Notes

Homologs of Idoxifene: Variation of Estrogen Receptor Binding and Calmodulin Antagonism with Chain Length

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A series of homologs of idoxifene [**1a**, (*E*)-1-[4-(*N*-pyrrolidinoethoxy)phenyl]-1-(4-iodophenyl)-2-phenyl-1-butene] and selected homologs of 4-iodotamoxifen [**2a**, (*E*)-1-[4-[(*N*-dimethylamino)-ethoxy]phenyl]-1-(4-iodophenyl)-2-phenyl-1-butene] with the side chain $(CH_2)_n$ varying in length from $n = 3$ (**1b**, **2b**) to $n = 10$ (**1i**, **2i**) have been synthesized and tested for antagonism of the calmodulin-dependent activity of cAMP phosphodiesterase and for binding affinity to rat uterine estrogen receptor. Compared with **1a** ($IC_{50} = 1.5$ μM), the homologs showed a progressive increase in calmodulin antagonism with a maximum inhibition at $n = 7-9$ (**1f**−**h**) ($IC_{50} = 0.2$ μM), declining at $n = 10$ (**1i**) to $IC_{50} = 1.6$ μM. In the pyrrolidino series, estrogen receptor binding affinity peaked at $n = 3$ (**1b**, RBA = 23; estradiol = 100), declining by $n = 10$ (**1i**) to RBA = 0.4, but the homolog $n = 8$ (**1g**, RBA = 3.5) was still comparable to tamoxifen (RBA = 3.9). A similar pattern of activity was seen for the dimethylamino counterparts. These compounds represent a new class of antiestrogens with potent calmodulin antagonism.

Introduction

We recently reported that the 3- and 4-carbon homologs of idoxifene [**1a**, (*E*)-1-[4-(*N*-pyrrolidinoethoxy)-phenyl]-1-(4-iodophenyl)-2-phenyl-1-butene] and 4-iodotamoxifen [**2a**, (*E*)-1-[4-[(*N*-dimethylamino)ethoxy]-phenyl]-1-(4-iodophenyl)-2-phenyl-1-butene] were more potent calmodulin antagonists than the parent compounds while retaining their estrogen receptor binding affinity.[1] We now report the extension of the homologous series and the identification of the 8-carbon homologs in both series as potent calmodulin antagonists.

Nonsteroidal antiestrogens such as **1a**, in phase II clinical trials,[2] and its parent tamoxifen [**3**, *trans*-1-[4-[2-(dimethylamino)ethoxy]phenyl]-1,2-diphenyl-1-butene], widely used in the treatment of breast cancer, are believed to act principally by the displacement of the growth promoting hormone estradiol from its protein receptor.[3] Both compounds also exhibit a number of hormone-independent effects which may contribute to their therapeutic action. These include calmodulin antagonism[4−6] and inhibition of protein kinase C.[7−10]

The exact role of calmodulin in the functioning of the estrogen receptor has not been defined. However, an estrogen receptor−calmodulin−estradiol complex has been identified, and its formation has been shown to be antagonized by the addition of some antiestrogens.[11] Also, a calmodulin-dependent protein tyrosine kinase responsible for phosphorylating the estrogen receptor has been identified.[12] Antagonism of calmodulin-dependent processes appears to correlate with cytotoxicity in estrogen receptor (ER) positive cell lines for some antiestrogens.[1,6]

Idoxifene 1a   **4-Iodotamoxifen 2a**

Tamoxifen 3
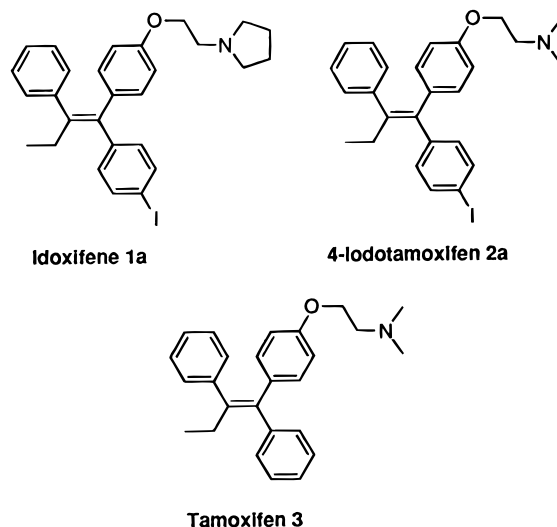
The attractiveness of calmodulin as a target is enhanced by the fact that its structure is well known, and so computerized molecular modeling can be used as part of a rational drug design strategy. Our modeling studies to date[1,13] have used the open form of calmodulin seen in X-ray crystal structures of the native protein.[14−17] This work modeled the interactions of 3- and 4-carbon homologs of **1a** and **2a** with calmodulin and suggested that they would be more potent calmodulin antagonists. The compounds **1b,c** and **2b,c** displayed increased antagonism, although the maximum antagonism appeared not to have been reached. More recent studies (to be reported elsewhere) have taken the "closed" calmodulin structures seen in crystal structures of complexes with peptides and drugs,[18−21] with analogous results to those

Scheme 1. Synthesis of Homologs of Idoxifene (**1a**) and 4-Iodotamoxifen (**2a**)
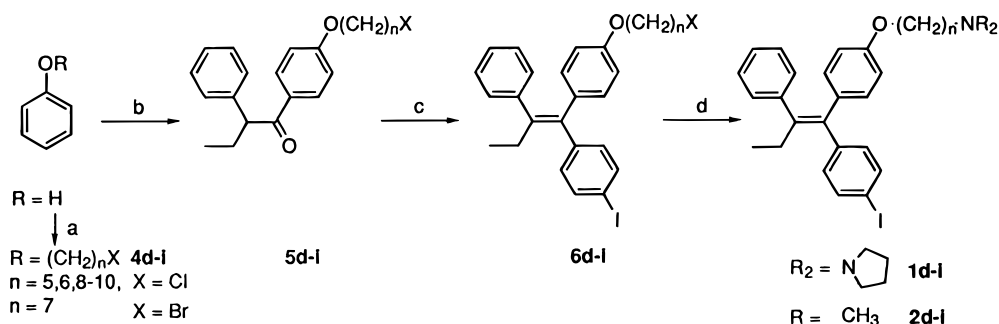
with the open form.[1,13] We have extended the homologous series for **1a** and **2a** to explore further the structure–activity relationships for calmodulin antagonism and estrogen receptor binding properties. In this paper we report that the C_{7-9} homologs **1f**–**h** and **2g** are potent calmodulin antagonists with estrogen receptor binding affinities (RBA) similar to that of **3**.

Results and Discussion

Synthesis. Homologs of Idoxifene (1a) and 4-Iodotamoxifen (2a). The synthesis of the 4-iodo homologs is outlined in Scheme 1. The procedure essentially follows that reported for the synthesis of 3- and 4-carbon homologs **1b,c** and **2b,c**,[1] substituting the appropriate [(ω-haloalkyl)oxy]benzene **4d–i** in the Friedel–Crafts acylation of 2-phenylbutanoic acid to give the 1,2-diarylbutanones **5d–i**, respectively. Reaction of the ketones **5d–i** with 4-iodophenyllithium, readily generated by treatment of 1,4-diiodobenzene with 1 equiv of *n*-butyllithium, and subsequent dehydration of the resulting tertiary alcohols gave the triarylbutenes **6d–i**, respectively, as a mixture of *E* and *Z* isomers which were separable by fractional crystallization. (*Trans* and *cis* are used in this paper to designate the relative positions of the ethyl group and the ring bearing the basic side chain.) The desired *E* (*trans*) isomers were then treated with pyrrolidine or dimethylamine to give the pyrrolidino compounds **1d–i** or dimethylamino compounds **2d–i**, respectively.

Biological Evaluation and General Discussion. Compounds were assayed for the inhibition of calmodulin-dependent cyclic AMP phosphodiesterase (cAMP → AMP) (none of the compounds inhibited the calmodulin-independent component of cAMP phosphodiesterase when assayed at the final concentrations of 10 and 20 μM), and the binding affinities of the compounds toward rat uterine cytosolic estrogen receptor were determined relative to estradiol (RBA = 100) following the published procedures.[6,22] The results are summarized in Tables 1 and 2.

In the pyrrolidino series there is a gradual rise in relative potency from 1.4 to 3.0 for $n = 3$–6 (**1b**–**e**) followed by a sharp jump at $n = 7$ (**1f**) to 7.5 ($IC_{50} = 0.2$ μM). The 8- and 9-carbon homologs **1g,h** display similar activity. The calmodulin antagonism drops off sharply at $n = 10$ (**1i**) to that of the parent compound **1a**.

Selected compounds in the dimethylamino series **2a–i** display a similar pattern with the C_8 compound **2g** being the most potent ($IC_{50} = 0.30$ μM). All the compounds in this series displayed similar or worse activity compared with their pyrrolidino counterparts.

Elongation of the basic side chain has been seen to produce an increase in potency in the naphthylsulfon-

Table 1. Antagonism of Calmodulin-Dependent cAMP Phosphodiesterase and Receptor Binding Affinities for Homologs of Idoxifene (**1a**)

compound	n	antagonism of CaM-dependent PDE,[a] $IC_{50} \pm SE$ (μM)	potency relative to parent compound[b]	binding affinity for ER
1a	2	1.5 ± 0.1	1	12
1b	3	1.1 ± 0.1	1.4	23
1c	4	1.0 ± 0.1	1.5	9
1d	5	0.8 ± 0.08	1.9	8
1e	6	0.5 ± 0.05	3.0	4
1f	7	0.2 ± 0.05	7.5	3
1g	8	0.2 ± 0.05	7.5	3.5
1h	9	0.2 ± 0.05	7.5	1.5
1i	10	1.6 ± 0.1	0.9	0.4

[a] None of the compounds gave significant inhibition of the calmodulin-independent activity of cAMP PDE when assayed at final concentrations of 10 and 20 μM. [b] Relative potency = IC_{50}(parent)/IC_{50}(compound).

Table 2. Antagonism of Calmodulin-Dependent cAMP Phosphodiesterase and Receptor Binding Affinities for Homologs of 4-Iodotamoxifen (**2a**)

| compound	n	antagonism of CaM-dependent PDE,[a] $IC_{50} \pm SE$ (μM)	potency relative to parent compound[b]	binding affinity for ER
2a	2	2.3 ± 0.4	1	8
2b	3	2.0 ± 0.2	1.2	8
2c	4	2.2 ± 0.2	1.1	25
2d	5	0.6 ± 0.05	3.8	6
2e	6	0.4 ± 0.05	5.8	8
2g	8	0.3 ± 0.05	7.7	2
2i	10	1.2 ± 0.1	1.9	0.4

[a] None of the compounds gave significant inhibition of the calmodulin-independent activity of cAMP PDE when assayed at final concentrations of 10 and 20 μM. [b] Relative potency = IC_{50}(parent)/IC_{50}(compound).

amide antagonists **7** including the calmodulin antagonist W-7.[23,24] In the case of the iodo-substituted compounds, a jump in activity was seen from $n = 10$ ($IC_{50} = 4$ μM) to $n = 12$ ($IC_{50} = 0.7$ μM), although no explanation was offered.
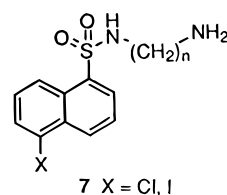
Examination of the computer model of calmodulin bound with **1a**[1] does not readily explain why such a dramatic increase in potency is seen from C_6 to C_{7-9} or why the activity should be much lower for C_{10}. However, recent studies in which calmodulin is crystallized with a peptide ligand, such as the calmodulin-binding

domain of smooth muscle myosin light chain kinase[17,25] or protein kinase IIa,[18] or the drug trifluoperazine[20,21] have shown calmodulin in a more compact globular structure with the ligand enclosed by the N- and C-terminal lobes. It seems likely that the binding of **1a** or **2a** and their homologs to calmodulin would produce a similar conformational change in the protein. Molecular modeling and crystallographic studies are underway in our laboratories to provide detailed structural information which can be used to rationalize these interactions.

Estrogen receptor binding affinity for both series drops with extending chain length beyond C_6. However, the most potent calmodulin antagonists (**1f**−**h** and **2g**) still have RBA values similar to that of **3**. This suggests that these compounds may be capable of antagonizing both estrogen- and calmodulin-dependent processes *in vivo*. Receptor binding affinity is lost at C_{10} (**1i** and **2i**) in both series.

Conclusions

Extension of the homologous series for **1a** and **2a** has revealed that elongation of the basic side chain results in greater antagonistic potency for calmodulin while the estrogen receptor binding affinity falls. The C_{7-9} homologs in both series (**1f**−**h** and **2g**) are potent calmodulin antagonists ($IC_{50} = 0.2$ μM) and have similar estrogen receptor binding affinities to **3** (RBA ∼ 2). These compounds represent a new class of antiestrogens which are potent calmodulin antagonists. They will be useful in elucidating the complex interactions of calmodulin and the estrogen receptor and may be novel therapeutic agents for the treatment of cancer.

Experimental Section

Chemical Methods. General Procedures. ^{1}H NMR spectra (internal Me₄Si) were obtained with a Bruker AC250 instrument. Melting points were obtained on a Reichert hotstage and are uncorrected. Chromatography refers to flash column chromatography on silica gel (Merck 15111) with the eluant indicated applied at a positive pressure of 0.5 atm. All reactions performed under an inert atmosphere were carried out in oven-dried glassware (110 °C, 24 h). Ether refers to diethyl ether. Hexane for chromatography was purchased from Romil (super purity grade). Anhydrous tetrahydrofuran (THF) was obtained by distillation from potassium and benzophenone. Purification of *E* and *Z* geometrical isomers was monitored by ^{1}H NMR spectroscopy and was carried out until none of the undesired isomer could be detected. Elemental analyses were determined by CHN Analysis Ltd., South Wigston, Leicester, England.

General Method for Preparation of 1-Chloro-ω-phenoxyalkanes. A two-phase mixture of phenol (5 g, 53 mmol), α,ω-dihaloalkane (30 mL), tetrabutylammonium hydrogen sulfate (0.3 g, 1 mmol), and 3 M NaOH (25 mL) was heated to reflux for 16 h. The mixture was diluted with ether (50 mL) and washed with HCl (1 M, 30 mL) and water (2 × 25 mL). The ether layer was dried (MgSO₄) and concentrated. Chromatography (CH₂Cl₂−hexane, 1:10) gave the products as colorless oils.

1-Chloro-5-phenoxypentane (4d): 9.32 g, 94%; bp 110 °C (0.1 mmHg); ^{1}H NMR (CDCl₃) δ 1.55−1.91 (m, 6, CH₂(C*H₂*)₃-CH₂), 3.56 (t, *J* = 6.7 Hz, 2, ClC*H₂*), 3.95 (t, *J* = 6.3 Hz, 2, OC*H₂*), 6.87−6.96 (m, 3, Ar*H*), 7.24−7.31 (m, 2, Ar*H*); MS (EI) *m/z* 198 (M⁺, 65).

1-Chloro-6-phenoxyhexane (4e): 9.21 g, 82%; bp 130 °C (0.1 mmHg); ^{1}H NMR (CDCl₃) δ 1.43−1.83 (m, 8, CH₂(C*H₂*)₄-CH₂), 3.54 (t, *J* = 6.7 Hz, 2, ClC*H₂*), 3.95 (t, *J* = 6.4 Hz, 2, OC*H₂*), 6.85−6.96 (m, 3, Ar*H*), 7.22−7.31 (m, 2, Ar*H*); MS (EI) *m/z* 212 (M⁺, 15).

1-Chloro-8-phenoxyoctane (4g): 9.20 g, 72%; bp 180 °C (0.3 mmHg); ^{1}H NMR (CDCl₃) δ 1.28−1.85 (m, 12, CH₂(C*H₂*)₆-CH₂), 3.54 (t, *J* = 6.4 Hz, 2, ClC*H₂*), 3.94 (t, *J* = 6.4 Hz, 2, OC*H₂*), 6.86−6.92 (m, 3, Ar*H*), 7.20−7.31 (m, 2, Ar*H*); MS (EI) *m/z* 240 (M⁺, 35).

1-Chloro-9-phenoxynonane (4h): 4.6 g, 61%; bp 175 °C (0.4 mmHg); ^{1}H NMR (CDCl₃) δ 1.26−1.49 (10, m, O(CH₂)₂-(C*H₂*)₅(CH₂)₂Cl), 1.71−1.83 (m, 4, OCH₂C*H₂*(CH₂)₅C*H₂*CH₂Cl), 3.53 (t, *J* = 6.4 Hz, 2, ClC*H₂*), 3.95 (2, t, *J* = 6.4 Hz, OC*H₂*), 6.88−6.96 (m, 3, Ar*H*), 7.24−7.31 (m, 2, Ar*H*); MS (EI) *m/z* 254 (M⁺ − 1, 50).

1-Chloro-10-phenoxydecane (4i): 9.8 g, 69%; bp 185 °C (0.2 mmHg); ^{1}H NMR (CDCl₃) δ 1.2−1.8 (m, 16, OCH₂(C*H₂*)₈-CH₂Cl), 3.51 (t, *J* = 6.4 Hz, 2, ClC*H₂*), 3.93 (t, *J* = 6.4 Hz, 2, OC*H₂*), 6.82−6.94 (m, 3, Ar*H*), 7.20−7.30 (m, 2, Ar*H*); MS (EI) *m/z* 268 (M⁺, 5).

1-Bromo-7-phenoxyheptane (4f). A mixture of phenol (1.7 g, 10 mmol), 1,7-dibromoheptane (10 g, 39 mmol), NaOH solution (1 M, 20 mL), and tetrabutylammonium hydrogen sulfate (0.05 g) was refluxed for 16 h and then diluted with ether (50 mL) and partitioned. The ether layer was washed with HCl (1 M, 25 mL) and water (25 mL), dried (Na₂SO₄), and concentrated. Distillation gave **4f** as a colorless oil (2.38 g, 90%): bp 180 °C (0.2 mmHg); ^{1}H NMR (CDCl₃) δ 1.21−1.54 and 1.73 −1.93 (m, 10, CH₂(C*H₂*)₅CH₂), 3.42 (t, *J* = 6.4 Hz, 2, C*H₂*Br), 3.95 (t, *J* = 6.4 Hz, 2, PhOC*H₂*), 6.87−6.96 (m, 2, Ar*H*), 7.24−7.32 (m, 3, Ar*H*); MS (EI) *m/z* 270 (M⁺ − 1, 20).

General Preparation of 1-[4-(ω-Chloroalkoxy)phenyl]-2-phenyl-1-butanones 5d−i. To a stirred solution of 2-phenylbutyric acid (20 mmol) and trifluoroacetic anhydride (5 mL) was added (ω-haloalkoxy)benzene **4d−i** (24 mmol). The resulting mixture was stirred for 16 h and then poured into saturated aqueous NaHCO₃ solution (30 mL) and extracted with ether (30 mL). The organic extracts were washed with water (2 × 30 mL), dried (MgSO₄), and concentrated.

1-[4-[(5-Chloropentyl)oxy]phenyl]-2-phenyl-1-butanone (5d). Crystallization (hexane) gave **5d** as fine white needles (5.49 g, 80%): mp 60−61 °C; ^{1}H NMR (CDCl₃) δ 0.87 (t, *J* = 7.5 Hz, 3, CH₂C*H₃*), 1.56−1.90 (m, 7, CH₂(C*H₂*)₃CH₂, part of CHOC*H₂*), 2.11−2.22 (m, 1, part of CHOC*H₂*), 3.54 (t, *J* = 6.4 Hz, 2, ClC*H₂*), 3.96 (t, *J* = 6.4 Hz, 2, OC*H₂*), 4.38 (t, *J* = 7.3 Hz, 1, C*H*OCH₂), 6.82 (d, *J* = 9.0 Hz, 2, Ar*H ortho* to OCH₂), 7.13−7.28 (m, 5, Ph*H*), 7.93 (d, *J* = 8.8 Hz, 2, Ar*H meta* to OCH₂). Anal. (C₂₁H₂₅O₂Cl) C, H, Cl.

1-[4-[(6-Chlorohexyl)oxy]phenyl]-2-phenyl-1-butanone (5e). Chromatography (CH₂Cl₂−hexane, 1:5) gave **5e** as white amorphous crystals (6.00 g, 84%): mp 43−45 °C (hexane); ^{1}H NMR (CDCl₃) δ 0.87 (t, *J* = 7.4 Hz, 3, CH₂C*H₃*), 1.45−1.87 (m, 9, CH₂(C*H₂*)₄CH₂, part of CHOC*H₂*), 2.11−2.22 (m, 1, part of CHOC*H₂*), 3.52 (t, *J* = 7.7 Hz, 2, ClC*H₂*), 3.95 (t, *J* = 6.4 Hz, 2, OC*H₂*), 4.38 (t, *J* = 7.3 Hz, 1, C*H*OCH₂), 6.82 (d, *J* = 7.9 Hz, 2, Ar*H ortho* to OCH₂), 7.16−7.28 (m, 5, Ph*H*), 7.93 (d, *J* = 9.0 Hz, 2, Ar*H meta* to OCH₂); MS (CI) *m/z* 359 (M⁺, 12). Anal. (C₂₂H₂₇O₂Cl) C, H, Cl.

1-[4-[(10-Chlorodecyl)oxy]phenyl]-2-phenyl-1-butanone (5i). Chromatography (CH₂Cl₂−petroleum ether, 1:5) gave **5i** as white crystals (5.83 g, 70%): mp 42−44 °C (hexane); ^{1}H NMR (CDCl₃) δ 0.89 (t, *J* = 7.3 Hz, 3, CH₂C*H₃*), 1.20−1.92 (m, 17, CH₂(C*H₂*)₈CH₂, part of CHOC*H₂*), 2.13−2.27 (m, 1, part of CHOC*H₂*), 3.53 (t, *J* = 6.6 Hz, 2, ClC*H₂*), 3.96 (t, *J* = 6.5 Hz, 2, OC*H₂*), 4.39 (t, *J* = 7.3 Hz, 1, C*H*OCH₂), 6.84 (d, *J* = 8.8 Hz, 2, Ar*H ortho* to OCH₂), 7.18−7.32 (m, 5, Ph*H*), 7.92 (d, *J* = 8.9 Hz, 2, Ar*H meta* to OCH₂); MS (CI) *m/z* 415 (M⁺, 72). Anal. (C₂₆H₃₅O₂Cl) C, H, Cl.

1-[4-[(8-Chlorooctyl)oxy]phenyl]-2-phenyl-1-butanone (5g). 2-Phenylbutyric acid (7.48 g, 46 mmol), **4g** (9.1 g, 38 mmol), and trifluoroacetic anhydride (7.5 mL, 52 mmol) were treated as in the general procedure. Chromatography (CH₂Cl₂−petroleum ether, 1:5) gave **5g** as white crystals (11.86 g, 81%): mp 56−57 °C (hexane); ^{1}H NMR (CDCl₃) δ 0.87 (t, *J* = 7.3 Hz, 3, CH₂C*H₃*), 1.26−1.50 and 1.69−1.90 (m, 14, CH₂(C*H₂*)₆CH₂Cl, part of C*H₂*CH₃), 2.08−2.26 (m, 1, part of C*H₂*CH₃), 3.51 (t, *J* = 6.6 Hz, 2, (CH₂)₆C*H₂*Cl), 3.94 (t, *J* = 6.6 Hz, 2, OC*H₂*(CH₂)₆), 4.38 (t, *J* = 7.3 Hz, 1, C*H*CO), 6.83 (d, *J* = 8.8 Hz, 2, Ar*H meta* to OCH₂), 7.15−7.20 (m, 1, Ph*H*), 7.20−

7.28 (m, 4, Ph*H*), 7.93 (d, *J* = 8.8 Hz, 2, Ar*H ortho* to OCH$_2$); IR (liquid, cm^{-1}) 2933, 2858, 1673, 1600, 1574, 1510; MS (CI) *m/z* 387 (M$^+$ + 1, 10). Anal. (C$_{24}$H$_{31}$O$_2$Cl) C, H, Cl.

1-[4-[(9-Chlorononyl)oxy]phenyl]-2-phenyl-1-butanone (5h). 2-Phenylbutyric acid (3.28 g, 20 mmol), **4h** (4.60 g, 18 mmol), and trifluoroacetic anhydride (20 mL) were treated as above. Chromatography (CH$_2$Cl$_2$–petroleum ether, 3:10) gave **5h** as white crystals (5.83 g, 70%): mp 42–44 °C (hexane); ^{1}H NMR (CDCl$_3$) δ 0.89 (t, *J* = 7.4 Hz, 3, CH$_2$C*H*$_3$), 1.22–1.50 and 1.71–1.89 (m, 15, CH$_2$(C*H*$_2$)$_7$CH$_2$Cl, part of C*H*$_2$-CH$_3$), 2.13–2.24 (m, 1, part of C*H*$_2$CH$_3$), 3.53 (t, *J* = 6.8 Hz, C*H*$_2$Cl), 3.96 (t, *J* = 6.5 Hz, OC*H*$_2$), 4.40 (t, *J* = 7.3 Hz, 1, C*H*CO), 6.84 (d, *J* = 8.8 Hz, 2, Ar*H meta* to OCH$_2$), 7.17–7.32 (m, 5, Ph), 7.95 (d, *J* = 9.0 Hz, 2, Ar*H ortho* to OCH$_2$); MS (CI) *m/z* 401 (M$^+$, 15). Anal. (C$_{25}$H$_{33}$O$_2$Cl) C, H, Cl.

1-[4-[(7-Bromoheptyl)oxy]phenyl]-2-phenyl-1-butanone (5f). 2-Phenylbutyric acid (2.02 g, 10 mmol), **4f** (1.70 g, 6.3 mmol), and trifluoroacetic anhydride (10 mL) were treated as above. Chromatography (CH$_2$Cl$_2$–petroleum ether, 1:4) gave **5f** as a colorless oil (2.85 g, 77%): mp 41–43 °C (hexane); ^{1}H NMR (CDCl$_3$) δ 0.89 (m, 3, CH$_2$C*H*$_3$), 1.25–1.51 and 1.69–1.92 (m, 10, OCH$_2$(C*H*$_2$)$_5$CH$_2$Br), 2.03–2.27 (m, 2, C*H*$_2$CH$_3$), 3.41 (t, *J* = 6.8 Hz, 2, C*H*$_2$Br), 3.96 (t, *J* = 6.5 Hz, OC*H*$_2$), 4.40 (t, *J* = 7.2 Hz, 1, COC*H*), 6.84 (d, *J* = 8.9 Hz, 2, Ar*H ortho* to OCH$_2$), 7.15–7.35 (m, 5, Ph*H*), 7.95 (d, *J* = 8.8 Hz, 2, Ar*H meta* to OCH$_2$); MS (EI) *m/z* 418 (M$^+$, 100); accurate mass (C$_{23}$H$_{29}$O$_2$Br) found 417.1423, calcd 417.1419.

General Preparation of (E)-1-[4-(ω-Chloroalkoxy)-phenyl]-1-(4-iodophenyl)-2-phenyl-1-butenes 6d–i. To a solution of 1,4-diiodobenzene (3.629 g, 11 mmol) in THF (30 mL) was added *n*-butyllithium (1.6 M in hexane, 6.9 mL, 11 mmol) at −78 °C. The resulting mixture was stirred for 10 min; then a solution of the ketone **5d–i** (11 mmol) in THF (20 mL) was added, and stirring continued at −78 °C for 1 h followed by 16 h at ambient temperature. Ammonium chloride solution (2 mL) was added; the mixture was diluted with ether (100 mL) and then washed with brine (100 mL) and water (2 × 100 mL). The organic layer was dried (MgSO$_4$) and concentrated. The residues were dissolved in ethanol (30 mL) and HCl (30%, 10 mL). The mixture was heated to reflux for 90 min, allowed to cool, diluted with ether (50 mL), and washed with water (50 mL), aqueous sodium thiosulfate solution (5 M, 50 mL), and water (50 mL). The organic layer was dried (MgSO$_4$) and concentrated.

(E)-1-[4-[(5-Chloropentyl)oxy]phenyl]-1-(4-iodophenyl)-2-phenyl-1-butene (6d). Chromatography (CH$_2$Cl$_2$–hexane, 1:5) followed by fractional crystallization (ethanol) gave **6d** as white crystals (2.402 g, 47%): mp 94–96 °C; ^{1}H NMR (CDCl$_3$) δ 0.89 (t, *J* = 7.5 Hz, 3, CH$_2$C*H*$_3$), 1.50–1.82 (m, 6, CH$_2$(C*H*$_2$)$_3$-CH$_2$), 2.42 (q, *J* = 7.4 Hz, 2, C*H*$_2$CH$_3$), 3.52 (t, *J* = 6.6 Hz, 2, C*H*$_2$Cl), 3.81 (t, *J* = 6.2 Hz, 2, C*H*$_2$O), 6.51 (d, *J* = 8.7 Hz, 2, Ar*H ortho* to OCH$_2$), 6.71 (d, *J* = 8.5 Hz, 2, Ar*H meta* to OCH$_2$), 6.97 (d, *J* = 8.4 Hz, 2, Ar*H meta* to I), 7.07–7.19 (m, 5, Ph*H*), 7.65 (d, *J* = 8.4 Hz, 2, Ar*H ortho* to I). Anal. (C$_{27}$H$_{28}$OClI) C, H, Cl, I.

(E)-1-[4-[(6-Chlorohexyl)oxy]phenyl]-1-(4-iodophenyl)-2-phenyl-1-butene (6e). Chromatography (CH$_2$Cl$_2$–hexane, 1:5) followed by fractional crystallization (ethanol) gave **6e** as white crystals (2.903 g, 53%): mp 58–62 °C; ^{1}H NMR (CDCl$_3$) δ 0.89 (t, *J* = 7.5 Hz, 3, CH$_2$C*H*$_3$), 1.36–1.80 (m, 8, CH$_2$(C*H*$_2$)$_4$-CH$_2$), 2.41 (q, *J* = 7.5 Hz, 2, C*H*$_2$CH$_3$), 3.51 (t, *J* = 6.4 Hz, 2, C*H*$_2$Cl), 3.80 (t, *J* = 6.4 Hz, 2, C*H*$_2$O), 6.51 (d, *J* = 9.0 Hz, 2, Ar*H ortho* to OCH$_2$), 6.71 (d, *J* = 9.0 Hz, 2, Ar*H meta* to OCH$_2$), 6.97 (d, *J* = 8.5 Hz, 2, Ar*H meta* to I), 7.06–7.20 (m, 5, Ph*H*), 7.64 (d, *J* = 8.5 Hz, 2, Ar*H ortho* to I); MS (FAB) *m/z* 545 (M$^+$, 100). Anal. (C$_{28}$H$_{30}$OClI) C, H, N, Cl, I.

(E)-1-[4-[(7-Bromoheptyl)oxy]phenyl]-1-(4-iodophenyl)-2-phenyl-1-butene (6f). Chromatography (CH$_2$Cl$_2$–hexane, 1:10) followed by fractional crystallization (ethanol) gave **6f** as white crystals (0.197 g, 9%): mp 54–57 °C; ^{1}H NMR (CDCl$_3$) δ 0.91 (t, *J* = 7.3 Hz, 3, CH$_2$C*H*$_3$), 1.30–1.51 and 1.62–1.97 (m, 10, CH$_2$(C*H*$_2$)$_5$CH$_2$), 2.44 (q, *J* = 7.3 Hz, 2, C*H*$_2$CH$_3$), 3.40 (t, *J* = 8.6 Hz, 2, C*H*$_2$Br), 3.81 (t, *J* = 8.6 Hz, 2, OC*H*$_2$), 6.53 (d, *J* = 8.8 Hz, 2, Ar*H meta* to OCH$_2$), 6.73 (d, *J* = 9 Hz, 2, Ar*H meta* to OCH$_2$), 6.99 (d, *J* = 8.5 Hz, 2, Ar*H meta* to I), 7.12–7.25 (m, 5, Ph*H*), 7.67 (d, *J* = 8.5 Hz, 2, Ar*H ortho* to I);

MS (EI) *m/z* 604 (M$^+$, 10); accurate mass (C$_{29}$H$_{32}$O^{79}BrI) found 602.0681, calcd 602.0686.

(E)-1-[4-[(8-Chlorooctyl)oxy]phenyl]-1-(4-iodophenyl)-2-phenyl-1-butene (6g). 1,4-Diiodobenzene (9.9 g, 30 mmol) in THF (100 mL), *n*-butyllithium (1.6 M in hexane, 18.75 mL, 30 mmol), and **5g** (11.18 g, 29 mmol) in tetrahydrofuran (50 mL) were treated as in the general procedure. Chromatography (CH$_2$Cl$_2$–hexane, 1:10) followed by fractional crystallization gave **6g** as white crystals (5.35 g, 31%): mp 55–58 °C; ^{1}H NMR (CDCl$_3$) δ 0.89 (t, *J* = 7.5 Hz, 3, CH$_2$C*H*$_3$), 1.26–1.46 and 1.60–1.80 (m, 12, CH$_2$(C*H*$_2$)$_6$CH$_2$), 2.415 (q, *J* = 7.4 Hz, 2, C*H*$_2$CH$_3$), 3.50 (t, *J* = 6.6 Hz, 2, C*H*$_2$Cl), 3.79 (t, *J* = 6.6 Hz, 2, OC*H*$_2$), 6.505 (d, *J* = 8.7 Hz, 2, Ar*H meta* to OCH$_2$), 6.705 (d, *J* = 8.7 Hz, 2, Ar*H meta* to OCH$_2$), 6.965 (d, *J* = 8.4 Hz, 2, 7.07–7.19 (m, 5, Ph*H*), 7.65 (d, *J* = 8.2 Hz, Ar*H ortho* to I); *υ*$_{max}$ (film) 2931, 2857, 1606, 1509; MS (CI) *m/z* 574 (M$^+$ + 1, 10). Anal. (C$_{30}$H$_{34}$OClI) C, H, Cl, I.

(E)-1-[4-[(9-Chlorononyl)oxy]phenyl]-1-(4-iodophenyl)-2-phenyl-1-butene (6h). Chromatography (CH$_2$Cl$_2$–hexane, 1:5) followed by fractional crystallization (hexane) gave **6h** as white crystals (1.96 g, 30%): mp 73–75 °C; ^{1}H NMR (CDCl$_3$) δ 0.91 (t, *J* = 7.5 Hz, 3, CH$_2$C*H*$_3$), 1.25–1.88 (m, 14, CH$_2$(C*H*$_2$)$_7$-CH$_2$), 2.43 (q, *J* = 7.4 Hz, 2, C*H*$_2$CH$_3$), 3.52 (t, *J* = 6.7 Hz, 2, C*H*$_2$Cl), 3.81 (t, *J* = 6.6 Hz, 2, C*H*$_2$O), 6.53 (d, *J* = 8.8 Hz, 2, Ar*H ortho* to OCH$_2$), 6.73 (d, *J* = 8.8 Hz, 2, Ar*H meta* to OCH$_2$), 6.98 (d, *J* = 8.3 Hz, 2, Ar*H meta* to I), 7.09–7.21 (m, 5, Ph*H*), 7.67 (d, *J* = 8.5 Hz, 2, Ar*H ortho* to I); MS (EI) *m/z* 586 (M$^+$ − 1, 100). Anal. (C$_{31}$H$_{36}$OClI) H, Cl, I; C: calcd, 63.43; found, 64.11.

(E)-1-[4-[(10-Chlorodecyl)oxy]phenyl]-1-(4-iodophenyl)-2-phenyl-1-butene (6i). Chromatography (20% CH$_2$Cl$_2$, hexane) followed by fractional crystallization (ethanol) gave **6i** as white crystals (2.35 g, 35%): mp 75–78 °C; ^{1}H NMR (CDCl$_3$) δ 0.89 (t, *J* = 7.5 Hz, 3, CH$_2$C*H*$_3$), 1.26–1.77 (m, 16, CH$_2$(C*H*$_2$)$_8$-CH$_2$), 2.42 (q, *J* = 7.4 Hz, 2, C*H*$_2$CH$_3$), 3.51 (t, *J* = 6.8 Hz, 2, C*H*$_2$Cl), 3.79 (t, *J* = 6.6 Hz, 2, C*H*$_2$O), 6.51 (d, *J* = 8.5 Hz, 2, Ar*H ortho* to OCH$_2$), 6.70 (d, *J* = 8.7 Hz, 2, Ar*H meta* to OCH$_2$), 6.97 (d, *J* = 8.4 Hz, 2, Ar*H meta* to I), 7.07–7.15 (m, 5, Ph*H*), 7.65 (d, *J* = 8.5 Hz, 2, Ar*H ortho* to I). Anal. (C$_{32}$H$_{38}$OClI) C, H, Cl, I.

General Procedure for Preparation of (E)-1-[4-(ω-N-Pyrrolidinoalkoxy)phenyl]-1-(4-iodophenyl)-2-phenyl-1-butenes 1d–i. A mixture of butene **6d–i** (1 mmol), pyrrolidine (2 mL), and ethanol (10 mL) was heated in a bomb at 100 °C for 4 h and then concentrated. Chromatography (ether) gave the title compounds.

(E)-1-[4-[(5-N-Pyrrolidinopentyl)oxy]phenyl]-1-(4-iodophenyl)-2-phenyl-1-butene (1d): white crystals (0.60 g, 98%); mp 88–91 °C; ^{1}H NMR (CDCl$_3$) δ 0.89 (t, *J* = 7.5 Hz, 3, CH$_2$C*H*$_3$), 1.18–1.80 and 2.37–2.57 (m, 16, CH$_2$(C*H*$_2$)$_3$C*H*$_2$N, N(C*H*$_2$C*H*$_2$)$_2$), 3.79 (t, *J* = 6.5 Hz, 2, OCH$_2$), 6.50 (d, *J* = 8.7 Hz, 2, Ar*H ortho* to OCH$_2$), 6.70 (d, *J* = 8.7 Hz, 2, Ar*H meta* to OCH$_2$), 6.97 (d, *J* = 8.3 Hz, 2, Ar*H meta* to I), 7.07–7.19 (m, 5, Ph*H*), 7.65 (d, *J* = 8.2 Hz, 2, Ar*H ortho* to I); MS *m/z* 565 (M$^+$, 35). Anal. (C$_{31}$H$_{36}$NOI) C, H, N, I.

(E)-1-[4-[(6-N-Pyrrolidinohexyl)oxy]phenyl]-1-(4-iodophenyl)-2-phenyl-1-butene (1e): off-white crystals (0.56 g, 96%); mp 38–42 °C; ^{1}H NMR (CDCl$_3$) δ 0.91 (t, *J* = 7.4 Hz, 3, CH$_2$C*H*$_3$), 1.63–1.79 and 2.37–2.50 (m, 20, N(C*H*$_2$C*H*$_2$)$_2$, C*H*$_2$-CH$_3$, CH$_2$(C*H*$_2$)$_4$C*H*$_2$N), 3.81 (t, *J* = 6.5 Hz, 2, OC*H*$_2$), 6.52 (d, *J* = 8.8 Hz, 2, Ar*H ortho* to OCH$_2$), 6.72 (d, *J* = 8.8 Hz, 2, Ar*H meta* to OCH$_2$), 6.98 (d, *J* = 8.5 Hz, 2, Ar*H meta* to I), 7.08–7.16 (m, 5, Ph*H*), 7.66 (d, *J* = 8.3 Hz, 2, Ar*H ortho* to I); MS *m/z* 579 (M$^+$, 80). Anal. (C$_{32}$H$_{38}$NOI) C, H, N; I: calcd, 22.93; found, 22.11.

(E)-1-[4-[(7-N-Pyrrolidinoheptyl)oxy]phenyl]-1-(4-iodophenyl)-2-phenyl-1-butene (1f): white crystals (0.136 g, 91%); mp 45–50 °C; ^{1}H NMR (CDCl$_3$) δ 0.85–0.95, 1.26–1.88 and 2.36–2.47 (m, 25, N(C*H*$_2$C*H*$_2$)$_2$, C*H*$_2$CH$_3$, CH$_2$(C*H*$_2$)$_5$C*H*$_2$N), 3.80 (t, *J* = 6.5 Hz, 2, OC*H*$_2$), 6.52 (d, *J* = 8.8 Hz, 2, Ar*H ortho* to OCH$_2$), 6.72 (d, *J* = 8.7 Hz, 2, Ar*H meta* to OCH$_2$), 6.98 (d, *J* = 8.2 Hz, 2, Ar*H meta* to I), 7.11–7.27 (m, 5, Ph*H*), 7.66 (d, *J* = 8.3 Hz, 2, Ar*H ortho* to I); MS (FAB) *m/z* 594 (M$^+$, 100); accurate mass (C$_{33}$H$_{42}$NOI) found 595.2816, calcd 595.2811.

(E)-1-[4-[(8-N-Pyrrolidinooctyl)oxy]phenyl]-1-(4-iodophenyl)-2-phenyl-1-butene (1g). A mixture of butene **6g**

(2.0 g, 3.5 mmol), pyrrolidine (15 mL), and ethanol (75 mL) was heated in a bomb at 100 °C for 4 h and then concentrated. Chromatography (ether) gave **1g** as a slightly brown oil (1.92 g, 90%); recrystallization (methanol) gave the title compound as off-white crystals: mp 55−58 °C; ^{1}H NMR (CDCl$_3$) δ 0.89 (t, J = 7.4 Hz, 3, CH$_2$CH$_3$), 1.22−1.80 and 1.60−1.80 (m, 16, CH$_2$(CH$_2$)$_6$CH$_2$, N(CH$_2$CH$_2$)$_2$), 2.31−2.50 (m, 8, N(CH$_2$CH$_2$)$_2$, CH$_2$CH$_3$, NCH$_2$), 3.78 (t, J = 6.5 Hz, 2, OCH$_2$), 6.51 (d, J = 8.7 Hz, 2, ArH *ortho* to OCH$_2$), 6.705 (d, J = 8.6 Hz, 2, ArH *meta* to OCH$_2$), 6.965 (d, J = 8.2 Hz, ArH *meta* to I), 7.07−7.19 (m, 5, PhH), 7.645 (d, J = 8.2 Hz, 2, ArH *ortho* to I); m/z (EI) 608 (M$^+$, 20). Anal. (C$_{34}$H$_{42}$NOI) C, H, N, I.

(*E*)-1-[4-[(9-*N*-Pyrrolidinononyl)oxy]phenyl]-1-(4-iodophenyl)-2-phenyl-1-butene (1h): off-white crystals (0.588 g, 96%); mp 69−71 °C; ^{1}H NMR (CDCl$_3$) δ 0.91 (t, J = 7.4 Hz, 3, CH$_2$CH$_3$), 1.28−1.80 (m, 18, N(CH$_2$CH$_2$)$_2$, CH$_2$(CH$_2$)$_7$CH$_2$), 2.37−2.48 (m, 8, N(CH$_2$CH$_2$)$_2$, CH$_2$CH$_3$, CH$_2$N), 3.81(t, J = 6.5 Hz, 2, OCH$_2$), 6.53 (d, J = 8.7 Hz, 2, ArH *ortho* to OCH$_2$), 6.73 (d, J = 8.8 Hz, 2, ArH *meta* to OCH$_2$), 6.98 (d, J = 8.5 Hz, 2, ArH *meta* to I), 7.08−7.15 (m, 5, PhH), 7.66 (d, J = 8.4 Hz, 2, ArH *ortho* to I); MS m/z 621 (M$^+$, 20). Anal. (C$_{35}$H$_{44}$NOI) C, H, N, I.

(*E*)-1-[4-[(10-*N*-Pyrrolidinodecyl)oxy]phenyl]-1-(4-iodophenyl)-2-phenyl-1-butene (1i): white needles (0.579 g, 91%); mp 75−76 °C (MeOH); ^{1}H NMR (CDCl$_3$) δ 0.90 (t, J = 7.3 Hz, 3, CH$_2$CH$_3$), 1.21−1.81 (m, 20, N(CH$_2$CH$_2$)$_2$, CH$_2$(CH$_2$)$_8$CH$_2$), 2.40−2.52 (m, 8, N(CH$_2$CH$_2$)$_2$, CH$_2$CH$_3$, CH$_2$N), 3.80 (t, J = 6.5 Hz, 2, OCH$_2$), 6.53 (d, J = 8.2 Hz, 2, ArH *ortho* to OCH$_2$), 6.72 (d, J = 8.5 Hz, 2, ArH *meta* to OCH$_2$), 6.98 (d, J = 7.7 Hz, 2, ArH *meta* to I), 7.08−7.17 (m, 5, PhH), 7.66 (d, J = 7.6 Hz, 2, ArH *ortho* to I); MS m/z 636 (M$^+$, 100). Anal. (C$_{36}$H$_{46}$NOI) C, H, N, I.

General Procedure for Preparation of (*E*)-1-[4-[ω-(*N*-Dimethylamino)alkoxy]phenyl]-1-(4-iodophenyl)-2-phenyl-1-butenes 2d−i. A mixture of butene **6d−i** (1 mmol) and dimethylamine (30% in ethanol, 20 mL) was heated in a bomb at 100 °C for 4 h and then concentrated. Chromatography (methanol−ether, 1:20) gave **2d−i**.

(*E*)-1-[4-[[5-(*N*-Dimethylamino)pentyl]oxy]phenyl]-1-(4-iodophenyl)-2-phenyl-1-butene (2d): colorless oil (0.412 g, 76%); ^{1}H NMR (CDCl$_3$) δ 0.89 (t, J = 7.3 Hz, 3, CH$_2$CH$_3$), 1.40−1.86 (m, 8, N(CH$_2$)$_4$CH$_2$), 2.23 (s, 6, (CH$_3$)$_2$N), 2.42 (q, J = 6.5 Hz, 2, CH$_2$CH$_3$), 3.80 (t, J = 6.4 Hz, 2, OCH$_2$), 6.50 (d, J = 8.9 Hz, 2, ArH *ortho* to OCH$_2$), 6.70 (d, J = 8.8 Hz, 2, ArH *meta* to OCH$_2$), 6.96 (d, J = 8.2 Hz, 2, ArH *meta* to I), 7.06−7.19 (m, 5, PhH), 7.64 (d, J = 8.3 Hz, 2, ArH *ortho* to I); MS m/z 539 (M$^+$, 100). Anal. (C$_{29}$H$_{34}$NOI) H, N, I; C: calcd, 64.56; found, 64.11.

(*E*)-1-[4-[[6-(*N*-Dimethylamino)hexyl]oxy]phenyl]-1-(4-iodophenyl)-2-phenyl-1-butene (2e): colorless oil (0.515 g, 93%); ^{1}H NMR (CDCl$_3$) δ 0.91 (t, J = 7.4 Hz, 3, CH$_2$CH$_3$), 1.15−1.52 and 1.68 (m, brs, 10, N(CH$_2$)$_5$CH$_2$), 2.21 (s, 6, N(CH$_3$)$_2$), 3.81 (t, J = 6.4 Hz, 2, OCH$_2$), 6.53 (d, J = 8.8 Hz, 2, ArH *ortho* to OCH$_2$), 6.72 (d, J = 8.8 Hz, 2, ArH *meta* to OCH$_2$), 6.98 (d, J = 8.5 Hz, 2, ArH *meta* to I), 7.09−7.21 (m, 5, PhH), 7.67 (d, J = 8.3 Hz, 2, ArH *ortho* to I); MS m/z 553 (M$^+$, 100). Anal. (C$_{30}$H$_{36}$NOI) C, H, N; I: calcd, 22.93; found 22.11.

(*E*)-1-[4-[[8-(*N*-Dimethylamino)octyl]oxy]phenyl]-1-(4-iodophenyl)-2-phenyl-1-butene (2g). **6g** (0.51 g, 0.89 mmol) gave **2g** as off-white crystals (0.446 g, 86%): mp 65−68 °C; ^{1}H NMR (CDCl$_3$) δ 0.91 (t, J = 7.4 Hz, 3, CH$_2$CH$_3$), 1.21−1.83 (m, 12, part of CH$_2$(CH$_2$)$_6$CH$_2$, N(CH$_3$)$_2$), 2.34−2.52 (m, 8, part of NCH$_2$(CH$_2$)$_6$CH$_2$, CH$_2$CH$_3$), 3.80 (t, J = 6.6 Hz, 2, OCH$_2$), 6.53 (d, J = 8.8 Hz, 2, ArH *ortho* to OCH$_2$), 6.72 (d, J = 8.8 Hz, 2, ArH *meta* to OCH$_2$), 6.98 (d, J = 8.3 Hz, 2, ArH *meta* to I), 7.09−7.21 (m, 5, PhH), 7.66 (d, J = 8.5 Hz, 2, ArH *ortho* to I); MS m/z 581 (M$^+$, 50). Anal. (C$_{32}$H$_{40}$NOI) C, H, N; I: calcd, 21.82; found 21.12.

(*E*)-1-[4-[[10-(*N*-Dimethylamino)decyl]oxy]phenyl]-1-(4-iodophenyl)-2-phenyl-1-butene (2i): white crystals (0.412 g, 68%); mp 70−72 °C; ^{1}H NMR (CDCl$_3$) δ 0.91 (t, J = 7.4 Hz, 3, CH$_2$CH$_3$), 1.23−1.74 (m, 16, CH$_2$(CH$_2$)$_8$CH$_2$), 2.19−2.28 (m, 8, NCH$_2$(CH$_2$)$_5$CH$_2$, N(CH$_3$)$_2$), 2.67 (q, J = 7.6 Hz, 2, CH$_2$CH$_3$), 3.80 (t, J = 7.6 Hz, 2, OCH$_2$), 6.53 (d, J = 8.5 Hz, 2, ArH *ortho* to OCH$_2$), 6.72 (d, J = 8.8 Hz, 2, ArH *meta* to OCH$_2$), 6.98 (d, J = 8.4 Hz, 2, ArH *meta* to I), 7.11−7.18 (m, 5, PhH), 7.66 (d,

J = 8.1 Hz, 2, ArH *ortho* to I); MS m/z 610 (M$^+$, 100). Anal. (C$_{34}$H$_{44}$NOI) C, H, N, I.

Estrogen Receptor Binding Assay. The affinity of the antiestrogens for the ER was measured using a competitive binding assay as described by Wakeling.[22] Immature rat cytosol was incubated at 4 °C for 16 h with 5 nM 17β-[2,4,6,7-^{3}H]estradiol in the presence of increasing amounts (0.1−100 000 nM) of test compounds dissolved in dimethylformamide or unlabeled estradiol (control). The nonspecific binding was quantified by a parallel set of tubes containing a 200-fold excess (with respect to [^{3}H]estradiol) of diethylstilboestrol. Unbound compounds were removed with dextran-coated charcoal, and the receptor-bound [^{3}H]estradiol was determined. The relative concentrations of estradiol and test compound required to achieve 50% inhibition of [^{3}H]estradiol binding give the RBA which is [IC$_{50}$ (estradiol)/IC$_{50}$ (test compound)] × 100.

Calmodulin Antagonism. This was determined using the calmodulin-dependent cyclic AMP phosphodiesterase as previously described.[6,26] The enzyme was assayed using 8-[^{3}H]-cAMP as substrate. The tritiated AMP formed during the incubation was converted into tritiated adenosine by the 5′-nucleotidase in snake venom. Product nucleosides were separated from unreacted substrate by batch elution with Dowex anion exchange resin with 3 mM acetic acid. The basal activity of cAMP phosphodiesterase (calmodulin independent) was determined by adding 1 mM EGTA to the assay medium. Assays were carried out in the presence and absence of different concentrations of the compounds dissolved in dimethyl sulfoxide. The results are expressed as the concentration of inhibitor giving 50% inhibition of the calmodulin-dependent cAMP phosphodiesterase and are the mean of triplicate determinations ± standard error.

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