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Promising core structure for nuclear receptor ligands: Design and synthesis of novel estrogen receptor ligands based on diphenylamine skeleton

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ABSTRACT

Novel diphenylamine-type estrogen receptor ligands were designed and synthesized, and their biological activities were evaluated by means of binding assays for estrogen receptor-α and -β and cell proliferation assay using MCF-7 cells. Compounds **4f**, **11b**, **12c**, and **8** showed moderate estrogenic activities. We propose that the diphenylamine skeleton may be a privileged structure for various nuclear receptor ligands, including RAR, RXR, and AR ligands.

Efficient synthetic methods for diphenylamines were developed by Buckwald et al.[1] and Hartwig,[2] and the diphenylamine skeleton has proved useful in medicinal chemistry.[3] The nitrogen atom has weak basicity (pK_b = 13.2) and the two benzene rings are sterically bulky, so diphenylamines do not readily form hydrogen bonds with amino acid residues of receptors. Among related structures, diphenylether and diphenylsulfide skeletons have less potential because it is not possible to introduce a substituent on the heteroatoms. In the case of diphenylmethanes, it is difficult to introduce substituents on the linker carbon atom, and the C-substituted products can be enantiomeric if the aromatic rings are different. Thus, the diphenylamine skeleton has the advantages that the linker nitrogen atom can be readily modified with various substituents and the products are not enantiomeric, unless an asymmetric substituent is introduced, so that synthesis is more straightforward. Thus, diphenylamine structure is expected to be a useful skeleton for new drugs.

We have already reported retinoic acid receptor (RAR)[4] and retinoid X receptor (RXR) modulators[5] based on the diphenylamine skeleton. In addition, an androgen receptor (AR) modulator based on the diphenylamine skeleton has been reported.[6] Since nuclear transfer is an important process for nuclear receptor ligands, the weak basicity of the nitrogen atom of diphenylamine (in other words, the low polarity of its structure) should be favorable for developing novel nuclear receptor ligands.

The estrogen receptor (ER) is a member of the superfamily of nuclear receptors. Endogenous estrogen, 17β-estradiol, plays important roles in the regulation of the female and male reproductive systems, as well as in bone metabolism, the cardiovascular systems, and the central nervous system. The first step in the appearance of estrogenic activity is the binding of agonist ligands to ERα[7] and ERβ,[8] resulting in a conformational change. The resulting ligand-bound ER then dimerizes, forms complexes with various cofactors, and binds to specific promoter elements of DNA to initiate gene transcription.[9]

A synthetic estrogen, diethylstilbestrol **1**, acts like a native ligand, estradiol. Hydroxytamoxifen **2** has potent antagonistic activ-

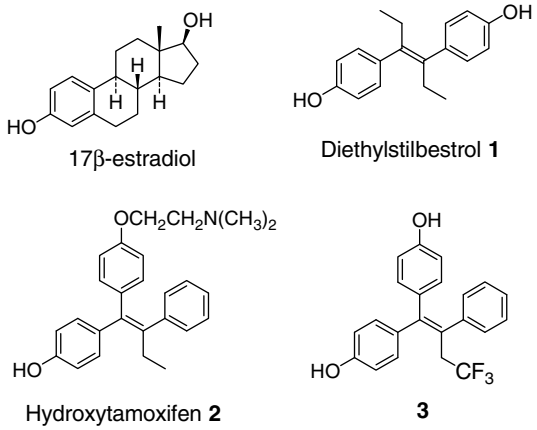

Figure 1. Structures of the native ligand and synthetic ligands for ER.

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ity for ER and acts as a selective estrogen receptor modulator (SERM).[10] Currently, various third- or fourth-generation SERMs are under development.[10] Recently, compound **3**, which is a potent antiestrogen without the alkylamino residue found in typical SERMs, was reported[11](see Fig. 1).

The development of ER ligands remains an important issue in the field of medicinal chemistry because novel functions of estrogen are still being found.[12] Therefore, we initially designed and synthesized N-alkylated diphenylamine derivatives **4** as ER ligand candidates based upon their attractive chemical properties and easy availability (Fig. 2). Here, we describe the synthesis and the structure–activity relationships of these diphenylamine derivatives.

The synthesis of N-alkylated compounds **4a–4j** is summarized in Scheme 1. Using the usual conditions of Buckwald–Hartwig amination, a key intermediate, bis(4-methoxyphenyl)diphenylamine **5**, was synthesized from 4-bromoanisole and *p*-anisidine, which are commercially available. N-alkylated compounds **6** were obtained by the reaction of **5** with the corresponding alkyl halides in the presence of NaH as a base. Compounds **6** were reacted with BBr_3 to afford N-alkylated derivatives **4a**–**4j**.

Scheme 2 summarizes the synthesis of compounds **4k**–**4m**. Compound **4k** was synthesized by N-acylation of **5**, demethylation, and reduction of the amide group with $LiAlH_4$, because *N*-(4-methoxybenzyl)diphenyl-amine was decomposed in the demethylation with BBr_3 as shown in Scheme 1. Compound **4l** was synthesized from cyclohexylamine by double Buckwald–Hartwig amination using $^tBu_3P$ as a ligand, followed by demethylation with BBr_3, since the sterically bulky *N*-cyclohexyl group could not be introduced into the intermediate **5** shown in Scheme 1. An *N*-phenyl derivative **4m** was synthesized by Buckwald–Hartwig amination of the intermediate **5** with bromobenzene, followed by demethylation with BBr_3.

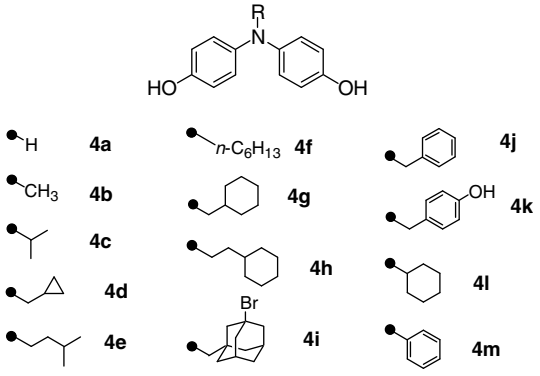
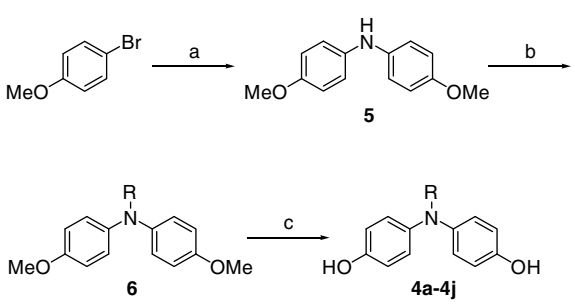
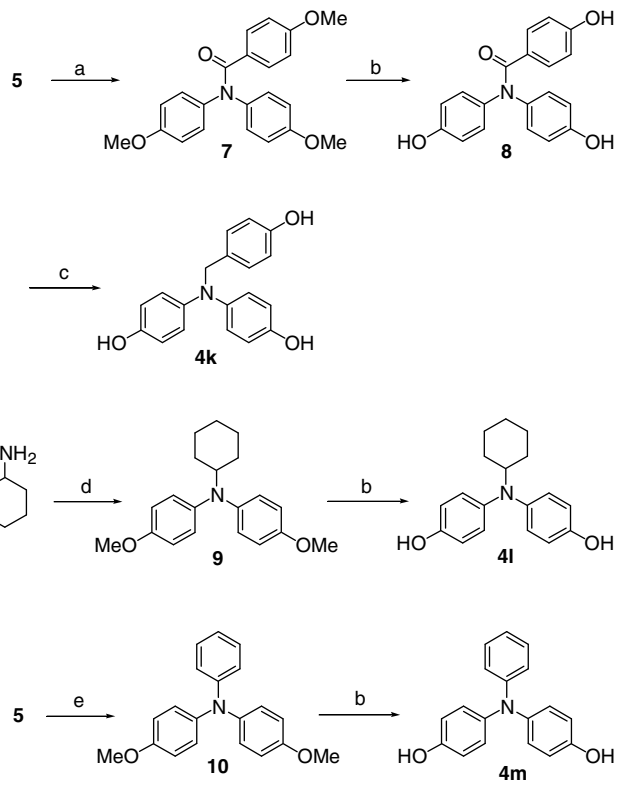


Figure 2. Designed compounds as ER ligand candidates based on diphenylamine skeleton.



Scheme 1. Synthetic scheme of N-alkylated diphenylamine derivatives **4a–4j**. Reagents and conditions: (a) *p*-anisidine, $Pd_2(dba)_3$, *rac*-BINAP, NaO^tBu, toluene, reflux, 67%; (b) NaH, RX, DMF, 22—97%; (c) BBr_3, CH_2Cl_2, 74%-quant.



Scheme 2. Synthetic scheme of **4k–4m**. Reagents and conditions: (a) 4-methoxybenzoyl chloride, toluene, reflux, 87%; (b) BBr_3, CH_2Cl_2, 53—97%; (c) $LiAlH_4$, THF, 58%; (d) *p*-anisidine, $Pd_2(dba)_3$, $^tBu_3P$, NaO^tBu, toluene, reflux, 91%; (e) bromobenzene, $Pd_2(dba)_3$, *rac*-BINAP, NaO^tBu, toluene, reflux, 71%.

All of the synthesized compounds were evaluated with a competitive binding assay using [2,4,6,7-^{3}H]17β-estradiol (4 nM) and human recombinant ERα and ERβ as an initial screening.[13] The results of binding assay are summarized as relative binding affinity (RBA) for ERα and ERβ, and selectivity ratio in Table 1. Compounds **4a**, **4b**, and **4c** did not bind to either of the ERs. When the hydrophobicity and bulkiness of the substituents on the nitrogen atom were increased, binding affinities for ERα and ERβ increased. Compound **4h** and **4k** showed moderate binding affinity to ERα and ERβ. Compound **4j** bound to ERβ about 30 times more strongly than to ERα.

The biological activities were evaluated by means of cell proliferation assay using human breast cancer MCF-7 cells.[14] Although

Table 1
Binding affinity and receptor selectivity of compounds **4** for the human recombinant ERα or ERβ

Compound	RBA[a] for ERα	RBA[a] for ERβ	Selectivity ERβα
4a	<0.001	<0.001	—
4b	<0.001	<0.001	—
4c	<0.001	<0.001	—
4d	0.054	<0.001	—
4e	0.129	2.339	18.132
4f	0.153	0.102	0.667
4g	0.890	6.696	7.523
4h	3.643	6.836	1.876
4i	0.551	0.565	1.025
4j	0.655	18.134	27.685
4k	4.260	4.050	0.951
4l	1.130	4.299	3.804
4m	1.295	1.912	1.476

[a] All binding assays were performed in triplicate ($n = 3$). IC_{50} value of estradiol for ERα and ERβ is each 4 nM. Relative binding affinity (RBA) is IC_{50} (estradiol)/IC_{50} (compound) × 100.

compounds **4a**–**4c** were inactive, compounds **4d**–**4m** allowed MCF-7 cells to grow in dose-dependent manner. The EC_{50} values of the tested compounds are summarized in Table 2. None of the synthesized compounds exhibited antiestrogenic activity (data not shown). Compounds **4f** and **4j** showed the best estrogenic activity ($EC_{50} = 1.1 \times 10^{-8}$ M and 6.7×10^{-8} M, respectively), although they were less potent than the native ligand, estradiol. The estrogenic activity of compounds **4f** and **4g** showed higher efficacy than that of estradiol.

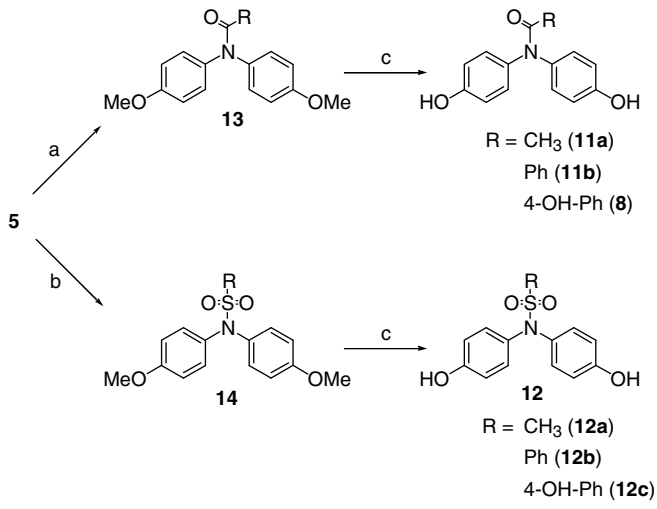
Next, *N*-acyl **8**[15] and **11** and *N*-sulfonyl **12** derivatives were designed and synthesized to evaluate the effect of the linking group on the activity. Based on the results of binding assay and cell proliferation assay, compound **4j** and **4k** with the less flexible *N*-benzyl substituents were selected. The methyl group as shown in the structure of **4a** was chosen as a negative control.

The structures and synthetic scheme of compounds **8**, **11a**, **11b**, and **12a**–**12c** are summarized in Scheme 3. The structure and synthetic scheme of compound **8** are shown in Scheme 2. As described in the synthesis of compound **8**, the reaction of **5** with the corresponding acyl chlorides in toluene afforded the *N*-acylated compounds, and demethylation with BBr_3 gave compounds **11a** and **11b**. *N*-Sulfonylated compounds **12** were obtained by the reaction of **5** with the corresponding sulfonyl chlorides in the presence of NaH in THF, followed by demethylation with BBr_3.

Table 2
EC_{50} values of N-alkylated diphenylamine derivatives **4a**—**4m** in cell proliferation assay using MCF-7 cells[a]

Compound	EC_{50}[b] (M)	Compound	EC_{50}[b] (M)
4a	Inactive	**4h**	5.0×10^{-7} (71)
4b	Inactive	**4i**	1.1×10^{-5} (77)
4c	Inactive	**4j**	6.7×10^{-8} (85)
4d	Compound	**4k**	3.7×10^{-7} (42)
4e	5.7×10^{-7} (31)	**4l**	5.9×10^{-7} (64)
4f	1.1×10^{-8} (130)	**4m**	1.2×10^{-7} (87)
4g	2.1×10^{-7} (109)	Estradiol	3.7×10^{-12} (100)

[a] MCF-7 cells were incubated with the test compounds for 5 days. Cell proliferation 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. The values in parentheses indicate the efficacy for cell proliferation with the value for estradiol taken as 100.



Scheme 3. Structures and synthetic scheme of *N*-acylated **11** and *N*-sulfonylated diphenylamine derivatives **8**, **12a**, and **12b**. Reagents and conditions: (a) RCOCl, toluene, reflux, 55—87%; (b) NaH, RSO_2Cl, THF, reflux, 43—69%; (c) BBr_3, CH_2Cl_2, 25—97%.

Table 3
Binding affinity and receptor selectivity of compounds **8**, **11a**, **11b**, and **12a**—**12c** for the human recombinant ERα or ERβ

Compound	RBA[a] for ERα	RBA[a] for ERβ	Selectivity ERβα
11a	<0.001	<0.001	—
11b	0.193	1.258	6.518
8	0.336	0.063	0.188
12a	<0.001	<0.001	—
12b	0.040	<0.001	—
12c	0.352	0.004	0.011

[a] All binding assays were performed in triplicate ($n = 3$). IC_{50} value of estradiol for ERα and ERβ is each 4 nM. Relative binding affinity (RBA) is IC_{50} (estradiol)/IC_{50} (compound) × 100.

Table 3 summarizes the binding affinity of *N*-acyl and *N*-sulfonyl compounds. As expected, methyl derivatives **11a** and **12a** did not bind to either ERα or ERβ. *N*-Acyl compounds showed higher binding affinity to both ERs than did the corresponding *N*-sulfonyl compounds. It seems that the binding affinity of these compounds was decreased compared to those of the parent compounds owing to the high hydrophilicity of the *N*-acyl and *N*-sulfonyl groups.[16]

The cell proliferation activity of compounds **8**, **11a**, **11b,** and **12a**–**12c** was evaluated using MCF-7 cells as described above. Dose–response curves and EC_{50} values of the test compounds are summarized in Figure 3 and Table 4, respectively. As expected, the methyl derivatives **11a** and **12a** showed weak estrogenic activity ($EC_{50} = 4.3 \times 10^{-5}$ M and 6.9×10^{-6} M, respectively), being more potent than the parent compound **4a**. Both derivatives, *N*-acyl and *N*-sulfonyl, showed the tendency that the compounds with a 4-hydroxyphenyl substituent, **8** and **12c**, exhibited more potent estrogenic activity than the phenyl derivatives **11b** and **12b**, although the parent 4-hydroxyphenyl compound **4k** showed weaker estrogenic activity than the phenyl compound **4j**. Com-

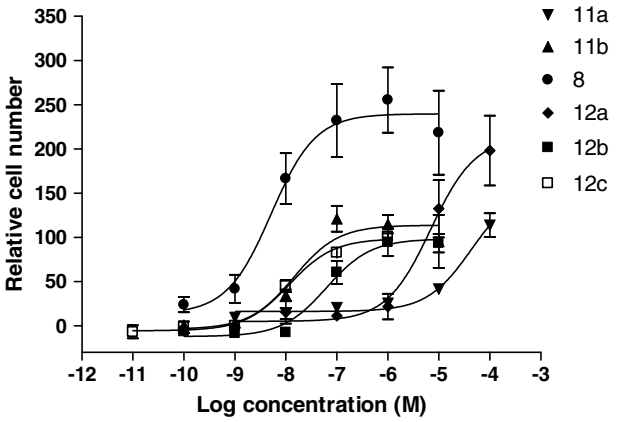


Figure 3. MCF-7 cell growth in the presence of the synthesized compounds. MCF-7 cells were incubated with the test compounds (1×10^{-4} to 1×10^{-11} M) for 4 days, and the results are shown as relative cell number, with the value for estradiol taken as 100. Cell proliferation assay was performed in triplicate ($n = 3$). Values are means ± SD for separate experiments.

Table 4
EC_{50} value of *N*-acylated and *N*-sulfonylated diphenylamine derivatives **8**, **11a**, **11b**, and **12a**—**12c** in cell proliferation assay using MCF-7 cells[a]

Compound	EC_{50} (M)[a]	Compound	EC_{50} (M)[a]
11a	4.3×10^{-5} (114)	**12a**	6.9×10^{-6} (213)
11b	1.4×10^{-8} (114)	**12b**	6.3×10^{-8} (98)
8	5.2×10^{-9} (240)	**12c**	1.2×10^{-8} (99)

[a] All experiments were performed according to the same method described in Table 2.

pound **8** showed the most potent estrogen activity among the test compounds, and the efficacy was 2.4 times higher than that of estradiol.

These results suggest that the diphenylamine skeleton is a suitable structure for the expression of estrogenic activity. The greatest advantage of the diphenylamine skeleton as a platform for drug design is the potential for control of the activity by easy modification of *N*-substituents and aromatic substituents. The diphenylamine skeleton appears to be a promising core structure for ligands of various nuclear receptors, including ER, AR, RAR, and RXR.

In conclusion, novel ER ligands were designed and synthesized based on the diphenylamine skeleton. Compounds **4f**, **8**, **11b**, and **12c** showed moderate estrogenic activities. It is noteworthy that the diphenylamine skeleton appears to be a suitable core structure for ER ligands, as well as for RAR, RXR, and AR ligands, and we suggest that this skeleton may be a privileged structure for nuclear receptor ligands. Further, structural modification of **4**, such as introduction of a basic side chain as in **2**, or a heteroatom containing-substituents on the nitrogen atom, may afford potent ER antagonists, SERMs or ERβ-selective modulators. It may prove possible to develop a wide range of nuclear receptor ligands based upon the diphenylamine skeleton.

Acknowledgments

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13. Endo, Y.; Iijima, T.; Yamakoshi, Y.; Fukasawa, H.; Miyaura, C.; Inada, M.; Kubo, A.; Itai, A. *Chem. Biol.* **2001**, *8*, 341: The ligand-binding activity of ERα and ERβ was determined by the nitrocellulose filter binding assay method. ERα and ERβ (0.5 μg/tube) were 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 [2,4,6,7-^{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 binding assay buffer. The membrane was washed twice with buffer (20 mM Tris–HCl, pH 8.0, 0.3 M NaCl), and then with 25% ethanol in distilled water. Radioactivity that remained on the membrane was measured in Atomlight using a liquid scintillation counter.
14. Human breast adenocarcinoma cell line MCF-7 was routinely cultivated in DMEM (High Glucose) supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/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% dextran-coated charcoal-stripped FBS (sFBS), 100 IU/mL penicillin, and 100 mg/mL streptomycin. Cells were trypsinized from the maintenance dish with phenol red-free 0.25% trypsin-EDTA and seeded in a 96-well plate at a density of 2×10^3 cells per final volume of 100 μL DMEM Low Glucose phenol red-free supplemented with 5% sFBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. After 24 h, the medium was replaced with 90 μL of fresh DMEM Low Glucose phenol red-free supplemented with 5% sFBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin, and 10 μL of the drug solution, which was supplemented with serial dilutions of compounds or DMSO as the diluent control, was prepared and added in the presence or absence of 1×10^{-10} M estradiol to triplicate microcultures. Cells were incubated for 6 days, and medium with compounds was changed once after 3 days. At the end of the incubation time, proliferation was assessed using WST-8 according to the manufacturers' instructions; 10 μL of WST-8 was added to microcultures and cells were incubated for 2–4 h. The absorbance at 450 nm was measured. This parameter relates to the number of living cells in the culture.
15. Compound **8** has been the subject of a PCT application during our studies of these compounds. However, their drug design was focused on benzanilide structure, not on the diphenylamine skeleton.Dalton, J. T.; Barrett, C.; He, Y.; Hong, S.-S.; Miller, D. D.; Mohler, M. L.; Narayanan, R.; Wu, Z. *PCT Int. Appl.* **2007**, WO2007062230 A2.
16. The *C*log*P* values of *N*-acyl and *N*-sulfonyl compounds and the parent compounds were calculated with ChemDraw Ultra 6.0. *C*log*P*: **4j** (4.51), **4k** (3.84), **11b** (3.84), **8** (3.71), **12b** (3.37), and **12c** (3.23).