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# Characterization of the Pharmacophore Properties of Novel Selective Estrogen Receptor Downregulators (SERDs)

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Selective estrogen receptor (ER) down-regulators (SERDs) reduce ERα protein levels as well as block ER activity and therefore are promising therapeutic agents for the treatment of hormone refractory breast cancer. Starting with the triarylethylene acrylic acid SERD **4**, we have investigated how alterations in both the ligand core structure and the appended acrylic acid substituent affect SERD activity. The new ligands were based on high affinity, symmetrical cyclofenil or bicyclo[3.3.1]nonane core systems, and in these, the position of the carboxyl group was extended from the ligand core, either retaining the vinylic linkage of the substituent or replacing it with an ether linkage. Although most structural variants showed binding affinities for ERα and ERβ higher than that of **4**, only the compounds preserving the acrylic acid side chain retained SERD activity, although they could possess varying core structures. Hence, the acrylic acid moiety of the ligand is crucial for SERD-like blockade of ER activities.

## Introduction

A novel class of compounds capable of modulating the level and activity of the estrogen receptor (ER[a]) are termed selective estrogen receptor downregulators (SERDs). These compounds are mechanistically distinct from ER ligands such as 4-hydroxytamoxifen, which act as either agonists or antagonists, depending upon the target tissue, and are termed selective estrogen receptor modulators (SERMs).[1,2] Although certain SERMs are clinically useful for menopausal hormone replacement and for the prevention and treatment of breast cancer,[3] there is a need for new ER ligands that are capable of overcoming acquired endocrine resistance in breast cancer. Current SERDs, such as ICI 182,780 (ICI, Figure 1), have a more antagonistic profile than SERMs and are capable of inhibiting the growth of tamoxifen-resistant breast cancer cells.[4]

SERDs are currently used in the treatment of metastatic breast cancer,[4,5] and their improved clinical activity is thought to be derived from their ability to down-regulate the ER protein as well as to block ER action.[6−9] ICI, however, has poor oral bioavailability,[10] and a search for alternative drugs led to the identification of GW-5638 (compound **1**, Figure 1), a SERD that shares the triarylethylene ligand core scaffold of tamoxifen but has the basic side chain replaced by an acrylic acid unit. Compound **1** has an agonist profile in bone but acts as an antagonist in the breast[11−13] and can inhibit the growth of tamoxifen-resistant breast tumors.[9] GW-7604 (compound **4**, Figure 1) is a hydroxylated analogue of compound **1** with higher ER affinity,[11,12] analogous to 4-hydroxytamoxifen, being a high affinity analogue and metabolite of tamoxifen.[14]

It has been proposed that the SERD activities of both **4** and ICI result from their distortion of the conformation of the ER ligand binding domain (LBD) so that hydrophobic surfaces become exposed, thereby promoting accelerated degradation of the ER protein.[15,16] The X-ray crystallographic structure of an ERα−LBD complex with a structural analogue of ICI shows the ligand bound in an inverted mode relative to estradiol, with the long 7α-substituent projecting outward from the ligand binding pocket, displacing helix-12 of the LBD and occupying the coactivator binding groove.[15] A recent structure of **4** with ERα shows that helix-12 is tilted from the normal SERM position through interaction of the carboxylic acid group of the ligand with the positive end of the helix-12 dipole.[16] In both cases, fluorescent probes of exposed protein hydrophobic regions have revealed that these ER−SERD structures are more hydrophobic than are complexes with estrogen agonists (estradiol, DES) or SERMs (hydroxytamoxifen and raloxifene).[16]

Although the distortion of helix-12 in the ERα structure complexed with **4** suggests an important role for the carboxylic acid function of the acrylic acid side chain, it is not clear whether this function is positioned optimally or might be improved by presentation in an alternative manner, through other linkages, or at different distances from the ligand core. Similarly, it is not known whether the triarylethylene core structure is essential or might be replaced by alternative moieties with higher ER binding affinities. The hydroxytriarylethylene (TAE) core of **4**, in particular, might be subject to cis−trans isomerization in solution because it shares this structural feature with hydroxytamoxifen and diethylstilbestrol, both of which do undergo this isomerization in a facile manner.[17,18]

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[a]Abbreviations: BN, bicyclononane; CF, cyclofenil; DES, diethylstilbestrol; E2, estradiol; ER, estrogen receptor; SERD, selective estrogen receptor downregulators; SERM, selective estrogen receptor modulators; TAE, triarylethylene.

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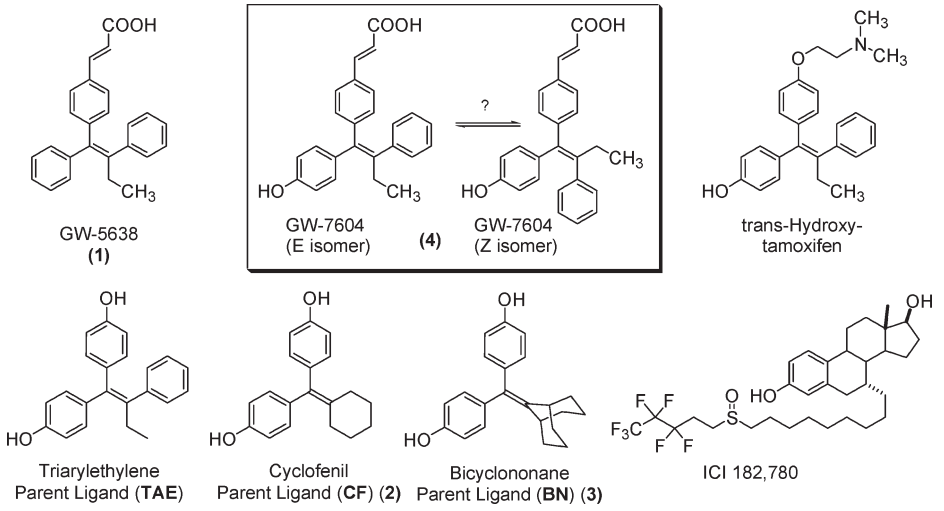



**Figure 1.** Selective estrogen receptor down-regulators (SERDs) and modulators (SERMs) and symmetrical ligands. The SERD GW-5638 (**1**) and its hydroxylated metabolite, GW-7604 (**4**), can potentially undergo cis−trans isomerization (boxed structures, see text). The GW compounds, as well as *trans*-hydroxytamoxifen, share a triaryethylene (TAE) structural core. The cyclic-core ligands, cyclofenil (CF, **2**) and bicyclononane (BN, **3**), are symmetrical and thus do not exist as cis−trans isomers. ICI is a known SERD.

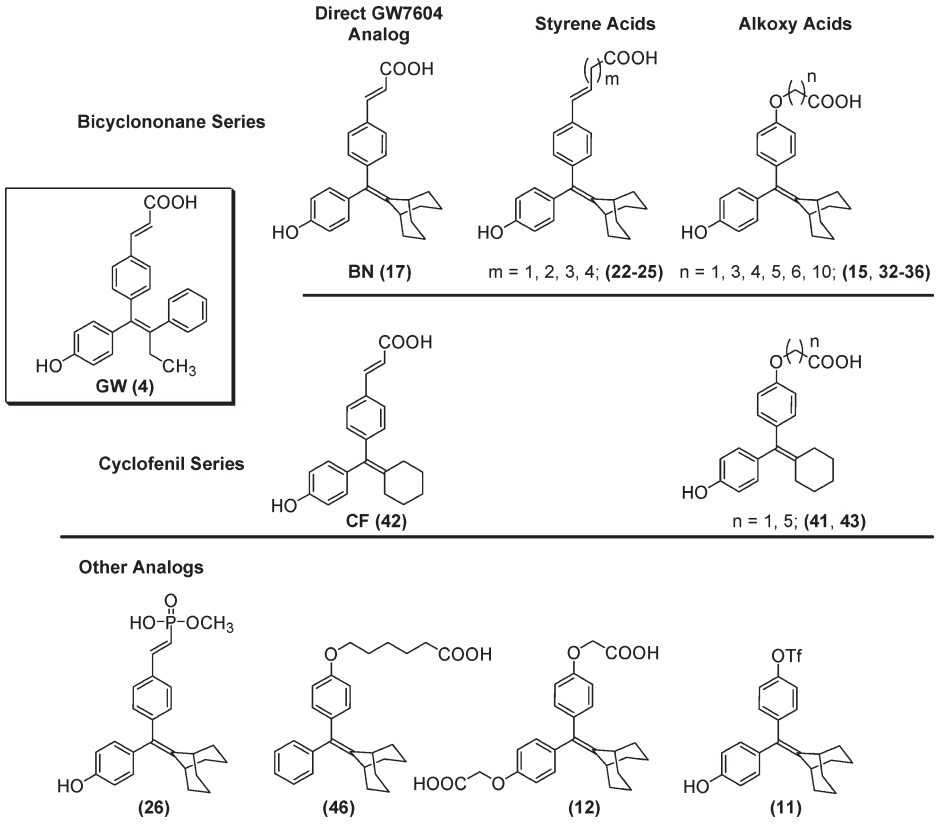


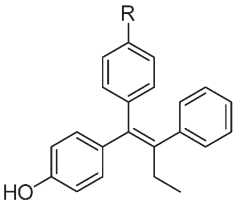
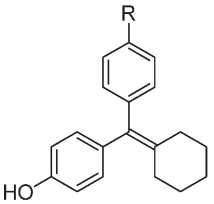
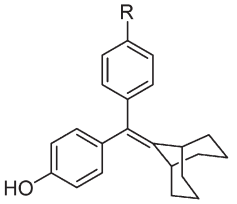

**Figure 2.** Series of SERD compound **4** analogues based on cyclofenil (CF) and bicyclononane (BN) and other analogues.

Therefore, to investigate to what extent the SERD activity of **4** is dependent on the hydroxytamoxifen-type core, the vinyl linkage, or the acrylic acid side chain, we prepared and evaluated the SERD activity of a new series of ER ligands having acidic side chains of varying length and linkages and built on different ligand core structures, cyclofenil (compound **2**) and bicyclononane (compound **3**), that are symmetrical and therefore do not exist as stereoisomers (Figures 1 and 2). From our initial series, we selected analogues having high binding affinity for ERα and ER$\beta$, and we further characterized their ability to alter the subcellular localization of ERα and to down-regulate its protein levels and inhibit gene stimulation by estrogen (SERD activity). The two new compounds having the most favorable SERD-like activity retained the acrylic acid side chain, although they had different core structures.

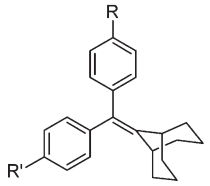
This study expands our current understanding of the optimal pharmacophore requirements for SERD activity and their capabilities in regulating the activity of the estrogen receptor. It also highlighted the importance of the acrylic acid side chain of **4** for engendering SERD-like activity but revealed that alternative ligand core structures can be used in place of the tamoxifen-like triarylethylene core used in the GW series.

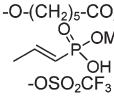
**Table 1.** Estrogen Receptor Binding Assay[e]

(a) Structure and Binding Affinity of Basic TAE, CF, and BN Systems

| | Triarylethylene (TAE) | | | | Cyclofenil (CN) | | | | Bicyclononane (BN) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| |  | | | |  | | | |  | | | |
| R | ligand | RBA[a] ERα | RBA[a] ERβ | β/α[b] | ligand | RBA[a] ERα | RBA[a] ERβ | β/α[b] | ligand | RBA[a] ERα | RBA[a] ERβ | β/α[b] |
|  | **TAE** | 377 ± 106 | 238 ± 48 | 0.6 | **CF, 2** | 68 ± 18 | 334 ± 54 | 4.9 | **BN, 3** | 513 ± 17 | 301 ± 54 | 0.6 |
|  | **4**[c,d] | 20.3 ± 6 | 28.3 ± 7 | 1.4 | **42**[c] | 2.1 ± 5 | 11.3 ± 3 | 5.4 | **17**[c] | 17.7 ± 4 | 25.9 ± 5 | 1.5 |
| | **4'**[c] | 3.6 ± 1 | 4.2 ± 0.6 | 1.2 | -- | -- | -- | -- | -- | -- | -- | -- |
|  | -- | -- | -- | -- | **44**[c] | 0.63 ± 0.1 | 1.9 ± 0.6 | 3.0 | **15**[c] | 3.6 ± 1 | 3.1 ± 0.3 | 0.9 |
|  | -- | -- | -- | -- | **43**[c] | 8.2 ± 0.5 | 28.8 ± 3 | 3.5 | **34**[c] | 52.0 ± 6 | 89.1 ± 9 | 1.7 |

(b) ERα and ERβ Binding Affinity of Other Ligands with a Bicyclononane Core



| R' | R | ligand | RBA[a] ERα | RBA[a] ERβ | β/α[b] |
|---|---|---|---|---|---|
| -H | -OH | **46** | 0.14 ± 0.02 | 0.30 ± 0.06 | 2.1 |
| -O-$CH_2$-$CO_2H$ | -O-$CH_2$-$CO_2H$ | **12** | 0.26 ± 0.05 | 0.40 ± 0.05 | 1.5 |
| -OH | -O-$(CH_2)_5$-$CO_2$Et | **29** | 15.2 | 30.8 | 2.0 |
| -OH |  | **26**[c] | 7.5 ± 1 | 7.7 ± 0.7 | 1.0 |
| -OH | -$OSO_2CF_3$ | **11** | 4.2 ± 1 | 8.9 ± 0.7 | 2.1 |

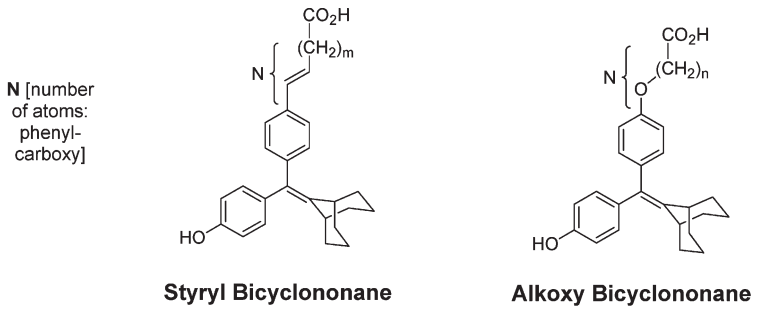
[a] Relative binding affinity (RBA) values are determined by competitive radiometric binding assays and are expressed as $IC_{50}^{[estradiol]}/IC_{50}^{[compound]} \times 100$ (RBA, estradiol = 100). In these assays the $K_d$ for estradiol is 0.2 nM for ERα and 0.5 nM for ER$\beta$. [b] For each value, the $\beta/\alpha$ ratio is calculated such that the ratio is >1 for compounds having higher affinity on ER$\beta$ than on ERα. [c] Compounds selected for further studies are indicated by a bold underline. [d] Compound **4** exists as a mixture of *E* and *Z* isomers. See Figure 1. [e] The ERα and ER$\beta$ binding affinities of SERDs with the triarylethylene (TAE), cyclofenil (CF), and bicyclononane (BN) cores with the acrylic acid substituent are presented in the top part of the table (part a). Some individual variants with the bicyclononane core are presented in the lower part of the table (part b).

## Results

**Chemical Synthesis.** The structures of the SERD **4** analogue series that we prepared are summarized in Figure 2, and those studied in greater detail are shown in Tables 1 and 2 (see compound numbers with underline). Some analogues (**42**, **43**, **44**) are based on a well-known, high affinity nonsteroidal estrogen, cyclofenil (**2**, Figure 1), but the majority (**11**, **12**, **15**, **17**, **22**−**26**, **29**, **32**−**36**, **46**) are based on bicyclononane (**3**, Figure 1), a related nonsteroidal estrogen that has exceptionally high binding affinity for both ERα and ER$\beta$ (Table 1a).[19] Notably, neither of these symmetrical ligand cores have cis−trans isomers, as does the TAE-based compound (compound **4**, Figure 1). In both the cyclofenil and bicyclononane series (designated CF and BN for simplicity), we prepared the direct analogue of **4** containing the acrylic acid substituent (**17**, **42**), as well as a series with carboxy-terminated alkoxy substituents (Figure 2, "alkoxy acids"). Because members of the bicyclononane series proved to have higher affinity, we prepared additional homologues of the acrylate-substituted system, having longer chain acids linked through a styryl function (Figure 2, "styrene acids"), as well as some individual variants, bis-carboxymethyl and a deoxy analogue or members with vinylphosphonate or trifluoromethanesulfonyloxy groups (Figure 2, bottom).

The synthetic routes used to prepare these compounds are shown in Schemes 1−4. McMurry coupling was used to prepare the parent cyclofenil and bicyclononane systems (compounds **7**−**9**) from their appropriate benzophenone precursors (compounds **5** and **6**, Scheme 1). Members of the alkoxy ether series were prepared from the parent cyclofenil and bicyclononane ligands by Williamson ether synthesis using various $\omega$-bromo esters, followed by ester hydrolysis (compounds **32**−**36** (Scheme 2) and compound **43** (Schemes 3 and 4)). Good yields of monosubstitution on the bisphenol parent ligands could be obtained in all cases except with ethyl bromoacetate. In this case, the bisphenols were first protected as the mono-TBDMS ethers (compounds **10**, **37**); monoalkylation followed by saponification/deprotection gave the desired monoalkoxymethyl ethers (compounds **15**,

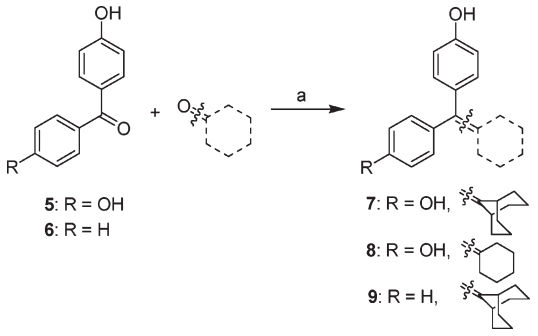
**Table 2.** Structure and Binding Affinity of the Extended Series of Styryl and Alkoxy Bicyclononanes[d]




| N | ligand | RBA[a] ERα | RBA[a] ERβ | β/α[b] | ligand | RBA[a] ERα | RBA[a] ERβ | β/α[b] |
|---|---|---|---|---|---|---|---|---|
| 2 | **17**[c] (m = 0) | 17.7 ± 4 | 25.9 ± 5 | 1.5 | **15**[c] (n = 1) | 3.6 ± 1 | 3.1 ± 0.3 | 0.86 |
| 4 | **22** (m = 2) | 9.0 ± 2 | 23.1 ± 6 | 2.6 | **32** (n = 3) | 5.6 ± 1 | 10.5 ± 1 | 1.9 |
| 5 | **23** (m = 3) | 13.1 ± 2 | 24.6 ± 7 | 1.9 | **33**[c] (n = 4) | 18.3 ± 3 | 38.3 ± 11 | 2.1 |
| 6 | **24**[c] (m = 4) | 38.5 ± 0. 2 | 62.8 ± 14 | 1.6 | **34**[c] (n = 5) | 52.0 ± 6 | 89.1 ± 9 | 1.7 |
| 7 | **25**[c] (m = 5) | 87.4 ± 10 | 81.1 ± 14 | 0.93 | **35** (n = 6) | 70.4 ± 2 0 | 139 ± 0 | 2.0 |
| 11 | -- | -- | -- | | **36**[c] (n = 10) | 95.6 ± 1 0 | 63.9 ± 17 | 0.67 |

[a] Relative binding affinity (RBA) values are determined by competitive radiometric binding assays and are expressed as $IC_{50}^{[estradiol]}/IC_{50}^{[compound]} \times 100$ (RBA, estradiol = 100). In these assays the $K_d$ for estradiol is 0.2 nM for ERα and 0.5 nM for ER$\beta$. [b] For each value, the $\beta/\alpha$ ratio is calculated such that the ratio is >1 for compounds having higher affinity on ER$\beta$ than on ERα. [c] Compounds selected for further studies are indicated by a bold underline. [d] The ERα and ER$\beta$ binding affinity of both the styryl and alkoxy bicyclononane series was determined and expressed similarly to the compounds in Table 1.

**Scheme 1.** Synthesis of Cyclofenil and Bicyclononane Analogues[a]




[a] Reagents: (a) $TiCl_4$, Zn, THF, reflux, 10 h.

**44**). The mono-TBDMS ethers were also used to prepare the monotrifluormethane sulfonates (compounds **11**, **38**), which where were used in Suzuki coupling to prepare members of the styryl series (compounds **17**, **22**−**25** (Scheme 2) and compound **42** (Schemes 3 and 4)). A complete description of the syntheses with experimental details and spectroscopic characterization is given in the Supporting Information.

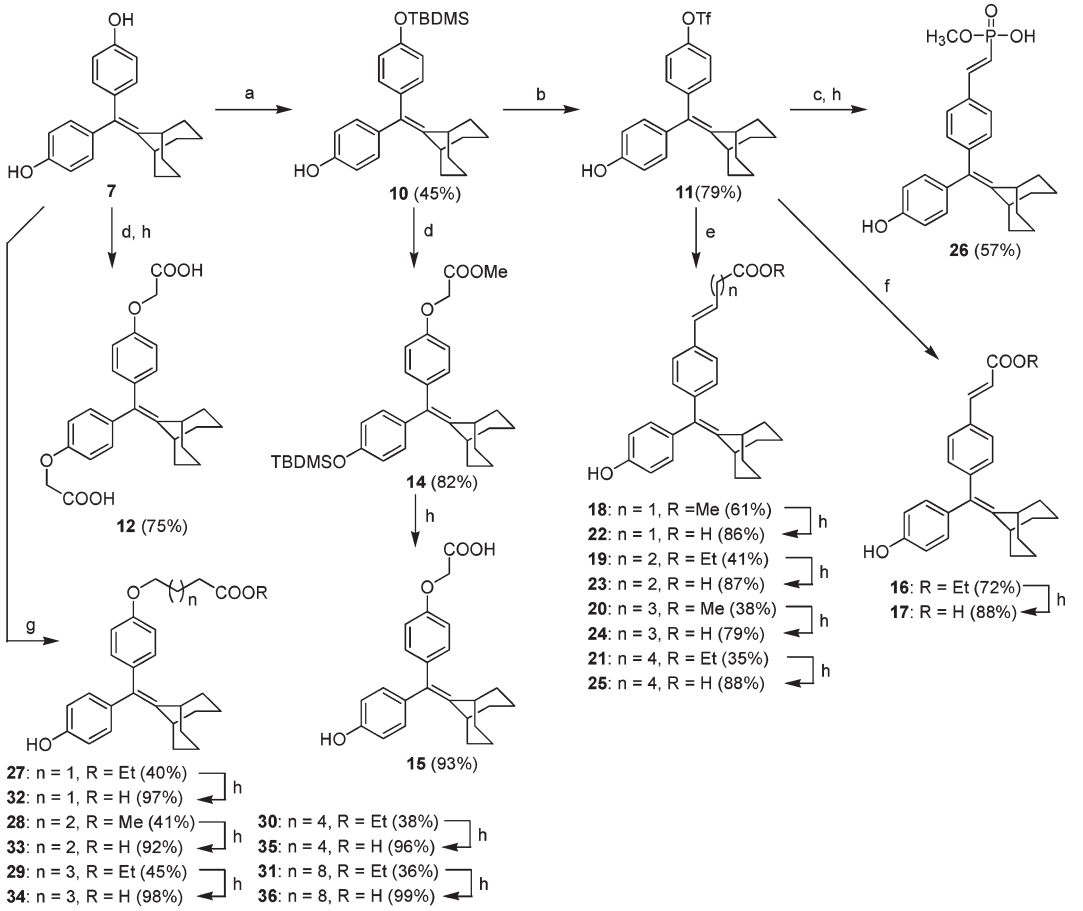
**Biological Results. Estrogen Receptor Binding Assays and Structure−Affinity Relationships.** The compounds were assayed for their binding affinity for human ERα and ER$\beta$ in a radiometric competitive binding assay using [$^3$H]estradiol as tracer and estradiol as the reference standard.[20,21] Binding affinities are expressed as relative binding affinity (RBA) values, with estradiol set at 100. Results are given in Table 1 (comparison of triarylethylene (TAE), cyclofenil (CF) and bicyclononane (BN) series) and Table 2 (extended BN styryl and alkoxy series).

The binding affinities of the parent phenols in the triarylethylene (TAE), cyclofenil (CF), and bicyclononane (BN) systems are presented in Table 1a. All three parent compounds have very high binding affinity for ERα and ER$\beta$, but the highest affinity is shown by the parent bicyclononane (**3**). As with hydroxytamoxifen,[14] compound **4** exists as a cis−trans isomer mixture. After careful recrystallization, we were able to obtain only the *Z* isomer in pure form (**4′**). The lower affinity of the pure Z isomer (Table 1a) suggests that the *E* isomer is predominant in binding to the ERs. This is consistent with the X-ray crystal structures of this compound,[16] as well as binding affinity measurements and X-ray structures for hydroxytamoxifen and diethylstilbestrol, where the isomer with the corresponding trans-4-hydroxystilbene isomer is the active one.[14,17,22]

In the acrylic acid series (Table 1), the affinity of the cyclofenil analogue (**42**) is considerably lower than that of the GW compound (**4**); the bicyclononane analogue (**17**), however, binds with comparable affinity. The binding affinities of two alkoxy analogues prepared in both the cyclofenil and bicyclononane series (compounds **44** and **43**, and compounds **15** and **34**, respectively) provided additional evidence for the higher affinity of members of the bicyclononane series; therefore, further synthetic efforts focused on this series. Also of note are the binding affinities of certain other bicyclononane analogues (Table 1, Figure 2, bottom) designed to test other side chain functional groups. Compounds in which the OH is missing (**46**) or substituted with a second alkoxy group (**12**) have poor binding affinities. The ester (**29**) has lower affinity than the corresponding acid **34**; the methyl phosphonate **26** binds less well than the corresponding carboxylate **17**, and the trifluoromethane sulfonate (**11**) binds only moderately well.

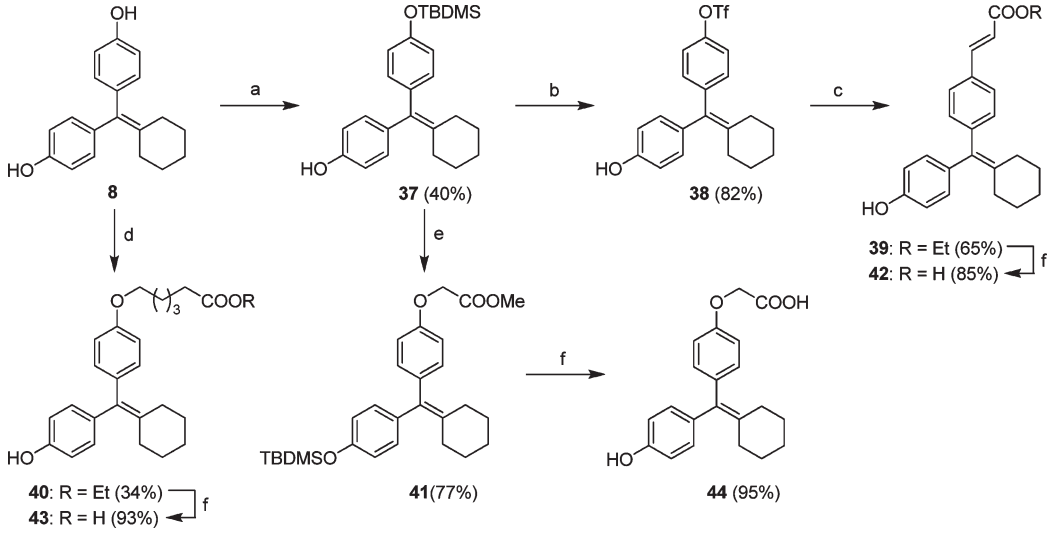
Binding affinities of the extended bicyclononane series of styryl and alkoxy analogues are given in Table 2. In both

**Scheme 2.** Synthesis of Acid Substituted Cyclononane Analogues[a]




[a] Reagents: (a) TBDMSCl, imidazole, room temp, THF, 24 h; (b) (i) $Tf_2O$, triethylamine, 0 °C to room temp, 8 h; (ii) TBAF, room temp, 15 min; (c) dimethyl vinylphosphonate, $Pd(PPh_3)_2Cl_2$, triethylamine, DMF, 120 °C, 24 h; (d) $BrCH_2COOCH_3$, $Cs_2CO_3$, $CH_3CN$, room temp, 24 h; (e) $CH_2{=}CH(CH_2)_nCOOR$, $Pd(PPh_3)_2Cl_2$, triethylamine, DMF, 120 °C, 24 h; (f) ethyl acrylate, $Pd(PPh_3)_2Cl_2$, triethylamine, DMF, 120 °C, 24 h; (g) $BrCH_2(CH_2)_nCH_2COOR$, $Cs_2CO_3$ or $K_2CO_3$, $CH_3CN$, room temp, 24 h; (h) 2 N KOH, MeOH, room temp, 24 h.

**Scheme 3.** Synthesis of Acid Substituted Cyclofenil Analogues[a]



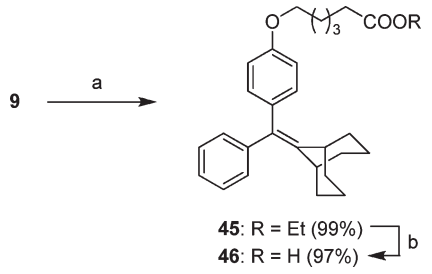

[a] Reagents: (a) TBDMSCl, imidazole, room temp, THF, 24 h; (b) (i) $Tf_2O$, triethylamine, 0 °C to room temp, 8 h; (ii) TBAF, room temp, 15 min; (c) ethyl acrylate, $Pd(PPh_3)_2Cl_2$, triethylamine, DMF, 120 °C, 24 h; (d) ethyl 6-bromohexanoate, $Cs_2CO_3$, $CH_3CN$, room temp, 24 h; (e) $BrCH_2COOCH_3$, $Cs_2CO_3$, $CH_3CN$, room temp, 24 h; (f) 2 N KOH, MeOH, room temp, 24 h.

series and for both receptors, binding affinities (with but one exception) increase with increasing chain length, reaching affinities much greater than that of **4** and even rivaling that of estradiol. This is shown graphically in Figure 3. None of the compounds in either Table 1 or Table 2 showed significant selectivity for either ERα or ERβ, although those in the

cyclofenil series (Table 1a) tended to be more ER$\beta$ selective, with the cyclofenil acrylic acid (**42**) being ~5-fold in favor of ER$\beta$.

**Western Immunoblotting.** We selected 10 compounds with good binding affinity for ER for further analysis of their SERD character (see underlined compound numbers in Tables 1 and 2). These compounds were the initial SERD compound (**4**), the direct analogues of **4** in both the cyclofenil (**42**) and bicyclononane (**17**) series, the high affinity analogues in the bicyclononane styryl (**24** and **25**) and alkoxy (**15**, **33**, **34**, and **36**) series, and the BN vinyl methyl phosphonate (**26**). The well established SERD ICI was also included for comparison.

**Scheme 4.** Synthesis of the Deoxy Analogue (**46**) of Bicyclononane Compound **34**[a]




[a] Reagents: (a) ethyl 6-bromohexanoate, $Cs_2CO_3$, $CH_3CN$, room temp, 24 h; (b) 2 N KOH, MeOH, room temp, 24 h.

MCF-7 breast cancer cells were treated with these compounds, and the ER$\alpha$ protein was extracted and analyzed by SDS−PAGE and Western immunoblotting. Because SERDs are known not only to lower ER levels in cells but also to affect the subcellular distribution of ER,[6−8] we monitored ER levels in whole cell extracts (WC) and in cytoplasmic (C), nuclear (N), matrix (M), and insoluble (I) fractions after treatment with these various compounds. The procedures used to obtain these extracts are given in the Experimental Section, and representative Western immunoblots are shown in Figure 4.

The well characterized SERD ICI markedly reduced ER$\alpha$ protein levels in the cytoplasmic fraction and did not raise ER$\alpha$ levels in the nuclear fraction; the slight elevation of ER$\alpha$ in the matrix and insoluble fractions is consistent with reports that this compound does redistribute ER$\alpha$ into the less soluble cell fractions (Figure 4A).[6−8] The known SERD **4** also reduces cytoplasmic ER$\alpha$ protein, but ER$\alpha$ levels in the nuclear, matrix, and insoluble fractions are elevated, consistent with what others have shown.[23] The bicyclononane analogue (**17**) redistributed and down-regulated of ER in a manner very similar to that of the SERD **4**, as did the cyclofenil analogue (compound **42**), though the latter to a somewhat lesser degree (Figure 4A).

Surprisingly, although they have higher ER$\alpha$ binding affinities than the three compounds discussed above, none of the bicyclononane acrylate homologues (styryl compounds

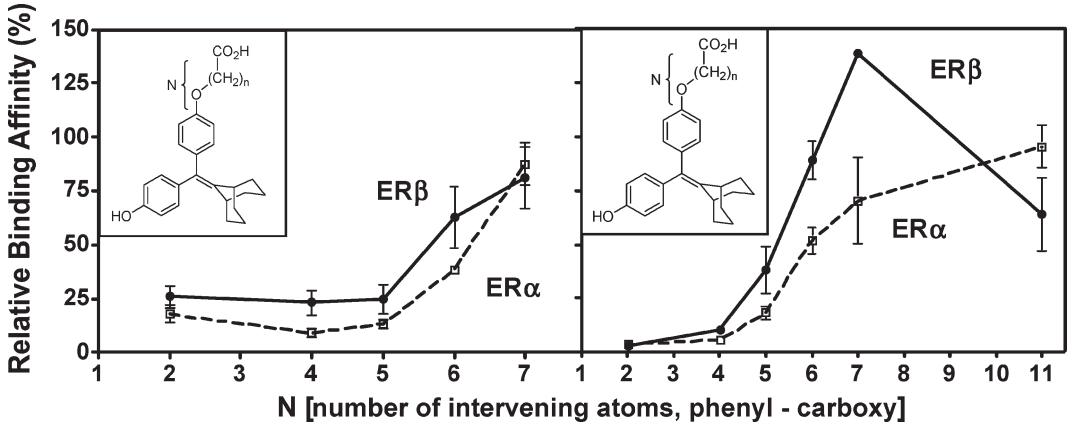



**Figure 3.** Dependence of ligand relative binding affinity on acid chain length. *N* represents the number of intervening atoms between the phenyl and the carboxyl moieties, irrespective of whether they are carbon or oxygen.

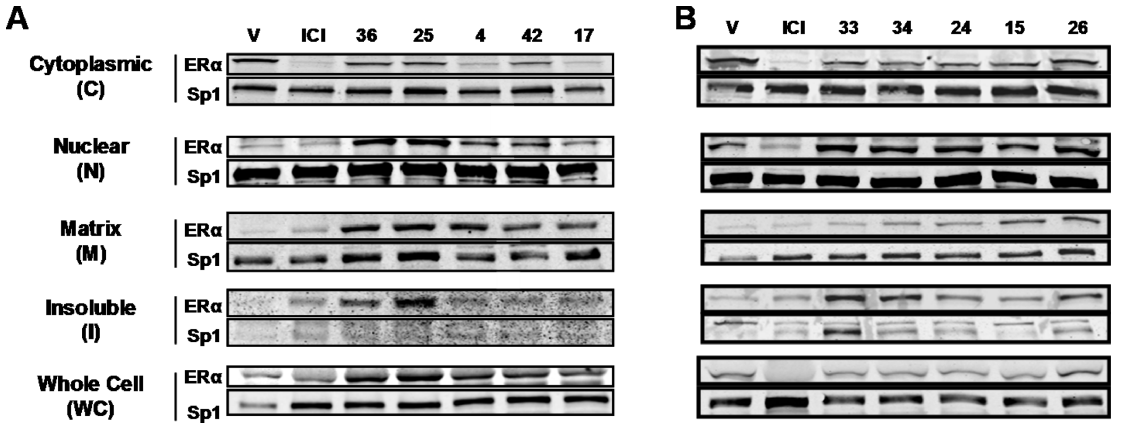



**Figure 4.** Subcellular distribution and down-regulation of ER$\alpha$ protein by ICI and compounds **36**, **25**, **4**, **42**, **17** (A) and **33**, **34**, **24**, **15**, and **26** (B). MCF-7 cells were treated with 1 $\mu$M compounds for 16 h, and the level of cellular ER$\alpha$ in the indicated subcellular fraction and in whole cell extracts was measured by Western immunoblotting. Sp1 was monitored as an internal reference for gel loading.

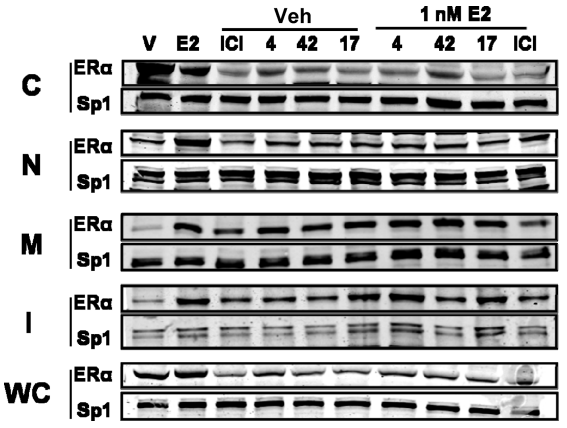



**Figure 5.** Western immunoblot analysis of of ERα protein level and subcellular distribution with compound and estradiol treatment. MCF-7 cells were treated with 1 $\mu$M compound for 16 h and then ±1 nM estradiol for 4 h prior to cell fractionation and monitoring of ERα by Western immunoblots. Leftmost two lanes show vehicle or E2 treatment for 20 h. Sp1 was monitored as an internal reference for gel loading.

**24** and **25**) or the carboxyalkoxy analogues (compounds **15**, **33**, **34**, and **36**) that we tested demonstrated substantial SERD-like activity (Figure 4). While cytoplasmic ERα levels were somewhat lower, nuclear ER, and in some cases also matrix and insoluble fractions, showed substantially increased ERα levels. These compounds all lack the acrylic acid side chain moiety which is present in compounds **4**, **17**, and **42**.[24]

Western immunoblot analysis of MCF-7 cells treated with ICI and compounds **4**, **42**, and **17**, alone or with E2, are shown in Figure 5. In the presence of E2, all of the SERDs tested were capable of lowering ERα protein levels. This down-regulation was seen particularly in the whole cell lysate and the cytoplasmic and the nuclear fractions. The cyclofenil acrylic acid (**42**) showed lower activity (i.e., less ER protein reduction), especially in the cytoplasmic fraction. The sequestration of ERα protein in the insoluble fraction increased upon concomitant E2 treatment. This may represent ligand-dependent turnover of the receptor,[25] as it is well-known that E2 enhances ER turnover.[26]

**Analysis of ERα Mediated Transcription Regulation.** Transcriptional analysis of established ERα target genes was carried out on MCF-7 cells treated with the best three compounds in the absence or presence of E2. The RNA levels of three E2-upregulated genes (pS2, progesterone receptor, carbonic anhydrase 12) were in most cases reduced by the SERDs **4**, **17**, and **42** (Figure 6A). Compounds **4** and **17** significantly blocked the E2-mediated stimulation of the pS2 and PR genes, but compound **42** was ineffective, consistent with its less effective down-regulation of the ER protein. However, **42** did block the E2-mediated activation of the CA12 gene even more so than did compound **17**. Compound **4** was the most effective in blocking estrogen action on all three of these E2-stimulated genes.

The three SERDs were also effective in reversing estrogen action on two genes that are repressed by E2, namely, BLNK and ENC1 (Figure 6B).[27] Compounds **4** and **17** did, in fact, reduce the E2-mediated repression of BLNK and ENC1 gene expression (Figure 6B), increasing RNA levels for these genes relative to the E2-repressed state, but the repression was not fully reversed to the control, basal level of expression.

## Discussion

In this study, we have examined a range of compounds structurally related to the selective estrogen receptor down-regulator (SERD), compound **4**. In all of these analogues, the isomerization-prone hydroxyltriarylethylene ligand core of **4** was replaced by two symmetrical cyclic systems, cyclofenil and bicyclononane, that are known to engender high ligand binding affinity for both estrogen receptors, ERα and ER$\beta$. In addition, homologues were prepared in which the carboxylic acid group on the acrylic acid side chain was extended (keeping the styrene linkage) or analogues in which it was replaced by an ether-linked carboxyalkyl substituent. While in both analogue series, extension of the acid function away from the ligand core increased ER binding affinity substantially, it did not result in compounds having SERD activity. In fact, the only compounds capable of reducing levels of ERα protein, bicyclononane **17** and cyclofenil **42**, were ones that retained the acrylic acid group present in **4**. Of the two new SERDs, **17** and **42**, the higher affinity bicyclononane compound (**17**) proved to be the better, and in most respects it closely replicated the activity of **4**. The marked dampening of ER action shown by compounds **4** and **17** indicates an obliteration of transactivation from both AF-1 and AF-2, a known effect of pure antagonist/SERD binding to the ER.[28−30]

Structural studies have suggested that the acrylic acid side chain of **4** down-regulates ERα protein levels by interactions with specific amino acids, namely, D351 in the ligand binding domain of the receptor.[31] This charge−charge repulsion blocks the proper positioning of the critical helix 12,[31,32] preventing it from adopting either the folded-back orientation found in agonist structures or the extended conformation found in SERM structures, where helix 12 occludes the hydrophobic groove of the AF-2 function, blocking interaction with coregulators.[16] With helix 12 thus mal-disposed, hydrophobic patches are exposed, and the receptor is thought to become a target for ubiquitination and processing by the ubiquitin−proteasome pathway.[33] A related mechanism is proposed for the activity of ICI-type SERDs,[16] although in this case, helix-12 appears to be completely disordered,[34] with even greater exposure of hydrophobic regions for targeting ubiquitination.[16]

Remarkably, the SERD activity of the compounds in this series appears to be uniquely associated with the acrylic acid unit that is common to compound **4**, cyclofenil **42**, and bicyclononane **17**. Even replacing it with a carboxymethyl ether, which enables the carboxyl group to access the same space as it occupies in the acrylic acid, did not suffice to engender SERD activity. This suggests that the rigidity of the acrylate unit, in fact, might be essential for maintaining the critical charge−charge repulsion with D351. Thus, the higher binding affinity found when the carboxylic acid group is spaced further from the aromatic ring might indicate relief of this ligand carboxylate−ERα D351 Coulombic repulsion, and it suggests that the critical interaction needed to distort the position of helix 12 and induce ER degradation (the very essence of SERD activity) comes with a cost in binding energy. Relevant to this point, others have found that maintaining the double bond but replacing the carboxylic acid group with a carboxamide or methyl ketone, which would maintain rigidity but remove the charge, also results in loss of SERD activity.[24]

While activation of the ubiquitin−proteasome pathway is a plausible explanation for the down-regulation of ERα protein, Wijayaratne and McDonnell did not find a large increase in ERα ubiquitination upon treatment with compound **4**.[35]

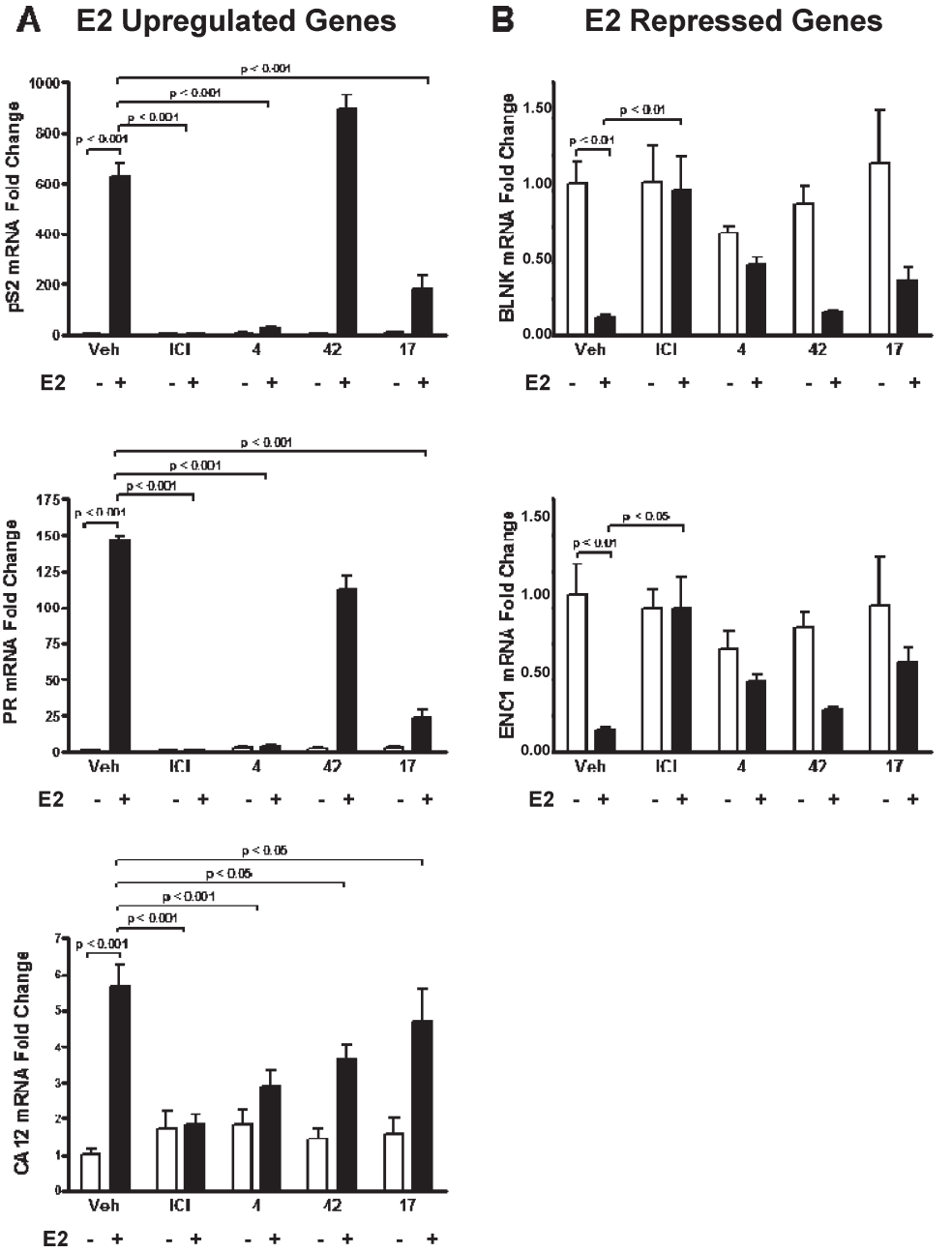



**Figure 6.** Impact of different compounds on ERα mediated transcriptional activity. MCF-7 cells were treated with 1 $\mu$M compounds for 16 h and then with vehicle (0.1% ethanol) or 1 nM estradiol for 4 h. Real-time PCR analysis was performed for E2-stimulated ERα-target genes (A) or E2-repressed ERα-target genes (B) as a measure of compound ability to antagonize estradiol action.

There are, however, other reports implicating the proteasome−ubiquitin pathway in ER turnover. Duong et al. described a ternary complex between ERα, p53, and the oncogenic ubiquitin-ligase Mdm2 in MCF-7 cells.[36] Also, receptor down-regulation is blocked by proteasome-inhibitor MG132 treatment.[37−39] It has been suggested that the 26S proteasome is necessary for the cycling of ERα protein needed to maintain transcription of target genes.[25] These studies all suggest the involvement of the ubiquitin−proteasome pathway in the down-regulation of ERα protein.

As others have seen, SERDs such as ICI not only reduce ERα protein levels overall but also redistribute it from the most soluble cellular compartments, the cytoplasm and nucleus, to less soluble compartments, the matrix and insoluble fractions. Presumably, at these locations it cannot function in its role as a ligand-modulated transcription factor. In fact, the movement of ER into these compartments might be a consequence of its ubiquitination, as others have noted.[40−42] Our observations of such down-regulations and alterations in intracellular distribution of ER after treatment with these new SERDs are consistent with their prevention of estrogen-regulated gene expression and highlight the importance of the acrylic acid side chain in enabling SERD activity.

## Conclusion

Compounds capable of suppressing estrogen action by reducing estrogen receptor levels appear to have particular promise for novel endocrine therapies in breast cancer. It is

hoped that they might not succumb to the development of acquired resistance that typically follows treatment with SERMs, and this appears to be the case in model systems.[43] Our study has demonstrated that other ligand core structures, in addition to the triarylethylene ligand core in traditional SERMs like tamoxifen and in the original GW SERD (**4**), can be used to produce SERD compounds (namely, the cyclofenil compound **42** and the bicyclononane compound **17**) that are still very effective at moderating the activity of ER. Our biological analyses of these novel SERDs underscore the importance of the acrylic side chain in effecting the down-regulation of ERα and the suppression of ER-regulated gene expression.

## Experimental Section

**Synthesis Materials and Methods.** See Supporting Information for details on synthesis, spectroscopic characterizations, and purity determination by normal and reversed phase HPLC analysis. All compounds assayed were >95% pure in both HPLC systems.

**Biological Materials and Methods. Compounds.** E2 and ICI 182,780 (ICI) were purchased from Sigma Chemical Co. (St. Louis, MO).

**Cell Culture.** The human breast cancer MCF-7 cell line was maintained in culture as previously described.[44] For Western analysis, cells were steroid depleted in phenol red-free media supplemented with 5% charcoal−dextran-treated calf serum for 5 days prior to the indicated ligand treatment. Media were changed on day 2 of culture, and cells were then treated with ligands. For real-time PCR gene expression analysis, cells were steroid depleted in phenol red-free media supplemented with 5% charcoal−dextran-treated calf serum for 7 days prior to the indicated ligand treatment. Media were changed on days 2 and 4 of culture, and cells were then treated with ligands.

**Cellular Fractionation.** MCF-7 cells were plated on 10 $cm^2$ plates and grown to ∼80% confluency. Cells were treated with either vehicle (0.1% EtOH) or 1 μM SERDs for 16 h ± 1 nM E2 for 4 h and harvested by scraping in 100 μL of buffer. Cellular fractionation was carried out as previously described.[23,45] Whole cell lysates were obtained by harvesting in 100 μL of whole cell lysis buffer [50 mM Tris (pH 8), 1 mM EDTA, 150 mM NaCl, 1% IGEPAL, 1% SDS, 5% glycerol]. Protein concentrations of the whole cell lysates and cytoplasmic and nuclear extracts were determined by BCA Protein Assay (Pierce, Rockford, IL).

**SDS−PAGE and Western Analysis.** Amounts of 20 μg of whole cell lysate and 40 μg of cytoplasmic and nuclear extracts were loaded on Tris-HCl SDS−PAGE gels (Bio-Rad, Hercules, CA). Equivalent fraction volumes of the detergent-extractable nuclear matrix and insoluble fractions were loaded. Proteins were transferred to nitrocellulose membranes (Pall Corporation, Pensacola, FL). Membranes were blocked in Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE). Overnight primary antibody (α-F10 [Santa Cruz Biotechnology, Santa Cruz, CA] and α-Sp1 [Upstate/Millipore, Bedford, MA] at 1:1000 dilution) and secondary antibody (Li-Cor Biosciences, Lincoln, NE, at 1:15,000 dilution) incubations were done in the presence of 0.1% Tween-20. PBST (PBS buffer with Tween) was used to wash off unbound antibody, and subsequent PBS washes were performed to limit the cross-reactivity of Tween-20 with the LiCor infrared imaging system.

**Total RNA Isolation and Real-Time PCR Analysis.** MCF-7 cells were plated in six-well plates and grown to ∼80% confluency. Cells were pretreated with 1 μM SERDs or 0.1% EtOH vehicle for 1 h and then treated with SERDs alone or concomitantly with 1 nM E2 for 16 h. Total RNA was extracted, reverse transcribed, and analyzed via real-time PCR as previously described.[44] Primers used are as follows: ENC1 forward (f), GGCCTCCCCCTCAGTCTCT; ENC1 reverse (r), GCACTCACTACTGCGGCGT. The primers used for pS2, progesterone receptor, CA12, and BLNK were those previously described.[27,44,46,47] Statistical analysis was performed with repeated measures one-way ANOVA using the Tukey's *t* test.

**Estrogen Receptor Binding Affinity Assays.** Relative binding affinities were determined by a competitive radiometric binding assay, as previously described,[20,21] using 10 nM [$^3$H]estradiol as tracer (GE Healthcare, Piscataway, NJ) and purified full-length human ERα and ERβ (PanVera/InVitrogen, Carlsbad, CA). Incubations were for 18−24 h at 0 °C. Then the receptor−ligand complexes were absorbed onto hydroxyapatite (BioRad, Hercules, CA) and unbound ligand was washed away. The binding affinities are expressed as relative binding affinity (RBA) values, with the RBA of estradiol set to 100. The values given are the average ± range or SD of two or more independent determinations. Estradiol binds to ERα with a $K_d$ of 0.2 nM and to ERβ with a $K_d$ of 0.5 nM.

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**Supporting Information Available:** Synthesis and spectroscopic characterization (**9**−**12**, **14**−**46**), $^1$H and $^{13}$C NMR spectra (**9**−**12**, **14**−**46**), and normal and reversed phase HPLC chromatograms (**11**, **15**, **17**, **22**−**26**, **29**, **31**−**36**, **42**−**44**, **46**). This material is available free of charge via the Internet at http://pubs.acs.org.

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