

Aliphatic Substitution of *o*-Carboranyl Phenols Enhances Estrogen Receptor Beta Selectivity

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The two subtypes of estrogen receptor (ER), ER α and ER β , differ greatly in expression pattern and biological functions, and ER β -selective ligands candidates to treat immune-related disorders. ER β -selective ligands have mostly been designed based the idea of introducing a substituent that interferes sterically with the ligand's interaction with Met421 to selectively decrease the affinity for ER α (the equivalent residue in ER β is Ile373). Therefore, we designed and synthesized a series of carboranyl phenol derivatives bearing an aliphatic substituent as candidate ER β -selective ligands. Introduction of a longer aliphatic substituent into the carboranyl moiety enhanced the ER β selectivity of *o*-carboranyl phenol derivatives **4**, but not *m*-carboranyl bisphenol derivatives **5**. Compound **4c** showed 7.4-fold ER β selectivity in ER-binding assay and exhibited moderate estrogenic activity in cell proliferation assay using MCF-7 cell line.

Key words carborane; estrogen receptor; subtype selectivity; estrogenic activity; docking study

We have shown that the carborane cage is available as a novel hydrophobic pharmacophore for drug development,¹⁾ and since then, various carborane-containing bioactive compounds have been reported.^{2,3)} Prior to that, carboranes had been used only as boron carriers for boron neutron capture therapy (BNCT), based on their high boron content.⁴⁾ We also designed and synthesized various carborane-containing bioactive compounds as candidate nuclear receptor ligands.^{5–8)} One derivative, 1-(4-hydroxyphenyl)-12-hydroxymethyl-*p*-carborane (BE120, **1**), shows more potent estrogenic activity than the endogenous female hormone estrogen, estradiol (E2, **2**, Fig. 1).^{1,9,10)} Subsequently, many carboranyl phenol derivatives have been synthesized and their estrogen-related biological activities examined.^{11–18)} Compounds that either induce or inhibit cellular estrogenic responses are used for estrogen replacement therapy after menopause or the treatment of breast cancer, respectively.^{19–21)} Tissue-selective ligands, which act as agonists for bone metabolism and as antagonists for the female reproductive systems (known as selective estrogen receptor modulators, SERMs) are widely used for the treatment of osteoporosis.²²⁾

The estrogen receptor subtypes ER α ²³⁾ and ER β ²⁴⁾ are expressed in breast and uterus and in bone, colon, endothelial cells, bladder, and areas of the brain important for cognition, respectively. Most ER modulators are non-selective for ER subtypes, but it has been proposed that compounds with ER subtype selectivity would be useful as third-generation SERMs.^{25,26)} Harris *et al.* showed that ER β -selective ligands

may be therapeutically useful to treat chronic intestinal and joint inflammation by modulating the immune response.²⁷⁾ Thus, ER β -selective ligands are of interest as potential clinical candidates and also as probes for ER β -related molecular biology.

The X-ray crystal structures of receptor-ligand complexes of ER α and ER β show that their binding pockets are quite similar.²⁸⁾ Binding of **2** to the ligand binding domains (LBDs) of the two ERs is illustrated in Fig. 2. The same amino acid residues are involved in hydrogen bonding with the two hydroxyl groups of E2 in the LBDs of ER α and ER β , and thus E2 is accommodated equally well by the two LBDs. However, in the hydrophobic pocket of the LBDs, Leu384 and Met421 of ER α are substituted by Met336 and Ile373 in ER β , respectively (Fig. 2). Compound **3** is an ER β -selective ligand which was designed based the idea of decreasing the affinity for ER α by introducing a *n*-butyl group that interferes sterically with Met421 of ER α ²⁹⁾ (Fig. 3).

We have synthesized a range of carboranyl phenol derivatives and have discovered various types of ER modulators, partial agonists, super-agonists, antagonists, and SERMs.^{11–18)} In addition, our quantitative structure–activity relationship (QSAR) studies of very simple carboranyl phenols showed that the hydrophobicity of the carborane cage is significantly correlated with ER α -binding affinity.^{30,31)} Although carborane-

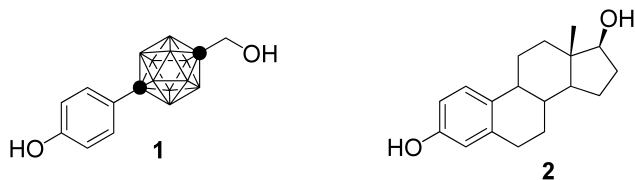


Fig. 1. Structures of Carborane-Containing Estrogen BE120 **1** and Estradiol **2**

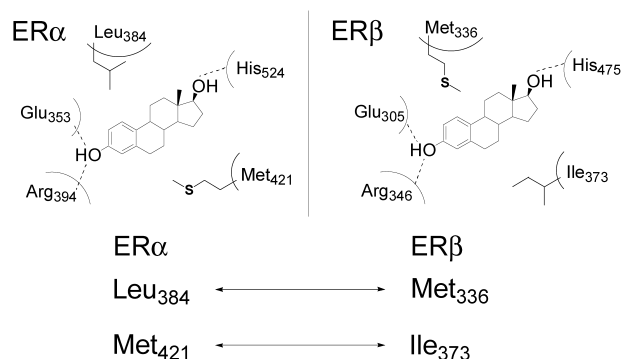


Fig. 2. Differences of Amino Acid Residues around the ER LBDs

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nyl phenol is a privileged structure as an ER ligand, little is known about the effect of aliphatic substituents on the carborane cage. Therefore, we designed and synthesized carboranyl phenol derivatives **4** and **5** with aliphatic substituents on the carborane cage for examination of their ER β selectivity and estrogenic activities. From the viewpoints of ease of synthesis and the directions of the aliphatic substituents, we focused on *o*-carboranyl phenol and *m*-carboranyl bisphenol as being most relevant to drug design.

Synthesis of *o*-carboranyl phenol derivatives **4a–c** is summarized in Chart 1. 4-Methoxyphenyl-*o*-carborane **6**, which

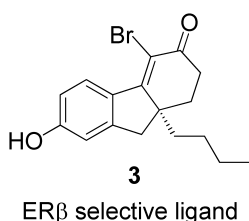
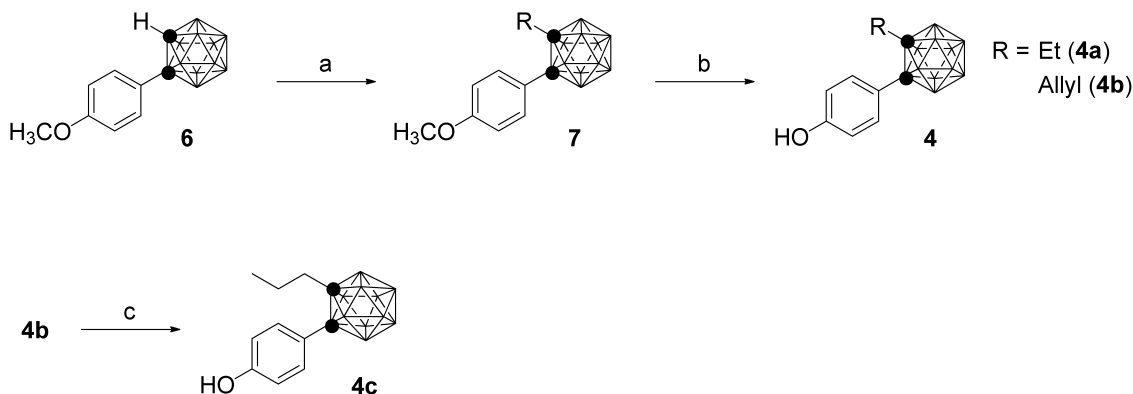
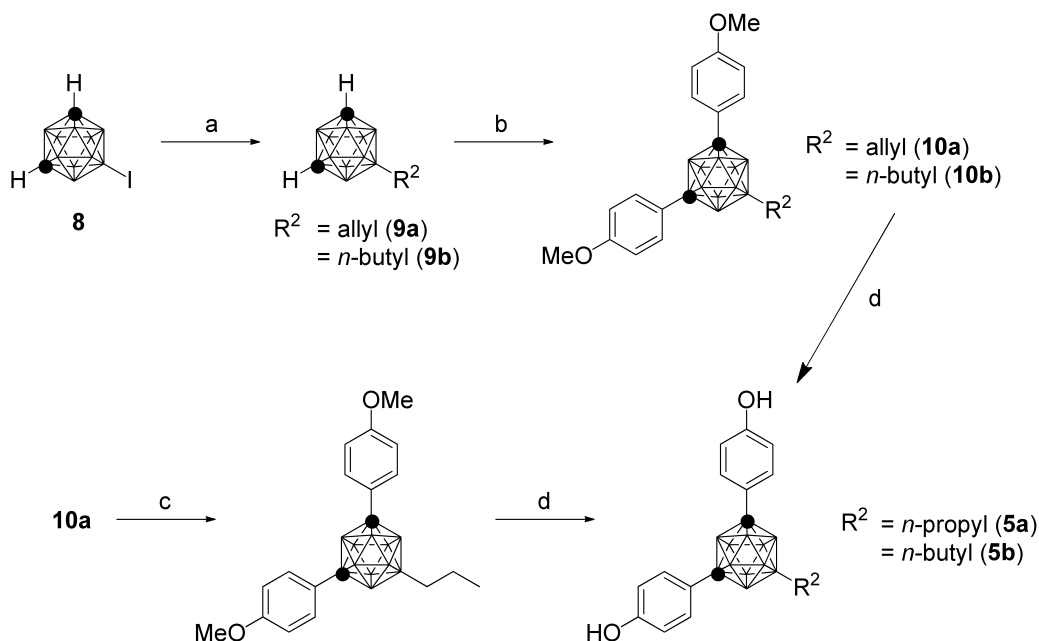


Fig. 3. Structure of ER β Selective Ligand **3**



Reagents and conditions: a) NaH, ethyl iodide or allyl bromide, DMF, rt; b) BBr₃, CH₂Cl₂, rt; c) Pd/C, H₂, MeOH/AcOEt, rt.

Chart 1. Synthesis of *o*-Carborane Derivatives **4a–c**



Reagents and conditions: a) Grignard reagents, Pd(PPh₃)₂Cl₂, Cu(I)I, THF, reflux; b) i) *n*-BuLi, DME, rt, ii) Cu(I)Cl, pyridine, 4-iodoanisole, 100°C; c) Pd/C, H₂, MeOH/AcOEt, rt; d) BBr₃, CH₂Cl₂, rt.

Chart 2. Synthesis of *m*-Carborane Derivatives **5a** and **b**

was synthesized by the literature procedure, was reacted with ethyl iodide or allyl bromide in the presence of NaH as a base to afford *C*-ethyl or *C*-allyl intermediates in 77% or 50% yield, respectively. Demethylation of the methoxy group of **7** with BBr₃ afforded the target compounds **4a** and **b** in 96% and 60% yields, respectively. The allyl group of compound **4b** was reduced by catalytic hydrogenation using Pd/C to afford the corresponding *C*-*n*-propyl derivative **4c** in 80% yield.

Chart 2 summarizes the synthesis of *m*-carboranyl bisphenol derivatives **5a** and **b**. 7-Iodo-*m*-carborane **8** was obtained by iodination of *m*-carborane.³²⁾ Grignard reagents, which were freshly prepared from Mg turnings and the corresponding halide, were reacted with **8** by Pd-catalyzed coupling reaction to afford *B*-allyl and *B*-*n*-butyl *m*-carborane in 99% and 61% yields, respectively.³³⁾ They were treated with *n*-butyl lithium to obtain the lithium salts, which were transformed into copper salts with Cu(I)Cl, and converted to the diaryl-*m*-carboranes **10a** and **b** in 37% and 39% yields, respectively.^{34,35)} *B*-*n*-Propyl derivative **5a** was synthesized by catalytic hydrogenation of the allyl group of **10a**, followed by deprotection of the phenol group with BBr₃ in 45% yield over

the 2 steps. Compound **5b** was obtained by deprotection of the phenol group of **10b** in 82% yield.

The ER binding affinities of the synthesized compounds were evaluated by means of competitive ER binding assays using [2,4,6,7-³H]17 β -estradiol and human recombinant ER α and ER β .³⁶⁾ Table 1 summarized the relative binding affinity (RBA) values, estimated based on the RBA of estradiol **2**, taken as 100. All the test compounds **4** and **5** bound to the ERs in a dose-dependent manner. Introduction of substituents onto the carborane cage decreased the binding affinity for both ERs. As the substituent was lengthened, the *o*-carboranyl phenol derivatives **4** showed a decrease of binding affinity for both ERs, but the decrease was more marked for ER α . Compound **4a** showed weak binding affinity for ER α but its binding affinity for ER β was higher than that of E2. This may suggest that ethyl group has an unfavorable steric interaction with the hydrophobic pocket of ER α , but not that of ER β . The RBA values of allyl derivative **4b** were decreased for both ERs. The allyl group is more polar than a *n*-propyl group and would be disfavored in a hydrophobic environment. Compound **4c** showed a high RBA value with 7.4-fold selectivity for ER β . These results show that introduction of an alkyl group onto the carborane cage markedly influences the ER β selectivity. On the other hand, *m*-carborane bisphenol derivatives **5a** and **b** showed little ER-subtype selectivity. We previously found that non-substituted *m*-carborane bisphenol had a similar RBA value to E2, while introduction of an alkylamino group into the second phenol group greatly decreased the binding affinity and changed the functional activity from ER agonist to ER antagonist.³⁷⁾ It seems that the phenol group is accommodated in a secondary hydrophobic pocket and forms hydrogen bonds in both ER LBDs.

A docking simulation of hER α (PDB: 1ERE) and hER β (PDB: 2IOG) with the carborane containing ligands, **4c** and **5b**, was performed with an automatic docking program (GOLD 5.2).³⁸⁾ A docking mode of **4c** in the hER β LBD is shown in Fig. 4a. The highly hydrophobic *o*-carborane cage and *n*-propyl group are located in the hydrophobic pocket of the receptor, but the *o*-carborane cage of **4c** is close to Ile373 of hER β , contrary to our hypothesis (Fig. 4a). Interestingly, the arrangement of **4c** in the LBDs of ER α is different from that in ER β (Fig. 4b) and *n*-propyl group of **4c** is close to Met421 in the hER α LBD (Fig. 4a). We suggested that the ER β selectivity of **4c** is caused by the steric repulsion between

the *n*-propyl group and Met421 of hER α . A docking study of **5b** with hERs showed that the second phenol group is accommodated in the secondary hydrophobic pocket of both ER LBDs (data not shown). The decrease of binding affinity and the poor selectivity of **5b** for ERs may suggest that the direction of *n*-butyl group is not suitable for steric repulsion to occur with Met421 of ER α .

Next, we evaluated the agonist and antagonist activities of the most ER β -selective compound **4c** by means of cell proliferation assay using the MCF-7 cell line, which shows estrogen-dependent growth.³⁶⁾ Compound **4c** promoted cell proliferation in a dose-dependent manner and the EC₅₀ value was 3.0×10^{-9} M (Fig. 5). When the maximum potency (E_{\max}) of E2 was taken as 100%, that of **4c** was 110%. Thus, compound **4c** is simply a weaker ER full agonist.

In conclusion, we examined the effect of introducing an aliphatic substituent into the carborane cage of *o*- or *m*-carboranyl phenol derivatives based on the structure of a well-known ER β -selective ligand **3**. Interestingly, the ER β selectivity of *o*-carboranyl phenol derivatives **4** was enhanced as the length of the substituent was increased, whereas *m*-carboranyl phenol derivatives **5** showed little ER β selectivity. The *C*-*n*-propyl *o*-carboranyl phenol **4c** showed 7.4-fold ER β selectivity in ER-binding assay and moderate estrogenic activity in MCF-7 cell proliferation assay. These results provide useful information on substituent effects to guide research aimed at discovery of selective carborane-containing estrogen modulators.

Experimental

General Considerations Melting points were determined with a Yanaco micro melting point apparatus and were not corrected. ¹H- and ¹³C-NMR spectra were recorded with JEOL JNM-EX-270 and JNM-LA-400 spectrometers. Chemical shifts of ¹H-NMR signals were referenced to tetramethylsilane (0.0 ppm) as an internal standard. Chemical shifts of ¹³C-NMR signals were referenced to residual ¹³C present in deuterated solvents. The splitting patterns are designated as follows: s (singlet), d (doublet), t (triplet), m (multiplet), q (quartet), and br (broad). Mass spectra were recorded on a JEOL JMS-DX-303 spectrometer. Column chromatography was carried out using Fuji Silysia BW80s and TLC was performed on Merck silica gel F₂₅₄. Carboranes were purchased from Katchem s.r.o. Other chemicals were purchased from Aldrich or Tokyo Kasei Ltd. and used without further purification, or

Table 1. Binding Affinity for ER α and ER β and Subtype Selectivity of Compounds **4** and **5**

Compound	R	RBA ^{a)}		Selectivity ER β /ER α
		ER α	ER β	
4a	Ethyl	43.7	140.7	3.2
4b	Allyl	9.3	44.9	4.8
4c	<i>n</i> -Propyl	11.8	87.0	7.4
5a	<i>n</i> -Propyl	27.6	21.7	0.8
5b	<i>n</i> -Butyl	32.4	43.5	1.3
E2 (1)		100	100	1.0

^{a)} Relative binding affinity with respect to that of estradiol **2** taken as 100%. All binding assays were performed in duplicate ($n=2$) and the numbers are average values.

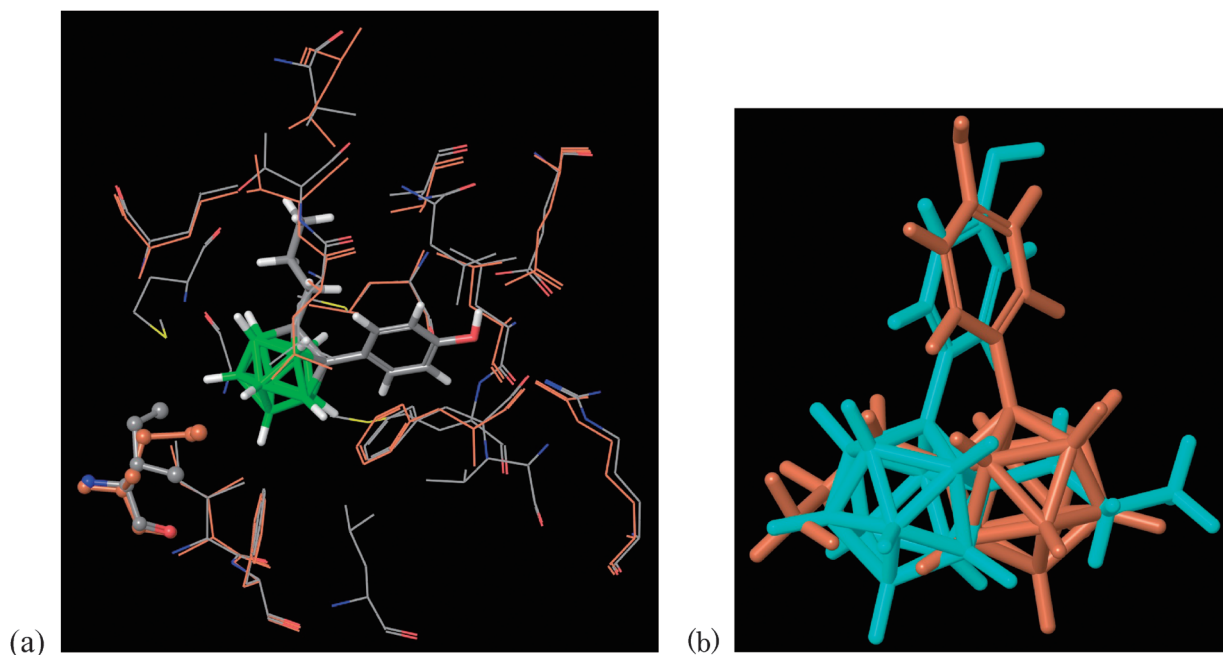


Fig. 4. Docking Model of **4c** to the LBDs of ER α and ER β

(a) Docking mode of **4c** in the hER β LBD (atom colors: H (white) B (green), C (gray), N (blue), O (red) and S (yellow)). The corresponding amino acid residues of hER α are colored orange. Both Ile373 of ER β and Met421 of ER α are displayed with a ball and stick model. (b) The arrangements of **4c** in the LBDs of ER α (brown) and ER β (aqua).

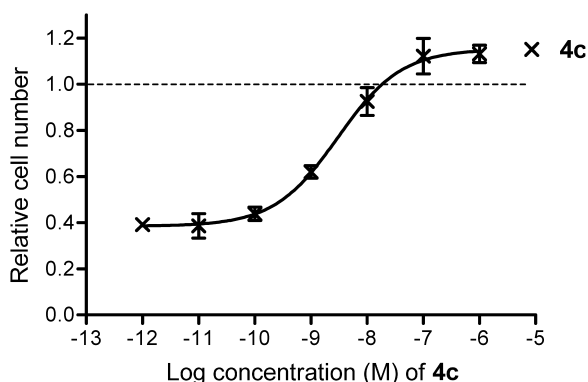


Fig. 5. Dose-Response Curve of Compound **4c** in MCF-7 Cell Proliferation Assay

MCF-7 cells were incubated with **4c** (1×10^{-6} to 1×10^{-12} M) for 4 d, and the results are shown as relative cell number, with the value for 0.1 nM estradiol taken as 1. Dashed line shows E_{\max} of E2. Cell proliferation assays were performed in triplicate ($n=3$). Values are means \pm S.D. for separate experiments.

were prepared according to procedures described in the literature. All solvents were commercial products of reagent grade, and were used without further purification.

General Procedure of C-Alkylation of 6 To a solution of **6** in dry *N,N*-dimethylformamide (DMF) was added NaH (1.2 eq) at 0°C under an argon (Ar) atmosphere, and the mixture was stirred for 30 min at the same temperature. Alkyl halide (1.2 eq) was added, and stirring was continued at room temperature for 2.5 h. The reaction mixture was poured onto ice and extracted with Et₂O. The organic layer was washed with brine, dried over Na₂SO₄, and concentrated. The residue was purified by silica gel column chromatography with 1:20 AcOEt-*n*-hexane to give the corresponding C-alkylated compound.

1-(4-Methoxyphenyl)-2-ethyl-1,2-dicarba-*closo*-dodecaborane: Colorless solid; 77% yield; ¹H-NMR (400 MHz, CDCl₃)

δ (ppm): 0.97 (t, $J=7.7$ Hz, 3H), 1.60–3.60 (brm, 10H), 1.85 (q, $J=7.7$ Hz, 2H), 3.82 (s, 3H), 6.87 (d, $J=9.2$ Hz, 2H), 7.53 (d, $J=9.2$, 2H); MS (electron ionization (EI)) m/z 278 (M^+ , 100%).

1-(4-Methoxyphenyl)-2-allyl-1,2-dicarba-*closo*-dodecaborane: Colorless solid; 50% yield; ¹H-NMR (400 MHz, CDCl₃) δ (ppm): 1.60–3.60 (brm, 10H), 2.53 (d, $J=7.7$ Hz, 2H), 3.83 (s, 3H), 4.73–4.79 (m, 1H), 5.03 (d, $J=10.1$ Hz, 1H), 5.56–5.68 (m, 1H), 6.88 (d, $J=8.7$ Hz, 2H), 7.54 (d, $J=8.7$, 2H); MS (EI) m/z 290 (M^+ , 100%).

General Procedure of Demethylation of 7 To a solution of **6** in CH₂Cl₂ was added a solution of 1 M BBr₃ (1.2 eq) in CH₂Cl₂ at -78°C, and the mixture was stirred at room temperature for 30 h, then poured onto ice and extracted with AcOEt. The organic layer was washed with brine, dried over Na₂SO₄, and concentrated. The residue was purified by silica gel column chromatography with 1:5 AcOEt-*n*-hexane to give the phenol derivative **4**.

1-(4-Hydroxyphenyl)-2-ethyl-1,2-dicarba-*closo*-dodecaborane (**4a**): 96% yield; colorless needles (AcOEt-*n*-hexane); mp 106.5–108.0°C; ¹H-NMR (400 MHz, CDCl₃) δ (ppm): 0.97 (t, $J=7.7$ Hz, 3H), 1.60–3.60 (brm, 10H), 1.85 (q, $J=7.7$ Hz, 2H), 5.25 (brs, 1H), 6.81 (d, $J=9.2$ Hz, 2H), 7.50 (d, $J=8.7$, 2H). MS (EI) m/z 264 (M^+ , 100%); high resolution (HR)-MS Calcd for C₁₀H₂₀B₁₀O: 264.2517, Found: 264.2521.

1-(4-Hydroxyphenyl)-2-allyl-1,2-dicarba-*closo*-dodecaborane (**4b**): 60% yield; colorless needles (CH₂Cl₂-*n*-hexane); mp 96.0–97.5°C; ¹H-NMR (400 MHz, CDCl₃) δ (ppm): 1.60–3.60 (brm, 10H), 2.54 (d, $J=7.2$ Hz, 2H), 4.72–4.79 (m, 1H), 5.03 (d, $J=10.1$ Hz, 1H), 5.34 (brs, 1H), 5.56–5.68 (m, 1H), 6.81 (d, $J=8.7$ Hz, 2H), 7.50 (d, $J=8.7$, 2H); MS (EI) m/z 276 (M^+ , 100%); HR-MS Calcd for C₁₁H₂₀B₁₀O: 276.2517, Found: 276.2516.

1-(4-Hydroxyphenyl)-2-*n*-propyl-1,2-dicarba-*closo*-dodecaborane (**4c**): To a solution of **4b** (25 mg) in AcOEt (5 mL) and MeOH (5 mL) was added Pd on carbon (5 mg) and the mixture

was stirred at room temperature for 30 min under a hydrogen atmosphere. The mixture was filtered, the residue was washed with AcOEt, and the organic layer was concentrated. The residue was purified by silica gel column chromatography with 1:5 AcOEt-*n*-hexane to give **4c** (21 mg, 80%) as colorless solid. Colorless oil; $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ (ppm): 0.74 (t, $J=7.2$ Hz, 3H), 1.60–3.60 (brm, 10H), 1.41 (sext, $J=7.7$ Hz, 2H), 1.73 (t, $J=8.7$ Hz, 2H), 5.45 (brs, 1H), 6.82 (d, $J=8.7$ Hz, 2H), 7.49 (d, $J=8.7$ Hz, 2H); MS (EI) m/z 278 (M^+ , 100%); HR-MS Calcd for $\text{C}_{11}\text{H}_{22}\text{B}_{10}\text{O}$: 278.2674, Found: 278.2678.

General Procedure of Synthesis of 9 A solution of Grignard reagent (4eq) was added dropwise to a stirred dry tetrahydrofuran (THF) solution (10 mL) of **8** (1.0 g, 3.70 mmol) at 0°C under an Ar atmosphere, and stirring was continued at room temperature for 30 min. Bis(triphenylphosphine)-palladium(II) dichloride (260 mg, 0.37 mmol) and copper(I) iodide (70.5 mg, 0.37 mmol) were added in one portion, and the reaction mixture was refluxed for 60 h. After removal of the solvent, Et_2O was added to the residue and the excess Grignard reagent was destroyed by slow addition of 10% HCl aqueous solution. The organic phase was separated from the mixture, and the aqueous layer was extracted with Et_2O . The combined organic phase was washed with water and brine, dried over Na_2SO_4 , and concentrated. The residue was purified by column chromatography on silica gel with *n*-hexane to afford the corresponding **9**.

9-Allyl-1,7-dicarba-*closo*-dodecaborane (**9a**): 99% yield; $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ (ppm): 1.40–3.50 (brm, 9H), 1.78 (brs, 2H), 2.88 (brs, 2H), 4.75–4.90 (m, 2H), 5.86 (m, 1H); MS (EI) m/z 184 (M^+ , 100%).

9-*n*-Butyl-1,7-dicarba-*closo*-dodecaborane (**9b**): 61% yield; $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ (ppm): 0.80–0.95 (m, 5H), 1.30–1.40 (m, 5H), 1.40–3.50 (brm, 9H), 2.85 (brs, 2H); MS (EI) m/z 200 (M^+ , 100%).

General Procedure of Synthesis of 10 To a solution of **9** (350 mg) in 1,2-dimethoxyethane (DME) (3 mL) was added dropwise a solution of 2.66 M *n*-BuLi in hexane (2.2 eq) at 0°C under an Ar atmosphere, and the mixture was stirred for 30 min. Cu(I)Cl (2.2 eq) was added in one portion, and stirring was continued at room temperature for 1 h. Pyridine (15 eq) and *p*-iodoanisole (2 eq) were added in one portion, and the mixture was refluxed for 20 h, then cooled to room temperature, and diluted with AcOEt. Insoluble materials were filtered off through Celite. The filtrate was washed with 10% HCl aqueous solution, 10% $\text{Na}_2\text{S}_2\text{O}_3$ aqueous solution, water and brine, dried over Na_2SO_4 , and concentrated. The residue was purified by silica gel column chromatography with 1:30 to 1:20 AcOEt-*n*-hexane to give the corresponding **10**.

1,7-Bis-(4-methoxyphenyl)-9-allyl-1,7-dicarba-*closo*-dodecaborane (**10a**): 37% yield; $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ (ppm): 1.60–3.60 (brm, 9H), 1.86 (d, $J=7.2$ Hz, 2H), 3.76 (s, 6H), 4.80–4.93 (m, 2H), 5.91 (m, 1H), 6.75 (d, $J=8.7$ Hz, 4H), 7.36 (d, $J=9.2$ Hz, 4H); MS (EI) m/z 396 (M^+ , 100%).

1,7-Bis-(4-methoxyphenyl)-9-*n*-butyl-1,7-dicarba-*closo*-dodecaborane (**10b**): 39% yield; $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ (ppm): 0.87–0.96 (m, 5H), 1.31–1.41 (m, 4H), 1.60–3.60 (brm, 9H), 3.78 (s, 6H), 6.76 (d, $J=8.7$ Hz, 4H), 7.37 (d, $J=8.7$ Hz, 4H); MS (EI) m/z 412 (M^+ , 100%).

1,7-Bis-(4-methoxyphenyl)-9-*n*-propyl-1,7-dicarba-*closo*-dodecaborane The title compound was prepared by the same method as described for the synthesis of **4c**. 55% yield;

$^1\text{H-NMR}$ (400 MHz, CDCl_3) δ (ppm): 0.92 (t, $J=8.3$ Hz, 2H), 0.97 (t, $J=7.3$ Hz, 3H), 1.43 (sext, $J=7.8$ Hz, 2H), 1.60–3.60 (brm, 9H), 3.77 (s, 6H), 6.76 (d, $J=8.8$ Hz, 4H), 7.37 (d, $J=8.8$ Hz, 4H).

1,7-Bis-(4-hydroxyphenyl)-9-*n*-propyl-1,7-dicarba-*closo*-dodecaborane (**5a**): 82% yield; colorless oil; $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ (ppm): 0.91 (t, $J=7.7$ Hz, 2H), 0.96 (t, $J=7.2$ Hz, 3H), 1.42 (sext, $J=7.2$ Hz, 2H), 1.60–3.60 (brm, 9H), 5.32 (brs, 2H), 6.69 (d, $J=8.7$ Hz, 4H), 7.31 (d, $J=8.7$ Hz, 4H); MS (EI) m/z 370 (M^+ , 100%); HR-MS Calcd for $\text{C}_{17}\text{H}_{26}\text{B}_{10}\text{O}_2$: 370.2936, Found: 370.2943.

1,7-Bis-(4-hydroxyphenyl)-9-*n*-butyl-1,7-dicarba-*closo*-dodecaborane (**5b**): Quant; colorless oil; $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ (ppm): 0.86–0.96 (m, 5H), 1.30–1.43 (m, 4H), 1.60–3.60 (brm, 9H), 5.82 (brs, 2H), 6.69 (d, $J=8.7$ Hz, 4H), 7.31 (d, $J=8.7$ Hz, 4H); MS (EI) m/z 384 (M^+ , 100%); HR-MS Calcd for $\text{C}_{18}\text{H}_{28}\text{B}_{10}\text{O}_2$: 384.3092, Found: 384.3094.

Competitive Binding Assay for ERs Ligand binding to estrogen receptors α (ER α) and β (ER β) was determined by the nitrocellulose filter binding assay method. ER (0.5 μg /tube, PanVera Co., Ltd.) was diluted with binding assay buffer (20 mM Tris-HCl pH 8.0, 0.3 M NaCl, 1 mM ethylenediamine-tetraacetic acid (EDTA) pH 8.0, 10 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride) and incubated with 4 nM [$6,7\text{-}^3\text{H}$]17 β -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 Atom-light (NEN) by using a liquid scintillation counter.

Cell Culture At 80% confluence, cells of the human breast adenocarcinoma cell line MCF-7 were 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 Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (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 2 d or 3 d later.

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 an incubator under an atmosphere of 5% CO_2 in air. On the day before 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 4 d, and medium with 4-OH-

Tam in the presence or absence of 1×10^{-11} M E2 was replaced once after 3 d. At the end of the incubation time, proliferation was evaluated by using a cell counting kit. WST-8 ($10 \mu\text{M}$) 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.

Docking Study Three-dimensional (3D) structures of protein-ligand complexes were predicted by GOLD 5.2 software with default settings.³⁸⁾ The 3D structure of human ER α and β used in this study was retrieved from the Protein DataBank (PDB) (PDB ID: 1ERE and 2IOG, respectively). Missing hydrogen atoms in the crystal structure were computationally added by Hermes.³⁹⁾ The center of the active site was defined as the center of the ligand in 1ERE and 2IOG for ER α and β , respectively, and the active site radius was set to 10.0 Å. The structural optimizations of ligands were carried out by B3LYP/6-31G(d,p) using Gaussian 09, Revision C.01.⁴⁰⁾ The cavities of ER were detected by Q-SiteFinder.⁴¹⁾ PDB entries 1ERE and 2IOG were used as 3D structures of ER α and β similar to the docking study. Ten sites were output for each protein and the sites were ranked by volume (default setting).

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