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Article

Elemental Isomerism: A Boron-Nitrogen Surrogate for a Carbon-Carbon Double Bond Increases the Chemical Diversity of Estrogen Receptor Ligands

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SUMMARY

To increase the chemical diversity of bioactive molecules by incorporating unusual elements, we have examined the replacement of a C=C double bond with the isoelectronic, isostructural B-N bond in the context of nonsteroidal estrogen receptor (ER) ligands. While the B-N bond was hydrolytically labile in the unhindered cyclofenil system, the more hindered anilino dimesitylboranes, analogs of triarylethylene estrogens, were easily prepared, hydrolytically stable, and demonstrated substantial affinity for ERs. X-ray analysis of one ERα-ligand complex revealed steric clashes with the *para* methyl groups distorting the receptor; removal of these groups resulted in an increase in affinity, potency, and transcriptional efficacy. These studies define the structural determinants of stability and cellular bioactivity of a B-N for C=C substitution in nonsteroidal estrogens and provide a framework for further exploration of "elemental isomerism" for diversification of drug-like molecules.

INTRODUCTION

The preparation of small molecules with cell-regulatory activities—agonists, antagonists, drugs, etc.—is a major component of "chemical genomics" and has as its ultimate goal the development of ligands capable of selective regulation of the activity of all important protein targets [1]. Efforts to prepare small-molecule regulators of increasingly broad biological activity demand new approaches and concepts to increase the structural and chemical diversity; these efforts have included high-throughput combinatorial synthesis and diversity-oriented synthesis [2]. Less developed have been efforts to increase the elemental diversity of drug-like molecules. In this work, we have examined the introduction of boron—specifically the isoelectronic and isostructural replacement of a C=C bond with a B-N bond—as a core structural element in bioactive ligands and have used nonsteroidal ligands for the estrogen receptor (ER) as a context. In these examples of "elemental isomerism," we have addressed the questions of whether the B-N analogs can be readily prepared, are stable, and retain structural characteristics consistent with biological activity.

Although boron is not an element commonly found in natural products or drugs, bioactive boron-containing compounds are known (Figure 1). The natural product boromycin and its relatives [3] are antibiotics and potential antivirals [4]. Borinic esters and oxazaborolidines with antibacterial activity are known [5, 6], and a benzoxaborole is being studied for onychomycosis [7]. Certain α-aminoboronic acids [8] and related agents have anticancer, hypolipidemic, and antifungal activity; diazaborines are antimalarials [9, 10], and others are protease [11] and proteosome inhibitors [12]. L-*p*-borono-phenylalanine and molecules with the compact $B_{10}H_{10}C_2$ carborane unit are of interest in boron-neutron capture therapy [13, 14]; the carborane has also been used in some nonsteroidal estrogens [15, 16].

Because of its inherently electrophilic character, the boron atom in most of these structures is tethered to a small-molecule unit through multiple single bonds to oxygen- or nitrogen-donor atoms, with boron thus being—formally—a boronate anion or a mixed boronic acid amide-ester. In some boronic acids, the electrophilic nature of the boron is also the key to its bioactivity [11, 12].

We were intrigued by another boron-containing entity, the boron-nitrogen (B-N) bond. The B-N bond is isoelectronic with the C=C double bond, and, curiously, at least with boron-10, which accounts for 20% of boron at natural abundance, the ^{10}B-^{14}N system has the same number of protons and neutrons as the ^{12}C-^{12}C system. Structural

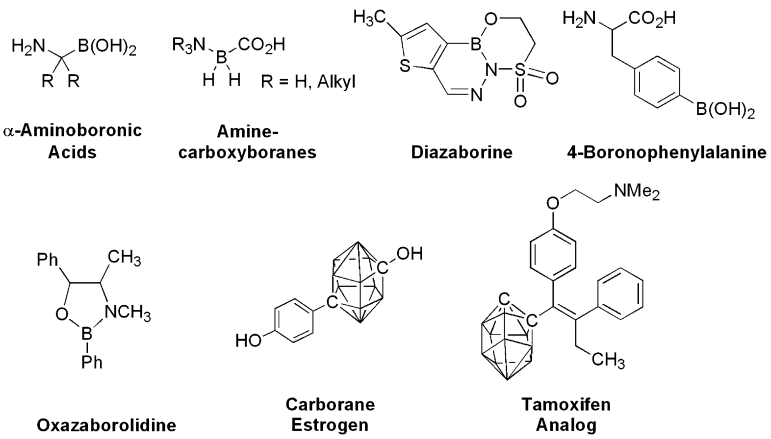


Figure 1. Boron-Containing Bioactive Molecules

studies have also shown that p_π-p_π bonding in B-N compounds gives partial double-bond character to the B-N bond that restricts rotation (Figure 2, left). This suggests that the isoelectronic substitution of a B-N bond for a C=C bond will also be isostructural [17–19]. Although it is not clear how hydrolytically stable the B-N bond will be, the electrophilicity of the boron center should be moderated to a considerable degree by electron donation from the nitrogen, and steric factors could play a role. As far as we are aware, a B-N bond has not been used as a surrogate for a C=C bond for the construction of bioactive molecules. Certain nonsteroidal ligands for the ER that have a C=C double bond at their core, exemplified by the 1,1-diarylethylenes and the triarylethylenes (Figure 2, right, top), provide convenient vehicles for examination of this isostructural-elemental replacement (Figure 2, bottom, right).

The ERs, members of the superfamily of nuclear receptors, have emerged as attractive pharmaceutical targets for therapeutic intervention in a wide variety of diseases, including osteoporosis and breast cancer [20]. Estrogens act through two ER subtypes, ERα and ERβ, which have different tissue distributions and sequence differences in their ligand-binding domain [21]. Estrogens can have remarkable tissue-selective effects, which has led to the development of compounds, termed selective ER modulators (SERMs) [22], that function as estrogen agonists in some tissues (bone, brain, and the cardiovascular system), but as antagonists in others (uterus and breast) [23, 24]. The ERs can bind a variety of steroidal and nonsteroidal ligands, and the search for better SERMs has driven efforts to increase the chemical diversity of these ligands, especially the nonsteroidal ones.

In this report, we describe the facile synthesis of elemental analogs of the di- and triarylethylene moieties of nonsteroidal estrogens, wherein the central C=C bond has been replaced by a B-N bond. We anticipated that the aminoborane structural surrogates shown in Figure 2 would possess structures similar to those of the known C=C ligands on which they were based, and we hoped that they would be stable compounds with interesting biological activity. We have found that these compounds can be readily prepared, that the hydrolytic lability of the B-N bond can be moderated by steric shielding of the boron center, and that ligands with high ER affinity and good

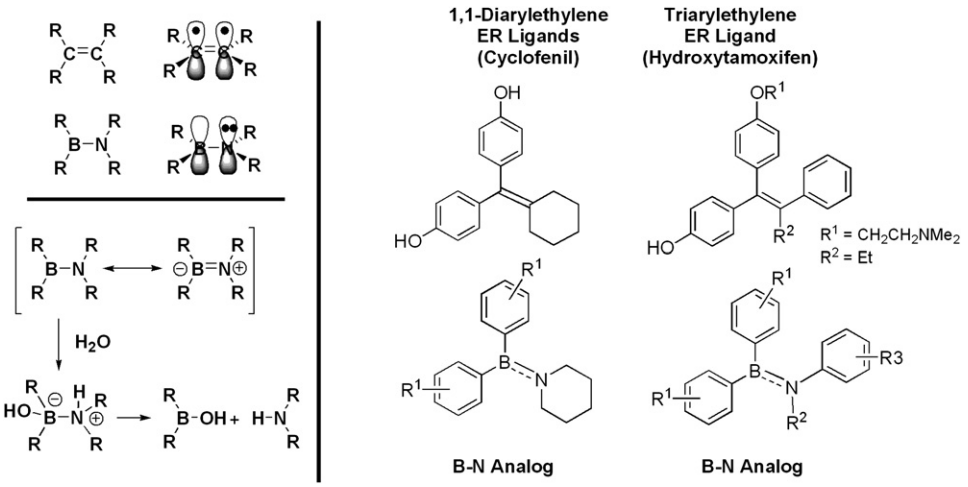


Figure 2. B-N Bond for C=C Bond Replacement and Di- and Triarylethylene

Comparison of π-bond systems, resonance forms, proposed mechanism of B-N hydrolysis, and nonsteroidal estrogens and their B-N analogs.

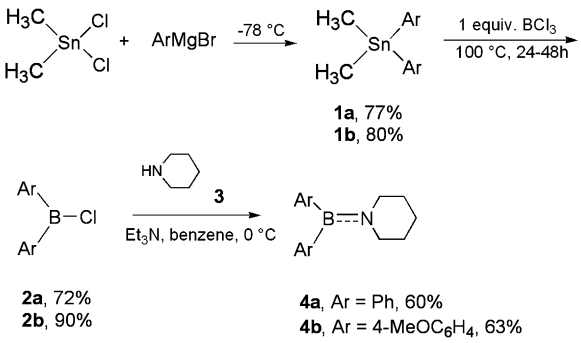


Figure 3. Synthesis of Piperidino Diarylboranes

potency and efficacy can be produced. Our optimization of the binding affinity and efficacy of these ER ligands in which the B-N bond was substituted for the C=C double bond was aided by X-ray crystallographic analysis of the complex of one B-N analog with ERα.

RESULTS

Chemical Synthesis

Piperidino Diarylboranes, B-N Analogs of 1,1-Diarylethylene Estrogens

To prepare compounds **4** (Figure 3), dimethyldiaryltins **1** were treated with BCl_3, furnishing diarylchloroboranes **2**, which reacted with piperidine (**3**) to give compounds **4**. These piperidine diarylboranes, however, were very sensitive to moisture and quickly decomposed in methanol.

To block hydroxyl attack on the unhindered boron center (Figure 2), we replaced the B-aryl groups with the hindered 2,4,6-trimethylphenyl (mesityl, Mes) groups. Dimesitylborane derivatives are known to be quite hydrolytically stable [17, 18]. We also replaced the piperidine with *p*-hydroxyanilines, to make analogs of triarylethylene estrogens.

Anilino Dimesitylboranes, B-N Analogs of Triarylethylene Estrogens

To synthesize anilino dimesiylboranes **8** and **9** (Figure 4A), fluoro dimesitylborane (**6**), prepared by a literature method from boron trifluoride etherate [18], was treated with the lithium anions of the various anilines **7** [17]. Standard product isolation gave the anilino boranes **8**, which could be purified by chromatography over neutral alumina. Demethylation of **8c–8g** with $BF_3 \cdot SMe_2$ proceeded at room temperature without product decomposition.

Gratifyingly, the anilino dimesitylboranes (**9c–9g**) were stable to air, water, dilute acid, and base (as well as to the more vigorous phenol-deprotection conditions). Also of note is the ease with which these highly hindered B-N compounds can be prepared, especially compared to their direct C=C analogs (see below).

Based on a crystal structure of compound **9e** bound to ERα, described below (Figure 5), we modified the aryl groups attached to the boron atom: the two *ortho* methyl groups were retained to stabilize the boron center against hydrolysis, but the *para* methyl groups were either substituted with methoxyl groups or were deleted to leave the rings unsubstituted at this site. The *p*-methoxyl analogs were to be precursors of the corresponding triols (**14**) in which one of the B-phenols might substitute for the A-ring phenol of an estrogen (Figure 4B).

Bis(2,6-dimethy-4-methoxyphenyl)fluoroborane, **10** [25], was treated with anilines **7c** and **7e** to give the anilino boranes **11a** and **11b**. We were, however, unable to prepare the corresponding anilino borane analogs **14** that had three free phenols, because these derivatives decomposed during demethylation. Notably, these B-N compounds were unstable to the same phenol-demethylation conditions used to convert methyl ethers **8c–8g** to phenols **9c–9g**; they were also unstable under milder deprotection conditions (Me_3SiI, etc.). Thus, introduction of a hydroxy group in the *para* position of the phenyl borane system must engender some instability. Reaction of **10** with 4-methylamino-phenol **12a** afforded the bismethoxy-monophenol analog, **13**.

The less bulky anilino boranes **16a–16c** were also synthesized in reasonable yields, respectively, by reaction of **7b**, **12a**, and **12c** with bis(2,6-dimethyl)fluoroborane, **15** (Figure 4C) [26]. Remarkably and conveniently, the B-N bonds in **13** and **16a–16c** can be formed in the presence of a free phenol.

Attempted Synthesis of the Carbon-Carbon and Carbon-Nitrogen Analogs of the Anilino Bis(2,6-dimethylphenyl)boranes

Because of the interesting binding and bioactivity of the anilino diarylboranes (see below), we attempted to prepare the corresponding C=C triarylethylene analog (compound **17**; Figure S1; see the Supplemental Data available with this article online) to evaluate more directly the effect of the isoelectronic B-N for C=C substitution on these biological parameters. The desired target compound (**17**) is, however, a very highly hindered, tetrasubstituted alkene, and thus far, we have been unable to prepare this molecule by using either Grignard addition, McMurray coupling, or thiadiazoline decomposition approaches, methods we have used to prepare highly hindered, tetrasubstituted diarylethylene nonsteroidal estrogens [27].

We also attempted to prepare a benzhydryl amine analog of the B-N compounds (**19**; Figure S1) by reacting *N*-methyl-*p*-methoxyaniline, **7c**, with the benzhydryl chloride, **18**. Upon demethylation, this would give a C-N analog of compound **16b**. This reaction was very sluggish, and after 3 days of reflux in acetonitrile, we obtained a new compound in low yield that was not the desired amine but rather the related imine (compound **20**), which could be characterized unmistakably by ^1H and ^{13}C NMR and mass spectrometry. Compound **20** represents a product of formal oxidative *N*-demethylation, and the mechanism for its formation is not obvious. The corresponding free phenol, **21**, was obtained by the demethylation of compound **20**.

X-Ray Crystal Structure of Dimesitylborane 9e

We were able to obtain a crystal structure of anilino borane **9e** as a complex with the ERα-ligand-binding domain (LBD) (Figure 5). With a resolution cutoff of 2.7 Å, the structure was refined to an R factor/R_{free} of 23.1%/29.7% (Table 1), indicating an acceptable fit of the structural

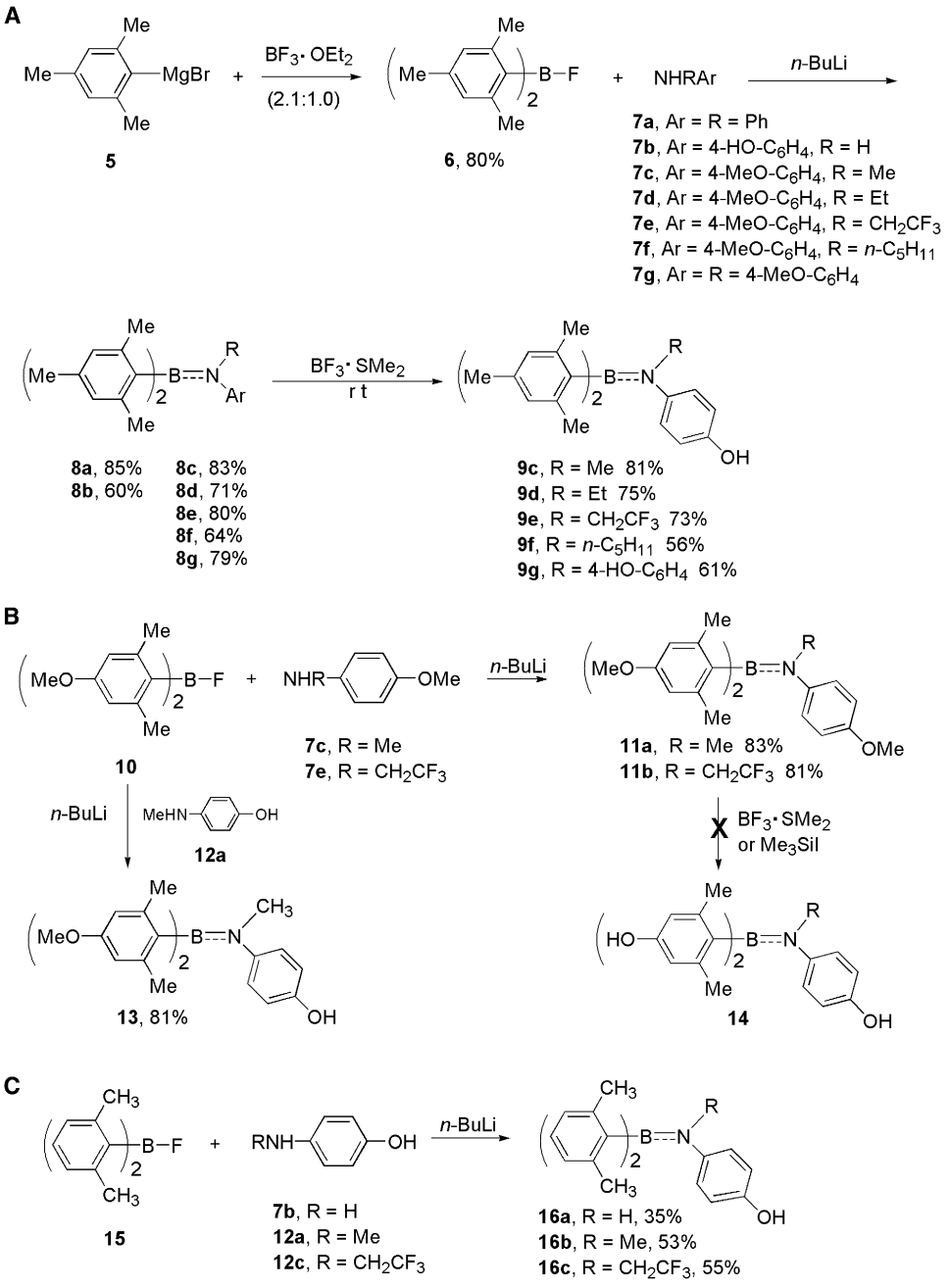


Figure 4. Synthesis of Anilino Diarylboranes

model into the electron density data. This structure, obtained in the presence of a coactivator peptide from the NR2 box of the SRC-2/GRIP-1 coactivator [28], which assists in crystallization of some ER-ligand complexes, helped us understand how these novel B-N analogs of triarylethylene estrogens bind to the ER. The ERα-LBD used for this structure work contained an engineered point mutation (Y537S) on the surface of the receptor [29]. Because it is more stable, this ERα-LBD can be expressed at very high levels in an active, unliganded form, which is a great advantage for crystallographic studies, and elsewhere we have shown that this ER mutant retains ligand-binding behavior equivalent to that of wild-type ER [30]. Further description of the utility of the Y537S ER mutant in crystallographic studies and the comparability of its structures with ligands to wild-type ER will be described elsewhere (K.W.N et al., unpublished data).

In the anilino borane **9e**-ERα-LBD complex, the receptor adopts a conformation that superficially resembles those of other ER agonist complexes (Figure 5A), but with distortions of the protein that are suggestive of a suboptimal fit of the ligand. The anilino borane molecule is roughly X

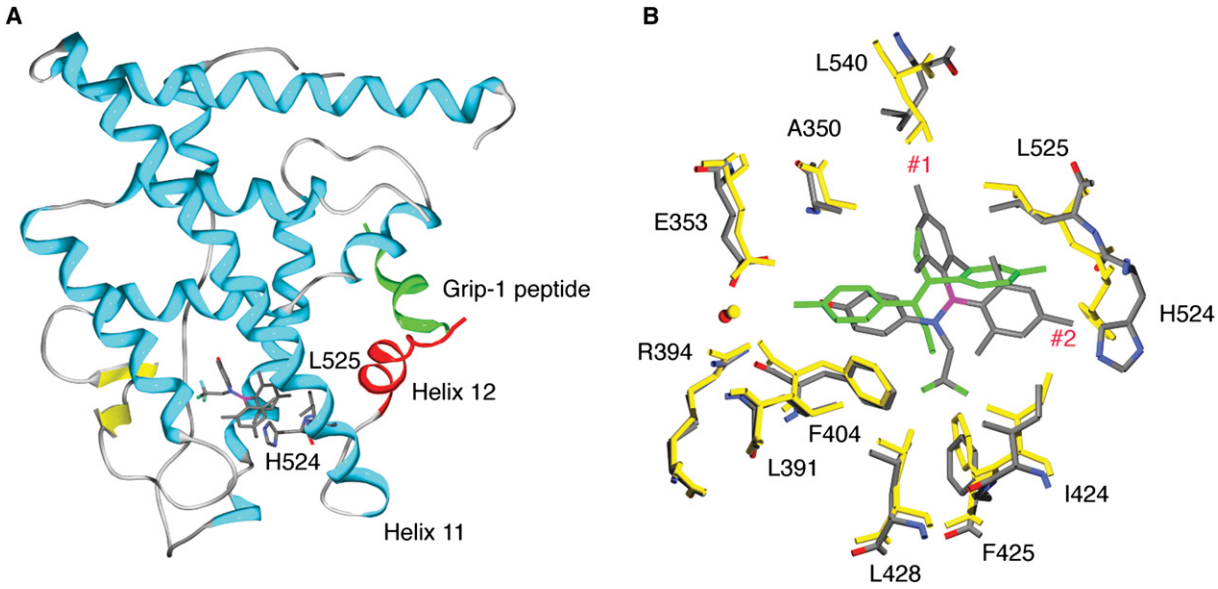


Figure 5. X-Ray Structure of Compound 9e Bound to ERα

(A) The ligand-binding domain of ERα is shown as a ribbon diagram; the bound compound **9e** is shown as a stick figure.

(B) Comparison of the X-ray crystallographic structure of anilino dimesitylborane (**9e**) complexed with ERα-ligand-binding domain with the diethylstilbestrol (DES) ERα structure [28]. The compound **9e**-ER structure is atom color coded by CPK; the ligand and selected residues in the pocket are shown. For the ligand, the boron is colored purple, and the fluorines are colored green. The DES molecule is colored green, and the corresponding ER amino acids are colored yellow. The structures were superimposed over all backbone residues by using SwissPDBViewer.

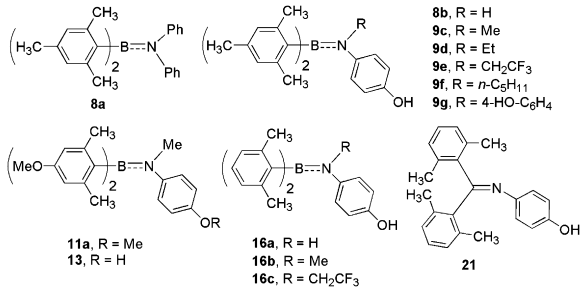
shaped (atom-colored structure, Figure 5B), and each axis of the "X" distorts the ligand-binding pocket because of certain unfavorable steric interactions. As a result, the ligand-binding pocket is enlarged (717 Å^3 with compound **9e**), which is ca. 200 Å^3 greater than what is typical for ER-agonist ligand complexes: 501 Å^3 for genistein-ERα-LBD (also with Y537S ERα; unpublished data) and 517 Å^3 for DES-ERα-LBD (wild-type sequence) [28].

Table 1. Summary of Crystallographic Statistics

Data Collection	
Beamline	APS-19BM
Space group	P 1 21 1
Unit cell (Å)	55.55 × 79.80 × 58.33
Resolution (Å)	12–2.70
Unique reflections	12,322
Average redundancy	3.6/3.1
Completeness (%)	93.2/82.3
R_{merge}	0.109/0.4
I/σ	11.4/2.27
Refinement	
Resolution (Å)	19–2.7
R_{free}/R factor	23.1/29.7
Average B factor	22
RMS deviations	
Bonds	0.010
Angles	1.381
Ramachandran analysis	
Disallowed	0

In all of the published ER structures, a phenol group forms a strong hydrogen bond network with E353, R394 (in ERα), and a highly conserved water molecule. In our structure, however, the phenolic oxygen of compound **9e** is a full angstrom further away from E353 (3.5 Å), compared to the typical distance of 2.5 Å seen in other structures (Figure 5B, compare compound **9e** in gray with diethylstilbestrol in green). Methyl group #1 prevents the ligand from entering more deeply into the pocket because of a close contact with A350. Methyl #1 and the trifluoromethyl group also distort the pocket, increasing the distance between L540 and I424, F425, and L428. A second distortion of the pocket occurs from the steric influence of Methyl #2, which repositions the backbone of helix 11 and the side chains of helix 11 residues H524 and L525. Electron density maps of the ligand and receptor-binding site residues illustrating these steric interactions are shown in the Supplemental Data (Figure S2). Thus, although the complex of anilino borane **9e** with ERα-LBD has an overall agonist-like topology, the pocket is considerably distorted from that typically seen with good ER agonists, because of the steric clashes between binding pocket residues and the two *para*-disposed methyl groups (Figure 5B, #1 and #2). These two steric interactions would be expected to reduce ligand-binding affinity, and the distortions that they induce in the ligand-binding pocket, particularly the

Table 2. ER Relative Binding Affinity of Anilino Diarylboranes and Imine Analog, 21



Entry	Compound	ERα	ERβ	α/β Ratio
1	**8a**	<0.003	0.008	—
2	**8b**	0.013 ± 0.001	0.183 ± 0.006	0.07
3	**9c**	1.3 ± 0.2	3.1 ± 0.8	0.4
4	**9d**	1.25 ± 0.01	1.12 ± 0.01	1.1
5	**9e**	2.24 ± 0.08	1.34 ± 0.38	1.7
6	**9f**	0.16 ± 0.04	0.120 ± 003	1.3
7	**9g**	0.17 ± 0.03	0.29 ± 0.09	0.6
8	**11a**	0.01	0.009	1
9	**13**	2.0 ± 0.51	1.3 ± 0.5	1.5
10	**16a**	1.67 ± 0.05	0.996 ± 0.05	1.7
11	**16b**	23 ± 0.01	16 ± 3.7	1.4
12	**16c**	10.15 ± 0.02	5.8 ± 1.5	1.7
13	**21**	0.21 ± 0.05	0.45 ± 0.26	0.47

Relative binding affinity (RBA) is $IC_{50}^{estradiol}/IC_{50}^{compound} \times 100$. The K_D for estradiol is 0.2 nM (ERα) and 0.5 nM (ERβ) [30, 32]. K_i values for the new compounds can be readily calculated by using the formula: $K_i = (K_D[estradiol]/RBA) \times 100$.

movement of helix 11 [31], also suggest that the agonist activity of this compound is not likely to be optimal.

The two "offending" *para*-disposed methyl groups, unlike the *ortho*-disposed methyls, are not essential for protecting the boron center from hydrolysis and thus could be removed without affecting the hydrolytic stability of the anilino borane functionality. We anticipated that their removal would relieve the unfavorable ligand-protein steric interactions and thus result in a tighter, more compact complex, which should have the more typical 500 Å^3 internal volume found with ER-agonist structures. We also thought that this change in ligand structure might allow the phenolic hydroxyl to interact in a more normal manner with E353, giving a stronger electrostatic interaction that should increase affinity. In addition, because we previously identified helix 11 as an allosteric modulator of helix 12 [31], we imagined that the more appropriately sized, undistorted ligand-binding pocket expected to form around the smaller anilino borane would have helix 11 properly positioned, and thus might give a complex that would produce a higher level of efficacy in transcription assays. Therefore, we prepared the additional series of aniline bis(2,6-dimethylphenyl)borane derivatives, as we have described (**16a**–**16c**; Figure 4C), analogs of the dimesitylboranes in which the *p*-methyl groups had been removed. The consequence of this structure-guided design modification on binding affinity and efficacy is detailed below.

It is of note that the orientation of the anilino borane **9e** in the ERα ligand-binding pocket is also unusual. Whereas other triarylethylene ER ligands, such as hydroxytamoxifen, place one of the geminally substituted phenyl groups in the A-ring-binding subpocket [28], the anilino borane uses the *N*-aryl group for this purpose. Undoubtedly, this results from the fact that, in the anilino boranes, we can prepare this as the only ring with a phenolic hydroxyl group; we have been unable to prepare anilino diarylboranes with hydroxyl groups on the B-aryl rings.

Binding Affinity

The binding affinities of the anilino diarylborane ligands for both ERα and ERβ, determined by a competitive radiometric binding assay [30, 32], are expressed as relative binding affinity (RBA) values, in which estradiol has an affinity of 100% (Table 2). Affinities depend on the nature of the substituents on both the nitrogen atom and B-diaryl groups. As is expected from the binding of many estrogens [33, 34], good binding requires an *N*-monophenol (**11a** versus **13**, entries 8 and 9), with a strong dependence on the nature

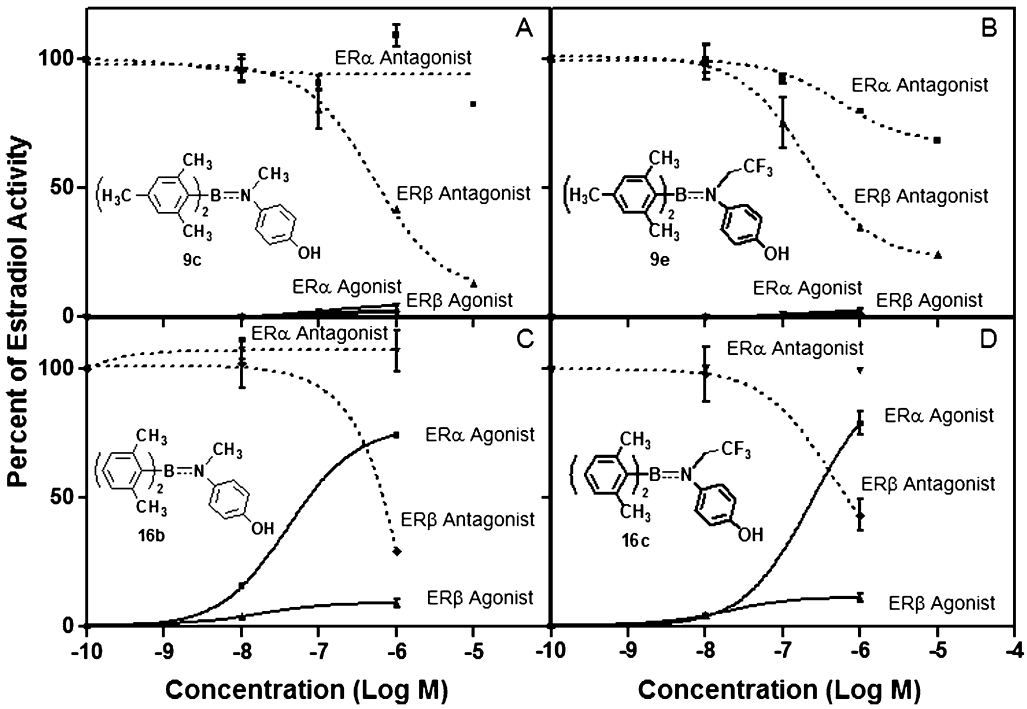


Figure 6. Transcription Activation through ERα and ERβ of Compounds 9c, 9e, 16b, and 16c

Human endometrial cancer cells (HEC-1) were transfected with expression vectors for ERα or ERβ and the estrogen responsive gene 2xERE-pS2-Luc and were incubated with the indicated ligand for 24 hr. Agonist assays are shown as solid lines, and antagonist assays (done in the presence of 1 nM estradiol, E2) are shown as dashed lines. Values are the mean ± SD of two or more experiments, expressed as a percent of the activity of ERα and ERβ with 10^{-9} M E2, which is set at 100%.

of the *N*-substituent. Regarding the nitrogen substituent, the highest affinity is observed in ligands with small *N*-alkyl groups. For example, ligand **9c**, with an *N*-methyl group, gave RBA values of 1.3% and 3.1% for ERα and ERβ, respectively (Table 2, entry 3), but binding affinity and subtype selectivity then decreases with an increase in alkyl chain length (namely, compounds **9d**, **9f**, and **9g**), the exception being the *N*-trifluoroethyl compound **9e**, which showed somewhat improved affinity and selectivity for ERα.

As anticipated from the X-ray structure, the *para* substituent on the B-aryl group has a big effect on binding affinity: analogs with *para*-disposed Me and MeO groups give similar, relatively modest affinities (**9c** and **13**), whereas deletion of the *para* groups resulted in a very marked increase in binding affinity (**16b** versus **9c** and **16c** versus **9e**), which, in the first case, was 20- and 14-fold on ERα and ERβ, respectively. Thus, elimination of the two groups responsible for steric clashes noted in the crystal structure resulted in significant increases in ER-binding affinities. In the one comparison we can make between a B-N compound and a C=N analog, it is apparent that the B-N compound (**16a**) binds better than the C=N analog (**21**), although the comparison is not exact, because compound **16a** has an N-H group, whereas compound **21** has only a nitrogen lone pair.

Transcriptional Activity

Some of the higher-affinity compounds were assayed for transcriptional activity through ERα and ERβ by cotransfection assays in human endometrial cancer cells (HEC-1) [35]. Neither of the dimesitylborane compounds (**9c** and **9e**; Figures 6A and 6B) showed substantial agonist activity, consistent with the proposed steric clash by the two *para* methyl groups that distorts the ligand-binding pocket, partially displacing helix 11 [31], and they appeared to be ERβ-selective antagonists, which, in themselves, comprise an interesting pharmacological class.

Also of interest were the activities of the less bulky anilino borane derivatives, (**16b** and **16c**) with the offending *p*-methyl groups removed, which were predicted to form a more compact structure with less distortion of helix 11. Both of these compounds showed good ERα agonist activity, but they were still nearly complete antagonists on ERβ (Figures 6C and 6D). Their potencies were also higher than those of the more bulky dimesitylboranes (**9c** and **9e**), consistent with their higher binding affinity.

DISCUSSION

In this study, we have expanded the chemical diversity of drug-like molecules by making an elemental substitution of a B-N bond for a C=C double bond, a molecular replacement that is isoelectronic and potentially isostructural, but that heretofore has not been widely examined. As a test system for this elemental substitution, we selected nonsteroidal ligands for the ER because these molecules are structurally simple, have a C=C double bond at

their core, and can be evaluated easily for their receptor-binding affinity and cellular bioactivity.

We found that the synthesis of B-N for C=C substitution analogs of members of the di- and triarylethylene nonsteroidal estrogens could easily be accomplished, and that the hydrolytic stability of the products was critically controlled by steric hindrance of the boron atom of the B-N bond. Although the electrophilic nature of the boron center in the B-N bond is reduced by electron donation through resonance from the nitrogen lone pair, it is still sufficiently reactive to undergo hydrolysis when unhindered. Introduction of flanking *ortho* methyl groups on both B-phenyl substituents, however, provided sufficient steric screening to make these substituted analogs very stable in aqueous and other harsh environments, including the acidic conditions used to deprotect aryl methyl ethers. Other aspects of their synthesis and stability were interesting. We could prepare diaryl-B-N-alkyl/aryl systems in which the aniline unit had a free *p*-hydroxy group simply by reacting a fluoro diarylborane with an unprotected *N*-alkyl-*p*-hydroxyaniline, yet we were unable to prepare analogs having a *p*-hydroxyl group on the B-phenyl groups because these latter B-N compounds were unstable. Also, while we could readily prepare the very hindered anilino diarylboranes with four *ortho* methyl groups flanking the boron center, we were unable to prepare the corresponding C=C (**17**) or C-N analogs (**19**). Attempts to prepare the latter produced the diaryl-C=N-aryl compound through an unusual net oxidation with loss of the *N*-alkyl group.

We were gratified that despite the extra alkyl substitution needed to stabilize the boron center from hydrolysis, some of the anilino dimesitylboranes demonstrated significant, though not high binding affinity for the ERs. Fortuitously, we were able to obtain a crystal structure of one of these compounds (**9e**) complexed with ERα that was quite revealing. Because the only phenol we could introduce into these systems was on the *N*-phenyl group, this molecule was of necessity oriented in the ligand-binding pocket in a manner essentially the reverse that of other triarylethylenes. Nevertheless, because of the open space in the core of the ligand-binding pocket in the ERs, the four *ortho* methyl groups did not suffer steric clashes with the receptor [28, 36]. On the other hand, the two *para* methyl groups in the mesityl substituents each appeared to engage in steric clashes that distorted ligand binding and receptor conformation.

We were able to eliminate these hypothesized disruptive interactions by removing the *para* methyl groups from the two B-aryl groups, and as predicted, the binding affinity of all of these desmethyl analogs was greatly improved, especially with ERα because the ligand-binding pocket of ERα is larger than that of ERβ [37]. Notably, as well, the potency and level of agonism of the highest binding analogs (**16b,c**) in cellular assays of ERα-regulated transcription was also greatly improved, giving a compound with ERα agonist and ERβ antagonist activity, related to that of R,R-tetrahydrochrysene, a compound described by us earlier [38]. Thus, we were able to achieve a structure-guided optimization of the biological activity of these B-N core ER ligands. Despite extensive efforts, however, we have been unable to make a final verification of the structural basis of ligand improvement by obtaining a crystal structure of ERα complexed with the desmethyl B-N analog (**16c**).

SIGNIFICANCE

The regulation of all cellular activities by using small molecules, the implicit goal of chemical genomics, presents a challenge to chemists to create small-molecule libraries of increasing structural and functional diversity. In this work, we have examined a different approach—that of preparing elemental analogs of known bioactive molecules by the isoelectronic and isostructural replacement of a C=C bond with a B-N bond. We have demonstrated the scope and limitation of this approach in the context of nonsteroidal ligands for the ER to determine whether the B-N analogs could be readily prepared, would be stable, and would retain biological activity. These questions were all answered in the affirmative, but with limitations of structure required to sterically protect the electrophilic boron center. Nevertheless, we were able to prepare analogs of triarylethylene nonsteroidal estrogens that retained nanomolar binding affinity for both ERα and ERβ. Our optimization of affinity and bioactivity was assisted by a crystal structure we obtained of an anilino dimesitylborane-ERα complex that revealed what appeared to be unfavorable ligand-receptor steric clashes. With this structural insight, we were able to redesign our ligands, removing the groups responsible for these unfavorable interactions, with the result that affinity, potency, and bioactivity were greatly enhanced. The favorable results we have obtained in challenging biomedically important receptor targets with B-N for C=C substitution elemental analogs of ligands should provide encouragement for further evaluation of this isoelectronic and isostructural bond replacement in other biological contexts. The compounds are easy to synthesize, in some cases much easier than their C=C double bond analogs, and, with appropriate considerations for moderating hydrolytic stability, they are stable and bioactive. The introduction of this replacement of the C=C bond with the B-N elemental analogs should facilitate progress toward reaching the holy grail of chemical genomics.

EXPERIMENTAL PROCEDURES

Materials and Methods

All reagents and solvents were obtained from Aldrich. Tetrahydrofuran, diethyl ether, toluene, and dichloromethane were obtained prior to use from a solvent-dispensing system [39]. Glassware was oven dried, assembled while hot, and cooled under an inert atmosphere. Unless otherwise noted, all reactions were conducted in an inert atmosphere. Reaction progress was monitored with analytical thin-layer chromatography (TLC) on 0.25 mm Merck F-254 silica gel glass plates. Visualization was achieved by either UV light (254 nm) or a potassium permanganate indicator. Flash chromatography was performed with

neutral aluminum (0.040–0.063 mm) packing. Fluoro dimesitylborane, **6**; bis(2,6-dimethylphenyl)fluoroborane, **15**; bis(2,6-dimethy-4-methoxyphenyl)fluoroborane, **10**; and anilines **7d**–**7g** were synthesized according to the literature (see Supplemental Data for the details).

^{1}H NMR, ^{13}C, ^{19}F, and ^{11}B NMR spectra were obtained on a 500, 400, or 300 MHz instrument. The chemical shifts are reported in ppm and are referenced to either tetramethylsilane or the solvent. Mass spectra were recorded under electron impact conditions at 70 eV. Melting points were obtained on a Thomas-Hoover MelTemp apparatus and are uncorrected.

Synthesis of Anilino Diarylboranes

A solution of aniline **7** or **12** (0.1 mmol) in 5 ml ether was treated with 1.05 equivalents of a hexane solution of *n*-butyllithium (2.1 eq. for *p*-hydroxylaniline **7b** and **12a** and **12c**) under nitrogen atmosphere at room temperature. A solution of diarylborane **6**, **10**, or **15** (1 eq.) in dry ether was added to this solution, and the mixture was stirred for 30 min [17]. The solution was then quenched by water, extracted three times with 10 ml ethyl acetate, dried with potassium carbonate, and evaporated to yield the crude product. The crude anilino borane products were purified by passage through a column of neutral alumina with hexanes-ethyl acetate as eluant. Demethylation of **8c**–**8g** (0.1 mmol) was conducted by using 30 equivalents of $BF_3 \cdot SMe_2$ in 5 ml dichloromethane at room temperature overnight. The reaction mixture was quenched by 2 ml methanol and 5 ml water, extracted three times with 10 ml ethyl acetate, and dried over K_2CO_3, and the pure product was obtained by flash chromatography over neutral alumina or by preparative alumina thin-layer chromatography.

(N-Methyl-p-methoxylanilino)dimesitylborane, 8c

Compound **8c** was purified by flash chromatography over neutral alumina (30% ethyl acetate/hexanes) to give a light-yellow needle (83% yield) (mp 105°C–107°C); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.97 (d, *J* = 9.0 Hz, 2H), 6.81 (s, 2H), 6.64 (d, *J* = 9.0 Hz, 2H), 6.56 (s, 2H), 3.73 (s, 3H), 3.21(s, 3H), 2.28 (s, 3H), 2.27 (s, 6H), 2.14 (s, 3H), 2.08 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.39, 142.64, 140.71, 140.44, 137.22, 136.78, 128.13, 127.82, 125.74, 113.30, 55.50, 40.58, 22.81, 22.61, 22.30, 21.33; ^{11}B NMR (96 MHz, $CDCl_3$) δ 43.69; MS (EI) *m/z* 385 (M^+, 10). HRMS (EI) calcd for $C_{26}H_{32}BNO$ 385.257695, found 385.257084.

(N-Ethyl-p-methoxylanilino)dimesitylborane, 8d

Compound **8d** was purified by flash chromatography over neutral alumina (30% ethyl acetate/hexanes) to give a light-yellow solid (71% yield) (mp 129°C–130°C); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.96 (d, *J* = 9.0 Hz, 2H), 6.80 (s, 2H), 6.63 (d, *J* = 9.0 Hz, 2H), 6.54 (s, 2H), 3.72 (s, 3H), 3.64 (q, *J* = 7.2 Hz, 2H), 2.31 (s, 6H), 2.28 (s, 3H), 2.14 (s, 3H), 2.10 (s, 6H), 1.16 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 156.34, 141.62, 140.39, 137.04, 136.68, 128.15, 127.88, 126.16, 113.22, 55.45, 47.46, 22.93, 22.68, 21.27, 21.15, 16.44; ^{11}B NMR (96 MHz, $CDCl_3$) δ 45.44; MS (EI) *m/z* 399 (M^+, 100). HRMS (EI) calcd for $C_{27}H_{34}BNO$ 399.27334, found 399.27469.

(N-2,2,2-Trifluoro-ethyl-p-methoxylanilino)dimesitylborane, 8e

Compound **8e** was purified by flash chromatography over neutral alumina (30% ethyl acetate/hexanes) to give a light-yellow solid (80% yield) (mp 38°C–40°C); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.15 (d, *J* = 9.0 Hz, 2H), 6.82 (s, 2H), 6.66 (d, *J* = 9.0 Hz, 2H), 6.58 (s, 2H), 4.11 (q, *J* = 9.0 Hz, 2H), 3.71 (s, 3H), 2.29 (s, 6H), 2.28 (s, 3H), 2.17 (s, 6H), 2.15 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 157.20, 142.01, 140.42, 140.32, 137.94, 137.43, 128.62, 128.19, 126.80, 125.44 (q, *J* = 280.8 Hz), 113.62, 55.44, 54.99 (q, *J* = 128.5 Hz), 22.77, 22.66, 21.99, 21.17; ^{19}F NMR (470 MHz, $CDCl_3$) δ −67.91; ^{11}B NMR (96 MHz, $CDCl_3$) δ 53.83; HRMS (ESI) calcd for $C_{27}H_{31}BF_3NO$ 453.24508, found 453.2429.

(N-Methyl-p-hydroxylanilino)dimesitylborane, 9c

Compound **9c** was purified by preparative alumina thin-layer chromatography (30% EtOAc/hexanes) to give a light-yellow solid (81% yield) (mp 58°C–60°C); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.90 (d, *J* = 9.0 Hz, 2H), 6.79 (s, 2H), 6.55 (d, *J* = 9.0 Hz, 2H), 6.53 (s, 2H), 4.58 (s, br, 1H), 3.18 (s, 3H), 2.26 (s, 3H), 2.25 (s, 6H), 2.13 (s, 3H), 2.05 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 152.29, 142.82, 140.70, 140.41, 137.24, 136.82, 128.12, 127.80, 127.94, 114.84, 113.48, 40.51, 22.74, 22.23, 21.26, 21.18; ^{11}B NMR (96 MHz, $CDCl_3$) δ 42.64; HRMS (ESI) calcd for $C_{25}H_{30}BNO$ 371.24204, found 371.2430.

(N-Ethyl-p-hydroxylanilino)dimesitylborane, 9d

Compound **9d** was purified by preparative alumina thin-layer chromatography (30% EtOAc/hexanes) to give a light-yellow solid (75% yield) (mp 56°C–58°C); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.90 (d, *J* = 8.5 Hz, 2H), 6.78 (s, 2H), 6.54 (d, *J* = 8.5 Hz, 2H), 6.52 (s, 2H), 4.54 (s, br, 1H), 3.61 (q, *J* = 7.2 Hz, 2H), 2.29 (s, 6H), 2.26 (s, 3H), 2.12 (s, 3H), 2.08 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 152.20, 141.75, 140.36, 137.04, 136.71, 128.13, 127.85, 126.35, 114.78, 113.48, 47.39, 22.90, 22.66, 21.25, 21.15, 16.36; ^{11}B NMR (96 MHz, $CDCl_3$) δ 44.37; MS (EI) *m/z* 385 (M^+, 100). HRMS (EI) calcd for $C_{26}H_{32}BNO$ 385.25769, found 385.257851.

(N-2,2,2-Trifluoro-ethyl-p-hydroxylanilino)dimesitylborane, 9e

Compound **9e** was purified by preparative alumina thin-layer chromatography (30% EtOAc/hexanes) to give a light-yellow solid (73% yield) (mp 75°C–77°C); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.06 (d, *J* = 8.5 Hz, 2H), 6.79 (s, 2H), 6.56 (d, *J* = 9.0 Hz, 2H), 6.54 (s, 2H), 4.67 (s, br, 1H), 4.06 (q, *J* = 9.0 Hz, 2H), 2.26 (s, 3H), 2.25 (s, 6H), 2.13 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 153.18, 142.14, 140.37, 140.29, 137.94, 137.43, 128.58, 128.14, 127.03, 126.49 (q, *J* = 282.2 Hz), 115.17, 54.91 (q, *J* = 128.5 Hz), 22.71, 22.66, 21.98, 21.15; ^{19}F NMR (470 MHz, $CDCl_3$) δ −67.91; ^{11}B NMR (96 MHz, $CDCl_3$) δ 52.50; MS (EI) *m/z* 439 (M^+, 39). HRMS (EI) calcd for $C_{26}H_{29}BF_3NO$ 439.22943, found 439.22868.

(N-Methyl-p-hydroxylanilino)bis(2,6-dimethyl-4-methoxylphenyl)borane, 13

Compound **13** was purified by flash chromatography over neutral alumina (20% ethyl acetate/hexanes) to give a light-yellow solid (81% yield) (mp 173°C–175°C); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.95 (d, *J* = 8.8 Hz, 2H), 6.63 (d, *J* = 8.8 Hz, 2H), 6.54 (s, 2H), 6.30 (s, 2H), 3.78 (s, 3H), 3.72 (s, 3H), 3.68 (s, 3H), 3.19 (s, 3H), 2.27 (s, 6H), 2.07 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.83, 141.82, 127.78, 125.93, 122.67, 114.85, 113.48, 112.71, 112.37, 111.86, 55.30, 40.56, 22.62, 21.65; ^{11}B NMR (96 MHz, $CDCl_3$) δ 44.36; MS (EI) *m/z* 403 (M^+, 10). HRMS (EI) calcd for $C_{25}H_{30}BNO_3$ 403.23187, found 403.23277.

(N-Methyl-p-hydroxylanilino)bis(2,6-dimethylphenyl)borane, 16b

Compound **16b** was purified by preparative alumina thin-layer chromatography (30% EtOAc/hexanes) to give a light-yellow solid (53% yield) (mp 65°C–67°C); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.11 (t, *J* = 7.2 Hz, 1H), 6.06 (d, *J* = 7.2 Hz, 2H), 6.92 (t, *J* = 7.2 Hz, 1H), 6.91 (d, *J* = 8.8 Hz, 2H), 6.72 (d, *J* = 7.6 Hz, 2H), 6.55 (d, *J* = 8.8 Hz, 2H), 4.52 (s, 1H), 3.20 (s, 3H), 2.30 (s, 6H), 2.10 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 152.57, 140.74, 140.50, 128.83, 127.88, 127.56, 127.27, 126.91, 125.99, 114.86, 40.52, 22.88, 22.37; ^{11}B NMR (96 MHz, $CDCl_3$) δ 45.18; MS (EI) *m/z* 343 (M^+, 100). HRMS (EI) calcd for $C_{23}H_{26}BNO$ 343.21074, found 343.210505.

(N-2,2,2-Trifluoro-ethyl-p-hydroxylanilino)bis(2,6-dimethylphenyl)borane, 16c

Compound **16c** was purified by preparative alumina thin-layer chromatography (30% EtOAc/hexanes) to give a light-yellow solid (55% yield) (mp 113°C–115°C); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7 .14 (t, *J* = 8.0 Hz, 1H), 7.07 (d, *J* = 8.5 Hz, 2H), 6.97 (d, *J* = 7.5 Hz, 2H), 6.94 (t, *J* = 7.5 Hz, 2H), 6.73 (d, *J* = 8.0 Hz, 2H), 6.56 (d, *J* = 8.0 Hz, 2H), 4.62 (s, 1H), 4.07 (q, *J* = 9.5 Hz, 2H), 2.31 (s, 6H), 2.17 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 153.32, 141.90, 140.41, 140.33, 128.54, 128.08, 127.73, 127.26 (q, *J* = 281.7 Hz), 127.25, 127.03, 115.19, 54.94 (q, *J* = 32.2 Hz), 22.80, 22.13; ^{19}F NMR (470 MHz, $CDCl_3$) δ −67.89; ^{11}B NMR (96 MHz, $CDCl_3$) δ 48.56; HRMS (ESI) calcd for $C_{24}H_{25}BF_3NONH_4$ 429.23250 ($M + NH_4^+$), found 429.2303.

Synthesis of Carbon-Nitrogen Analog of the Anilino Bis(2,6-dimethylphenyl)boranes, 21

A mixture of *N*-methyl-*p*-hydroxyaniline **7c** (0.5 mmol) and the benzhydryl chloride, **18** (0.5 mmol), in 10 ml acetonitrile was heated at reflux for 3 days and concentrated in vacuo; the residue was dissolved in ethyl acetate. The mixture was washed with 0.25 M aqueous NaOH, dried over sodium sulfate, and concentrated in vacuo. The crude product, **20**, was purified by preparative silica gel thin-layer chromatography

(20% EtOAc/hexanes). Demethylation of **20** (0.1 mmol) was conducted by using 30 equivalents of $BF_3 \cdot SMe_2$ in 5 ml dichloromethane at room temperature for 48 hr. The reaction mixture was quenched by 2 ml methanol and 5 ml water and extracted three times with 10 ml ethyl acetate; the organic layer was washed by brine and dried over K_2CO_3; and the pure product was obtained by preparative silica gel thin-layer chromatography (30% EtOAc/hexanes).

(Bis-[2,6-dimethyl-phenyl]-methylene)-(4-methoxy-phenyl)-amine, 20

Light yellow viscous oil (25% yield); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.12–7.15 (m, 2H), 7.08–7.11 (m, 1H), 6.88–6.90 (m, 1H), 6.88 (d, J = 8.0 Hz, 2H), 6.71–6.75 (m, 2H), 6.69 (d, J = 8.0 Hz, 2H), 3.74 (s, 3H), 2.61 (s, 3H), 1.96 (s, 6H), 1.82 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 168.22, 156.82, 142.90, 139.94, 139.32, 137.77, 137.16, 136.69, 129.79, 129.57, 128.95, 128.73, 128.43, 123.27, 113.82, 55.54, 22.51, 21.22, 20.65; HRMS (ESI) calcd for $C_{24}H_{24}ONH$ 344.20143 (M + H$^+$), found 344.2014.

(Bis-[2,6-dimethyl-phenyl]-methylene)-(4-hydroxy-phenyl)-amine, 21

Light-yellow solid (55% yield) (mp 58°C–60°C); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.14–7.16 (m, 2H), 7.08–7.11 (m, 1H), 6.89–6.91 (m, 1H), 6.88 (d, J = 8.0 Hz, 2H), 6.67–6.69 (m, 2H), 6.62 (d, J = 8.0 Hz, 2H), 4.83 (s, br, 1H), 2.61 (s, 3H), 1.96 (s, 6H), 1.81 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 165.50, 153.52, 141.59, 139.84, 136.86, 135.00, 133.62, 129.87, 129.68, 128.64, 128.43, 127.29, 123.76, 120.92, 115.62, 22.50, 21.20, 20.66; HRMS (ESI) calcd for $C_{23}H_{23}ONH$ 330.18578 (M + H$^+$), found 330.1858.

Estrogen Receptor-Binding Affinity

Relative binding affinities were determined by a competitive radiometric binding assay as previously described [30, 32], by using 10 nM [^{3}H]estradiol as tracer ([6,7-^{3}H]estra-1,3,5,[10]-triene-3,17β-diol, 51–53 Ci/mmol, Amersham BioSciences, Piscataway, NJ); purified full-length human ERα and ERβ were purchased from PanVera/Invtrogen (Carlsbad, CA). Incubations were conducted for 18–24 hr at 0°C. Hydroxyapatite (BioRad, Hercules, CA) was used to absorb the receptor-ligand complexes, and free ligand was washed away. The binding affinities are expressed as relative binding affinity (RBA) values; the RBA of estradiol was set to 100%. The values given are the average ± range or SD of 2–3 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.

Gene Transcriptional Activity

Assays were performed as previously described [35, 40]. Human endometrial cancer (HEC-1) cells were maintained in minimum essential medium (MEM) plus phenol red supplemented with 5% calf serum and 5% fetal calf serum. Cells were plated in phenol red-free Improved MEM and 5% charcoal dextran-treated calf serum (CDCS) and were given fresh medium 24 hr before transfection. Transfection assays were performed in 24-well plates with a mixture of 0.35 ml serum-free Improved MEM medium and 0.15 ml Hank's balanced salt solution containing 5 μl lipofectin (Life Technologies, Inc., Gaithersburg, MD), 1.6 μg transferrin (Sigma, St. Louis, MO), 200 ng pCMV β-galactosidase as internal control, 1 μg 2ERE-pS2-Luc, and 100 ng ER expression vector per well. The cells were incubated at 37°C in a 5% CO_2-containing incubator for 5 hr. The medium was then replaced with fresh Improved MEM supplemented with 5% CDCS plus the desired concentrations of ligands. Cells were harvested 24 hr later. Luciferase and β-galactosidase activity was assayed as described [40].

Protein Purification and X-Ray Crystallography

The ERα-LBD (298–554) Y537S [29] was expressed in BL21 cells as a fusion protein with a 6xHis tag and a TEV protease site. After chromatography over a nickel-NTA column and tag cleavage, the protein was dialyzed into 20 mM Tris-HCl (pH 8), 50 mM NaCl, 10% glycerol, and 50 mM β-mercaptoethanol. The protein was then diluted twice in water and purified with Q-ion exchange chromatography, eluting at ~175 mM NaCl. The protein was concentrated to 10 mg/ml and incubated overnight with 1 mM compound **9e** and 1–2 mM GRIP peptide [28]. The protein-ligand slurry was centrifuged at 15,000 × g for 15 min, and the supernatant was used for crystallization trials. The protein crystallized in 25%–28% (w/v) PEG monomethyl ether 2000, 0.1 M Bis Tris (pH 6.5). Data were collected at the Structural Biology Center at the Advanced Photon Source. The data were processed with HKL2000, and the structure was solved by using molecular replacement with the CCP4 software package. The structure was refined and rebuilt with CCP4, CNS, XtalView, and Coot software.

Supplemental Data

Supplemental Data include experimental methods for the preparation and characterization of compounds **6**, **7c–7g**, **8a**, **8b**, **8f**, **8g**, **9f**, **9g**, **10**, **11a**, **11b**, **15**, and **16a** and a figure showing the electron density of the ligand and ERα-LBD-binding pocket residues and are available at http://www.chembiol.com/cgi/content/full/14/6/659/DC1/.

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