Design and Synthesis of Phthalimide-Based Fluorescent Liver X Receptor Antagonists

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Based on our structure–activity relationship study of liver X receptor (LXR) ligands, we designed and synthesized fluorescent LXR antagonists containing an unsubstituted or substituted amino group on a phthalimide unit.

Key words liver X receptor; liver X receptor antagonist; fluorescent ligand

Liver X receptors (LXR) are members of the nuclear receptor superfamily, and are involved in the regulation of cholesterol, lipid, and glucose metabolism.[1,2] Two subtypes, LXRα and LXRβ, which have characteristic distribution patterns, are known so far. LXRα is expressed abundantly in liver, adipose tissue and macrophages, while LXRβ is ubiquitously distributed in organs and tissues. The endogenous ligands of LXRs are reported to be cholesterol metabolites (oxysterols), such as 22(*R*)-hydroxycholesterol (**1**) and 24(*S*),25-epoxycholesterol (**2**) (Fig. 1).[3] In macrophages, liver, and intestine, activation of LXRs induces expression of genes involved in cholesterol homeostasis.[4] It may be possible to prevent or even reverse atherosclerosis by modulating the expression or activity of LXRs, which therefore represent attractive potential therapeutic targets. Much research has been done to discover LXR ligands suitable for the treatment of metabolic disorders, as exemplified by the representative compound T-090317 (**3**) (Fig. 1).[5]

We have studied the design and synthesis of ligands of various nuclear receptors, such as peroxisome proliferator-activated receptor (PPAR) and farnesoid X receptor (FXR), and recently we expanded our focus to include LXR.[6—8] We have also demonstrated that the 5-substituted phenanthridin-6-one derivative (**4**), which is a conformationally restricted heterocyclic analogue of T0901317, lacks LXR-agonistic activity, but shows LXR-antagonistic activity.[9] This prompted us to speculate that the nature of the LXR-modulating activity elicited by the conformationally restricted T0901317 derivative depends on the three-dimensional geometry of the two phenyl ring moieties. Based on this concept, we performed further structural modification of T0901317, and found that other types of conformationally restricted analogs of dibenz[*b*,*f*][1,4]oxazepin-11-one, 11,12-dihydrodibenz[*b*,*f*]-azocin-6-one, and (*Z*)-dibenz[*b*,*f*]azocin-6-one derivatives, exhibited LXR-agonistic activity.[10]

As a part of our continuing structural development of subtype-selective LXR ligands, we required a rapid and simple method to determine LXR-binding activity without using isotope-labeled material(s). Here, we report the design and the synthesis of fluorescent LXR antagonists suitable for use in a fluorescent ligand binding assay system for the identification of endogenous and exogenous LXR ligands.

We have already reported structurally simple LXR antagonists with a phthalimide skeleton, such as **5** and **6** (Fig. 1).[6—8] The phthalimide structure is not fluorescent itself, but the introduction of an amino group at the benzene ring of phthalimide was reported to be fluorogenic, probably due to intramolecular charge transfer.[11,12] Based on these insights, we anticipated that if such amino group-bearing phthalimide derivatives exhibit LXR activity, we would be able to obtain fluorescent LXR ligands.

Chemistry **11a—d** were prepared from 2- (or 4)-nitrobenzyltriphenylphosphonium bromide (**7**), which was itself prepared by the reaction of 2- (or 4)-nitrobenzyl bromide with triphenylphosphine. Wittig reaction of **7** with benzaldehyde derivative in the presence of NaOMe as a base, and sub-

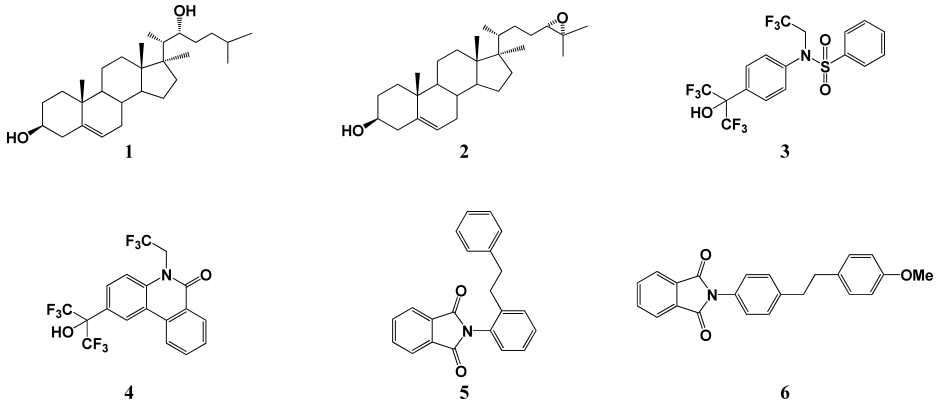

Fig. 1. Structures of Known LXR Ligands

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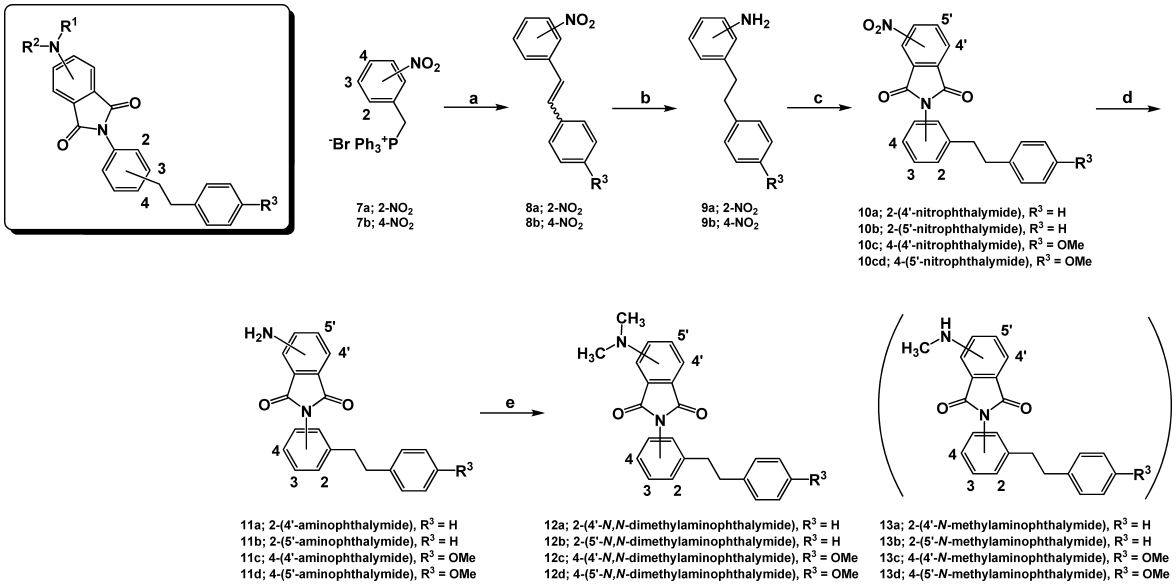
sequent reduction with 10% Pd–C, afforded the aniline derivatives (**9a**, **b**). **9a**, **b** were treated with nitrophthalic anhydride in acetic acid to afford **10a**—**d**. Hydrogenation of **10a**—**d** with 10% Pd–C afforded the desired amino derivatives (**11a**—**d**). The amino group of **11a**—**d** was methylated with trioxane in the presence of $NaBH(OAc)_3$ as a reducing agent to afford *N*,*N*-dimethyl derivatives (**12a**—**d**) and *N*-monomethyl derivatives (**13a**—**d**).[13)]

Cell Culture and Cotransfection Assay Human embryonic kidney HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum and antibiotic–antimycotic mixture (Nacalai) at 37 °C in a humidified atmosphere of 5% CO_2 in air. Transfections were performed by calcium phosphate coprecipitation. Eight hours after transfection, LXR full agonist, T0901317 with or without the test compound were added. Cells were harvested approximately 16—20 h after the treatment, and luciferase and β-galactosidase activities were assayed using a luminometer and a microplate reader. DNA cotransfection experiments included 50 ng of reporter plasmid, 20 ng of pCMX®-β-galactosidase, 15 ng of each LXR 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 β-galactosidase control and reported values are the means of triplicate assays.

Fluorescence Measurements Fluorescence spectra were measured at 25 °C on a HITACHI F2500 spectrofluoro-Photometer with a 1-cm path-length quartz cell.

Results and Discussion

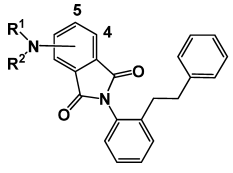
As can be seen from Table 1, the position and shape of the amino substituents located on the benzene ring of the synthesized compounds is important for the inhibitory activity towards both LXR subtypes. In the case of **5** derivatives, introduction of an unsubstituted amino group at the 4- and 5-positions did not result in apparent LXRα- or LXRβ-inhibitory

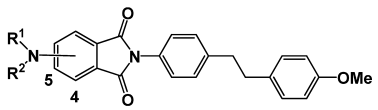


Reagents and conditions: (a) benzaldehyde, MeONa, dehydrated THF, rt., 3 d, 80—90%; (b) H_2, 10% Pd–C, AcOEt, rt, 1 h, 74—89%; (c) nitrophthalic anhydride, AcOH, reflux, 3 h, quant.; (d) H_2, 10% Pd–C, AcOEt, rt, 1 h, 64%-quant.; (e) trioxane, $NaBH(OAc)_3$, AcOH, rt., 3—7 d, **12a**—**d** 6—61%, **13a**—**d** 16—61%.

Chart 1. Synthesis of the Present Series of Amino-Substituted Phthalimides

Table 1. LXR-Antagonistic Activities of the Amino-Substituted Phthalimides





Compd.	NR^1R^2	IC_{50} (μM)		Compd.	NR^1R^2	IC_{50} (μM)	
		LXR α	LXR β			LXR α	LXR β
11a	4-NH_2	>30	>30	**11c**	4-NH_2	>30	>30
13a	4-$NHCH_3$	2.9	10	**13c**	4-$NHCH_3$	>30	>30
12a	4-$N(CH_3)_2$	1.6	21	**12c**	4-$N(CH_3)_2$	>30	>30
11b	5-NH_2	>30	>30	**11d**	5-NH_2	1.8	5.3
13b	5-$NHCH_3$	6.9	20	**13d**	5-$NHCH_3$	0.75	4.2
12b	5-$N(CH_3)_2$	4.2	17	**12d**	5-$N(CH_3)_2$	1.0	5.0
5		9.8	>30	**6**		0.2	>30

activity at the concentration of 30 μM. Methylation of the amino group caused the appearance of LXR-antagonistic activity. All the methylated derivatives (**12a**, **12b**, **13a**, **13b**) exhibited more potent LXR antagonistic activity than the lead compound **5**. They exhibited partial subtype preference for LXRα, as did **5**. However, in the **6** series, a somewhat different structure–activity relationship was obtained. All the 4-substituted derivatives (**11c**, **12c**, **13c**) lacked LXR-antagonistic activity at the concentration of 30 μM, whereas the lead **6** exhibited LXRα-selective antagonistic activity. On the other hand, the 5-amino derivative (**11d**) exhibited apparent LXR-antagonistic activity towards both subtypes; the LXRα-antagonistic activity of **11d** was decreased to some extent, while the LXRβ-antagonistic activity was increased considerably, as compared with those of **6**. Consequently, **11d** exhibited non-selective LXR antagonistic activity. The presence of a methyl group did not affect the LXR-antagonistic activity, *i.e.*, all the 5-amino-substituted derivatives **11d**, **12d**, and **13d** exhibited equipotent non-selective LXR-antagonistic activity. These structure–activity relationship studies indicated that the binding modes of amino-**5** derivatives and amino-**6** derivatives are somewhat different, even though these compounds have similar structural motifs.

Next, the absorption and fluorescence spectra of representative LXR-positive compounds (**12a**, **12b**, **11d**, **12d**) were examined in dimethyl sulfoxide (DMSO), which is a co-solvent frequently used in bioassay and for preparing stock solutions. The excitation and fluorescence spectra of 5 μM concentration of each compound were depicted in Fig. 2. In the case of **6** derivatives, the 5-unsubstituted amino derivative

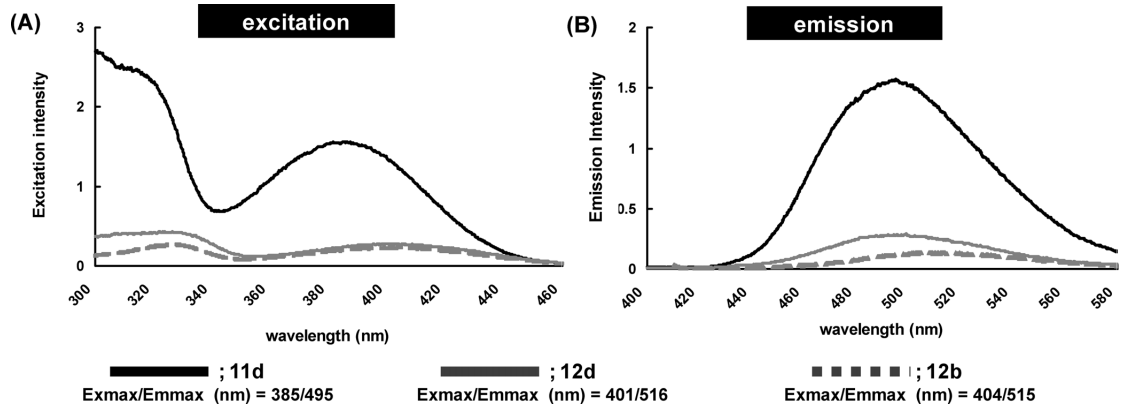


Fig. 2. (A) Excitation and (B) Fluorescence Spectra of Amino-Substituted Phthalimides

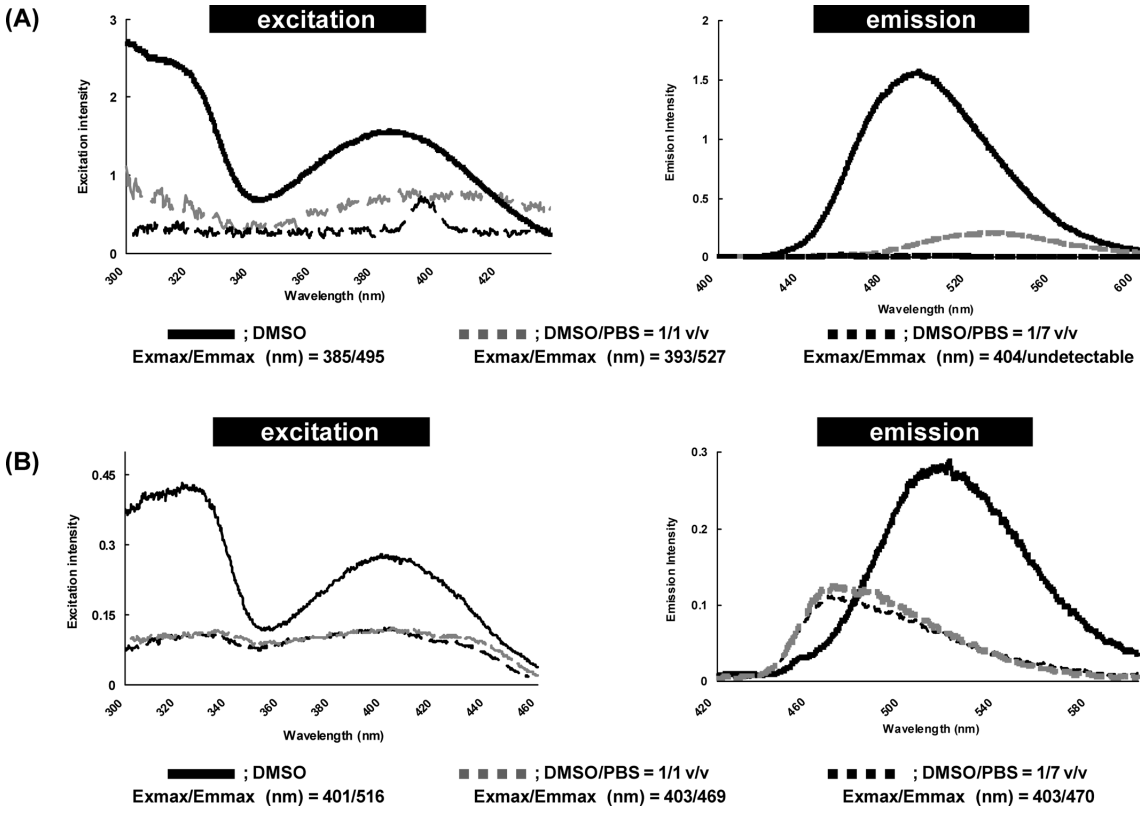


Fig. 3. Effect of PBS on the Excitation and Fluorescence Spectra of (A) **11d** and (B) **12d** in DMSO

(**11d**) exhibited excitation and emission spectra, with maxima at 385 nm and 495 nm, respectively. The 5-*N*,*N*-dimethylamino derivative **12d** exhibited weak excitation and emission spectra, as compared to **11d**, with maxima at 401 nm and 516 nm, respectively. The excitation and emission intensities of **11d** are about 6 greater than those of **12d**. In the **5** series, the 5-*N*,*N*-dimethylamino derivative **12b** exhibited weak excitation and emission as compared to **12d**, with maxima at 404 nm and 515 nm, respectively. It is of interest that the 4-*N*,*N*-dimethylamino derivative **12a** did not exhibit apparent excitation or emission (data not shown). The rank order of the fluorescence intensity decreased in the order of 5-NH_2>5-$N(Me)_2$≫4-$N(Me)_2$. This prompted us to speculate that methylation of the 5-amino group of phthalimide decreased both excitation and emission, probably due to loss of the hydrogen-bonding nature of the amino group and/or change of the electron donor–acceptor balance of the phthalimide ring.[11] The shift of the *N*,*N*-dimethylamino group from the 5-position to the 4-position of the phthalimide ring decreased both excitation and emission, but the exact reason unknown. Based on the spectral intensity, we selected the amino-**6** series for further study.

For binding assay application, we have to use aqueous solution, because the active conformation of LXR protein is unstable in organic media. Therefore, we investigated the effect of the addition of phosphate-buffered saline (PBS) (pH 7.4). In the case of **11d**, the excitation and emission intensity decreased dramatically, especially in the case of its fluorescence, and exhibited red shift of fluorescence, depending on the solvent polarity. That is, the excitation and emission maxima (nm) shifted from 385/495 (DMSO) to 393/527 (DMSO : PBS = 1 : 1 v/v). The excitation and emission maxima (nm) of **11d** in DMSO : PBS = 1 : 7 v/v was undetectable. This bathochromic shift was consistent with the reported result for an aza-crowned phthalimide.[14] To the contrary, a hypochromic shift of fluorescence was observed with increasing solvent polarity in the case of **12d**. The excitation and emission maxima (nm) shifted from 401/516 (DMSO) to 403/469 (DMSO : PBS = 1 : 1 v/v) and 403/470 (DMSO : PBS = 1 : 7 v/v). The excitation and emission intensities of **12d** were also decreased in polar solvents, but the degree of the decrease was less than in the case of **11d**. The mechanisms underlying these results remain unclear.

In conclusion, fluorescent LXR antagonists based on previously reported *N*-phenylphthalimide were developed. The fluorescence intensity and emission maxima of the present series of probes were dependent on environmental polarity. As the binding site of LXR is highly hydrophobic, an increase in fluorescence intensity is expected to occur upon specific binding of the present series of compounds to LXR,[15] so **11d** and **12d** should be good candidate probes for an LXR-binding assay system. Optimization of the assay format is in progress.

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13) **11a**; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 7.49 (d, J=8.4 Hz, 1H), 7.06—7.40 (m, 10H), 6.92 (d, J=8.4 Hz, 1H), 5.30 (s, 2H), 2.84 (m, 4H); FAB-MS m/z: 343 $(M+H)^+$. **11b**; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 7.74 (d, J=8.4 Hz, 1H), 7.05—7.40 (m, 10H), 6.89 (dd, J=8.4, 2.1 Hz, 1H), 4.40 (s, 2H), 2.80 (m, 4H); FAB-MS m/z: 343 $(M+H)^+$. **11c**; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 6.80—7.50 (m, 11H), 5.31 (s, 2H), 3.80 (s, 3H), 2.92 (m, 4H); FAB-MS m/z: 373 $(M+H)^+$. **11d**; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 6.80—7.50 (m, 11H), 4.40 (s, 2H), 3.80 (s, 3H), 2.90 (m, 4H); FAB-MS m/z: 373 $(M+H)^+$. **12a**; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 7.57 (d, J=5.1 Hz, 1H), 7.00—7.40 (m, 11H), 3.13 (s, 6H), 2.83 (m, 4H); FAB-MS m/z: 371 $(M+H)^+$. **12b**; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 7.71 (d, J=8.4 Hz, 1H), 7.05—7.40 (m, 10H), 6.80 (dd, J=8.4, 2.4 Hz, 1H), 4.60 (s, 1H), 2.98 (d, J=4.8 Hz, 3H), 2.83 (m, 4H); FAB-MS m/z: 371 $(M+H)^+$. **12c**; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 6.84—7.54 (m, 11H), 3.80 (s, 3H), 3.13 (s, 6H), 2.90 (m, 4H); FAB-MS m/z: 401 $(M+H)^+$. **12d**; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 7.74 (d, J=8.4 Hz, 1H), 6.83—7.34 (m, 10H), 3.80 (s, 3H), 3.15 (s, 6H), 2.90 (m, 4H); FAB-MS m/z: 401 $(M+H)^+$. **13a**; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 7.56 (d, J=8.4 Hz, 1H), 7.00—7.40 (m, 11H), 3.53 (q, J=7.5 Hz, 1H), 3.05 (s, 3H), 2.81 (m, 4H); FAB-MS m/z: 357 $(M+H)^+$. **13b**; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 7.71 (d, J=8.4 Hz, 1H), 7.05—7.40 (m, 10H), 6.80 (dd, J=8.4, 2.4 Hz, 1H), 4.60 (s, 1H), 2.98 (d, J=4.8 Hz, 3H), 2.83 (m, 4H); FAB-MS m/z: 357 $(M+H)^+$. **13c**; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 6.83—7.53 (m, 11H), 3.80 (s, 3H), 3.35 (q, J=7.5 Hz, 1H), 3.09 (s, 3H), 2.90 (m, 4H); FAB-MS m/z: 387 $(M+H)^+$. **13d**; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 7.73 (d, J=8.4 Hz, 1H), 6.82—7.35 (m, 10H), 3.80 (s, 3H), 3.55 (q, J=7.5 Hz, 1H), 3.09 (s, 3H), 2.90 (m, 4H); FAB-MS m/z: 387 $(M+H)^+$.
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