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Novel estrogen receptor (ER) modulators: Carbamate and thiocarbamate derivatives with *m*-carborane bisphenol structure

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ABSTRACT

Novel carborane-containing estrogen receptor (ER) modulators, carbamate and thiocarbamate derivatives **5** and **6**, were designed and synthesized based upon the *m*-carborane bisphenol skeleton. Their activities were evaluated by competitive binding assay with recombinant human ERα, transcriptional activation assay and cell proliferation assay. All test compounds dose-dependently bound to human ERα and showed potent estrogenic activity. The binding affinities of thiocarbamates **6a** and **6b** are higher than those of the alkyl carbamates **5a**–**5d** and are similar to that of the phenyl carbamate **5e**. The binding affinity was well correlated with the acidity of the NH proton, indicating the existence of an interaction between the NH proton and amino acid residue(s) of the ERα ligand binding domain. The amino acid residue(s) interacting with the NH proton appears to be different from Asp351, which is known to play an important role in the expression of antiestrogenic activity. The side chain of the *m*-carborane bisphenol structure strictly controls the balance of estrogenic and antiestrogenic activities, and the (thio)carbamates can be classified as an agonist group.

1. Introduction

Steroid hormones, estrogens, influence the growth, differentiation, and functioning of many target tissues.[1] 17β-Estradiol (E2) is an endogenous estrogen that plays important roles in the female and male reproductive systems, as well as in bone maintenance, in the central nervous system, and in the cardiovascular system.[1] The first step in the appearance of estrogenic activity is mediated by the binding of agonist ligands to estrogen receptors (ER) α and β, resulting in a conformational change. The ligand-bound ER dimerizes, forms huge complexes with various cofactors, and binds to specific promoter elements of DNA to initiate gene transcription.[1] Compounds that either induce or inhibit cellular estrogen responses have potential value as biochemical tools and candidates for drug development. Since the discovery of the non-steroidal estrogens, many estrogen agonists and antagonists have been developed as agents for regulating fertility, preventing and controlling hormone-responsive breast cancer, and post-menopausal hormone replacement.[2]

Binding of ligands to the ER ligand binding domain (LBD) primarily requires a phenolic ring, which hydrogen-bonds with Glu353 and Arg394 of the hER LBD (Fig. 1).[3] It is also well-known that the secondary alcohol group of E2 interacts with His524 of hER (Fig. 1).[3] The hydrophobic group should closely match the hydrophobic surface of the ER, so as to increase the binding affinity. The hydrophobic structure also plays a role as a scaffold, fixing the spatial positions of the hydrogen-bonding functional groups. In our studies to develop new hydrophobic structures for use in drug design, we have focused on the exceptional hydrophobic character and spherical geometry of carboranes, and utilized them as a hydrophobic component of biologically active molecules.[4]

The carboranes (dicarba-*closo*-dodecaboranes) are chemical building blocks of remarkable thermal stability and high boron content; they are resistant to attack by most types of reagent, and are generally inactive towards biological systems.[6] One of their most striking features is the ability of the two carbon atoms and ten boron atoms to adopt icosahedral geometry, in which the carbon and boron atoms are hexa-coordinated. This feature of the structure gives rise to the unusual properties of such molecules and their carbon and boron derivatives. Their properties make them uniquely suitable for various specialized applications, including synthesis of polymers for high temperature use and neutron shielding.[7] In the fields of medicinal chemistry and pharmaceutical sciences, incorporation of large numbers of boron atoms into tumor calls for boron neutron capture therapy (BNCT) has become of interest in recent years.[8] Most carborane-containing compounds that have so far been synthesized are composed of cellular building blocks (nucleic acids,[9] amino acids,[10] sugars,[11] and so on), to which carborane units are added.

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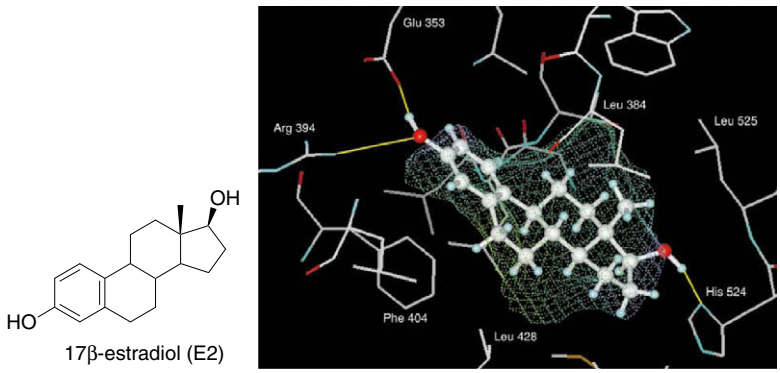


Figure 1. The structure of native estrogen, E2, and the binding mode to hERα LBD. The illustration is based on the reported docking study of E2 with hERα LBD.[5]

In our previous studies on carborane-containing ER modulators, we showed that the carborane cage plays an important role in controlling activity. The *p*-carborane cage is suitable for ER agonists, and the *o*-carborane cage for partial agonists and antagonists.[5,12] Previously, we have reported that a *m*-carborane bisphenol, **BE260** (**1**), exhibited potent estrogenic activity and an *o*-carborane 3,6-bisphenol (**2**), which has the same geometry as **1**, showed no estrogenic activity (Fig. 2).[13] That is because the *o*-carborane cage of **2** could not enter the hydrophobic pocket of ERα due to the existence of acidic C–H hydrogens. Additionally, mono-alkylaminated *m*-carborane bisphenol derivatives **3b** and **3d** behaved as partial ER agonists, while other similar derivatives, **3a** and **3c**, exhibited ER agonistic activity. They showed no ER antagonistic activity and acted as ER agonist (Fig. 2).[13,14] The side chain of the bisphenol derivatives tightly controls the estrogenic and antiestrogenic activities via interactions with the amino acid residues of the ER LBD. Thus, *m*-carborane bisphenol structure appears to be suitable for the development of various types of estrogen ligands, including agonists, partial agonists and selective estrogen receptor modulators (SERMs).

The alkylamino side chain of tamoxifen **4**, which is a clinically used antiestrogen, is protonated and electrostatically interacts with a negatively charged amino acid residue of Asp351 in the ER LBD.[15] The functional importance and spatial relationship of Asp351 for antiestrogenic activity have been well established.[3,16] Therefore, it is important to examine the structure–activity relationships of the side chains of the *m*-carborane bisphenol structure for the development of promising candidates for carborane-containing ER modulators. We focused on the interaction of the side chain of the ligands with the amino acid residues of the ER LBD. The NH proton of amide, urea and carbamate groups can interact with the carboxylate anion of the Asp residue through hydrogen bond formation.[17] Therefore, we designed and synthesized carbamate **5** and thiocarbamate derivatives **6** from **1**, and evaluated their activities by means of competitive binding assay with hER, transactivation assay and cell proliferation assay (Fig. 3).

2. Results and discussion

2.1. Chemistry

The designed compounds **5a**–**5d** were readily synthesized from **1** according to a general method for the synthesis of carbamates from phenols. Compound **1** was synthesized from *m*-carborane via two steps; the Ullmann type coupling reaction with 4-iodoanisole followed by deprotection of two methoxy groups.[18] Compound **1** reacted with a variety of isocyanates in the presence of triethylamine as a base in toluene to afford the corresponding mono-carbamates in 36–66% yield (Scheme 1).

On the other hand, the reaction of **1** with isothiocyanates did not proceed at all under the conditions used for the synthesis of carbamates derivatives **5**. The desired thiocarbamate derivatives **6a** and **6b** were synthesized from **1** by stepwise synthesis in one pot (Scheme 2). Compound **1** was treated with 10% NaOH aqueous solution, reacted with thiophosgene to generate *O*-alkyl chloromethanethioate in situ, and condensed with methylamine or cyclohexylamine to afford methylthiocarbamate **6a** or cyclohexylthiocarbamate **6b** in 72% or 32% yield, respectively.[19] All desired compounds were easily separated from the starting material and di-carbamates formed as by-products by means of silica gel

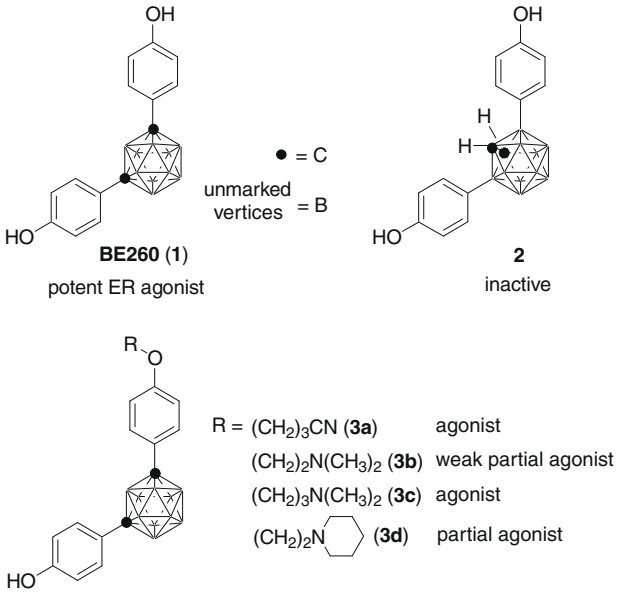


Figure 2. Profiles of *m*-carborane bisphenol derivatives.

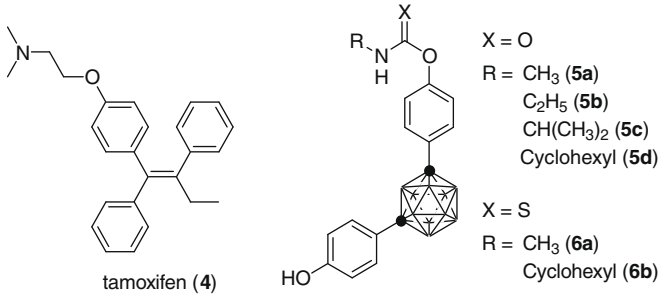
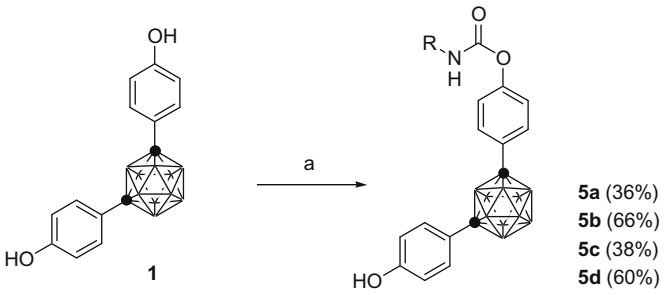
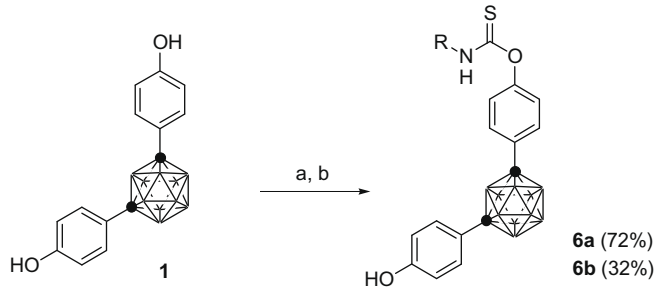


Figure 3. The structures of tamoxifen **4** and the designed compounds **5** and **6**.



Scheme 1. Synthesis of carbamate derivatives **5a**–**5d**. Reagents and conditions: (a) isocyanates, triethylamine, dry toluene, reflux.



Scheme 2. Synthesis of thiocarbamate derivatives **6a** and **6b**. Reagents and conditions: (a) 10% NaOH aqueous solution, thiophosgene ($CSCl_2$), $CHCl_3$, rt; (b) amines, rt.

column chromatography. The structures of the compounds were confirmed by melting point, NMR spectrum (^1H and ^{13}C), MS spectrum, IR spectrum, and elemental analysis (see Section 4). In the IR spectrum, the stretching vibrations of N–H, C=O and C=S were observed at around 3300, 1700 and 1200 cm^{-1}, respectively.

2.2. Competitive binding assay of the synthesized compounds with hERα

The binding affinity of the synthesized compounds for hERα was evaluated by means of competitive binding assay using [6,7-^{3}H]17β-estradiol ([^{3}H]E2) and recombinant hERα.[5] Compounds **5a**–**5d**, **6a** and **6b** were incubated with 4 nM of [^{3}H]E2 at 4 °C for 18 h, followed by filtration and measurement of the radioactivity remaining on a nitrocelluloses membrane. All the test compounds competed with [^{3}H]E2 and bound to hERα in a dose-dependent manner. Table 1 summarizes the relative binding affinity (RBA) of the synthesized compounds. All compounds showed weaker binding affinity to hERα than that of E2. The RBA value of the carbamates **5a**–**5d** was around 10 and there were no significant substituent effects. On the other hand, thiocarbamates **6a** and **6b** showed three or four times more potent binding affinity for ERα than the carbamates **5a**–**5d**, although they are weaker binders than E2. It is an interesting result that the small difference between carbonyl and thiocarbonyl groups markedly affected the binding affinity. This suggests that the difference of binding affinity between carbamate and thiocarbamate derivatives arises from either the difference of physical properties between oxygen and sulfur atoms or the acidity of the NH proton of the carbamate or thiocarbamate group. The acidity of the PhC(=S)NH_2 proton (pK_a 16.9) is much higher than that of an oxo amide (pK_a 23.4),[20] and the thiocarbamate group should interact more strongly than the carbamate group with Asp351 of the ERα LBD. Although it is not established whether the carbamate and thiocarbamate groups interact with Asp351 or some other amino acid residue(s) of the ERα LBD, the NH proton of the thiocarbamate should be involved in hydrogen bond formation with an amino acid residue(s) of the LBD.

Table 1
Relative binding affinity (RBA) of carbamate and thiocarbamate derivatives

Compound	Substituent	RBA[a]
E2	–	100
3b	$CH_2CH_2N(CH_3)_2$	1[b]
5a	$CONHCH_3$	9
5b	$CONHC_2H_5$	7
5c	$CONHCH(CH_3)_2$	8
5d	CONH-Cyclohexyl	10
6a	$CSNHCH_3$	39
6b	CSNH-Cyclohexyl	33

[a] The relative binding affinity of estradiol is taken as 100. Values represent the average of duplicate experiments.
[b] See Ref. 13.

2.3. Transcriptional activation assay of synthesized compounds

To evaluate the activity of the synthesized compounds as agonists and antagonists, transcriptional assay was done with ERE/Luci (firefly luciferase) and phRL/CMV (*Renilla* luciferase) plasmids, which were transiently transfected into human breast cancer MCF-7 cells.[21] Unexpectedly, all of the test compounds showed ER agonistic activity. None of them showed ER antagonistic activity (data not shown). That is, all the compounds act as agonists. Table 2 summarizes the EC_{50} values of **5a**–**5d**, **6a** and **6b** in ER-activation assay. The carbamate derivatives **5a**–**5d** showed sub-nanomolar-order EC_{50} values having higher efficacy than E2. Thiocarbamate derivatives **6a** and **6b** showed more potent ER agonistic activity than the carbamate derivatives, the estrogenic activity of these compounds being correlated with the order of the binding affinity for ERα.

It is noteworthy that the carbamate and thiocarbamate derivatives showed agonistic activity. As described above, the introduction of a side chain into the *m*-carborane bisphenol **1** sometimes produces an ER agonist (Fig. 2). Compound **3a**, which has a butyronitrile chain, showed ER agonistic activity in transactivation assay. In addition, **3c** acts as an ER agonist even though it has an alkylamino side chain that should be able to interact with Asp351. These results suggest that the pocket in which the Asp351 residue is located strictly controls the activity and that an optimal spatial relationship between the alkylamino chain and Asp351 residue is very important for the expression of ER antagonistic activity. The result of transactivation assay suggests that the carbamate group of the test compounds interacts with some other amino acid residue(s), not Asp351.

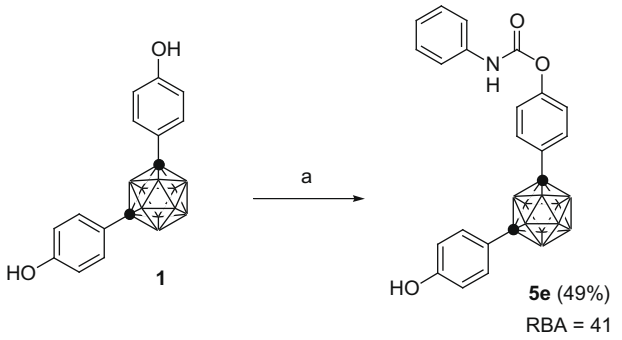
2.4. Design, synthesis and biological evaluation of phenyl carbamate derivative

A C–H···π interaction between an aromatic ring and a C–H moiety such as CH_3, CH_2 or CH group, is also important in the ligand-receptor complexation process.[22] The Leu354 amino acid residue lies near the Asp351 amino acid residue of ERα LBD, and introduction of an aromatic ring into the side chain of the carbamate compounds is expected to enhance the binding affinity through C–H···π interaction with Leu354. Moreover, aryl carbamate has a more acidic NH proton than alkyl carbamate, which is a great advantage for hydrogen bond formation. Thus, phenyl carbamate derivative **5e** was redesigned with the aim of increasing the binding affinity and synthesized in 49% yield using the same method as shown in Scheme 1 (Scheme 3). The binding affinity of **5e** was evaluated using the same method as described above. Interestingly, the RBA

Table 2
EC_{50} values of carbamate and thiocarbamate derivatives in transcriptional activation assay[a]

Compound	EC_{50} (M)[b]
E2	$2.02 \pm 0.32 \times 10^{-12}$ (100)
5a	$1.46 \pm 0.12 \times 10^{-10}$ (131)
5b	$3.82 \pm 0.11 \times 10^{-10}$ (152)
5c	$1.98 \pm 0.06 \times 10^{-10}$ (155)
5d	$4.30 \pm 0.23 \times 10^{-10}$ (171)
6a	$9.88 \pm 0.13 \times 10^{-11}$ (170)
6b	$1.15 \pm 0.19 \times 10^{-10}$ (137)

[a] Transactivation assay was performed in triplicate ($n = 3$).
[b] EC_{50} values of the test compounds were estimated from the sigmoidal dose–response curves using GraphPad Prism 4 software. Values are means ± SD for separate experiments. The values in parentheses indicate the efficacy for cell proliferation with the value for E2 taken as 100.



Scheme 3. Synthesis of phenyl carbamate derivative **5e**. Reagents and conditions: (a) phenyl isocyanate, triethylamine, dry toluene, reflux.

value of **5e** is 41, being much higher than those of carbamate derivatives **5a**–**5d** and similar to those of thiocarbamates **6a** and **6b**.

2.5. Cell proliferation assay using MCF-7 cell line

From the results of binding assay and transactivation assay, compounds **5c**, **5e**, and **6a** were selected for further evaluation, using cell proliferation assay with MCF-7 cells.[23] The compounds were incubated with MCF-7 cells for four days at 37 °C in humidified 5% CO_2 in air. All the test compounds showed potent estrogenic activity and no antiestrogenic activity (Fig. 4). Compound **6a** showed the most potent estrogen agonistic activity among them. Although compound **5e** showed similar potent binding affinity for ERα to the thiocarbamate **6a**, the estrogenic activity of **5e** was weaker than that of **6a** and similar to that of **5c** in cell proliferation assay.

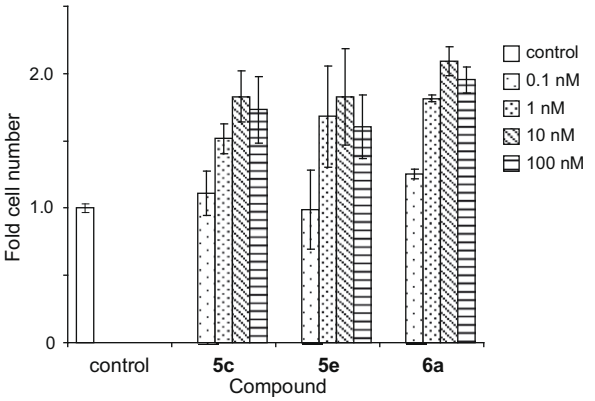


Figure 4. MCF-7 cell proliferation induced by the selected compounds **5c**, **5e**, and **6a**. The control was 0.5% DMSO, which was included in all test compound solutions. MCF-7 cells were incubated with the test compounds (1×10^{-10} to 1×10^{-7} M) for four days, and the results are shown as fold cell number, with the value for the control as taken 1. Cell proliferation assay was performed in triplicate ($n = 3$).

3. Conclusion

In conclusion, we have designed and synthesized carbamate and thiocarbamate derivatives based upon the *m*-carborane bisphenol structure as novel ER modulators, and evaluated their biological properties. The synthesized compounds **5** and **6** bound to recombinant hERα and showed potent estrogenic activity in transactivation and cell proliferation assays. The binding affinities correlated with the acidity of the NH proton, which may suggest an interaction between the NH proton and an amino acid residue(s) of the ERα LBD. The estrogenic activity of these compounds suggests that the amino acid residue interacting with the NH proton is different from Asp351, which plays an important role in the expression of antiestrogenic activity. Further studies of the structure–activity relationships and the influence of novel functional groups on the side chain of the *m*-carborane bisphenol structure should contribute significantly to the development of novel types of carborane-containing ER modulators.

4. Experimental

4.1. General considerations

Melting points were determined with a Yanaco micro melting point apparatus and were not corrected. ^{1}H NMR and ^{13}C NMR spectra were recorded with JEOL JNM-EX-270 and JNM-LA-400 spectrometers. Chemical shifts for ^{1}H NMR spectra were referenced to tetramethylsilane (0.0 ppm) as an internal standard. Chemical shifts for ^{13}C NMR spectra were referenced to residual ^{13}C present in deuterated solvents. The splitting patterns are designated as follows: s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet). Mass spectra were recorded on a JEOL JMS-DX-303 spectrometer. Elemental analyses were performed with a Perkin Elmer 2400 CHN spectrometer. Column chromatography was carried out using Merck silica gel 60 (0.063–0.200 μm) and TLC was performed on Merck silica gel F_{254}. *m*-Carborane was purchased from Katchem s.r.o. (Prague, Czech Republic). Other reagents were purchased from Wako Pure Chemical Industries, Ltd., Sigma-Aldrich Co., and Tokyo Chemical Industry, Ltd. (TCI). All solvents were commercial products of reagent quality, and were used without further purification.

4.2. Synthesis

4.2.1. 1-(4-Methylcarbamoyloxyphenyl)-7-(4-hydroxyphenyl)-*m*-carborane (5a)

To a stirred solution of 1 (500 mg, 1.52 mmol) in dry toluene (15 mL), methyl isocyanate (0.11 mL, 1.83 mmol) and triethylamine (10 mg) were added at room temperature. The mixture was refluxed for 1 h, then cooled to room temperature and concentrated. The residue was purified by silica gel column chromatography with 1:30 MeOH/$CHCl_3$ to give **5a** (220 mg, 36%) as a colorless solid. Colorless cubes (CH_2Cl_2-*n*-hexane) mp 173.5–175.0 °C; ^{1}H NMR (270 MHz, $CDCl_3$) δ (ppm) 1.40–3.80 (br m, 10H), 2.89 (d, $J = 4.8$ Hz, 3H), 5.04 (m, 1H), 5.85 (s, 1H), 6.58 (d, $J = 8.6$ Hz, 2H), 7.01 (d, $J = 8.6$ Hz, 2H), 7.26 (d, $J = 8.7$ Hz, 2H), 7.43 (d, $J = 8.6$ Hz, 2H); ^{13}C NMR (67.8 MHz, CD_3OD) δ (ppm) 27.6, 78.8, 80.1, 116.1, 122.7, 127.2, 130.0, 130.1, 133.3, 153.2, 157.1, 159.4; MS (FAB) *m*/*z* 444 (M^++1), 154 (100%); IR (KBr) ν (cm^{-1}): 3249 (NH), 1721 (C=O); Anal. Calcd for $C_{16}H_{23}B_{10}NO_3$: C, 49.85; H, 6.01; N, 3.63. Found: C, 49.91; H, 6.18; N, 3.70.

4.2.2. 1-(4-Ethylcarbamoyloxyphenyl)-7-(4-hydroxyphenyl)-*m*-carborane (5b)

To a stirred solution of 1 (500 mg, 1.52 mmol) in dry toluene (15 mL) and ethyl isocyanate (0.15 mL, 1.83 mmol) and triethylamine (10 mg) were added. This mixture was refluxed for 10 h, then further ethyl isocyanate (0.06 mL, 0.76 mmol) was added. The mixture was refluxed for 15 h, cooled to room temperature, and concentrated. The residue was purified by silica gel column chromatography with 1:30 MeOH/$CHCl_3$ to give **5b** (400 mg, 66%) as a colorless solid. Colorless needles (CH_2Cl_2-*n*-hexane) mp 135.0–136.5 °C; ^{1}H NMR (270 MHz, $CDCl_3$) δ (ppm) 1.21 (t, J = 7.3 Hz, 3H), 1.40–3.80 (br m, 10H), 3.31 (quint, J = 6.9 Hz, 2H), 5.05 (m, 1H), 5.89 (s, 1H), 6.58 (d, J = 8.7 Hz, 2H), 7.02 (d, J = 8.9 Hz, 2H), 7.26 (d, J = 8.7 Hz, 2H), 7.43 (d, J = 8.6 Hz, 2H); ^{13}C NMR (67.8 MHz, CD_3OD) δ (ppm) 15.1, 36.8, 78.8, 80.2, 116.1, 122.7, 127.3, 130.0, 130.1, 133.3, 153.2, 156.5, 159.5; MS (FAB) m/z 400 (M^++1), 154 (100%); IR (KBr) ν (cm^{-1}): 3345 (NH), 1715 (C=O); Anal. Calcd for $C_{17}H_{25}B_{10}NO_3$: C, 51.11; H, 6.31; N, 3.51. Found: C, 50.87; H, 6.15; N, 3.70.

4.2.3. 1-(4-Isopropylcarbamoyloxyphenyl)-7-(4-hydroxyphenyl)-*m*-carborane (5c)

Compound **5c** was prepared by the same method as described for the synthesis of **5a**. 38% yield; colorless needles (CH_2Cl_2-*n*-hexane); mp 155.5–157.0 °C; ^{1}H NMR (270 MHz, $CDCl_3$) δ (ppm) 1.24 (d, J = 6.6 Hz, 6H), 1.40–3.80 (br m, 10H), 3.88 (m, 1H), 4.88 (d, J = 7.6 Hz, 1H), 5.32 (s, 1H), 6.63 (d, J = 8.9 Hz, 2H), 7.02 (d, J = 8.7 Hz, 2H), 7.29 (d, J = 8.7 Hz, 2H), 7.43 (d, J = 8.9 Hz, 2H); ^{13}C NMR (67.8 MHz, CD_3OD) δ (ppm) 22.7, 44.4, 78.8, 80.1, 116.1, 122.7, 127.2, 129.9, 130.1, 133.2, 153.2, 155.7, 159.4; MS (FAB) m/z 414 (M^++1), 154 (100%); IR (KBr) ν (cm^{-1}): 3333 (NH), 1706 (C=O); Anal. Calcd for $C_{18}H_{27}B_{10}NO_3$: C, 52.28%; H, 6.58; N, 3.39. Found: C, 52.04; H, 6.57; N, 3.41.

4.2.4. 1-(4-Cyclohexylcarbamoyloxyphenyl)-7-(4-hydroxyphenyl)-*m*-carborane (5d)

Compound **5d** was prepared by the same method as described for the synthesis of **5a**. 60% yield; colorless needles (CH_2Cl_2-*n*-hexane) mp 180.0–181.0 °C; ^{1}H NMR (270 MHz, $CDCl_3$) δ (ppm) 1.10–1.46 (m, 4H), 1.40–3.80 (br m, 10H), 1.50–1.80 (m, 4H), 2.00 (m, 2H), 3.55 (m, 1H), 4.95 (d, J = 7.7 Hz, 1H), 5.53 (s, 1H), 6.60 (d, J = 8.7 Hz, 2H), 7.02 (d, J = 8.7 Hz, 2H), 7.28 (d, J = 8.7 Hz, 2H), 7.43 (d, J = 8.7 Hz, 2H); ^{13}C NMR (67.8 MHz, CD_3OD) δ (ppm) 26.1, 26.6, 33.9, 51.6, 78.9, 80.2, 116.1, 122.7, 127.3, 130.0, 130.1, 133.3, 153.2, 155.8; MS (FAB) m/z 454 (M^++1), 154 (100%); IR (KBr) ν (cm^{-1}): 3272 (NH), 1715 (C=O); Anal. Calcd for $C_{21}H_{31}B_{10}NO_3$: C, 55.61; H, 6.89; N, 3.09. Found: C, 55.68; H, 7.09; N, 3.00.

4.2.5. 1-(4-Phenylcarbamoyloxyphenyl)-7-(4-hydroxyphenyl)-*m*-carborane (5e)

Compound **5e** was prepared by the same method as described for the synthesis of **5a**. 49% yield; colorless needles (CH_2Cl_2-*n*-hexane) mp 164.0–165.5 °C; ^{1}H NMR (270 MHz, $CDCl_3$) δ (ppm) 1.40–3.80 (br m, 10H), 5.23 (s, 1H), 6.68 (d, J = 8.9 Hz, 2H), 6.95 (br s, 1H), 7. 10 (d, J = 8.9 Hz, 2H), 7.31 (d, J = 8.7 Hz, 2H), 7.33–7.46 (m, 5H), 7.48 (d, J = 9.1 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 77.2, 78.2, 115.1, 119.0, 121.4, 124.3, 127.3, 129.1, 129.1, 129.2, 129.3, 132.8, 136.8, 150.7, 156.3; MS (FAB) m/z 448 (M^++1), 154 (100%); IR (KBr) ν (cm^{-1}): 3321 (NH), 1703 (C=O); HRMS Calcd for $C_{21}H_{25}B_{10}NO_3$: 448.2926. Found: 448.2906.

4.2.6. 1-(4-Methylthiocarbamoyloxyphenyl)-7-(4-hydroxyphenyl)-*m*-carborane (6a)

To a mixture of 1 (300 mg, 0.91 mmol) and 10% NaOH aqueous solution (0.72 mL, 1.83 mmol) was added thiophosgene (91 μL, 1.19 mmol) in $CHCl_3$ (5 mL), and the reaction mixture was stirred for 30 min at room temperature. Then 40% methylamine in water (0.11 mL, 1.37 mmol) was added, and the mixture was stirred for 1 h at room temperature. More 40% methylamine in water (0.11 mL, 1.37 mmol) was added and stirring was continued for 30 min. The mixture was poured into water and extracted with AcOEt. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated. The residue was purified by silica gel column chromatography with 1:30 MeOH/CH_2Cl_2 to give **6a** (0.28 g, 76%) as a colorless solid. Colorless cubes (CH_2Cl_2-*n*-hexane); mp 144.0–145.5 °C; ^{1}H NMR (270 MHz, $CDCl_3$) δ (ppm) 1.40–3.80 (br m, 10H), 3.18 (d, J = 4.9 Hz, 3H), 3.49 (q, J = 7.1 Hz, 1H), 4.97 (s, 1H), 6.70 (d, J = 8.9 Hz, 2H), 6.99 (d, J = 8.7 Hz, 2H), 7.33 (d, J = 8.7 Hz, 2H), 7.47 (d, J = 8.9 Hz, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ (ppm) 30.9, 77.5, 78.7, 114.6, 122.5, 125.8, 128.3, 128.7, 132.3, 154.1, 158.0, 189.6; MS (FAB) m/z 402 (M^++1), 154 (100%); IR (KBr) ν (cm^{-1}): 3306 (NH), 1176 (C=S); Anal. Calcd for $C_{16}H_{23}B_{10}NO_2S$: C, 47.86; H, 5.77; N, 3.49. Found: C, 47.75; H, 5.39; N, 3.34.

4.2.7. 1-(4-Cyclohexylthiocarbamoyloxyphenyl)-7-(4-hydroxyphenyl)-*m*-carborane (6b)

To a mixture of 1 (300 mg, 0.91 mmol) in 10% NaOH aqueous solution (1 mL) was added thiophosgene (0.11 mL, 1.37 mmol) in $CHCl_3$ (3 mL), and the mixture was stirred for 1 h at room temperature. Then cyclohexylamine (0.20 mL, 1.83 mmol) was added, and the mixture was stirred for 4 h at room temperature, poured into water, and extracted with AcOEt. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated. The residue was purified by silica gel column chromatography with 1:30 MeOH/$CHCl_3$ to give **6b** (137 mg, 32%) as a colorless solid. Colorless cubes (CH_2Cl_2-*n*-hexane); mp 191.0–193.0 °C; ^{1}H NMR (270 MHz, $CDCl_3$) δ (ppm) 1.15–1.80 (m, 8H), 1.40–3.80 (br m, 10H), 2.00–2.20 (m, 2H), 3.86 (m, 1H), 4.07 (m, 1H), 4.86 (s, 1H), 6.70 (d, J = 8.9 Hz, 2H), 6.98 (d, J = 8.7 Hz, 2H), 7.33 (d, J = 8.7 Hz, 2H), 7.46 (d, J = 8.9 Hz, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ (ppm) 24.7, 25.1, 31.2, 54.7, 77.5, 78.7, 114.7, 122.5, 125.9, 128.2, 128.7, 132.2, 153.9, 158.0, 187.4; MS (FAB) m/z 470 (M^++1), 154 (100%); IR (KBr) ν (cm^{-1}): 3245 (NH), 1176 (C=S); Anal. Calcd for $C_{21}H_{31}B_{10}NO_2S$: C, 53.70; H, 6.65; N, 2.98. Found: C, 53.61; H, 6.39; N, 3.05.

4.3. Competitive binding assay with hAR

The ligand binding activity of estrogen receptor α (ERα) was determined by the nitrocellulose filter binding assay method. ERα (0.5 μg/tube, PanVera Co., Ltd.) was diluted with a binding assay buffer (20 mM Tris–HCl pH 8.0, 0.3 M NaCl, 1 mM EDTA pH 8.0, 10 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride) and incubated with 4 nM [6,7-^{3}H]17β-estradiol in the presence or absence of an unlabeled competitor at 4 °C for 18 h. The incubation mixture was absorbed by suction onto a nitrocellulose membrane that had been soaked in binding assay buffer. The membrane was washed twice with buffer (20 mM Tris–HCl pH 8.0, 0.15 M NaCl) and then with 25% ethanol in distilled water. Radioactivity that remained on the membrane was measured in Atomlight (NEN) by using a liquid scintillation counter.

4.4. Transfection and transactivation assay

Human breast cancer cell line MCF-7 cells were maintained in DMEM (Sigma Chemical Co.) containing penicillin and streptomycin with 5% fetal bovine serum (FBS; Life Technologies, Rockville, MD). ERE-luciferase reporter assay using MCF-7 cells was performed according to the reported method. Briefly, transient transfections in MCF-7 cells were performed using Transfast™ (Promega Co., Madison, WI), according to the manufacturer's protocol. Trans-

fections were done in 96-well plates at 8000 cells/well with 0.1 μg of $p(ERE)_3$-SV40-luc and 0.3 ng of phRL/CMV (Promega Co.) as internal standards. Twenty-four hours after addition of the sample, the assay was performed with a Dual Luciferase assay kit™ (Promega Co.). For the assay of antiestrogens, the inhibitory effect of test compounds on the estrogenic activity of E2 at the concentration of 1×10^{-10} M was examined.

4.5. MCF-7 Cell proliferation assay

4.5.1. Cell culture

At 80% confluence, cells of the human breast adenocarcinoma cell line MCF-7 was trypsinized from the maintenance dish with 0.25% trypsin-EDTA and collected by centrifugation (4 °C, 1500 rpm, 5 min). The supernatant was removed, 1 mL of DMEM supplemented with 10% FBS, 100 IU/mL penicillin and 100 mg/mL streptomycin was added, and cell aggregates were broken up by thorough pipetting. Cells were seeded in a dish at a concentration of 4×10^4 cell/mL or 8×10^4 cell/mL, and cultivated at 37 °C in a 5% CO_2 humidified incubator. Cells were routinely cultivated two days or three days later.

4.5.2. Cell proliferation assay

Cells of the human breast adenocarcinoma line MCF-7 were routinely cultivated in DMEM supplemented with 10% FBS, 100 IU/mL penicillin and 100 mg/mL streptomycin at 37 °C in a 5% CO_2 humidified incubator. On the day before an assay, MCF-7 cells were switched to DMEM (low glucose phenol red-free supplemented with 5% sFBS, 100 IU/mL penicillin and 100 mg/mL streptomycin). Cells were trypsinized from the maintenance dish into phenol red-free trypsin-EDTA and seeded in a 96-well plate at a density of 2×10^3 cells per final volume of 100 μL DMEM supplemented with 5% sFBS, 100 IU/mL penicillin and 100 mg/mL streptomycin. After 24 h, the medium was replaced with 90 μL of fresh DMEM and 10 μL of drug solution, supplemented with serial dilutions of 4-OH-Tam or DMSO as the dilution control in the presence or absence of 1×10^{-11} M E2, was added to triplicate microcultures. Cells were incubated for four days, and medium with 4-OH-Tam in the presence or absence of 1×10^{-11} M E2 was replaced once after three days. At the end of the incubation time, proliferation was evaluated by using WST-8. WST-8 (10 μM) was added to microcultures and cells were incubated for 2 h. The absorbance at 450 nm was measured. This parameter is related to the number of living cells in the culture.

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