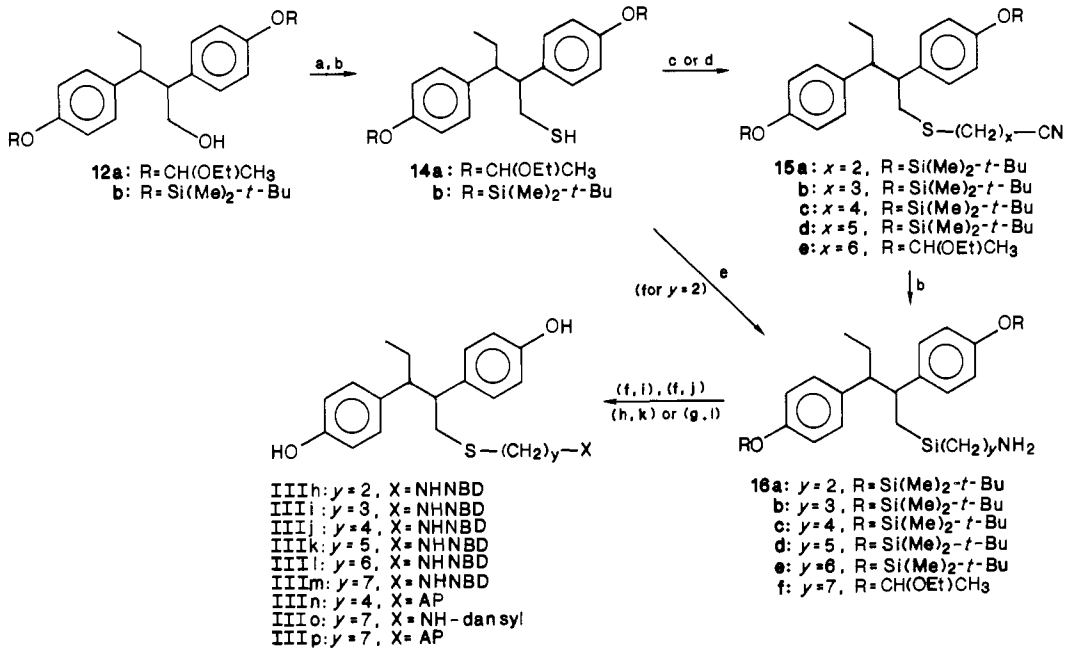
Estrogen Receptor Binding Affinity. The estrogen receptor binding affinities of the hexestrol derivatives we have prepared are summarized in Tables I–III. These values were determined in a radiometric receptor competition assay, using [3H]estradiol as a tracer.[6a] This assay provides estimates of the estrogen receptor binding affinity

(10) Caswell, L. R.; Campbell, J. B.; Cecil, R. *J. Heterocycl. Chem.* **1979**, *16*, 225.

(11) Dermer, O. C.; Ham, G. E. *Ethylenimine and Other Aziridines*; Academic Press: New York, 1969; p 232.

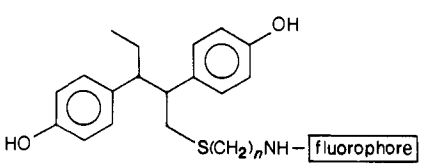
(12) Rapoport, L.; Smith, A.; Newman, M. S. *J. Am. Chem. Soc.* **1947**, *69*, 693.

Scheme IV[a]



[a] (a) Ph_3P, diisopropyl azodicarboxylate, CH_3COSH; (b) $LiAlH_4$; (c) CH_2=CHCN, NaOMe; (d) KH, $Br(CH_2)_xCN$ (x = 3–6); (e) ethylenimine, THF/EtOH, Δ; (f) NBD-Cl, Et_3N or K_2CO_3; (g) dansyl-Cl, Et_3N; (h) 3-nitrophthalic anhydride, HOAc, Δ; then TsOH, Δ; (i) TsOH; (j) Dowex-50; (k) H_2, Pd/C.

Table III. Estrogen Receptor Binding Affinity and Fluorescence Quantum Yield of Thioether-Linked Norhexestrol–Fluorophore Conjugates



compd no.	n	fluorophore	RBA	Φ_F
IIIh	2	NBD	0.85	0.15
IIIi	3	NBD	4.3	0.35
IIIj	4	NBD	3.9	0.37
IIIk	5	NBD	3.5	0.43
IIIl	6	NBD	2.9	0.46
IIIm	7	NBD	3.4	0.63
IIIn	4	AP	3.6	
IIIo	7	dansyl	2.6	
IIIp	7	AP	1.6	

of these compounds relative to that for tracer estradiol; therefore, the affinities are expressed as RBA or "relative binding affinity" values, where, by definition, estradiol has a value of 100. In each case the value shown is the average of duplicate determinations that vary from one another by less than 25%.

As mentioned, it was apparent from the behavior of the ring-substituted hexestrol derivatives (If–i) that longer chains were not well-tolerated. Therefore, we selected the preparation of NBD, dansyl, and AP derivatives from *o*-(3-aminopropyl)hexestrol. While the last of these has reasonable affinity for the estrogen receptor (RBA = 7.4), the fluorescence properties of the AP group (cf. below and Table IV) will limit the utility of this compound.

It was on the basis of the high receptor binding affinity of side-chain-substituted hexestrol and norhexestrol derivatives that we had previously prepared (e.g., IC,d, IIIc–e) that we began a more extensive investigation of the potential of these analogues as estrogen–fluorophore conjugates. The RBA values of the chloroethoxy ethyl ether and butyl thioether derivatives (IIf,g, IIIf,g) suggested that optimal binding would be attained with the thioether lmkage in the norhexestrol series. Therefore, we examined quite a number of norhexestrol–fluorophore conjugates in which the length of the (aminoalkyl)thio group was varied progressively from two to seven methylene groups.

Because the fluorophore unit adds considerable bulk to the molecule, it is not surprising that all of these derivatives have a receptor affinity lower than that of the butyl thioether IIIg. Once beyond the two-carbon chain length, the NBD derivatives display a remarkably constant binding affinity (RBA = 2.9–4.3).

Because of the large number of derivatives that would be involved, we have not prepared the other fluorophore conjugates with each chain-length analogue. Rather, with the four-carbon amino thioether, we also prepared the AP derivative, since in the ring-substituted series, this fluorophore conjugate had shown substantially higher affinity than the corresponding NBD derivative (cf. Table I, Io vs. Im); in the case of the norhexestrol aminobutyl thioether,

Table IV. Fluorescent Spectroscopic Characteristics of Selected Fluorophores[a]

fluorophore	λ_{max}^{excit}, nm	λ_{max}^{emiss}, nm	molar absorptivity, ϵ	quantum yield, Φ_f (solvent)	environmental sensitivity
nitrobenzoxadiazole (NBD)	460	520	20 000	0.65 (THF)	good
aminophthalimide (AP)	390	460	5000	0.30 (THF)	good
dansyl	336	500	5000	0.35 (THF)	poor
fluorescein	490	510	30 000	0.92 (0.01 N aqueous NaOH)	

[a] The excitation and emission spectra and quantum yields were determined on a Spex Fluorolog 2 (Model IIIC) instrument in THF. The molar absorptivities were measured on a Hewlett-Packard 8451 UV–vis spectrophotometer in ethanol solution.

the AP and the NBD derivatives had nearly equal affinities for receptor. With the seven-carbon-linked amino thioether, we prepared, in addition to the NBD derivative, the dansyl and AP derivatives. The NBD compound displayed the highest affinity.

Fluorophore Evaluation. Fluorescein isothiocyanate (FITC) has frequently been used to prepare fluorescent estrogen conjugates;[2] however, the receptor binding affinities of these derivatives are generally low. Attributing this to the large size and high polarity of the fluorescein moiety, we have chosen to study three relatively small, non-ionic fluorophores.

The fluorescence properties of the NBD, AP, and dansyl fluorophores are summarized in Table IV, along with the properties of fluorescein. Compared to fluorescein as the "standard", the dansyl and AP fluorophores have much less favorable spectroscopic characteristics: Their quantum yields and molar absorptivities are considerably lower, and their emission maxima are shifted toward the blue (and hence are more subject to interference by cellular autofluorescence). By contrast, the NBD fluorophore has the most closely matched spectroscopic characteristics: It has a high quantum yield and molar absorptivity, and its excitation and emission maxima are similar to those of fluorescein. In addition, NBD derivatives are very stable, easy to prepare and purify, and sterically compact, which, we expect, allows for higher binding affinity than would the corresponding fluorescein analogues.

One factor affecting the fluorescence properties of estrogen–fluorophore conjugates is the distance separating the two groups. An example is seen with the norhexestrol side chain substituted NBD derivatives (Table III), where the quantum yield increases with increasing chain length. This suggests that the phenolic unit can act as an excited state quencher, since, in the seven carbon analog IIIm, wherein the fluorophore is far from the phenol, a value consistent with that determined for a simpler NBD derivative (the NBD derivative of *n*-butylamine, $\Phi_F = 0.65$) was obtained (see Table III).[13] Consistent with this proposed mechanism is the relatively low quantum yield of the ring-substituted NBD derivative (Im), in which the phenol–fluorophore distance is relatively small.

Conclusion

In our efforts to prepare fluorescent estrogens having good spectroscopic characteristics and high affinity for receptor, we have synthesized hexestrol–fluorophore conjugates, since our previous studies have shown that hexestrol can accomodate relatively bulky substituents while retaining significant affinity for the receptor. We evaluated the effect of the site of attachment and the nature of the attaching chain on the binding affinity, ultimately finding the norhexestrol thioether-linked derivatives to be superior, and we found the NBD group to have good fluorescence characteristics, which, coupled with its small size and ease of handling, made it most favorable overall. Combining these findings, we prepared the NBD derivatives shown in Table III. All of these compounds display favorable spectroscopic characteristics. Also, they are stable compounds that do not contain readily hydrolyzable links between fluorophore and ligand; they have been obtained in pure form (combustion or HPLC analysis), and some of their binding affinities (i.e., IIIi and IIIj) are among the highest reported for fluorescent estrogen conjugates. We are currently investigating whether they will be suitable for our intended application.

(13) Other examples of intramolecular quenching in fluorophore–ligand conjugate systems have been described; see, for example: Scopes, D. I. C.; Barrio, J. R.; Leonard, N. J. *Science (Washington, D.C.)* **1977**, *195*, 296.

Experimental Section

General Procedures. The fluorescence derivatizing agents (NBD-Cl, dansyl-Cl, 3-nitrophthalic anhydride) were obtained from Aldrich.

Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Proton magnetic resonance (^{1}H NMR) spectra were obtained on Varian EM-390 (90 MHz), XL-200 (200 MHz), and HR-220 (220 MHz), GE QE-300 (300 MHz), or Nicolet NT-360 (360 MHz) spectrometers using tetramethylsilane as an internal standard. The data are reported in the form: δ value of signal (peak multiplicity, number of protons, coupling constant (if applicable), assignment). Low-resolution electron-impact mass spectra were obtained on a Finnigan MAT CH-5 spectrometer. High-resolution electron-impact spectra were performed on a Finnigan MAT 731 instrument. Fast atom bombardment (FAB) mass spectra were obtained on a V. G. Instruments ZAB HF mass spectrometer. Elemental analyses were performed by the Microanalytical Services Laboratory of the University of Illinois School of Chemical Sciences.

Flash chromatography[14] was performed with use of Woelm silica gel (32–64 μm). High-performance liquid chromatography (HPLC) was performed on either a Spectra-Physics 8700 solvent delivery system using a Beckman 153 ultraviolet detector (280 nm) or a Varian 5060 system using a Perkin-Elmer LC-75 spectrophotometric detector (275 nm). Columns and solvent systems used were as follows: column A (Alltech Reosil), column B (Whatman PXS 10/50), column C (Whatman ODS-2), and column D (Spheriprep (SiO_2)); solvent systems A (hexane/CH_2Cl_2/*i*-PrOH, ratios given in parentheses for each compound), B (CH_3CN/H_2O, 60/40), and C (cyclohexane/CH_2Cl_2/*i*-PrOH, 80/16/4).

Except where mentioned otherwise, a standard procedure was used for product isolation; this involved quenching by addition to water or an aqueous solution, exhaustive extraction with an organic solvent, washing the extracts with aqueous solutions if necessary, drying with $MgSO_4$ or Na_2SO_4, filtration, and evaporation of solvent under reduced pressure. The particular solvents and aqueous washes used are mentioned in parentheses after the phrase "product isolation".

Although only selected spectral data are presented, all isolated, purified compounds were fully characterized (IR, NMR, MS, and HRMS or combustion analysis), and their structural assignments are considered unambiguous. All the hexestrol derivatives showed NMR signals for the CH_3CH_2CH (Ar) systems at δ 0.45–0.60 (t, $J = \sim 7$ Hz), 0.95–1.65 (m), and 2.4–3.8 (m). All compounds submitted for biological testing either gave acceptable combustion analyses (±0.4% of calculated values) or migrated as single components on HPLC.

A complete description of the preparation and characterization of compounds i, 1, If–k, 7, IIf, 8, IIg, **10a**, **11a**, **12a**, IIIf, 13, IIIg, **14a**, **15e**, **16f**, IIIn–p is given in the supplementary material.

(3*R,4*S**)-3-[3-[3-(*p*-Toluenesulfonyloxy)propyl]-4-(2-tetrahydropyranyloxy)phenyl]-4-[4-(2-tetrahydropyranyloxy)phenyl]hexane (2).** A solution of alcohol 1 (812 mg, 1.64 mmol) in 10 mL of dry pyridine was cooled to 0 °C in a flask under nitrogen. *p*-Toluenesulfonyl chloride (623 mg, 3.28 mmol) was added in one portion, and the solution was stirred at 0 °C for 8 h. After product isolation (H_2O, Et_2O, H_2O, brine), the crude material was chromatographed (hexane/EtOAc, 4:1). There was obtained 935 mg (88%) of toluenesulfonate **2**, which, due to its limited stability, was used promptly in subsequent reactions: IR (film) 1200, 1190 cm^{-1}, no OH band; ^{1}H NMR ($CDCl_3$) δ 2.43 (m, 5 H, Ar C*H*, Ar CH_3), 4.02 (t, 2 H, $J = 6$ Hz, CH_2OS), 7.52 (AA′BB′, 4 H, $J = 9$ Hz, $\Delta\nu = 0.49$, Ar *H*, tosyl ring).

(3*R,4*S**)-3-[3-(3-Phthalimidopropyl)-4-(2-tetrahydropyranyloxy)phenyl]-4-[4-(2-tetrahydropyranyloxy)-phenyl]hexane (4).** Into a flask fitted with a reflux condenser were placed toluenesulfonate **2** (935 mg, 1.43 mmol), potassium phthalimide (794 mg, 4.29 mmol), and 25 mL of *N*,*N*-dimethylformamide (DMF). The light yellow, heterogeneous reaction mixture was stirred at 70 °C for 3 h and then cooled.

(14) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.

Product isolation (H_2O, Et_2O, H_2O, brine) and chromatography (hexane/EtOAc, 3:1) furnished phthalimide **4** (807 mg, 90%) as a colorless gum: IR (film) 1760, 1700 cm^{-1}.

(3*R,4*S**)-3-[3-(3-Aminopropyl)-4-(2-tetrahydropyranyloxy)phenyl]-4-[4-(2-tetrahydropyranyloxy)phenyl]hexane (5).** Compound **4** (626 mg, 1.00 mmol) was dissolved in absolute EtOH (50 mL) and treated with hydrazine hydrate (590 mg, 10 mmol). The mixture was stirred at 25 °C in a stoppered flask for 48 h. The thick white precipitate of phthalhydrazide (88% recovery) was removed by filtration, and the filtrate was evaporated to dryness. There remained crude amine **5**, an off-white solid, which was used in subsequent reactions without further purification: yield 499 mg (100%); HRMS, calcd/found ($C_{31}H_{45}NO_4$) 495.3346/495.3347.

(3*R,4*S**)-3-[3-[3-[(7-Nitro-2,1,3-benzoxadiazol-4-yl)amino]propyl]-4-hydroxyphenyl]-4-(4-hydroxyphenyl)hexane (Im).** Amine **5** (104 mg) was dissolved in 15 mL of MeOH in a flask with a nitrogen inlet. The solution was treated with triethylamine (0.15 mL, ~5 equiv) and NBD-Cl (50 mg, ~1.2 equiv), and the mixture was stirred at room temperature. The reaction mixture was initially bright yellow, but gradually became black. After 4 h, the solvent was removed under a stream of dry nitrogen and replaced with ether. Filtration through silica gel followed by flash chromatography (hexane/Et_2O/CH_2Cl_2, 4:2:1) gave 85 mg (62% from **4**) of an orange oil. Deprotection of this material with TsOH (2 equiv) yielded 57 mg (90%) of an orange solid (Im), mp 145–147 °C dec, after purification by preparative TLC (hexane/EtOAc, 3:2). The purified material migrated as a single component on HPLC (column B, solvent system A (60/32/18)). Im: IR (film) 3310, 1540, 1350 cm^{-1}; ^1H NMR (acetone-d_6) δ 3.65 (m, 2 H, CH_2N), 6.30 (d, 1 H, J = 9 Hz, Ar *H*, *ortho* to amine), 7.95–8.30 (m, 3 H, O*H*, N*H*), 8.46 (d, 1 H, J = 9 Hz, Ar *H*, ortho to nitro); MS (10 eV), m/z (relative intensity) 460 (1, $M^+ - NO$); HRMS (FAB), calcd/found ($C_{27}H_{30}N_4O_5$ + H) 491.2294/491.2295.

(3*R,4*S**)-3-[3-[3-[5-(*N*,*N*-Dimethylamino)naphthalene-1-sulfonamido]propyl]-4-hydroxyphenyl]-4-(4-hydroxyphenyl)hexane (In).** Amine **5** (112 mg) was dissolved in 10 mL of MeOH and 0.16 mL (~5 equiv) of triethylamine, and the mixture was cooled to 0 °C. In one portion, solid dansyl-Cl (91 mg, ~1.5 equiv) was added. The yellow reaction mixture was stirred at 0 °C for 9 h and then allowed to reach room temperature overnight. An additional 55 mg of dansyl-Cl was added, and stirring was continued at room temperature for 4 h. After product isolation (half-saturated $NaHCO_3$, CH_2Cl_2, H_2O, brine), the residue was chromatographed (hexane/Et_2O/CH_2Cl_2, 4:2:1) to give 120 mg (73% from **4**) of a blue-green oil. Deprotection of this material with TsOH (2 equiv) afforded the pure bisphenol (In) after chromatography (hexane/Et_2O/CH_2Cl_2, 3:2:1) and recrystallization from hexane/Et_2O: yield 75 mg (81%) of yellow crystals; mp 199–201 °C; IR (film) 3300, 1310, 1140 cm^{-1}; ^1H NMR (acetone-d_6) δ 2.87 (s, 6 H, CH_3N), 2.94 (t, 2 H, J = 7 Hz, CH_2N), 7.01 (s, 1 H, O*H*), 7.06 (s, 1 H, O*H*), 7.25–7.64 (m, 3 H, Ar *H*, ring bearing NMe_2), 8.02–8.58 (m, 3 H, Ar *H*, ring bearing sulfonyl); MS (70 eV), m/z (relative intensity) 560 (4, M^+). Anal. ($C_{33}H_{40}N_2O_4S$) C, H, N, S.

(3*R,4*S**)-3-[3-[3-(Aminophthalimido)propyl]-4-hydroxyphenyl]-4-(4-hydroxyphenyl)hexane (Io).** Amine **5** (94 mg) and 3-nitrophthalic anhydride (70 mg, ~2 equiv) were dissolved in 10 mL of HOAc and refluxed under nitrogen for 3 h. The yellow reaction mixture was cooled, and most of the solvent was evaporated. Product isolation (Et_2O, saturated $NaHCO_3$, H_2O) and chromatography (hexane/EtOAc, 2:3) gave 79 mg (82%) of a yellow oil, which, upon trituration with hexane/$CHCl_3$, yielded a pale yellow solid (Il), mp 178–181 °C, which was pure by HPLC (column B, solvent system A (60/32/8)). A portion of this material (23 mg) was taken up in HOAc and hydrogenated over 5% Pd/C (5 mg) for 3 h at 25 °C (atmospheric pressure of H_2). The catalyst was removed by filtration, the solvent was evaporated, and the residue was purified by chromatography (hexane/Et_2O/CH_2Cl_2, 2:2:1) and recrystallization from hexane/Et_2O to give 18 mg (83%) of a bright yellow solid (Io), mp 173–175 °C. The purified product migrated as a single component on HPLC (column B, solvent system A (60/32/8)). Io: IR (film) 3480, 3370, 1750, 1690 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.69 (t, 2 *H*, J = 7 Hz, CH_2N), 5.00 (s, 1 H, O*H*), 5.14 (s, 1 H, O*H*), 5.23 (br s, 2 H, NH_2), 7.17–7.45 (m, 3 H, Ar *H*, phthalimide ring); MS (10 eV), m/z (relative intensity) 337 (100, benzylic cleavage); HRMS, calcd/found ($C_{29}H_{32}N_2O_4$) 472.2332/472.2347.

1,2-Bis[4-[(*tert*-butyldimethylsilyl)oxy]phenyl]-1-butanone (10b). Sodium hydride (3.38 g, 50% in oil, 70.3 mmol) was placed in a flask under nitrogen, washed with hexane, and then suspended in 100 mL of dry DMF. The mixture was cooled to 0 °C, and ethanethiol (5.21 mL, 70.3 mmol) was added slowly. After hydrogen evolution was complete, the mixture was stirred at room temperature as 1,2-bis(4-methoxyphenyl)-1-butanone (**9**)[7a] (5 g, 17.6 mmol) in 20 mL of DMF was added dropwise. The reaction was stirred for 19 h at 120 °C and then cooled to room temperature. Product isolation (saturated NH_4Cl, EtOAc) and chromatography (hexane/EtOAc, 1:1) gave a pale yellow oil (4.5 g, 99%). A portion of this material (4.00 g, 15.6 mmol) was dissolved in 16 mL of dry DMF in a flask under nitrogen. To the stirred solution were added *tert*-butyldimethylsilyl chloride (7.065 g, 46.87 mmol) and imidazole (5.312 g, 78.12 mmol). After 1 h, product isolation (saturated $NaHCO_3$, EtOAc, H_2O) and chromatography (hexane/EtOAc, 9:1) afforded 6.73 g (89%) of the bis(silyl ether) **10b** as a clear, colorless oil: IR (neat) 1690 cm^{-1}, no OH band; ^1H NMR ($CDCl_3$) δ 0.18 and 0.22 (2 s, 12 H, Si(CH_3)$_2$), 0.81 (t, 3 H, J = 6 Hz, CH_3CH_2), 0.91 (s, 18 H, SiC(CH_3)$_3$), 1.52–2.10 (m, 2 H, CH_2CH_3), 4.24 (t, 1 H, $CHCH_2$), 6.66–7.85 (m, 8 H, Ar *H*); MS (70 eV), m/z (relative intensity) 484 (100, M^+). Anal. ($C_{28}H_{44}O_3Si_2$) C, H.

2,3-Bis[4-[(*tert*-butyldimethylsilyl)oxy]phenyl]-1-pentene (11b). Into a flask under nitrogen were placed previously dried methyltriphenylphosphonium bromide (9.96 g, 27.9 mmol) and 100 mL of dry Et_2O. The suspension was cooled to 0 °C, and *n*-butyllithium (11.7 mL, 2.37 M in hexane, 27.9 mmol) was added dropwise via syringe. After the addition was complete, the cooling bath was removed, and the reaction mixture was stirred for 0.5 h, whereupon a clear, bright orange solution was obtained. To this solution, ketone **10b** (5.4 g, 11.2 mmol), in a minimum amount of dry Et_2O, was added. After the rection was stirred for 20 h at room temperature, product isolation (saturated NH_4Cl, Et_2O) and chromatography (hexane/Et_2O, 49:1) gave a pale yellow oil (4.16 g, 77%). **11b**: IR (neat) 1650–1610 cm^{-1}, no carbonyl band; ^1H NMR ($CDCl_3$) δ 3.54 (t, 1 H, J = 6 Hz, $CHCH_2$), 5.10, 5.33 (2 s, 2 H, C=CH_2); MS (70 eV), m/z (relative intensity) 482 (23, M^+). Anal. ($C_{29}H_{46}O_2Si_2$) C, H.

(2*R,3*S**)-2,3-Bis[4-[(*tert*-butyldimethylsilyl)oxy]phenyl]-1-pentanol (12b).** Alkene **11b** (502.6 mg, 1.04 mmol) was placed in a flask under nitrogen and dissolved in 5 mL of dry THF. The solution was cooled to 0 °C, and BH_3–THF complex (1.01 mL, 1.03 M in THF, 1.04 mmol) was added. The reaction mixture was stirred for 4.5 h at 0 °C, then quenched by sequential addition of water (2 mL), sodium hydroxide (3.2 mL, 3 N), and hydrogen peroxide (2.2 mL, 30% aqueous), and heated at 45–50 °C for 1 h. The mixture was cooled and the product extracted into Et_2O. Purification by flash chromatography (hexane/EtOAc, 4:1) afforded **12b** (the 2*R**,3*R** isomer) as a white solid (82% yield): mp 73–75 °C; IR (neat) 3500–3200 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.54 (t, 3 H, J = 6 Hz, CH_2CH_3), 1.10–1.41 (m, 2 H, CH_2CH_3), 2.51–3.10 (m, 2 H, Ar C*H*), 3.46–3.53 (m, 2 H, CH_2OH); MS (70 eV), m/z (relative intensity) 500 (100, M^+); HRMS, calcd/found ($C_{29}H_{48}O_3Si_2$) 500.3142/500.3139.

(2*R,3*S**)-2,3-Bis[4-[(*tert*-butyldimethylsilyl)oxy]phenyl]-1-pentanethiol (14b).** Triphenylphosphine (603 mg, 2.30 mmol) was placed in a flask under nitrogen and dissolved in 2 mL of dry THF. The solution was cooled to 0 °C and diisopropyl azodicarboxylate (0.45 mL, 2.30 mmol) was added dropwise with stirring, forming a thick, white precipitate in a yellow solution. After the reaction mixture was stirred for 30 min, thiolacetic acid (0.16 mL, 2.30 mmol) and alcohol **12b** (553 mg, 1.105 mmol) were dissolved in 1 mL of THF and added to the reaction mixture dropwise. The reaction mixture was stirred for 5 h at 0 °C and then quenched with 1 mL of EtOH. Concentration under a stream of dry nitrogen and chromatographic (hexane/CH_2Cl_2/Et_2O, 11:3:1) purification yielded a pale yellow oil (469 mg, 76%). A portion of this material (154 mg, 0.276 mmol) was placed in a flask under nitrogen and dissolved in 1 mL of dry Et_2O. The solution was cooled to 0 °C, and a solution of lithium aluminum hydride (0.2 mL, 1.5 M in THF, 0.30 mmol) was added dropwise. Upon stirring for 5 min, the reaction was quenched

with sodium phosphate (Na_2HPO_4, 5% aqueous), and the product was isolated (CH_2Cl_2). Purification by flash chromatography (hexane/Et_2O, 9:1) gave **14b** in 58% yield: IR (Nujol), no OH band; ^1H NMR ($CDCl_3$) δ 2.42–2.65 (m, 4 H, CH_2S, Ar CH); MS (70 eV), m/z (relative intensity) 516 (1, M^+). Anal. ($C_{29}H_{48}O_2SSi_2$) C, H, S.

(2*R,3*S**)-2,3-Bis[4-[(*tert*-butyldimethylsilyl)oxy]-phenyl]pentyl 2-Cyanoethyl Sulfide (15a).** To a solution of thiol **14b** (500 mg, 0.969 mmol) in anhydrous dioxane (5 mL) under nitrogen at 45 °C were added acrylonitrile (257 mg, 4.85 mmol) and solid sodium methoxide (6 mg, 0.11 mmol). The reaction mixture was stirred for 1 h at 45 °C, cooled to room temperature, and applied directly to flash chromatography (hexane/Et_2O, 4:1), affording nitrile **15a** as a clear oil (332 mg, 60%): IR ($CHCl_3$) 2255 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.22 (overlapping t, 2 H, J = 6 Hz, CH_2CN), 2.36 (overlapping t, 2 H, J = 6 Hz, SCH_2); MS (70 eV), m/z (relative intensity) 569 (1, M^+). Anal. ($C_{32}H_{51}NO_2SSi_2$) C, H, N, S.

(2*R,3*S**)-2,3-Bis[4-[(*tert*-butyldimethylsilyl)oxy]-phenyl]pentyl [3-Cyanopropyl, 4-Cyanobutyl, 5-Cyanopentyl] Sulfide (15b–d). General Procedure.** To a cold (−78 °C) solution of thiol **14b** (600 mg, 1.16 mmol) in dry THF (5 mL) under nitrogen were added 4-bromobutyronitrile, 5-bromopentanenitrile, or 6-bromohexanenitrile (11.62 mmol) and subsequently an excess of potassium hydride (~100 mg, 2.50 mmol, rinsed with THF). After 15 min at −78 °C, the solution was stirred for 1 h, while being allowed to warm slowly to room temperature. Product isolation (5% Na_2HPO_4, EtOAc, brine) and flash chromatography (hexane/Et_2O, 9:1) gave the desired nitriles **15b**, **15c**, and **15d** in 90%, 50%, and 58% yields, respectively: IR ($CHCl_3$) 2250 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.15–1.70 (m, 4–8 H, CH_2CH_3, $SCH_2(CH_2)_nCH_2CN$), 2.23 (**15b**) or 2.11 (**15c** and **15d**) (t, 2 H, J = 7 Hz, CH_2CN), 2.27 (**15b**) or 2.24 (**15c** and **15d**) (t, 2 H, J = 7 Hz, SCH_2CH_2), 2.48 (distorted d, 2 H, $CHCH_2$).

Nitrile 15b: MS (FAB), m/z (relative intensity) 568 (10, $M^+ - CH_3$). Anal. ($C_{33}H_{53}NO_2SSi_2$) C, H, N, S.

Nitrile 15c: MS (FAB), m/z (relative intensity) 597 (7, M^+).

Nitrile 15d: MS (FAB), m/z (relative intensity) 596 (5, $M^+ - CH_3$). Anal. ($C_{35}H_{57}NO_2SSi_2$) C, H, N, S.

(2*R,3*S**)-2,3-Bis[4-[(*tert*-butyldimethylsilyl)oxy]-phenyl]pentyl 2-Aminoethyl Sulfide (16a).** To a THF/EtOH (1:5) solution of thiol **14b** (160 mg, 0.310 mmol) was added ethylenimine (600 μL, 11.58 mmol) at room temperature under nitrogen. The reaction was stirred for 48 h at reflux, whereupon TLC analysis indicated no further consumption of starting material (50% completion). After the solution was concentrated (stream of N_2 in the hood), purification by preparative TLC (EtOAc/Et_3N, 4:1) furnished **16a** as a pale yellow oil (45 mg, 26%): IR ($CHCl_3$) 1600 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.15–2.25 (m, 2 H, SCH_2CH_2), 2.50 (distorted d, 2 H, $CHCH_2S$), 2.55–2.66 (m, 3 H, CH_2NH_2, Ar CH); MS (FAB), m/z (relative intensity) 560 (25, M^+ + H); HRMS (FAB), calcd/found ($C_{31}H_{53}NO_2SSi_2$ + H) 560.3426/560.3420.

(2*R,3*S**)-2,3-Bis[4-[(*tert*-butyldimethylsilyl)oxy]-phenyl]pentyl [3-Aminopropyl, 4-Aminobutyl, 5-Aminopentyl, 6-Aminohexyl] Sulfide (16b–e). General Procedure.** To a solution of nitrile (1 mmol) in dry THF under nitrogen was added lithium aluminum hydride (15.0 mL of a 1 M THF solution filtered through dry Celite under nitrogen) in one portion. After 24 h at room temperature, the product was isolated (5% Na_2HPO_4, EtOAc, brine). Crude yields of the desired amines were 60% (**16b**), 68% (**16c**), 55% (**16d**), and 85% (**16e**): IR ($CHCl_3$) 1600 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.15–1.57 (m, 4–10 H, CH_2CH_3, $SCH_2(CH_2)_nCH_2NH_2$), 2.17 (**16b**), or 2.12 (**16c**, **16d**, and **16e**) (t, 2 H, J = 6 Hz, SCH_2CH_2), 2.48 (distorted d, 2 H, $CHCH_2S$), 2.50–2.89 (m, 4 H, Ar CH, CH_2NH_2).

Amine 16b: MS (FAB), m/z (relative intensity) 574 (50, M^+ + H); HRMS (FAB), calcd/found ($C_{32}H_{55}NO_2SSi_2$ + H) 574.3570/574.3594.

Amine 16c: MS (FAB), m/z (relative intensity) 588 (29, M^+ + H); HRMS (FAB), calcd/found ($C_{33}H_{57}NO_2SSi_2$ + H) 588.3813/588.3770.

Amine 16d: MS (FAB), m/z (relative intensity) 602 (39, M^+ + H); HRMS (FAB), calcd/found ($C_{34}H_{59}NO_2SSi_2$ + H) 602.3907/602.3895.

Amine 16e: MS (FAB), m/z (relative intensity) 616 (33, M^+ + H); HRMS (FAB), calcd/found ($C_{35}H_{61}NO_2SSi_2$ + H) 616.4088/616.4064.

(2*R,3*S**)-2,3-Bis(4-hydroxyphenyl)pentyl [*N*-(7-Nitro-2,1,3-benzoxadiazol-4-yl)-2-aminoethyl, -3-aminopropyl, -4-aminobutyl, -5-aminopentyl, -6-aminohexyl] Sulfide (IIIh–l). General Procedure.** To a solution of the amine (1 equiv) in MeOH (~1 mL/25 mg of amine) was added solid NBD-Cl (3.0 equiv) in one portion. The reaction mixture was stirred at room temperature under nitrogen for 2 h. The product was isolated (brine, EtOAc, brine) and purified by preparative TLC (hexane/EtOAc, 4:1) to give an orange oil. This was taken up in EtOAc and treated with a large excess (~25 equiv) of TsOH. The solvent was removed, and the oily mixture was heated at 40 °C for 10 min. Preparative TLC (hexane/EtOAc, 2:3) afforded an orange oil. Yields of the fluorescent products prepared in this manner were: 31% (IIIh), 22% (IIIi), 53% (IIIj), 31% (IIIk), and 34% (IIIl). Each of the purified compounds migrated as a single component on HPLC (IIIh, i, column A, solvent system A (60/35/5); IIIj–l, column C, solvent system B).

Compound IIIh: ^1H NMR (acetone-d_6) δ 3.47–3.55 (br t, 2 H, CH_2NH), 6.25 (d, 1 H, J = 8.5 Hz, Ar H, ortho to amine), 8.52 (d, 1 H, J = 8.5 Hz, Ar H, ortho to nitro); MS (FAB), m/z 495 (M^+ + H).

Compound IIIi: ^1H NMR (acetone-d_6) δ 3.57 (dt, 2 H, J = 7, 7 Hz, CH_2NH), 6.37 (d, 1 H, J = 9 Hz, Ar H, ortho to amine), 8.53 (d, 1 H, J = 9 Hz, Ar H, ortho to nitro); MS (FAB), m/z (relative intensity) 509 (12, M^+ + H); HRMS (FAB), calcd/found ($C_{26}H_{28}N_4O_5S$ + H) 509.1859/509.1861.

Compound IIIj: ^1H NMR (acetone-d_6) δ 3.50–3.65 (m, 2 H, CH_2NH), 6.42 (d, 1 H, J = 9 Hz, Ar H, ortho to amine), 8.15 (s, 1 H, OH), 8.17 (s, 1 H, OH), 8.52 (d, 1 H, J = 9 Hz, Ar H, ortho to nitro); MS (FAB), m/z (relative intensity) 523 (21, M^+ + H).

Compound IIIk: ^1H NMR (acetone-d_6) δ 3.50–3.65 (m, 2 H, CH_2NH), 6.46 (d, 1 H, J = 9 Hz, Ar H, ortho to amine), 8.14 (s, 1 H, OH), 8.16 (s, 1 H, OH), 8.52 (d, 1 H, J = 9 Hz, Ar H, ortho to nitro); MS (FAB), m/z (relative intensity) 537 (18, M^+ + H).

Compound IIIl: IR (KBr) 3550, 1358 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.44 (dt, 2 H, J = 7, 7 Hz, CH_2NH), 5.03 (s, 1 H, OH), 5.23 (s, 1 H, OH), 6.17 (d, 1 H, J = 9 Hz, Ar H, ortho to amine), 8.48 (d, 1 H, J = 9 Hz, Ar H, ortho to nitro); MS (FAB), m/z 551 (M^+ + H); HRMS (FAB), calcd/found ($C_{29}H_{34}N_4O_5S$ + H) 551.2334/551.2331.

(2*R,3*S**)-2,3-Bis(4-hydroxyphenyl)pentyl *N*-(7-Nitro-2,1,3-benzoxadiazol-4-yl)-7-aminoheptyl Sulfide (IIIm).** Potassium carbonate (73.6 mg, 0.533 mmol), amine **16f** (96.9 mg, 0.178 mmol), and 5 mL of EtOH were placed in a flask and cooled to 0 °C. An ethanolic solution of NBD-Cl (70.8 mg, 0.355 mmol) was added dropwise, and the mixture was stirred for 17 h at 0 °C. Concentration under a stream of nitrogen and chromatography (hexane/EtOAc, 3:1) yielded a brown oil (65 mg, 52%). A portion of this material (38.4 mg, 0.054 mmol) was placed in a flask and dissolved in CH_2Cl_2. Dowex-50 cation exchange resin was added, and the mixture was stirred for 3 h at room temperature. The reaction mixture was filtered, and the Dowex was washed with 1:1 CH_2Cl_2/MeOH. The solvent was removed to yield a brown oil (25.6 mg, 84%). The pure 2*R**,3*S** isomer IIIm was obtained by HPLC (column B, solvent system A (85/12/3)): IR (film) 3400 cm^{-1}; ^1H NMR (acetone-d_6) δ 3.69 (m, 2 H, CH_2NH), 6.52 (d, 1 H, J = 9 Hz, Ar H, ortho to amine), 8.50 (d, 1 H, J = 9 Hz, Ar H, ortho to nitro); MS (FAB), m/z (relative intensity) 587 (14, M^+ + Na); HRMS (FAB), calcd/found ($C_{30}H_{36}N_4O_5S$ + Na) 587.2304/587.2391.

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Registry No. 1, 104946-83-0; 1 (methyl ether), 104947-16-2; 2, 104946-84-1; 4, 104946-85-2; 5, 104946-86-3; 6, 74536-73-5; 7,

104946-87-4; **7** (methyl ester), 104975-65-7; **8**, 104946-88-5; **9**, 4390-94-7; **9** (diol), 76579-69-6; **10a**, 104947-29-7; **10b**, 85546-02-7; **11a**, 104947-30-0; **11b**, 104946-95-4; **12a**, 104947-31-1; **12b**, 104946-96-5; **13**, 104947-34-4; **14a**, 104994-60-7; **14a** (acetylthio deriv), 104947-37-7; **14b**, 104946-98-7; **14b** (acetylthio deriv), 104946-97-6; **15a**, 104946-99-8; **15b**, 104947-00-4; **15c**, 104947-01-5; **15d**, 104947-02-6; **15e**, 104947-38-8; **16a**, 104947-03-7; **16b**, 104947-04-8; **16c**, 104947-05-9; **16d**, 104947-06-0; **16e**, 104947-07-1; **16f**, 104947-39-9; **16f** (NBD deriv), 104947-13-9; **If**, 104947-17-3; **Ig**, 104947-18-4; **Ih**, 104947-20-8; **Ih** (THP deriv), 104947-19-5; **Ii**, 104947-21-9; **Ij**, 104947-22-0; **Ik**, 104947-24-2; **Ik** (THP deriv), 104947-23-1; **Il**, 104946-93-2; **Im**, 104946-90-9; **Im** (THP deriv), 104946-89-6; **In**, 104946-92-1; **In** (THP deriv), 104946-91-0; **Io**, 104946-94-3; **IIf**, 104947-26-4; **IIf** (benzyl deriv), 104947-25-3; **IIg**, 104947-28-6; **IIg** (benzyl deriv), 104947-27-5; **III** (Y = 4, X = 3-nitrophthalic anhydride), 104947-40-2; **IIIf**, 104947-33-3; **III** (di(*t*-BuMe$_2$Si ether)), 104947-32-2; **IIIg**, 104947-36-6; **IIIg** (ethylvinyl ether deriv), 104947-35-5; **IIIh**, 104947-08-2; **IIIi**, 104947-09-3; **IIIj**, 104947-10-6; **IIIk**, 104947-11-7; **IIIl**, 104947-12-8; **IIIm**, 104947-14-0; **IIIn**, 104947-41-3; **IIIo**, 104947-43-5; **IIIo** (ethylvinyl deriv), 104947-42-4; **IIIp**, 104975-78-2; *i*, 104947-15-1; dansyl-Cl, 605-65-2; NBD-Cl, 10199-89-0; 4-MeC$_6$H$_4$SO$_2$Cl, 98-59-9; *t*-BuMe$_2$SiCl, 18162-48-6; MePh$_3$PBr, 1779-49-3; MeCOSH, 507-09-5; H$_2$C = CHCN, 107-13-1; Br(CH$_2$)$_3$CN, 5332-06-9; Br(CH$_2$)$_4$CN, 5414-21-1; Br(CH$_2$)$_5$CN, 6621-59-6; MeI, 74-88-4; PhCH$_2$Br, 100-39-0; i (diol), 76412-01-6; i (hexestrol monoallyl ether), 76411-99-9; BuSH, 109-79-5; PhCH$_2$Cl, 100-44-7; EtOCH=CH$_2$, 109-92-2; MeSO$_2$Cl, 124-63-0; Br(CH$_2$)$_6$CN, 20965-27-9; potassium phthalimide, 1074-82-4; 3-nitrophthalic anhydride, 641-70-3; ethylenimine, 151-56-4; dihydropyran, 25512-65-6; 2,4-dinitrofluorobenzene, 70-34-8; 2,2′-dichlorodiethyl ether, 111-44-4.

Supplementary Material Available: Complete experimental procedures and spectroscopic characterizations of the following compounds: i, **1**, If–k, **7**, IIf, **8**, IIg, **10a**, **11a**, **12a**, IIIf, **13**, IIIg, **14a**, **15e**, **16f**, IIIn, IIIo, and IIIp (18 pages). Ordering information is given on any current masthead page.

Double-Headed Haptens with Pyrocatechol (Poison Ivy like) and Methylene Lactone Functional Groups: A Search for Skin-Tolerance Inducers

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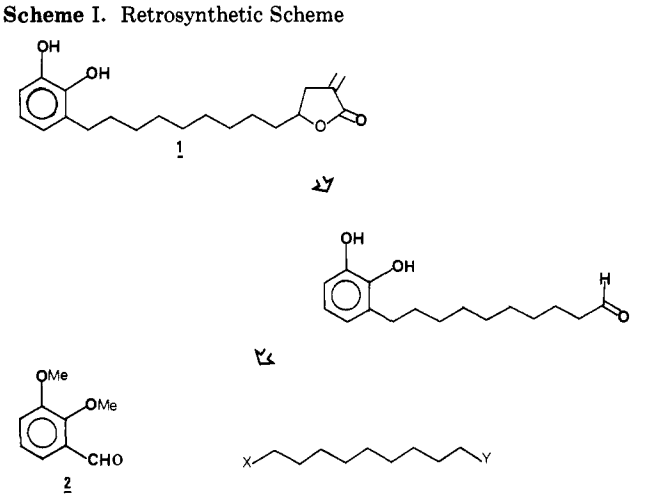
In order to develop new theories for desensitization, a potential skin sensitizer, **1**, with two different haptenic ends, (a) a pyrocatechol group and (b) an α-methylene-γ-butyrolactone moiety, separated by a straight nine-carbon-atom chain has been prepared and used to sensitize guinea pigs. A "monohapten" **8** containing an electrophylic α-methylene-γ-butyrolactone, connected to a dimethoxybenzene group, has also been prepared. Both the "bihapten" **1** and the "monohapten" **8** were shown to be sensitizers. Bihapten **1** sensitized animals recognized only bihapten **1** (and not the lactone **8**) while monohapten **8** sensitized guinea pigs reacted to both **8** and **1**. Bihapten **1** treated animals were further sensitized to monohapten **8** and challenged to the latter: the skin intensity reaction was significantly lower than the test to **8** in **8**-sensitized animals. Bihapten **1** seems therefore to "tolerize" against the α-methylene-γ-butyrolactone end.

Molecular aspects of allergic contact dermatitis (ACD) are now better understood, thanks to structure–activity relationships studies.[1,2] In particular, it seems that induction of sensitization is dependent on the ability of the sensitizer to form covalent bonds with epidermal proteins; most of the skin haptens are therefore electrophilic.[2] However, apparently nonelectrophilic skin sensitizers do exist;[3,4] their activity can be rationalized either by an in vivo transformation into electrophiles (in this case they are called prohaptens) or their capacity to form strong hydrophobic bonds, in particular by insertion into cell membranes.

Once that bond is formed, accessory cells (Langerhans cells, LC[5]) have to take up the foreign complete antigen (hapten + carrier protein), process them, and present them on their surface in association with Ia.[6] LC are essential to the induction of a contact allergy by an epicutaneous pathway.[7] It appears that a specific tolerance may be induced by application of a hapten to a skin area in which a fair number of LC are either absent naturally (mouse tail skin)[8] or artificially (anti-Ia antisera and complement)[7] or

Scheme I. Retrosynthetic Scheme



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