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Symmetric 4,4′-(piperidin-4-ylidenemethylene)bisphenol derivatives as novel tunable estrogen receptor (ER) modulators

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ABSTRACT

We designed and synthesized 4,4′-(piperidin-4-ylidenemethylene)bisphenol derivatives as novel tunable estrogen receptor (ER) modulators. The introduction of hydrophobic substituents on the nitrogen atom of the piperidine ring enhanced ERα binding affinity. In addition, the introduction of four methyl groups adjacent to the piperidine ring nitrogen atom remarkably enhanced ERα binding affinity. *N*-Acetyl-2,2,6,6-tetramethylpiperidine derivative **3b** showed high ERα binding affinity, high MCF-7 cell proliferation inducing activity, and high metabolic stability in rat liver S9 fractions.

1. Introduction

Tamoxifen (Fig. 1) has been used worldwide for more than 40 years for the treatment of breast cancer.[1] It exhibits competitive antagonism against endogenous estrogen 17β-estradiol (E2, Fig. 1) on the estrogen receptor (ER). Tamoxifen produces a powerful effect against ER-positive but not ER-negative breast cancer.[1] In addition, since E2 is an endogenous estrogen that plays important roles in the female and male reproductive systems as well as in bone maintenance, the central nervous system, and the cardiovascular system, tamoxifen also exerts some biological actions in those tissues.[2] Interestingly, tamoxifen acts as either an agonist or an antagonist depending on tissue type; it exhibits anti-estrogenic action in breast cancer and hot flashes, and estrogenic action in bone and cholesterol metabolism, and is a selective estrogen receptor modulator (SERM).[3]

Following the success of tamoxifen, several triphenylethylene derivatives, such as toremifene[4] and clomifene[5], were developed as novel SERMs (Fig. 1). An alkylamino chain is attached to the terminal of benzene ring where it plays a strategic role in the expression of antagonistic activity.[6] The three SERMs show quite varied biological activities because of the different hydrophobic side chains attached to the ethylene moiety.[7] The hydrophobic side chain plays an important role in controlling SERM activity. The triphenylethylene structure has geometric isomers *E* and *Z*, which are due to the key alkylamino chain and the asymmetric hydrophobic part. As the isomers easily isomerize between the *E* and *Z* forms, problems arise in the synthesis, purification, and preservation of the SERMs.[8] Indeed, 4-hydroxytamoxifen, an active

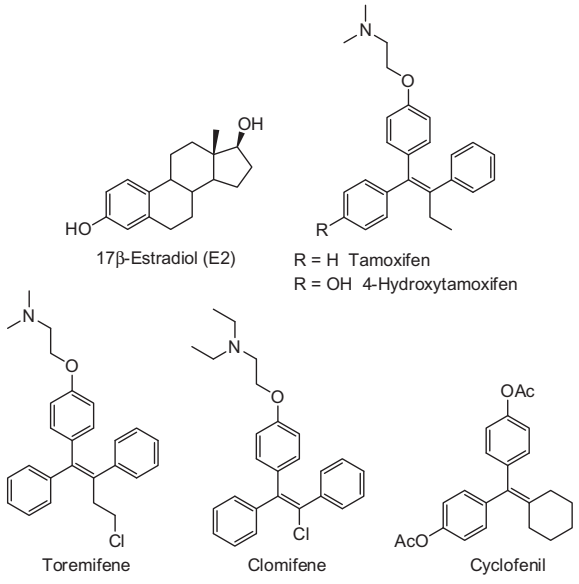

Figure 1. Chemical structures of 17β-estradiol (E2) and clinically used SERMs.

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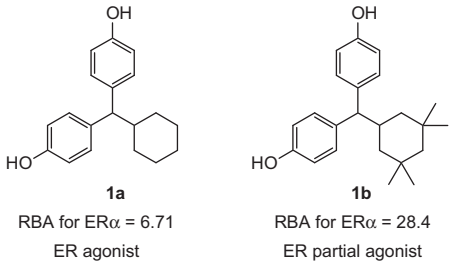


Figure 2. Chemical structures of cyclofenil derivatives **1a** and **1b**.

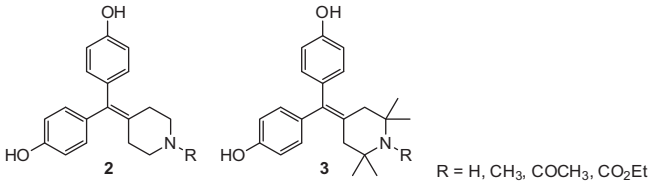


Figure 3. Novel ER modulator candidates **2** and **3** having a tunable piperidine ring.

metabolite of tamoxifen, easily isomerizes from the active *Z* form to the inactive *E* form in solution and in vitro studies (Fig. 1).[9] In this regard, novel SERMs having other skeletons, such as benzothiophene,[10] dihydronaphthalene,[11] benzopyrane,[12] and steroid,[13] have been developed and used as antagonistic SERMs.

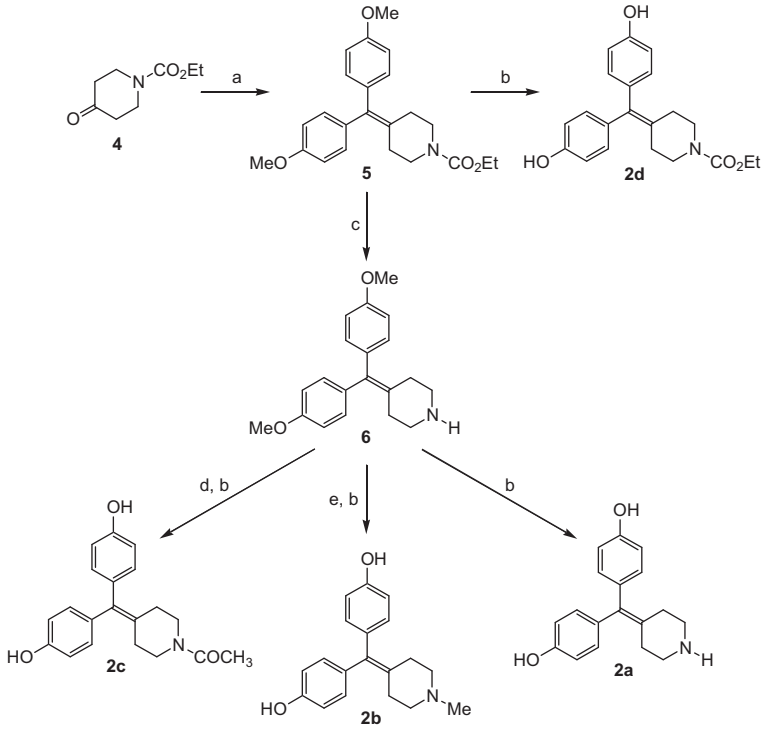
On the other hand, cyclofenil shows weak estrogenic activity because of the absence of the alkylamino chain, and thus it has been developed as an agonistic SERM (Fig. 1).[14] Cyclofenil has a C-2 symmetric structure and no *E* and *Z* isomer. Recently, we have reported an SAR study of the cyclohexane ring of cyclofenil reductant **1a**, in which the diphenylmethane skeleton and the cyclohexane ring are linked by a single bond (Fig. 2).[15] Transformation of the cyclohexane ring of **1a** into the 3,3,5,5-tetramethylcyclohexane ring enhanced ERα binding affinity because of the favorable hydrophobic interaction with the ERα ligand binding domain (LBD). 3,3,5,5-Tetramethylcyclohexane derivative **1b** acts as an ER partial agonist, whereas compound **1a** is an ER agonist.

The cyclohexyl moiety in **1a** and **1b** plays an important role in the hydrophobic interactions with the hydrophobic amino acid residues of ERα LBD, but is not easy tunable. A multi-tunable symmetric structure would be a very attractive tool for ER ligand studies because it would enable detailed SAR studies and not require isomer separation, and its physicochemical properties would be controllable for optimum ADME. Therefore, we focused on the discovery of readily available ER modulators with multi-tunable symmetric structures and designed novel ER ligand candidates **2a**–**2d**, **3a**, and **3b** containing a piperidine ring (Fig. 3). In this paper, we describe the synthesis of those compounds, their biological activities, such as ERα binding affinity and ER-dependent proliferation of MCF-7 cell line, and a metabolic study in rat liver S9 fractions.

2. Results and discussion

2.1. Chemistry

A structure common to compounds **2a**–**2d** was synthesized from piperidinone derivatives and 4,4′-dimethoxybenzophenone by means of the McMurry coupling.[16] Scheme 1 summarizes the synthesis of *N*-substituted piperidine derivatives **2a**–**2d**. Commercially available *N*-ethoxycarbonyl piperidin-4-one **4** was reacted with 4,4′-dimethoxybenzophenone to afford **5** in 90% yield, which was then demethylated with BBr_3 to afford **2d** in 78% yield. McMurry coupling product **5** was hydrolyzed with KOH and then subjected to spontaneous decarboxylation to afford key intermediate **6** in 90% yield. Compound **6** was transformed into **2a** with BBr_3 in 54% yield. *N*-Methylated derivative **2b** was obtained by reductive amination using paraformaldehyde and $NaBH_4$ in trifluoroethanol,



Scheme 1. Synthesis of the designated derivatives **2a**–**2d**. Reagents and conditions: (a) $TiCl_4$, Zn, 4,4′-dimethoxybenzophenone, THF, 90%; (b) BBr_3, CH_2Cl_2, 23–90%; (c) KOH, EtOH, 90%; (d) Ac_2O, 93%; (e) $(CHO)_n$, $NaBH_4$, CF_3CH_2OH, 87%.

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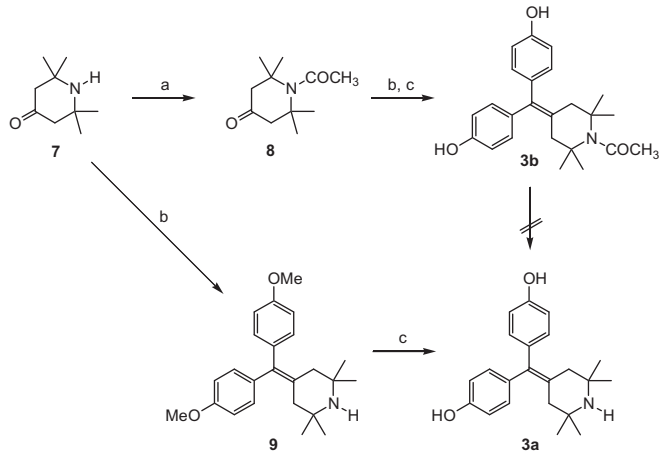

Scheme 2. Synthesis of 2,2,6,6-tetramethylpiperidine derivatives **3a** and **3b**. Reagents and conditions: (a) Ac_2O, 88%; (b) $TiCl_4$, Zn, 4,4′-dimethoxybenzophenone, THF, 48–75%; (c) BBr_3, CH_2Cl_2, 75–82%.

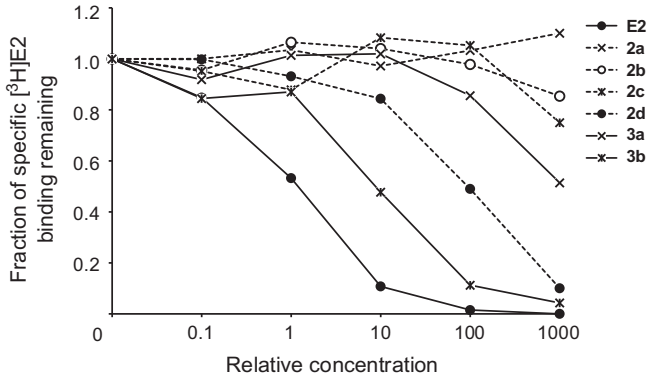

Figure 4. Binding affinities of test compounds **2a**–**2d**, **3a**, and **3b**. Competitive binding assay of compounds **2a**–**2d**, **3a**, and **3b** with [^{3}H]E2 (4 nM) for ERα. Binding assays of the test compounds (0.4–4 μM) were conducted in the presence of [6,7-^{3}H]17β-estradiol (4 nM). The assays were performed in duplicate ($n = 2$).

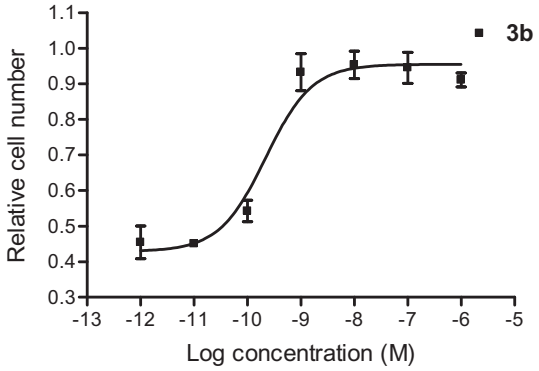

Figure 5. Dose–response curve of **3b** in the MCF-7 cell proliferation assay. MCF-7 cells were incubated with **3b** (1×10^{-6} to 1×10^{-12} M) for 5 days, and the results are shown by relative cell number with the value for E2 taken as 1. Cell proliferation assay was performed in triplicate ($n = 3$). Values are means ± SD of separate experiments.

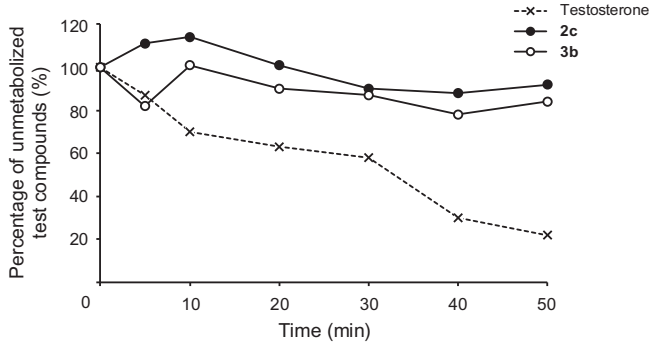

Figure 6. Percentages of **2c** and **3b** remaining in rat liver S9 fractions. The percentages were estimated from peak area ratios of corticosterone as an internal standard to the tested compounds.

followed by deprotection with BBr_3 in 20% yield over two steps. *N*-Acetylated derivative **2c** was synthesized by acetylation and subsequent demethylation in 84% yield over two steps.

The McMurry coupling of 4,4′-dimethoxybenzophenone with *N*-acetyl tetramethylpiperidinone **8**, which was obtained through the acetylation of commercially available 2,2,6,6-tetramethylpiperidin-4-one **7** followed by deprotection with BBr_3, afforded **3b** in 38% yield over three steps (Scheme 2). To prepare **3a**, we tried to remove the *N*-acetyl group from **3b**. However, deacetylation did not proceed under both acidic and basic conditions due to the steric hindrance of the four methyl groups adjacent to the nitrogen atom of the piperidine ring. Therefore, direct McMurry coupling of 2,2,6,6-tetramethylpiperidine with 4,4′-dimethoxyphenylbenzophenone was carried out to afford corresponding coupling product **9** in 75% yield. Compound **9** was transformed into **3a** by demethylation with BBr_3 in 82% yield.

2.2. Biological evaluations

The ERα-binding affinity of the synthesized compounds was evaluated in a competitive binding assay using tritium-labeled E2 and recombinant hERα (Fig. 4).[17] Unsubstituted piperidine derivative **2a** showed no binding affinity for ERα LBD, whereas *N*-methyl and *N*-acetyl derivatives **2b** and **2c** bound very weakly to ERα LBD. Aliphatic amines **2a** and **2b** would be present in the protonated form in this assay buffer. Therefore, the extremely low binding affinity of **2a** and **2b** might be caused by the polarity around the protonated nitrogen atom on the piperidine ring rather than the basicity of the nitrogen atom. *N*-Acetylated derivative **2c** also showed low binding affinity because of the high polarity (*C*Log*P*: 1.72).[18] Compound **2d** having an ethyl carbamate group dose-dependently bound to ERα and showed higher ERα binding affinity than **2a**–**2c** owing to the increase in hydrophobicity caused by the ethyl group. Indeed, compound **2d** had the highest *C*Log*P* value at 3.90 among **2a**–**2d**.[18] In addition, ethyl group of **2d** would be accommodated in the hydrophobic pocket of ERα. The steric hinderance of the four methyl groups in **3a** and **3b** obscured the physicochemical properties of the nitrogen atom, such as basicity and hydrophilicity. Thus, **3a** and **3b** would show higher binding affinity than corresponding normal piperidine derivatives **2a** and **2c**, if the sterically bulky tetramethyl moiety were tolerated in the hydrophobic space of ERα LBD. Although the binding affinity of **3a** was low, it seemed that the four methyl groups improved incompatibility between the piperidine ring and ERα LBD. Interestingly, compound **3b** showed more than 100 times higher binding affinity than corresponding piperidine derivative **2c**. The four methyl groups exerted a strong positive effect on the binding of the piperidine derivatives to ERα LBD.

Next, cell functional assays of **3b** were performed using the MCF-7 cell line that showed ERα-dependent growth.[17] Compound **3b** demonstrated high cell proliferation inducing activity in a dose dependent manner (Fig. 5), but no inhibition of cell proliferation activity inducing by 0.1 nM of E2 (data not shown). The EC_{50} value

of **3b** estimated from the sigmoidal dose response curve was 0.22 nM. The maximal efficacy for MCF-7 cell proliferation inducing activity of **3b** was similar to that of E2. Interestingly, **3b** acted as ER full agonist, unlike the lead compound **1b**. We suggested that ER full agonist activity of **3b** would be caused by the nitrogen atom or the spatial configuration of 2,2,6,6-tetramethylpiperidine ring.

To examine the effect of the four methyl groups on metabolic stability, we measured the elimination rates of piperidine derivatives **2c** and **3b** in rat liver S9 fractions (Fig. 6).[19] The percentage (%) of unmetabolized test compounds was estimated from the peak areas of **2c** and **3b** in HPLC analyses. Approximately 80% of testosterone used as a positive control was metabolized at 50 min under the assay conditions. Compounds **2c** and **3b** showed low metabolic rates; approximately 10% and 20% were metabolized at 50 min, respectively. Compound **3b** was more rapidly metabolized than **2c** and the tetramethyl moiety had no effect on the metabolic stability of these compounds. It seems that the piperidine rings of **2c** and **3b** are quite stable under the assay conditions.

3. Conclusion

In conclusion, we designed and synthesized novel tunable ER modulators having the 4,4′-(piperidin-4-ylidenemethylene) bisphenol structure. In contrast to the unsubstituted piperidine ring that was unfavorable for ERα LBD, the introduction of hydrophobic substituents on the nitrogen atom or the four methyl groups adjacent to the nitrogen atom improved the binding affinity of the piperidine ring for ERα LBD. Compound **3b** showed high binding affinity for ERα, high MCF-7 cell proliferation inducing activity, and good metabolic stability in rat liver S9 fractions. We are of the opinion that 2,2,6,6-tetramethylpiperidine ring is a promising core skeleton for ER modulator studies. Further investigations focusing on the chemical modification of the substituents on the nitrogen atom of the 2,2,6,6-tetramethylpiperidine ring, the introduction of alkylmino chains on the phenol group for the development of ER antagonists and SERMs, and the biological evaluation of ER subtype and tissue selectivity, are in progress.

4. Experimental section

4.1. General

Melting points were determined with a Yanaco micro melting point apparatus and were uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded with JEOL 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 designed as follows: s (singlet), d (doublet), t (triplet), and q (quartet). 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 Fuji Silysia silica gel BW-80S and TLC was performed on Merck silica gel F_{254}. Reagents were purchased from Wako Pure Chemical Industries, Ltd, Sigma-Aldrich Co., and Tokyo Chemical Industry, Ltd (TCI). All solvents were of reagent quality, purchased commercially, and were used without further purification.

4.2. Synthesis

4.2.1. Methyl 4-[(4-methoxyphenyl)(*N*-ethyloxy-carbonylpiperidin-4-ylidene)methyl]phenoxide (5)

To a stirred suspension of Zn powder (2.83 g, 43.2 mmol) in 30 mL of dry THF was added slowly titanium(IV) tetrachloride (2.37 mL, 21.6 mmol) under an argon (Ar) atmosphere. A mixture was refluxed for 2.5 h and cooled to room temperature. To the mixture was added a solution of 4,4′-dimethoxybenzophenone (1.42 g, 5.86 mmol) and ethyl 4-oxopiperidine-1-carboxylate (1.01 g, 5.90 mmol) in 20 mL of dry THF, and it was refluxed for 12 h. The reaction mixture was poured into saturated $NaHCO_3$ aqueous solution. Ether was added to the aqueous solution with vigorous stirring, and insoluble materials were filtered off through Celite. The filtrate was extracted with ether, dried over $MgSO_4$, and concentrated. The residue was purified by column chromatography on silica gel with 40:1 *n*-hexane/AcOEt to afford the title compound **5** (2.23 g, 99%) as a yellow liquid; ^{1}H NMR (395 MHz, $CDCl_3$) δ (ppm) 7.01 (d, 2H, J = 7.9 Hz), 6.82 (d, 2H, J = 7.9 Hz), 4.14 (q, 2H, J = 7.9 Hz), 3.79 (s, 6H), 3.49 (t, 4H, J = 4.0 Hz), 2.35 (t, 4H, J = 4.0 Hz), 1.26 (t, 3H, J = 7.9 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 158.1, 155.5, 136.5, 134.8, 132.9, 130.7, 113.3, 61.2, 55.0, 44.8, 31.5, 14.6; MS (EI) m/z 381 (M^+, 100%).

4.2.2. Methyl 4-[(4-methoxyphenyl)(piperidin-4-ylidene) methyl]phenoxide (6)

A mixture of **5** (7.54 g, 19.8 mmol) and potassium hydroxide (92.9 g, 1.66 mol) in 300 mL of ethanol was refluxed for 6 h under an Ar atmosphere. The solvent was evaporated, water (50 mL) was added to the residue, and then the aqueous mixture was extracted with AcOEt. The organic phase was dried over Na_2SO_4 and concentrated to afford the title compound **6** (5.52 g, 90%) as a yellow solid. ^{1}H NMR (395 MHz, $CDCl_3$) δ (ppm) 7.02 (d, 4H, J = 7.9 Hz), 6.82 (d, 4H, J = 7.9 Hz), 3.79 (s, 6H), 2.90 (t, 4H, J = 4.0 Hz), 2.33 (t, 4H, J = 4.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 157.9, 136.3, 135.2, 130.9, 129.2, 113.3, 55.1, 48.5, 33.5; MS (EI) m/z 309 (M^+), 267 (100%).

4.2.3. 4-[(4-Hydroxyphenyl)(piperidin-4-ylidene)methyl]phenol (2a)

To a solution of **6** (2.95 g, 9.54 mmol) in 20 mL of CH_2Cl_2 was added 1 M of BBr_3 solution (17.5 mL, 17.5 mmol) at 0 °C under Ar atmosphere. The mixture was stirred for 12 h at 0 °C, the solvent was removed, water was added to the residue, and the mixture was extracted with AcOEt. The organic phase was dried over Na_2SO_4 and concentrated to afford the title compound **2a** (1.44 g, 54%) as a yellow solid; colorless cubes (MeOH); mp 205–207 °C; ^{1}H NMR (395 MHz, CD_3OD) δ (ppm) 6.88 (d, 4H, J = 7.9 Hz), 6.69 (d, 4H, J = 7.9 Hz), 2.83 (t, 4H, J = 4.0 Hz), 2.32 (t, 4H, J = 4.0 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ (ppm) 157.2, 137.6, 135.3, 134.3, 131.9, 115.8, 33.6, 32.2; MS (EI) m/z 281 (M^+, 100%); Anal. Calcd for $C_{18}H_{19}NO_2$: C, 76.84; H, 6.81; N, 4.98. Found: C, 76.84; H, 6.81; N, 4.91.

4.2.4. 4-[(4-Hydroxyphenyl)(*N*-methylpiperidin-4-ylidene) methyl]phenol (2b)

A solution of paraformaldehyde (191 mg, 6.4 mmol) in 30 mL of trifluoroethanol was stirred at 35–40 °C. To the stirred solution was added **6** (1.18 g, 3.88 mmol), and $NaBH_4$ (185 mg, 4.89 mmol) was added 5 min later. After 30 min, the mixture was filtered through Celite and the filtrate was concentrated. The residue was purified by column chromatography on silica gel with AcOEt to afford *N*-methylated compound (1.09 g, 87%) as a yellow solid. ^{1}H NMR (395 MHz, DMSO) δ (ppm) 6.96 (d, J = 7.9 Hz, 4H), 6.85 (d, 4H, J = 7.9 Hz), 3.72 (s, 6H), 2.33 (t, 4H, J = 4.0 Hz), 2.25 (t, 4H, J = 4.0 Hz), 2.15 (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ (ppm) 159.8, 137.7, 136.2, 133.3, 131.8, 114.4, 58.0, 55.7, 45.9, 32.1; MS (EI) m/z 323 (M^+, 100%). The *N*-methylated compound was transformed into **2b** by the same method that described the synthesis of **2a**; 23% yield; colorless needles (MeOH–$CHCl_3$) mp 255–257 °C; ^{1}H NMR (395 MHz, CD_3OD) δ (ppm) 6.99 (d, 4H, J = 8.0 Hz), 6.70 (d, 4H, J = 8.0 Hz), 2.52 (t, 4H, J = 8.0 Hz), 2.41

(t, 4H, J = 4.0 Hz), 2.32 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ (ppm) 157.1, 138.0, 135.3, 132.9, 131.9, 115.7, 58.1, 46.1, 32.2; MS (EI) m/z 295 (M^+, 100%).; Anal. Calcd for $C_{19}H_{21}NO_2$: C, 76.45; H, 7.17; N, 4.74; Found: C, 76.45; H, 7.15; N, 4.69.

4.2.5. 4-[(4-Hydroxyphenyl)(*N*-acetylpiperidin-4-ylidene) methyl]phenol (2c)

A mixture of **6** (218 mg, 0.71 mmol) and 3 mL of acetic anhydride was heated at 100 °C for 6 h. After cooling to room temperature, 10% NaOH aqueous solution was added and stirred. The mixture was extracted with CH_2Cl_2, washed with brine, dried over Na_2SO_4, and then concentrated. The residue was purified by column chromatography on silica gel with 2:1 *n*-hexane/AcOEt to afford *N*-acetylated compound (230 mg, 93%) as a yellow solid. ^{1}H NMR (395 MHz, CD_3OD) δ (ppm) 7.02 (d, J = 7.9 Hz, 4H), 6.85 (d, 4H, J = 7.9 Hz), 3.77 (s, 6H), 3.60 (t, 2H, J = 4.0 Hz), 3.55 (t, 2H, J = 4.0 Hz), 2.43 (t, 2H, J = 4.0 Hz), 2.35 (t, 2H, J = 4.0 Hz), 2.11 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 168.7, 158.0, 136.7, 134.5, 132.0, 130.5, 113.2, 54.9, 47.3, 42.7, 31.8; MS (EI) m/z 351 (M^+, 100%). The *N*-acetylated compound was transformed into **2c** by the same method that described the synthesis of **2a**; 22% yield; colorless leaflets (MeOH–$CHCl_3$) mp 255–257 °C; ^{1}H NMR (395 MHz, CD_3OD) δ (ppm) 6.99 (d, 4H, J = 8.0 Hz), 6.70 (d, 4H, J = 8.0 Hz), 2.52 (t, 4H, J = 8.0 Hz), 2.41 (t, 4H, J = 4.0 Hz), 2.32 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ (ppm) 157.1, 138.0, 135.3, 132.9, 131.9, 115.7, 58.1, 46.1, 32.2; MS (EI) m/z 295 (M^+, 100%). Anal. Calcd for $C_{20}H_{21}NO_3$: C, 74.28; H, 6.55; N, 4.33; Found: C, 74.28; H, 6.58; N, 4.25.

4.2.6. 4-[(4-Hydroxyphenyl)(*N*-ethyloxycarbonyl-piperidin-4-ylidene)methyl]phenol (2d)

Compound **2d** was prepared by the same method that described the synthesis of **2a**; 90% yield; a yellow prisms (CH_2Cl_2) mp 105 °C; ^{1}H NMR (395 MHz, $CDCl_3$) δ (ppm) 6.96 (d, 4H, J = 7.9 Hz), 6.75 (d, 4H, J = 7.9 Hz), 4.14 (q, 2H, J = 4.0 Hz), 3.48 (t, 4H, J = 4.0 Hz), 2.34 (t, 4H, J = 4.0 Hz), 1.26 (t, 3H, J = 7.9 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ (ppm) 157.3, 157.2, 139.0, 135.2, 133.0, 131.9, 115.8, 62.7, 46.4, 32.6, 15.0; MS (EI) m/z 353 (M^+, 100%). Anal. Calcd for $C_{21}H_{23}NO_4$: C, 71.37; H, 6.56; N, 3.96; Found: C, 71.11; H, 6.59; N, 3.93.

4.2.7. *N*-Acetyl-2,2,6,6-tetramethyl-4-piperidone (8)

A mixture of 2,2,6,6-tetramethyl-4-piperidone (4.91 g, 31.6 mmol) and 20 mL of acetic anhydride was heated at 100 °C for 7 h. Solvent was removed, 10% NaOH aqueous solution was added, and the mixture was stirred. The mixture was extracted with CH_2Cl_2, washed with brine, dried over Na_2SO_4, and then concentrated. The residue was purified by column chromatography on silica gel with 3:1 *n*-hexane/AcOEt to afford the title compound **8** (5.49 g, 88%) as a colorless liquid. ^{1}H NMR (395 MHz, $CDCl_3$) δ (ppm) 2.59 (s, 4H), 2.23 (s, 3H), 1.54 (s, 12H); MS (EI) m/z 197 (M^+), 140 (100%).

4.2.8. Methyl 4-[(4-methoxyphenyl)(2,2,6,6-tetramethylpiperidin-4-ylidene)methyl]phenoxide (9)

Compound **9** was prepared by the same method that described the synthesis of **5**; 75% yield; ^{1}H NMR (395 MHz, CD_3OD) δ (ppm) 7.07 (d, 4H, J = 8.0 Hz), 6.82 (d, 4H, J = 8.0 Hz), 3.78 (s, 6H), 2.14 (s, 4H), 1.18 (s, 12H); ^{13}C NMR (CD_3OD) δ (ppm) 157.8, 137.3, 135.4, 132.4, 130.2, 113.4, 55.1, 52.8, 43.7, 31.2; MS (EI) m/z 365 (M^+), 98 (100%).

4.2.9. 4-[(4-Hydroxyphenyl)(2,2,6,6-tetramethylpiperidin-4-ylidene)methyl]phenol (3a)

Compound **3a** was prepared by the same method that described the synthesis of **2a**; 82% yield; colorless cubes (MeOH–$CHCl_3$) mp 110–112 °C; ^{1}H NMR (395 MHz, CD_3OD) δ (ppm) 6.96 (d, 4H, J = 8.0 Hz), 6.70 (d, 4H, J = 8.0 Hz), 2.18 (s, 4H), 1.18 (s, 12H); ^{13}C NMR (100 MHz, CD_3OD) δ (ppm) 182.2, 176.4, 157.2, 140.4, 134.7, 131.9, 115.7, 58.4, 46.1, 30.3, 28.4; MS (EI) m/z 379 (M^+), 224 (100%). Anal. Calcd for $C_{22}H_{27}NO_2$ 0.1 H_2O: C, 77.88; H, 8.08; N, 4.13. Found: C, 77.92; H, 8.19; N, 4.10.

4.2.10. 4-[(4-Hydroxyphenyl)(2,2,6,6-tetramethyl-*N*-acetylpiperidin-4-ylidene)methyl]phenol (3b)

The product obtained from McMurry coupling reaction of compound **9** with 4,4′-dimethoxybenzophenone was transformed into **3b** by the same method that described the synthesis of **2a**; 43% yield over two steps; colorless cubes (Et_2O) mp 138–140 °C; ^{1}H NMR (395 MHz, CD_3OD) δ (ppm) 6.93 (d, 4H, J = 7.9 Hz), 6.72 (d, 4H, J = 7.9 Hz), 2.64 (s, 4H), 2.17 (s, 3H), 1.45 (s, 12H); ^{13}C NMR (100 MHz, CD_3OD) δ (ppm) 182.2, 176.4, 157.2, 140.4, 134.7, 131.9, 115.7, 58.4, 46.1, 30.3, 28.4; MS (EI) m/z 379 (M^+), 224 (100%). Anal. Calcd for $C_{24}H_{29}NO_3$: C, 75.96; H, 7.70; N, 3.69. Found: C, 75.91; H, 7.83; N, 3.73.

4.3. ERα binding assay

The ligand binding affinity of ERα was determined by the previously reported method.[17] Briefly, ERα was diluted with an assay buffer, which contains 20 mM Tris–HCl, 0.3 M NaCl, 10 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and incubated with 4 nM [6,7-^{3}H]17β-estradiol in the presence or absence of an unlabeled competitor at 4 °C for 16 h. The incubation mixture was absorbed by suction onto a nitrocellulose membrane that had been soaked in assay buffer. The membrane was washed twice with buffer, and then with 25% EtOH in distilled water. Radioactivity that remained on the membrane was measured in Atomlight using a liquid scintillation counter.

4.4. MCF-7 cell proliferation assay

The human breast adenocarcinoma cell line, MCF-7 was 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. Before an assay, MCF-7 cells were switched to DMEM (low glucose phenol red-free supplemented with 5% FBS, 100 UI/mL penicillin and 100 mg/mL streptomycin). Cells were trypsinized from the maintenance dish with phenol red-free trypsin–EDTA and seeded in a 96-well plate at a density of 2000 cells per final volume of 100 μL DMEM supplemented with 5% FBS, 100 UI/mL penicillin and 100 mg/mL streptomycin. After 24 h, the medium was exchanged into 90 μL of fresh DMEM and 10 μL of the drug solution, supplemented with serial dilutions of **3b** or DMSO as dilute control in the presence or absence of 1 nM E2, was added to triplicate microcultures. Cells were incubated for 5 days, and medium with **3b** or DMSO as dilute control in the presence or absence of 1 nM E2 was exchanged once after 3 days. At the end of the incubation time, proliferation was evaluated by using the WST-8. 10 nM of WST-8 was added to microcultures and cells were incubated for 4 h. The absorbance at 450 nm was measured. This parameter relates to the number of living cells in the culture.

4.5. Metabolism study of compounds 2c and 3b in rat liver S9 fractions

For the evaluation of metabolic stability, 40 μL of compound was incubated with 1 mg/mL pooled rat liver S9 fractions in 0.1 M potassium phosphate buffer (pH 7.4) containing 3.3 mM $MgCl_2$, 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, and 0.4 U/mL glucose-6-phosphatedehydrogenase at 37 °C for 0, 5, 10,

20, 30, 40, and 50 min. The incubation was stopped by the precipitation of S9 enzymes with the same volume of cold acetonitrile containing corticosterone as the internal standard. The percentage of unmetabolized test compound at the different time points was estimated from the peak area in HPLC spectrum.

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