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## Design and synthesis of novel selective estrogen receptor degradation inducers based on the diphenylheptane skeleton†‡

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Estrogen receptors (ERs) are a family of nuclear receptors (NRs) that regulate physiological effects such as reproduction and bone homeostasis. It has been reported that approximately 70% of human breast cancers are hormone-dependent and ER $\alpha$ -positive. Recently, novel anti-breast cancer drugs based on different mechanisms of action have received significant attention. In this article, we have designed and synthesized a selective ER degradation inducer based on the diphenylheptane skeleton. Western blotting analysis revealed that PBP-NC10 degraded ER $\alpha$  through the ubiquitin–proteasome system. We also performed computational docking analysis to predict the binding mode of PBP-NC10 to ER $\alpha$ .

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### Introduction

Estrogen receptor (ER), a member of the nuclear receptor (NR) family of transcription factors, relates to several biological functions such as reproduction and bone physiology.<sup>1</sup> The binding of 17 $\beta$ -estradiol (E2) and its metabolites, estrone and estriol, to the ER ligand binding domain (LBD) induces the folding of Helix 12 (H12) and the subsequent interaction with co-activators along the hydrophobic groove, resulting in ER-mediated target gene transcriptions.<sup>2</sup> The ER is divided into two subtypes, ER $\alpha$  and ER $\beta$ , which are encoded by distinct genes but are highly conserved in their LBD.<sup>3</sup> It is well known that approximately 70% of human breast cancers are hormone-dependent and ER $\alpha$ -positive.<sup>4</sup> Therefore, several ER $\alpha$ -mediated transcriptional inhibitors such as tamoxifen (ER $\alpha$  antagonist)<sup>5</sup> and raloxifene<sup>6</sup> are widely used for ER $\alpha$ -positive breast cancer therapy (Fig. 1a).<sup>7</sup> Tamoxifen is metabolized to 4-hydroxytamoxifen (4-OHT) with a much higher binding affinity for ER $\alpha$ , and 4-OHT exerts its anti-estrogenic actions.<sup>8</sup> However, it has been problematic that long-term administration of tamoxifen might cause drug resistance in breast cancer cells and also increases the risk factors for uterine cancer because of its agonistic effects on the uterus. Therefore, novel anti-breast cancer drugs based on different mechanisms of action have received significant attention. Recently, several methods to induce targeted protein degrada-

tion using small molecules have been reported such as PROTAC,<sup>9</sup> SNIPER,<sup>10</sup> and hydrophobic tag systems.<sup>11</sup>

Among these, selective ER downregulators (SERDs) have been discussed as anti-breast cancer drugs. Fulvestrant (ICI182,780) and ICI164,384 (Fig. 1b), which are E2 derivatives bearing a long alkyl chain on the 7 $\alpha$ -position of E2, are representative SERDs and are capable of inducing ER degradation through the ubiquitin–proteasome system (UPS).<sup>12</sup> Based on the X-ray structure analysis of ICI164,384 bound to the ER-LBD, the ER-degradation mechanism of ICI164,384 is speculated to involve binding of the long alkyl chain of ICI164,384 to the hydrophobic groove of ER, sterically preventing H12 folding in ER and the co-activator interaction

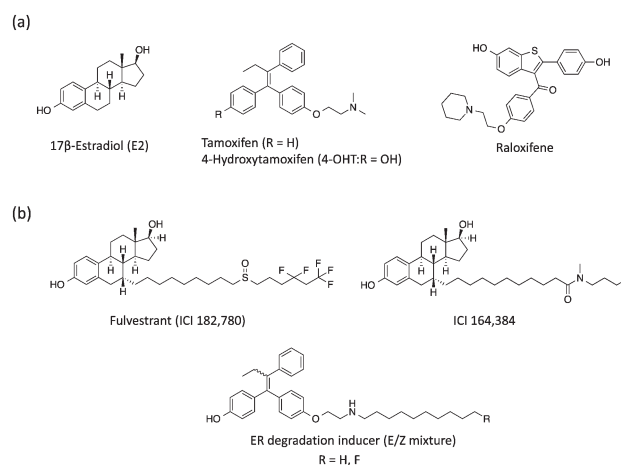


Fig. 1 (a) Chemical structures of 17 $\beta$ -estradiol, anti-estrogenic compounds and (b) selective estrogen receptor downregulators.

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with ER, resulting in destabilization of ER and subsequent degradation.<sup>13</sup>

Previously, we reported that 4-OHT derivatives bearing long alkyl chains (Fig. 1b) induced ER degradation similar to fulvestrant.<sup>14</sup> However, these synthesized compounds were obtained and assayed as a mixture of geometric isomer *E* and *Z* forms. It has been reported that the triphenylethylene skeleton of 4-OHT is easily isomerized from the active *Z* form to an inactive *E* form in solution.<sup>15</sup> Therefore, these findings raise the possibilities that its isomerization decreases the ER-antagonistic and ER-downregulating activities. On the other hand, Hashimoto and Ishikawa *et al.* recently reported that the diphenylalkane skeleton acted as a steroid skeleton mimic and could be a key to the development of several NR ligands.<sup>16</sup> Among these, the ER antagonist PBP based on the diphenylheptane skeleton has potent anti-estrogenic activity with an IC<sub>50</sub> value of 4.5 nM from a reporter gene assay.<sup>17</sup> Moreover, the diphenylalkane skeleton has an advantage of good chemical stability and for short-step synthesis over the triphenylethylene skeleton of 4-OHT. Herein, we described the development of PBP derivatives bearing several long alkyl chains on the hydroxy group (Fig. 2), and evaluated their biological properties such as ER degradation, binding affinity, transcriptional activity and anti-proliferation activity.

## Results and discussion

The synthetic routes to the designed compounds PBP-C2–C12, PBP-NC6–NC16, PBP-NH<sub>2</sub>, and PBP-tam are shown in Scheme 1. 4-Heptanone and *o*-cresol were combined under acidic conditions to give the diphenylheptane skeleton (PBP, 3). Alkylation of the 4-hydroxy group of PBP with the corresponding 1-bromoalkanes afforded alkylated products PBP-C2–C12. Compounds PBP-NC6–NC16 were synthesized by mono-alkylation of PBP with 1,2-dichloroethane and subsequent alkylation of alkylamines with compound 4. After alkylation of PBP with benzyl (2-bromoethyl)carbamate, the Cbz group was removed using Pd(OH)<sub>2</sub> under H<sub>2</sub> atmosphere to give PBP-NH<sub>2</sub>. PBP-tam was obtained by the treatment of PBP with 2-chloro-*N,N*-dimethylethylamine.

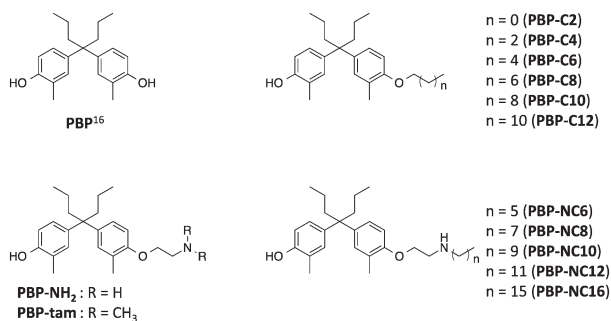
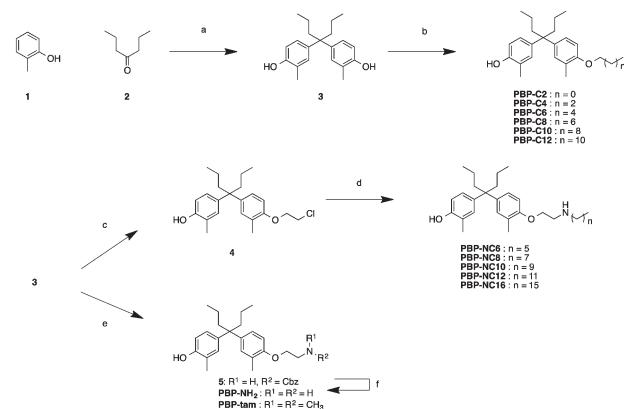


Fig. 2 Chemical structures of the diphenylheptane skeleton (PBP) and designed compounds PBP-NH<sub>2</sub>, PBP-tam, PBP-C2 to PBP-C12, and PBP-NC6 to PBP-NC16 in this study.

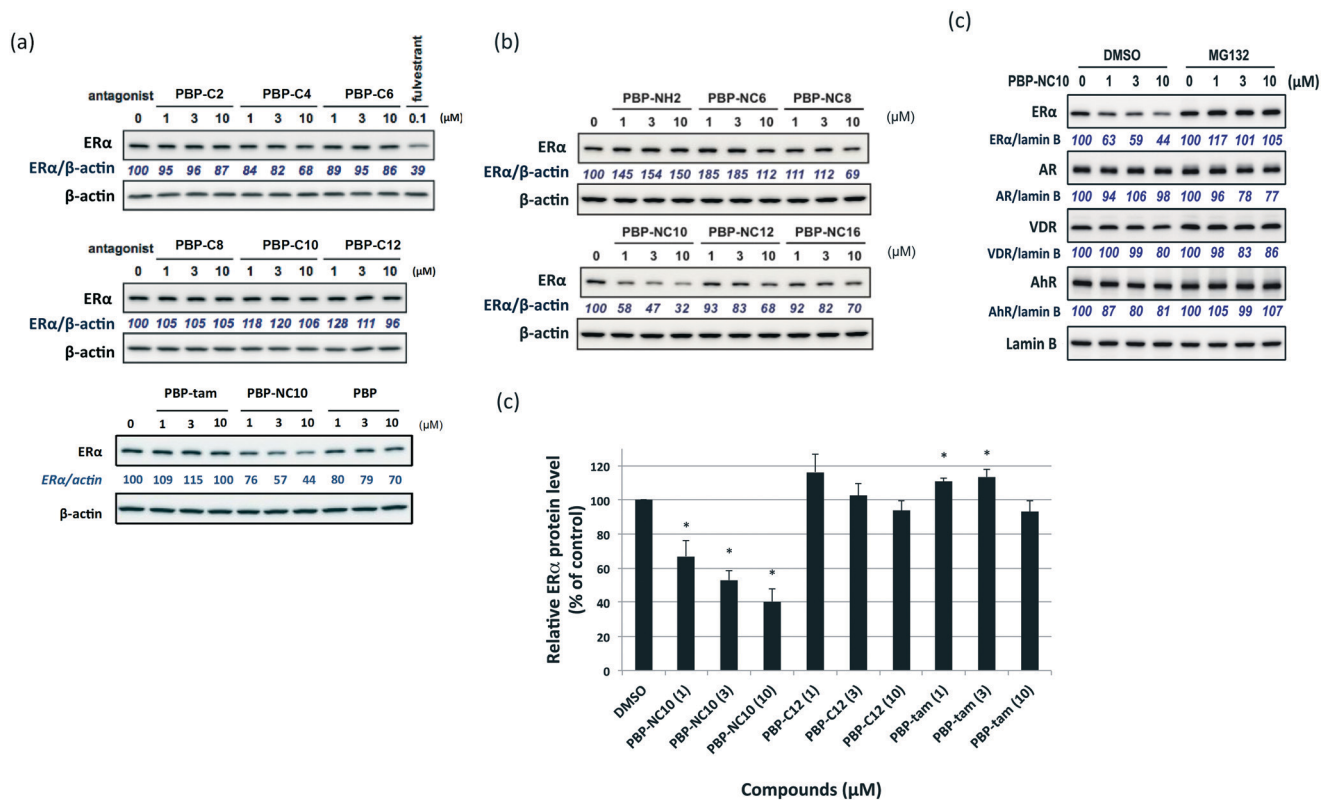


Scheme 1 Synthesis of compounds PBP-NH<sub>2</sub>, PBP-tam, PBP-C2 to PBP-C12, and PBP-NC6 to PBP-NC16. Reagents and conditions: (a) MsOH, rt, 3 days, 59%; (b) R-I (R = ethyl, *n*-hexyl, *n*-octyl, *n*-decyl, *n*-dodecyl), K<sub>2</sub>CO<sub>3</sub>, MeCN, 60–100 °C, 16–47%; (c) 1,2-dichloroethane, K<sub>2</sub>CO<sub>3</sub>, MeCN, 48 h, 19%; (d) R-NH<sub>2</sub> (R = *n*-hexyl, *n*-octyl, *n*-decyl, *n*-dodecyl, *n*-hexadecyl), MeCN, microwave, 120 °C, 4 h, 20–42%; (e) benzyl (2-bromoethyl)carbamate or 2-chloro-*N,N*-dimethylethylamine C<sub>5</sub>H<sub>9</sub>Cl, NaI, DMF, 100 °C, 21 h, 6%; (f) Pd(OH)<sub>2</sub>, H<sub>2</sub>, MeOH, rt, 26 h, 25%.

First, we investigated the effects of the synthesized compounds on ER $\alpha$ -downregulating activity by western blotting analysis using MCF-7 breast cancer cells. The potency of the ER $\alpha$ -downregulating activity is described as the ratio of ER $\alpha$  to standard proteins. As shown in Fig. 3, ER $\alpha$  downregulation was observed by treatment with PBP-NC8, PBP-NC10, and PBP-NC12 with amino alkyl chains (ER $\alpha$  downregulation ratio at 10  $\mu$ M; PBP-NC8: 69%, PBP-NC10: 32%, and PBP-NC12: 68%). In particular, the ER $\alpha$  expression level was dose-dependently reduced in the presence of PBP-NC10 (ER $\alpha$  downregulation ratio at 1  $\mu$ M: PBP-NC10: 58%). On the other hand, PBP-C2–C12, with simple alkyl chains, PBP-NC6 and PBP-NC16, with short or long alkyl chains, and PBP-tam, with a dimethylamino group, did not show ER $\alpha$ -downregulating activity (Fig. 3a and b). These results indicated that the nitrogen atom and the optimal length of the alkyl chain of PBP-NC derivatives play a pivotal role in the ER $\alpha$ -downregulating activity.

Next, we investigated the selectivity of PBP-NC10 over other NRs such as the androgen receptor (AR), vitamin D receptor (VDR), and aryl hydrocarbon receptor (AhR) because the diphenylalkane skeleton could act as several NR ligands.<sup>16</sup> Moreover, we also performed the western blotting analysis in the presence of proteasome inhibitor MG132 to investigate whether ER $\alpha$  downregulation by PBP-NC10 was mediated by UPS as hypothesized. As shown in Fig. 4, ER $\alpha$  was downregulated by treatment with PBP-NC10 in a dose-dependent manner, whereas the other NR expression levels were unaffected. Furthermore, reductions in the expression levels of ER $\alpha$  induced by PBP-NC10 were suppressed by MG132, indicating that PBP-NC10 induced proteasomal degradation.

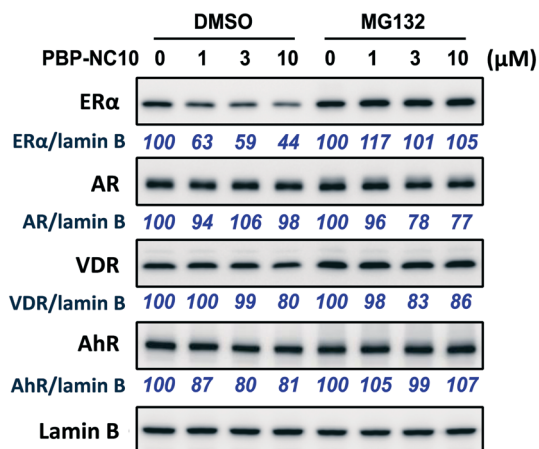
Next, we investigated the effects of the synthesized compounds on the binding affinity and transcriptional activity



**Fig. 3** (a and b) Western blotting analysis of estrogen receptor (ER)  $\alpha$  levels in MCF-7 cells after 6 h of incubation with the synthesized compounds. (c) Quantification of the levels of ER $\alpha$  is provided in a histogram. Data are expressed as means  $\pm$  S.E. of three independent experiments (\* $P < 0.05$  versus control).

against ER $\alpha$ . To evaluate the ability of the compounds to bind to ER $\alpha$ , a fluorescence polarization (FP)-based competitive binding assay was conducted using a PolarScreen nuclear receptor competitor assay kit (Life Technologies).<sup>14</sup> As reported previously, 4-OHT and PBP strongly bound to ER $\alpha$  with IC<sub>50</sub> values of 5.6 nM and 7.3 nM, respectively. Table 1

shows that PBP-C2 to PBP-C8 exhibited moderate binding affinity to ER $\alpha$  (IC<sub>50</sub> = 40–400 nM), whereas PBP-C10 and PBP-C12 did not bind to ER $\alpha$ . These data indicated that the introduction of longer alkyl chains rather than an *n*-octyl group lowered the binding affinity. On the other hand, PBP-NC8 to PBP-NC12 showed pronounced ER $\alpha$  binding affinity (IC<sub>50</sub> values of 2.4 nM for PBP-NC8, 4.9 nM for PBP-NC10, and 4.9 nM for PBP-NC12), which was similar to that of 4-OHT (IC<sub>50</sub>



**Fig. 4** The effects of PBP-NC10 on nuclear receptor levels in MCF-7 cells with or without proteasome inhibitor MG132. ER, estrogen receptor; AR, androgen receptor; VDR, vitamin D receptor; AhR, aryl hydrocarbon receptor.

**Table 1** ER $\alpha$  binding affinity of PBP derivatives

Entry	Binding affinity IC <sub>50</sub> (nM)
4-OHT	5.6
PBP	7.3
PBP-C2	40
PBP-C4	87
PBP-C6	168
PBP-C8	400
PBP-C10	NA <sup>a</sup>
PBP-C12	NA <sup>a</sup>
PBP-tam	313
PBP-NH <sub>2</sub>	78
PBP-NC6	78
PBP-NC8	2.4
PBP-NC10	4.9
PBP-NC12	4.9
PBP-NC16	20

<sup>a</sup> No activity at 1 μM.

value of 5.6 nM) in the FP assay. Moreover, **PBP-tam** exhibited lower binding affinity to ER $\alpha$  compared with **PBP-NH<sub>2</sub>** and **PBP-NC** derivatives (**PBP-tam**: IC<sub>50</sub> = 313 nM, **PBP-NH<sub>2</sub>**: IC<sub>50</sub> = 78 nM). These results revealed that the hydrogen atom on the nitrogen atom of **PBP-NC** derivatives was important for binding to ER $\alpha$ .

Furthermore, the ER $\alpha$  transcriptional activity was evaluated using a reporter gene assay with CMX-GAL4N-hER $\alpha$  as the recombinant receptor gene, TK-MH100x4-LUC as a reporter gene, and the CMX- $\beta$ -galactosidase gene for normalization, as reported previously.<sup>18</sup> Human Embryonic Kidney 293 (HEK293) cells were incubated with the synthesized compounds in the absence or presence of E2 (0.3 nM). After incubation, the cells were assayed for reporter gene and  $\beta$ -galactosidase activities. As shown in Fig. 5, it was demonstrated that **PBP-NC10** exhibited potent ER $\alpha$  antagonistic activity, whereas **PBP-C12**, which has the same length of alkyl chain as **PBP-NC10**, showed slight ER $\alpha$  antagonistic activity (ER $\alpha$  antagonistic activity: **PBP-C12**, 61% inhibition at 10  $\mu$ M; **PBP-NC10**, IC<sub>50</sub> = 45 nM).

Finally, we performed a computational docking analysis to predict the binding mode of **PBP-NC10** to ER $\alpha$  as follows; the X-ray co-crystal structure of ER $\alpha$  bound to 4-OHT was obtained from the Protein Data Bank (PDB: 3ERT) and used in docking studies.<sup>19</sup> Docking models of **PBP-NC10** bound to ER $\alpha$  were constructed *via* a conformational search analysis using Molecular Operating Environment (MOE). MMFF94S was used as a force field. In the modeled structures, the amino group of **PBP-NC10** interacted with the side-chain carboxyl group of Glu351 and the alkyl chain of **PBP-NC10** was included in the hydrophobic groove composed of hydrophobic amino acids such as Leu354, Met357, Ile358, Leu379, and Trp383 of ER $\alpha$ . Therefore, these results indicated that the amino group of **PBP-NC10** played an important role in leading the alkyl chain to the hydrophobic groove. Furthermore, the results of the docking analysis suggested that the length of the hydrophobic groove interacting with the long

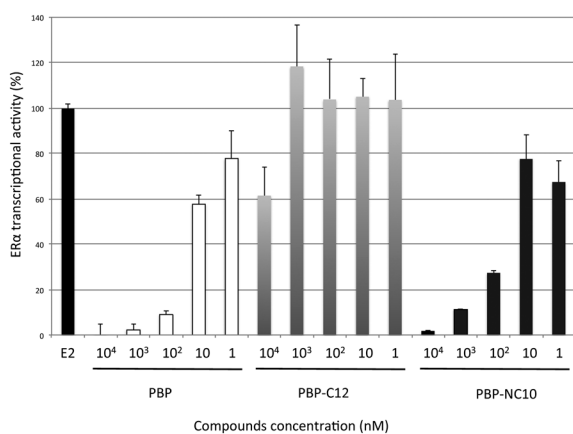


Fig. 5 ER $\alpha$  antagonistic activity of **PBP**, **PBP-C12** and **PBP-NC10** in HEK293 cells. Data are expressed as means  $\pm$  S.E. of three independent experiments.

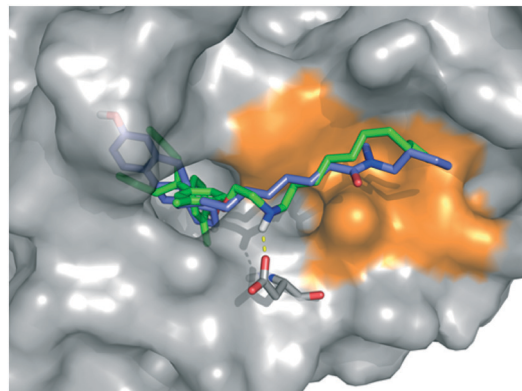


Fig. 6 Computational model of the estrogen receptor (ER)  $\alpha$  ligand binding domain and **PBP-NC10**, ICI164,384. The hydrophobic region is colored orange, **PBP-NC10** is colored green, and ICI164,384 is colored blue.

alkyl chain was the best around a decyl group on the amino group of **PBP-NC10** (Fig. 6).

## Conclusions

We have developed a novel selective estrogen degradation inducer **PBP-NC10**, which could be useful as a lead structure in the development of compounds with more potent and selective ER degradation-inducing activities. We also revealed that the amino group and optimal length of the long alkyl chain of **PBP-NC10** are essential for exerting its ER $\alpha$ -downregulating activity. Furthermore, a docking study was performed and predicted that the interaction between the hydrogen atom on the amino group of **PBP-NC10** and the carboxylic acid of Glu351 of ER $\alpha$  leads the long alkyl chain to bind to the hydrophobic groove of ER $\alpha$ .

## Experimental section

### Chemistry

4-Hydroxytamoxifen was purchased from Fluka. All other reagents and solvents were purchased from Sigma-Aldrich, Wako Pure Chemical and Tokyo Chemical Industry, and were used without purification. Analytical TLC was carried out using Merck silica gel 60 F254 pre-coated plates and visualized using a 254 nm UV lamp, phosphomolybdic acid, *p*-anisaldehyde or ninhydrin staining. Column chromatography was performed with silica gel (spherical, neutral) purchased from Kanto Chemical. The microwave reactions were carried out using a Biotage Initiator. <sup>1</sup>H and <sup>13</sup>C NMR spectra were obtained on a Varian AS 400 Mercury spectrometer (400 MHz for <sup>1</sup>H and 100 MHz for <sup>13</sup>C). FT-IR spectra were recorded on a JASCO FT/IR-4100 spectrometer at 1 cm<sup>-1</sup> resolution, with an average of 128 scans used for the solution (CDCl<sub>3</sub>) method and a 0.1 mm path length for NaCl cells. Chemical shifts are expressed as ppm downfield from a solvent residual peak or internal standard tetramethylsilane

(TMS). High-resolution mass spectra were obtained on a Shimadzu IT-TOF MS equipped with an electrospray ionization source.

**4-[4-(4-Ethoxy-3-methylphenyl)heptan-4-yl]-2-methylphenol (PBP-C2).** A solution of 321 (95.5 mg, 0.31 mmol) and  $K_2CO_3$  (47.4 mg, 0.34 mmol) in MeCN (2 mL) was added ethyl iodide (47 mg, 0.34 mmol) and stirred at 60 °C for 6 h. The reaction mixture was acidified with 3% HCl and extracted with  $Et_2O$ . The organic layer was washed with brine and dried over  $Na_2SO_4$ . After filtration, concentration *in vacuo*, and purification by silica gel flash column chromatography (AcOEt/hexane = 1/4), PBP-C2 (35.5 mg, 34%) was obtained as colorless oil; IR (CDCl<sub>3</sub>, cm<sup>-1</sup>): 3602, 2958, 2932, 2872, 1607, 1503, 1477; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 6.90–6.93 (m, 3H), 6.85 (d, *J* = 8.4 Hz, 1H), 6.67 (d, *J* = 8.4 Hz, 1H), 6.61 (d, *J* = 8.4 Hz, 1H), 4.72 (br s, 1H), 3.98 (q, *J* = 14.0 Hz, 2H), 2.18 (s, 3H), 2.16 (s, 3H), 1.92–1.96 (m, 4H), 1.39 (t, *J* = 7.2 Hz, 3H), 0.93–0.98 (m, 4H), 0.81–0.85 (m, 6H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ 154.9, 151.5, 142.1, 141.3, 130.6, 130.5, 126.8, 126.1, 125.7, 122.7, 114.3, 110.2, 63.7, 48.2, 40.6, 17.6, 16.9, 16.4, 15.3, 15.2; [HR-ESI(+)]: *m/z* calcd for C<sub>23</sub>H<sub>33</sub>O<sub>2</sub> [M + H]<sup>+</sup>: 341.2475, found: 341.2445.

**4-[4-(4-*n*-Butoxy-3-methylphenyl)heptan-4-yl]-2-methylphenol (PBP-C4).** Compound PBP-C4 was prepared from 3 (62.0 mg, 0.20 mmol) using the procedure described for PBP-C2. Yield 16%; colorless oil; IR (CDCl<sub>3</sub>, cm<sup>-1</sup>): 3603, 2959, 2933, 2872, 1605, 1503, 1468; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 6.89–6.93 (m, 3H), 6.86 (d, *J* = 8.0 Hz, 1H), 6.67 (d, *J* = 8.4 Hz, 1H), 6.64 (d, *J* = 8.4 Hz, 1H), 4.47 (br s, 1H), 3.93 (t, *J* = 6.2 Hz, 2H), 2.19 (s, 3H), 2.16 (s, 3H), 1.91–1.95 (m, 4H), 1.73–1.79 (m, 2H), 1.48–1.53 (m, 2H), 0.91–0.99 (m, 7H), 0.82–0.85 (m, 6H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ 154.8, 151.1, 141.9, 140.8, 130.3, 130.1, 126.6, 125.8, 125.4, 122.3, 114.0, 109.7, 67.4, 47.9, 40.3, 31.6, 19.4, 17.3, 16.5, 16.1, 14.9, 14.0; [HR-ESI(+)]: *m/z* calcd for C<sub>25</sub>H<sub>37</sub>O<sub>2</sub> [M + H]<sup>+</sup>: 369.2788, found: 369.2826.

**4-[4-(4-*n*-Hexyloxy-3-methylphenyl)heptan-4-yl]-2-methylphenol (PBP-C6).** Compound PBP-C6 was prepared from 3 (62.0 mg, 0.20 mmol) using the procedure described for PBP-C2. Yield 47%; colorless oil; IR (CDCl<sub>3</sub>, cm<sup>-1</sup>): 3602, 2958, 2932, 2871, 1607, 1503, 1468; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 6.89–6.93 (m, 3H), 6.86 (d, *J* = 8.4 Hz, 1H), 6.66 (d, *J* = 8.4 Hz, 1H), 6.62 (d, *J* = 8.4 Hz, 1H), 4.59 (br s, 1H), 3.92 (t, *J* = 6.6 Hz, 2H), 2.19 (s, 3H), 2.16 (s, 3H), 1.92–1.96 (m, 4H), 1.73–1.80 (m, 2H), 1.43–1.49 (m, 2H), 1.31–1.36 (m, 4H), 0.88–0.98 (m, 7H), 0.84 (t, *J* = 7.0 Hz, 6H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ 154.8, 151.2, 141.9, 140.9, 130.4, 130.2, 126.5, 125.8, 125.5, 122.4, 114.0, 109.8, 67.8, 47.9, 40.4, 31.7, 29.5, 25.9, 22.7, 17.3, 16.6, 16.1, 14.9, 14.1; [HR-ESI(+)]: *m/z* calcd for C<sub>27</sub>H<sub>41</sub>O<sub>2</sub> [M + H]<sup>+</sup>: 397.3101, found: 397.3078.

**4-[4-(4-*n*-Octyloxy-3-methylphenyl)heptan-4-yl]-2-methylphenol (PBP-C8).** Compound PBP-C8 was prepared from 3 (59.1 mg, 0.19 mmol) using the procedure described for PBP-C2. Yield 13%; colorless oil; IR (CDCl<sub>3</sub>, cm<sup>-1</sup>): 3602, 2958, 2931, 2871, 1607, 1503, 1468; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 6.91–6.93 (m, 3H), 6.85 (d, *J* = 8.4 Hz, 1H), 6.66 (d, *J* = 8.4 Hz,

1H), 6.62 (d, *J* = 8.4 Hz, 1H), 4.81 (br s, 1H), 3.91 (t, *J* = 6.2 Hz, 2H), 2.19 (s, 3H), 2.16 (s, 3H), 1.91–1.95 (m, 4H), 1.75–1.79 (m, 2H), 1.45–1.46 (m, 2H), 1.30–1.31 (m, 8H), 0.82–0.97 (m, 13H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ 155.1, 151.6, 142.2, 141.2, 130.7, 130.5, 126.9, 126.2, 125.8, 122.8, 114.3, 110.1, 68.2, 48.3, 40.7, 32.2, 29.9, 29.8, 29.7, 26.6, 23.1, 17.7, 16.9, 16.5, 15.3, 14.6, 14.5; [HR-ESI(+)]: *m/z* calcd for C<sub>29</sub>H<sub>45</sub>O<sub>2</sub> [M + H]<sup>+</sup>: 425.3414, found: 425.3392.

**4-[4-(4-*n*-Decyloxy-3-methylphenyl)heptan-4-yl]-2-methylphenol (PBP-C10).** Compound PBP-C10 was prepared from 3 (95.3 mg, 0.31 mmol) using the procedure described for PBP-C2. Yield 39%; colorless oil; IR (CDCl<sub>3</sub>, cm<sup>-1</sup>): 3602, 2957, 2929, 2871, 1607, 1503, 1468; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 6.90–6.93 (m, 3H), 6.85 (d, *J* = 8.4 Hz, 1H), 6.66 (d, *J* = 8.4 Hz, 1H), 6.61 (d, *J* = 8.4 Hz, 1H), 4.69 (br s, 1H), 3.91 (t, *J* = 6.8 Hz, 2H), 2.18 (s, 3H), 2.16 (s, 3H), 1.92–1.96 (m, 4H), 1.73–1.80 (m, 2H), 1.42–1.48 (m, 2H), 1.23–1.34 (m, 12H), 0.81–0.99 (m, 13H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ 154.8, 151.2, 141.9, 140.9, 130.4, 130.2, 126.5, 125.8, 125.5, 122.5, 114.0, 109.8, 67.8, 47.9, 40.4, 32.0, 29.7, 29.6, 29.5, 29.4, 26.2, 22.7, 17.3, 16.6, 16.1, 14.9, 14.2; [HR-ESI(+)]: *m/z* calcd for C<sub>31</sub>H<sub>49</sub>O<sub>2</sub> [M + H]<sup>+</sup>: 453.3727, found: 453.3767.

**4-[4-(4-*n*-Dodecyloxy-3-methylphenyl)heptan-4-yl]-2-methylphenol (PBP-C12).** Compound PBP-C12 was prepared from 3 (92.8 mg, 0.30 mmol) using the procedure described for PBP-C2. Yield 33%; colorless oil; IR (CDCl<sub>3</sub>, cm<sup>-1</sup>): 3607, 2927, 2855, 1604, 1505, 1457; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 6.89–6.93 (m, 3H), 6.86 (d, *J* = 8.4 Hz, 1H), 6.66 (d, *J* = 8.4 Hz, 1H), 6.64 (d, *J* = 8.4 Hz, 1H), 4.47 (br s, 1H), 3.91 (d, *J* = 6.4 Hz, 2H), 2.19 (s, 3H), 2.16 (s, 3H), 1.91–1.95 (m, 4H), 1.75–1.79 (m, 2H), 1.44–1.46 (m, 2H), 1.24–1.34 (m, 16H), 0.82–0.98 (m, 13H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ 154.8, 151.1, 141.9, 140.8, 130.3, 130.1, 126.6, 125.8, 125.4, 122.3, 114.0, 109.7, 67.7, 47.9, 40.3, 31.9, 29.7, 29.6, 29.5, 29.4, 26.2, 22.7, 17.3, 16.5, 16.1, 14.9, 14.1; [HR-ESI(+)]: *m/z* calcd for C<sub>33</sub>H<sub>53</sub>O<sub>2</sub> [M + H]<sup>+</sup>: 481.4040, found: 481.4002.

**4-[4-(4-(2-Chloroethoxy)-3-methylphenyl)heptan-4-yl]-2-methylphenol (4).** To a solution of 3 (525.8 mg, 1.7 mmol) in MeCN (10 mL) was added 1,2-dichloroethane (333 mg, 3.4 mmol) and  $K_2CO_3$  (705 mg, 5.1 mmol) at room temperature. The mixture was stirred for 48 h at room temperature, brine was added and the whole mixture was extracted with ethyl acetate. The organic layer was dried over  $Na_2SO_4$ , filtered, and then concentrated *in vacuo*. The residue was purified by column chromatography (ethyl acetate/*n*-hexane = 1/4) to give 4 (117 mg, 19%) as colorless oil; IR (CDCl<sub>3</sub>, cm<sup>-1</sup>): 3602, 2958, 2932, 2071, 1607, 1503, 1457; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 6.89–6.95 (m, 3H), 6.83 (d, *J* = 8.4 Hz, 1H), 6.65 (t, *J* = 7.8 Hz, 2H), 5.58 (br s, 1H), 4.18 (t, *J* = 5.8 Hz, 2H), 3.79 (t, *J* = 5.8 Hz, 2H), 2.18 (s, 6H), 1.92–1.96 (m, 4H), 0.82–0.99 (m, 10H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ 153.8, 151.6, 142.2, 141.4, 130.5, 130.3, 126.4, 125.9, 122.8, 114.0, 110.3, 68.1, 48.0, 42.3, 40.3, 31.6, 22.7, 17.3, 16.5, 16.2, 14.9; [HR-ESI(+)]: *m/z* calcd for C<sub>23</sub>H<sub>32</sub>ClO<sub>2</sub> [M + H]<sup>+</sup>: 375.2085, found: 375.2051.

**4-(4-[4-(2-(*n*-Hexylamino)ethoxy)-3-methylphenyl]heptan-4-yl)-2-methylphenol (PBP-NC6).** A solution of 4 (49.0 mg, 0.13

mmol) in MeCN (0.5 mL) was added *n*-hexylamine (66.0 mg, 0.908 mmol) and stirred at 120 °C for 4 h assisted microwave irradiation. The reaction mixture was concentrated *in vacuo* and purified by silica gel flash column chromatography (dichloromethane) to give **PBP-NC6** (24.2 mg, 0.055 mmol, 42%) as colorless oil. IR (CDCl<sub>3</sub>, cm<sup>-1</sup>): 3602, 2958, 2931, 2871, 1606, 1503, 1466; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 6.88–6.93 (m, 3H), 6.71 (d, *J* = 8.0 Hz, 1H), 6.63 (d, *J* = 8.4 Hz, 1H), 6.55 (d, *J* = 8.4 Hz, 1H), 4.05 (t, *J* = 4.4 Hz, 2H), 3.03 (t, *J* = 4.2 Hz, 2H), 2.73 (t, *J* = 7.0 Hz, 2H), 2.17 (s, 3H), 2.13 (s, 3H), 1.91–1.94 (m, 4H), 1.54–1.57 (m, 2H), 1.28–1.32 (m, 6H), 0.82–0.95 (m, 13H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ 154.7, 152.6, 142.2, 141.3, 130.9, 130.7, 127.0, 126.4, 125.8, 123.5, 114.6, 110.4, 66.9, 50.0, 49.1, 48.4, 40.9, 32.2, 29.9, 27.4, 23.1, 17.8, 17.1, 16.9, 15.4, 14.6; [HR-ESI(+)]: *m/z* calcd for C<sub>29</sub>H<sub>46</sub>NO<sub>2</sub> [M + H]<sup>+</sup>: 440.3523, found: 440.3482.

**4-(4-{4-[2-(*n*-Octylamino)ethoxy]-3-methylphenyl}heptan-4-yl)-2-methylphenol (PBP-NC8).** Compound **PBP-NC8** was prepared from **4** (57.0 mg, 0.15 mmol) using the procedure described for **PBP-NC6**. Yield 34%; colorless oil; IR (CDCl<sub>3</sub>, cm<sup>-1</sup>): 3606, 2958, 2930, 2871, 1605, 1504, 1457; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 6.89–6.93 (m, 3H), 6.78 (d, *J* = 8.4 Hz, 1H), 6.65 (d, *J* = 8.4 Hz, 1H), 6.59 (d, *J* = 8.4 Hz, 1H), 4.08 (t, *J* = 4.8 Hz, 2H), 3.06 (t, *J* = 4.8 Hz, 2H), 2.76 (t, *J* = 7.6 Hz, 2H), 2.18 (s, 3H), 2.15 (s, 3H), 1.91–1.95 (m, 4H), 1.56–1.60 (m, 2H), 1.26–1.29 (m, 10H), 0.82–0.98 (m, 13H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ 154.7, 152.1, 142.2, 141.8, 130.8, 130.7, 127.0, 126.4, 125.8, 123.2, 114.6, 110.5, 66.8, 50.0, 49.0, 48.4, 40.8, 32.3, 29.9, 29.8, 27.7, 23.2, 17.8, 17.1, 16.7, 15.4, 14.6; [HR-ESI(+)]: *m/z* calcd for C<sub>31</sub>H<sub>50</sub>NO<sub>2</sub> [M + H]<sup>+</sup>: 468.3836, found: 468.3815.

**4-(4-{4-[2-(*n*-Decylamino)ethoxy]-3-methylphenyl}heptan-4-yl)-2-methylphenol (PBP-NC10).** Compound **PBP-NC10** was prepared from **4** (33 mg, 0.09 mmol) using the procedure described for **PBP-NC6**. Yield 32%; colorless oil; IR (CDCl<sub>3</sub>, cm<sup>-1</sup>): 3603, 2957, 2929, 2856, 1606, 1503, 1466; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 6.88–6.93 (m, 3H), 6.74 (d, *J* = 8.4 Hz, 1H), 6.64 (d, *J* = 8.4 Hz, 1H), 6.57 (d, *J* = 8.4 Hz, 1H), 4.06 (br, 2H), 3.04 (br, 2H), 2.73 (t, *J* = 7.6 Hz, 2H), 2.18 (s, 3H), 2.14 (s, 3H), 1.91–1.94 (m, 4H), 1.54–1.56 (m, 2H), 1.25–1.29 (m, 14H), 0.82–0.95 (m, 13H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ 154.7, 152.4, 142.1, 141.4, 130.8, 130.6, 126.9, 126.4, 125.8, 123.4, 114.6, 110.4, 66.9, 50.0, 49.1, 48.4, 40.8, 40.2, 32.4, 30.1, 30.0, 29.8, 27.7, 27.4, 23.2, 17.8, 17.1, 16.8, 15.4, 14.6; [HR-ESI(+)]: *m/z* calcd for C<sub>33</sub>H<sub>54</sub>NO<sub>2</sub> [M + H]<sup>+</sup>: 496.4149, found: 496.4101.

**4-(4-{4-[2-(*n*-Dodecylamino)ethoxy]-3-methylphenyl}heptan-4-yl)-2-methylphenol (PBP-NC12).** Compound **PBP-NC12** was prepared from **4** (68.7 mg, 0.183 mmol) using the procedure described for **PBP-NC6**. Yield 26%; colorless oil; IR (CDCl<sub>3</sub>, cm<sup>-1</sup>): 3603, 2957, 2920, 2855, 1605, 1503, 1465; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 6.88–6.93 (m, 3H), 6.77 (d, *J* = 8.4 Hz, 1H), 6.65 (d, *J* = 8.4 Hz, 1H), 6.59 (d, *J* = 8.4 Hz, 1H), 4.07 (t, *J* = 4.8 Hz, 2H), 3.05 (t, *J* = 5.2 Hz, 2H), 2.75 (t, *J* = 7.6 Hz, 2H), 2.18 (s, 3H), 2.14 (s, 3H), 1.91–1.95 (m, 4H), 1.53–1.58 (m, 2H), 1.25–1.30 (m, 18H), 0.82–0.98 (m, 13H); <sup>13</sup>C NMR (100

MHz, CDCl<sub>3</sub>): δ 154.2, 151.6, 141.6, 141.3, 130.3, 130.2, 126.5, 125.9, 125.3, 122.7, 114.1, 109.9, 66.4, 49.5, 48.6, 47.9, 40.3, 39.7, 31.9, 29.7, 29.6, 29.5, 29.4, 29.3, 27.2, 26.9, 22.7, 17.3, 16.6, 16.2, 14.9, 14.1; [HR-ESI(+)]: *m/z* calcd for C<sub>35</sub>H<sub>58</sub>NO<sub>2</sub> [M + H]<sup>+</sup>: 524.4462, found: 524.4445.

**4-(4-{4-[2-(*n*-Hexadecylamino)ethoxy]-3-methylphenyl}heptan-4-yl)-2-methylphenol (PBP-NC16).** Compound **PBP-NC16** was prepared from **4** (28.1 mg, 0.08 mmol) using the procedure described for **PBP-NC6**. Yield 34%; colorless oil; IR (CDCl<sub>3</sub>, cm<sup>-1</sup>): 3603, 2957, 2927, 2855, 1606, 1503, 1466; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 6.88–6.93 (m, 3H), 6.75 (d, *J* = 8.4 Hz, 1H), 6.63 (d, *J* = 8.8 Hz, 1H), 6.58 (d, *J* = 8.4 Hz, 1H), 4.05 (t, *J* = 4.8 Hz, 2H), 3.04 (t, *J* = 5.0 Hz, 2H), 2.73 (t, *J* = 7.4 Hz, 2H), 2.18 (s, 3H), 2.13 (s, 3H), 1.91–1.95 (m, 4H), 1.25–1.28 (m, 28H), 0.82–0.98 (m, 13H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ 154.2, 151.7, 141.6, 141.1, 130.3, 130.2, 126.5, 125.9, 125.3, 122.7, 114.1, 109.9, 66.4, 49.5, 48.6, 47.9, 40.3, 31.9, 29.7, 29.6, 29.5, 29.4, 27.2, 22.7, 17.3, 16.6, 16.3, 14.9, 14.2; [HR-ESI(+)]: *m/z* calcd for C<sub>39</sub>H<sub>66</sub>NO<sub>2</sub> [M + H]<sup>+</sup>: 580.5088, found: 580.5077.

**4-(4-{4-[2-(*N*-Aminoethoxy)-3-methylphenyl]heptan-4-yl}-2-methylphenol (PBP-NH<sub>2</sub>).** To a solution of **3** (614.3 mg, 1.97 mmol) and Cs<sub>2</sub>CO<sub>3</sub> (321.3 mg, 0.99 mmol) in MeCN (5 mL) was added benzyl (2-bromoethyl)carbamate (264.7 mg, 1.03 mmol), and the mixture was stirred at 40 °C for 16 h. The reaction mixture was neutralized with 3% aqueous HCl and extracted with ethyl acetate. The organic layer was washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and then concentrated *in vacuo*. The residue was dissolved in MeOH (6 mL) and Pd(OH)<sub>2</sub> was added (*ca.* 20 mg). The solution was stirred at room temperature for 16 h under H<sub>2</sub> atmosphere. The reaction mixture was filtered through celite and concentrated *in vacuo*. The residue was purified with silica gel chromatography (AcOEt/*n*-hexane = 1/4) to afford **PBP-NH<sub>2</sub>** (175 mg, 25%) as colorless oil; IR (CDCl<sub>3</sub>, cm<sup>-1</sup>): 3602, 2958, 2932, 2871, 1606, 1503, 1465; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 6.91–6.94 (m, 3H), 6.80 (d, *J* = 8.4 Hz, 1H), 6.66 (d, *J* = 8.4 Hz, 1H), 6.61 (d, *J* = 8.4 Hz, 1H), 3.98 (t, *J* = 5.0 Hz, 2H), 3.11 (t, *J* = 5.0 Hz, 2H), 2.19 (s, 3H), 2.16 (s, 3H), 1.91–1.95 (m, 4H), 0.91–0.99 (m, 4H), 0.84 (t, *J* = 7.0 Hz, 6H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ 154.3, 151.7, 141.6, 141.2, 130.3, 130.2, 126.5, 125.9, 125.3, 122.7, 114.0, 109.8, 69.4, 47.9, 41.6, 40.3, 17.3, 16.6, 16.3, 14.9; [HR-ESI(+)]: *m/z* calcd for C<sub>23</sub>H<sub>34</sub>NO<sub>2</sub> [M + H]<sup>+</sup>: 356.2584, found: 356.2547.

**4-(4-{4-[2-(*N,N*-Dimethylamino)ethoxy]-3-methylphenyl}heptan-4-yl)-2-methylphenol (PBP-tam).** To a solution of **3** (265.4 mg, 0.85 mmol), K<sub>2</sub>CO<sub>3</sub> (351.6 mg, 2.5 mmol) and NaI (127.2 mg, 0.85 mmol) in DMF (5 mL) was added 2-chlorodimethylethylamine (122.2 mg, 0.85 mmol) and the mixture was stirred at 100 °C for 16 h. The reaction mixture was concentrated *in vacuo*, and the residue was purified with silica gel chromatography (AcOEt/MeOH = 9/1) to afford **PBP-tam** (18.8 mg, 0.05 mmol, 6%) as colorless oil; IR (CDCl<sub>3</sub>, cm<sup>-1</sup>): 3607, 3020, 2959, 1382, 1212; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 6.80–6.92 (m, 3H), 6.81 (d, *J* = 8.4 Hz, 1H), 6.65 (d, *J* = 8.4 Hz, 1H), 6.59 (d, *J* = 8.4 Hz, 1H), 4.05 (t, *J* = 6.0 Hz, 2H),

2.78 (t,  $J = 6.0$  Hz, 2H), 2.37 (s, 6H), 2.18 (s, 3H), 2.13 (s, 3H), 1.91–1.95 (m, 4H), 0.93–0.98 (m, 4H), 0.83 (t,  $J = 6.8$  Hz, 6H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  154.8, 151.1, 141.9, 140.8, 130.3, 130.1, 126.6, 125.8, 125.4, 122.3, 114.0, 109.7, 67.4, 47.9, 40.3, 31.6, 19.4, 17.3, 16.5, 16.1, 14.9, 14.0; [HR-ESI(+)]:  $m/z$  calcd for  $\text{C}_{25}\text{H}_{38}\text{NO}_2$  [ $\text{M} + \text{H}$ ] $^+$ : 384.2897, found: 384.2898.

## Biology

**Cell culture.** Human breast carcinoma MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 100  $\mu\text{g mL}^{-1}$  kanamycin with 10  $\mu\text{g mL}^{-1}$  insulin (Sigma, St. Louis, MO, USA). The cells were treated with various concentrations of the compounds for 6 h. Whole-cell lysates were analyzed using western blotting with the indicated antibodies.

**Western blotting.** Cells were lysed with SDS lysis buffer (0.1 M Tris-HCl at pH 8.0, 10% glycerol, 1% SDS) and boiled for 10 min. The protein concentration was measured by the BCA method (Pierce, Rockford, IL, USA) and the lysates containing an equal amount of protein were separated by SDS-PAGE, transferred to PVDF membranes (Millipore, Darmstadt, Germany) for western blotting using the appropriate antibodies. The immunoreactive proteins were visualized using the Immobilon Western chemiluminescent HRP substrate (Millipore) or Clarity Western ECL substrate (Bio-Rad, Hercules, CA, USA), and light emission was quantified with a LAS-3000 lumino-image analyzer (Fuji, Tokyo, Japan). The antibodies used in this study were anti-ER $\alpha$  rabbit monoclonal antibody (mAb) (Cell Signaling Technology, Danvers, MA, USA; 8644), anti- $\beta$ -actin mouse mAb (Sigma; A5316), anti-AR rabbit mAb antibody (Cell Signaling Technology; 5153), anti-AhR rabbit mAb antibody (Cell Signaling Technology; 13790), anti-VDR rabbit mAb antibody (Cell Signaling Technology; 12550), and anti-Lamin B goat pAb (Santa Cruz, Dallas, TX, USA; sc-6216).

**Fluorescence polarization assay.** Test compounds were dissolved in DMSO to prepare a stock solution (10 mM). Fluorescence polarization-based competition binding assays were conducted to determine the relative binding affinity of compounds for ER $\alpha$  using commercially available kits (P2698, Life Technologies) according to the manufacturer's instructions. Fluorescence polarization signals (mP values) were then measured using an EnVision multiple plate reader (Perkin Elmer) with a 470 nm excitation filter and a 535 nm emission filter. The fraction of compound bound to ER $\alpha$  was correlated with the mP value and plotted against values of competitor concentrations.

**Reporter gene assay.** ER-antagonistic activities of the compounds were evaluated by means of reporter gene assay using a Gal4-human ER $\alpha$  reporter system.<sup>18</sup> A ligand binding domain (LBD) of human ER $\alpha$  was inserted into the pCMX-GAL4 vector to obtain pCMX-GAL4-HER (pCMX-flag vector to make pCMX-ER $\alpha$ ). A GAL4-responsive MH100 (USA)x4-tk-LUK reporter was used. Human embryonic kidney (HEK 293) cells were cultured in Dulbecco's modified Eagle's medium

(DMEM) without phenol red containing 10% fetal bovine serum (FBS) and antibiotic-antimycotic (Nacalai) at 37 °C in a humidified atmosphere of 5% CO $_2$  in air. Transfections were performed by the calcium phosphate coprecipitation method. Test compounds were added at 8 h after transfection. Cells were harvested 16–20 h after the treatment, and luciferase and  $\beta$ -galactosidase activities were assayed using a luminometer and a microplate reader. DNA cotransfection experiments were performed with 50 ng of reporter plasmid, 15–20 ng of pCMX- $\beta$ -galactosidase, 10–15 ng of each receptor expression plasmid and pGEM carrier DNA to make a total of 150 ng of DNA per well in a 96-well plate. Luciferase data were normalized to an internal  $\beta$ -galactosidase control, and the reported values are means of triplicate assays. Antagonist activity was measured in the presence of 0.3 nM 17 $\beta$ -estradiol.

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